J. exp. Biol. **133**, 73–86 (1987)
Printed in Great Britain © *The Company of Biologists Limited 1987*

BRANCHIAL AND ANTENNAL GLAND Na^+/K^+-DEPENDENT ATPase AND CARBONIC ANHYDRASE ACTIVITY DURING SALINITY ACCLIMATION OF THE EURYHALINE CRAYFISH *PACIFASTACUS LENIUSCULUS*

By MICHÈLE G. WHEATLY
Zoology Department, University of Florida, Gainesville, FL 32611, USA

AND RAYMOND P. HENRY
Department of Zoology and Wildlife Science, Auburn University, Auburn, AL 36849, USA

Accepted 1 June 1987

SUMMARY

Haemolymph and urine electrolyte status and branchial and antennal gland activities of Na^+/K^+-ATPase and carbonic anhydrase (CA) were determined in the crayfish *Pacifastacus leniusculus* after 3 weeks acclimation in fresh water (FW) and 350 and 750 mosmol kg^{-1} sea water (SW). In FW the crayfish maintained haemolymph osmolality around 370 mosmol kg^{-1} due to hyperionic regulation of the major electrolytes. Involved in this are ion uptake mechanisms situated on the gills, and mechanisms of ion reabsorption from the primary urinary filtrate in the antennal gland (AG). Both of these processes are associated with high activities of Na^+/K^+-ATPase and CA. The two enzymes are uniformly distributed on gill sets 2–7, unlike the situation in euryhaline marine species. Additionally, activity levels of both enzymes are extremely high in the AG and can be correlated with the ability to produce a hypo-osmotic urine. In comparison, enzyme activity is negligible in marine species which produce isosmotic urine.

Crayfish continued to hyperosmoregulate in 350 mosmol kg^{-1} SW. High levels of Na^+/K^+-ATPase confirmed the presence of a component active in the uptake of major electrolytes in the gills and also in the AG, where ion reabsorption persisted. In 750 mosmol kg^{-1} SW crayfish became isosmotic. Since ATPase is regulated chiefly by deactivation/activation of pre-existing enzyme, overall activity was mostly unchanged. CA activity was significantly reduced in both 350 and 750 mosmol kg^{-1} SW and correlated with the transition from osmoregulation to osmoconformity, suggesting that it is regulated primarily by deinduction/induction of new enzyme. The difference in the mechanism of regulation exhibited by these two enzymes is believed to relate to their subcellular distribution.

INTRODUCTION

In recent years research on osmoregulation in decapod crustaceans has focused largely on euryhaline marine species such as *Callinectes* and *Carcinus* which are

Key words: Na^+/K^+-ATPase, carbonic anhydrase, crayfish, ion regulation.

isomotic in full-strength sea water but which hyper-regulate in dilute sea water (for reviews see Kirschner, 1979; Mantel & Farmer, 1983). Hyper-regulation requires active ion uptake, primarily at the gills, and elimination of the water load *via* the antennal gland. Since the urine produced is isosmotic it constitutes a significant route for counterproductive ion loss (Cameron & Batterton, 1978). Similar vectors of ion and water movement are evident in predominantly freshwater species such as the crayfish; these species, however, have lower concentrations of ions in the circulating body fluids, and they have developed the ability, unique amongst decapods, to produce a hypo-osmotic urine (Bryan, 1960*a*). Thus, they are extremely effective hyper-regulators. Certain freshwater crayfish (e.g. *Pacifastacus leniusculus*) are also quite euryhaline and can survive in 13 ‰ salinity (Miller, 1960). Furthermore, recent studies suggest that *P. leniusculus* regulates its haemolymph hypo-osmotically to more concentrated sea water (750 mosmol kg^{-1}), at least on a short-term basis (Kerley & Pritchard, 1967; Wheatly & McMahon, 1982). Thus, this animal affords the unique opportunity for studying the physiological mechanisms of both hyper- and hyporegulation.

The active transport of ions across epithelia is primarily attributable to cation- and anion-dependent ATPases (Towle, 1984). Carbonic anhydrase (CA) is indirectly involved in this process since it provides the counterions for Na^+ and Cl^- uptake (i.e. H^+ and HCO_3^-) from an intracellular pool of molecular CO_2 (Henry & Cameron, 1983; Henry, 1984). These two enzymes typically share a common distribution and are used as indicators of ion regulation. In euryhaline marine decapods activity is greatest in the posterior two or three gills which are the primary site of salt and water balance (Neufeld, Holliday & Pritchard, 1980; Henry & Cameron, 1982*a*). Increases in activity of both enzymes have been reported under conditions of hyper-regulation (Towle, Palmer & Harris, 1976; Neufeld *et al.* 1980; Henry & Cameron, 1982*b*); extrabranchial distribution is less well documented.

The present investigation of *P. leniusculus* had two original objectives: to compare branchial and antennal gland ATPase and CA activities with the known distribution in marine species; and to document long-term changes in haemolymph and urine ion status and tissue transport enzyme activities under conditions of both hyper- (FW) and hyporegulation (750 mosmol kg^{-1}) *vs* those for conformity (350 mosmol kg^{-1}).

MATERIALS AND METHODS

Crayfish, *Pacifastacus leniusculus leniusculus* (Dana) were obtained from the Sacramento–San Joaquin estuary in California. Prior to experiments, animals were housed 15 to a 20-l aquarium in 10 l of fresh water at 12 °C under a 12 h:12 h light:dark cycle and fed weekly on chopped liver. The water, which was changed weekly, was recycled through a bottom filter; PVC pipes were provided as refuges. Experimental crayfish were acclimated for 3 weeks to 350 (or 475 in some cases) or 750 mosmol kg^{-1} SW obtained by diluting sea water (see Table 1).

Branchial transepithelial potential (TEP), osmolality and electrolyte concentrations in external water, haemolymph and urine, and tissue Na^+/K^+-dependent

Table 1. *Ionic composition of experimental media*

Medium	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-
Fresh water (14)	0·717 ± 0·021	0·005 ± 0·000	0·052 ± 0·003	0·045 ± 0·002	0·531 ± 0·024
350 mosmol kg^{-1} SW (6)	184·6 ± 7·8	3·32 ± 0·02	6·82 ± 0·10	35·33 ± 0·85	184·4 ± 1·3
750 mosmol kg^{-1} SW (6)	391·0 ± 10·7	8·20 ± 0·13	15·60 ± 0·32	68·80 ± 1·47	410·0 ± 15·1

Values expressed in mequiv l^{-1} as mean ± S.E.M. with *N* values in parentheses.

ATPase and CA data were obtained for 90 intermoult crayfish of both sexes (mean mass 31·90 ± 0·92 g).

Measurement of transepithelial potential

The TEP between *Pacifastacus* haemolymph and the external solution was measured using a procedure modified from Evans, Cooper & Bogan (1976). Agar bridges (PE 160 tubing containing 2 % agar in crayfish saline) were inserted into the haemolymph (through a hole drilled in the carapace just anterior to the cephalic groove in the dorsal midline for recording) and into the bathing solution (for reference). These were connected to test tubes filled with 3 mol l^{-1} KCl and containing matched calomel electrodes, and the potential was recorded on a Keithley model 616 digital multimeter ($10^{13}\,\Omega$ input impedance). The water level was adjusted so that the dorsal cephalothorax was exposed while the branchial inhalant openings remained submerged; this arrangement prevented short-circuiting between the haemolymph and the bath. All measured TEPs were corrected for electrode, bridge and tip asymmetry.

Haemolymph and urine sampling

A prebranchial haemolymph sample (200–400 μl) was removed from the infrabranchial sinus of the cheliped using a Hamilton syringe. Urine samples were obtained by lifting the nephropore flap with a hooked needle and sucking fluid into a drawn-out Pasteur pipette.

Tissue preparation

Crayfish were killed and the following tissues dissected out and assayed for ATPase and CA activity: heart, both antennal glands, and seven individual sets of gills combined from both sides of the crayfish (numbered 2–7, anterior to posterior).

In contrast to brachyurans, as many as three trichobranchiate gills arise from each thoracic segment in macrurans. In segments 3–7 of the crayfish these consist of a large podobranch which arises from the base of the appendage and two smaller arthrobranchs arising from the arthrodial membrane. Preliminary assays revealed that Na^+/K^+-ATPase activity was uniformly distributed amongst the three filaments, and thus the filaments within each gill were pooled for both enzymatic assays.

Osmolality and inorganic ion concentrations

Osmolality was determined immediately using a vapour pressure osmometer (Wescor 5100B). Samples were subsequently frozen and thawed at a later date for determination of inorganic ion levels. Cl^- concentration was determined using either a digital chloridometer (Aminco-4-4417) or a coulometric titrator (Radiometer CMT 10). Cation concentrations (Na^+, K^+, Mg^{2+} and Ca^{2+}) were determined by atomic absorption spectrophotometry (Perkin Elmer 2380).

Na^+/K^+-ATPase assay

Tissues were quickly dissected out, blotted dry and placed in 10 volumes of ice-cold homogenizing solution ($0{\cdot}25\,mol\,l^{-1}$ sucrose, $6\,mmol\,l^{-1}$ EDTA, $20\,mmol\,l^{-1}$ imidazole, 0·1 % sodium deoxycholate) at pH 6·8. The tissues were homogenized in an iced ground-glass tissue homogenizer, the shaft of which was driven by a Talboys 134-1 motor at up to 2000 rev. min^{-1}. Homogenates were centrifuged for 2 min at 13 750 rev. min^{-1} in a Fisher 235B microcentrifuge, and the supernatant was saved on ice for the assay.

Na^+/K^+-ATPase activity was measured by determination of the amount of inorganic phosphate (Pi) liberated from the hydrolysis of the substrate adenosine triphosphate (ATP). The methodology was compiled from previous investigations by Towle *et al.* (1976), Neufeld *et al.* (1980) and Stagg & Shuttleworth (1982). Briefly, enzyme activity was determined as the difference between Pi liberated from ATP in the presence of K^+ (experimental) and in the absence of K^+ with ouabain present (control). Samples of $100\,\mu l$ of the fresh crude homogenate were preincubated with 2 ml of incubation medium containing either $100\,mmol\,l^{-1}$ NaCl, $30\,mmol\,l^{-1}$ KCl, $5\,mmol\,l^{-1}$ $MgCl_2$ and $20\,mmol\,l^{-1}$ imidazole at pH 7·8 (experimental) or $130\,mmol\,l^{-1}$ NaCl, $5\,mmol\,l^{-1}$ $MgCl_2$, $20\,mmol\,l^{-1}$ imidazole and $1\,mmol\,l^{-1}$ ouabain also at pH 7·8 (control). The reaction was initiated in all tubes by the addition of 0·2 ml of $50\,mmol\,l^{-1}$ disodium-ATP (neutralized with imidazole).

Following incubation for 45 min at 25 °C, the reaction was stopped by the addition of 2 ml of ice-cold ammonium molybdate [2 % $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ in $1{\cdot}8\,mol\,l^{-1}$ H_2SO_4]; Pi was determined on 1 ml of reaction volume as described by Atkinson, Gatemby & Lowe (1973). Protein concentration of the crude homogenate was determined using the Coomassie brilliant blue dye-binding technique (Bio-Rad Laboratories, Technical Bulletin 1051). The portion of the ATPase activity dependent on the simultaneous presence of Na^+ and K^+ was calculated as the difference between activities in experimental and control tubes and is expressed in nmol Pi mg $protein^{-1}$ min^{-1}.

Carbonic anhydrase assay

Tissues were dissected, quickly blotted dry and placed in approximately eight volumes of cold buffer ($225\,mmol\,l^{-1}$ mannitol, $75\,mmol\,l^{-1}$ sucrose, $10\,mmol\,l^{-1}$ Tris, adjusted to pH 7·40 with $HPO_4{}^{2-}$ – see Henry & Kormanik, 1985; Henry,

Dodgson, Storey & Forster, 1986). Tissues were homogenized with a motor-driven ground-glass homogenizer and centrifuged for 20 min at approximately 10 000 ***g*** at 4°C (Sorvall RC-5B). The resulting supernatant was assayed for CA activity according to the method of Henry & Kormanik (1985). The initial velocity of hydration was monitored by following the rapid decrease in pH using sensitive semi-micro electrodes (World Precision Instruments MEPH-2 Beetrode and MERE-2 reference) coupled to a null-point differential pH meter (University of Pennsylvania Biological Instrumentation Group). Velocity was measured for a decrease of approximately 0·15 pH units. By calibrating the system with samples of 0·1 $mol\,l^{-1}$ HCl, reaction velocity could be measured as $\mu mol\,CO_2\,min^{-1}$. Protein concentrations were determined for each sample with Coomassie brilliant blue (Bio-Rad Laboratories), and CA activity was reported as $\mu mol\,CO_2\,mg\,protein^{-1}\,min^{-1}$.

Statistical treatment of data

Data are expressed as mean ± S.E. (number of observations). Sample means were compared by Student's two-tailed *t*-test (unpaired variates) using $P < 0{\cdot}05$ as the confidence limit.

RESULTS

Transepithelial potential

The TEP in FW-acclimated crayfish was −13·9 ± 1·5 mV (6) (haemolymph relative to medium). Values recorded after acclimation in 350 and 750 $mosmol\,kg^{-1}$ media, respectively, were +0·5 ± 1·1 (4) and +1·0 ± 2·7 mV (4).

Haemolymph and urine ion regulation

Haemolymph osmolality and inorganic ion levels are plotted as a function of external levels in Figs 1–3. In FW, haemolymph osmolality was maintained approximately 365 $mosmol\,kg^{-1}$ above ambient due to hyperionic regulation of all major electrolytes. Crayfish acclimated to 350 $mosmol\,kg^{-1}$ SW were still hyperosmotic due to hyperionic regulation of Na^+, Cl^- and Ca^{2+}, although the haemolymph–medium differences were reduced by two-thirds. The difference was reduced further in crayfish acclimated to 475 $mosmol\,kg^{-1}$ SW. Crayfish acclimated to 750 $mosmol\,kg^{-1}$ SW were virtually isosmotic and isoionic with respect to $[Na^+]$ and $[K^+]$ although $[Cl^-]$ and $[Mg^{2+}]$ were maintained below ambient. In the present study the only invariant ion was $[Ca^{2+}]$ which was regulated consistently above ambient levels.

The urine produced was considerably hypo-osmotic to the haemolymph due to active reabsorption of all major electrolytes. This is reflected in urine:haemolymph ion ratios of less than 1·0 (Table 2). In the case of the major ions, Na^+ and Cl^-, reabsorption takes place against a 10-fold concentration gradient. In 350 mosmol

kg^{-1} SW these ratios increased although they remained significantly below 1·0. It was impossible to collect urine for analysis in crayfish acclimated to 750 mosmol kg^{-1} SW.

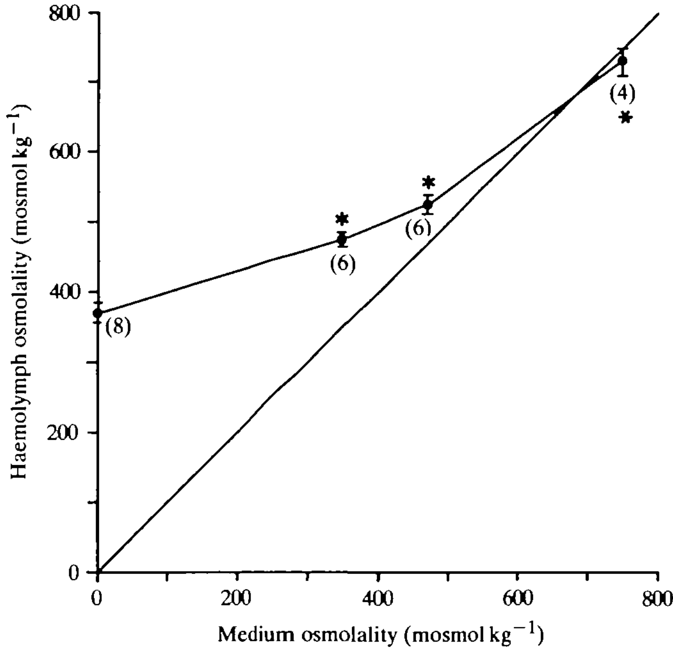

Fig. 1. Haemolymph osmolality in *Pacifastacus leniusculus* plotted as a function of external osmolality (isosmotic line indicated diagonally). Values are mean ± S.E.M. with N values indicated in parentheses. Asterisks denote significant departures from values determined in fresh water.

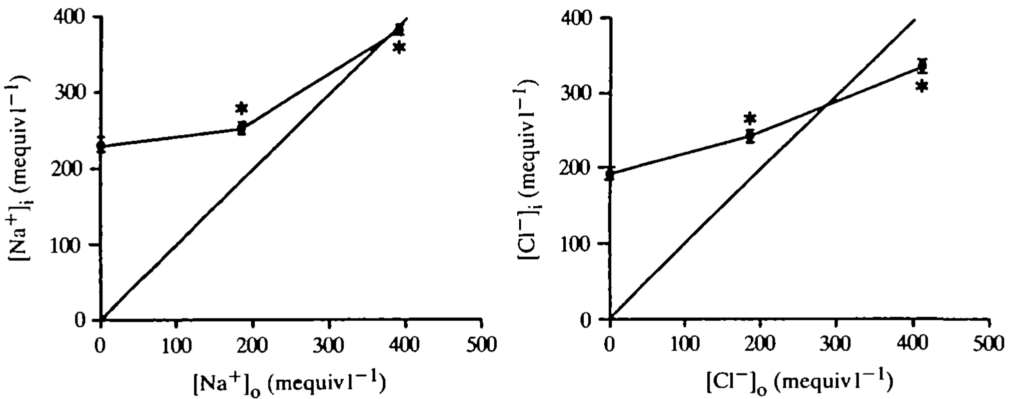

Fig. 2. Haemolymph (designated i) concentrations of Na^+ and Cl^- in *Pacifastacus leniusculus* plotted as a function of external levels (designated o). Values are mean ± S.E.M. Asterisks denote significant departures from values determined in fresh water.

Na^+/K^+-ATPase and carbonic anhydrase activities

Tissue Na^+/K^+-ATPase levels at each acclimation salinity are illustrated in Fig. 4. In FW-acclimated crayfish, there was no significant difference in ATPase

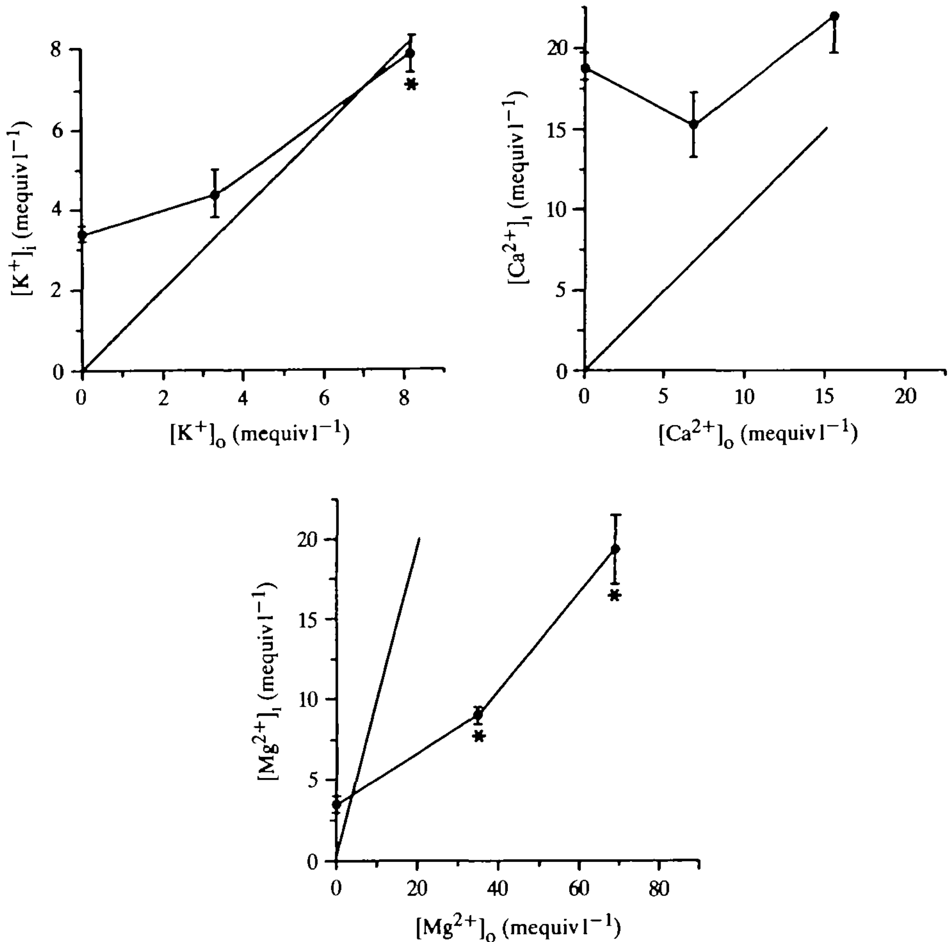

Fig. 3. Haemolymph (designated i) concentrations of K^+, Ca^{2+} and Mg^{2+} in *Pacifastacus leniusculus* plotted as a function of external levels (designated o). Values are mean ± S.E.M. Asterisks denote significant departures from values determined in fresh water.

Table 2. *Urine: haemolymph ratios of the major haemolymph ions of* Pacifastacus leniusculus *acclimated to fresh water and 350 mosmol kg^{-1} sea water for 3 weeks*

Salinity	Na^+	Cl^-	K^+	Ca^{2+}	Mg^{2+}
Fresh water (8)	0·094 ± 0·014	0·022 ± 0·005	0·192 ± 0·020	0·069 ± 0·005	0·156 ± 0·045
350 mosmol kg^{-1} (5)	0·192 ± 0·041	0·161 ± 0·030	0·385 ± 0·162	0·175 ± 0·019	0·454 ± 0·169

Values are mean ± S.E.M. N values given in parentheses.

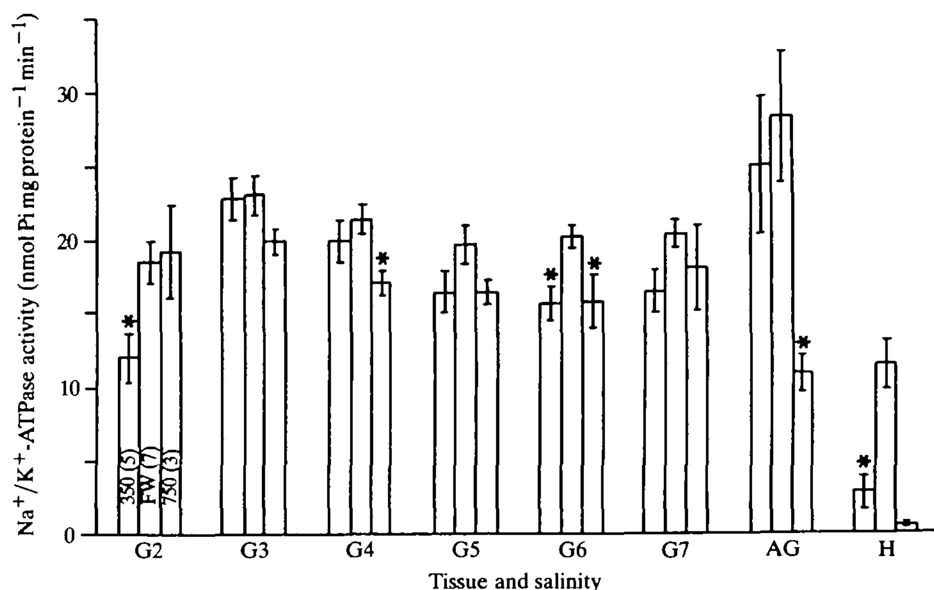

Fig. 4. Na^+/K^+-ATPase activity in various tissues of *Pacifastacus leniusculus* acclimated for 3 weeks in fresh water (FW), 350 or 750 mosmol kg^{-1} sea water. Values are mean ± S.E.M. with N values in parentheses. Asterisks denote significant departures from values determined in FW. G, AG and H refer, respectively, to gill, antennal gland and heart.

levels among gill pairs 2–7, indicating that enzyme activity is homogeneously distributed throughout the branchial tissue. Mean activity in the antennal gland was 50 % higher than in the gills; this was not significant, however, due to variation around mean values. Enzyme activity in both tissues was significantly greater than in the heart, a tissue which is not involved in ion regulation.

After acclimation for 3 weeks to 350 mosmol kg^{-1} SW there was no significant change in ATPase activity in the antennal gland and in most of the gills. In 750 mosmol kg^{-1} SW a 60 % reduction in activity was measured in the antennal gland and a 20 % reduction in two of the gill sets. Levels in the remaining gills were unchanged.

More pronounced changes were observed in CA activity (Fig. 5). In FW, a uniform distribution of enzyme activity was measured in all six sets of gills. Antennal gland activity, however, was almost three-fold greater than branchial activity (2200 and 800 μmol CO_2 mg protein^{-1} min^{-1}, respectively). Baseline levels of activity in the heart were very low in comparison (100 μmol CO_2 mg protein^{-1} min^{-1}).

On acclimation to either 475 mosmol kg^{-1} SW or 750 mosmol kg^{-1} SW there were comparable reductions in CA levels which were significant in all tissues except the heart. In the gills, activity was reduced to 25 % of the FW value and was once again uniformly distributed among the six gill sets. More pronounced changes in activity were measured in the antennal gland, where a greater reduction in CA activity was observed in 750 mosmol kg^{-1} SW (80 %) than in 475 mosmol kg^{-1} SW (64 %). Heart CA activity was unchanged.

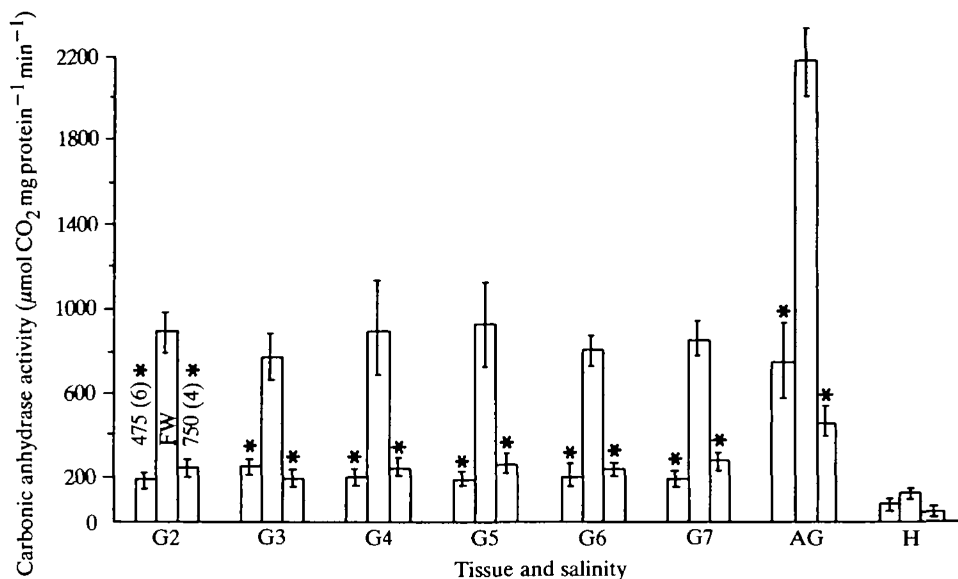

Fig. 5. Carbonic anhydrase activity in various tissues of *Pacifastacus leniusculus* acclimated for 3 weeks in fresh water (FW), 475 or 750 mosmol kg^{-1} sea water. Values are mean ± S.E.M. with N values in parentheses. Asterisks denote significant departures from values determined in FW. G, AG and H refer, respectively, to gill, antennal gland and heart.

DISCUSSION

Ion regulation with salinity acclimation

The ability of *Pacifastacus* to hyper-regulate effectively in FW (Figs 1–3) is due to a combination of factors, some of which diverge markedly from the adaptations seen in euryhaline marine species. The presence of active ion uptake mechanisms on the gills (Bryan, 1960*b*; Shaw, 1964; Ehrenfeld, 1974) is a common feature of both groups, and it is indicated in this study by the marked departure of a measured TEP from the calculated equilibrium potentials for both Na^+ (E_{Na} = −140·2 mV) and Cl^- (E_{Cl} = +146·5 mV). This is linked with a reduction in osmotic permeability, as shown by a significantly lower rate of urine production [3 % body mass day^{-1} (M. G. Wheatly, unpublished data) *vs* 20 % in *Callinectes* (Cameron, 1978)]. Furthermore, the antennal gland in crayfish eliminates this water load more efficiently since ions are reabsorbed from the urine (see Table 2).

As the external osmotic concentration was raised, haemolymph osmolality increased, probably due to a reduction in diffusional ion loss (Bryan, 1960*b*). In 350 mosmol kg^{-1} medium, E_{Na} and E_{Cl} were calculated as −7·7 and +6·0 mV, respectively, indicating that there was still an active component to uptake of the major electrolytes. Interestingly, the antennal gland also retained the ability to conserve ions (Table 2) under conditions approaching osmoconformity. However, urine flow rate decreased by 85 % (M. G. Wheatly, unpublished results) as one would predict from the reduced water load.

The ability to hyporegulate during the initial 48 h of exposure to 750 mosmol kg^{-1} SW (Wheatly & McMahon, 1982) did not persist, and crayfish were virtually isosmotic after 3 weeks (Fig. 1). This confirms that complete osmotic equilibration in decapods may require several days (see Bryan 1960*c*; Harris & Micallef, 1971). The close agreement between E_{Na} (+3·3 mV) and the measured TEP suggests that Na^+ was distributed passively (confirmed by Fig. 2). This agrees with Bryan's (1960*c*) results, which showed that active Na^+ movements ceased at external concentrations around 400 mequiv l^{-1}. Chloride ions, however, continue to be actively extruded at these levels ($E_{Cl} = -5{\cdot}2$ mV), as illustrated in Fig. 2, agreeing with the accepted model for salt secretion in teleost fish. Preliminary data (M. G. Wheatly, unpublished) indicate that urine flow rate is virtually undetectable under these conditions.

In addition, this study demonstrated the crayfish's ability to regulate divalent cation concentrations. Based on the present data, $[Ca^{2+}]$ must be actively taken up at all salinities (Greenaway, 1985), a process which is probably branchial in all cases. Magnesium, however, must be secreted when concentrations exceed 10 mequiv l^{-1}. In marine decapods this generally occurs at the antennal gland (Gross & Capen, 1966). However, since the urine:haemolymph ratio for Mg^{2+} (Table 2) never exceeded 1·0 at the higher salinities, we can only conclude that Mg^{2+} was secreted extrarenally.

Distribution of transport enzyme activities

Quantitatively, branchial ATPase and CA activities in *Pacifastacus* (Figs 4, 5) agree with values reported in other aquatic arthropods (see reviews by Henry, 1984; Towle, 1984). In marine species, branchial ATPase activity tends to be higher (around 300–400 nmol mg^{-1} min^{-1}; Neufeld *et al.* 1980; Towle, Mangum, Johnson & Mauro, 1982; Holliday, 1985) corresponding with increased branchial ion fluxes. Na^+ and Cl^- influxes are both around 40 μequiv g^{-1} h^{-1} in *Callinectes* in fresh water (Cameron, 1978) compared to 0·5 μequiv g^{-1} h^{-1} in *Pacifastacus* (M. G. Wheatly, unpublished results). Other authors (Horiuchi, 1977; Chapelle & Zwingelstein, 1984) have also reported low ATPase activities in freshwater decapods. The comparison is better documented, however, in teleost fish where Na^+/K^+-ATPase levels in the gills of marine species are typically 2- to 10-fold higher than in freshwater species (see reviews by Jampol & Epstein, 1970; de Renzis & Bornancin, 1984). This difference is less apparent for CA since isosmotic crayfish (475 mosmol kg^{-1} SW, this study) and blue crabs (full-strength SW, Henry & Cameron, 1982*b*) both have branchial CA activities in the range 100–300 μmol CO_2 mg $protein^{-1}$ min^{-1}. Furthermore, the increase observed in both species during hyper-regulation was comparable.

Irrespective of acclimation salinity, the distribution of transport enzymes in the crayfish is striking in two respects. First, activity is homogeneously distributed among the gill sets and, second, activity in the antennal gland is greater than in the gills.

In marine crabs such as *Callinectes*, ATPase activity is concentrated in the posterior two or three gill pairs where the chloride cells are located (Copeland &

Fitzjarrell, 1968). Anterior gills, which function primarily in gas exchange (Aldridge & Cameron, 1979), contain only baseline levels. CA activity is similarly distributed (Henry & Cameron, 1982*b*). Not surprisingly, this is the region of the branchial tissue where changes in transport enzyme activities are observed during hyper-regulation.

Dickson & Dillaman (1985) recently showed that chloride cells are homogeneously distributed throughout the gill pairs in crayfish. Histological findings would therefore tend to confirm the present biochemical evidence that all gills function in ion transport as well as gas exchange in this species. The increased demands of a predominantly freshwater existence may require all gills to function in ion regulation. Similarly, the uniform ATPase distribution among gill pairs reported in four species of land crabs (Towle, 1981; Henry & Cameron, 1982*b*) may enable the gills to take equal advantage of limited and periodically available fresh water for rehydration when severely challenged by arid environments. Thus the presence or absence of anterior/posterior differentiation probably relates to the environmental demands placed on the animal with respect to its ability to maintain proper salt and water balance.

Our second noteworthy finding in the crayfish was higher enzyme activity in the antennal gland than in the gills. The reverse is true in marine species where antennal gland levels (only documented for CA) are both very low and salinity-insensitive (Henry & Cameron, 1982*b*). This is consistent with the relative role of this organ in ion regulation; marine species produce isosmotic urine (Cameron & Batterton, 1978; Cameron, 1979), whereas the crayfish antennal gland has a demonstrated ability to reabsorb electrolytes from the primary filtrate against a concentration gradient (Table 2). Increased renal Na^+/K^+-ATPase activity has similarly been associated with Na^+ retention in the process of hypo-osmotic urine formation in other aquatic vertebrates (Saintsing & Towle, 1978; Towle *et al.* 1982). A parallel can again be seen with land crabs, which also possess high antennal gland ATPase activity, although their urine is isosmotic. In this case the antennal gland may limit water loss by reducing urinary flow.

Enzyme activity with acclimation salinity

Even though the haemolymph–medium osmolality gradient was greatly reduced in 350 mosmol kg^{-1} SW, crayfish were still hyperosmotic (Fig. 1) and hyperionic (Figs 2, 3). TEP measurements confirmed that there was still an active component to the uptake of major electrolytes. This explains why ATPase activities were largely unchanged at the gills (Fig. 4). The urinary data confirmed that *P. leniusculus* retained its ability to reabsorb ions from the primary urine even under near isosmotic conditions, as shown by urine:haemolymph ion ratios below 1·0 (Table 2). The active reabsorption of electrolytes requiring high levels of ATPase presumably contributed to the hyperosmotic condition in the haemolymph.

In contrast, in 750 mosmol kg^{-1} SW the haemolymph was virtually isosmotic and soionic. Associated with this transition from regulation to conformity was a significant reduction in ATPase activity in the antennal gland. While this suggests

that postfiltrational electrolyte reabsorption had declined, we were unable to collect urine to support this theory. A significant reduction in ATPase activity was only reported in two of the six sets of gills. Recently we have examined the dynamics of salinity acclimation in *P. leniusculus* (R. P. Henry & M. G. Wheatly, in preparation) and found that ATPase is regulated largely on a short-term basis by deactivation/activation of pre-existing enzyme. This fits in with its subcellular distribution since a basal–lateral membrane location (Towle, 1984) would expose it to haemolymph factors such as ions and biogenic amines which change early in the salinity response (R. P. Henry & M. G. Wheatly, in preparation). In support of this Mantel *et al.* (1986) have recently shown that dopamine increases the activity of Na^+/K^+-ATPase in the microsomal fraction of green crab, *Carcinus maenas*, gills by activating enzyme already present. Since induction/deinduction is not strongly implicated in ATPase regulation, one would not expect to observe long-term changes in overall enzyme activity.

Carbonic anhydrase activity was highest in fresh water, supporting the conclusion that branchial CA plays the same role in ion regulation previously outlined for *Callinectes* (Henry & Cameron, 1983; Henry, 1984), namely that it provides counterions for Na^+ and Cl^- uptake from intracellular CO_2. Furthermore, CA inhibition by acetazolamide has identical effects on Na^+ and Cl^- fluxes in the two species (Ehrenfeld, 1974; Cameron, 1979). Unlike ATPase in the present study, CA activity decreased significantly in both 475 and 750 mosmol kg^{-1} SW in gills and antennal gland (Fig. 5).

The CA activity at the antennal gland was greater in 475 than in 750 mosmol kg^{-1} SW, suggesting that it decreased with the haemolymph–medium osmolality gradient, although significant activity was still present in isosmotic crayfish. The role of CA in the antennal gland is presumably similar to that in the gills, namely to provide counterions, only this time for tubular reabsorption of Na^+ and Cl^-. If this is true, then the present data would suggest that tubular reabsorption occurs even in 750 mosmol kg^{-1} SW but at a reduced rate.

The fact that there was no significant difference in gill CA activity between animals in 475 and 750 mosmol kg^{-1} SW implies that the branchial enzyme was not involved in active Na^+ and Cl^- uptake in 475 mosmol kg^{-1} SW. A hyperosmotic condition in the blood could, as we have already suggested, have been attributed to postfiltrational electrolyte reabsorption.

The present findings demonstrate that CA activity is significantly reduced in all tissues as crayfish approach osmotic conformity with the environment after long-term acclimation. This suggests that activity is regulated by deinduction/induction of new enzyme. Recent measurements of the dynamics of this change (R. P. Henry & M. G. Wheatly, in preparation) confirm that it occurs after 7 days – corresponding with the time course for proliferation of chloride cells in the gills of other euryhaline species (Shirai & Utida, 1970). The fraction of CA that is involved in ion regulation is cytoplasmic (Henry, 1986) and, as such, is removed from the influence of transient changes in haemolymph ions and neurohormonal modulators. We believe that the

difference in the mechanism of enzyme regulation observed in the present investigation after long-term salinity acclimation of *P. leniusculus* is primarily attributable to differences in the subcellular distribution of the two enzymes.

We wish to thank Steve Shimizu at Pacific Crayfish Company, Gardena, California for providing an uninterrupted supply of animals. Marcia Schwassmann and Daniel Dent are thanked for help with the ATPase assays. MGW was supported by NSF Grant no. DCB 84-15373. RPH was supported by NSF Grant no. DCB 84-17379, Auburn University (Grant-in-Aid) and by funds from the Alabama Agricultural Experiment Station (AAES Jl. No. 15-861116).

REFERENCES

ALDRIDGE, J. B. & CAMERON, J. N. (1979). CO_2 exchange in the blue crab, *Callinectes sapidus* (Rathbun). *J. exp. Zool.* **207**, 321–328.

ATKINSON, A., GATEMBY, A. O. & LOWE, A. G. (1973). The determination of inorganic orthophosphate in biological systems. *Biochim. biophys. Acta* **320**, 195–204.

BRYAN, G. W. (1960*a*). Sodium regulation in the crayfish *Astacus fluviatilis*. I. The normal animal. *J. exp. Biol.* **37**, 83–99.

BRYAN, G. W. (1960*b*). Sodium regulation in the crayfish *Astacus fluviatilis*. II. Experiments with sodium-depleted animals. *J. exp. Biol.* **37**, 100–112.

BRYAN, G. W. (1960*c*). Sodium regulation in the crayfish *Astacus fluviatilis*. III. Experiments with NaCl-loaded animals. *J. exp. Biol.* **37**, 113–128.

CAMERON, J. N. (1978). NaCl balance in blue crabs, *Callinectes sapidus* in freshwater. *J. comp. Physiol.* **123**, 127–135.

CAMERON, J. N. (1979). Effects of inhibitors on ion fluxes, trans-gill potential and pH regulation in freshwater blue crabs, *Callinectes sapidus* (Rathbun). *J. comp. Physiol.* **133**, 219–225.

CAMERON, J. N. & BATTERTON, C. V. (1978). Antennal gland function in the freshwater blue crab, *Callinectes sapidus*: water, electrolyte, acid–base and ammonia excretion. *J. comp. Physiol.* **123**, 143–148.

CHAPELLE, S. & ZWINGELSTEIN, G. (1984). Phospholipid composition and metabolism of crustacean gills as related to changes in environmental salinities: relationship between Na^+-K^+-ATPase activity and phospholipids. *Comp. Biochem. Physiol.* **78**B, 363–372.

COPELAND, D. E. & FITZJARRELL, A. T. (1968). The salt absorbing cells in the gills of the blue crab (*Callinectes sapidus* Rathbun) with notes on modified mitochondria. *Z. Zellforsch. mikrosk. Anat.* **92**, 1–22.

DE RENZIS, G. & BORNANCIN, M. (1984). Ion transport and gill ATPases. In *Fish Physiology*, vol. Xb (ed. W. S. Hoar & D. J. Randall), pp. 65–104. New York: Academic Press.

DICKSON, J. S. & DILLAMAN, R. M. (1985). Distribution and ultrastructure of osmoregulation and respiratory filaments in the gills of the crayfish. *Am. Zool.* **24**, 214.

EHRENFELD, J. (1974). Aspects of ionic transport mechanisms in crayfish *Astacus leptodactylus*. *J. exp. Biol.* **61**, 57–70.

EVANS, D. H., COOPER, K. & BOGAN, M. B. (1976). Sodium extrusion by the sea-water acclimated fiddler crab *Uca pugilator*: comparison with other marine Crustacea and marine teleost fish. *J. exp. Biol.* **64**, 203–219.

GREENAWAY, P. (1985). Calcium balance and moulting in the Crustacea. *Biol. Rev.* **60**, 425–454.

GROSS, W. J. & CAPEN, R. L. (1966). Some functions of the urinary bladder in a crab. *Biol. Bull. mar. biol. Lab., Woods Hole* **131**, 272–291.

HARRIS, R. R. & MICALLEF, H. (1971). Osmotic and ionic regulation in *Potamon edulis* a freshwater crab from Malta. *Comp. Biochem. Physiol.* **38**A, 769–776.

HENRY, R. P. (1984). The role of carbonic anhydrase in blood ion and acid–base regulation. *Am. Zool.* **24**, 241–253.

HENRY, R. P. (1986). Subcellular distribution of carbonic anhydrase in the gills of the blue crab, *Callinectes sapidus*. *Am. Zool.* **26**, 216.

HENRY, R. P. & CAMERON, J. N. (1982*a*). Acid–base balance in *Callinectes sapidus* during acclimation from high to low salinity. *J. exp. Biol.* **101**, 255–264.
HENRY, R. P. & CAMERON, J. N. (1982*b*). The distribution and partial characterization of carbonic anhydrase in selected aquatic and terrestrial decapod crustaceans. *J. exp. Zool.* **221**, 309–321.
HENRY, R. P. & CAMERON, J. N. (1983). The role of carbonic anhydrase in respiration, ion regulation and acid–base balance in the aquatic crab *Callinectes sapidus* and the terrestrial crab *Geocarcinus lateralis*. *J. exp. Biol.* **103**, 205–225.
HENRY, R. P., DODGSON, S. J., STOREY, B. T. & FORSTER, R. E. (1986). Rat lung carbonic anhydrase: activity, localization and isozymes. *J. appl. Physiol.: Respirat. Environ. Exercise Physiol.* **60**, 638–645.
HENRY, R. P. & KORMANIK, G. A. (1985). Carbonic anhydrase activity and calcium deposition during the molt cycle of the blue crab, *Callinectes sapidus*. *J. crust. Biol.* **5**, 234–241.
HOLLIDAY, C. W. (1985). Salinity-induced changes in gill Na,K-ATPase activity in the mud fiddler crab, *Uca pugnax*. *J. exp. Zool.* **233**, 199–208.
HORIUCHI, S. (1977). Characterization of gill Na,K-ATPase in the freshwater crayfish *Procambarus clarkii* (Girard). *Comp. Biochem. Physiol.* **56B**, 135–138.
JAMPOL, L. M. & EPSTEIN, F. H. (1970). Sodium–potassium-activated adenosine triphosphatase and osmotic regulation by fishes. *Am. J. Physiol.* **218**, 607–611.
KERLEY, D. E. & PRITCHARD, A. W. (1967). Osmotic regulation in the crayfish *Pacifastacus leniusculus*, stepwise acclimated to dilutions of sea water. *Comp. Biochem. Physiol.* **20**, 101–113.
KIRSCHNER, L. B. (1979). Control mechanisms in crustaceans and fishes. In *Mechanisms of Osmoregulation in Animals* (ed. R. Gilles), pp. 157–222. Chichester, New York, Brisbane, Tokyo: John Wiley.
MANTEL, L. H. & FARMER, L. L. (1983). Osmotic and ionic regulation. In *The Biology of the Crustacea*, vol. V, *Internal Anatomy and Physiological Regulation* (ed. L. H. Mantel), pp. 53–161. New York: Academic Press.
MANTEL, L. H., SOMMER, M., NG, A., BUCK, L. & CURRAN, B. (1986). Neuroendocrine effects on osmoregulation in the green crab, *Carcinus maenas*. *Am. Zool.* **26**, 129.
MILLER, G. C. (1960). The taxonomy and certain biological aspects of the crayfish of Oregon and Washington. Master's thesis, Oregon State University, Corvallis, Oregon.
NEUFELD, G. J., HOLLIDAY, C. W. & PRITCHARD, J. B. (1980). Salinity adaptation of gill Na,K-ATPase in the blue crab, *Callinectes sapidus*. *J. exp. Zool.* **211**, 215–224.
SAINTSING, D. S. & TOWLE, D. W. (1978). Na^+-K^+-ATPase in the osmoregulating clam *Rangia cuneata*. *J. exp. Zool.* **206**, 435–442.
SHAW, J. (1964). The control of the salt balance in the Crustacea. *Symp. Soc. exp. Biol.* **18**, 237–254.
SHIRAI, N. & UTIDA, S. (1970). Development and degeneration of the chloride cell during saltwater and freshwater adaptation of the Japanese eel, *Anguilla japonica*. *Z. Zellforsch. mikrosk. Anat.* **103**, 247–264.
STAGG, R. M. & SHUTTLEWORTH, T. J. (1982). Na^+,K^+-ATPase, ouabain binding and ouabain-sensitive oxygen consumption in gills from *Platichthys flesus* adapted to seawater and freshwater. *J. comp. Physiol.* **147**, 93–99.
TOWLE, D. W. (1981). Transport-related ATPases as probes of tissue function in three terrestrial crabs of Palau. *J. exp. Zool.* **218**, 89–95.
TOWLE, D. W. (1984). Membrane-bound ATPases in arthropod ion-transporting tissues. *Am. Zool.* **24**, 177–185.
TOWLE, D. W., MANGUM, C. P., JOHNSON, B. A. & MAURO, N. A. (1982). The role of the coxal gland in osmotic and pH regulation in the horseshoe crab *Limulus polyphemus*. In *Physiology and Biology of the Horseshoe Crab: Studies on Normal and Environmentally Stressed Animals* (ed. J. Bonaventura, C. Bonaventura & S. Tesh), pp. 147–172. New York: Alan R. Liss, Inc.
TOWLE, D. W., PALMER, G. E. & HARRIS III, J. L. (1976). Role of gill $Na^+ + K^+$-dependent ATPase in acclimation of blue crabs (*Callinectes sapidus*) to low salinity. *J. exp. Zool.* **196**, 315–321.
WHEATLY, M. G. & MCMAHON, B. R. (1982). Responses to hypersaline exposure in the euryhaline crayfish *Pacifastacus leniusculus*. I. The interaction between ionic and acid–base regulation. *J. exp. Biol.* **99**, 425–445.