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EFFECTS OF WATER SALINITY ON ACID–BASE BALANCE IN DECAPOD CRUSTACEANS

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Summary

Extracellular acid–base balance in decapod crustaceans is influenced by water salinity, although the nature of this relationship is unclear. In euryhaline crabs, a decrease in salinity results in a metabolic alkalosis in the haemolymph and an increase in salinity results in a metabolic acidosis. Alterations in acid–base status by external changes in salinity are thought to be secondary to the adjustments required for ionic and osmotic regulation. In the present study, acid–base adjustments in the haemolymph of *Eriocheir sinensis* after transfer to 30 % sea water accompanied alterations in muscle pH and [HCO_3^-], as an initial acidosis coincided with an alkalosis in the leg muscle. By 48 h transfer, haemolymph pH increased as muscle pH and HCO_3^- declined. Haemolymph [Cl^-] decreased significantly 3 h after transfer to a new steady state but haemolymph [Na^+] and muscle [Na^+] and [Cl^-] remained unchanged. Muscle free amino acid concentration increased twofold 6 h after transfer, followed by a 2.5-fold increase in the haemolymph after 24 h. In contrast, 30 % sea water had no effect on haemolymph acid–base adjustments in the osmoconforming crab, *Necora puber*, which lacks ion and osmo-regulatory mechansims. Collectively these observations support the view that salinity-induced alterations in acid–base status are caused by adjustments consistent with cell volume regulation.

Key words: acid–base balance, ion regulation, osmoregulation, crustacean, *Eriocheir sinensis*, *Necora puber*, salinity change.

Introduction

It has been known for some time that external salinity can influence extracellular acid–base status in brachyuran decapod crustaceans (Truchot, 1973; Truchot, 1981; Truchot, 1992; Mangum et al., 1976; Henry and Cameron, 1982; Wheatly, 1985). Although the underlying mechanisms and the physiological consequences remain unclear, the basis for the relationship between acid–base status and ion regulation can be attributed to the catalysed hydration of CO_2 by carbonic anhydrase to give carbonic acid (H_2CO_3) and, subsequently, HCO_3^- and H^+ (Wheatly and Henry, 1992). HCO_3^- and H^+ not only affect acid–base equilibria, but also act as counterions in the transfer of Cl^- and Na^+ across plasma membranes via electroneutral ion transporters between the extracellular space and either the ambient water or the intracellular compartment. In aquatic crustaceans, gas and ion exchange between the animal and its environment occur predominantly over the gills because of the relative impermeability of the general body surface (Lignon, 1986; Lignon and Péqueux, 1990). In gill epithelia, HCO_3^- is usually exchanged for Cl^-, and H^+ for Na^+. These ion exchanges are driven by a basolateral Na^+/K^+-ATPase (Towle and Kays, 1986; Taylor and Taylor, 1992; Towle, 1997), and possibly, in the case of Cl^-/HCO_3^- exchange, an apically located H^+-ATPase (Onken and Putzenlechner, 1995).

In aquatic decapod crustaceans, the coupled branchial transfer of acid/base equivalents to electroneutral ion exchange is the principal mechanism of acid–base regulation (Wheatly and Henry, 1992). Evidence comes from a number of studies, in which acid–base and ion fluxes are modified during acid–base disturbances. For example, in the strongly euryhaline crab *Callinectes sapidus*, compensation for a hypercapnic acidosis was accompanied by apparent H^+ excretion due to the uptake of HCO_3^- and associated efflux of Cl^- (Cameron and Iwama, 1987). In the shore crab *Carcinus maenas* and the crayfish *Pacifasticus leniusculus*, the lowering of HCO_3^- in the haemolymph during recovery from hyperoxia or hypercapnia was attributed to the excretion of base equivalents into the external medium (Truchot, 1979; Wheatly, 1989). Moreover, crayfish placed in Cl^- free medium experienced a haemolymph alkalosis and a reduction in the efflux of base equivalents (Dejours et al., 1982). Collectively these experiments indicate that HCO_3^- or anion exchange is important to the restoration of acid–base status in decapod crustaceans after acid–base imbalance. In addition, it is thought that anion exchangers play an important role in the uptake of Cl^- in aquatic crustaceans exposed to dilute seawater (Péqueux, 1995), providing a potential link between acid–base balance and ion-uptake mechanisms. Consequently, compromises between acid–base compensation and the requirements of ionic and osmotic homeostasis must occur

often, as emphasised by Cameron and Iwama (Cameron and Iwama, 1989). However, the relative contribution of anion exchangers to both of these homeostatic processes remains unclear (Cameron and Iwama, 1987; Cameron and Iwama, 1989; Whiteley, 1999).

When exposed to diluted sea water, marine crustaceans are unusual in comparison to other marine invertebrates in that they can show a wide variety of responses, from crabs that are strong osmoregulators to those that are osmoconformers. In coastal marine crustaceans dilution of the ambient sea water results in water uptake down an osmotic gradient, followed by a loss of ions down a concentration gradient, coupled to salt loss via the increased production of iso-osmotic/isotonic urine (Mantel and Farmer, 1983). Osmoregulators maintain their haemolymph hyperosmotic to the external water by having an exoskeleton, which resists osmotic swelling and has a reduced permeability to ions to prevent ion leakage (Lignon and Péqueux, 1990), and by active and passive uptake mechanisms for specific ions. Active uptake of Na^+ and Cl^- is associated with the increased activity of the ion-transporting enzymes Na^+/K^+-ATPase and carbonic anhydrase, predominantly in the posterior gills. Studies carried out on crude gill homogenates, and more recently on partially purified membrane vesicles, show that Na^+/K^+-ATPase activities increase two- to fivefold on exposure to diluted sea water, usually 3 days after transfer (Holliday, 1985; Harris and Santos, 1993; Corotto and Holliday, 1996; Lucu and Devescovi, 1999). Branchial carbonic anhydrase activity increases over a similar time course and is critical for the adaptation of *Callinectes sapidus* to low salinity (Henry and Cameron, 1982). Although not directly involved in the ion exchange process, carbonic anhydrase is thought to maintain local supplies of HCO_3^- and H^+ for counterion exchange by the accelerated hydration of CO_2.

Animal cells act as near perfect osmometers, since cell membrane permeability to water is generally between five to eight orders of magnitude higher than the permeability to inorganic osmolytes (Na^+, K^+, Cl^-) (Rorive and Gilles, 1979). Consequently cell volume control is an important feature of osmoregulation in euryhaline crustaceans during osmotic stress. From the large number of mammalian studies carried out to date, it seems that animal cells utilise a multitude of volume regulatory mechanisms, including transport of inorganic and organic osmolytes across the cell membrane and alterations in metabolism to modify levels of organic metabolites (Lang et al., 1998). Several metabolic pathways are sensitive to cell volume changes, including glycogen synthesis and glycolysis, leading to changes in the amount of carbohydrate metabolites that contribute to cellular osmolarity (Al-Habori et al., 1992). Hypo-osmotic exposure results in cell swelling, which activates mechanisms involved in regulatory cell volume decrease. These include activation of separate K^+ and nonselective anion channels, which allow K^+ to move out of the cell down its concentration gradient followed by an efflux of Cl^- down the resulting electrochemical gradient (Lang and Paulmichl, 1995; Trachtman et al., 1993). The nonselective anion channels also allow outward movement of HCO_3^- (Weiss and Lang, 1992), and these fluxes, combined with an enhancement of Cl^-/HCO_3^- activity (Livne and Hoffman, 1990), cause cytosolic acidification during cell swelling. Cell shrinkage has the opposite effect because intracellular electrolyte accumulation involves a $Na^+/K^+/2Cl^-$ cotransporter moving K^+ into the cell against an electrochemical gradient and the activation of a Na^+/H^+ exchanger, resulting in an intracellular alkalosis (Grinstein et al., 1983). Experiments on isolated crab tissues demonstrate that K^+ plays an important role in the limitation of cell swelling with a rapid decrease in intracellular K^+ in crab axons during hypo-osmotic exposure (Gilles, 1980; Gilles and Péqueux, 1981). In contrast, the mechanisms involved in cell volume regulation after hyperosmotic exposure are poorly understood, as tissues of euryhaline crabs are unable to effect volume readjustment after shrinkage *in vitro* (Gilles, 1973).

In contrast to vertebrate tissues, in which cell osmolality is mostly determined by the inorganic ions Na^+, K^+ and Cl^-, small organic molecules play a more important role in cell volume regulation in decapod crustaceans (for reviews see Gilles, 1983; Péqueux, 1995). In particular, organic osmolytes appear to be more significant than K^+ in the re-adjustment of cell volume following hypo-osmotic exposure (Gilles, 1973). An increase in the production and efflux of non-essential amino acids from the tissues causes a decrease in intracellular osmotic concentration (reviewed by Gilles, 1980). The resulting deamination is extensive, causing an increase in NH_3 excretion in dilute medium, with the potential for an increase in proton excretion as NH_4^+ (Mangum et al., 1976; Truchot, 1992). NH_4^+ exchange may occur on the basolateral surface of the gill epithelia by substitution for K^+ in the Na^+/K^+-ATPase and on the apical surface by electroneutral Na^+/NH_4^+ exchange (Lucu, 1993; Towle and Hølleland, 1987; Towle, 1993). NH_4^+ is therefore included in both passive and active ion-uptake mechanisms in osmoregulating crustaceans during exposure to low salinity.

Our present understanding of the effects of salinity change on extracellular acid–base balance in crustaceans is influenced to a large extent by studies on weak and strong osmoregulating crabs carried out by Truchot (Truchot, 1981; Truchot, 1992) and by Henry and Cameron (Henry and Cameron, 1982). By subjecting the weak osmoregulating crab *Carcinus maenas* to various changes in salinity at constant water P_{CO_2}, Truchot (Truchot, 1981) was able to show that a decrease in salinity led to a transient respiratory acidosis in the prebranchial haemolymph, followed by a metabolic alkalosis that lasted several days. A similar response was found in the strong osmoregulator *Callinectes sapidus*, although the alkalosis in this species was persistent rather than transient (Henry and Cameron, 1982). The metabolic alkalosis in *Carcinus maenas* was associated with a large net base efflux from the crabs, leading Truchot (Truchot, 1981) to conclude that the acid–base disturbances originated in the tissue and were related to metabolic adjustments ensuring iso-osmotic intracellular regulation. In addition, Henry and Cameron (Henry and

Cameron, 1982) found that the alkalosis was correlated with an increase in Na^+–Cl^- difference, taken as an indicator of strong ion difference, and may also be related to changes in haemolymph protein levels during low salinity acclimation. Proteins carry a net negative charge and may serve to offset the increase in strong ion difference altering pH in *Callinectes sapidus*, as protein levels double on transfer to low salinity (Lynch and Web, 1973).

On transfer of *Eriocheir sinensis* from sea water to fresh water, Truchot (Truchot, 1992) detected the steady development of a metabolic alkalosis in the haemolymph despite a transient increase in P_{CO_2}, and in contrast to *Carcinus maenas*, a significant efflux of acid equivalents. Even though alterations in haemolymph pH and [HCO_3^-] were accompanied by adjustments in extracellular osmolarity and ion concentrations, the time course for these events differed, with acid–base adjustments peaking after 3 days and alterations in acid efflux and extracellular ion concentrations occurring within the first 2 days. In *Eriocheir sinensis*, haemolymph pH remained significantly higher than seawater values 29 days after transfer, indicating that the relative alkalosis is a permanent feature of seawater dilution. In contrast, transfer from fresh water to sea water induced a metabolic acidosis, which was almost fully compensated by a marked hypocapnia (Truchot, 1992). Conversely, transfer of *Carcinus maenas* from fresh water to sea water resulted in the development of a metabolic acidosis associated with an acid efflux.

The differences in timing of the various adjustments outlined above, and the conflicting data on the direction of whole-body acid equivalent fluxes, indicate that salinity-dependent acid–base imbalances are not due simply to passive ion fluxes and altered ionic gradients causing perturbed branchial Cl^-/HCO_3^- or Na^+/H^+ exchanges. Indeed, the situation is far more complex, as changes in external salinity also induce homeostatic adjustments in ion regulation, water balance and cell volume control, and all these mechanisms can potentially influence the flux of acid–base equivalents and strong ions between body compartments. In addition, changes in external salinity can alter the CO_2-combining properties of the water by changing the titratable carbonate alkalinity. These physico-chemical changes can in turn influence haemolymph P_{CO_2} levels, as an increase in water carbonate alkalinity at constant inspired, but low water, P_{CO_2} (Pw_{CO_2}) results in a respiratory alkalosis in *Carcinus maenas* (Truchot, 1984; Truchot and Forgue, 1998). However, the salinity-dependent changes in water titration alkalinity did not affect the metabolic alkalosis observed in *Carcinus maenas* (Truchot, 1981).

The complex interaction between acid–base and ion equilibria during salinity change was recognised by Truchot (Truchot, 1992), who stated that '*the exact nature of these various processes as well as their contribution to the development of salinity dependent acid–base disturbances in euryhaline animals clearly await further studies*'. The aim of the present investigation was to examine the short-term effects of salinity change on adjustments in ion and acid–base variables in the extra- and intracellular compartments of two species of crabs with differing abilities for osmoregulation. Experiments were carried out on the velvet swimming crab (*Necora puber*), a sublittoral marine species with little tolerance of salinity change, and the strong osmoregulating crab *Eriocheir sinensis*, which can tolerate both fresh water and full-strength sea water. The subsequent effects of seawater dilution were followed immediately after transfer and for up to 48 h, to establish whether salinity-induced adjustments in acid–base balance are dependent on the changes necessary for ionic and osmotic regulation.

Materials and methods

Chinese mitten crabs, *Eriocheir sinenesis* (Milne Edwards), were captured in baited traps in the Thames, Chelsea, London, and transferred to aquaria in Bangor. The crabs were caught in late summer when the males and females naturally migrate downstream to the tidal estuaries. On arrival at Bangor, the animals were held in recirculating, fully aerated sea water at 12 °C and 35 ‰ salinity (titration alkalinity 2.8 mequiv l^{-1}; pH 8.06) for 2 weeks before experiments commenced. *Necora puber* (L.) were obtained from a local wholesaler and maintained in sea water at 35 ‰ salinity as described for *Eriocheir sinensis*. 2 days before each experimental run, crabs were prepared for the withdrawal of post-branchial haemolymph by drilling a small hole into the carapace over the pericardial cavity behind the heart without puncturing the underlying hypodermis. The area was covered with a rubber patch and fixed into position with cryanoacrylate glue.

Experimental procedure

In the first set of experiments, 30 intermoult *Eriocheir sinensis* of either sex and with mean body mass of 55.4±3.6 g were transferred to experimental tanks supplied with running sea water at 12 °C and 35 ‰ salinity. Small postbranchial haemolymph samples (0.3–0.6 ml) were withdrawn from 6 crabs after the animals had been allowed to settle to the experimental conditions for 24 h. Samples were used for the immediate measurement of pH and total CO_2 levels. Remaining subsamples were frozen and stored at –20 °C for the determination of haemolymph Na^+, Cl^-, total ammonia and free amino acid levels. Subsequently, a walking leg was removed by autotomy, and the flexor and extensor muscles were dissected out from the merus segment. Tissues were isolated as quickly as possible and clamp-frozen for storage in liquid nitrogen to determine intracellular pH, Na^+, Cl^-, ammonia and amino acid levels. The holding water was subsequently diluted with dechlorinated fresh water to a salinity of 10 ‰ (30 % sea water) (titratable alkalinity 2.6 mequiv l^{-1}, pH 8.04). The remaining crabs were sampled in groups of six at 3, 6, 24 and 48 h after sea water dilution, as described above for the control group. In the second set of experiments 12 intermoult *Necora puber* (mean body mass 121±4.4 g) were exposed to sea water at 20 ‰ salinity (60 % SW) and a separate group of 10 crabs (mean body mass 112±5.6g) was transferred to sea water at 10 ‰ salinity (30 %

SW). Postbranchial haemolymph samples were withdrawn as described for *Eriocheir sinensis*, and used for the determination of haemolymph acid–base variables and Cl^- levels. All experiments were carried out at 11–13 °C.

Haemolymph analysis

Heamolymph pH levels were determined by injecting a small subsample past the face of a glass pH electrode and its reference electrode (E301 pH electrode) housed in a BC202 blood gas cell supplied with circulating water at 12 °C, and connected to a BGM200 blood gas meter (Cameron Instrument Company). The pH electrode was calibrated with precision buffers at regular intervals (Whatman International, pH 6.91 and pH 7.46 at 12 °C). Total carbon dioxide was determined on 30 µl subsamples injected into a 2 ml chamber containing a CO_2 electrode (Radiometer E5037) and filled with 0.01 mmol l^{-1} HCl maintained at 38 °C (Cameron, 1971). The P_{CO_2} electrode was connected to a Radiometer PHM73 pH/blood gas monitor and calibrated with standard $NaHCO_3$ solutions of 10, 20 and 40 mmol l^{-1}.

Cl^- concentrations were analysed using a chloride titrator (PCLM 3, Jenway), and haemolymph Na^+ concentrations were determined by atomic absorption spectrophotometry (Pye Unicam SP9). Total free amino acids in the haemolymph were determined using a standard Ninhydrin test (Lee and Takahashi, 1996), and ammonia levels were determined by a micro-modification of the salicylate hypochlorite assay (Verdouw et al., 1978).

Tissue analysis

Intracellular pH was estimated in the gill and leg muscle by the homogenised frozen tissue method as described by Pörtner et al. (Pörtner et al., 1990) and outlined for use on crustacean tissue (Whiteley et al., 1995). In summary, each sample was ground to a fine powder under liquid nitrogen using a precooled pestle and mortar, and added to 300–600 µl of inhibitor medium (130 mmol l^{-1} KF and 6 mmol l^{-1} nitriloacetic acid, adjusted to pH 7 with KOH). After thorough mixing and a brief centrifugation (15 s) at 3,000 ***g***, the pH values of the resulting supernatant were measured as described for the haemolymph samples.

Tissue samples were prepared for the determination of ammonia and free amino acid levels by homogenising tissue under liquid nitrogen in a pestle and mortar, and extracting the resultant powder with 80 % methanol. After centrifugation at 5,000 ***g*** for 20 min at 4 °C, the supernatants were analysed as described for the haemolymph samples.

Calculations

Measured values for pH and total CO_2 were used to derive partial pressures of CO_2 (P_{CO_2}) and combined bicarbonate and carbonate concentrations (referred to as $[HCO_3^-]$) by using the Henderson–Hasselbalch equation. The apparent first dissociation constant for carbonic acid (pK_1') and the CO_2 solubility coefficients (αCO_2) were taken from Truchot (Truchot, 1976) and adjusted for salinity and temperature. Intracellular HCO_3^- concentrations were calculated as described above, assuming that the values for intracellular P_{CO_2}, αCO_2 and pK_1' were the same as those determined in the haemolymph.

Statistical analyses

Values are expressed as means ± S.E.M. (N=6). Statistical differences between the means of the dependent variables were tested by one-way analysis of variance (ANOVA). Multiple comparisons were carried out using Dunnett's T3 test and Tukey's HSD test. Differences were accepted as significant at the 95 % level of confidence (P<0.05).

Results

Haemolymph acid–base balance and ion regulation

Mean acid–base variables in the haemolymph of *Eriocheir sinensis* and *Necora puber* before and after transfer to diluted

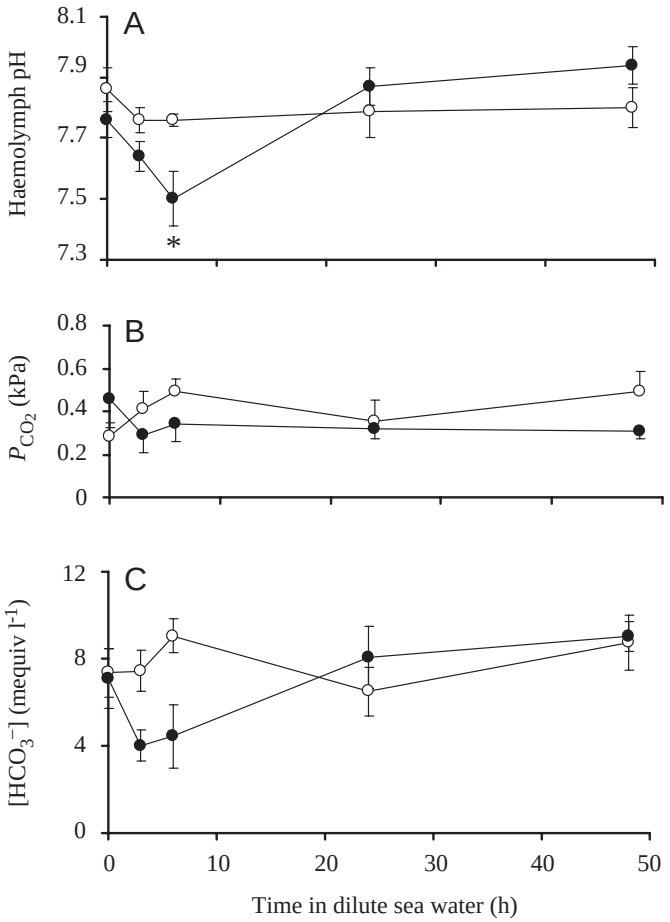

Fig. 1. Changes in haemolymph acid–base variables in *Eriocheir sinensis* (filled symbols) and *Necora puber* (open symbols) on transfer from full-strength sea water (time 0) to 30 % and 60 % sea water, respectively. Values are means ± S.E.M. (N=6) for (A) haemolymph pH, (B) partial pressure of CO_2 (P_{CO_2}) and (C) combined bicarbonate and carbonate concentrations ($[HCO_3^-]$). Significant differences (P<0.05) from the mean values obtained in full-strength sea water (time 0) are indicated with an asterisk.

sea water are displayed in Fig. 1. In *Eriocheir sinensis*, haemolymph pH decreased significantly (P=0.037) after transfer to 30 % sea water from a mean of 7.76±0.06 to 7.5±0.09 after 6 h, before gradually increasing (P=0.01) to a mean value of 7.9±0.06 after 48 h. The changes in pH occurred at constant P_{CO_2}, because the small drop in P_{CO_2} calculated 3 h after transfer from 0.46±0.11 kPa to 0.29±0.08 kPa was not significant. In addition, there was no significant change in calculated haemolymph [HCO_3^-] levels 48 h after transfer. In *Necora puber* there were no significant differences in haemolymph pH, P_{CO_2} or HCO_3^- after transfer to diluted sea water (Fig. 1).

Transfer of *Eriocheir sinesis* to diluted sea water caused a significant decline (P=0.046) in haemolymph [Cl^-] from 412±18.4 mequiv l^{-1} in 100 % sea water crabs to 332±31 mequiv l^{-1} 3 h after transfer. After this time [Cl^-] remained unchanged and significantly different (P<0.02) from the values in 100 % sea water crabs, being 336±20 mequiv l^{-1} after 48 h. In contrast, haemolymph [Na^+] was not influenced by seawater dilution, as mean values after 2 days (318±27 mmol l^{-1}) were similar to those obtained in full-strength sea water (333±43 mmol l^{-1}). On transfer of *Necora puber* to 60 % sea water, there was a significant decline (P=0.02) in haemolymph [Cl^-] from 506±12 mequiv l^{-1} in full-strength sea water to 418±64 mequiv l^{-1} 6 h after transfer. Mean [Cl^-] remained significantly lower (P<0.02), but unchanged in *Necora puber* for the remainder of the experiment, being 416±63 mequiv l^{-1} 48 h after transfer.

Intracellular changes in acid–base status and ion regulation

In the leg muscle of *Eriocheir sinensis* there was a transient and significant increase (P<0.01) in pHi over the first 6 h by 0.16 units (Fig. 2A). This was accompanied by a significant increase (P=0.013) in intracellular [HCO_3^-] from 3.08±0.6 mequiv l^{-1} to 5.5±0.4 mequiv l^{-1}, marking an intracellular alkalosis (Fig. 2B). More than 6 h after transfer to diluted sea water, pHi and [HCO_3^-] levels returned towards the values obtained in full-strength sea water. In contrast, there were no significant changes in muscle [Na^+] and [Cl^-] during seawater dilution with mean values in full-strength sea water at 47±26 mmol kg^{-1} tissue water and 27±15 mmol kg^{-1} tissue water, respectively. Changes in free amino acid pools (FAA) in the haemolymph and leg muscle of *Eriocheir sinensis* are shown in Fig. 3. Haemolymph [FAA] increased significantly

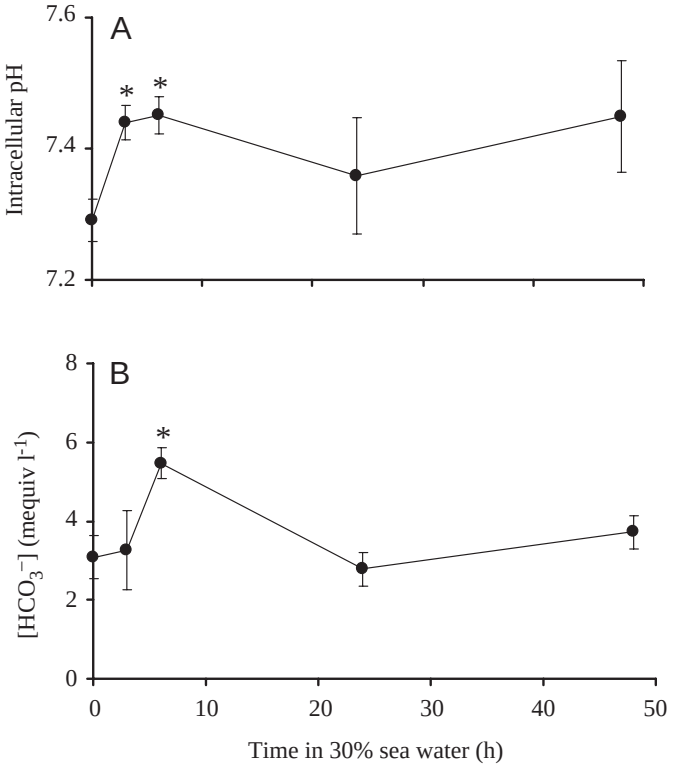

Fig. 2. Changes in (A) intracellular pH and (B) bicarbonate ([HCO_3^-]$_i$) levels in the leg muscle of *Eriocheir sinensis* on transfer from full-strength sea water (time 0) to 30 % sea water. Values are means ± S.E.M. and N=6 in all cases. Significant differences (P<0.05) from the mean values obtained in full-strength sea water (time 0) are indicated with an asterisk.

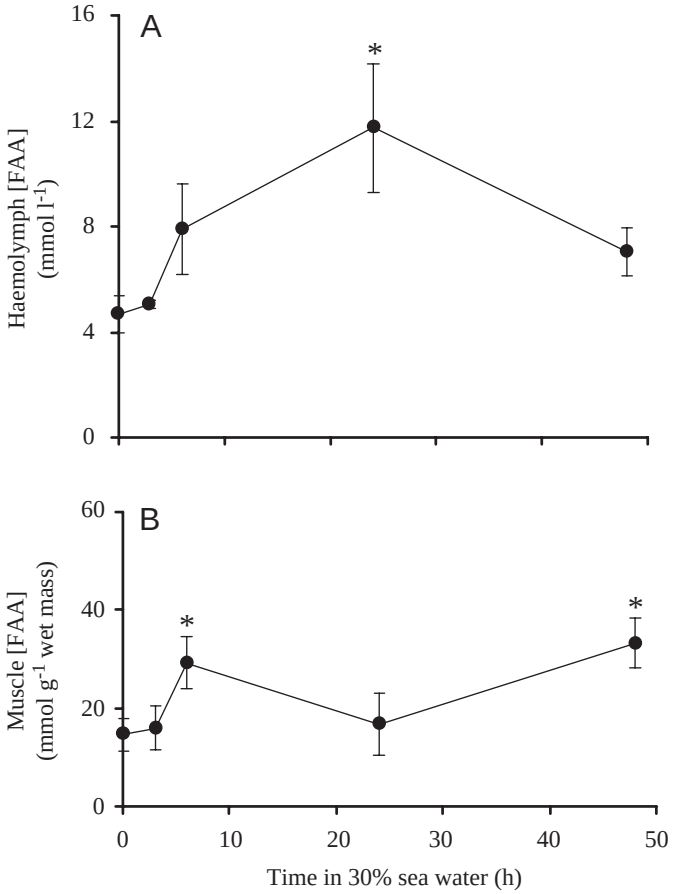

Fig. 3. Mean values (± S.E.M., N=6) for free amino acid concentrations [FAA] in the haemolymph (A) and leg muscle (B) of *Eriocheir sinensis* on transfer from full-strength sea water (time 0) to 30 % sea water. Significant differences (P<0.05) from the mean values obtained in full-strength sea water (time 0) are indicated with an asterisk.

(P=0.015) over the first day in diluted sea water from 4.67±0.72 mmol l^{-1} to 11.74±2.44 mmol l^{-1}. Muscle [FAA] increased from 14.58±3.44 to 29.21±5.38 μmol l^{-1} g^{-1} wet mass 6 h after transfer and doubled from 14.58±3.44 to 33±5 μmol l^{-1} g^{-1} wet mass 2 days after transfer (P<0.05) (Fig. 3B). Haemolymph ammonia levels showed little change at around 25 μmol l^{-1} over the 2 day transfer period. In contrast, muscle ammonia concentrations decreased significantly (P=0.014) on initial transfer from 237±17 μmol l^{-1} to 119±28 μmol l^{-1} after 3 h, before returning to values similar to those in 100 % sea water by 24 h transfer (206±42 μmol l^{-1}).

Discussion

On initial transfer to dilute sea water, *Carcinus maenas* and *Callinectes sapidus* experienced a small haemolymph acidosis associated with a transient hypercapnia (Truchot, 1981; Henry and Cameron, 1982). Truchot (Truchot, 1981) attributed this initial acidosis to changes in the acid–base status of the water. By changing water salinity and titration alkalinity (TA) simultaneously at constant P_{CO_2}, Truchot (Truchot, 1981) found that a reduction in water TA with no change in salinity resulted in a hypercapnic acidosis. This dependency arises from an associated decline in the water capacitance coefficient for CO_2 leading to an increase in haemolymph P_{CO_2} at constant inspired P_{CO_2} (Truchot and Forgue, 1998). In the present study, the transient acidosis observed in *Eriocheir sinensis* 6 h after seawater dilution was associated with a fall in [HCO_3^-] at constant P_{CO_2}. Interestingly, the heamolymph acidosis coincided with the development of an alkalosis in the leg muscle, suggesting the exchange of acid/base equivalents between the extra- and intracellular compartments within 6 h of transfer. Parallel measurements on whole-animal flux rates in *Eriocheir sinensis* showed a net acid efflux rate of 4.2±0.2 mequiv kg^{-1} h^{-1} 6 h after transfer to fresh water (R. Geoghegan and N. M. Whiteley, unpublished observations). In contrast, crabs in steady state acid–base exchange with the water appear to excrete base equivalents, or are in acid–base balance with zero excretion (summarised by Wheatly and Henry, 1992). Moreover, no consistent differences can be found between seawater and freshwater species. Therefore, seawater dilution in *Eriocheir sinensis* resulted in a large efflux of acid equivalents from the body, as found by Truchot (Truchot, 1992). All these observations suggest that low salinity induced an initial metabolic acidosis in the intracellular compartment, followed by a net efflux of acid equivalents from the tissues. The nature of this metabolic acidosis is unknown, but appears to have an overriding effect on acid–base balance on initial transfer.

In the present study there was no change in haemolymph [Na^+] after transfer to dilute seawater but [Cl^-] fell significantly to a new steady state after 3 h. In the strong osmoregulating crab *Callinectes sapidus*, transfer from high to low salinity resulted in a marked drop in haemolymph Na^+, Cl^- and osmolarity within the first 6 h. After this time all three values stabilised but the resulting [Cl^-] was lower than [Na^+], resulting in a positive difference between the two ions 96 h after transfer to low salinity. Henry and Cameron (Henry and Cameron, 1982) reasoned that the Na^+–Cl^- difference was a reliable indicator of strong ion difference and could be used to explain the observed changes in haemolymph pH during low salinity acclimation. Subsequent analysis of strong ion difference, however, revealed several problems in using this approach to explain acid–base changes, because of a number of theoretical and practical difficulties, including the measurement of the major dissociated cations and anions (for more details see Cameron and Iwama, 1989). Moreover, HCO_3^- is considered to be one of the anionic components of physiological fluids, and is a function of several other variables, such as pH (Truchot, 1987). In *Callinectes sapidus*, the Na^+–Cl^- difference was correlated with the increase in HCO_3^- concentrations (Henry and Cameron, 1982), but not in

Table 1. *Effects of 48 h of seawater dilution on acid–base variables and Cl^- concentrations in the haemolymph of a range of decapod crustaceans*

		Acid–base variable					
Species	Salinity (%)	pH	Δ[H^+] (nmoles l^{-1})	P_{CO_2} (kPa)	[HCO_3^-] (mequiv l^{-1})	Δ[HCO_3^-] (mequiv l^{-1})	[Cl^-] (mequiv l^{-1})
Carcinus maenas[a]	100	7.85	–5.8	0.21	5.5	2.5	525
	30	8.08		0.20	8.0		300
Callinectes sapidus[b]	100	7.75	–3.4	0.32	4.2	4.8	450
	30	7.84		0.40	9.0		340
Eriocheir sinensis[c]	100	7.89	–3.4	0.28	8.0	4.2	–
	FW	7.98		0.43	12.2		–
Eriocheir sinensis[d]	100	7.76	–5.8	0.46	7.09	1.93	412
	30	7.94		0.31	9.02		318

Δ[H^+], differences in H^+ concentrations between 100 % sea water (SW) values and either 30 % sea water (SW) or fresh water (FW) values; Δ[HCO_3^-], differences in [HCO_3^-] between 100 % SW values and either 30 % SW or FW values.

Data taken from [a]Truchot, 1981; [b]Henry and Cameron, 1982; [c]Truchot, 1992; [d]this study.

the present study where a negative Na^+–Cl^- difference prevailed 48 h after transfer to low salinity, accompanied by a small increase in HCO_3^- (see Table 1). Further measurements are needed before the role of strong ion difference can be determined during low salinity exposure. Interestingly, there are some remarkable similarities between acid–base adjustments in osmoregulating crabs after 48 h at low salinity, as summarised in Table 1. For instance, haemolymph $[H^+]$ in *Eriocheir sinensis* held in 30 % sea water (this study), decreased over 2 days by an equivalent amount to that observed in *Carcinus maenas* by Truchot (Truchot, 1981), with a similar increase in haemolymph $[HCO_3^-]$, suggesting that similar mechanisms are involved.

Previous studies have shown that the ion-transporting enzymes Na^+/K^+-ATPase and carbonic anhydrase increase in activity in the posterior gills of osmoregulating crabs 3 days after transfer (Holliday, 1985; Harris and Santos, 1993; Corotto and Holliday, 1996). Indeed, measurements of Na^+/K^+-ATPase activities in the posterior gills of *Eriocheir sinensis* showed little change over the 2 day transfer period (N. M. Whiteley, unpublished observations). Evidence for the involvement of carbonic anhydrase in osmoregulation was obtained by injecting *Callinectes sapidus* with the inhibitor acetazolamide (Henry and Cameron, 1982). After injection, osmoregulation failed and the crabs did not survive transfer to dilute sea water. Unfortunately, the corresponding changes in haemolymph acid–base status were not determined, although the present study shows that the acid–base adjustments observed in the first 2 days of transfer could not be caused by active alterations in ion regulation. Alternatively, passive exchange mechanisms may play a more important role, with a reduction in salinity decreasing the availability of Cl^- for branchial Cl^-/HCO_3^- exchange, leading to an accumulation of haemolymph $[HCO_3^-]$ and a reduction in base output. Although there is no evidence for such passive changes in the present study, a similar explanation was given by Truchot (Truchot, 1992) for the metabolic alkalosis observed in *Eriocheir sinenesis* after transfer to fresh water.

In the present study there was no change in muscle $[Na^+]$ and $[Cl^-]$ in *Eriocheir sinensis* after transfer to low salinity, even though muscle pHi and $[HCO_3^-]$ levels continued to change, suggesting that intracellular acid–base adjustments are secondary to the changes caused by ion regulation. In contrast, acid–base adjustments showed a similar time course to the alterations associated with cell volume control between 6 and 48 h after transfer to low salinity. For instance, by 6 h transfer there was a significant increase in free amino acid concentrations ([FAA]) in the leg muscle, which appeared as a 2.5-fold increase in haemolymph [FAA] after 24 h, indicating an increase in intracellular organic osmolytes (Gilles, 1983). Parallel measurements on whole-body flux rates showed that ammonia excretion rates increased significantly by 48 h transfer, indicating deamination of the increased free amino acid pool (R. Geoghegan and N. M. Whiteley, unpublished observations). In the present study, haemolymph ammonia levels remained unchanged during seawater dilution. In contrast, Mangum et al. (Mangum et al., 1976) found that after 2 days in dilute seawater, haemolymph ammonia concentrations and ammonia excretion rates increased in *Callinectes sapidus*. These authors concluded that ammonia output on exposure to low salinity was related to adjustments in ion regulation with increased ammonia excretion at the gills due to Na^+/NH_4^+ exchange, causing an increased loss of H^+ ions. In addition, the transfer of inorganic osmolytes from the cells during regulatory cell volume decrease may have contributed to the haemolymph metabolic alkalosis. The outward movement of HCO_3^- via nonselective anion channels during cell volume decrease has been shown to result in an intracellular acidosis in mammalian cells (Lang et al., 1998). In the present study, the recovery of HCO_3^- in the haemolymph 24 h after exposure to low salinity coincided with a fall in pHi and $[HCO_3^-]$ in the leg muscle, suggesting a similar response in crustacean cells.

In contrast to the acid–base adjustments recorded in the strong osmoregulating crabs *Eriocheir sinensis* and *Callinectes sapidus* (Henry and Cameron, 1982; Truchot, 1992), acid–base variables in the osmoconformer *Necora puber* were unaffected by transfer to low salinity (60 % sea water). In contrast, haemolymph $[Cl^-]$ fell to a new steady state 6 h after transfer, to equilibrate with the external change in $[Cl^-]$. Collectively these observations suggest that regulatory adjustments in ion levels and cell volume are important factors in causing acid–base imbalance during transfer to low salinity. Earlier experiments showed that active changes in ion regulation take approximately 3 days after salinity transfer, so it appears that the adjustments involved in cell volume control are more important in causing the pronounced metabolic alkalosis observed in most osmoregulating crabs 2 days after transfer to dilute seawater (Table 1).

In summary, transfer of marine omoregulating crabs to low salinity results in a metabolic alkalosis in the haemolymph that can be transient or persistent. This alkalosis has been attributed to perturbations of ion exchanges at the gill, changes in strong ion differences that accompany ion regulatory changes, an increase in ammonia excretion caused by increased deamination of organic osmolytes, and exchange of inorganic osmolytes for cell volume regulation (Mangum et al., 1976; Henry and Cameron, 1982; Truchot, 1992). In the present study it was found that haemolymph acid–base changes in *Eriocheir sinensis* during exposure to low salinity were secondary to ion regulation and cell volume control, as no acid–base adjustments were observed in the osmoconforming crab *Necora puber*. In osmoregulating crabs, cell swelling during low salinity exposure activates an increase in free amino acid, ammonia and HCO_3^- efflux from the cells. Ammonia excretion rates increase in both *Eriocheir sinensis* (R. Geoghegan and N. M. Whiteley, unpublished observations) and *Callinectes sapidus* (Mangum et al., 1979) and may increase H^+ efflux by Na^+/NH_4^+, although there is no evidence that Na^+/NH_4^+ exchange increases at low salinity. Consequently the relationship between water salinity and haemolymph acid–base status is complex, involving the

exchange of acid–base equivalents, electrolytes and osmolytes between body compartments and the surrounding medium, but only in crabs with an ability to maintain ionic and osmotic homeostasis.

This paper is dedicated to J. P. Truchot, whose many publications on acid–base regulation in decapod crustaceans have provided much inspiration. We also thank Roni Robbins at The Natural History Museum, London, for supplying *Eriochier sinensis* from the Thames.

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