Biogeosciences, 6, 1479–1489, 2009
www.biogeosciences.net/6/1479/2009/

Biogeosciences

The subtle effects of sea water acidification on the amphipod *Gammarus locusta*

C. Hauton, T. Tyrrell, and J. Williams

School of Ocean and Earth Science (SOES), University of Southampton, National Oceanography Centre (NOCS), European Way, Southampton, Hants, SO14 3ZH, UK

Received: 17 November 2008 – Published in Biogeosciences Discuss.: 15 January 2009
Revised: 9 June 2009 – Accepted: 16 June 2009 – Published: 7 August 2009

Abstract. We report an investigation of the effects of increases in pCO_2 on the survival, growth and molecular physiology of the neritic amphipod *Gammarus locusta*, which has a cosmopolitan distribution in estuaries. Amphipods were reared from juvenile to mature adult in laboratory microcosms at three different levels of pH in nominal range 8.1–7.6. Growth rate was estimated from weekly measures of body length. At sexual maturity the amphipods were sacrificed and assayed for changes in the expression of genes coding for a heat shock protein (*hsp70* gene) and the metabolic enzyme glyceraldehyde-3-phosphate dehydrogenase (*gapdh* gene). The data show that the growth and survival of this species is not significantly impacted by a decrease in sea water pH of up to 0.5 units. Quantitative real-time PCR analysis indicated that there was no significant effect of growth in acidified sea water on the sustained expression of the *hsp70* gene. There was a consistent and significant increase in the expression of the *gapdh* gene at a pH of ∼7.5 which, when combined with observations from other workers, suggests that metabolic changes may occur in response to acidification. It is concluded that sensitive assays of tissue physiology and molecular biology should be routinely employed in future studies of the impacts of sea water acidification as subtle effects on the physiology and metabolism of coastal marine species may be overlooked in conventional gross "end-point" studies of organism growth or mortality.

1 Introduction

It is almost universally accepted that the current atmospheric CO_2 concentration of ca. 380 ppmv is set to rise by an estimated 1% y^{-1} over the next decades (Houghton et al., 2001). This rate is approximately 100 times faster than has occurred over the past 650 k years and the rising CO_2 levels are irreversible on human timescales (Royal Society, 2005). Reductions in sea water pH have already been reported as a consequence of an increased level of CO_2 in the atmosphere; for example, long time-series data sets have already recorded a decline in both pH and dissolved carbonate since 1990 (Kleypas et al., 2006). Model predictions of the ΔpH of sea water by 2100 range between -0.4 and -0.5 units (Caldeira and Wickett, 2005).

It has been argued that this decrease in sea water pH will have two consequences for marine organisms (Royal Society, 2005). Firstly it has been predicted that a reduction in sea water pH will produce a reduction in the calcification rate of shelled marine organisms. Indeed, experimental exposure to reduced pH, and the associated changes in sea water carbonate chemistry, has been shown to reduce the calcification of various marine invertebrate species (Kleypas et al., 1999; Gazeau et al., 2007; Hall-Spencer et al., 2008). Secondly changes in the pH of sea water will potentially cause a disruption to the internal acid/base balance in both calcifying and non-calcifying species. The maintenance of internal acid/base balance is essential for maintaining protein conformation and subsequently enzyme function and metabolism. A number of studies have begun to report the effects of reductions in pH on the physiology, growth and life-history of marine species (Kurihara et al., 2004; Berge et al., 2006). Hypercapnia has been shown to cause metabolic suppression in protostome invertebrates (Portner et al., 2004) through disruptions in internal acid/base balance, although it is also clear that differences do occur and some species have a better tolerance of pCO_2-induced acidosis than others (Portner et al., 2004).

Whilst the published literature on the effects of acidification in marine invertebrates is growing rapidly we are still at an early stage in our understanding of the mechanisms involved. For example, earlier studies have sometimes been

Correspondence to: C. Hauton
(ch10@noc.soton.ac.uk)

restricted to adult life stages (Spicer et al., 2007) or used pCO_2 concentrations (e.g. 10 000 ppm) that far exceed future predicted environmental levels (Kurihara et al., 2004). Other researchers have designed experiments which have utilized inorganic acids to reduce pH rather than CO_2, a protocol which has been shown to underestimate toxic effect (Kikkawa et al., 2004). Extrapolation from these studies to draw substantive conclusions on the widespread future impacts of sea water acidification remains difficult, especially in coastal and estuarine species which may have evolved to tolerate episodic extreme low pH events (Attrill et al., 1999; but see also Ringwood and Keppler, 2002). Estuarine environments are known to be areas where pH varies over a large range. Al-Rasheid and Sleigh (1995) reported values in the range of 6.76–7.95 for Southampton Water (the site of this study) while Attrill et al. (1999) recorded transient pH values ranging between 6.7–8.9 for the Thames Estuary (UK) and Ringwood and Keppler (2002) have reported mean pHs in the range 7.2–7.8 (minimal pHs ranging from 6.9 to 7.6) for the Charleston Harbour Estuary (USA).

Herein we describe a study of the effects of exposure to acidic sea waters on juveniles through to adults of the neritic amphipod *Gammarus locusta*. This species is a ubiquitous member of the intertidal and subtidal marine communities in coastal and estuarine environments, habitats which are typified by a variable pH regime. As with many crustaceans, gammarid amphipods mineralize their chitinous cuticle by depositing calcium carbonate (Wheatly, 1999) and low environmental calcium concentrations have been shown to limit the distribution of a number of gammarid species (Wright, 1979; Rukke, 2002; Zehmer et al., 2002). In addition this species, as with all other crustaceans, has a requirement to regulate internal acid/base balance to maintain an efficient metabolism.

Based on the mensurative observations of this species in estuarine environments we hypothesized that this species would be tolerant of sustained decreases in sea water pH of at least 0.5 pH units. Studies on the mortality, growth and development were combined with an investigation of changes in the molecular physiology using quantitative real-time PCR. The expression of the genes coding for the 70 kDa heat shock protein and the metabolic enzyme glyceraldehyde-3-phosphate dehydrogenase were monitored. The 70 kDA heat shock protein is a molecular chaperone and is responsible for stabilizing or refolding proteins that are denatured by a variety of environmental stressors (e.g. pollution, hypoxia, temperature (Dalhoff, 2004)). Expression of this protein is induced as part of the general stress response of the organism and has been shown to protect the organism from subsequent exposure to even more extreme events (Daugaard et al., 2007). Glyceraldehyde-3-phosphate dehydrogenase is an essential glycolytic enzyme that catalyses the reversible oxidation and phosphorylation of D-glyceraldehyde-3-phosphate to D-glycerate 1,3-bisphosphate. An increase in the expression of the either of these genes, relative to control amphipods, would indicate that prolonged (generational) exposure to low pH may produce sub-lethal physiological impacts that lead to changes in overall lifetime fitness. To our knowledge this is the first published application of sensitive molecular techniques to probe the effects of ocean acidification on marine invertebrates through the detection of sub-lethal impacts.

2 Material and methods

2.1 Design of high-CO_2 microcosm systems

CO_2 microcosms were based on previously described systems (Widdicombe and Needham, 2007; Dupont et al., 2008) which regulate the delivery of 100% carbon dioxide gas to achieve a desired pH set point. Briefly, each microcosm consisted of a "mixing tank" and separate "incubation tank" that were maintained at 20°C using bench-top water baths. Sea water was continuously circulated between the 10-litre "mixing tank" and the two-litre "incubation tank" using peristaltic pumps (flow rate $1.5\,l\,h^{-1}$). On alternate days the incubation chambers were isolated, by switching off the peristaltic pumps, and 100% of the water was exchanged in each mixing tank. The pH and temperature in each mixing tank was allowed to equilibrate before the recirculating system was switched back on. In this way the accumulation of nitrogenous excretion was minimized without disturbing the amphipods. Microcosms were set up under a 12:12 h L:D regime. The sea water used for this experiment was sourced from Southampton Water and had been stored in a large-capacity recirculating research aquarium prior to its use in this study.

Three separate microcosms were set up and adjusted to different nominal pH regimes: pH 8.1 (the control), pH 7.8 (~550ppm pCO_2) and pH 7.6 (~980 ppm pCO_2). The most extreme Δ pH used in this study approximated the modelled decrease in surface ocean pH by 2100 under the IPCC A2 SRES scenario of CO_2 emissions (Caldeira and Wickett, 2005). pH was controlled using AquaMedic (Bissendorf, Germany) pH Computers which measured the pH in the mixing tanks via an electrode. When the measured pH rose above the setpoint the computer opened a gas solenoid valve to deliver 100% CO_2 directly into a 10-litre mixing tank. The water in each mixing tank was also vigorously aerated to ensure that it did not become undersaturated with oxygen. During each water change on alternate days the pH electrodes were recalibrated using pH buffers according to the manufacturer's instructions; the drift in each pH meter was never more than 0.02 units.

Temperature and salinity were measured in the mixing chambers on every day of the experiment using an independently calibrated LF323 combined conductivity meter (WTW, Weilheim, Germany)and pH was measured in the incubation chambers during the water changes using a

three-decimal-place SevenMulti pH meter (Mettler, Schwerzenbach, Switzerland). In addition water samples were routinely collected and fixed using mercuric chloride according to established protocols (Dickson et al., 2007). These samples were used to characterize the carbonate chemistry in each incubation (dissolved inorganic carbon (DIC) and total alkalinity), using a VINDTA 3C instrument (Marianda, Kiel, Germany).

Measurements of water nutrient concentrations were recorded at the end of this study. It was noted that both phosphate (263 μmol/kg) and nitrate levels (19.4 mmol/kg) in the water were extremely high, deviating from typical values of coastal waters.

2.2 Broodstock collection and maintenance

Adult specimens of *Gammarus locusta* were identified according to Lincoln (1979) and collected from the epifaunal community found on permanent moorings in Southampton Water in the UK (50° 52.2′ N 01° 23.0′ W). They were returned to the laboratory and maintained at 20°C in two 10-l plastic aquaria, being fed thrice weekly using dried oats. The broodstock tanks were also stocked with *Ulva lactuca* and this was also consumed by the amphipods. 90% water exchanges of the broodstock tanks were carried out on alternate days by siphoning water from the tanks through a 63 μm mesh and replacing it with temperature-equilibrated fresh aquarium water. Newly released juvenile amphipods, of mixed sex, were collected from the broodstock tank and used to populate the three experimental incubation chambers as required (see Sect. 2.3).

2.3 Effects of elevated CO_2 on amphipod survival and growth

For each experiment the three incubation tanks were stocked at equal density (range=20–30 individuals per incubation chamber) with cohorts of newly-released juveniles from the broodstock tank (see Sect. 2.2). Juvenile ampihpods were fed *ad libitum* with dried oats during the experiment. Each incubation tank was also stocked with fronds of the macroalgae *Ulva* spp. This macroalgae increased habitat complexity and reduced aggressive interactions between the amphipods and also provided an additional food source.

Four separate cohorts of amphipods were sequentially reared in the incubation tanks. Two were reared to sexual maturity (a 28-day experiment) and two cohorts were reared to adolescence (14 days). Amphipod growth was expressed as an increase in ash-free dry weight (AFDW) with time. At regular intervals during each experiment all animals were removed from each chamber, blotted dry with tissue, and quickly photographed using a Leica MZ8 dissecting microscope fitted with a Nikon CoolPix 990 digital camera. Total length (from the joint of the head with the peduncle of antenna 1 to the tip of the longest uropod) of each amphipod was measured in each image using SigmaScan Image Measurement Software. Total lengths were converted to ash-free dry weight using a relationship determined from an initial group of 20 amphipods that were measured, dried at 60°C to constant weight and then ashed at 550°C.

Percent survival in each incubation tank for each cohort was determined during body length measurement.

2.4 Molecular analyses of changes in gene expression

2.4.1 Isolation of *Gamnmarus locusta*-specific gene sequences

Gene sequences for *G. locusta* glyceraldehyde-3-phosphate dehydrogenase (*gapdh* gene) and the inducible 70 kDa heat shock protein (*hsp70* gene) were first determined. Gene fragments were isolated using degenerate primers designed against conserved amino acid sequences identified from CLUSTAL (Higgins and Sharp, 1988) alignments of published sequences. Each alignment was examined and any mismatches were corrected manually.

For *gapdh*, degenerate primers (sense 5′-CNG AYG CNC CAN TGT TYG T-3′, antisense 5′-ACR TCR TCY TCN GTR TAN CC-3′, Tm=51 °C) were designed against an amino acid alignment of 9 crustacean sequences. For *hsp70* an amino acid alignment of 16 crustacean heat shock sequences was used to identify conserved regions from which two primers were designed (sense 5′-GCN CAN AAR GAY GCN GG-3′, antisense 5′-ARD ATN CCR TTN GCR TCD AT-3′, Tm=51°C). Primers were produced by Eurofins MWG Operon (Ebersberg, Germany).

Degenerate primers were used in conventional PCR reactions to isolate fragments of each gene. PCR reactions were performed using 0.5 μl *Taq* DNA polymerase (5 Uμl^{-1}; Qiagen, Sussex, UK) in 25 μl reactions according to the manufacturer's protocol and containing 2 μM final concentration of each of the sense and antisense degenerate primers and 1 μl cDNA prepared from whole amphipods as described below (Sect. 2.4.3). The sequence for the *hsp70* gene was isolated from total RNA that had been extracted from a heat shocked amphipod (3 h at 30°C followed by 30 min at 20°C). The PCR protocol was 1 cycle of 95°C for 5 min, 30 cycles of 95°C for 30 s, annealing temperature for 30 s and 72°C for 1 min with a final extension of 72°C for 7 min. PCR products were gel-purified and positive gene fragments were identified based on predicted amplicon size and extracted using the Qiaquick gel extraction kit (Qiagen, Crawley, Sussex, UK).

Extracted PCR products were cloned using an Invitrogen TOPO TA cloning kit (pCR 2.1-TOPO vector and TOP 10F *E. coli*; Invitrogen, Glasgow, UK). Plasmid DNA was isolated from bacterial colonies that had an appropriately sized insert using the Qiaprep spin miniprep kit (Qiagen). A minimum of 3 separate colonies were picked at random and were sequenced using vector-specific (M13) primers by Geneservice Ltd (University of Oxford, Oxford, UK). The nucleotide

sequences were used to deduce amino acid sequences for each gene fragment and these were compared against the EMBL and SWISSPROT databases using a BLAST sequence similarity search (Altschul et al., 1997).

2.4.2 Rapid amplification of 5′ and 3′ cDNA ends (RACE)

5′ and 3′ cDNA sequences were generated using a SMART RACE amplification kit (Clontech, Saint-Germain-en-Laye, France). The nucleotide sequence of each gene fragment (Sect. 2.4.1) was used to design gene-specific 5′ and 3′ RACE and nested RACE primers for each of the two genes. RACE-ready 5′ and 3′ cDNAs were first generated using the kit reagents and total RNA (primer sequences and reaction conditions available on request). Separate RACE PCR reactions were performed for each cDNA end for each gene using the universal primer (UPM) and the gene-specific RACE primers. Similarly, nested RACE PCR reactions were performed for each end of each gene by diluting the primary RACE reactions and using them as a template with the nested universal primer (NUP) and the nested gene-specific primers. Both RACE and nested RACE PCR reaction conditions were as follows: 1 cycle of 94°C for 7 min, 40 cycles of 94°C for 30 s, 68°C for 30 s and 72°C for 3 min with a final extension at 72°C for 7 min. The 5′ and 3′ nested RACE PCR products were gel-purified, cloned and sequenced as described above. For each gene the separate fragments were assembled and used to deduce complete amino acid sequences which were then compared to published sequences. Both sequences have been published on the EMBL database (Sect. 3.3).

2.4.3 Manipulation of *G. locusta* and RNA extraction and production of cDNA

An initial experiment was conducted to confirm the function of the product of the putatively identified *hsp70* gene. Ten adult amphipods were taken from the broodstock and were exposed to a temperature of 30°C for 3 h before being allowed to recover for 30 min at a temperature of 20°C. None of the amphipods died during the course of the heat shock treatment. *Hsp70* expression was compared to ten amphipods which were held at the broodstock temperature of 20°C for the same time. At the end of this heat-shock experiment all amphipods were immediately homogenized in 1 ml of TRI reagent (Sigma-Aldrich, Dorset, UK).

At the end of each cohort of the experiment (two 14-day exposures and two 28-day exposures) all remaining amphipods were measured as described above and then immediately homogenized in 1 ml TRI reagent. Total RNA was extracted from each amphipod using TRI reagent according to the manufacturer's protocol, quantified by measuring the absorbance at 260 nm and stored at −80°C. Two μg of each RNA sample was DNase-treated (Sigma Aldrich) and the sample RNA concentration was then re-quantified using Ribogreen reagent (Invitrogen, Strathclyde, UK) according to the manufacturer's instructions. 300 ng of DNase-treated total RNA was reverse transcribed using Invitrogen SuperScript II reverse transcriptase primed using random nonamers.

2.4.4 Quantitative real-time PCR analysis of gene expression

Quantification of mRNA transcript abundance required a pair of primers to be designed for each gene. Primer pairs were designed against the complete nucleotide sequences using the software package Primer Express (Applied Biosystems, Cheshire, UK). Each primer pair was designed using the default parameters available using Primer Express. The sequences of the primers are summarised in Table 1 which includes details of the optimised primer conditions and assay validation.

Real-time PCR was conducted in 25 μl reactions containing 12.5 μl of Precision Mastermix with SYBR green (PrimerDesign, Hants, UK), 2 μl of each primer (concentrations optimised for each gene as shown in Table 1), 2 μl of sample cDNA and 6.5 μl sterile ultrapure water. Each sample was run in duplicate and "no template controls" (NTCs) were included for every primer pair in each run. Real time PCR reactions were run on a Corbett Rotorgene 3000 (Corbett Life Science, New South Wales, Australia) using the following standard reaction conditions: 1 cycle of 95°C for 10 min and 40 cycles of 95°C for 10s, 60°C for 1 min. The specificity of each reaction was determined by running a melt analysis at the end of each PCR cycle (Bustin, 2004). PCR products were also confirmed by agarose gel-purification of an aliquot of each real-time PCR reaction. Examination of these gels indicated that each reaction produced a single product of the correct size with no non-specific amplification (data not shown).

The number of copies of each gene was determined using an absolute quantification approach (Bustin et al., 2000). The Ct value of each gene in every sample was compared to a standard curve that was produced from a serial dilution of the respective pCR 2.1-TOPO plasmid according to an established protocol (Roche Molecular Biochemicals, 2003). For each gene an extracted plasmid that contained the target amplicon of each primer pair was identified. These plasmids were linearised using the restriction enzyme *Not* I (10U μl^{-1}, Sigma-Aldrich), which cut the pCR 2.1-TOPO vector but not the cloned nucleotide sequences. Once the plasmid had been cut, the DNA concentration was determined using PicoGreen dsDNA Quantitation reagent (Invitrogen). The concentration of each linearised plasmid was used to calculate the number of copies of that plasmid extracted from each clone using the relation described in Borg et al. (2003).

Table 1. Summary of real-time PCR primers and validated optimal reaction conditions.

Gene assay	Primer ID	Sequence (5′-3′)	Assay concentration (nM)	Amplicon size (nt)	Reaction efficiency	Regression relation	R^2
hsp70	*hsp70* rt F2	CAA AGA TGG ACA AGG GAC AGA TG	300	96	1.06	CT=(−3.188 x log dilution) + 9.521	0.998
	hsp70 rt R2	AGC TTC TGC ACC TTT GGA ATG	300				
gapdh	*gapdh* rt F1	AAC GCC TCC TGC ACC ACT AA	50	91	0.91	CT = (- 3.572 x log dilution) + 13.891	0.997
	gapdh rt F2	CCT CCT CAA TGC CGA AGC T	50				

2.5 Statistical analyses of data

Mortalities of *Gammarus locusta* juveniles during development were translated into survival curves and were analysed using Kaplan-Meier survivorship analysis (Lee, 1992). This analysis was performed using MedCalc software.

Total length measurements of amphipods were converted to ash-free dry weight. Data were $\log_{10}$-transformed to achieve normality and were then compared using linear regression analysis. The growth rate of amphipods (increase in AFDW with time) for each pH was compared within each cohort using analysis of covariance (ANCOVA) to compare the difference in slope of the regression lines (Sokal and Rohlf, 1995).

Gene expression data were first $\log_{10}$-transformed to meet normality and homoscedacity of variance criteria. Differences in the number of copies of each gene at each pH were assessed using nested analysis of variance (ANOVA) (Underwood, 1997). The first factor, pH (pH), had three levels representing the three different nominal set points used in this study. A second random factor, time (Ti), was nested within pH and had two levels to represent the different durations of the incubations (14- and 28-day). A third factor, batch (Ba), which was nested within both pH and Ti accounted for variation in gene expression within the two replicate batches run for each of the two incubation durations. The linear model for this analysis is given by the relation:

$$X = \text{MEAN} + \text{pH} + \text{Ti(pH)} + \text{Ba(pHxTi)} + \text{RES}$$

Nested ANOVAs were conducted using the software package GMAV5 (Underwood, 1997). Statistical significance was accepted at $P < 0.05$ for all tests.

3 Results

3.1 CO_2 microcosm performance

The temperature, salinity and pH measured within the incubation chambers during the experiments are summarised in Fig. 1A. The mean (±SD) temperatures in the control, nominal pH 7.8 and nominal pH 7.6 incubations were 20.2±0.9°C, 20.0±0.3°C and 20.1±0.4°C, respectively. Mean salinities in the three incubations were 33.9±0.89, 33.7±0.8 and 34.1±0.8, respectively. There were no significant differences (ANOVA) in the temperature or salinity of any of the three incubations over the course of the study. Mean pH in each condition was measured as 8.08 ± 0.06 (control), 7.83±0.04 (nominal set point pH 7.8) and 7.47±0.04 (nominal set point pH 7.6), respectively. As expected there was a very highly significant difference in the pH of the three different incubation tanks during the study. DIC increased from a mean of 2273.78±101.00 μg kg^{-1} in the control incubation to 2599.39±145.46 μg kg^{-1} in the nominal pH 7.6 incubation whilst total alkalinity fluctuated about a mean of 2869.45±110.94 μg kg^{-1} across all three incubations (see Fig. 1B). There was a significant increase in the mean DIC of the nominal pH 7.6 incubation when compared to the control incubation.

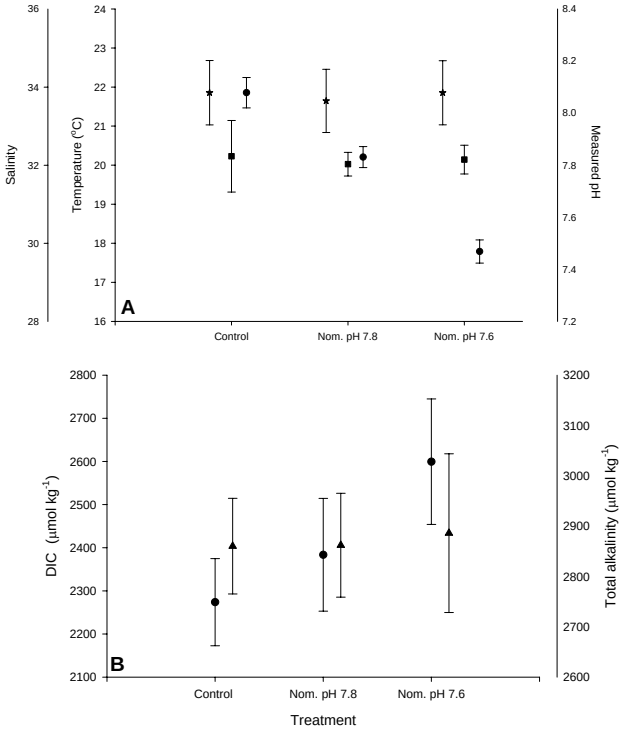

Fig. 1. Environmental parameters for each incubation showing the mean ±SD in each case. **(A)** temperature (squares), salinity (stars) and pH (circles) and **(B)** dissolved inorganic carbon (circles) and total alkalinity (triangles).

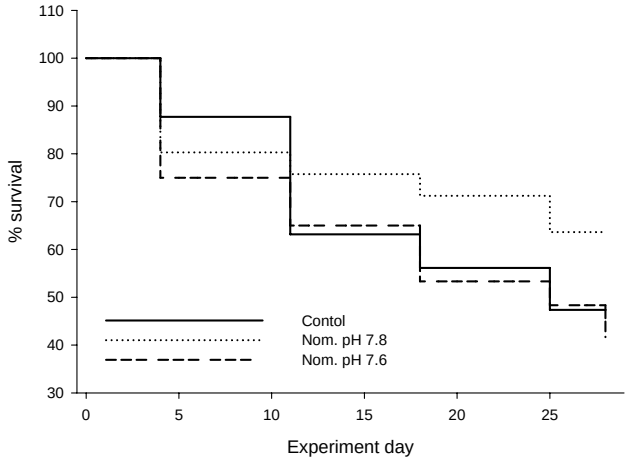

Fig. 2. Comparisons of amphipod survival at different incubation pH showing a significant improvement in survival rate of amphipods maintained at a nominal pH 7.8.

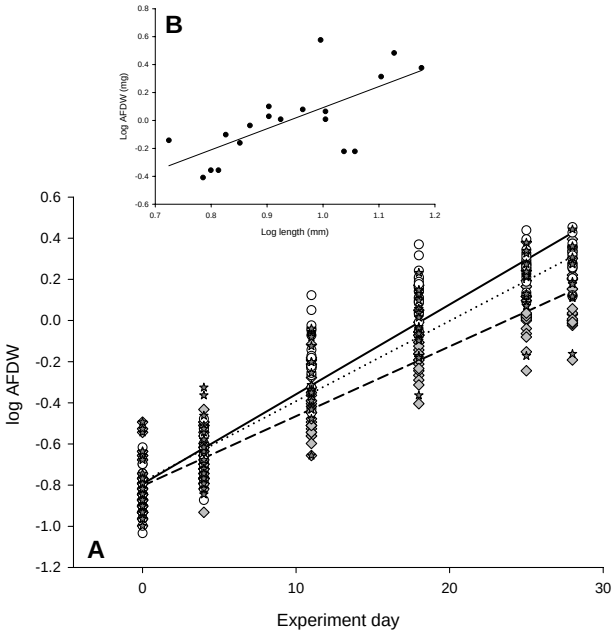

Fig. 3. (A) comparison of growth rate of amphipods grown at different pH. Grey diamonds and the dashed line represent control amphipods, circles and the solid line represent amphipods grown at nominally pH 7.8 and grey stars and dotted line represent pH 7.6. ANCOVA indicated that the slopes of the three regression lines were not significantly different. In the other three experimental cohorts the growth rates were more similar than shown here. **(B)** Length v AFDW relation for *G. locusta*; log AFDW=(1.5104 x log Length)-1.4817.

3.2 Amphipod survival and growth

Kaplan Meier analysis indicated that whilst there was no significant difference in the survival during the first 14 days of incubation (suppl. data available http://www.biogeosciences.net/6/1479/2009/bg-6-1479-2009-supplement.pdf) there was a significant improvement in the survival of amphipods held at nominally pH 7.8 after 28 days growth (compared to the control and pH 7.6 amphipods; Fig. 2).

There was no significant difference (ANCOVA) in the growth rate of amphipods during the first 14 days or over the full 28 days it took to reach sexual maturity (identified as pre-copulatory mating pairs seen in all incubation chambers). As an example, the data from the second cohort of the 28-day experiment are presented in Fig. 3A which show the most divergent growth rates (regression slopes) that were recorded; these slopes were not statistically different.

3.3 Changes in gene expression of CO_2-exposed amphipods

The genes coding for a 70 kDa heat shock protein (*hsp70* gene; FM165078) and glyceraldehyde-3-phosphate dehydrogenase (*gapdh* gene; FM165079) were identified in *Gammarus locusta*. The *hsp70* gene was 2522 nucleotides in length which coded for a 642 amino acid protein with a predicted molecular weight of 70.14 kDa and a theoretical isoelectric point of 5.37. The 5′ UTR was comprised of 249 nucleotides and the 3′ UTR consisted of 350 nucleotides. The predicted protein had an 80.9% identity with an *hsp70* gene isolated from the deep sea vent shrimp *Mirocaris fortunata* (A1XQQ5, Ravaux et al., 2007). PROSITE analysis (Sigrist et al., 2002) confirmed the presence of three *hsp70* signature domains in the predicted sequence: ile 10 – ser 17, ile 198 – leu 211, and ile 335 – lys 349. Furthermore, the absence of Karlin and Brocchieri's (1998) "domain 13" indicated that the protein coded by this gene was eukaryotic in origin and present in the cytoplasmic compartment of the cell.

The *gapdh* gene was 1264 nucleotides in length coding for a 334 amino acid protein which had an 86.0% identity to the *gapdh* gene from *Procambarus clarkii* (Q7YT60). PROSITE analysis confirmed the presence of a glyceraldehyde 3-phosphate dehydrogenase active site between ala 147 – leu 154. The gene started with a short 5′ UTR of 79 nucleotides and the 179-nucleotide 3′ UTR terminated with a polydenylated region.

To confirm the *hsp70* gene isolated from *G. locusta* encoded for a stress-inducible heat shock protein, rather than a constitutively-expressed heat shock cognate, a preliminary heat-shock experiment was conducted. The expressions of the *hsp70* and *gapdh* genes were compared between amphipods that had been incubated for three hours at 30°C and controls. There was a very highly significant 2000-fold increase in the expression of *hsp70* (Fig. 4) after treatment but no change in the expression of the *gapdh* gene.

Nested analysis of variance revealed that there was no significant effect of pH treatment on the expression of the *hsp70*

Table 2. Summary of nested ANOVA comparing pH, time (Ti, nested in pH) and batch (Ba, nested in pH and Time) for the *gapdh* gene expression data. Analysis conducted using GMAV5 (Underwood, 1997).

Source of variation	Sum of squares	Degrees of freedom	Mean square	F ratio	*P*
pH	3.149	2	1.5745	21.2	0.017*
Ti(pH)	0.2228	3	0.0743	0.27	0.8425
Ba(pHxTi)	1.6277	6	0.2713	4.06	0.0013*
Residual	5.6152	84	0.0668		
Total	10.6146	95			

Model for analysis: X=MEAN + pH + Ti(pH) + Ba(pHxTi) + Residual

over the whole experiment (Fig. 5). Further, there was no significant effect of the nested factors Time or Batch on the expression of this gene. Nested ANOVA demonstrated a small but significant and reproducible effect of incubation pH on the expression of the *gapdh* gene (Fig. 5, Table 2). *Post hoc* SNK analysis indicated that the expression of the *gapdh* gene was significantly higher in amphipods reared at nominally pH 7.6 than in those reared at pH 7.8 and 8.1. There was a mean 2.79-fold increase in the expression of the *gapdh* gene between the amphipods incubated at nominally pH 7.6 and the controls. There was no significant effect of incubation time on this relationship although there were significant but inconsistent batch effects in the expression of the *gapdh* gene (Table 2).

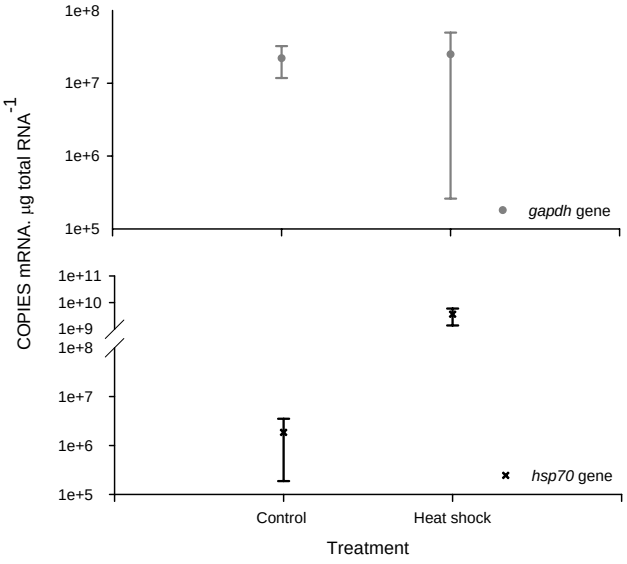

Fig. 4. Comparison of *hsp70* and *gapdh* gene expression in *G. locusta* recovering from exposure to heat shock, showing a very highly significant increase in the expression of the *hsp70* gene (black cross) in exposed amphipods but no change in expression of the *gapdh* gene (grey circles) (means ±SD, n=10).

4 Discussion

This study had two specific intentions. The main purpose of this study was to examine the potential resilience of eurytopic estuarine species to the impact of a reduction in sea water pH arising from the increases in dissolved CO_2 predicted under the IPCC SRES A2 scenario. A second goal of this research was to make use of sensitive molecular techniques to identify sub-lethal effects that might be undetected in whole organism "end-point" studies (e.g. individual mortality or growth). To that end, this study investigated the effects of CO_2-mediated changes in the future mean pH on the molecular physiology, growth and survival of the ubiquitous neritic marine amphipod *Gammarus locusta*.

As discussed, there are considerable difficulties in extrapolating from global predictions of future pH scenarios (Caldeira and Wickett, 2005) to the effects on marine species at local and regional scales. This is particularly the case in coastal and estuarine environments which are known to be areas where pH varies over a large range. Species in these environments would be expected to be eurytopic in nature and might be predicted to be physiologically or behaviourally resistant to CO_2-driven increases in sea water acidity. In support of this idea Spicer et al. (2007) have recently shown that the velvet swimming crab *Necora puber*, a coastal marine species, can effectively regulate its haemolymph pH in environments where the external pH is held at 6.74 for at least 16 days. The earlier work of Kroon (2005) has also shown that juvenile shrimp (*Metapenaeus macleayi*) demonstrate aversion behaviours to avoid waters of low pH.

Gammarus locusta occurs widely on all marine shores in north-west Europe and is often abundant from the middle shore to the sub-littoral associated with algae and under stones (Hayward and Ryland, 1995). *Gammarus locusta* has also been recorded in the north-eastern United States (Global

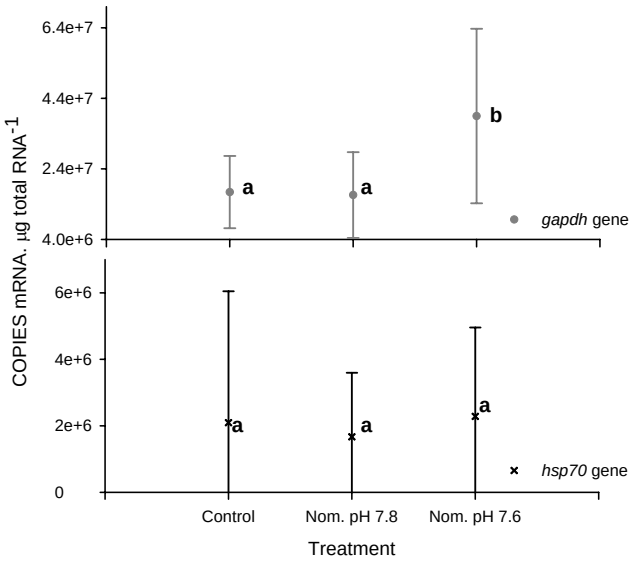

Fig. 5. Changes in *hsp70* and *gapdh* gene expression in *G. locusta* maintained at different pH during the experiment. Statistical analysis of both data sets revealed no significant "time effect" on the expression of either gene (Table 2 and Suppl. Table 8 http://www.biogeosciences.net/6/1479/2009/bg-6-1479-2009-supplement.pdf) and so presentation of the data has been simplified by pooling the results of the 14 day and 28 day experiments. Lower case letters denote statistical differences within each gene data set (*post hoc*. SNK test). Data presented as the mean ±SD (n=32). Raw data are provided as supplementary data (Suppl. Table 7). A summary of the *hsp70* nested ANOVA has been provided (Suppl. Table 8).

Biodiversity Information Facility; http://us.mirror.gbif.org). In light of the coastal habitats exploited by this species and the earlier data from other neritic species it was hypothesized that *G. locusta* would have evolved physiological or behavioural mechanisms to succeed within this variable environment. However, what was not certain from the published literature was whether or not the physiological or behavioural abilities of this or any coastal species could meet the physiological challenges imposed by generational exposure to chronic low pH or exposure to low pH within a restricted environment where behavioural avoidance was not possible.

Mortality during growth was recorded in all incubations including the control. Mortality of growing juveniles most often occurs during the moult, which is a physiologically stressful period in the life history of crustaceans (Factor, 1995; Charmantier-Daures and Vernet, 2004). In this study there was no significant difference in the percent mortality recorded during the first 14 days of growth. However, over the 28 days it took to reach sexual maturity there was an improvement in the survival in the pH 7.8 incubation compared to the other two incubations which might suggest that, when measured in terms of mortality during development, *Gammarus locusta* might have quite particular requirements for a limited pH regime. In retrospect, the choice of a pH set point of 8.1 for the control incubation in this study was not representative of the environment from which the organisms were collected. In light of this, it is clear that any experimental manipulations of pH should take in to account the sea water pH experienced by organisms in their natural environment. Assuming that these amphipods are "adapted" to an environmental pH that fluctuates below pH 7.95 (Al-Rasheid and Sleigh, 1995), the significant effects on metabolic gene expression (see below) at a measured pH of 7.47 (a decrease of ∼0.5 units) become noteworthy.

In addition to influences on survival, the frequency of moulting also determines the overall growth rate of juvenile crustaceans (Charmantier-Daures and Vernet, 2004). There was no significant difference in the growth rate of amphipods across the entire experiment suggesting that there was no detrimental impact of low pH exposure on the moult cycle of the amphipods. It is concluded that the moulting process and subsequent growth of these juvenile amphipods were unaffected by a sustained decrease of at least 0.5 pH units. These data validate the key hypothesis that *G. locusta* is a robust species that will be able to tolerate the current predictions of future changes in sea water pH as a function of changes in atmospheric CO_2 (Caldeira and Wickett, 2005).

As well as assessments of organism growth and survival we also investigated changes in the molecular physiology of *G. locusta* incubated under different pH regimes. For this first study the genes coding for the enzyme glyceraldehyde-3-phosphate dehydrogenase and the 70 kDa heat shock protein were sequenced. Whilst the existing literature on the various heat shock proteins and heat shock cognates is comprehensive, the identity and definitive role of some of the genes and peptides that have been sequenced remains uncertain (see Sanders, 1993; Karlin and Brocchieri, 1998 and Daugaard et al., 2007 for reviews). For example some authors have regarded the heat shock cognates as constitutively expressed peptides which maintain the proper folding of nascent proteins during their synthesis and translocation to different cellular compartments (Sanders, 1993; Liu et al., 2004) whilst other authors have reported 70 kDa heat shock cognates that are inducible and respond to stress (Lo et al., 2004; Wu et al., 2008). Analysis of the predicted amino acid translation of the *hsp70* gene suggested that it did code for a heat shock protein. As described in Sect. 3.3, it contained the three signature domains which characterize *hsp70* genes and had a high (up to ca. 81%) identity to other published *hsp70* gene sequences from diverse phyla. However, the problem of definitive identification of function based on sequence similarity alone has been raised before. Leignel et al. (2007) have recently argued that the "best criteria to use to distinguish between *hsc70* and *hsp70* still seem to be gene expression studies." As a consequence an initial heat shock experiment was conducted to investigate the induced expression of this gene following stress. Exposure to a +10°C heat-shock for

3 h produced a very significant increase in the expression of *Gammarus locusta hsp70* which indicates that the gene isolated in this species does indeed code for an inducible heat shock protein.

There was no significant increase in the expression of the *hsp70* gene after 14 and 28 days growth at reduced pH (to a minimum of pH 7.47). From these data it can be concluded that prolonged exposure to low pH in this experiment did not induce a sustained acid/base imbalance which denatured cytoplasmic proteins to an extent that the induction of heat shock proteins was required to refold them. This suggests that the physiological buffering in the tissues of *G. locusta* was sufficient to cope with this pH decrease over a prolonged period (juvenile to adult). The HSP70 protein is just one of a number of cellular chaperones that have been shown to respond to various stressors in a variety or organisms (see review by Sanders, 1993) and it is important to clarify that, whilst no long-term up-regulation of this gene was recorded in this current study other cellular chaperones, for example members of the HSP90, HSP60 or small molecular weight heat shock proteins, may have been up-regulated at these times. Furthermore, the time course of expression was not followed in this initial study and it is possible that there may have been a transient increase (e.g. over the first few hours) in expression of the *hsp70* gene as part of an initial shock response to low pH that then diminished as the amphipod physiology acclimated to the new environment. Nevertheless, the challenges of extrapolating from an ephemeral "shock response" imposed by an immediate reduction in pH to the more "realistic" scenario of a gradual reduction in pH over a longer time span are not trivial. As such, selecting model species with short-generation times, such as amphipods, may allow the potential for "adaptation with time" to be investigated and this is an area of research that certainly warrants additional work. Species which can successfully adapt to gradual changes in sea water pH will undoubtedly have an ecological advantage in comparison to those with long generation times or with only a stenotopic physiology. It is clear that there is scope for future more in-depth studies, making use of whole transcriptome approaches such as suppressive subtractive hybridisation (Pan et al., 2005), of the complete suite of molecular chaperones involved in the initial shock response to low pH and the subsequent extended period of physiological acclimation.

Maintenance of acid/base status in marine invertebrates does not occur spontaneously and is an energy requiring process (Portner et al., 2004). Published data (Wood et al., 2008) have already shown that exposure to decreases in environmental pH causes a disturbance to the metabolism of marine invertebrates. Wood et al. (2008) have demonstrated an increased metabolic rate in the ophiuroid, *Amphiura filiformis,* in response to exposure to low pH waters. This was recorded as a significant increase in the rate of oxygen uptake in the pH range 7.7–6.8. However, the authors did report that this physiological response may have compromised organism survival and overall fitness. Data from the current study provides some preliminary molecular data which also lends support to this idea; at a mean pH of 7.47 we found a significant and consistent increase in the expression of the *gapdh* gene in *Gammarus locusta*. Whilst these two studies suggest sub-lethal physiological changes occur in marine invertebrates in response to prolonged exposure to reduced pH it is clear, however, that any hypothesis requires more exhaustive evaluation. Caution should be exercised when interpreting the current data as any one of the many enzymes involved in glycolysis or the TCA might exert a greater limitation on overall organism metabolism than glyceraldehyde-3-phosphate dehydrogenase. It is well known, for example, that the overall basal aerobic metabolic rate of any organism is a function of multiple enzyme reactions, each with their own rate constants and feedback interactions (Alberts et al. 2008). The future validation of this idea will require the study of multiple additional genes encoding the many other enzymes within the glycolytic or TCA pathways, such as pyruvate decarboxylase and citrate synthase.

It is important to stress that in the current experiments the *G. locusta* were fed *ad libitum;* no attempt was made to control or quantify the amphipods' energy intake. Further work in this laboratory is assessing the physiological response of amphipods in a food limited environment; a situation in which negative impacts on organism physiology might be more apparent. This research will investigate changes in organism scope for growth as well energy partitioning in response to demands placed on acid/base homeostasis.

Sub-lethal and lethal effects of exposure to acid pH environments have been recorded at other critical stages of development. At extreme pH values (pH 6.2) Kikkawa et al. 2004) demonstrated that there was 85.8% mortality of eggs in red sea bream (*Pagrus major*) whilst Kurihara et al. (2004) have shown that at pH 6.8 the production of eggs was reduced in the copepod *Acartia steueri* and fertilization success was decreased in the sea urchins *Menicentrotus pulcherrimus* and *Echinometra mathaei*. It is clear from these earlier studies that all life stages need to be studied to understand the full potential toxic effect of exposure to reduced pH brought about by increased pCO_2. However, this present study has demonstrated that by utilizing sensitive molecular approaches the effects of exposure to more acidic sea water can be recorded at much less extreme pH values than have been used previously.

5 Conclusions

In conclusion, this study has presented data which support the hypothesis that the ubiquitous coastal amphipod *Gammarus locusta* is a robust species that will not suffer a reduction in juvenile growth rates or an increased rate of mortality as a result of the current predictions for future acid sea water scenarios. The acid/base homeostatic ability of

this species is sufficient to cope with such changes without apparently inducing "classic" stress responses, evidenced in this study as the expression of the gene coding for the 70kDa heat shock protein. However, there is a significant and sustained change in the expression of the *gapdh* gene in this species that, in combination with the data from earlier studies of other research groups, suggest subtle changes in organism physiology do take place.

As argued by others, such indirect effects, which are not necessarily immediately apparent in short-term "end-point" studies, may impact the ecological fitness of a population and its long-term persistence within an ecosystem. Clearly, the use of sensitive molecular and physiological approaches to identify such indirect and sub-lethal responses represents a powerful technique with which to realize the full spectrum of possible impacts of future increases in sea water acidity.

Acknowledgements. This research was funded by the UK Natural Environment Research Council (NE/E008305/1). The authors would like to acknowledge the advice of P. Challenor (NOCS, UK) on the statistical analysis of amphipod growth and the assistance of J. S. Riley and E. A. Achterberg (SOES – NOCS, UK) for analyzing the carbonate chemistry samples. The authors gratefully acknowledge the assistance of four anonymous reviewers, J. P. Gattuso, S. Dupont, M. C. Thorndyke and H.-O. Pörtner in improving on earlier versions of this manuscript.

Edited by: H.-O. Pörtner

References

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. Molecular Biology of the Cell. Garland Science, Abingdon, New York, 1268 pp. 2002.

Al-Rasheid, K. A. S. and Sleigh, M. A.: Distribution and abundance of interstitial ciliates in Southampton Water in relation to physicochemical conditions, metal pollution and the availability of food organisms, Est. Coast. Shelf Sci., 41, 61–80, 1995.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J. H., Zhang, Z., Miller, W., and Lipman, D. J.: Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, Nuc. Acids Res., 25, 3389–3402, 1997.

Attrill, M. J., Power, M., and Thomas R. M.: Modelling estuarine Crustacea population fluctuations in response to physicochemical trends, Mar. Ecol. Prog. Ser., 178, 89–99, 1999.

Berge, J. A., Bjerkeng, B., Pettersen, O., Schaanning, T., and Øxnevad, S.: Effects of increased sea water concentrations of CO2 on growth of the bivalve *Mytilus edulis* L. Chemosphere, 62, 681–687, 2006.

Borg, I., Rohde, G., Löseke, S., Bittscheidt, J., Schultze-Werninghaus, G., and Bufe, S. A.: Evaluation of a quantitative real-time PCR for the detection of respiratory syncytial virus in pulmonary diseases, Eur. Resp. J., 21, 944-951, 2003.

Bustin, S. A.: A to Z of Quantitative PCR. IUL Biotechnology Series, International University Line, La Jolla California, 882 pp., 2004.

Bustin, S. A.: Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays, J. Mol. Endocrin., 25, 169–193, 2000.

Caldeira, K. and Wickett, M. E.: Ocean model predictions of chemistry changes from carbon dioxide emissions to the atmosphere and ocean, J. Geophys. Res., 110, C09S04, doi:10.1029/2004JC002671, 2005.

Charmantier Daures, M. and Vernet, G.: Moulting, autotomy and regeneration, in: The Crustacea, edited by: Forest, J. and von Vaupel Klein, J. C., 1, Brill Publishers, Leiden, 161–255, 2004.

Dalhoff, E. P.: Biochemical indicators of stress and. metabolism: applications for marine ecological studies. Annu. Rev. Physiol., 66, 183–207, 2004.

Daugaard, M., Rohde, M. and Jaattela, M.: The heat shock protein 70 family: highly homologous proteins with overlapping and distinct functions, FEBS Lett., 581, 3702–3710, 2007.

Dickson, A. G., Sabine, C. L., Christian, J. R.: Guide to Best Practices for Ocean CO_2 Measurements. PICES Special Publication 3, IOCCP Report No. 8, 2007.

Dupont, S., Havenhand, J., and Thorndyke, M.: CO_2-driven acidification radically affects larval survival and development in marine organisms, Comp. Biochem. Physiol., 150A, S170–S170, 2008.

Factor J. R.: Biology of the lobster, Academic Press, San Diego, 528 pp., 1995.

Gazeau, F., Quiblier, C., Jansen J. M., Gattuso, J.-P., Middleburg, J. J., and Heip, C. H. R.: Impact of elevated CO2 on shellfish calcification, Geophys. Res. Lett., 34, L07603, doi:10.1029/2006GL028554, 2007.

Hall-Spencer, J. M., Rodolfo-Metalpa, R., Martin, S., Ransome, E., Fine, M., Turner, S. M., Rowley, S. J., Tedesco, D., and Buia, M.-C.: Volcanic carbon dioxide vents show ecosystem effects of ocean acidification, Nature 454, 96–99, 2008.

Hayward, P. J. and Ryland, J. S.: Handbook of the Marine Fauna of North-West Europe, Oxford University Press, Oxford, 812 pp., 1995.

Higgins, D. G. and Sharp, P. M.: CLUSTAL: a package for performing multiple sequence alignment on a microcomputer, Gene, 73, 237–244, 1988.

Houghton, J. T., Ding, Y., Griggs, D. J., Noguer, M., van der Linden, M., Dai, X., Maskell, K. and Johnson, C. A.: Climate change 2001: the scientific basis (Contribution of WG1 to the IPCC Third Assessment) Cambridge, Cambridge University Press, 2001.

Karlin, S. and Brocchieri, L.: Heat shock protein 70 family: multiple sequences comparisons, function and evolution, J. Mol. Evol., 47, 565–577, 1998.

Kikkawa, T., Kita, J., and Ishimatsu, A.: Comparison of lethal effect of CO_2 and acidification on red sea bream (*Pagrus major*) during the early developmental stages, Mar. Poll. Bull., 48, 108–110, 2004.

Kleypas, J. A., Buddemeier, R. W., Archer, D., Gattuso, J. P., Langdon, C., and Opdyke, B. N.: Geochemical consequences of increased atmospheric carbon dioxide on coral reefs, Science 284, 118–120, 1999.

Kleypas, J. A., Feely, R. A., Fabry, C., Langdon, C. L., Sabine C. L., and Robbins, L. L.: Impacts of ocean acidification on coral reefs and other marine calcifiers: a guide for future research, report of a workshop held 18–20 April 2005, St. Petersburg, Florida, sponsored by NSF, NOAA and the US Geological Survey, 88pp,

2006.
Kroon, F. J.: Behavioural avoidance of acidified water by juveniles of four commercial fish and prawn species with migratory life stages, Mar. Ecol. Prog. Ser., 285, 193–204, 2005.
Kurihara, H., Shimode, S., and Shirayama, Y.: Sub-lethal effects of elevated concentration of CO_2 on planktonic copepods and sea urchins, J. Oceanogr., 60, 743–750, 2004.
Lee, E. T.: Statistical Methods for Survival Data Analysis, 2nd ed., New York, John Wiley & Sons, 513 pp., 1992.
Leignel, V., Cibois, M., Moreau, B., and Chénais, B.: Identification of a new subgroup of HSP70 in Bythograeidae (hydrothermal crabs) and Xanthidae, Gene, 396, 84–92, 2007.
Lincoln R. J.: British Marine Amphipoda: Gammaridea: British Museum (Natural History), London, 658 pp., 1979.
Liu, J., Yang, W.-J., Zhu, X.-J., Karouna-Renier, N. K., and Rao, R. K.: Molecular cloning and expression of two HSP70 genes in the prawn, *Macrobrachium rosenbergii*, Cell Stress Chap., 9, 313–323, 2004.
Lo, W.-Y., Liu, K.-F., Liao, I.-C., and Song Y.-L.: Cloning and molecular characterization of heat shock cognate 70 from tiger shrimp (*Penaeus monodon*) Cell Stress Chap. 9, 332–343, 2004.
Pan, D., He, N., Yang, Z., Liu, H., and Xu X.: Differential gene expression profile in hepatopancreas of WSSV-resistant shrimp (*Penaeus japonicus*) by suppression subtractive hybridisation, Dev. Comp. Immunol., 29, 103–112, 2005.
Pörtner, H. O., Langenbuch, M., and Reipschläger, A.: Biological impact of elevated CO_2 concentrations: lessons from animal physiology and earth history, J. Oceanogr., 60, 705–718, 2004.
Ravaux, J., Toullec, J.-Y., Leger, N., Lopez, P., Gaill, F., and Shillito, B.: First hsp70 from two hydrothermal vent shrimps, *Mirocaris fortunata* and *Rimicaris exoculata*: characterization and sequence analysis, Gene 386, 162–172, 2007.
Ringwood, A. H. and Keppler, C. J.: Water quality variation and clam growth: is pH really a non-issue in estuaries?, Estuaries, 25, 901–907, 2002.
Royal Society: Ocean acidification due to increasing atmospheric carbon dioxide, *Policy Document 12/05*, The Royal Society, London, 60 pp., 2005.
Rukke, N. A.: Effects of low calcium concentrations on two common freshwater crustaceans, *Gammarus lacustris* and *Astacus astacus*, Funct. Ecol., 16, 357–366, 2002.
Sanders, B. M.: Stress proteins in aquatic organisms: an environmental perspective, Crit. Rev. Toxicol., 23, 49–75, 1993.
Sigrist, C. J. A., Cerutti, L., Hulo, N., Gattiker, A., Falquet, L., Pagni, M., Bairoch, A., and Bucher, P.: PROSITE: a documented database using patterns and profiles as motif descriptors, Brief Bioinform., 3, 265–274, 2002.
Sokal, R. R. and Rolf, F. J.: Biometry, 3rd ed. W. H. Freeman and Company, New York, 887 pp., 1995.
Spicer, J. I., Raffo, A., and Widdicombe, S.: Influence of CO_2-related seawater acidification on extracellular acid-base balance in the velvet swimming crab Necora puber, Mar. Biol., 151, 1117–1125, 2007.
Truchot, J. P.: Mechanisms of compensation of blood respiratory acid-base disturbances in the shore crab *Carcinus maenas* (L.), J. Exp. Zool., 210, 407–416, 1979.
Underwood, A. J.: Experiments in Ecology. Cambridge, Cambridge University Press, 504 pp., 1997.
Wheatly, M. G.: Calcium homeostasis in Crustacea: the evolving role of branchial, renal, digestive and hypodermal epithelia, J. Exp. Zool., 283, 620–640, 1999.
Widdicombe, S. and Needham, H. R.: Impact of CO2-induced seawater acidification on the burrowing activity of *Nereis virens* and sediment nutrient flux, Mar. Ecol. Prog. Ser., 341, 111–122, 2007.
Wood, H. L., Spicer, J. I., and Widdicombe, S.: Ocean acidification may increase calcification raters, but at a cost, Proc R. Soc. B., 275, 1767–1773, 2008.
Wright, D. A.: Calcium regulation in intermoult *Gammarus pulex*, J. Exp. Biol., 83, 131–144, 1979.
Wu, R., Sun, Y., and Lei, L. M.: Molecular identification and expression of heat shock cognate 70 (HSC70) in the Pacific white shrimp *Litopenaeus vannamei*, Mol. Biol., 42, 234–242, 2008.
Zehmer, J. K., Mahon, S. A., and Capelli, G. M.: Calcium as a limiting factor in the distribution of the amphipod *Gammarus pseudolimnaeus*, American Mid. Natural., 148, 350–362, 2002.