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Ocean acidification and host–pathogen interactions: blue mussels, *Mytilus edulis*, encountering *Vibrio tubiashii*

Maria E. Asplund,[1]* Susanne P. Baden,[1] Sarah Russ,[1] Robert P. Ellis,[2] Ningping Gong[3] and Bodil E. Hernroth[4,5]

[1]*Department of Biological and Environmental Sciences, University of Gothenburg, Kristineberg 566, SE – 451 78 Fiskebäckskil, Sweden.*
[2]*College of Life and Environmental Sciences, University of Exeter, Exeter, Devon, EX4 4QD, UK.*
[3]*Department of Biological and Environmental Sciences, University of Gothenburg, Box 463, SE – 405 30 Gothenburg, Sweden.*
[4]*The Royal Swedish Academy of Sciences, Kristineberg 566, SE – 451 78 Fiskebäckskil, Sweden.*
[5]*Department of Natural Science, Inst. Biomedicine, Kristianstad University, SE – 291 88 Kristianstad, Sweden.*

Summary

Ocean acidification (OA) can shift the ecological balance between interacting organisms. In this study, we have used a model system to illustrate the interaction between a calcifying host organism, the blue mussel *Mytilus edulis* and a common bivalve bacterial pathogen, *Vibrio tubiashii*, with organisms being exposed to a level of acidification projected to occur by the end of the 21st century. OA exposures of the mussels were carried out in relative long-term (4 months) and short-term (4 days) experiments. We found no effect of OA on the culturability of *V. tubiashii*, in broth or in seawater. OA inhibited mussel shell growth and impaired crystalline shell structures but did not appear to affect mussel immune parameters (i.e haemocyte counts and phagocytotic capacity). Despite no evident impact on host immunity or growth and virulence of the pathogen, *V. tubiashii* was clearly more successful in infecting mussels exposed to long-term OA compared to those maintained under ambient conditions. Moreover, OA exposed *V. tubiashii* increased their viability when exposed to haemocytes of OA-treated mussel. Our findings suggest that even though host organisms may have the capacity to cope with periods of OA, these conditions may alter the outcome of host–pathogen interactions, favouring the success of the latter.

Received 5 July, 2013; accepted 13 October, 2013. *For correspondence. E-mail maria.asplund@bioenv.gu.se; Tel. (+46) 31786 95 26; Fax (+46) 31786 39 90.

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Introduction

Ocean acidification (OA), caused by anthropogenic CO_2 emissions, has been proposed as one of the greatest threats marine ecosystems face (Halpern *et al*., 2008). Seawater pH has fallen by 0.1 units since the industrial revolution (The Royal Society, 2005), and is predicted to decrease by a further 0.4 units by the end of the 21st century (Caldeira and Wickett, 2003). Meta-analysis of recent research revealed overall strong negative effect of future OA with interspecific differences (Kroeker *et al*., 2010), significantly impacting marine biodiversity (Widdicombe and Spicer, 2008). In particular calcifying organisms, such as bivalves, are suggested to be negatively influenced due to their evident dependence on calcium carbonate (e.g. Orr *et al*., 2005; Gazeau *et al*., 2007), while to date there is little evidence of the impact of OA on potentially associated heterotrophic bacteria, despite many physiological processes in bacteria being dependent on external pH levels (Liu *et al*., 2010). For instance, it is suggested that production of protease (Grossart *et al*., 2006) and glycosidase (Piontek *et al*., 2010) will increase in marine bacterioplankton communities with high pCO_2/low pH conditions. These enzymes are important in natural degradation of biomass to generate energy for heterotrophic bacteria, but could also be associated with their pathogenicity against other organisms (Ridgway *et al*., 2008). Since OA appears to influence organisms differently, the interactive processes between organisms will also be expected to vary, such as in the interaction between pathogens and their host organisms. Although effects of OA on invertebrate immune defence systems have been described and tested in a number of studies (e.g. Bibby *et al*., 2008; Hernroth *et al*., 2011; 2012; Matozzo *et al*., 2012), the simultaneous interactive processes and potential dis-

turbance of the ecological balance between hosts species and their pathogens during periods of OA has not yet been widely considered.

In marine ecosystems, microbes can easily be transported in the water column and filter-feeding invertebrates, like bivalves, are able to accumulate high numbers of pathogenic bacteria (Hernroth *et al*., 2002) without getting infected (Nottage and Birkbeck, 1990; Venier *et al*., 2011). Adult bivalves generally possess a number of defence mechanisms against invasive microorganisms, such as physicochemical barriers of shells, cuticle and mucus, chemical protection by lyzosomal enzymes and anti-microbial peptides (AMP) and cellular defence by phagocytotic haemocytes (Canesi *et al*., 2002; Girón-Pérez, 2010). Microbial pathogens on the other hand have a high evolutionary potential for invading their hosts due to enhanced mutation rates, shorter generation times and abilities for horizontal gene-transfer. In this so called arms-race, Roth and colleagues (2012) suggest that hosts' defence systems are well adapted to their local common pathogens while they can be more susceptible to pathogens not commonly encountered in their habitats. However, since immune defence is energetically costly for the host (Sheldon and Verhulst, 1996), it is possible that the equilibrium level in this arms race might shift if the host is exposed to other stressors, such as OA. During long-term impacts of OA, the host may have to concentrate its energy on other processes like shell formation or growth.

In shallow coastal ecosystems, most organisms are used to coping with a natural variability in seawater pH and carbonate chemistry that vary with depth, season and productivity level. Therefore, we would assume that both host organisms and pathogens living in these areas will have a wide pH-tolerance, at least during temporary pH-stress. However, with respect to the changes in seawater carbonate chemistry projected to occur by the end of the century (Caldeira and Wickett, 2003), these organisms will likely need to adapt to more long-term shifts in the seawater pH/CO_2 dynamics. In this predictive framework, it is of ecological significance not only to understand the consequences for individual species but also the interaction between host and pathogen.

In this study, we aimed to illustrate a host–pathogen interaction between adult specimens of a calcifying bivalve host, the blue mussel *Mytilus edulis*, and a common bivalve pathogen, *Vibrio tubiashii*, when exposed to a future scenario of OA projected to occur by the end of the 21st century. Members of *Mytilus* spp and *Vibrio* spp. are attractive as a host–pathogen model due to their wide distribution and high abundance in the coastal environment, and are thus expected to have developed adaptations for encountering each other, as well as to fluctuations in seawater pH. *V. tubiashii* is particularly known to infect and cause severe impact on early life stages of bivalves (Tubiash *et al*., 1970; Hada *et al*., 1984) but is so far not reported to infect adult specimens. In specific, we examined the influence of OA on (i) the pathogen growth, viability and virulence, (ii) the host growth, immune defence and stress response and (iii) the interaction between these organisms.

Results and discussion

Climate driven environmental changes, such as ocean acidification, could either directly impact the physiology of the host or pathogen, or indirectly affect the hosts' normal microbial flora, potentially facilitating colonization by opportunistic pathogens (Mouchka *et al*., 2010), but so far such interactions are rarely studied (but see Meron *et al*., 2011; 2012). Therefore, our study on the interaction between *M. edulis* and *V. tubiashii* could serve as a model for discussing the influence of OA on host–pathogen interactions.

OA influence on the pathogen

Vibrio spp. are ubiquitous, opportunistic in the marine environment and contain a number of pathogenic species (Thompson *et al*., 2004). Their abundance and toxicity are suggested to follow environmental cues (e.g. Martin *et al*., 2002; Asplund *et al*., 2011), but little is known about how *Vibrio* bacteria will react to shifts in seawater carbonate chemistry induced by OA. However, there are indications that prevalence of *Vibrio* may increase in microbial communities at lower pH (Meron *et al*., 2012). Vibrios generally have wide pH growth optimum which may be an adaptation to the dynamic pH conditions of the coastal waters that many vibrios experience (e.g. Merrell *et al*., 2002; Rhee *et al*., 2002; Wang and Gu, 2005). Accordingly, growth rate of *V. tubiashii* NCIMB 1337 cultured in peptone medium, in the present study, showed no apparent differences between the control (Cont) and OA pH treatments (*t*-test, $P = 0.26$, Fig. 1A). According to Temperton and colleagues (2011), this strain has a biphasic optimal growth at pH 8.2 and 6.5, and a declined growth between pH 7.0 and 7.5, and therefore our OA pH treatment is outside the range where reduced grow were expected. Further, bacterial growth in our study did not affect the ΔpH between treatments even though there was a small parallel decline in pH during the exponential phase of the culture development (Fig. 1A).

In the pasteurized seawater, *V. tubashii* culturability was stable during the first days, but after 7 days of incubation, it declined by approximately 70% and 78% in OA and Cont treatments respectively (Fig. 1B). Pasteurized seawater seemingly has the potential to support the bacterial demands since no microcell formation was regis-

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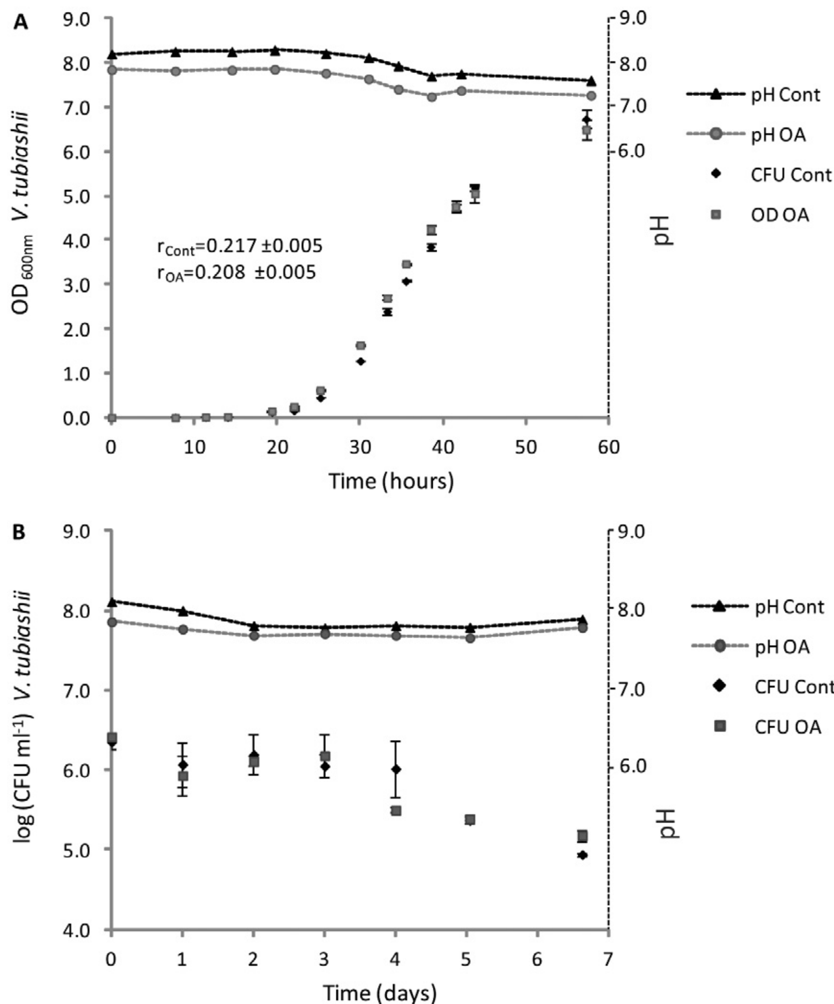

Fig. 1. (A) Development of the pH and *V. tubiashii* culture (measured as optical density at 600 nm, OD600, $n = 3$) in peptone yeast broth with initial pH set to Cont and OA conditions. Exponential growth rate, r, is given in OD-units h^{-1} (B) Development of pH and the persistence of culturalable *V. tubiashii* (log_{10}-transformed, CFU ml^{-1} seawater)in pasteurized seawater initially set at Cont and OA condition.

tered, as reported during formation of stress resistant vibrios suffering from multiple nutrient or carbon starvation (Srinivasan and Kjelleberg, 1997).

Haemolytic and proteolytic properties of *V. tubiashii* are associated with their pathogenicity to bivalves (Hasegawa *et al.*, 2008). In the virulence assay in the present study, there was a tendency for OA-treated cultures and supernatants to demonstrate haemolytic activity more often, and quicker, than control cultures. However, after 2 days of incubation, there were no visible differences between treatments (Table 1). For the proteolytic activity, the results were highly variable with no apparent differences between OA and Cont treatments (Table 1). However, activities were tested from cultures in a pre-stationary phase which may be less optimal for the proteolytic activity since it could be underdeveloped at this stage, possibly because of higher requirement of haemolysin in the initial stage of the infection process (Hasegawa *et al.*, 2008; Hasegawa and Häse, 2009). Production of such extracellular enzymes could also be a primary mechanism for breaking down proteins to release amino acids, which gram-negative bacteria, like *Vibrio*, commonly uses in their catabolism to neutralize a low pH environment (Jones and Oliver, 2009). Although we could not draw distinct conclusions of the OA effect in our virulence assay, it showed, in agreement with Hasegawa and Häse (2009), that both the haemolytic and proteolytic activities were inhibited by ethylenediaminetetraacetic acid (EDTA) (Table 1). This demonstrates a metal ion dependency of these enzymes which seems to be common for many virulence factors in vibrios (e.g. Norqvist *et al.*, 1990; Lee *et al.*, 1995).

OA influence on the host

Many marine invertebrates have calcified structures as shells, plates and inner or outer skeletons. In bivalves, the shell acts as protection against predators (Gutiérrez *et al.*, 2003) and can also be kept closed as a first-line defence against pathogenic *Vibrio* sp. (Collin *et al.*, 2012). Increased pCO_2 in the sea reduces the amount of bioavailable calcium carbonate, and calcifying processes

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Table 1. Summary of haemolytic and proteolytic activity ($n = 2$) of *V. tubiashii* cultures (Cult.) grown in pH corresponding to OA and Cont conditions respectively, 0.2 μm filtered supernatants obtained from the *V. tubiashii* cultures (Sup.) and supernatants mixed with EDTA (Sup. + EDTA) detected on horse-blood agar (BAP) and brain heart infusion agar with gelatine (BHI + gel). Any activity were visually detected 1 or 2 days after plating or addition into cut out wells in the agar.

	Haemolytic activity on BAP				Proteolytic activity on BHI + gel.	
	+ 1 day		+ 2 days		+ 2 days	
	plated	wells	plated	wells	plated	wells
Cont.						
Cult.	var	+	++	++	var	+
Sup.	–	–	var	+	–	+
Sup. + EDTA	–	–	–	–	–	–
OA						
Cult.	++	++	++	++	+	+
Sup.	var	var	var	+	var	+
Sup. + EDTA	–	var	–	var	var	–

Negative (–), positive (+), highly positive (++), varied results, both positive and negative (var).

are strongly dependent on the carbonate saturation state of seawater (Caldeira, 2007). In the experimental tanks saturation (Ω) of both calcite and aragonite were only 44% of that of the controls, and ΩAr was below the critical value for shell dissolution ($\Omega < 1$) (Table 2). To compensate for low extracellular pH, it is suggested that mussels buffers carbonate from the shell (Michaelidis *et al.*, 2005). In theory, the $Ca(HCO_3)_2$-$CaCO_3$ equilibrium in the extrapallial fluid (between the shell and the mantle), which has a similar inorganic composition as haemolymph, would be slightly affected after OA exposure (Crenshaw, 1972; Allam *et al.*, 2000). In our current study, calcium levels in the haemolymph were maintained during the long-term (4 months) exposure of OA (mM, mean ± SE; Cont: 7.41 ± 0.06 and OA: 7.43 ± 0.05), which follows the pattern reported for Norway lobsters (Hernroth *et al.*, 2012). However, OA had a negative effect on the average shell growth (Δshell length) which after 4 months of OA exposure was 40% lower than in the control group (Mann–Whitney *U*-test, $P = 0.008$, Fig. 2B). Reduced growth due to OA has earlier been shown for juvenile *M. edulis* (Berge *et al.*, 2006) and Mytilus *galloprovincialis* (Michaelidis *et al.*, 2005). The shell length of the OA-treated mussels was probably affected by increased calcium carbonate dissolution at the shell edge, as confirmed by the scanning electron microscopy (SEM) images showing that the prisms of OA-exposed shells were partially dissolved and had lost their consistent sizes (Fig. 2D). This is the growth zone of the shell and cannot be repaired by the mantle tissue.

In agreement with studies of seastars (Hernroth *et al.*, 2011) and Norway lobsters (Hernroth *et al.*, 2012), we found a slight decrease in haemolymph pH (~ – 0.1 pH units) both for the short- (4 days) and long-term (4 month) OA exposed mussels compared to the 4 days Cont treatment [Fig. 2A, analysis of variance (ANOVA), $P = 0.027$]. However, Lannig and colleagues (2010) demonstrated a more pronounced pH_e drop (– 0.5 pH units) in Japanese oyster, *Crassostera gigas*, after exposure to OA. The capacity to preserve homeostasis, regardless of external conditions, is important for the maintenance of health but is also coupled to high energy costs (Wood *et al.*, 2008).

During stress, energy is allocated to maintain protein repair via chaperones, like heat shock proteins (HSPs). These stabilize and refold denatured proteins (Mayer and Bukau, 2005) and transfer peptides throughout the cell, which in turn also affect the immune defence (Moseley, 2000). In agreement to what was found for *Asterias rubens* (Hernroth *et al.*, 2011) in the current study, *Hsp70* was induced in the short-term OA-exposed mussels (units mg^{-1} protein, mean ± SE, 131 ± 17) compared to the controls [75 ± 5, ANOVA, $P = 0.034$, Student–Newman–Keuls (SNK)-test], but these high levels were not sustained

Table 2. Mean values (± STD) of temperature (°C), $pH_{total\ scale}$, alkalinity (A_T μmol kg^{-1}) and calculated values (CO2sys software) of *p*CO2 (μatm), calcite saturation, Ω Ca and aragonite saturation, Ω Ar.

Treatment	*in situ* Temp	*in situ* $pH_{total\ scale}$	STP $pH_{total\ scale}$	A_T μmol kg^{-1}	*p*CO2 μatm	Ω Ca	Ω Ar
Cont 14°C	13.6 ± 0.01	7.95 ± 0.01	8.17 ± 0.01	2241 ± 22	511 ± 15	2.84 ± 0.01	1.82 ± 0.01
OA 14°C	13.6 ± 0.02	7.56 ± 0.04	7.74 ± 0.04	2233 ± 4	1358 ± 119	1.25 ± 0.09	0.80 ± 0.06

In situ are values at ambient temperature, STP (standard temperature and pressure) are values as if to be measured at standard temperature (0°C) and pressure (1atm).

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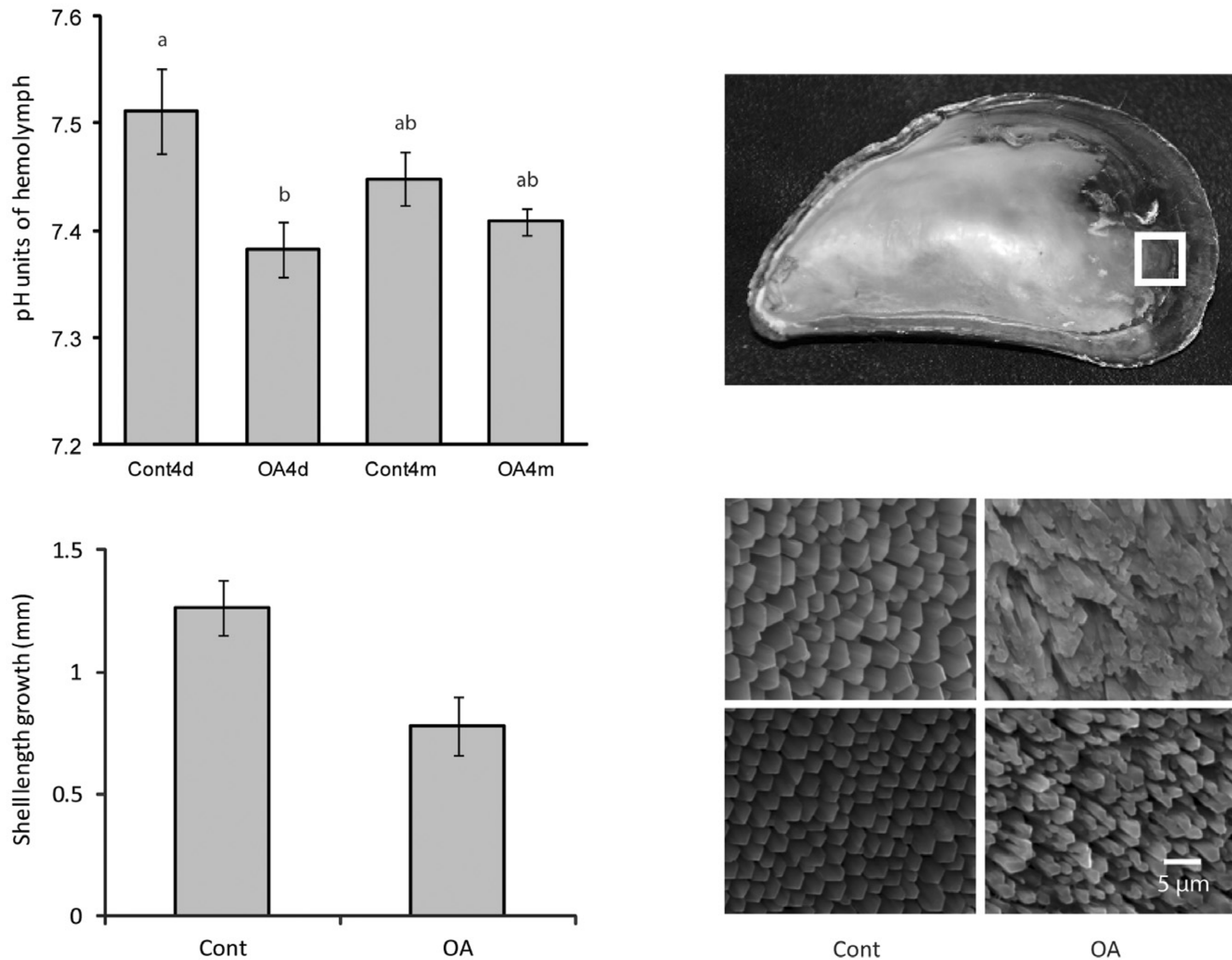

Fig. 2. (A) pH_{NBS} in cell free mussel haemolymph. Different letters above bars show significantly different means at $P < 0.05$. (B) Mussel average shell length growth (mean ± SE, $n = 39$) based on the difference in ranked shell length before and after 4-month exposure to OA and Cont respectively. (C) Edge regions of the shell, outside the mussel's mantle attachment area, in White Square, were taken for SEM imaging (D) SEM imaging microstructures of prismatic shells from Cont and OA groups, (bar = 5 μm).

during a long-term exposure (4 months OA: 89 ± 5; 4 months Cont: 72 ± 5, Fig. S1C). Elevated *Hsp70* levels are probably too costly in the long run, as also shown for *Drosophila* (Krebs and Feder, 1998) and for Antarctic marine invertebrates living on the margin of their distribution (Clark *et al.*, 2007).

Mussel haemocytes and humoral compounds such as antimicrobial peptides (AMP) are shown to be efficient against pathogen infections (Canesi *et al.*, 2002). The immune response of *M. edulis*, in terms of haemocyte numbers (μl^{-1} haemolymph, mean ± SE 4 days Cont: 972 ± 284; 4 days OA: 794 ± 198; 4 months Cont: 857 ± 157; 4 months OA: 974 ± 193, Fig. S1A), and their phagocytotic capacity (units mean ± SE, 4 days Cont: 936 ± 322; 4 days OA: 1250 ± 251; 4 months Cont: 1424 ± 129 and 4 months OA: 1599 ± 119, Fig. S1B) were in our present investigation seemingly unaffected by OA. These results contrast findings from seastars (Hernroth *et al.*, 2011), Norwegian lobster, (Hernroth *et al.*, 2012) and other bivalves like *Chamelea gallina* and *Mytilus galloprovincialis* (Matozzo *et al.*, 2012), where these immune factors were suppressed during exposure to OA. Moreover, Bibby and colleagues (2008) demonstrated that phagocytic capacity declined with decreasing pH and with longer exposure times in a population of *M. edulis* specimens collected from the Cornish coast, south west England. We therefore suggest that there might be discrepancies in immune response between mussel populations due to local adaptations or to variable food availability (Thomsen and Melzner, 2010). High food supply has been shown to clearly counteract the influence of OA on growth and calcification of juvenile *M. edulis* (Thomsen *et al.*, 2013) and may also positively effect the number of haemocytes

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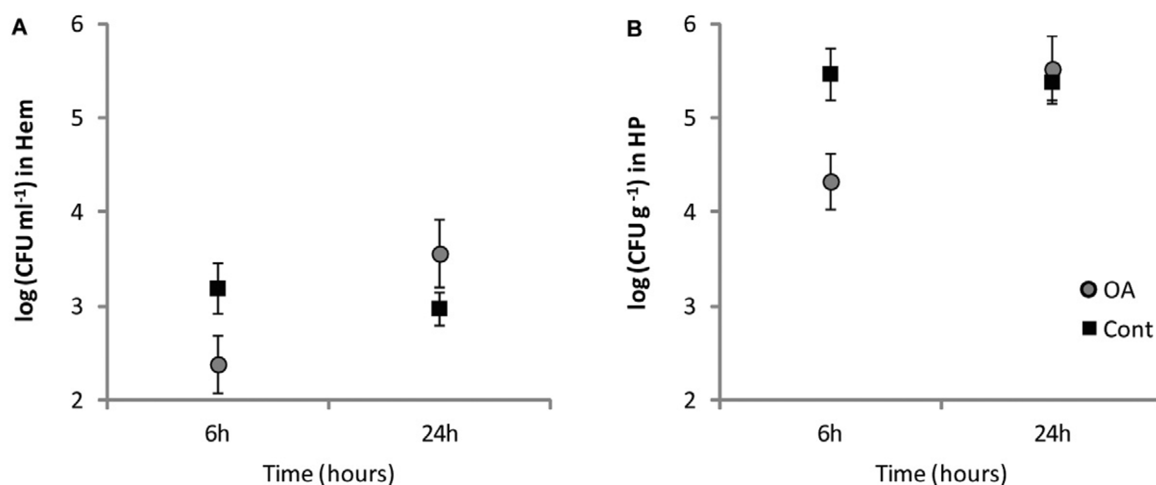

Fig. 3. Yellow colony forming units (log_{10}-tranformed CFU, mean ± SE, $n = 8$), detected on TCBS-agar, per ml haemolymph (Hem) and per g hepatopancreas (HP) from mussels exposed for 4 months of OA and control conditions (Cont) respectively. Samples were extracted 6 and 24 h post-injection of 10^5 *V. tubiashii* g^{-1} ww mussel.

as shown for the pacific oyster *Crassostera gigas* (Delaporte *et al*., 2006).

OA influence on host–pathogen interactions. *Vibrio* bacteria coexist in the marine environment with their host organisms, with adult bivalves usually resisting infections (Paillard *et al*., 2004). In our study, it was necessary to inject 10^6 *V. tubiashii* cells g^{-1} ww into the mussel to receive any visual effect, in terms of reduced production of byssus, on adult *M. edulis* in control conditions indicating a high host resistance against this bacterium (Table S2). However, our results showed that a lower dose (10^5 cells g^{-1} ww) of *V. tubiashii,* corresponding to levels that have been found in seawater (Elston *et al*., 2008), successfully infect *M. edulis* when this host–pathogen combination is exposed to environmental stressors such as OA. In both the haemolymph and hepatopancreas of the long-term OA exposed mussels, there was a clear growth of *V. tubiashii* between 6 and 24 h post-injection (ANOVA Hem: $P = 0.018$, Fig. 3A, Hep: $P = 0.011$, Fig. 3B), when injected with OA acclimatized bacteria, while in the control system, which had higher bacterial numbers in the earlier phase compared to the OA-treated group, the mussels seemed to be capable of keeping the bacteria from multiplying or potentially even suppress the bacterial numbers (Fig. 3A and B). The exposure of mussel to *V. tubiashii* was limited to 24 h post-injection to keep it within the time frame where the persistence of culturability and ΔpH in pasteurized seawater was stable as shown in Fig. 1B. Ninety-five per cent of the colony forming units (CFUs) on the thiosulfate-citrate-bile salts-sucrose (TCBS)-agar from both haemolymph and hepatopancreas had the same morphology and yellow colour as *V.tubiashii* plated directly from cultures. Background bacteria [counted in the phosphate-buffered saline (PBS)-injected mussels] did not affect the relative outcome of the generalized linear model (GLM) analysis and were therefore omitted from the analysis.

This interactive effect on the host–pathogen system was further supported by the short-term *in vitro* challenge where viability of OA exposed *V. tubiashii* clearly increased when encountering haemocytes from 4 days OA exposed mussels and was significantly higher than all other combinations (ANOVA, $P < 0.001$, SNK-test, Fig. 4). In contrast, when both organisms were exposed to control conditions, the haemocytes seemed to be able to keep

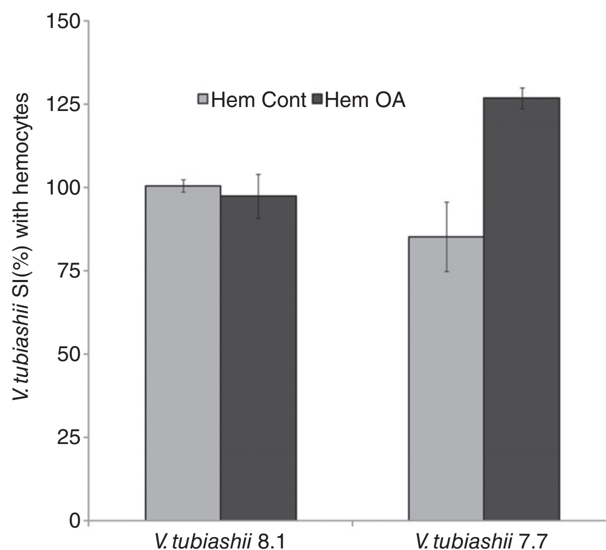

Fig. 4. Survival index (%, mean ± SE) of *V. tubiashii* cultured in pH 7.7 and 8.1 respectively, exposed to *M. edulis* haemocytes treated with Cont and OA conditions.

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the bacteria in a steady state. This agrees with earlier studies demonstrating that *Vibrio* can survive in the presence of mussel haemocytes (Hernroth *et al*., 2010; Collin *et al*., 2012), while other bacteria are easily degraded (Hernroth *et al*., 2010), probably depending on the virulence potential of the bacteria (Hernroth, 2003; Pruzzo *et al*., 2005; Collin *et al*., 2012). However, we found that when *V. tubiashii* was acclimatized to OA conditions and subsequently exposed to haemocytes extracted from mussels maintained under control conditions, the viability of the bacteria decreased (Fig. 4). This clearly highlights the importance of prior adaptation of the bacteria to experimental conditions, and that when the host is in good vigour, and not stressed by OA, it may invest in its immune response to combat bacteria.

Our results further stress the importance of studying the impact of climate change on the interaction between different organisms and suggest that the response noted may be significantly different from what would be expected by studying each organism separately. Even though we found no apparent effect of OA on either culturability of *V. tubiashii* or the immune response of *M. edulis*, when investigated separately, we clearly showed that *V. tubiashii* was more successful in this interaction when exposed to OA conditions. This may have several explanations. For example, the condition of the host when exposed to OA was seemingly negatively affected, shown by the inability to maintain high Hsp70 levels, growth inhibition and shell structure impairment during long-term stress, and this may influence its susceptibility to pathogen infection. Opportunistic bacteria, like members of the *Vibrio* genus, may take advantage of disturbances in certain mechanisms of mussel immunity that were not covered by this study, such as dysregulation of signalling pathways, humoral responses, AMP expression, etc. Furthermore, pathogens might express other virulence factors to overcome changes in their outer milieu that were overlooked in our study. It has also been suggested that even the normal microflora in marine bivalves may become more virulent, and lethal, due to environmental changes (Pruzzo *et al*., 2005), and that bacteria are masters in monitoring the surroundings, regulating their expression of their genes to adapt to changes in their environment (Skorupski and Taylor, 1997). Bacterial success lies primarily in an ability to effectively invade an organism and then multiply using the resources that the host provides, not necessarily inducing disease or killing the host. Therefore, disease is often a secondary consequence of the enzymes produced as a result of the interaction with the host's immune defence (Mekalanos, 1992).

Although the direct mechanisms that led to the success of the *V. tubiashii* in its interaction with this bivalve host, when exposed to OA, are yet unexplored, our study indicates that OA may favour the bacteria. Our study was carried out at 14°C which is within the temperature range where *V. tubiashii* occur in temperate regions (Temperton *et al*., 2011). The growth rate at this temperature was both in the Cont and OA treatments as high as previously shown for *V. cholera* cultured at 30°C (Wang and Gu, 2005). Rising temperature regimes due to global warming have been noted as a promoter for *Vibrio*-associated diseases (Harvell *et al*., 2002; Baker-Austin *et al*., 2012; Vezzulli *et al*., 2012) and may further increase the advantages of the pathogen.

Concluding remarks

This study has shown that a level of ocean acidification projected to occur in our near future could influence the interaction between *M. edulis* and *V. tubiashii,* though OA alone does not seem to influence either the host immune response or the pathogen virulence respectively. Our results emphasize the necessity of exploring host–pathogen interactions and not only effects on host and pathogen separately when evaluating climate induced ocean acidification as a driving force for infectious diseases. The ability of bacterial pathogens to adapt faster to changing conditions, compared to their hosts, provides them with a potentially significant advantage in this evolutionary arms race when challenged by future climate change. If the rate of OA occurs according to current predictions, we may face a future where opportunistic pathogens may thrive and cause severe threats to their host organisms. The emergence of microbial pathogens that proliferate at faster rates in the marine environment due to higher temperatures but also due to their ability for fast adaptation to OA conditions could have major implications for many calcifying host organisms. The combinative effect of OA and pathogen may cause the tipping point for adult host specimens to be infected by local occurring bacteria that they generally have a well-established immune system against. Especially vulnerable are hosts occurring in high densities, where the bacteria can easily spread, such as among habitat-structuring and reef-building organisms, as well as those in aquaculture where bivalves are important examples. If carbon dioxide induced acidification of oceans have the hereby suggested impact on habitat-building key organisms, serious consequences may be expected for all other species utilizing these habitats, which will in turn also impact human food security.

Experimental procedures

Experiment 1: effects on growth and viability of V. tubiashii

Growth of *V. tubiashii* NCIMB 1337 (ATCC19106, provided by Plymouth Marine Laboratory, UK) were followed at 14°C in a saline (32PSU) peptone culture medium with pH adjusted to

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8.1 and 7.7 respectively, representing ambient pH (control; Cont) and ocean acidification (OA) pH-conditions. Development of the cultures was monitored by optical density (OD_{600nm}) and number CFU ml^{-1} by culturing [room temperature (RT), 24 h] on TCBS-agar (Table S1). pH was monitored during the culturing process.

Survival and viability (CFU ml^{-1}) of *V. tubiashii* and pH were followed daily over one week (14°C) after inoculation into pasteurized seawater (~ 10^6 bacteria ml^{-1}), adjusted to Cont and OA conditions, in culturing flasks (*n* = 3). In the OA treatment seawater was bubbled with CO_2 lowering the pH by 0.4 units and equilibrated to a stable pH prior to addition of the bacteria. Flasks with pasteurized seawater without inoculated *V. tubiashii* showed there were no background bacteria.

Experiment 2: effects on immune and stress responses of M. edulis

Experimental setup. Mytilus edulis (~ 5–7 cm), collected from 0.5–2 m depth in Gullmarsfjord (Swedish west coast) were used in short-term (4 days) and long-term (4 months) experiments. Mussels (*n* = 20) were placed in four exposure tanks (200 l) with flow-through (1 l min^{-1}) of fjord seawater (32 m depth, ~ 33 PSU), thermo regulated to 14°C, providing the mussel with a natural food source and maintaining circulation. In two of the tanks, pH of the seawater was reduced by 0.4 units, via bubbling of CO_2 controlled by pH electrodes connected to a computerized feedback system (Fig. S2, Table S1), regulating gas flow. In the other two tanks, which were bubbled only with air, there were no pH adjustments (Cont). After 4 months of exposure (November 2009–February 2010), another set of mussels (*n* = 8) was added into the duplicate tanks and exposed to exposure conditions (Cont/OA) for four days. Tanks were cleaned weekly from detritus and faeces. During the experiments temperature, salinity and pH were monitored daily. Alkalinity (A_T μmol kg^{-1}) was measured twice a week (for details see supplementary experimental procedures *SExp. proc.*) using an Eppendorf BioPhotometer (Table S1) and analysed according to Sarazin and colleagues (1999). $pH_{total\ scale}$, pCO_2 (μatm) and carbonate chemistry, in terms of calcite and aragonite (crystal forms of $CaCO_3$) saturation, was calculated using CO_2sys software (Pierrot *et al.*, 2006). These measurements secured the maintenance of the experimental treatment conditions (Table 2).

After the exposures haemolymph was withdrawn from the adductor muscle (4 days *n* = 4; 4 months *n* = 18) for microscopic determination of total haemocyte counts (THC μl^{-1}) and their phagocytic capacity (*SExp. proc.*) were analysed in accordance with Oweson and colleagues (2008).The mantel was dissected, instantly frozen and kept at –80°C until used for *Hsp70* analyses, supernatants of tissue homogenates (*SExp. proc.*) were then analysed by using an enzyme-linked immunosorbent assay (ELISA) method previously applied on mantel tissue of scallops (Brun *et al.*, 2008). Absorbance of the samples (4 days and 4 months *n* = 4) was measured in a microplate reader (Table S1) and presented as mean Abs units μg^{-1} protein.

Mussel homeostasis, growth and shell structure. Haemolymph was centrifuged (5000 g, 14°C, 5 min) for determination of pH_{NBS} in cell free haemolymph using a Methrom pH electrode (Table S1) calibrated with NIST buffers (Riebesell *et al.*, 2010). Calcium concentrations of the haemolymphs' were analysed using a flame photometer (Table S1). Shell lengths were registered before and at the end of the 4 months exposure. Shell prismatic microstructures of eight randomly chosen mussels from Cont and OA treatment respectively were analysed with a scanning electron microscopy, SEM (Table S1). Shell pieces (~ 0.5 cm^2), from the area outside where the mantel attaches (Fig. 2C), were glued to aluminium stubs (Table S1) with conductive carbon cement (Table S1) and covered by gold in a JFC-1000 ion sputter (Table S1), and photos were obtained at 2000 × magnification.

Experiment 3: effects on host–pathogen interaction

Experimental setup. A second set of mussels was exposed to the same exposure conditions (Cont/OA) with flow-through (2.5 l min^{-1}) water supplied from 200 l header tanks for 4 months (*n* = 20, October 2010–January 2011) and 4 days (*n* = 8), as described for experiment 2. Mussels from the 4-month exposure were used for infectivity studies *in vivo*, for determining haemolymph calcium concentration and effects on their shell growth and structure (see above). Mussels from the 4-day exposure were used for *in vitro* interactions between mussel haemocytes and *V. tubashii*.

Host–pathogen interaction – in vivo*.* After the 4-month exposure, eight mussels from each tank were injected (into the adductor muscle) with 10^5 *V. tubiashii* g^{-1} ww of mussel and eight mussels with PBS-NaCl (Table S1) maintaining the same Cont/OA conditions. This sub-lethal bacterial exposure level was determined as a non-visual effect dose by testing effects of different doses (i.e. 10^5–10^8 cells g^{-1} ww mussel) on mussel mortality, firmness of the adductor muscle and production of byssus threads prior to the start of the experiment (Table S2). The bacteria were cultured as in experiment 1 until OD_{600nm} of ~ 3 under and the same pH conditions (8.1 or 7.7) as the mussels they were going to be injected into. Half of the injected mussels, four bacteria-injected and four PBS-injected from each tank, were maintained under experimental conditions (with water collected from the header tanks supplying experiment 3) for 6 h, and the remaining mussels were maintained for 24 h, in 2 l enclosed tanks (OA and Cont respectively), on an orbital shaking table. The mussels were shut with rubber bands for 1 h post-injection to secure distribution of bacteria into the haemolymph (St-Jean *et al.*, 2002). After the incubation, aliquots of haemolymph and homogenized hepatopancreas were diluted with PBS (maintaining the same pH) and cultured on TCBS agar plates. Leftover haemolymph from the PBS-injected mussels was instantly frozen (– 80°C) and later used for analysis of calcium levels (see above).

Host–parasite interaction – in vitro*.* The survival of *V. tubashii,* following incubation together with mussel haemocytes, was investigated by *in vitro* exposures in 96-well microplates. *V. tubashii* was cultured as in Experiment 1 until OD_{600nm}~ 2 and then diluted with PBS to 10^7 cells ml^{-1}. Mussel haemocytes, from 4-day Cont/OA-exposed mussels were harvested through centrifugation of haemolymph. The experiment was carried out in a crossed setup; bacteria from

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control conditions were exposed to haemocytes from control mussels and from OA exposed mussels, and those bacteria cultured in OA conditions were likewise exposed to haemocytes of Cont and OA-mussels respectively. A colourometric Cell Proliferation Assay (Table S1), based on the enzymatic reduction of tetrazolium and phenylmethasulphazone to formazan by living cells, was used for analysing the survival (survival index; SI%) of bacteria according to Hernroth (2003).

Virulence properties. Virulence properties were investigated as haemolytic and proteolytic activity of *V. tubiashii* cultures and its supernatants obtained when cultured for the *in vitro* interaction experiment. Cultures, sterile filtered culture supernatants and supernatants mixed with EDTA were plated on horse blood agar and applied into 50 µl punched wells. Haemolytic activity, judged as colourless zones, was observed after 1 and 2 days incubation at RT. Protease activity in cultures, supernatants and supernatants mixed with EDTA were judged by plating on brain heart agar (5% w/v) supplemented with gelatine (1% w/v) according to a protocol by Vermelho and colleagues (1996). After 2-day incubation at RT, the protease activity was detected as colourless zones in the Comassie stained gel.

Data analysis

Differences between *V. tubiashii* growth rate in OA and Cont treatments were analysed with *t*-test (experiment 1). A Mann–Whitney Rank Sum Test was used to test for differences in shell growth (Δshell length, experiment 2). All other categorized data (experiment 2 and 3) were analysed using GLMs of variances with Tank (2 levels) nested within treatment (OA/Cont). Mussel responses to OA/Cont conditions (THC, phagocytotic capacity, Hsp70 and pH) in experiment 2 included short-term and long-term responses (four levels). The crossed set-up of the *in vitro* experiment also generated four levels, and the infectivity experiment included an extra factor time (two levels, experiment 3). Interactions between factors were tested and Student–Newman–Keuls (SNK) was used as a post-hoc test. Adjustment of the significance level for multiple testing was made according to Holm–Bonferroni (Holm 1979). Prior to all analyses, data were tested to meet the assumption of homogeneity of variances (Levene, 1960) and $\log_{10}$ transformed for CFU data, experiment 3.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. (A) Total hemocyte counts μl^{-1} (THC, mean ± SE) in the hemolymph (B) Phagocytic capacity units (mean ± SE) of the hemocytes (C) *Hsp70* units mg^{-1} protein (mean ± SE) from mantle tissue from mussels exposed to OA and Cont conditions for 4 days and 4 month (mo) respectively. Different letters above bars show significantly different means at $P < 0.05$.

Fig. S2. Details on the experimental CO_2 bubbling, controlled by pH electrodes which were connected to a computerized feedback system.

Table S1. Details of buffers, chemicals and consumables as well as instruments used in the experiments.

Table S2. Below visible effect dose test. Visual condition of mussels ($n = 3$) 4 days post-injection of *Vib rio tubiashii* at four different concentrations levels respectively.

Supplementary experimental procedures (*SExp. proc.*).

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