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RESEARCH ARTICLE

The effects of elevated temperature and P_{CO_2} on the energetics and haemolymph pH homeostasis of juveniles of the European lobster, *Homarus gammarus*

Daniel P. Small^{1,2,*}, Piero Calosi^{1,2}, Samuel P. S. Rastrick³, Lucy M. Turner², Stephen Widdicombe⁴ and John I. Spicer²

ABSTRACT

Regulation of extracellular acid–base balance, while maintaining energy metabolism, is recognised as an important aspect when defining an organism’s sensitivity to environmental changes. This study investigated the haemolymph buffering capacity and energy metabolism (oxygen consumption, haemolymph [L-lactate] and [protein]) in early benthic juveniles (carapace length <40 mm) of the European lobster, *Homarus gammarus*, exposed to elevated temperature and P_{CO_2} . At 13°C, *H. gammarus* juveniles were able to fully compensate for acid–base disturbances caused by the exposure to elevated seawater P_{CO_2} at levels associated with ocean acidification and carbon dioxide capture and storage (CCS) leakage scenarios, via haemolymph [HCO_3^-] regulation. However, metabolic rate remained constant and food consumption decreased under elevated P_{CO_2} , indicating reduced energy availability. Juveniles at 17°C showed no ability to actively compensate haemolymph pH, resulting in decreased haemolymph pH particularly under CCS conditions. Early benthic juvenile lobsters at 17°C were not able to increase energy intake to offset increased energy demand and therefore appear to be unable to respond to acid–base disturbances due to increased P_{CO_2} at elevated temperature. Analysis of haemolymph metabolites suggests that, even under control conditions, juveniles were energetically limited. They exhibited high haemolymph [L-lactate], indicating recourse to anaerobic metabolism. Low haemolymph [protein] was linked to minimal non-bicarbonate buffering and reduced oxygen transport capacity. We discuss these results in the context of potential impacts of ongoing ocean change and CCS leakage scenarios on the development of juvenile *H. gammarus* and future lobster populations and stocks.

KEY WORDS: Developmental physiology, Ocean acidification, Ocean warming, Early benthic juvenile, Acid–base balance, Metabolism

INTRODUCTION

The ability of marine invertebrates to respond to the effects of environmental change on extracellular acid–base balance while

maintaining energy metabolism is recognised as important in defining an organism’s vulnerability or resilience to future predicted global change (Melzner et al., 2009; Wittmann and Pörtner, 2013). Understanding the extent to which marine invertebrates can maintain acid–base homeostasis and ion regulation during environmental change is crucial if we are to predict the biological consequences of elevated seawater CO_2 partial pressure (P_{CO_2} ; e.g. Rastrick et al., 2014; Small et al., 2016a; Lee et al., 2019). Unless there is physiological compensation, elevated seawater P_{CO_2} results in decreases in haemolymph pH (Michaelidis et al., 2005; Pane and Barry, 2007). Decreased haemolymph pH can impact functional processes such as the oxygen affinity of the respiratory pigment (Pörtner, 1990), while an increase in intracellular H^+ can disrupt cellular processes such as metabolism, protein synthesis, ion regulation and cell volume control (Grainger et al., 1979; Madhus, 1988; Whiteley, 1999; Whiteley, 2011). Therefore, the ability to regulate haemolymph pH is crucial for marine invertebrates to function under conditions of elevated seawater P_{CO_2} and may, in part, determine present and future species distribution (e.g. Calosi et al., 2013a; Rastrick et al., 2014; Calosi et al., 2017; Small et al., 2016a). Furthermore, elevated P_{CO_2} can impact upon an animal’s aerobic scope in relation to temperature (Metzger et al., 2007; Pörtner, 2008; Pörtner and Farrell, 2008; Walther et al., 2009; cf. Gräns et al., 2014).

Marine crustaceans possess effective extracellular buffering mechanisms to maintain haemolymph acid–base homeostasis, predominantly involving HCO_3^- regulation (Cameron, 1978; Truchot, 1979; Cameron, 1985). Thus, crustaceans are considered to be amongst the most ‘tolerant’ groups of marine invertebrates to elevated P_{CO_2} (Melzner et al., 2009; Whiteley, 2011). HCO_3^- regulation across gill membranes utilises in part electroneutral ion exchange of HCO_3^- for Cl^- and H^+ for Na^+ by Na^+/K^+ - and H^+ -ATPases (Cameron, 1978; Henry and Wheatly, 1992; Wheatly and Henry, 1992). Ion, including HCO_3^- , regulation is metabolically expensive (Whiteley, 2011). For example, increased rates of protein synthesis and ion transport accounted for 84% of available ATP in developing larvae of the sea urchin *Strongylocentrotus purpuratus* exposed to 800 μatm P_{CO_2} compared with 40% in control larvae (Pan et al., 2015). Therefore, the use of such regulatory mechanisms can have energetic implications and may result in trade-offs with other energy-demanding processes (Calow and Forbes, 1998). Species considered tolerant to elevated P_{CO_2} tend to maintain metabolic rate, energy metabolism and aerobic capacity upon exposure, while more sensitive species experience either increased or decreased metabolic rate with decreased aerobic capacity and increased metabolic costs (Calosi et al., 2013b; Turner et al., 2015; Calosi et al., 2017). The up-regulation of metabolic rate is well documented for marine invertebrates exposed to elevated P_{CO_2} (e.g. Wood et al., 2008, 2010; Beniash et al., 2010; Small et al., 2015;

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Calosi et al., 2013b). It is proposed that increased metabolic rate arises from an increased energy demand due to the need to maintain acid–base homeostasis together with other physiological functions such as mineralisation (Wood et al., 2008, 2010; Beniash et al., 2010). However, while increased metabolic rate in the fan worm *Sabella spallanzanii* can be linked to increased ATP production and aerobic capacity (Calosi et al., 2013b; Turner et al., 2015), it appears to come at a cost to homeostatic capabilities, in this case of carbonic anhydrase concentration (Turner et al., 2015). Conversely, metabolic depression is proposed to have evolved as a mechanism to conserve ATP in times of acute stress (e.g. Reipschläger and Pörtner, 1996; Langenbuch and Pörtner, 2002; Calosi et al., 2013b), thus maintaining a positive balance between energy supply and demand (Bishop and Brand, 2000; Seibel and Walsh, 2003).

Changes in metabolic rate and aerobic capacity due to alterations in energy demand via increased homeostatic regulation may still have an underlying functional effect even on such a ‘tolerant’ group of organisms (Widdicombe and Spicer, 2008; Whiteley, 2011). Such changes will impact other physiological aspects related to oxygen and energy availability such as thermal sensitivity (Metzger et al., 2007; Walther et al., 2009), scope for aerobic activity and osmo-/iono-regulation (Dissanayake et al., 2010; Dissanayake and Ishimatsu, 2011). When considering the combined effects of elevated P_{CO_2} and temperature, elevated temperature appears to have a negative effect on a species’ ability to regulate acid–base balance (Zittier et al., 2013; Rastrick et al., 2014). Thus, there is a pressing need to understand how organism physiology responds under combinations of elevated temperature and P_{CO_2} .

The juvenile stages of marine invertebrates have, until recently, been thought to be the most tolerant life stage to the effects of global change because of their presumed wide aerobic scope compared with that of other stages (Pörtner and Farrell, 2008). However, juveniles are now beginning to be considered as a potentially very sensitive life history stage, at least in part as a result of the significant physiological development associated with this stage of their life cycle (Long et al., 2013; Small et al., 2016b). In homarid lobsters, the early benthic juvenile phase is defined as post-settled individuals, usually less than 40 mm in carapace length, demonstrating cryptic behaviours (Wahle and Steneck, 1991; Incze and Wahle, 1992). This is to distinguish them from later adolescent phase and reproductive phase individuals, which exhibit markedly different behaviour and habitat use (Wahle and Steneck, 1991). In crustaceans generally, early benthic juveniles tend to be physiologically distinct from older immature juveniles and adults because of the continuous development of physiological functions, such as osmoregulation (Charmantier et al., 1998, 2001, 2002; Cieluch et al., 2004), oxyregulation (Spicer and Eriksson, 2003), ion regulation and haemolymph haemocyanin structure and function (e.g. Brown and Terwilliger, 1992; Terwilliger and Dumler, 2001; Terwilliger and Ryan, 2001). Despite such important developmental differences, the sensitivity of specific juvenile stages within this developmental trajectory has rarely been considered when assessing juvenile responses to global change drivers such as temperature and P_{CO_2} (Small et al., 2016b).

The European lobster is an ecologically and economically important species, so its ability to compensate for environmental changes, and the associated energetic costs, will potentially have wide socio-economic impacts. If we are to understand the effects of complex global change scenarios on marine organisms, we need to understand what makes some species tolerant and others susceptible (Widdicombe and Spicer, 2008): a concept that must also be applied to different life history stages, such as those of the lobster species

studied here, to acquire a more comprehensive understanding of species sensitivity to global change (e.g. Long et al., 2013; Small et al., 2016b; Swiney et al., 2016). The aim of the present study was to investigate the ability of early benthic juvenile *Homarus gammarus* to regulate haemolymph acid–base status when challenged with elevated P_{CO_2} levels at both current and elevated environmental temperatures, whilst maintaining a positive energy metabolism. To achieve this, early benthic (12 month old) juvenile lobsters (*H. gammarus* <40 cm carapace length, as defined by Wahle and Steneck, 1992), were exposed to three P_{CO_2} treatments at two temperatures for 14 days. Temperature treatments were a control of 13°C, representing the current summer average in the UK, and 17°C (i.e. +4°C) representing ocean warming scenarios from Sokolov et al. (2009). Control P_{CO_2} treatments were 450 μatm , representing current atmospheric P_{CO_2} , and 1100 and 8000 μatm , in line with end of century business as usual predictions of P_{CO_2} increases due to ocean acidification (OA) and carbon dioxide capture and storage (CCS) leakage scenarios (Caldeira and Wickett, 2003, 2005; Raven et al., 2005; Blackford et al., 2009; Kano et al., 2010). A simulated leak from such infrastructure can disrupt sediment carbonate chemistry and significantly alter the benthic macrofaunal community in close proximity to the release, although it seems to be able to recover in as little as 18 days once the leak ceases (Widdicombe et al., 2015). It is therefore possible that shallow sub-tidal benthic communities are at risk of acute exposure to high CO_2 levels as a result of leakage from sub-surface infrastructure involved in proposed CCS activities. In the present study, the haemolymph acid–base status of juvenile lobsters was assessed after the 14 day exposure period by measuring haemolymph pH, P_{CO_2} and HCO_3^- concentration. Furthermore, to explore the energetic implications of haemolymph acid–base regulation, oxygen consumption was measured as a proxy for metabolic energy demand, food ingestion rate as a proxy for energy acquisition, and epipodite Na^+/K^+ -ATPase activity as a proxy for energy utilisation. Haemolymph ion levels were also measured as a proxy for ion homeostasis, while the main anaerobic end product of crustaceans, L-lactate, and protein concentration were measured in the haemolymph as proxies for condition.

MATERIALS AND METHODS

Animal collection, husbandry and exposure

Early benthic juvenile *Homarus gammarus* (Linnaeus 1758) ($N=54$, 11 months old, carapace length 11.5 ± 0.1 mm) were supplied by the National Lobster Hatchery (Padstow, UK) as described in Small et al. (2016b). Briefly, larvae hatched from wild-caught ovigerous females kept in an aquarium (volume 1200 l) were reared in a batch culture system (Burton, 2003) as described by Scolding et al. (2012) under constant environmental conditions (salinity 35, temperature 17–19°C, dissolved oxygen 8 mg l^{-1}). After metamorphosis, juveniles were transferred to individual ‘Orkney’ lobster pots. These are small plastic pots (volume 100 ml) with holes in the bottom, which are connected together and suspended in a shallow aquarium (volume 250 l) supplied with constantly aerated, recirculated and mechanically and biologically filtered seawater. Individuals were fed every 3 days throughout the experimental period with a selection of squid (*Logilo vulgaris*), mussel (*Mytilus edulis*) and krill (*Euphausia superba*) *ad libitum*. All uneaten food was removed from the chambers after 3 h.

Individuals were transported by car to the Plymouth Marine Laboratory Seawater Facility (PML, Plymouth, UK), where, upon arrival, temperature was adjusted to 15°C at a rate of 0.5°C day^{-1} . Juveniles were kept under these temperature conditions for 3 weeks prior to elevated P_{CO_2} exposure to minimise the effect of sudden

temperature changes on proxies measured during the experimental period, before being haphazardly assigned to one of two temperature treatments: 13°C and 17°C ($N=27$ per treatment). The 13°C treatment represented current seasonal temperatures in the coastal water of southwest England, and the 17°C treatment represented a 4°C increase associated with future predictions of ocean warming (Sokolov et al., 2009). Temperature was once more increased at a rate of 0.5°C day⁻¹ to reach experimental temperatures from the holding temperature.

The experimental system was similar to that described by Small et al. (2015) modified by Small et al. (2016b). Briefly, the system consisted of six aquaria, three per temperature; within each were nine pots, three per P_{CO_2} treatment each containing one individual juvenile lobster ($N=9$ individuals per temperature \times P_{CO_2} combination). Each lobster was individually supplied with a constant flow (10 ml min⁻¹) of recirculated, mechanically filtered, aerated seawater at the appropriate temperature. The temperature of the whole system was maintained at 13°C (measured 13.19±0.02°C), while water inflow into the ‘warm’ tanks was heated to 17°C (measured 17.67±0.03°C) using an aquarium heater (3614 Aquarium Heater, Eheim GmbH & Co. KG, Deizisau, Germany) in conjunction with a chiller (L Series, Guangdong Boyu Group Co. Ltd, Guangdong, China).

In order to achieve the elevated P_{CO_2} scenarios detailed in the Introduction, three nominal pH treatments were chosen. A control treatment of pH 8.1 (450 µatm P_{CO_2}) and two experimental treatments of pH 7.7 (1100 µatm P_{CO_2}) and pH 6.9 (8000 µatm P_{CO_2}). To achieve the two low pH conditions, air mixed with pure CO₂ was bubbled into each pot following the methods of Findlay et al. (2008). The CO₂ content of the air was adjusted until the desired pH levels were achieved and remained stable. Air CO₂ levels were continuously measured using a CO₂ gas analyser (Li-820, Li-Cor Biosciences, Lincoln, NE, USA). Because of the use of pH to monitor the system, and the slight temperature effect on P_{CO_2} concentrations needed to achieve this pH (Table 1), nominal values for pH are used throughout the Results section to refer to the different elevated P_{CO_2} /decreased pH treatments.

Seawater pH (NBS scale, 826 Mobile pH meter, Metrohm, Switzerland) was checked against a reference pH buffer each morning (pH 7.00, pH Buffer Solution, Mettler Toledo, Columbus, OH, USA) and calibrated if necessary against three pH buffer solutions (pH 4.01, pH 7.00, pH 9.21; Mettler Toledo). Temperature and salinity were measured daily using a thermocouple (HH802U Omega Engineering Inc., Stamford, CT, USA) and a hand-held refractometer (S/Mill Hand Refractometer, Atago, Tokyo, Japan),

respectively. Water samples (volume 150 ml) were collected every 5 days and poisoned with HgCl₂⁻ to prevent microbial activity (Riebesell et al., 2010) for subsequent determination of seawater total alkalinity (A_T). All water samples and measurements for seawater carbonate chemistry were removed from the individual pots within which individual lobsters resided. A_T was measured by the gran titration method using an alkalinity titrator (As-Alk2 Titrator, Apollo SciTech Inc., Bogart, GA, USA) and, together with values of pH, temperature and salinity, was used to calculate seawater P_{CO_2} (µatm), total carbon dioxide (TCO₂, µmol kg⁻¹), bicarbonate concentration ([HCO₃⁻], µmol kg⁻¹), carbonate concentration ([CO₃²⁻], µmol kg⁻¹), aragonite saturation (Ω_{ara}) and calcite saturation (Ω_{cal}) using the CO2SYS program (Lewis and Wallace, 2006) with constants provided by Mehrbach et al. (1973), refitted by Dickson and Millero (1987), and KSO₄ constants from Dickson, (1990). Seawater physico-chemical parameters throughout the exposure period are summarised in Table 1.

Determination of rates of oxygen and food consumption

After a 2 week exposure to elevated temperature and P_{CO_2} , the rate of oxygen consumption was determined for individual juvenile lobsters, as described in Small et al. (2016b). Individuals were starved for 48 h prior to measuring oxygen and food consumption to minimise the influence of feeding history on the rates measured. Individuals were transferred into stop flow respiration chambers (volume 195 ml) blacked out with black plastic sheet, where they were allowed to settle for 30 min in their designated treatment conditions. After 30 min undisturbed, water flow to each respirometer was stopped, and rate of oxygen consumption recorded for 2 h by determining oxygen partial pressure (P_{O_2}) every 15 min. P_{O_2} was recorded using an oxygen analyser system (101, OxySense, Dallas, TX, USA) as described in Rastrick and Whiteley (2011). After measuring oxygen consumption, individuals were returned to their designated treatment containers, where they were left to rest for 1 h before rates of food consumption were determined. Individuals were fed with pre-weighed fresh squid, and left to feed for 1 h. At the end of the trial, any remaining squid was removed and weighed, and consumption rates were calculated (g squid eaten g⁻¹ body mass h⁻¹).

Determination of haemolymph acid-base status

After feeding trials had finished, lobsters were kept in their designated treatments for a further 48 h before determination of haemolymph acid-base status to minimise any effects of digestion

Table 1. Water chemistry parameters measured and characterised throughout the 2 week exposure period

Parameter	13°C			17°C		
	pH 8.1	pH 7.7	pH 6.9	pH 8.1	pH 7.7	pH 6.9
Temperature (°C)	13.1±0.02 ^A	13.2±0.04 ^A	13.2±0.06 ^A	17.6±0.06 ^B	17.7±0.04 ^B	17.7±0.07 ^B
pH (NBS scale)	8.12±0.01 ^A	7.73±0.01 ^B	6.91±0.01 ^C	8.06±0.01 ^A	7.71±0.01 ^B	6.88±0.01 ^C
Salinity	34.1±0.06	34.0±0.03	34.1±0.05	34.0±0.04	34.0±0.03	34.1±0.05
A_T (µequiv kg ⁻¹)	2198±11	2195±11	2195±7	2194±17	2170±14	2210±21
TCO ₂ (µmol kg ⁻¹)*	2023±10 ^A	2153±14 ^B	2497±1 ^C	2019±16 ^A	2120±13 ^B	2508±26 ^C
P_{CO_2} (µatm)*	430±6 ^A	1156±38 ^B	7974±139 ^C	517±11 ^D	1247±23 ^B	9025±181 ^E
[HCO ₃ ⁻] (µmol kg ⁻¹)*	1877±9 ^A	2049±14 ^B	2172±7 ^C	1871±16 ^A	2014±12 ^B	2185±21 ^C
[CO ₃ ²⁻] (µmol kg ⁻¹)*	128±2.1 ^A	58±1.7 ^B	9.3±0.2 ^C	130.1±1.8 ^A	62.8±1.2 ^B	10.2±0.2 ^C
Ω_{cal} *	3.08±0.05 ^A	1.39±0.04 ^B	0.22±0.01 ^C	3.13±0.04 ^A	1.51±0.03 ^B	0.25±0.01 ^C
Ω_{ara} *	1.97±0.03 ^A	0.89±0.03 ^B	0.14±0.01 ^C	2.02±0.03 ^A	0.97±0.02 ^B	0.16±0.01 ^C

A_T , total alkalinity; TCO₂, total carbon dioxide content; P_{CO_2} , carbon dioxide partial pressure; [HCO₃⁻], bicarbonate concentration; [CO₃²⁻], carbonate concentration; Ω_{cal} , calcite saturation; and Ω_{ara} , aragonite saturation. Data are means±s.e.m. Superscript capital letters indicate significant differences between treatments. *Parameters calculated using CO₂SYS program (Lewis and Wallace, 2006) with constants provided by Mehrbach et al. (1973) refitted by Dickson and Millero (1987).

on any of the parameters measured. Haemolymph samples (volume 100 μl) were obtained by direct cardiac puncture via the membrane between the carapace and abdomen using a gastight syringe (Gastight 1710 100 μl syringe, Hamilton Co., Bonaduz, Switzerland) and needle (RN Needle, Hamilton Co.). Samples were obtained anaerobically while the individuals were still submerged in treatment water to avoid any effects of emersion on haemolymph acid–base parameters (Calosi et al., 2013a). A subsample of 70 μl of haemolymph was transferred within 2 s into a microcentrifuge tube (Eppendorf, volume 1.5 ml), the size and shape of which allowed for a tight fit onto the end of a micro-pH electrode (Micro-InLab pH combination electrode, Mettler Toledo) connected to a calibrated pH meter (Seven Easy pH Meter, Mettler Toledo) in order to anaerobically determine haemolymph pH (NBS scale) (see also Donohue et al., 2012; Rastrick et al., 2014; Small et al., 2016b). During pH measurements, the sample was placed in a waterbath set to the designated treatment temperature. Any remaining haemolymph from pH measurements (~ 60 μl) was immediately frozen at -80°C for subsequent analysis. The remaining 30 μl was injected into a CO_2 analyser (965D, Corning Diagnostics, Cambridge, MA, USA) within 2 s of pH measurements to determine haemolymph TCO_2 .

Haemolymph P_{CO_2} and HCO_3^- were calculated from measured pH and TCO_2 values using the Henderson–Hasselbalch equation in the following forms:

$$P_{\text{CO}_2} = \text{TCO}_2 / \alpha (10^{\text{pH} - \text{p}K'_1} + 1), \quad (1)$$

$$[\text{HCO}_3^-] = \text{TCO}_2 - \alpha P_{\text{CO}_2}, \quad (2)$$

where α is the solubility coefficient of CO_2 in *Carcinus maenas* haemolymph (13°C , $\alpha=0.4050$ $\text{mmol l}^{-1} \text{ kPa}^{-1}$; 17°C , $\alpha=0.3375$ $\text{mmol l}^{-1} \text{ kPa}^{-1}$; calculated from Truchot, 1976) and $\text{p}K'_1$ is the first apparent dissociation constant of carbonic acid in *C. maenas* haemolymph (13°C , $\text{p}K'_1=6.04$; 17°C , $\text{p}K'_1=6.015$; calculated from Truchot, 1976).

Determination of epipodite Na^+/K^+ -ATPase activity

Immediately following haemolymph acid–base status measurements, all epipodites were carefully removed from each individual, using watchmakers' forceps, for the determination of Na^+/K^+ -ATPase activity using the method of Brooks and Mills (2003) with the modifications described below. Epipodites were chosen for this analysis as they are the primary site of Na^+/K^+ -ATPase osmo- and iono-regulatory activity in lobster gills (Lucu and Devescovi, 1999; Flik and Haond, 2000). All epipodites from each individual were sonicated (Vibracell, Sonics and Materials Inc., Danbury, CT, USA) in 250 μl of ice-cold sonication buffer containing 100 mmol l^{-1} Hepes, 100 mmol l^{-1} NaCl and 0.1% sodium deoxycholate, pH 7.2. Activity was determined in two different buffers: 30 μl of sonicate was added to 500 μl of (1) buffer containing 10 mmol l^{-1} MgCl_2 , 100 mmol l^{-1} NaCl, 15 mmol l^{-1} KCl and 100 mmol l^{-1} Hepes, pH 7.2, and (2) the same buffer without KCl but containing 10 mmol l^{-1} ouabain, which specifically inhibits Na^+/K^+ -ATPase. Samples were prepared in triplicate. The reaction was started by the addition of 27 μl ATP (100 mmol l^{-1}) followed by incubation in a hot block (Dry Block Thermostat, Grant, Cambridge, UK) at 37°C for 20 min. After 20 min, the reaction was stopped with 1 ml Bonting's reagent (560 mmol l^{-1} H_2SO_4 , 8.1 mmol l^{-1} ammonium molybdate and 176 mmol l^{-1} FeSO_4). Colour, arising from the reaction of free phosphate with Bonting's reagent, was allowed to develop for 20 min at room temperature before absorbance was measured at

700 nm using a spectrophotometer (Novaspec II, Pharmacia LKB Biochrom Ltd, Cambridge, UK) using 0.65 mmol l^{-1} phosphorus standard solution (Sigma-Aldrich, St Louis, MO, USA) as the standard. The difference between ATP concentrations in the two buffers can be attributed to Na^+/K^+ -ATPase activity. Protein concentration was also determined for the epipodite sonicate in a microplate format (VersaMax microplate reader, Molecular Devices, San Jose, CA, USA) using the method of Bradford (1976) with 200 mg ml^{-1} bovine serum albumin (Sigma-Aldrich) as the standard.

Ionic and biochemical analysis of haemolymph

Frozen haemolymph samples were thawed and centrifuged at 10,000 rpm for 5 min to remove cells and coagulates before protein, L-lactate and ionic concentrations were quantified as follows. [L-Lactate] was determined using 8 μl of haemolymph from each individual, diluted ($\times 3$) and mixed with L-lactate reagent (Sigma-Aldrich), then read at $\lambda=550$ nm using a plate reader (VersaMax Microplate Reader, Molecular Devices).

Haemolymph protein concentration was determined in 5 μl haemolymph diluted ($\times 10$) using the Coomassie Brilliant Blue dye binding method (Bradford, 1976) with bovine serum albumin (Sigma-Aldrich) as the standard. Optical density was read at $\lambda=595$ nm using a microplate reader (VersaMax Microplate Reader, Molecular Devices). A further 10 μl of haemolymph from each individual was diluted ($\times 150$), then analysed for concentrations of Ca^{2+} , Mg^{2+} , Na^+ , K^+ , P^+ and Cu^{2+} using an ICP-OES (Varian 725-ES, Agilent Technologies Inc., Santa Clara, CA, USA).

Statistical analysis

The effects of temperature, pH and temperature \times pH were analysed using two-way ANCOVA tests with the term tank as a random factor nested within temperature \times pH treatments and wet body mass (WBM) as a covariant. There was no significant effect of tank or WBM throughout, so consequently these terms were removed from the analysis and an ANOVA performed. Assumptions of normality or distribution, using a Kolmogorov–Smirnov test, and homogeneity of variances, using a Levene's test of equality of error, were always met and therefore no transformations were required. Differences between treatments were determined by estimated marginal means. All data are presented as means \pm s.e.m. All statistical analyses were performed using SPSS software v.22 (SPSS, Chicago, IL, USA).

RESULTS

Haemolymph acid–base status

At 17°C , haemolymph pH (pH_e) decreased from 7.33 ± 0.04 in individuals exposed to pH 8.1 to 7.08 ± 0.08 and 6.58 ± 0.18 in individuals exposed to pH 7.7 and 6.9, respectively. This represents decreases of 3% and 10%. There was no difference in pH_e of individuals exposed to 13°C (all pH treatments) nor between control pH treatments of the two temperatures. This resulted in a significant interaction between seawater temperature and pH on pH_e ($F_{2,49}=10.314$, $P<0.001$; Fig. 1A) along with a significant effect of seawater temperature ($F_{1,49}=10.407$, $P=0.005$; Fig. 1A) and seawater pH ($F_{2,49}=7.691$, $P=0.004$; Fig. 1A) in isolation.

At 13°C , haemolymph P_{CO_2} (P_{CO_2e}) increased from 0.41 ± 0.04 kPa in individuals exposed to pH 8.1 to 0.69 ± 0.16 and 1.19 ± 0.16 kPa in individuals exposed to pH 7.7 and 6.9, respectively. This represents an increase of 60% and 102%, respectively. At 17°C , P_{CO_2e} increased from 0.34 ± 0.03 kPa in individuals exposed to pH 8.1 to 0.69 ± 0.14 and 2.94 ± 0.72 kPa in individuals exposed to pH 7.7 and 6.9, respectively. This represents

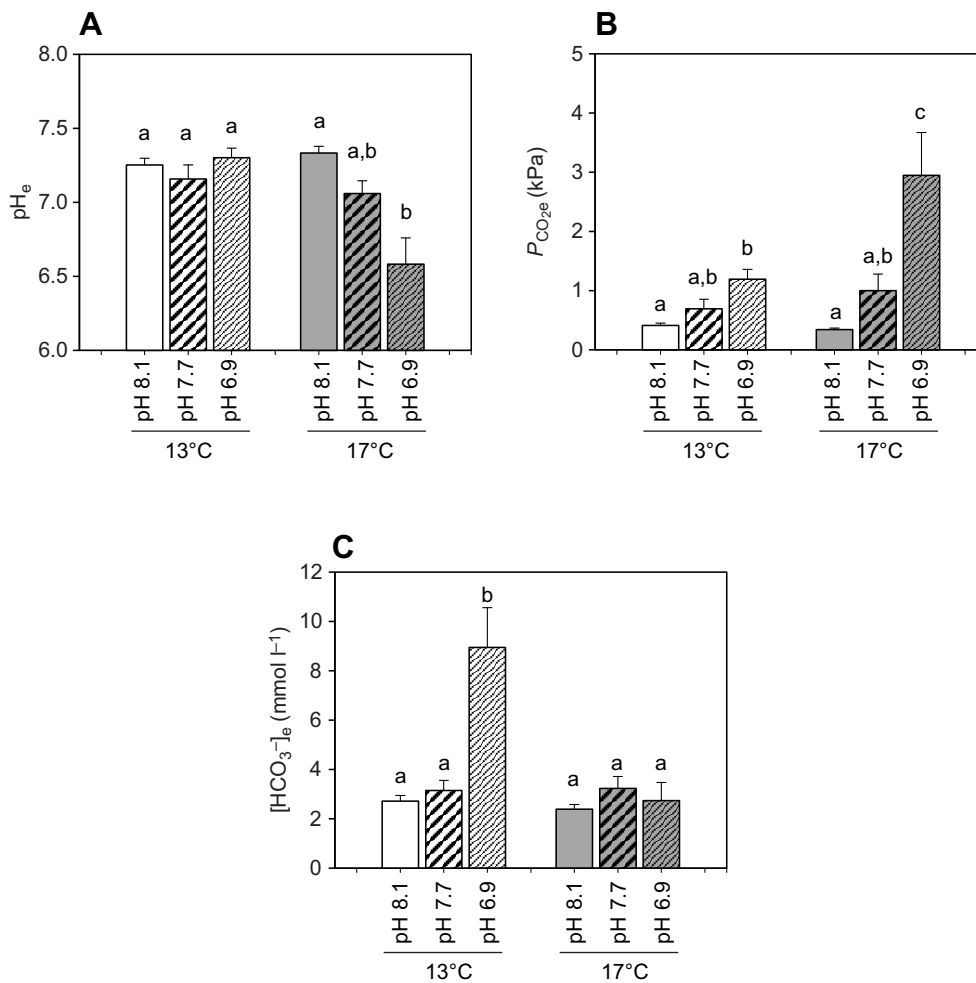


Fig. 1. Effect of exposure to elevated temperature and decreased pH on the mean haemolymph acid–base status of early benthic juvenile European lobster, *Homarus gammarus*.

(A) Haemolymph pH (pH_e ; NBS scale). (B) Haemolymph carbon dioxide partial pressure (P_{CO_2e}). (C) Haemolymph bicarbonate concentration ($[HCO_3^-]_e$). Data are means \pm s.e.m. Bar colour indicates temperature treatment (white, 13°C; grey, 17°C); striping indicates pH treatment (no stripes, pH 8.1; thick stripes, pH 7.7; thin stripes, pH 6.9). Different lowercase letters indicate significant differences between treatments ($P < 0.05$).

an increase of 190% and 770%, respectively. This resulted in a significant interaction between seawater temperature and pH on P_{CO_2e} ($F_{1,49}=6.559$, $P=0.002$; Fig. 1B) along with a significant effect of seawater temperature ($F_{1,49}=4.083$, $P=0.03$; Fig. 1B) and seawater pH ($F_{2,49}=20.273$, $P<0.001$; Fig. 1B) in isolation.

At 13°C, haemolymph $[HCO_3^-]_e$ concentration ($[HCO_3^-]_e$) increased from 2.71 ± 0.24 mmol l⁻¹ in individuals exposed to pH 8.1 to 8.94 ± 1.52 mmol l⁻¹ in individuals exposed to pH 6.9. This represents an increase of 230%. There were no differences in $[HCO_3^-]_e$ between any other treatment. This resulted in a significant interaction between seawater temperature and pH on $[HCO_3^-]_e$ ($F_{2,49}=9.618$, $P<0.001$; Fig. 1C) along with a significant effect of seawater temperature ($F_{1,49}=15.103$, $P<0.001$; Fig. 1C) and seawater pH ($F_{1,49}=11.299$, $P<0.001$; Fig. 1C) in isolation.

Rates of oxygen and food consumption

The oxygen consumption rate of individuals exposed to pH 8.1 at 13°C was 0.11 ± 0.02 $\mu\text{mol min}^{-1} \text{g}^{-1}$. There was no significant effect of seawater temperature or pH, either in isolation or in combination, on the rate of oxygen consumption ($P>0.05$; Fig. 2A). The rate of food consumption decreased from 0.12 ± 0.04 mg mg⁻¹ WBM h⁻¹ in individuals exposed to pH 8.1 to 0.04 ± 0.02 mg mg⁻¹ WBM h⁻¹ in individuals exposed to pH 6.9, both at 13°C. When exposed to 17°C, food consumption rate increased from 0.7 ± 0.2 mg mg⁻¹ WBM h⁻¹ in individuals exposed to pH 8.1 to 0.13 ± 0.2 mg mg⁻¹ WBM h⁻¹ in individuals exposed to pH 7.7, while decreasing to 0.05 ± 0.2 mg mg⁻¹ WBM h⁻¹ in individuals

exposed to pH 6.9. This resulted in a significant effect of seawater pH on the rate of food consumption ($F_{1,50}=5.732$, $P=0.005$; Fig. 2B). There was no significant interaction between seawater temperature and pH, nor a significant effect of seawater temperature in isolation, on the rate of food consumption ($P>0.05$; Fig. 2B).

Epipodite Na⁺/K⁺-ATPase activity

Epipodite Na⁺/K⁺-ATPase activity at 13°C ranged from 2.51 ± 0.66 nmol mg⁻¹ h⁻¹ in individuals exposed to pH 8.1 to 4.02 ± 0.75 nmol mg⁻¹ h⁻¹ in individuals exposed to pH 7.7 and 5.77 ± 1.32 nmol mg⁻¹ h⁻¹ in individuals exposed to pH 6.9. At 17°C, epipodite Na⁺/K⁺-ATPase activity was 5.03 ± 1.30 nmol mg⁻¹ h⁻¹. Despite these apparent increases in epipodite Na⁺/K⁺-ATPase activity (37%, 56% and 43%, respectively), there was no significant effect of seawater temperature or pH, in isolation or in combination ($P>0.05$; Fig. 3).

Haemolymph ions and biochemistry

Concentrations of haemolymph ions, protein and lactate are displayed in Table 2. Haemolymph protein concentration ($[\text{protein}]_e$) increased by 235% between individuals exposed to pH 8.1 at 13°C and those exposed to pH 8.1 at 17°C. This resulted in a significant effect of seawater temperature on $[\text{protein}]_e$ ($F_{1,47}=5.144$, $P=0.030$; Table 2), with no significant interaction between seawater temperature and pH, nor a significant effect of seawater pH in isolation, on $[\text{protein}]_e$ ($P>0.05$; Table 2). Likewise, concentrations of haemolymph Cu²⁺ ($[\text{Cu}^{2+}]_e$) increased by 50%

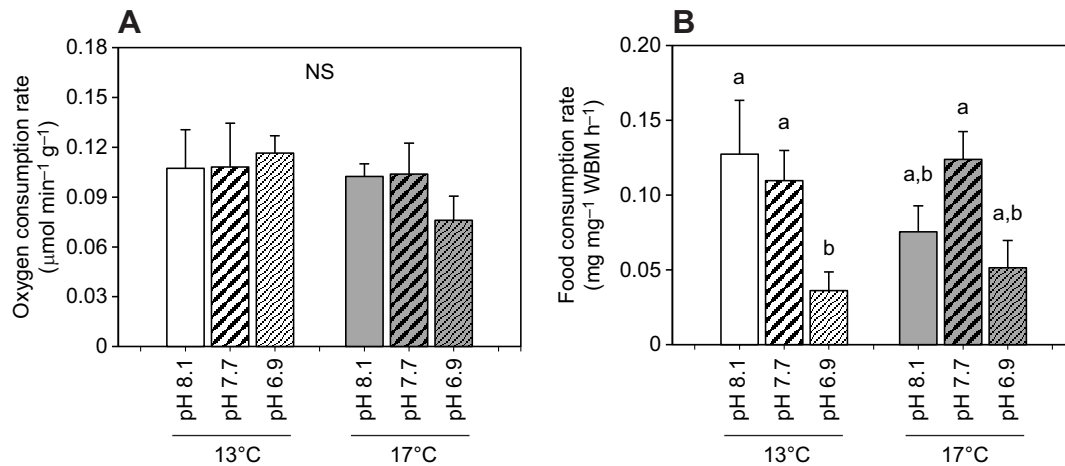


Fig. 2. Effect of exposure to elevated temperature and decreased pH on mean metabolic rate and feeding rate of early benthic juvenile *H. gammarus*. (A) Metabolic rate measured as oxygen consumption at standard temperature and pressure (STP). (B) Feeding rate (WBM, wet body mass). Data are means \pm s.e.m. Bar colour indicates temperature treatment (white, 13°C; grey, 17°C); striping indicates pH treatment (no stripes, pH 8.1; thick stripes, pH 7.7; thin stripes, pH 6.9). Different lowercase letters indicate significant differences between treatments ($P < 0.05$). NS, not significant.

between individuals exposed to pH 8.1 at 13°C and those exposed to pH 8.1 at 17°C. This resulted in a significant effect of seawater temperature on $[Cu^{2+}]_e$ ($F_{48,1}=4.057$, $P=0.049$; Table 2), with no significant interaction between seawater temperature and pH, nor a significant effect of seawater pH in isolation, on $[Cu^{2+}]_e$ ($P > 0.05$; Table 2). There was a significant effect of seawater pH on haemolymph Na^+ concentration ($[Na^+]_e$, $F_{48,1}=4.626$; $P=0.015$; Table 2), with no significant interaction between seawater temperature and pH, nor a significant effect of seawater pH in isolation, on $[Na^+]_e$ ($P > 0.05$; Table 2). There were no further significant effects of seawater pH or temperature, in isolation or in combination, on the concentrations of haemolymph L-lactate, Ca^{2+} , Mg^{2+} or K^+ ($P > 0.05$; Table 2).

DISCUSSION

Early benthic juvenile European lobster, *Homarus gammarus*, were able to fully compensate for acid–base disturbances during exposure to elevated seawater P_{CO_2} conditions, associated with OA and CCS

leakage scenarios, at the lower environmental temperature. Exposure to elevated temperature, however, compromised this compensatory response as indicated by a decrease in haemolymph pH, particularly under CCS conditions. Despite the increase in buffering effort upon exposure to elevated P_{CO_2} at control temperature, rates of oxygen consumption were unchanged, whilst rates of food consumption decreased. Coupled with high haemolymph L-lactate levels and low haemolymph protein levels compared with later stage juvenile and adult lobsters, this could suggest that the early benthic juvenile stage of *H. gammarus* is energetically limited. The implication of these results for the development of early benthic juvenile *H. gammarus* under conditions of elevated temperature and P_{CO_2} can be better understood by first describing the haemolymph physiology and biochemistry of early benthic juvenile lobsters, and then comparing these traits with our existing knowledge of later stage juveniles and adults.

Haemolymph acid–base homeostasis

Early benthic juvenile *H. gammarus* exposed to 13°C possessed effective active haemolymph buffering compensation under both OA and CCS P_{CO_2} conditions. At this temperature, increased $P_{CO_2,e}$ due to elevated seawater P_{CO_2} conditions was accompanied by an increase in $[HCO_3^-]_e$, resulting in complete buffering of pH_e (Fig. 1). However, at 17°C, haemolymph bicarbonate buffering was compromised, resulting in a significant decrease in pH_e at $P_{CO_2,e}$ conditions mimicking CCS leakage scenarios, and a more moderate decrease in pH_e under OA conditions. This is similar to the breakdown of acid–base buffering capacity reported in adult spider crab *Hyas araneus* when exposed to elevated P_{CO_2} and temperature levels, despite effective buffering of the acid–base balance when exposed to elevated P_{CO_2} at control temperature (Zittier et al., 2013). $[HCO_3^-]_e$ regulation from the surrounding seawater is achieved via Na^+/H^+ and Cl^-/HCO_3^- exchange found in the gills, with H^+ -ATPase and Na^+/K^+ -ATPase playing a key role (Wheatly and Henry, 1992; Freire et al., 2008). Subsequently, acid–base buffering capabilities have been linked to ionic and osmotic regulatory capabilities (Widdicombe and Spicer, 2008; Melzner et al., 2009). Changes in haemolymph metabolic profiles in *C. maenas* exposed to elevated P_{CO_2} were similar to those observed under hypo-osmotic stress (Hammer et al., 2012). Lobsters are osmoconformers in seawater and possess poor osmo-regulatory ability (Lucu and

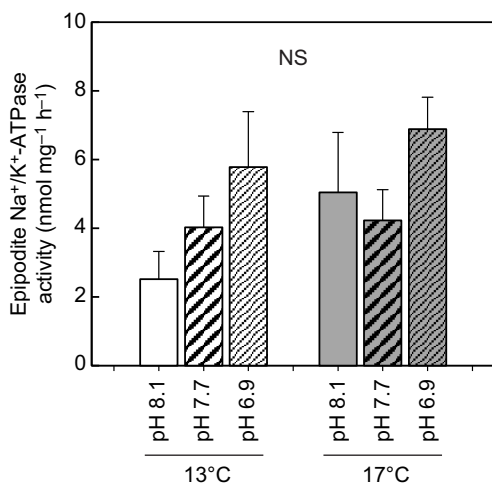


Fig. 3. Effect of exposure to elevated temperature and decreased pH on mean epipodite Na^+/K^+ -ATPase activity of early benthic juvenile *H. gammarus*. Bar colour indicates temperature treatment (white, 13°C; grey, 17°C); striping indicates pH treatment (no stripes, pH 8.1; thick stripes, pH 7.7; thin stripes, pH 6.9). NS, not significant.

Table 2. Haemolymph biochemistry of early benthic juvenile European lobster, *Homarus gammarus*, after a 2 week exposure to elevated temperature and decreased pH

Haemolymph ions and biochemistry	13°C			17°C		
	pH 8.1	pH 7.7	pH 6.9	pH 8.1	pH 7.7	pH 6.9
[Ca ²⁺] _e (μmol l ⁻¹)	11.27±0.3	11.12±0.5	11.14±0.4	10.95±0.6	12.39±0.4	12.13±0.2
[Mg ²⁺] _e (μmol l ⁻¹)	33.34±2.8	29.70±3.2	35.54±2.0	29.10±3.0	27.90±2.3	34.16±2.8
[Cu ²⁺] _e (μmol l ⁻¹)	0.10±0.03 ^A	0.11±0.03 ^A	0.10±0.02 ^A	0.19±0.06 ^B	0.15±0.03 ^B	0.13±0.02 ^B
[Na ⁺] _e (μmol l ⁻¹)	385±6 ^A	373±3 ^B	383±5 ^A	395±9 ^A	374±5 ^B	384±5 ^A
[K ⁺] _e (μmol l ⁻¹)	13.0±1.9	14.8±2.1	15.0±3.1	18.2±2.7	16.0±2.4	18.9±2.3
[protein] _e (mg ml ⁻¹)	3.93±0.96 ^A	5.46±1.58 ^A	4.75±0.75 ^A	9.21±2.79 ^B	6.31±0.95 ^B	6.92±1.49 ^B
[L-lactate] _e (mmol l ⁻¹)	1.91±0.66	1.28±0.22	1.64±0.40	2.67±1.36	1.82±1.17	1.89±0.76

Data are means±s.e.m. Superscript capital letters indicate significant differences between treatments ($P<0.05$).

Devescovi, 1999; Charmantier et al., 2001), and in the present study we find a limited increase in Na⁺/K⁺-ATPase activity under elevated P_{CO_2} . This indicates that when exposed to hypercapnic water, other ion exchange channels not measured here such as carbonic anhydrase drive the uptake of HCO₃⁻ from the surrounding medium. The short exposure time of the present study (14 days) suggests that at 13°C, *H. gammarus* juveniles can effectively cope with extreme levels of P_{CO_2} ; however, over longer time frames this may come at some cost, e.g. carapace demineralisation and subsequent increase in moult-related mortalities (Small et al., 2016b; Swiney et al., 2016), as seen in other marine invertebrates (Wood et al., 2008, 2010).

Energy metabolism

Haemolymph acid–base regulation, whether via carapace dissolution or acquisition from the surrounding water, is presumed to be an energetically expensive process and therefore may have energetic repercussions even in species that possess good regulatory capabilities (Pörtner et al., 2004; Whiteley, 2011). The maintenance of internal homeostasis and processes under unfavourable conditions often results in costs associated with other traits, which in turn helps to define an organism's performance and fitness levels (Calow and Forbes, 1998; Turner et al., 2015). In the present study, juvenile lobsters at 13°C maintained metabolic rate when exposed to elevated P_{CO_2} despite increased haemolymph acid–base regulation. While it may therefore seem that ocean acidification has no effect on energy metabolism of lobsters, the absence of any increase in metabolism could indicate a reallocation of the energy budget toward ATP-demanding activities, such as activity and/or protein and lipid synthesis/turnover (Langenbuch and Pörtner, 2002; Dissanayake et al., 2010; Dissanayake and Ishimatsu, 2011). Pan et al. (2015) demonstrate that while larvae of the sea urchin *S. purpuratus* do not exhibit a response to ocean acidification in terms of metabolic rate, the investment of ATP into protein synthesis and ion regulation increased from ~50% under control conditions to ~85% under acidified conditions.

The absence of a change in metabolic rate in this instance could suggest that ATP availability remains constant despite changes in other processes. Ultimately, this may result in reductions in other longer-term energy demands (Pörtner et al., 2004; Pan et al., 2015), including reductions in growth and survival (Long et al., 2013; Small et al., 2016b) and reproduction (Kurihara et al., 2004), as seen in other phyla (Michaelidis et al., 2005; Wood et al., 2008; Beniash et al., 2010; Stumpp et al., 2012). Indeed, early benthic juveniles of the American lobster, *Homarus americanus*, also showed no change in metabolic rate with decreasing pH despite changes in metabolic apparatus (Menu-Courey et al., 2019). In the present study, feeding rate also decreased as a result of exposure to elevated P_{CO_2} at 13°C. Decreased feeding would bring a reduction to the overall energy budget of the

organism, limiting the amount of energy available for the above-mentioned trade-offs for increased acid–base regulation. Feeding rate has been demonstrated to be reduced in decapod crustacean species in response to elevated P_{CO_2} , including *H. gammarus* (Small et al., 2016b) and *C. maenas* (Appelhans et al., 2012).

The interaction of elevated temperature and elevated P_{CO_2} is rather more complicated. Firstly, metabolic rate was temperature insensitive in juvenile lobsters. Within optimal temperature ranges, metabolic rate increases as temperature increases as a result of the increase in cell kinetics as well as energy demand. However, once passed the pejus limits (*sensu* Pörtner and Farrell, 2008), metabolic rate ceases to rise or even decrease as temperature increases (Dehnel, 1960; Schatzlein and Costlow, 1978; Vernberg et al., 1981; Anger, 1987; Storch et al., 2009a,b). It is important to note that measurements of metabolic rate in the present study represent a measurement for juvenile lobster routine metabolism rate. Consequently, the lack of increase in oxygen consumption between temperature treatments probably indicates a mismatch in energy supply and demand, resulting in energy limitation of early benthic juvenile *H. gammarus* at 17°C, which is enhanced by the decrease in food consumption observed at elevated temperature. This in turn may explain the absence of the bicarbonate buffering response at 17°C, as juveniles have less available energy to satisfy the demands of acid–base regulation. Foregut clearance rates of juvenile lobsters are estimated at 80% clearance in 2 h and lobsters require an almost constant supply of food (Mente et al., 2001). It is therefore possible that the absence of temperature effects could be due to the feeding trials being too short (~1 h). This is only enough time for only one foregut fill. Further examination of lobster feeding rates and gut clearance times is required to better understand temperature and P_{CO_2} effects on food consumption.

Haemolymph biochemistry

Changes in energy metabolism and haemolymph homeostasis of early benthic juvenile lobsters reported in this study can partially explain the energy limitations of this stage with regards to exposure to elevated temperature and P_{CO_2} . Compared with adult lobsters under normocapnic conditions, juvenile lobsters exhibit higher levels of haemolymph L-lactate and Mg²⁺, but much lower levels of haemolymph pH, HCO₃⁻ and Cu²⁺ (Taylor and Whiteley, 1989; Whiteley and Taylor, 1990). However, these lobsters also experienced a decrease in [Na⁺]_e under OA, but not CCS conditions, regardless of exposure temperature. Haemolymph cation concentrations increase under elevated seawater P_{CO_2} conditions in a range of decapod crustaceans (e.g. Maus et al., 2018; Small et al., 2010; Spicer et al., 2007); thus, [Na⁺]_e is implicated in acid–base regulation. The reason for this is not clear (Hans et al., 2014) but probably indicates upregulation in Na⁺/K⁺-ATPase activity (see Turner et al., 2015).

Why the $[Na^+]_e$ reported in the present study does not follow this trend of increasing with increasing seawater P_{CO_2} is unclear and needs further investigation but may be indicative of a hermitic response to seawater P_{CO_2} coupled with Na^+/K^+ -ATPase activity, while trending upwards, not being significantly different and thus probably not the primary route of $[HCO_3^-]$ regulation. Compared with those of later stage juveniles (WBM 40–50 g), haemolymph protein levels of juveniles in the present study are considerably lower (Hagerman, 1983). The reason for the low haemolymph pH values is not clear, but it may be due to lower protein (haemocyanin) concentrations, which may reduce the non-bicarbonate buffering capacity of the haemolymph. Therefore, it is possible that the high L-lactate levels in juvenile lobsters described in this study, when compared with those of crabs and adult lobsters (Taylor and Whiteley, 1989; Whiteley and Taylor, 1990; Watt et al., 1999), may indicate greater reliance on anaerobic metabolism and but would also result in an allosteric increase in haemocyanin oxygen affinity, improving oxygen uptake and transport (Bridges, 2001). While changes to haemolymph homeostasis during crustacean ontogeny have not yet, to our knowledge, been explored in relation to sensitivity to elevated P_{CO_2} , it is suggested that they will result in changing sensitivity as lobsters develop.

Conclusions

During the short exposure period used in this study (14 days), early juvenile *H. gammarus* exhibited successful acid–base regulatory capabilities at 13°C when exposed to both OA and CCS seawater P_{CO_2} conditions. Such successful extracellular buffering capacity is perhaps unsurprising, as juvenile lobsters can inhabit a range of soft benthic sediments, including burrows they excavate beneath stones in sandy sediments and complex tunnel networks they can construct in fine mud sediments (D.P.S., personal observation). These burrow environments are potentially higher in P_{CO_2} and lower in pH than the surrounding seawater (Widdicombe et al., 2009, 2011). Given that other crustacean species which permanently reside in sediment burrows, such as the burrowing shrimp, *Upogebia deltaura*, also have low pH_e and exhibit a high level of tolerance to elevated P_{CO_2} conditions (Donohue et al., 2012), it seems reasonable to assume that the physiological response of juvenile *H. gammarus* to elevated P_{CO_2} has potentially evolved under the selective pressure to cope with the environmental conditions imposed by this habitat. How juvenile lobster can sustain exposure to elevated P_{CO_2} over the long term is currently unclear, especially given how energetically expensive this process can be. The high energy demand required to maintain acid–base balance, coupled with the early benthic juvenile *H. gammarus* being an energetically limited stage, may explain the high rates of moult death observed in Small et al. (2016b), and the breakdown of acid–base buffering capacity at elevated temperatures. We recommend longer-term studies to further assess the potential impacts of elevated P_{CO_2} levels upon lobster development, and ultimately recruitment to the adult stages and thus the population dynamics during the potentially vulnerable period between the end of the pelagic larval development phase and the transition to benthic-living juveniles.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: D.P.S., P.C., S.W., J.I.S.; Methodology: D.P.S., P.C., S.P.S.R., L.M.T., S.W., J.I.S.; Formal analysis: D.P.S.; Investigation: D.P.S., S.P.S.R., L.M.T.; Resources: P.C.; Writing - original draft: D.P.S., P.C., S.P.S.R., L.M.T., J.I.S.; Writing - review & editing: D.P.S., P.C., S.P.S.R., L.M.T., S.W., J.I.S.; Visualization: S.W., J.I.S.; Supervision: P.C., S.W., J.I.S.; Project administration: S.W., J.I.S.; Funding acquisition: P.C., S.W., J.I.S.

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