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

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Received: 17 November 2008 – Accepted: 17 November 2008 – Published: 15 January 2009

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Published by Copernicus Publications on behalf of the European Geosciences Union.

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## Abstract

We report an investigation of the effects of increases in  $p\text{CO}_2$  on the 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 rate 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 expression of the *hsp70* gene. However, there was a consistent and significant increase in the expression of the *gapdh* gene at a pH of  $\sim 7.5$  which indicated a possible disruption to oxidative metabolic processes. It was concluded that future predicted changes in sea water pH may have subtle effects on the physiology and metabolism of coastal and marine species which may be overlooked in studies of whole organism response.

## 1 Introduction

It is almost universally accepted that the current atmospheric  $\text{CO}_2$  concentration of ca. 380 ppmv is set to rise by an estimated  $1\% \text{y}^{-1}$  over the next decades (Houghton et al., 2001). This rate is approximately 100 times faster than has occurred over the past 650 000 years and the rising  $\text{CO}_2$  levels are irreversible on human timescales (Royal Society, 2005). Reductions in seawater pH have already been reported as a consequence of an increased level of  $\text{CO}_2$  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  $\Delta\text{pH}$  of sea water by 2100

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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 seawater 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 a wider range of marine 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  $p\text{CO}_2$ -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 restricted to adult life stages (Spicer et al., 2007) or used  $p\text{CO}_2$  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  $\text{CO}_2$ , 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 Keppeler, 2002). Estuarine environments are known to be areas where pH varies over a large range. Al-Rasheid

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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 Keppeler (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).

To this end 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 exists as 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. Any increase

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in the expression of the either of these genes, relative to control amphipods, would indicate either general sub-lethal physiological or metabolic stress associated with exposure to reduced pH. 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<sub>2</sub> microcosm systems

CO<sub>2</sub> 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-l “mixing tank” and the 2-l “incubation tank” using peristaltic pumps (flow rate 1.5 l h<sup>-1</sup>). 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.

Three separate microcosms were set up and adjusted to different nominal pH regimes: pH 8.1 (the control), pH 7.8 (~550 ppm pCO<sub>2</sub>) and pH 7.6 (~980 ppm pCO<sub>2</sub>). The most extreme ΔpH used in this study approximated the modelled decrease in surface ocean pH by 2100 under the IPCC A2 SRES scenario of CO<sub>2</sub> 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

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deliver 100% CO<sub>2</sub> directly into a 10-l 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 and pH was measured in the incubation chambers on alternate days during the water change using a three-decimal-place SevenMulti pH meter (Mettler, Schwerzenbach, Switzerland).

### 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<sub>2</sub> 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 amphipods 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 complex-

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ity 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 *Gammarus 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', anti-sense 5'-ACR TCR TCY TCN GTR TAN CC-3',  $T_m=51^\circ\text{C}$ ) 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

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which two primers were designed (sense 5'-GCN CAN AAR GAY GCN GG-3', anti-sense 5'-ARD ATN CCR TTN GCR TCD AT-3',  $T_m=51^\circ\text{C}$ ). 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 U µl<sup>-1</sup>; 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

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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

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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 non-amers.

#### 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 10 s, 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

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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 ( $10 \text{ U } \mu\text{l}^{-1}$ , 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).

## 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:

$$X = \text{MEAN} + \text{pH} + \text{Ti}(\text{pH}) + \text{Ba}(\text{pH} \times \text{Ti}) + \text{RES} \quad (1)$$

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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 $\text{CO}_2$ microcosm performance

The temperature, salinity and pH measured within the incubation chambers during the experiments are summarised in Fig. 1. The mean ( $\pm$ SD) temperatures in the control, nominal pH 7.8 and nominal pH 7.6 incubations were  $20.23 \pm 0.92^\circ\text{C}$ ,  $20.03 \pm 0.30^\circ\text{C}$  and  $20.14 \pm 0.37^\circ\text{C}$ , respectively. Mean salinities in the three incubations were  $33.86 \pm 0.82$ ,  $33.65 \pm 0.81$  and  $34.1 \pm 0.82$ , 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  $8.078 \pm 0.058$  (control),  $7.831 \pm 0.040$  (nominal set point pH 7.8) and  $7.468 \pm 0.044$  (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.

### 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 (data not shown) 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.

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### 3.3 Changes in gene expression of CO<sub>2</sub>-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: isoleu 10–ser 17, isoleu 198–leu 211, and isoleu 335–lys 349. Furthermore, the absence of Karlin and Brocchieri's (1998) "domain 13" (gly pro lys his) indicated that the protein coded by this gene was eukaryotic in origin and present in the cytoplasmic compartment of the cell (as opposed to the mitochondria).

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 expression 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* over the whole experiment (data not shown).

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Further, there was no significant effect of the nested factors Time or Batch on the expression of this gene. Nested ANOVA demonstrated a significant effect of incubation pH on the expression of the *gapdh* gene (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 no significant effect of incubation time on this relation although there were significant but inconsistent batch effects in the expression of the *gapdh* gene (Table 2).

## 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 drop in sea water pH arising from the increases in dissolved CO<sub>2</sub> 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 impacts that might be undetected in studies of whole organism responses. To that end, this study investigated the effects of CO<sub>2</sub>-mediated changes in the future mean pH on the molecular physiology, growth and survival of the ubiquitous neritic marine amphipod *Gammarus locusta*.

As discussed earlier, there is considerable difficulty 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<sub>2</sub>-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.

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*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 Biodiversity Information Facility; <http://us.mirror.gbif.org>). In light of the distribution of this species and the earlier data from other species it was hypothesized that *G. locusta* would also 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 long-term (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 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 difference in the moulting frequency between treatments. It can be concluded that the moulting frequency and subsequent growth of these juvenile amphipods were unaffected by a sustained decrease of at least 0.5 pH units. These data confirm the working 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

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a function of changes in atmospheric CO<sub>2</sub> (Caldeira and Wickett, 2005).

In addition to assessments of organism growth and survival we also investigated changes in the molecular stress physiology of *G. locusta* incubated under different pH regimes. For this 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; 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, phylogenetic comparison of this sequence with other published heat shock protein and heat shock cognate sequences for crustaceans and other taxa did not definitively support this identity. In this analysis, which included the 23 heat shock sequences (*hsps* and *hscs*) used by Lo et al. (2004) and the top 20 hits identified by a BLAST search using FM165078, the *Gammarus locusta* sequence was consistently placed in a mixed clade of metazoan *hsp* and *hsc* sequences (data not shown). The problem of definitive identification 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

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*Gammarus locusta hsp70* which indicates that the gene isolated in this species does indeed code for an inducible heat shock protein.

There was, however, no significant increase in the expression of the *hsp70* gene after 14 and 28 days growth at reduced pH (to a minimum of pH 7.468). From these data it can be concluded that the acid/base disturbance in this experiment did not denature cytoplasmic proteins to an extent that heat shock proteins were required to refold them. This indicates that the intracellular buffering systems in the tissues of *G. locusta* were sufficient to cope with this level of acidosis over a prolonged period (juvenile to adult). Maintenance of acid/base status in marine invertebrates is reliant upon a system of membrane bound proteins including v-type H<sup>+</sup>-ATPases as well as sodium/proton pumps and sodium-dependent chloride/bicarbonate exchange (Portner et al., 2004). In marine crustaceans in particular bicarbonate anion exchange plays a major role in the restoration of acid/base balance (Truchot, 1979). It can be predicted that the induction of these membrane bound pumps and exchange processes will have an energetic cost in terms of the utilisation of molecules of ATP. Upstream of these processes, therefore, will be the induction of pathways which generate ATP, namely glycolysis and the tricarboxylic acid cycle.

These predictions are supported by the observation that, at a mean pH of 7.468, there was a significant increase in the expression of the *gapdh* gene. This implies that the rates of oxidative metabolic processes in the amphipod tissues were increased to furnish more ATP molecules to support the energy consuming processes of acid/base balance. Evidence of an increased metabolic rate in response to exposure to low pH waters (7.7–6.8) has recently been shown for the ophiuroid *Amphura filiformis* (Wood et al., 2008). The fact that in this study, and at the pH range studied, the increase in oxidative metabolic processes did not result in an increase in growth rate supports our contention that in this species the increase in metabolism supports acid/base homeostasis. It is conceivable that the metabolic costs associated with acid/base homeostasis might impact other processes not measured in this study, such as immune response or maturation of sexual tissues or future reproductive output. These predictions are currently

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being tested as the part of on-going studies in this laboratory.

Lethal and sub-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*<sub>2</sub>. However, this present study has demonstrated that by utilizing sensitive molecular approaches the costs of exposure to acidic sea water can be recorded at much less extreme pH values than have been used by previous workers.

## 5 Conclusions

In conclusion, this study has presented preliminary 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 inducing “classic” stress responses such as the expression of the 70 kDa heat shock protein to act as a molecular chaperone. However, there is a significant change in the metabolism of this species which may indicate that energy is being diverted to drive acid/base homeostasis, potentially to the detriment of future reproductive success of this species.

*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.

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**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	$R^2$
<i>hsp70</i>	<i>hsp70</i> rt F2	CAA AGA TGG ACA AGG GAC AGA TG	300	96	1.06	CT=(-3.188× log dilution)+9.521	0.998
	<i>hsp70</i> rt R2	AGC TTC TGC ACC TTT GGA ATG	300				
<i>gapdh</i>	<i>gapdh</i> rt F1	AAC GCC TCC TGC ACC ACT AA	50	91	0.91	CT=(-3.572× log dilution)+13.891	0.997
	<i>gapdh</i> rt F2	CCT CCT CAA TGC CGA AGC T	50				

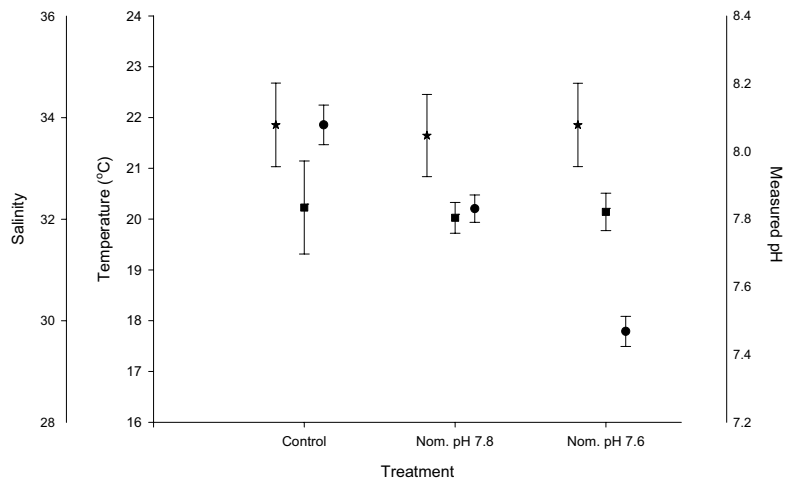
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**Table 2.** Summary of nested ANOVA comparing pH, time (Ti, nested in pH) and batch (Ba, nested in pH and Time) for the *gapdh* gene expression data. Analysis conducted using GMAV (Underwood, 1997).

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(pH×Ti)	1.6277	6	0.2713	4.06	0.0013*
Residual	5.6152	84	0.0668		
Total	10.6146	95			

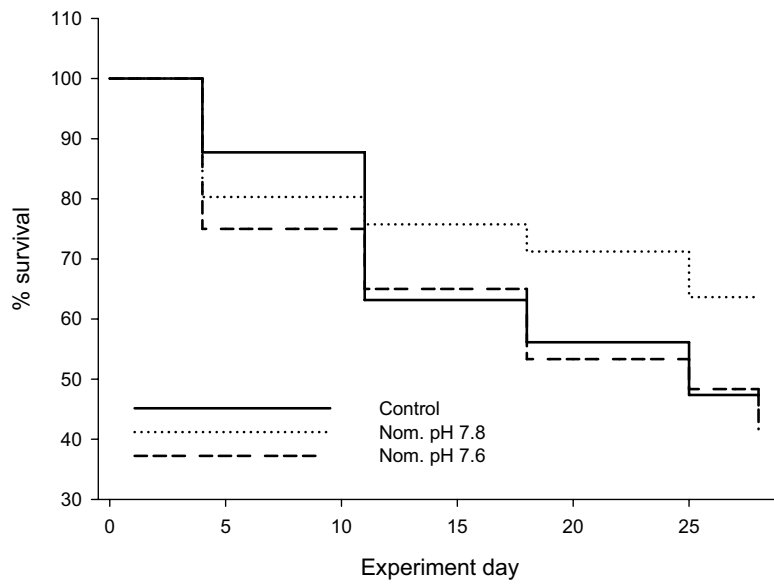
Model for analysis:  $X = \text{MEAN} + \text{pH} + \text{Ti}(\text{pH}) + \text{Ba}(\text{pH} \times \text{Ti}) + \text{Residual}$

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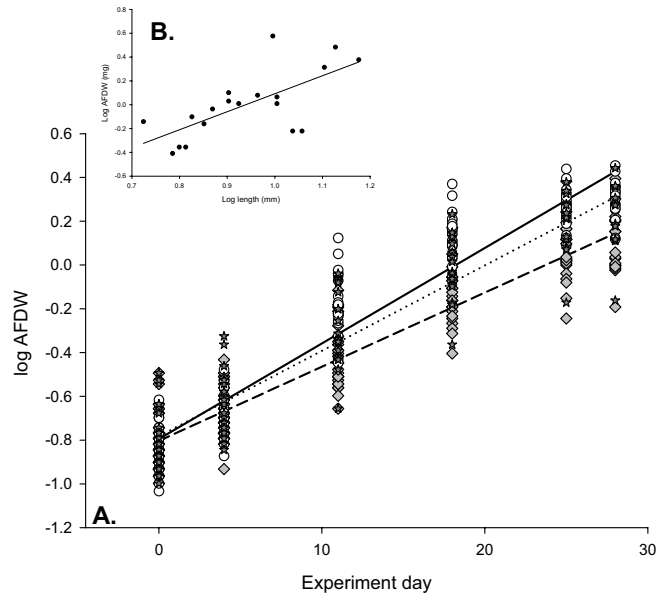
**Fig. 1.** Summary of incubation conditions during the study showing the mean  $\pm$ SD of temperature (squares), salinity (stars) and pH (circles) for each incubation.

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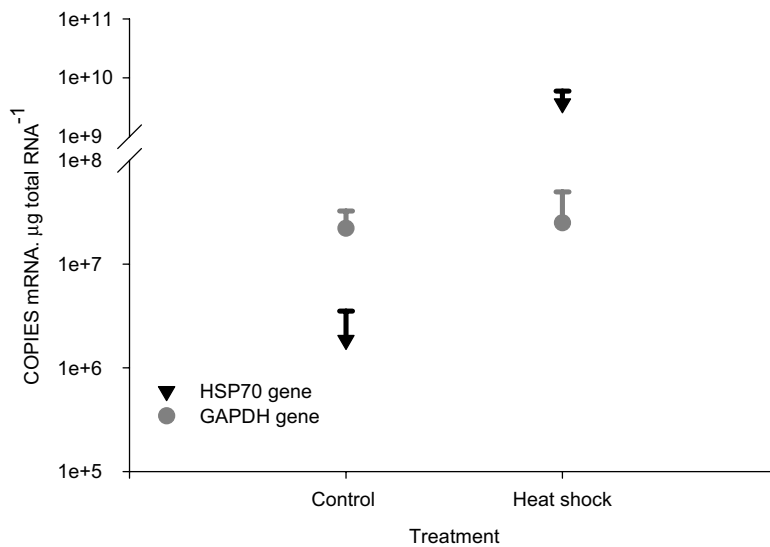
**Fig. 2.** Comparisons of amphipod survival at different incubation pH showing a significant improvement in survival rate of amphipods maintained at a nominal pH 7.8.

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**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*;  $\log \text{AFDW} = (1.5104 \times \log \text{Length}) - 1.4817$ .

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**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 triangles) in exposed amphipods but no change in expression of the *gapdh* gene (grey circles) (means+SD,  $n=10$ ).

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