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RESEARCH ARTICLE

Living in warmer, more acidic oceans retards physiological recovery from tidal emersion in the velvet swimming crab, *Necora puber*

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ABSTRACT

The distribution patterns of many species in the intertidal zone are partly determined by their ability to survive and recover from tidal emersion. During emersion, most crustaceans experience gill collapse, impairing gas exchange. Such collapse generates a state of hypoxemia and a hypercapnia-induced respiratory acidosis, leading to hyperlactaemia and metabolic acidosis. However, how such physiological responses to emersion are modified by prior exposure to elevated CO_2 and temperature combinations, indicative of future climate change scenarios, is not known. We therefore investigated key physiological responses of velvet swimming crabs, *Necora puber*, kept for 14 days at one of four pCO_2/temperature treatments (400 µatm/10°C, 1000 µatm/10°C, 400 µatm/15°C or 1000 µatm/15°C) to experimental emersion and recovery. Pre-exposure to elevated pCO_2 and temperature increased pre-emersion bicarbonate ion concentrations [HCO_3^-], increasing resistance to short periods of emersion (90 min). However, there was still a significant acidosis following 180 min emersion in all treatments. The recovery of extracellular acid–base via the removal of extracellular pCO_2 and lactate after emersion was significantly retarded by exposure to both elevated temperature and pCO_2. If elevated environmental pCO_2 and temperature lead to slower recovery after emersion, then some predominantly subtidal species that also inhabit the low to mid shore, such as *N. puber*, may have a reduced physiological capacity to retain their presence in the low intertidal zone, ultimately affecting their bathymetric range of distribution, as well as the structure and diversity of intertidal assemblages.

KEY WORDS: Climate change, Ocean acidification, OA, Crustacea, Acid–base balance, Lactate

INTRODUCTION

Since pre-industrial times, global mean atmospheric carbon dioxide partial pressure (pCO_2) has increased from ~300 to 400 µatm and is predicted to rise to 800 µatm or more by the end of the century [‘business-as-usual’ CO_2 emission scenario (Houghton et al., 2001)]. Because of the insulating effect of CO_2 in the atmosphere, this is predicted to increase global sea-surface temperatures by 4–5°C (Sokolov et al., 2009). Furthermore, almost half (48%) of the anthropogenically derived CO_2 produced to date has been absorbed by the oceans (Sabine et al., 2004). This has altered the carbonate system, resulting in a 30% increase in H^+ concentration (0.1 pH unit) and a 16% decrease in carbonate ion concentrations (Feely et al., 2004; Fabry et al., 2008). This phenomenon, termed ocean acidification (OA), is predicted to reduce ocean pH (currently 8.1) to 7.8–7.7 by the end of the century (Caldeira and Wickett, 2003). The future survival and distributions of marine species will, in part, be determined by species-specific levels of physiological responses to these environmental changes (e.g. Stillman et al., 2008; Pörtner and Farrell, 2008; Melzner et al., 2009; Rastrick and Whiteley, 2011; Rastrick and Whiteley, 2013; Calosi et al., 2013a; Calosi et al., 2013b). These responses define the range over which cellular homeostasis can be maintained in response to elevated pCO_2 and temperature. In part, this involves adjustments in acid–base balance, which are crucial to maintaining protein stability and enzyme function (Somero, 1986). Changes in extracellular acid–base balance are dependent on respiratory [i.e. changes in extracellular pCO_2 (PCO_2)] and metabolic (i.e. changes in metabolic proton production, e.g. anaerobic lactate fermentation) processes. CO_2 produced in the mitochondria via respiration diffuses into the cytoplasm and then into the extracellular fluid, where it hydrates and dissociates into HCO_3^- and H^+, decreasing extracellular pH (pHe). In many marine invertebrates, including crustaceans, acid–base homeostasis is maintained via the constant diffusion of respiratory CO_2 across the gills into the surrounding water, although this can be impeded by increases in environmental pCO_2 associated with OA (Whiteley, 2011; Melzner et al., 2013).

Predicted changes in environmental temperature may also affect the acid–base regulation of marine ectotherms, with pHe decreasing at higher temperatures because of increased ionisation at higher kinetic energies. This relationship has been reported in a number of crustaceans under controlled conditions, with gradients ranging from −0.015 to −0.023 (pH units °C^{-1}) (reviewed by Whiteley, 1999), and could lead to a decrease in organismal pHe of between 0.06 and 0.115 units by the end of the century, based on a temperature increase of 4–5°C. Although these changes, as predicted by the alpha-stat hypothesis (Reeves, 1972), are natural and help maintain protein ionisation and thus function (Reeves, 1972; Cameron, 1989), little is still known about how this drop in pHe may affect pH sensitive pathways. Temperature also affects the kinetics of metabolism, affecting rates of O_2 uptake and CO_2 excretion (Rahn, 1966). At higher temperatures this can lead to a mismatch between O_2 supply and demand, leading to metabolic acidosis. Such acidosis could be exacerbated by increased environmental and respiratory PCO_2, leading to hypercapnia and respiratory acidosis that, in turn, may affect aerobic scope and thermal tolerance, and potentially therefore the future distribution of species (Pörtner and Farrell, 2008; Calosi et al., 2013a; Calosi et al., 2013b; Melzner et al., 2013).

During short- to medium-term disturbances in acid–base status, crustaceans buffer pHe via increases in extracellular [HCO_3^-]

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($[HCO_3^-]e$), whilst in the longer term, protein buffering is probably employed [mainly using haemocyanin (see Whiteley, 2011)]. During immersion, pH compensation is accomplished by ion exchange across the gill epithelia with 93% of $[HCO_3^-]e$ being taken up from the surrounding seawater, and only 7% coming from internal stores (Cameron, 1985). This involves branchial pumping mechanisms, such as Na^+/K^+-ATPase, that maintain haemolymph osmotic and acid–base homeostasis when exposed to disturbances via variations in abiotic drivers, such as salinity, pH, pCO_2, pO_2 and temperature (Wood and Cameron, 1985; Whiteley, 1999; Whiteley et al., 2001; Freire et al., 2003; Lucu and Towle, 2003; Masui et al., 2005; Mendonça et al., 2007; Santos et al., 2007; Masui et al., 2009). These are characteristic of environments typified by rapidly fluctuating physico-chemical factors, such as the intertidal (Newell, 1979), and may be more pronounced in the future (IPCC, 2013). This has led to the suggestion that strong ion-regulators/osmoregulators that are more typically found in the intertidal zone may be more tolerant to future climate change than weak ion-regulators/osmoconformers (Widdicombe and Spicer, 2008; Melzner et al., 2009; Whiteley, 2011).

At present, most studies have focused on either elevated pCO_2 or temperature in isolation (e.g. Spicer et al., 2007; Small et al., 2010; cf. Melzner et al., 2013), whilst comparatively few studies have investigated the combined effects of multiple climate drivers on marine invertebrates' physiological responses (e.g. Dissanayake and Ishimatsu, 2011; Melatunan et al., 2011; Calosi et al., 2013c). In addition, previous studies have not accounted for the combined effects of routine acid–base disturbance associated with living in the intertidal. Most subtidal or low-intertidal crustaceans experience gill collapse during emersion, which impairs gas exchange, leading to hypoxia, hypercapnia and respiratory acidosis (e.g. Truchot, 1975; Taylor and Innes, 1988; Taylor and Whiteley, 1989). This is accompanied by systemic hypoxia (hypoxemia) and increased anaerobic lactate fermentation, leading to hyperlactaemia and metabolic acidosis (e.g. Taylor and Whiteley, 1989). At higher temperatures this can even be exacerbated by increased metabolic demand (Rahn, 1966). In addition, as during emersion, the branchial route of ion exchange is impaired and $[HCO_3^-]$ buffering must be sourced by mobilising HCO_3^- from exoskeletal $CaCO_3$ (e.g. Truchot, 1975; Taylor and Whiteley, 1989; Taylor and Spicer, 1991), an ability that, in part, determines emersion resistance and possibly intertidal distribution (Whiteley, 1999). The ability to recover acid–base homeostasis following re-immersion, which involves the removal of PCO_2 and lactate before the next low tide, may also play a role in defining species' intertidal distributions, with species whose distribution can extend up to the high shore (e.g. *Carcinus maenas*) exhibiting enhanced pathways for lactate metabolism via gluco- and glyconeogenesis (Johnson and Uglow, 1985; Johnson and Uglow, 1987). However, to date, it is not known how these physiological responses that allow crustaceans to occur in the intertidal zone and survive routine acid–base disturbances associated with emersion are affected by additional acid–base disturbance associated with OA and warming.

Therefore, the aim of this study was to investigate how future climate change scenarios might affect the physiological responses of a species that occurs in the intertidal, when a realistic period of emersion followed by re-immersion is considered. We exposed individuals of the velvet swimming crabs, *Necora puber* (Linnaeus 1767), to future predicted OA and warming scenarios and then investigated the effect of emersion and re-immersion on their acid–base physiology.

Necora puber is an ecologically important member of rocky shore intertidal assemblages (Silva et al., 2008; Griffin et al., 2008). Despite being a relatively weak ion-regulator/osmoconformer, *N. puber* can maintain its extracellular acid–base status when immersed during short-term exposure to pCO_2 values higher than those predicted for the end of the century *via* $[HCO_3^-]e$ buffering associated with active ion-exchange with seawater, and is therefore considered a 'tolerant' species (Spicer et al., 2007; Small et al., 2010). *Necora puber* is most common subtidally, but also inhabits the low to mid intertidal, where it experiences emersion-related fluctuations in environmental pH and temperature (Newell, 1979). Like other decapods that are predominantly subtidal, but can inhabit the intertidal, *N. puber* experiences gill collapse and has a limited ability to mobilise $[HCO_3^-]$ from its exoskeleton when emersed, with metabolic acidosis proving fatal after just 4 h of air exposure (Whiteley, 1999). While some decapods that can occur higher up on the shore (e.g. *C. maenas*) can show full acid–base compensation during emersion (Truchot, 1975), many species (such as *N. puber*) that are also considered 'tolerant' to OA lack such an adaptation. Thus it is possible that low-shore species presently considered to be relatively 'tolerant' to climate change may actually be greatly affected by global change drivers when exposed to routine acid–base disturbances associated with the natural intertidal systems.

RESULTS

Acid–base parameters following acclimatisation

Values for haemolymph pH (pHe), total dissolved and bound CO_2 (TCO_2), pCO_2 (PCO_2) and $[HCO_3^-]$ ($[HCO_3^-]e$) measured and calculated after 2 weeks of previous exposure to different combinations of elevated pCO_2 and temperature are presented in Table 1. There were no significant effects of elevated pCO_2 and temperature on pHe and PCO_2 (P>0.05). However, mean $[HCO_3^-]e$ buffering was greater in elevated compared with ambient pCO_2 treatments ($F_{1,48}$=32.15 P<0.001), and this difference was more pronounced at elevated temperature ($F_{1,48}$=4.40, P<0.05). There was no significant difference in non-bicarbonate buffering between treatments with non-bicarbonate buffer lines ranging from 1.9 to 2.9 mmol l^{-1} 0.1 pH $^{-1}$ (P>0.05).

Acid–base parameters during emersion and recovery

Fig. 1 shows pHe, $[HCO_3^-]e$ and PCO_2 values for *N. puber* during 180 min of emersion and following 420 min of re-immersion recovery, for crabs exposed for 2 weeks to each pCO_2 temperature

Table 1. Haemolymph pH (pHe), $[HCO_3^-]$ ($[HCO_3^-]e$) and pCO_2 (PCO_2), and total dissolved and bound CO_2 (TCO_2) in *Necora puber* following 2 weeks exposure to each of the four pCO_2/temperature treatments

Nominal treatment	400 µatm pCO_2/10°C	400 µatm pCO_2/15°C	1000 µatm pCO_2/10°C	1000 µatm pCO_2/15°C
pHe	7.84±0.05	7.89±0.07	7.87±0.06	7.95±0.05
$[HCO_3^-]e$ (mmol l^{-1})	6.56±0.42^{A}	7.14±0.64^{A}	9.19±0.64^{B,1}	11.03±0.75^{B,2}
PCO_2 (kPa)	0.29±0.04	0.30±0.06	0.38±0.06	0.37±0.05
TCO_2 (mmol l^{-1})	6.68±0.43^{A}	7.25±0.45^{A}	9.36±0.64^{B,1}	11.17±0.76^{B,2}

Values are means ± s.e.m. Significant differences between pCO_2 treatments are represented by different letters, and significant differences between temperature treatments are represented by different numbers (F-test, P<0.05).

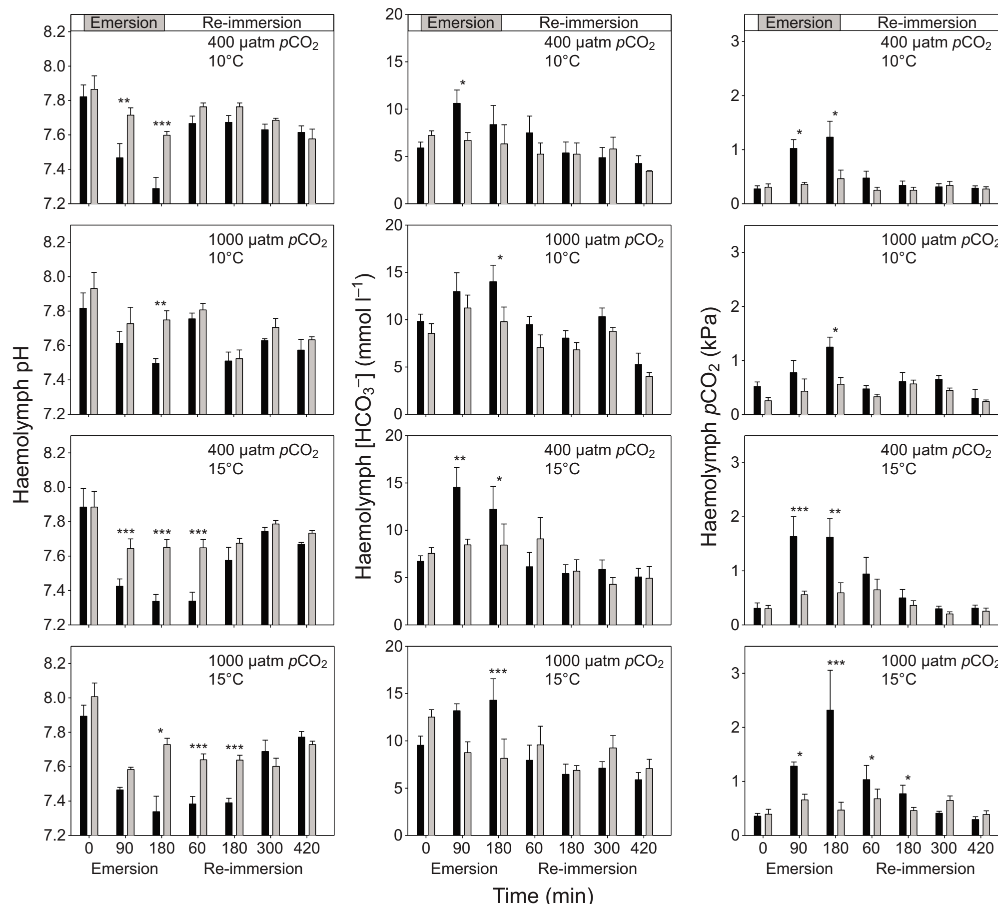

Fig. 1. Haemolymph pH, pCO_2 (kPa) and $[HCO_3^-]$ (mmol l^{-1}) in *Necora puber* as a function of time (min) in emersed/re-immersed experimental crabs (black bars) and immersed control crabs (grey bars) for each of the acclimatisation treatments. The acclimatisation temperature and pCO_2 are shown in the top right corner of each graph. Horizontal bars at the top of the figure represent the emersion (grey) and re-immersion (white) phases for the experimental crabs. Bars show means ± s.e.m. Significant increases in haemolymph pCO_2 and $[HCO_3^-]$ or decreases haemolymph in pH in emersed/re-immersed crabs compared with control crabs at a given time are indicated by asterisks (*P<0.05; **P<0.01; ***P<0.001). Pairwise comparisons are based on F-tests generated from the estimated marginal means of the repeated-measures ANCOVA and adjusted for multiple comparisons using Bonferroni correction (α<0.05).

combination. Across all treatments there was a significant emersion-related reduction in pHe and significant increases in PCO_2 and $[HCO_3^-]$e. During re-immersion, mean pHe, and PCO_2 and $[HCO_3^-]$e all returned to values not significantly different from the control crabs. However, the onset of acidosis during emersion and rates of recovery following re-immersion differed amongst the pCO_2/temperature treatments.

During emersion, pHe significantly decreased in all treatments ($F_{2,24}$=49.56, P<0.001; Fig. 1). pHe also exhibited some variation in the control groups ($F_{2,24}$=14.75, P<0.01), possibly as a result of repeated sampling; however, this decrease was significantly smaller than in the emersed crabs ($F_{2,48}$=5.50, P<0.01; Fig. 1). To account for this, all further comparisons between treatments are based on the statistical difference in responses between emersed crabs and immersed control crabs with time. The significantly greater decreases in pHe of emersed compared with control crabs during emersion was significantly affected by treatment pCO_2, with pHe decreasing more slowly at higher pCO_2, shown by a significant interaction between the terms 'emersion' (emersed or control crabs), 'time' and 'pCO_2' ($F_{2,48}$=3.43, P<0.05). PCO_2 also increased significantly during emersion compared with control crabs ($F_{2,48}$=3.45, P<0.05), although this was not significantly affected by treatment. After emersion, the highest PCO_2 levels were recorded in the elevated pCO_2 and temperature treatment; despite this, pHe was not significantly lower in this treatment at the end of emersion (P>0.05). This is associated with a significant increase relative to controls in $[HCO_3^-]$e during emersion, with concentrations rapidly returning to those of control crabs within 60 min of re-immersion ($F_{6,48}$=6.504, P<0.001). Although increases in $[HCO_3^-]$e were limited to ~5 mmol l^{-1} in all treatments during emersion, higher pre-emersion $[HCO_3^-]$e led to significantly higher $[HCO_3^-]$e with elevated pCO_2 and temperature ($F_{1,24}$=4.96, P<0.05).

In the elevated pCO_2 treatments, the recovery time for mean pHe (i.e. the time necessary for mean pHe of crabs that were emersed and then re-immersed and control crabs, that were continuously immersed, to show no statistical difference) was delayed (<60 min). Furthermore, recovery time following re-immersion was longer in individuals kept at elevated temperature (60 min for ambient pCO_2 and 120 min in elevated pCO_2) when compared with those kept under low-temperature conditions, which recovered within 60 min of re-immersion. This complex pattern in the response of mean pHe was identified by the presence of a four-way interaction between the terms 'time', 'emersion', 'pCO_2' and 'temperature' ($F_{4,48}$=2.83, P<0.05).

In the elevated temperature and pCO_2 treatment, recovery of PCO_2 following emersion also took longer compared with all other treatments, with PCO_2 in emersed crabs taking on average 240 min longer to decrease to control levels. All other crabs recovered within 60 min of re-immersion. This is evidenced by the significant four-way interaction between 'emersion', 'time', 'pCO_2' and 'temperature' ($F_{4,48}$=3.06, P<0.05).

Haemolymph lactate concentrations during emersion and recovery

Haemolymph lactate concentrations ([lactate]e) increased during emersion but, with the exception of the elevated temperature and pCO_2 treatment, decreased to control values after re-immersion (Fig. 2). In the elevated pCO_2 treatments, [lactate]e remained significantly higher in emersed compared with control crabs for at least 240 min longer during recovery, detectable as a significant

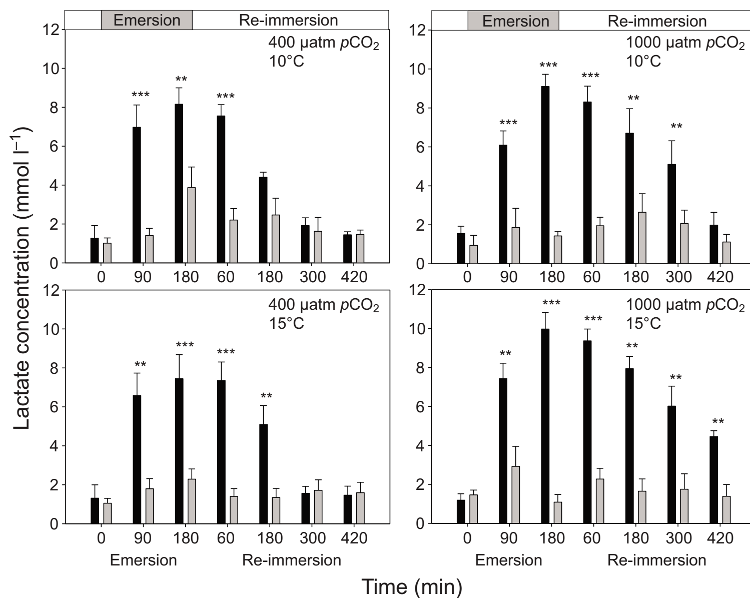

Fig. 2. Haemolymph lactate concentrations (mmol l^{-1}) in *N. puber* as a function of time (min) for emersed/re-immersed experimental crabs (black bars) and immersed control crabs (grey bars) for each of the acclimatisation treatments. The acclimatisation temperature and pH are shown in the top right corner of each graph. Horizontal bars at the top of the figure represent the emersion (grey) and re-immersion (white) phases for the experimental crabs. Bars show means ± s.e.m. Significant increases in the haemolymph lactate concentrations of emersed/re-immersed crabs compared with control crabs at any given time are indicated by asterisks (**P<0.01; ***P<0.001). Pairwise comparisons are based on F-tests generated from the estimated marginal means of the repeated-measures ANCOVA and adjusted for multiple comparisons using Bonferroni correction (α<0.05).

three-way interaction between the terms 'emersion', 'time' and 'pCO$_2$' ($F_{6,48}$=3.01, P<0.05). Recovery from reduced pHe and elevated PCO$_2$ coincided with the recovery of [lactate]e and took longest in the elevated temperature and pCO$_2$ treatment, where lactate concentrations remained significantly above control levels even after 420 min ($F_{1,48}$=11.28, P<0.01) of re-immersion.

DISCUSSION

Previous studies have suggested an inherent tolerance of intertidal organisms to predicted future pCO$_2$ and temperature conditions (Widdicombe and Spicer, 2008; Melzner et al., 2009; Whiteley, 2011), generally because of the presence of physiological mechanisms that allows them to survive in a eurythermal and eurycapnic environment (e.g. Stillman, 2008; Whiteley et al., 2011). This was found previously to be the case also for the swimming crab *N. puber* (Spicer et al., 2007; Small et al., 2010). Here, in common with Small et al. (Small et al., 2010), we describe in *N. puber* complete acid–base compensation following mid-term acclimatisation (in our case 2 weeks) to high pCO$_2$. Interestingly, this compensation, achieved via [HCO_3^-] buffering, may increase the resistance of this species to extracellular acidosis during short periods of emersion (<90 min) at both control and elevated temperatures. However, importantly, following an 'ecologically realistic' emersion of 180 min, all treatments showed metabolic and respiratory acidosis, and the recovery of acid–base homeostasis was significantly delayed by both increased seawater pCO$_2$ and temperature.

Below we discuss the onset and recovery from respiratory and metabolic acidosis during routine emersion and re-immersion before going on to consider how these acid–base parameters were adjusted by exposure to elevated pCO$_2$ and temperature conditions. Finally, we suggest some putative mechanisms that may underlie these adjustments and may affect intertidal distribution.

Emersion

After 3 h emersion, the pHe of *N. puber* decreased by 0.3–0.6 pH units in all treatments, and was similar, though not as pronounced, to that found by Whiteley (Whiteley, 1999) for this species (0.8 pH units after 4 h emersion). This emersion-related reduction in pHe was also comparable to that observed after 2 h emersion in the spider crab *Maja squinado* (Taylor and Innes, 1988) and 14 h emersion in the edible crab *Cancer pagurus* (Whiteley, 1999). The latter, like *N. puber*, is a weak ion-regulator/osmoconformer that is predominantly a subtidal species that also occurs in the low intertidal. In contrast, high-shore species such as *Carcinus maenas* can show fully compensated hypercapnic acidosis after 100 h emersion. This is, in part, due to the fact that this species possesses strengthened gill lamellae that do not collapse when not supported by water, thus allowing some gas exchange to continue when immersed (Truchot, 1975). However, in most intertidal and subtidal species, the collapse of gill lamellae during emersion compromises gas exchange (e.g. DeFur and McMahon, 1984). Accordingly, in the present study, PCO$_2$ increased 2.5- to 6.5-fold during 3 h of emersion, a value consistent with that reported by Whiteley (Whiteley, 1999) for *N. puber* (sixfold after 4 h of emersion) and by Taylor and Innes (Taylor and Innes, 1988) for *M. squinado* (after 2 h of emersion).

As well as compromising CO$_2$ excretion, leading to extracellular hypercapnia and respiratory acidosis, gill collapse during emersion also limits O$_2$ diffusion, resulting in hypoxemia and metabolic acidosis as a result of hyperlactaemia. Haemolymph lactate concentrations in the present study increased 3.8–8.8 mmol l^{-1} above control levels after 3 h emersion. A similar increase in lactate levels was reported for *Homarus gammarus* (Taylor and Whiteley, 1989) and the freshwater crayfish *Austropotamobius pallipes* (Taylor and Wheatly, 1980). Indeed, the importance of both respiratory (increased PCO$_2$) and metabolic (increased L-lactate) components to acidosis is evident from the modified Davenport diagrams (Fig. 3), with haemolymph acid–base adjustments occurring along or below the non-bicarbonate buffer line. This may also suggest the importance of non-bicarbonate (protein) buffering, particularly in species such as *N. puber* that are poor at sourcing [HCO_3^-]e during emersion. Protein buffers are important in routinely active crustaceans such as *N. puber*, as they help buffer acid–base disturbances as a result of increased [lactate]e during anaerobic exercise (Watt et al., 1999; Melzner et al., 2009; Whiteley, 2011). As haemocyanin accounts for 80–95% of haemolymph protein (Truchot, 1978; Claybrook, 1983), high protein levels in *N. puber* compared with other decapods are also associated with increased oxygen transport efficiency, decreasing basal levels of anaerobic metabolism and thus [lactate]e (Watt et al., 1999). However,

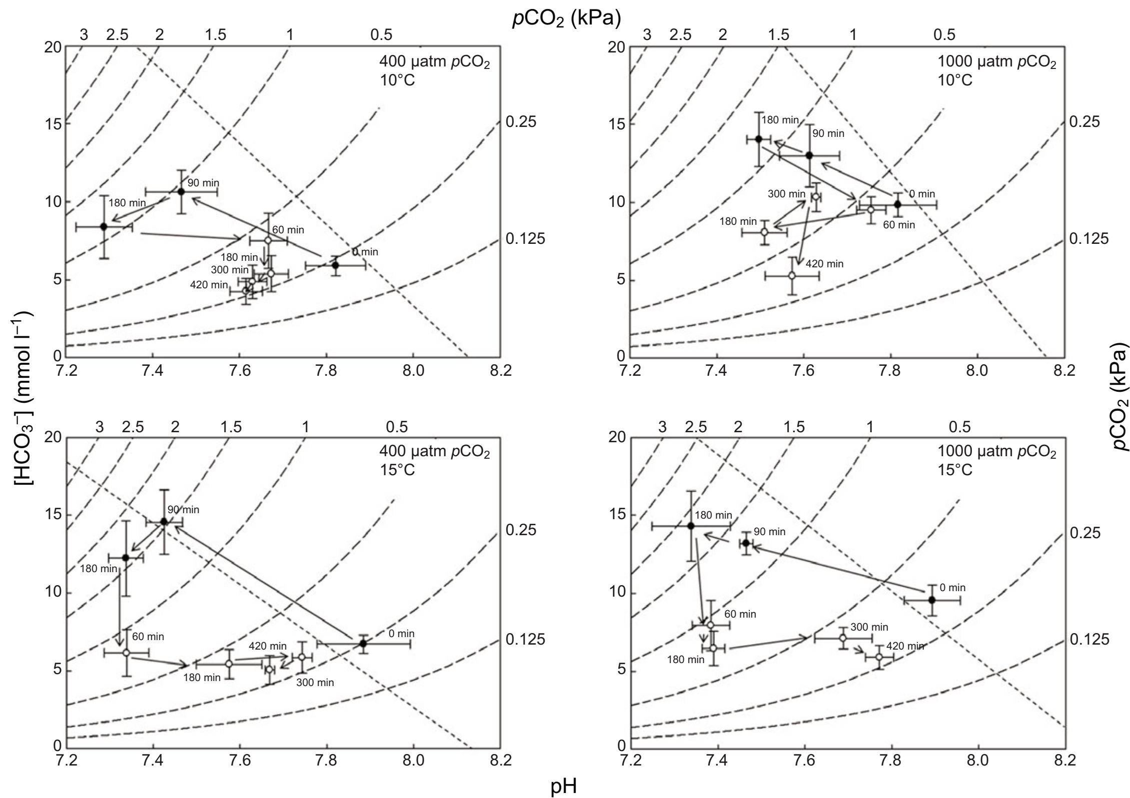

Fig. 3. Davenport diagrams showing the relationships between haemolymph pH (pH units), pCO_2 (kPa) and $[HCO_3^-]$ (mmol l^{-1}) in *N. puber* during emersion (filled circles) and re-immersion (open circles) in each of the acclimatisation treatments. The acclimatisation temperature and pH are shown in the top right corner of each diagram. Time (min) next to each point shows the duration of emersion or re-immersion. Dashed diagonal line represents the non-bicarbonate buffer line determined for *N. puber* in each treatment. All data points are means ± s.e.m. (α<0.05).

in the present study, 2 weeks acclimatisation to elevated temperature and pCO_2 had no significant effect on the non-bicarbonate buffering capacity, suggesting that, at least in the short-term, differences between treatments are driven by compensatory changes in $[HCO_3^-]$e (discussed below) (Spicer et al., 2007). However, it is possible that protein buffering would become more important with longer exposure (Small et al., 2010; Whiteley, 2011; Donohue et al., 2012). Between treatments, the highest levels in both [lactate]e and PCO_2 during emersion were measured in the combined elevated pCO_2 and temperature treatment, probably because of increased metabolic demand at high temperature. However, despite these higher levels of lactate and PCO_2, there was no significant difference in pH between treatments after 180 min. The rate of the onset of extracellular acidosis was also slower in the elevated pCO_2 treatment at both temperatures. This is most likely due to the varying availability of internal $[HCO_3^-]$e between treatments. After 2 weeks acclimation to elevated pCO_2 and temperature, *N. puber* showed a significant increase in $[HCO_3^-]$e buffering, suggesting that a new set point in acid–base homeostasis had been reached. In addition to buffering changes in pHe associated with elevated environmental pCO_2, $[HCO_3^-]$e also increased significantly with temperature. This appears to buffer passive changes in pHe associated with the alpha-stat hypothesis (Reeves, 1972; Cameron, 1989; reviewed by Whiteley, 1999).

In *N. puber*, $[HCO_3^-]$ was most likely taken up from the surrounding seawater, via electroneutral ion exchange across the gill epithelia (Whiteley, 1999). Previous studies have shown that increased $[HCO_3^-]$e was not associated with a corresponding increase in Ca^{2+}, suggesting that very little if any additional $[HCO_3^-]$ had originated from the dissolution of $CaCO_3$ structures (Spicer et al., 2007; Small et al., 2010). In species such as *N. puber* that are poor at utilising internal HCO_3^- stores, and so are unable to achieve complete acid–base compensation during routine tidal emersion (Whiteley, 1999), pre-emersion $[HCO_3^-]$e accumulation is likely to be of greater importance. Increased metabolic acidosis between 90 and 180 min of emersion also suggests $[HCO_3^-]$e limitation at control PCO_2 levels (Fig. 3). Therefore, increased $[HCO_3^-]$e buffering capacity associated with acclimatisation to higher pCO_2 and temperature may explain the slower onset of acidosis in these treatments. This suggests that pre-exposure to elevated pCO_2 and temperature are important determinants of compensatory ability during emersion. Pre-exposure to predicted future levels of CO_2 increases *N. puber* resilience to short periods of emersion (up to 90 min) at both temperatures. Despite these changes in pre-emersion $[HCO_3^-]$e levels, crabs still incurred respiratory and metabolic acidosis after 180 min of emersion. It should also be noted that at the site of collection, natural emersion periods routinely exceed 90 min.

Recovery

Recovery from acidosis following re-immersion was also dependent on previous exposure to both elevated seawater pCO_2 and temperature. In crustaceans, the recovery of pHe levels following hypoxia (either environmental or systemic) depends on the species-specific rate of removal of respiratory PCO_2 and metabolic lactate (Whiteley, 1999).

Recovery from metabolic and respiratory acidosis in the present study took between 1 and 5 h, with recovery taking 2 h in crabs acclimatised to elevated temperature and up to 4 h longer in crabs

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acclimatised to both elevated pCO_2 and temperature. This is probably due, at least in part, to the pHe and temperature dependence of haemolymph PCO_2 and lactate removal (see below), the rate of which depends on the levels reached during emersion, as well as the efficiency of the pathways responsible for their removal. Removal rates of PCO_2 may be increased by hyperventilation (e.g. Massabuau et al., 1984; Donohue et al., 2012). In marine crustaceans, like other 'water breathers', hyperventilation is usually considered a response to low O_2 tensions and increased anaerobic metabolism rather than to remove PCO_2, although this is undeniably a consequence (Massabuau et al., 1984; Wheatly and Henry, 1992b). Indeed, it is possible that emersion-related decreases in PO_2, corresponding to elevated hemolymph lactate, may drive hyperventilation upon re-immersion [observed in *A. pallipes* (Taylor and Wheatly, 1980)]. However, acid–base values do not recover along the non-bicarbonate buffer line but below it, i.e. metabolic acidosis persists, particularly at the elevated temperature, suggesting that recovery time is determined more by metabolism (i.e. anaerobic dependence) than respiration (Fig. 3). As the highest levels of PCO_2 and [lactate]e were observed in the combined elevated pCO_2 and temperature treatment, it is not surprising that acid–base status took longer to return to control levels in this treatment. However, the pathways responsible for removal may also be sensitive to changes in pHe and temperature. Removal of PCO_2, and thus recovery from respiratory acidosis, in crustaceans is dependent on the passive diffusion of CO_2 across the gill epithelia and to some extent the action of carbonic anhydrase, the gill enzyme that can catalyse the production of HCO_3^- from CO_2 (e.g. Burnett and McMahon, 1985; Burnett and McMahon, 1987; Wheatly and Henry, 1992a). The recovery of PCO_2 levels in the present study took under an hour in most treatments, which was not inconsistent with recovery from similar PCO_2 levels by *H. gammarus* (Taylor and Whiteley, 1989) and *C. maenas* (Truchot, 1975) following emersion. Because of higher levels of PCO_2 reached during emersion, diffusion gradients were actually higher in the elevated temperature and pCO_2 treatment (2.2 kPa) than in the other treatments (1.2–1.6 kPa). Although this led to faster PCO_2 removal over the first 60 min (1.3 kPa compared with 0.7–0.8 kPa in the other treatments), overall, PCO_2 remained higher than controls up to 4 h longer in this treatment. This may be due, in part, to the higher metabolic rates that crabs experience at elevated temperature, and the associated increased respiratory CO_2 levels during recovery (Melzner et al., 2013).

Recovery from metabolic acidosis following emersion, which is in part reliant on the removal of lactate, occurs on a more protracted time scale than CO_2 removal and appears to be the rate-limiting pathway slowing the recovery of acid–base homeostasis, a pattern also shown following exercise in crabs (e.g. Booth et al., 1985). Here in *N. puber*, lactate recovery times ranged between 3 and over 7 h depending on the treatment (Fig. 2). It has been suggested that the removal of extracellular H^+ ions occurs more rapidly than the removal of lactate H^+, which is retained be the tissues/haemolymph, and additional H^+ ions associated with the intercellular dissociation of lactic acid, which are metabolised more slowly (Booth et al., 1985; Booth and McMahon, 1985). Johnson and Uglow (Johnson and Uglow, 1985; Johnson and Uglow, 1987) suggested that rapid recovery from metabolic acidosis in *C. maenas* following hypoxia was associated with rapid lactate removal because of enhanced pathways for the re-synthesis of glucose from accumulated L-lactate, via gluco- and glyconeogenesis (see also Hervant et al., 1999). It is possible that these pathways are both temperature and pH dependent, leading to the longer recovery times from metabolic acidosis observed under future OA and warming scenarios in the present study. At control temperature, acid–base status recovers directly towards resting levels (Fig. 3). This response has previously been shown at natural temperatures in *C. pagurus* and *H. gammarus* (Taylor and Whiteley, 1989; Whiteley, 1999). However, at elevated temperature, pHe did not return to resting levels along the non-bicarbonate buffer line, but dropped below it, demonstrating continued metabolic acidosis during recovery at the higher temperature (Fig. 3). Associated lactate recovery was also achieved 2 h faster at 10°C compared with 15°C at either pCO_2 level tested (Fig. 3). Higher metabolic demand of the tissues at the higher temperature could lead to a mismatch between O_2 supply and demand, forcing reliance on anaerobic lactate fermentation and continued metabolic acidosis during recovery. Indeed, it has been suggested that lactate-supported glyconeogenesis is more efficient at lower temperatures, and that ectotherms experiencing hyperlactaemia in a thermal gradient select lower temperatures in which to recover (Kieffer et al., 1994; Gleeson, 1996). Gluconeogenesis is also suggested to be inhibited at low pH (Hems et al., 1966; Iles et al., 1977; Kashiwagura et al., 1984) because of the inhibition of the enzyme pyruvate carboxylase (Scrutton and Utter, 1967; Iles et al., 1977; reviewed by Zammit, 1999) associated with crustacean lactate metabolism (Hervant et al., 1999). This could, in part, explain the depression of lactate-supported glyconeogenesis reported in the muscle of ectotherms at decreased pH (Bendall and Taylor, 1970), and the slower rates of lactate removal in the present study at elevated pCO_2. Although it is clear that lactate removal and, consequently, recovery from metabolic acidosis will be retarded by elevated environmental pCO_2 and temperature predicted by 2100 (Caldeira and Wickett, 2003; Sokolov et al., 2009), more work is required to understand the role of elevated pCO_2 and temperature on the pathways and rates of lactate and CO_2 removal in marine ectotherms (cf. Melzner et al., 2013).

Conclusions and ecological implications

Intertidal assemblages and the species that comprise them, including some with a predominantly subtidal distribution, may be more tolerant to OA and warming than their strictly subtidal relatives (Pane and Barry, 2007; Spicer et al., 2007; Melzner et al., 2009; Small et al., 2010; Whiteley, 2011). We suggest that in response to the combined pre-exposure to OA and warming, some low-shore intertidal crustaceans are relatively poor at regulating their acid–base status during emersion (Whiteley, 1999). These species may show increased resistance to short periods of emersion (up to 90 min) as a result of pre-exposure to elevated CO_2 and temperature, but retarded recovery of acid–base status following longer-term emersion (natural emersion periods are routinely 180 min). This suggests that in the future, recovery periods may be longer because of elevated pCO_2 and temperature, and this could drive individuals of predominantly subtidal species out of the lower intertidal where they may be found, i.e. *N. puber* could become strictly subtidal, or at least limit the duration of time when they can forage. The loss or reduction of foraging activity of a major predator in the rocky shore low- to mid-intertidal assemblages could affect the structure of these ecosystems, with possible implications for biodiversity and ecosystem function (e.g. Hale et al., 2011; Christen et al., 2013). *Necora puber*, as for other predators, is an important top-down controller of grazing invertebrates and thus affects biodiversity and productivity levels of rocky shore intertidal habitats (Silva et al., 2008; Griffin et al., 2008). Although *N. puber* and similar species are inactive during low tide and it is possible that they migrate up the shore to feed at high tide, this is unlikely as differences in the

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vertical distribution of crabs have been shown to affect community structure, with subtidal crabs mostly feeding in the subtidal despite their ability to invade the intertidal at high tide (e.g. Yamada and Boulding, 1996). Indeed, even if crabs did spend time migrating up the shore to feed this would still decrease feeding time and so the function of *N. puber* as an ecologically important predator in the intertidal. It is therefore imperative that future studies on single and multistressors also consider routine physiological fluctuations associated with natural systems in order to make robust predictions of species and community putative tolerance to future climate change drivers.

MATERIALS AND METHODS

Animal collection and acclimatisation

Adult male *N. puber* were collected by hand from the low intertidal zone at Mountbatten, Plymouth, Devon (50°21′34″N, 04°07′45″W), in March 2011. Forty-eight individuals (intermolt, carapace width 4.71±0.11 cm) were transported to the Plymouth Marine Laboratories (Plymouth, UK) within 30 min of collection, inside cool-boxes and covered by damp seaweed, to prevent thermal shock and desiccation. Crabs were placed individually in 48 aquaria (1 l volume), each supplied with recirculating aerated seawater (salinity=35, temperature=10°C, pH=8.1), and were left to recover for 24 h before being acclimatised to the conditions described below. The 48 aquaria, each containing one crab, were then haphazardly assigned to one of four temperature and pCO_2 combinations (N=12 individuals per treatment), where nominal pCO_2 and temperature treatments were chosen as representative of current seasonal habitat environmental conditions and those predicted to occur by the end of this century (Caldeira and Wickett, 2003; Orr et al., 2005; Sokolov et al., 2009). The four treatments employed were: ‘ambient’=10°C and 400 µatm pCO_2 (pH 8.1); ‘elevated pCO_2’=10°C and 1000 µatm pCO_2 (pH 7.8); ‘elevated temperature’=15°C and 400 µatm pCO_2 (pH 8.1); and ‘combined’=15°C and 1000 µatm pCO_2 (pH 7.8). Ambient pCO_2 treatments were maintained by bubbling untreated air through the water in each aquarium. Elevated pCO_2 treatments were maintained by enriching the air with CO_2 before bubbling (after Findlay et al., 2008; Pistevos et al., 2011). pCO_2 levels of both the untreated and CO_2-enriched air were monitored using a CO_2 Analyser (LI-820, Li-Cor, Lincoln, NB, USA). Seawater was continuously supplied to each aquarium (2 ml min^{-1}), and allowed to overflow into a common sump where it was collected, degassed by vigorous bubbling of untreated air, filtered (Filter Systems Incorporated, Warrington, UK), and cooled (Industrial Cooling Systems, New Milton, UK) before being recirculated to each aquarium. The temperature of each aquarium was maintained using heated water baths [Aqua One, 150W, Kong’s (UK) Limited, Romsey, UK]. Maximum temperature fluctuation experience over the exposure period was ±0.5°C. Crabs were acclimatised to one of the four pCO_2/temperature treatments for 2 weeks before being subjected to emersion stress (see below). Following the methods of Small et al. (Small et al., 2010), they were fed on *Mytilus edulis* on days 0 and 8 of the experiment. The pH, temperature and salinity of water in each aquarium were monitored every 24 h, and total alkalinity every 7 days, during this acclimatisation period. pH was measured using a pH combination electrode (Micro-Inlab pH, Mettler-Toledo, Leicester, UK) and meter (Seven Easy pH meter, Mettler-Toledo, Leicester, UK). Temperature and salinity were monitored daily using a handheld multimeter (325, WTW GmbH, Weilheim, Germany); total alkalinity was determined by Gran titration (As-Alk2, Apollo SciTech Inc., Bogart, GA, USA). Values for the physico-chemical parameters (and the carbonate chemistry values calculated from them) in the aquaria in this system are presented in Table 2.

Experimental setup and procedure

Six individuals were selected haphazardly from each of the four treatments. Individuals were removed from their acclimatisation chambers in order to obtain a clean, anaerobic sample of haemolymph from each individual (see below), to provide baseline values for the parameters measured at the start of the experiment (time 0). These samples were also used to examine differences in acid–base parameters after 2 weeks acclimatisation to each treatment. Three crabs from each treatment were then immersed in their acclimatisation chambers (controls) whilst the other three individuals from each treatment were exposed to air in chambers of the same volume as those used for immersed individuals. An emersion time of 180 min was used as it represented the emersion time in the mid- to low-shore where the animals were collected (S.P.S.R., personal observation). These chambers were supplied with untreated (pCO_2=400 µatm) or CO_2-enriched air (pCO_2=1000 µatm) to match future predicted atmospheric pCO_2 for 2100 (Houghton et al., 2001). Air was bubbled through seawater (depth=2 cm) at the bottom of each emersion chamber, which helped equilibrate the gases in the chamber whilst maintaining appropriate humidity levels, preventing desiccation. Individuals were placed onto a perforated platform in the emersion chamber, which separated them from the seawater at all times. The chambers were also covered with black plastic to reduce internal light levels to mimic the humid, dark natural microhabitat that occurs under brown seaweed in rock crevasses, where the crabs are found when emersed. Air pCO_2 levels in the chambers were continuously monitored using a CO_2 analyser (LI-820, Li-Cor). Air temperature during emersion matched acclimatisation treatments (10 or 15°C) by means of a water jacket surrounding the chambers and connected to a water bath (Grant Cambridge Ltd, Cambridge, UK). Air temperature was monitored using a K-type thermocouple in each chamber connected to a temperature logger (Omega, HH806AU, Manchester, UK). After 180 min of emersion, crabs were re-immersed in their original acclimatisation chambers. Hemolymph was sampled at 90 and 180 min from emersed and control (immersed) individuals of each treatment to determine extracellular acid–base parameters (pHe, PCO_2, $[HCO_3^-]$e and [lactate]e). Following re-immersion, haemolymph was sampled in control and re-immersed individuals at 60, 180, 300 and 420 min to assess the recovery of acid–base parameters. This experiment was repeated the following day on different animals, producing

Table 2. Physico-chemical measurements from seawater from each of the four pCO_2/temperature treatments over the 14 day acclimatisation period

Nominal treatment	400 µatm pCO_2/10°C	400 µatm pCO_2/15°C	1000 µatm pCO_2/10°C	1000 µatm pCO_2/15°C
pH	8.09±0.008^A	8.08±0.007^A	7.83±0.004^B	7.82±0.01^B
Temperature (°C)	10.01±0.5^A	15.71±0.13^B	9.98±0.06^A	15.58±0.15^B
Salinity	34.91±0.12	35.06±0.14	35.00±0.17	35.10±0.19
TA (µEq kg^{-1})	2496±48	2705±109	2641±86	2636±156
DIC (µmol kg^{-1})	2327±49	2482±94	2568±88	2548±148
pCO_2 (µatm)	500±18^A	546±25^A	988±65^B	1136±67^B
Ω_{calc}	3.06±0.02	4.19±0.36	1.96±0.07	2.22±0.22
Ω_{arag}	1.94±0.03	2.7±0.22	1.25±0.04	1.43±0.14
HCO_3^- (µmol kg^{-1})	2178±48	2288±83	2443±85	2415±139
CO_3^{2-} (µmol kg^{-1})	127±1	174±14	81±3	92±9

Temperature, salinity and pH (NBS scale) were measured daily. Total alkalinity (TA) was measured weekly. All other parameters [pCO_2; calcite and aragonite saturation state (Ω_{calc} and Ω_{arag}, respectively); HCO_3^-; and CO_3^{2-}] were calculated from pH and TA with CO2SYS (Pierrot et al., 2006) using the dissociation constants of Mehrbach et al. (Mehrbach et al., 1973) as refitted by Dickson and Millero (Dickson and Millero, 1987). Values are means ± s.e.m. Different letters indicate significant variation between treatments (ANOVA, P<0.05).

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six control and six emersed/re-immersed replicates for each of the pCO_2 temperature combinations.

Haemolymph sampling and determination of acid–base parameters

At each time point, crabs were removed from their experimental chambers for less than 20 s and a clean, clear and anaerobic 60 µl haemolymph sample was obtained from each crab using a gas-tight, 100 µl microsyringe (Hamilton, GASTIGHT 1710RN, Bonaduz, Switzerland), the needle of which was inserted into the infrabranchial sinus, via the arthrodial membrane at the base of the first or second pereopod. To account for handling stress, all comparisons were made between control (constantly immersed) and emersed/re-immersed crabs at each time point. A subsample of haemolymph (30 µl) was dispensed immediately and anaerobically from the gas-tight syringe into a CO_2 analyser (965D, Corning Diagnostics, Halstead, UK) for the determination of total dissolved and bound CO_2 (TCO_2). The remaining 30 µl sample of haemolymph was carefully and rapidly injected into a 0.5 ml microcentrifuge tube (Fisher Scientific, TUL-649-010L, Loughborough, UK), into which was immediately inserted a micro-pH electrode (Micro-Inlab pH, Mettler-Toledo) that formed a gas-tight seal between the sample and the air. The electrode was connected to a pH meter (Seven Easy pH meter, Mettler-Toledo) and calibrated using Mettler-Toledo pH_{NBS} standards (pH 4.01, 7.00 and 9.21 at 25°C) (after e.g. Donohue et al., 2012) and checked against precision pH buffers (pH 8 and 7.4 at 25°C; Whatman International Ltd, Maidstone, UK). Sample temperature was maintained by partially immersing the microcentrifuge tube containing the sample and micro-pH electrode in a water bath at the respective treatment temperature. After pH determination, haemolymph was stored at −20°C for 5–6 weeks before being used for the determination of lactate (see below). To determine non-bicarbonate buffer lines (NBLs) for the modified Davenport diagrams (Fig. 3), an extra 100 µl of haemolymph was extracted from all crabs at time 0 (as described above) and frozen at −20 for 6 weeks. Each sample was defrosted and injected into a glass diffusion chamber and equilibrated to 0.1, 1.0 and 2.5% CO_2 (mixed with O_2 balanced N_2) in turn, using gas supplied by a gas mixing pump (Wösthoff pump, Wösthoff GmbH, Bochum, Germany). pH was measured using a micro pH electrode (MI-413, Microelectrodes Inc., Bedford, NH, USA) inserted into the diffusion chamber and attached to a pH meter (Seven Easy pH meter, Mettler-Toledo) and calibrated as described above. The sample was agitated with a magnetic stirrer and was considered equilibrated when the pH value stopped decreasing (after ~15 min); this value was recorded for each level of pCO_2 and used to calculate NBLs (see below). To match the temperature treatment of 10 or 15°C, the temperature of the diffusion chamber was monitored and control by a water bath (described above).

Determination of haemolymph lactate concentrations

Haemolymph lactate concentrations ([lactate]e) were determined using the lactate oxidase method (after Webster, 1996). Haemolymph (20 µl) was centrifuged (5 min at 12,100 ***g***) to remove haemocytes. A 10 µl sub-sample of the supernatant was then diluted (5×) and 10 µl of the dilution was applied to microtitre plates filled with 100 µl of reagent (Trinity Biotech, 735-10, Wicklow, Ireland). The plate was read at λ=540 nm using a VersaMax Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). The absorbance of these samples was compared with that of lactate standards (40 mg dl^{-1}, 826-10, Trinity Biotech) diluted to 0, 0.14, 0.28, 0.56, 1.11, 2.22 and 4.44 mmol l^{-1}, on the same plate. This allowed interpolation of haemolymph lactate concentration from a standard curve.

Calculation of PCO_2 and $[HCO_3^-]e$

PCO_2 and $[HCO_3^-]$e were calculated using the Henderson–Hasselbalch equation in the forms:

$$PCO_2 = TCO_2 / \alpha\,(10^{pH-pK'_1} + 1)\,, \tag{1}$$

$$[HCO_3^-] = TCO_2 - \alpha PCO_2\,, \tag{2}$$

where α is the solubility coefficient of CO_2 (0.435 mmol l^{-1} kPa^{-1} at 10°C, 0.375 mmol l^{-1} kPa^{-1} at 15°C, salinity=33) and pK'_1 is the first apparent dissociation constant of carbonic acid in crab haemolymph (6.025 at 10°C, 6.055 at 15°C, salinity=33). Values of α and pK'_1 are for haemolymph from *C. maenas* (Truchot, 1976) and have previously been successfully applied to *N. puber* (e.g. Watt et al., 1999; Spicer et al., 2007; Small et al., 2010). To construct NBLs, $[HCO_3^-]$ levels were calculated from empirically derived pH and PCO_2 values using the Henderson–Hasselbalch equation in the form:

$$[HCO_3^-] = 10^{pH-pK'_1}\,\alpha PCO_2\,. \tag{3}$$

The gradient of pH plotted against $[HCO_3^-]$ for each treatment was use to describe the NBLs shown in the modified Davenport diagrams (Fig. 3).

Statistical analysis

Repeated-measures designs have been used previously in acid–base studies (e.g. Calosi et al., 2013a) as they allow the incorporation of individuals' information whilst investigating species' response to environmental stress (Aldrich, 1975; Aldrich, 1989; Bennett, 1987; Calosi et al., 2013c). In doing so, this avoids the problem of natural variation in acid–base parameters that can mask subtle changes in individual responses (Taylor and Whiteley, 1989). However, because of the possible experimental effects associated with taking multiple samples from a same individual (Truchot, 1975), all comparisons between treatments are based on the statistical difference in responses between emersed crabs and immersed control crabs that are exposed to exactly the same sampling procedure (i.e. the same sampling stress). A repeated-measures ANCOVA was used to test for between-treatment differences in each acid–base parameter throughout emersion and recovery, using Mauchly's test for sphericity to confirm conformity to analytical assumptions. Day and carapace width were both included as covariates but as there was no significant effects of these parameters, they were removed from subsequent analyses. F-tests were generated to detect any significant differences in each of the acid–base parameters between emersed and control groups, for each independent combination of treatment temperature and treatment pCO_2 across time. These tests were based on linearly independent pairwise comparisons generated from the estimated marginal means of the repeated-measures ANCOVA. Pairwise comparisons were adjusted for multiple comparisons using Bonferroni correction, assuming α=0.05. All statistical analyses were performed using SPSS software v. 18 (SPSS, Chicago, IL, USA). All values are presented as means ± s.e.m.

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Competing interests

The authors declare no competing financial interests.

Author contributions

S.P.S.R. developed the original concept and experimental design, supported by P.C., J.I.S. and S.W. S.P.S.R. also carried out the experiments. Animals and seawater parameters were maintained by R.C.-P. under supervision of S.P.S.R. Lactate determination was carried out by R.C.-P. and G.N. under supervision of S.P.S.R. Data analysis was carried out by A.F. and S.P.S.R. All authors contributed to the final manuscript.

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