The Journal of Experimental Biology 204, 991–1002 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
JEB3012

ENVIRONMENTALLY MEDIATED CARBONIC ANHYDRASE INDUCTION IN THE GILLS OF EURYHALINE CRUSTACEANS

RAYMOND P. HENRY*

Department of Biological Sciences, 131 Cary Hall, Auburn University, Auburn, AL 36849, USA

*e-mail: henryrp@mail.auburn.edu

Accepted 1 December 2000; published on WWW 12 February 2001

Summary

The enzyme carbonic anhydrase appears to be a central molecular component in the suite of physiological and biochemical adaptations to low salinity found in euryhaline crustaceans. It is present in high activities in the organs responsible for osmotic and ionic regulation, the gills, and more specifically, the individual gills that are specialized for active ion uptake from dilute sea water. Within those gills carbonic anhydrase is distributed among different subcellular pools, the cytoplasm, mitochondria and microsomes. The cytoplasmic pool represents the largest subcellular fraction of carbonic anhydrase activity, and it is this fraction that undergoes a tenfold induction during acclimation to low salinity. Carbonic anhydrase activity is present in excess of that needed to support the general ion-transport processes, and so it is doubtful that carbonic anhydrase activity itself is a point of short-term regulation in response to salinity changes. Rather, upregulation of carbonic anhydrase appears to be a result of selective gene expression, representing a permanent response to long-term adaptation to low salinity. The exact signal that initiates the induction of carbonic anhydrase, and the pathway through which that signal is transduced to the activation of the carbonic anhydrase gene, are unknown, but two promising avenues of research exist. First, induction of carbonic anhydrase is immediately preceded by hemodilution and subsequent cell swelling, a potential initiating event in the process. Second, recent work indicates that expression of carbonic anhydrase is under the control of a repressor substance, located in the eyestalk, whose effect is removed upon exposure to low salinity.

Key words: carbonic anhydrase, salinity, crustacean, osmoregulation.

Introduction

Euryhalinity, the ability of an aquatic organism to tolerate large changes in environmental salinity without compromising its survival or critical life processes, is based on a coordinated set of morphological, physiological and biochemical adaptations. These adaptations result in one or both of two strategies being found in euryhaline invertebrates: (i) iso-osmotic intracellular regulation (also called cell volume regulation) and (ii) aniso-osmotic extracellular regulation (also called osmoregulation) (Florkin and Schoffeniels, 1969).

The invasion of dilute estuarine waters by marine invertebrates is accompanied by the uptake of water into both the extracellular and intracellular fluid compartments, and it results in tissue and cell swelling. All species have mechanisms of cell volume readjustment that allow for continuity of function (for reviews, see Pierce and Amende, 1981; Henry, 1995), but some species can also reduce the degree of fluid compartment dilution and cell swelling by regulating the osmotic concentration of their hemolymph. These species are termed osmotic and ionic regulators, and they have the ability to maintain the concentration of their hemolymph significantly above that in the external medium. By maintaining relatively stable hemolymph osmotic concentrations in the face of decreasing ambient salinity, intracellular water gain and cell swelling are kept to a minimum. Crustaceans, especially decapods, are known to be strong regulators.

Osmotic regulation is accomplished by the active uptake of salts (primarily Na^+ and Cl^-) from the medium across the gills (for reviews, see Mantel and Farmer, 1983; Pequeux, 1995). This process is silent in marine invertebrates acclimated to high salinity (e.g. 35‰) in which the osmotic and ionic concentrations of the extracellular fluid (hemolymph) of marine invertebrates passively reflects that of the surrounding sea water. However, at a critical low salinity, usually near 25‰, branchial ion-uptake mechanisms are activated, and the organism makes the transition from conformity to regulation. One of the most prominent features of this transition is the upregulation of a host of transport-related proteins, specifically the Na^+/K^+-ATPase and carbonic anhydrase (Towle, 1984; Towle, 1997; Henry, 1984; Henry, 1988a; Henry, 1988b). For these two enzymes, the majority of the evidence supports the hypothesis that upregulation is a result of *de novo* synthesis of new protein (see below). So, it appears that the onset of osmoregulation is contingent upon the activation of the specific genes responsible for the synthesis of these transport-related

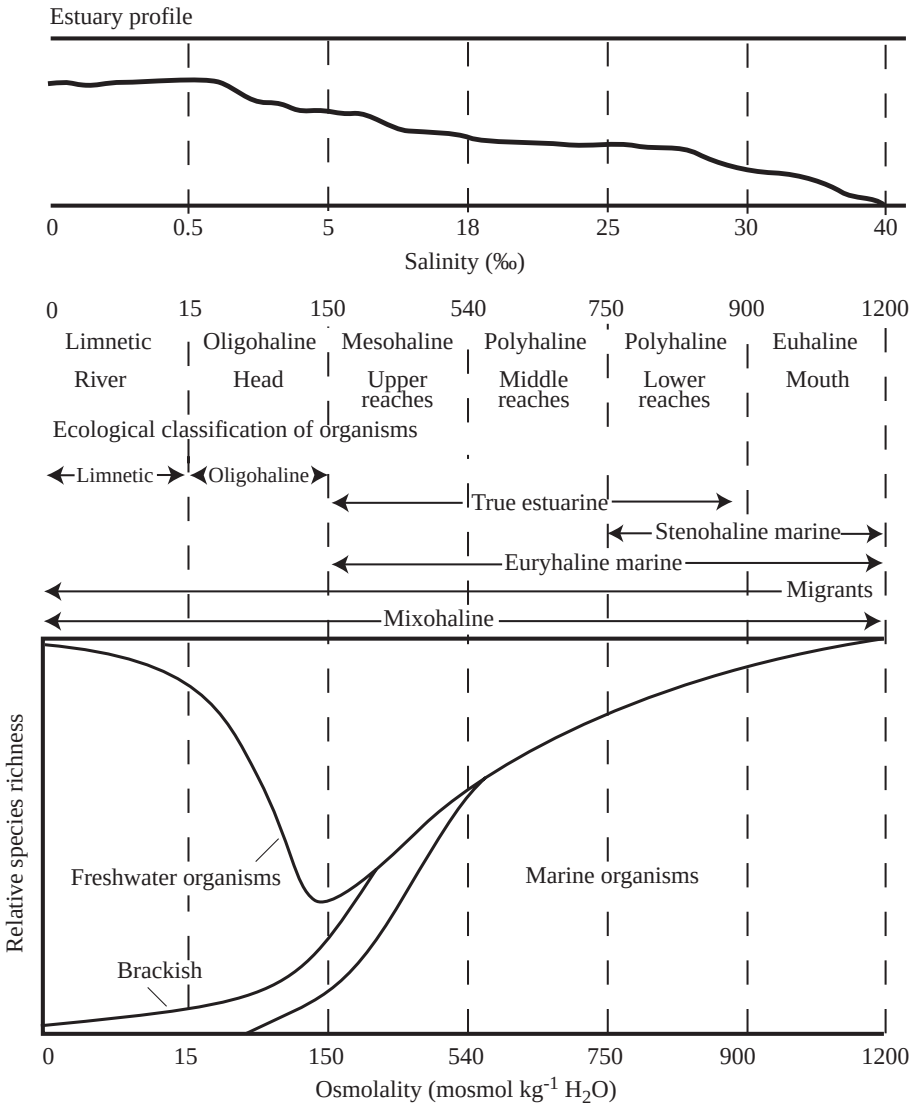

Fig. 1. Schematic representation of a typical coastal plain estuary. Top panel: longitudinal profile of the estuary; the solid sloping line represents the division between the water column above and sediment below. The longitudinal salinity gradient of the estuary runs from riverine fresh water at 0‰ salinity at the far left to full-strength sea water at up to 40‰ at the far right. Salinity is also shown as mosmol kg^{-1} water. Specific zones of the estuary are based on annual salinity variation. Middle panel: ecological classification of the distributions of organisms that spend either part or all their life cycle along the salinity gradient of the estuary. Physical and ecological classification based Carriker, 1967. Bottom panel: relative number of species of different ecological classification found in different salinity zones of the estuary. (Redrawn from Gainey and Greenberg, 1977, for the distribution of benthic molluscan species.)

proteins. And while, ultimately, this is a question of how an organism interprets an environmental signal and transduces that signal into selective gene expression, the question starts with the interaction between the organism and the physical variables in its environment, the estuary.

Life in the estuary

Estuaries can be broadly defined as bodies of water in which fresh water, originating from inland, mixes with salt water from oceans or bays (Gross, 1972). There are many specific types of estuaries, from drowned river beds to lagoons and fjords, but they typically share one common feature: a longitudinal salinity gradient from the head of the estuary (where fresh water enters) to the mouth (where estuarine waters empty into full-strength sea water) (Carriker, 1967). This gradient can range from 0‰ to 40‰ salinity (0–1200 mosmol kg^{-1} water) and can be divided into discrete zones on the basis of annual variation in salinity (Fig. 1). Furthermore, the ecological distribution of organisms in the estuary appears to fall along the boundaries of these salinity zones.

Gainey and Greenberg (Gainey and Greenberg, 1977) reviewed the distribution of benthic molluscan species and found that the number of marine species is highest in the euhaline zone, decreases in the polyhaline and drops off dramatically in the mesohaline. The stenohaline marine species, those with a narrow salinity tolerance, appear to be restricted to the euhaline and lower polyhaline zones, while the more euryhaline species are found down through the mesohaline (Fig. 1). True estuarine species appear to be distributed within the polyhaline and mesohaline zones. Very few species, however, seem to be able to survive in the more extreme low-salinity region of the oligohaline zone. Even the number of brackish-water species declines between the mesolahine and oligohaline zones. The oligohaline zone is therefore a major physical barrier to the invasion of the most dilute waters of the estuary (see also Deaton and Greenberg, 1986). It also appears to be a physical barrier to the invasion of the estuary from fresh water because few

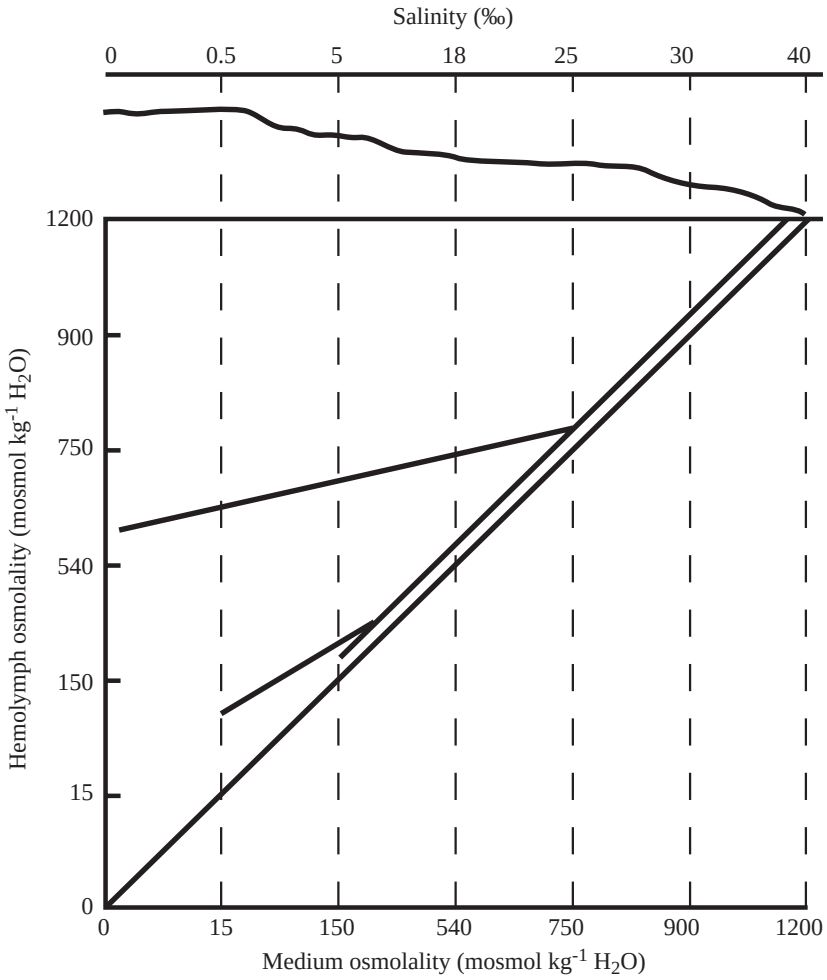

Fig. 2. Schematic representation of hemolymph *versus* ambient medium osmotic concentration (mosmol kg^{-1} water) in euryhaline organisms superimposed on the salinity gradient of a typical coastal plain estuary. The diagonal line running the length of the gradient from the origin represents the iso-osmotic line. Osmotic and ionic conformers are represented by the line running parallel and slightly above the iso-osmotic line and ending at the oligohaline zone. Strong osmotic and ionic regulators are depicted by the upper line that diverges from the iso-osmotic line, and weak regulators are shown by the lower divergent line. Data for strong regulators are taken from the blue crab *Carcinus maenas* (see text for details), and data for weak regulators are taken from the oligohaline bivalve *Rangia cuneata* (Henry and Mangum, 1980).

limnetic species are found above the upper salinity value of 5 ‰ (Fig. 1).

Interestingly, of the two physiological strategies of adaptation to low salinity, osmotic regulation appears to be a common thread among the most euryhaline invertebrates, and it seems to be necessary to the ability to survive into the most dilute regions of the estuary, particularly in the oligohaline zone (Fig. 2). Osmoconforming species, whose hemolymph osmotic concentration parallels that of the iso-osmotic line, have a lower lethal salinity limit in the range 8–10 ‰ (Kinne, 1971). Invertebrate species that survive below those values can be classified as either strong or weak osmoregulators, depending on the magnitude of the hemolymph–medium difference in osmotic concentration.

Among crustaceans, there is a continuum of euryhalinity, from complete passive osmotic and ionic conformity to very strong regulation. Marine stenohaline species (such as *Libinia emarginata* and *Cancer irroratus*) are osmotic and ionic conformers over their entire salinity range, and their lower lethal limit is usually near 18 ‰ (for a complete review, see Mantel and Farmer, 1983). Weak osmotic and ionic regulators, such as *Callinectes similis*, the lesser blue crab, have a lower limit of salinity in the field near 15 ‰ (Tagatz, 1967; Hsueh, 1992); mortality was reported to be 80 % at 5 ‰ in laboratory experiments, and calculated hemolymph osmolality was maintained at a maximum of 250 mosmol kg^{-1} water above that in the medium (Engel, 1977).

The most euryhaline species are the migrants, organisms that spend part of their life cycle in the estuary. These are highly mobile species, primarily portunid crustaceans that invade the estuary in spring and summer as part of their reproductive life cycle. They are typically strong osmotic and ionic regulators. Two of the most extensively studied crustacean species have been the blue crab *Callinectes sapidus* and the green shore crab *Carcinus maenas*. The green crab can survive salinities as low as 8 ‰, and it is considered a moderately strong regulator, maintaining its hemolymph osmolality approximately 350 mosmol kg^{-1} water above ambient at 8 ‰ (Zanders, 1980; Henry et al., 1998). Adapted for both low and fluctuating salinity, the blue crab can survive the full range of the estuarine gradient in nature as well as large step changes in salinity imposed by laboratory experiments. One of the strongest documented regulators, *Callinectes sapidus*, when acclimated to fresh water, can maintain a gradient of more than 600 mosmol kg^{-1} water between its hemolymph and the surrounding medium (Cameron, 1978a). Some species,

primarily crayfish, are found most commonly in fresh water where they maintain their hemolymph osmolality approximately 370–450 mosmol kg^{-1} water above that in the water (e.g. Mantel and Farmer, 1983). Euryhaline crayfish, such as *Pacifastacus leniusculus*, make the transition from regulation to conformity when acclimated to salinities above 13‰ (Wheatly and Henry, 1987).

Physiological mechanism of ion regulation

Euryhaline crustaceans, such as the blue crab, maintain hemolymph osmotic and ionic concentrations above those in the ambient medium by active uptake of salts (primarily Na^+ and Cl^-) across the gill (e.g. Cameron, 1978a; Cameron, 1978b; Cameron, 1979). There is conflicting evidence concerning the exact mechanism of branchial ion transport, but the current ideas can be summarized as follows. Na^+ uptake across the apical surface of the gill occurs *via* a combination of Na^+/H^+ and Na^+/NH_4^+ exchange (e.g. Kirschner, 1979; Evans and Cameron, 1986), and possibly also *via* Na^+ uptake linked to H^+ extrusion by an apical V-type ATPase (as is believed to occur in fish gills; Lin and Randall, 1991). A Na^+/H^+ exchange protein has been identified in crustacean gills (Towle et al., 1997), and evidence has also been presented suggesting the presence of a $Na^+/K^+/2Cl^-$ exchange protein (Riestenpatt et al., 1996). A basolaterally localized Na^+/K^+-ATPase is believed to be responsible for Na^+ transport from the branchial cytoplasm to the hemolymph and also for establishing the inward gradient across the apical membrane (Towle, 1984; Towle, 1997). Apical uptake of Cl^- is believed to take place, at least in part, *via* Cl^-/HCO_3^- exchange, with transport to the hemolymph occurring passively down an electrochemical gradient (e.g. Pequeux, 1995). The enzyme carbonic anhydrase is believed to support the general ion-transport process by supplying H^+ and HCO_3^-, *via* the intracellular catalysis of respiratory CO_2, for counterions in cation and anion uptake, respectively (Henry, 1984; Henry, 1988b).

Marine crustaceans evolved in an aquatic medium, in which their hemolymph osmotic and ionic concentrations were in passive equilibrium with those in the surrounding sea water. The incorporation of branchial ion-uptake mechanisms allowed them to invade estuarine waters. However, marine species have retained a relatively high degree of branchial permeability in low salinity and, therefore, they also tend to lose a significant amount of salt across the gills *via* passive diffusion. The blue crab is a perfect example of this (Fig. 3), with high rates of diffusive salt loss (Cameron, 1978a). Total Na^+ efflux is 583 μmol kg^{-1} h^{-1} (Fig. 3), while Na^+ efflux in a freshwater crayfish, *Astacus leptodactylus*, is 136 μmol kg^{-1} h^{-1}, reduced by a factor of approximately 4 (Ehrenfeld, 1974). Furthermore, the blue crab also loses a large amount of salt through the excretion of copious volumes of iso-osmotic/iso-ionic urine from the antennal gland, accounting for 41% of the total Na^+ loss to the medium (Cameron and Batterton, 1978). In contrast, urinary salt loss in freshwater

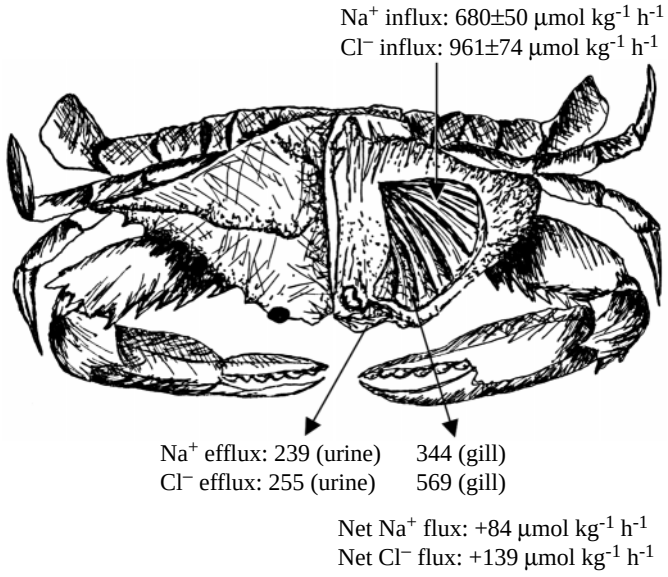

Fig. 3. Branchial and urinary ion fluxes (μmol kg^{-1} h^{-1}) in the blue crab acclimated to low salinity. Ion influx occurs across the posterior gills only; efflux occurs across all gills and through the urine. (Data from Cameron, 1978a; Cameron and Batterton, 1978.)

crayfish (*Astacus fluviatilis*) accounts for only 5–8% of the total loss (Bryan, 1960). To remain in positive salt balance (i.e. net ion influx), blue crabs maintain very high rates of Na^+ and Cl^- uptake in what can be called a rapid 'pump and leak' strategy of low-salinity adaptation (Cameron, 1978a). As a result, euryhaline crustaceans, such as the blue crab, need high levels of activity of the two major transport-related enzymes, the Na^+/K^+-ATPase and carbonic anhydrase.

Carbonic anhydrase as a central transport enzyme

The enzyme carbonic anhydrase (CA) functions in support of a host of transport processes through the catalyzed hydration of molecular CO_2:

$$CO_2 + H_20 \xleftrightarrow{CA} H^+ + HCO_3^- .$$

CA is one of the fastest enzymes known, and the near instantaneous maintenance of the equilibrium among the chemical species in the reaction ensures that the supply of counterions for both cation and anion transport will not be limiting.

Discovered in crustaceans over 60 years ago (Ferguson et al., 1937), CA has been shown to be centrally important in the uptake and regulation of Na^+ and Cl^-. The bulk of CA activity is concentrated in the gills of euryhaline species, even when they are acclimated to high salinity (Henry and Cameron, 1982a). Furthermore, branchial CA activity in stenohaline osmotic and ionic conformers, such as *Libinia emarginata*, *Chaceon fenneri* and *C. quinquedens*, acclimated to 32–35‰, is only approximately 10% of that found in the gills of *Callinectes sapidus* acclimated to similar salinity (Henry and Cameron, 1982a; Henry et al., 1990). CA activity in euryhaline crabs is salinity-sensitive, being induced in the gills of animals transferred

to low salinity (Henry and Cameron, 1982a; Henry and Cameron, 1982b). More specifically, this induction takes place within individual gills that have been shown physiologically and ultrastructurally to be the specific sites of ion uptake (Henry, 1984). These are typically the posterior 3–4 gill pairs, which are also characterized by having a thick (10 μm) epithelium, an extensive system of intracellular tubules arising from infoldings of the basolateral membrane, a dense mitochondrial population and high activities of the Na^+/K^+-ATPase (Copeland and Fitzjarrell, 1968; Finol and Croghan, 1983; Cioffi, 1984; Towle, 1984; Compere et al., 1989). Selective induction of CA activity results in a heterogeneous distribution of activity among gills of euryhaline species acclimated to low salinity, with the posterior gills having significantly higher levels of activity (Henry, 1984). This induction appears to be present in all but the most stenohaline species. There is a tenfold increase in CA activity in the posterior gills of both *C. sapidus* and *C. similis* and approximately equivalent values of CA activity in those gills (1500–2000 μmol CO_2 mg^{-1} protein min^{-1}), despite the different degrees of euryhalinity expressed by the two species (Henry, 1988a; Piller et al., 1995).

CA activity in the euryhaline freshwater crayfish *Pacifasticus leniusculus* is also labile in response to changes in salinity but there are, however, some significant differences from the pattern seen in marine species. First, in crayfish acclimated to fresh water, all seven gill pairs have high levels of CA activity (900 μmol CO_2 mg^{-1} protein min^{-1}; Wheatly and Henry, 1987), which are correlated with the presence of salt-absorbing 'chloride cells' in all gills (Dickson et al., 1991). This activity is altered by reciprocal changes in salinity: it decreases after transfer to either 450 or 750 mosmol kg^{-1} water and is induced upon re-introduction to fresh water (Henry and Wheatly, 1988). Second, CA activity in the antennal gland, which is low and salinity-insensitive in marine species (Henry and Cameron, 1982a), is actually higher in crayfish than that in the gills (Wheatly and Henry, 1987) and is also salinity-sensitive (Henry and Wheatly, 1988). The differences in CA tissue distribution and salinity-sensitivity reflect a different physiological 'strategy' of osmotic and ionic regulation employed by freshwater crustaceans. These species have reduced branchial permeability and, therefore, much less diffusive loss of hemolymph ions, and they have the ability to produce a hypo-osmotic/hypo-ionic urine through active reabsorption of salts by the antennal gland (Bryan, 1960; Harris and Micallef, 1971; Ehrenfeld, 1974). The high levels of CA activity in the antennal gland probably serve to support the multiple ion-reabsorption mechanisms, much as branchial CA activity supports ion uptake in the gills.

So, while there may be different physiological 'strategies' of adaptation to low salinity, and while the presence of specific ion-transport proteins is both tissue-specific and variable among crustacean species, the presence of high and inducible levels of CA activity seems to be a universal molecular feature (along with that of the Na^+/K^+-ATPase) of euryhalinity. This idea is further reinforced by the absence of this feature in stenohaline marine species. For example, *Cancer irroratus*, a stenohaline osmotic and ionic conformer, does not display any degree of CA induction in its posterior gills when transferred from 35 ‰ to 18 ‰ salinity (Henry et al., 2000). Crabs such as these lack the physiological and biochemical mechanisms needed for active uptake of ions from the ambient medium in low-salinity sea water that are necessary for the invasion of the more dilute waters of the estuary.

Surprisingly, CA induction in the posterior gills of the green crab *Carcinus maenas*, which is almost as euryhaline as *Callinectes sapidus* and more so than *C. similis*, was reported to be at most twofold after transfer from 40 ‰ to 10 ‰ (Bottcher et al., 1990a). While the actual values for CA activity in *C. maenas* and *C. sapidus* are not directly comparable because of the non-standard units reported in the former study, the degrees of induction are, and they are very different. The low degree of salinity-sensitivity, among other things (see below) has been used as an argument in downplaying the role of CA in ion transport in *Carcinus*. However, a re-examination of this phenomenon has shown that CA activity in the posterior gills (G7–G9) undergoes an eightfold increase in *Carcinus* transferred from 32 ‰ to 12 ‰, a degree of induction that is wholly consistent with that found in other euryhaline crustacean species (Henry et al., 1999a).

The discrepancy is possibly a result of the CA assay used by Bottcher et al. (1990a). Those authors measured the time needed for a change in pH to occur from 9.4 to 6.7 as the CO_2 hydration reaction proceeded, a drop in nearly 3 pH units. Carbonic anhydrase is highly pH-sensitive: maximum activity of the catalyzed hydration reaction occurs at pH 9 and drops to nearly zero at pH 6 (Coleman, 1980). The progressive decrease in the CO_2 hydration activity of CA with the progressive decrease in pH over the course of the assay would result in an underestimation of the true activity of the sample, with the error becoming larger as the amount of CA activity being measured increases. The delta pH method described by Henry (1991) measures CA activity over a change in pH of only 0.15 units, eliminating any potential pH artifacts in measuring changes in CA activity.

Physiologically and pharmacologically, CA has also been directly shown to be involved in the ion-transport processes of crustacean gills. Treatment with the CA inhibitor acetazolamide, in blue crabs and other crustacean species, results in a disruption of the normal Na^+ and Cl^- uptake mechanisms (Ehrenfeld, 1974; Cameron, 1979), with Na^+ uptake being significantly reduced and Cl^- efflux being enhanced, resulting in the crabs being put into a position of negative salt balance (i.e. the rate of diffusive salt loss exceeding the rate of salt uptake). Treatment with acetazolamide also results in a dose-dependent depression in the osmotic and ionic concentrations in the hemolymph of blue crabs acclimated to low salinity (Henry and Cameron, 1983). Furthermore, when branchial CA activity is inhibited in blue crabs acclimated to 28 ‰, they do not survive past 48 h during an acute transfer to low salinity, and hemolymph osmotic and ionic concentrations during that time are significantly lower than in control crabs (see Fig. 6 below).

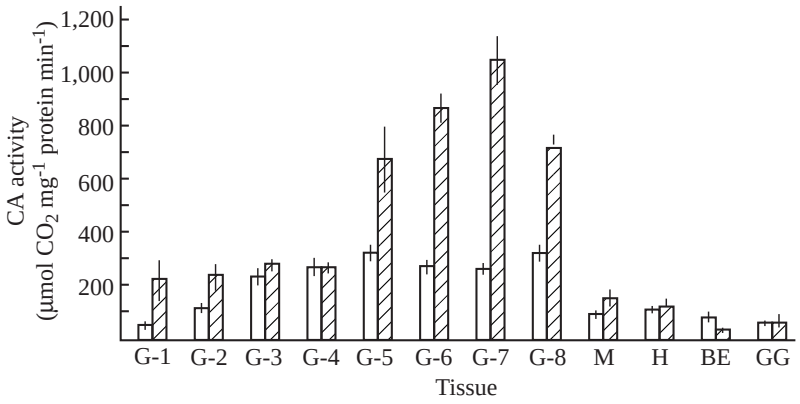

Fig. 4. Tissue carbonic anhydrase (CA) activity in the blue crab (*Callinectes sapidus*) acclimated to 28‰ (open columns) and 4‰ (hatched columns). G1–G8, individual gills from the anterior to the posterior of the crab; M, backfin muscle; H, heart; BE, branchial epithelium; GG, green gland (antennal gland). Values are means ± S.E.M. (*N*=10). (Data redrawn from Henry and Cameron, 1982a.)

Recently, however, a report on the effects of acetazolamide on ion transport in the isolated perfused gill of *C. maenas* cast doubt on the role of CA in ion regulation. Bottcher et al. (Bottcher et al., 1991) found no effects of 100 μmol l^{-1} acetazolamide when applied to both sides of the gill on either Na^+ or Cl^- uptake. Unfortunately, these results are questionable because of the short incubation time used for the inhibitor (20 min). Acetazolamide has a low lipid solubility (Maren, 1967) and, as such, it permeates membranes slowly. Previous work on blue crabs showed that with an initial circulating concentration of 1 mmol l^{-1} in the hemolymph it still took 4–6 h before branchial CA was fully inhibited and hemolymph ion regulation was disrupted (Henry and Cameron, 1983; Henry, 1988a). The results of Bottcher et al. (Bottcher et al., 1991) were similar to those seen in blue crabs treated with a membrane-impermeant CA inhibitor, quaternary ammonium sulfanilamide (QAS) (Henry, 1987), indicating that the absence of effect on ion transport was a result of an incubation time that was insufficient to allow enough acetazolamide to accumulate within the intracellular compartment of the gill to fully inhibit the cytoplasmic pool of CA. Interestingly, a re-examination of the role of CA in *C. maenas*, using 1 mmol l^{-1} acetazolamide injected into the hemolymph, showed a reduction in osmotic and ionic concentrations that was initiated at 4–6 h post-injection (Henry et al., 1998).

Environmentally mediated induction of carbonic anhydrase

One of the most interesting aspects of branchial CA and its role in ion transport in crustaceans is the fact that exposure to low salinity stimulates a large induction of the enzyme (see above). This was originally reported as an approximately fourfold increase in CA activity (as measured by the pH-stat assay of Henry and Cameron, 1982a) in the posterior, ion-transporting gill pairs of the blue crab *C. sapidus* (Fig. 4).

Further investigation of branchial CA revealed that there are multiple subcellular pools of the enzyme. Homogenization of gill tissue and fractionation, either by density gradient or by differential centrifugation, localized CA in *C. sapidus* to at least two subcellular fractions, the cytoplasm and the microsomes (Henry, 1988a). The cytoplasmic CA pool is the larger of the two, being more than 90 % of the total branchial CA activity. It is also this enzyme pool that is salinity-sensitive; advances in tissue handling and assay sensitivity revealed a tenfold increase in cytoplasmic CA activity in crabs transferred from 35‰ to 8‰ salinity. The general pattern of subcellular CA distribution was confirmed for another euryhaline species, *C. maenas* (Bottcher et al., 1990a; Bottcher et al., 1990b), although the magnitude of the microsomal CA pool was reported to be as high as 70 % of the total branchial CA activity. Results from physiological and pharmacological experiments have shown that the cytoplasmic fraction is involved in ion transport and that the microsomal fraction functions in facilitated CO_2 transport from the hemolymph across the gills (for a review, see Henry, 1988b). Because the different subcellular pools of CA appear to have different physiological roles, and because of the putatively large amount of membrane-associated CA, it was suggested that branchial CA in *C. maenas* functioned primarily in respiratory CO_2 excretion (Bottcher et al., 1990a; Bottcher et al., 1990b; Bottcher et al., 1991). A recent re-investigation of the subcellular distribution of branchial CA in *C. maenas*, however, indicated that it was, in fact, similar to that reported for *C. sapidus*. CA activity in the cytoplasmic fraction of gills from crabs acclimated to 10‰ was fivefold higher than that for the microsomal fraction. Cytoplasmic CA activity was induced over tenfold, but only in the posterior gills, and while there was also a twofold induction in CA activity in the microsomal fraction in the posterior gills in low *versus* high salinity, that fraction never made up more than 10 % of the total CA activity in the gill under any conditions (R. P. Henry, unpublished results).

Again, it is possible that methodological differences could have produced the reported differences in results of the two studies on *C. maenas*. Subcellular fractionation is usually carried out by three steps of differential centrifugation, isolating each fraction at each step (e.g. Henry, 1988a). The initial step, a low-speed centrifugation, produces a so-called ‘cellular debris’ pellet, which contains high levels of CA activity that cannot be localized to any specific fraction; this is true for both invertebrate and vertebrate gills (Henry et al., 1988; Henry et al., 1993; Henry, 1988a). Bottcher et al.

(Bottcher et al., 1990a; Bottcher et al., 1990b) centrifuged the crude gill homogenate at 100 000 ***g*** without any of the intermediate steps. Thus, they produced a single pellet containing microsomes, mitochondria and cellular debris, all of which contain CA activity; this could have resulted in an overestimation of the contribution of membrane-associated CA to the total.

Two of the major questions in the study of CA as it relates to ion transport and regulation in aquatic organisms are whether the levels of activity are sufficient to account for the actual uptake rates of the different ions and to what extent the large inductions of CA activity are necessary for adaptation to low salinity. It is possible only to get an approximate comparison of the rates of CA activity and those of ion uptake, but when that is attempted for both *Callinectes sapidus* and *Pacifastacus leniusculus*, it appears that CA activity is present well in excess of that needed for ion uptake. For example, Na^+ and Cl^- uptake rates for *C. sapidus* acclimated to fresh water are 608 and 961 μmol kg^{-1} whole animal fresh mass h^{-1}, respectively (Fig. 3, data from Cameron, 1978a), while CA activity in the posterior gills is approximately 10 mmol CO_2 g^{-1} tissue fresh mass min^{-1} for crabs at 28 ‰ (Henry and Cameron, 1982a). Converting these values to the same units, and assuming that the gills comprise about 0.1 % of the total weight of the crab, CA activity is present in excess by a factor of approximately 1000 compared with the rates of ion uptake. A similar estimate has been done for *P. leniusculus*, and the same relationship was found to exist in both gills and antennal gland (Henry and Wheatly, 1988). Obviously, the situation in the gill is more complex because the ion-uptake processes take place across the apical surface of the gill and the supply of counterions must be made available to the intracellular boundary layer of the apical membrane. If CA is uniformly distributed throughout the cytoplasm, then only a fraction of that pool may have a role in the ion-uptake process. Soluble CA is known to function in a number of cellular transport and metabolic processes (for a review, see Henry, 1996), and the high levels of activity in the branchial cytoplasm may be necessary for more than just ion regulation. The question of CA induction is more intriguing.

The increase in CA activity in the posterior gills is directly related to the decrease in acclimation salinity, as seen in blue crabs *C. sapidus* (Fig. 5). At 35 ‰, the blue crab is an osmotic and ionic conformer, and there is no difference in CA activity between anterior (G3) and posterior (G7) gills. The critical salinity for the onset of CA induction is 26 ‰ (R. Henry, unpublished data); transfer from 35 ‰ to any salinity above that value has no effect. Between 35 and 25 ‰, hemolymph osmolality decreases by approximately 250 mosmol kg^{-1} water, and a significant increase in branchial CA activity occurs in the posterior gills. From that point on, however, CA activity continues to increase in the posterior gills with progressively lower salinity, while hemolymph osmolality remains relatively stable. It is not, therefore, hemolymph osmotic or ionic concentrations *per se* that initiate and control CA induction, rather it is either a direct effect of

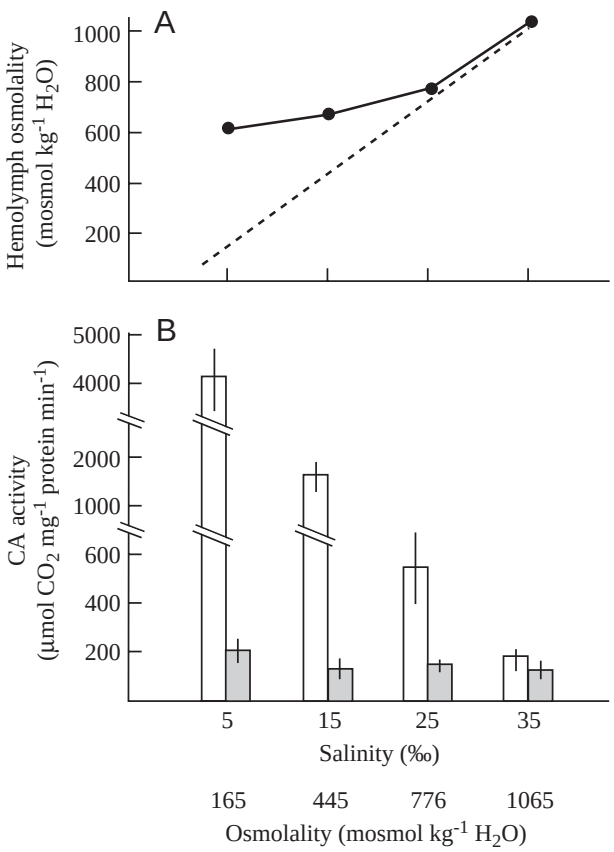

Fig. 5. (A) Hemolymph osmotic concentration of blue crabs (*Callinectes sapidus*) acclimated for 3 weeks to various salinities. Values are means ± S.E.M., *N*=5–6. Error bars are not shown because they were smaller than the size of the circle used to denote the data point. Dashed line represents the iso-osmotic line. (B) Branchial carbonic anhydrase activity in anterior (G3; hatched columns) and posterior (G7; open columns) gills of blue crabs acclimated for 3 weeks to various salinities. Values are means ± S.E.M., *N*=5–6. (Data redrawn from Henry and Watts, 2001.)

lowered ambient salinity or a result of the progressive increase in the concentration difference between the medium and the hemolymph (Fig. 5). As has been reported previously (Henry and Cameron, 1982a; Henry and Cameron, 1982b; Henry, 1988a), there is no increase in CA activity in the anterior gills for any acclimation salinity.

As discussed above, *Callinectes sapidus* can survive direct transfer from 35 ‰ to fresh water, an ability that makes them particularly well adapted to estuarine conditions of both low and fluctuating salinity. Since the blue crab is exposed to rapid changes in salinity, it also means that the physiological and biochemical mechanisms of ion transport must be activated quickly if they are to be ecologically relevant. It was originally reported that branchial CA induction in the blue crab *C. sapidus* took of the order of 4–7 days (Fig. 6) (Henry and Cameron, 1982b), a time course that was inconsistent with the establishment of new steady-state hemolymph osmotic and ionic concentrations. The latter values stabilized within 24 h after transfer to low salinity (Fig. 6). CA is absolutely

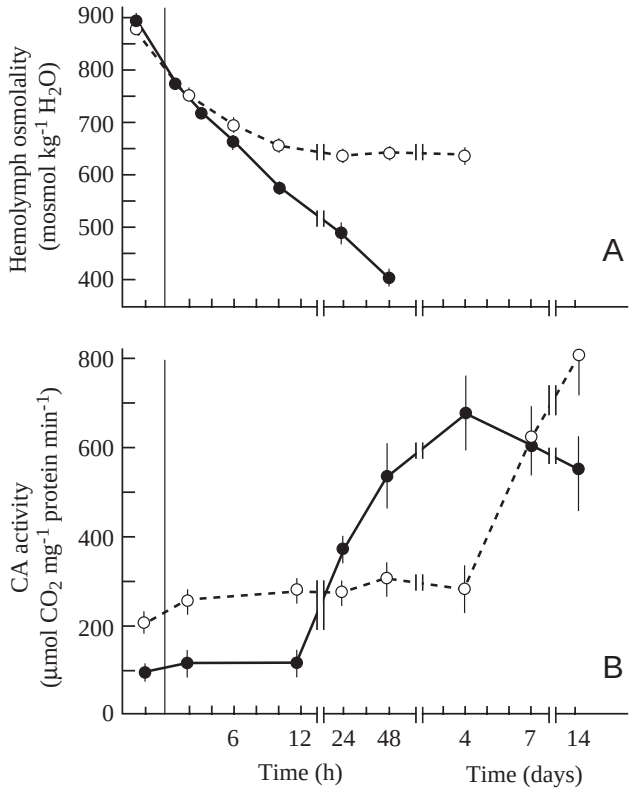

Fig. 6. (A) Time course of readjustment of hemolymph osmotic concentration in blue crabs (*Callinectes sapidus* acclimated to a salinity of 28‰ for 3 weeks and acutely transferred to 8‰. Open circles represent control crabs; filled circles represent crabs that had been treated with 1 mmol l^{-1} acetazolamide prior to transfer (all these crabs died by 96 h after transfer). Values are means ± S.E.M., *N*=6. (Data redrawn from Henry and Cameron, 1982b.) (B) Time course of carbonic anhydrase induction in posterior gills (G7) in blue crabs acclimated to 28‰ for 3 weeks and acutely transferred to 12‰ (open circles; values are means ± S.E.M., *N*=6; data redrawn from Henry and Cameron, 1982b), or acclimated to 35‰ for 3 weeks and transferred to 25‰ (filled circles; values are means ± S.E.M., *N*=6; data redrawn from Henry and Watts, 2001).

necessary for acclimation to low salinity, however; inhibition of CA activity with acetazolamide in crabs acclimated to 28‰ resulted in both an inability to re-establish stable hemolymph osmotic concentrations after an acute transfer to 8‰ and 100 % mortality 48 h post-transfer (Fig. 6A). This discrepancy, an apparent paradox in the field for years, was finally resolved as advances in CA assay sensitivity and tissue handling revealed that the first significant increase in CA activity occurs at 24 h after transfer to low salinity (Fig. 6B) (Henry and Watts, 2001). This more rapid time course of CA induction is congruent with the onset of hemolymph osmotic and ionic regulation.

The apparent discrepancy still exists, however, in *Carcinus maenas*, whose hemolymph osmotic and ionic concentrations stabilize within 24 h after transfer from 32 to 12‰ but whose levels of branchial CA activity do not increase until 96 h post-transfer (Henry et al., 1998; Henry et al., 1999a). The resolution to this apparent paradox may lie in the differential expression and regulation of the two major transport-related enzymes, CA and the Na^+/K^+-ATPase. In green crabs acclimated to 32‰, a salinity in which they are osmotic and ionic conformers, CA activity is minimal but activity of the Na^+/K^+-ATPase is high and similar to that found in other species (e.g. *C. sapidus*) under conditions in which they are osmotic and ionic regulators. In fact, green crabs *Carcinus maenas* had to be acclimated in the laboratory to 40‰, a salinity that is hardly ever encountered in their natural habitat, before a reduction in Na^+/K^+-ATPase activity was seen. Furthermore, while CA activity undergoes a tenfold induction upon transfer to 12‰, there is no parallel increase in the Na^+/K^+-ATPase activity (Henry et al., 1998; Henry et al., 1999a). Rather, for *C. maenas*, the Na^+/K^+-ATPase appears to be fully expressed even in high salinity. It is generally believed that the Na^+/K^+-ATPase provides the driving force behind the overall ion uptake process and, therefore, modulation of that enzyme's activity can regulate the rates of branchial ion uptake. There is also a growing body of evidence suggesting that the Na^+/K^+-ATPase can be regulated over the short term by a variety of factors, including an unidentified hemolymph-borne factor (Savage and Robinson, 1983), dopamine (Sommer and Mantel, 1988), polyamines (Lee, 1992), fatty acids (Morohashi et al., 1991), sinus gland extract (Eckhardt et al., 1995), cyclic-AMP-dependent protein kinase A (Lucu and Flik, 1999) and crustacean hypoglycemic hormone (Spannings-Pierrot et al., 1999). The combination of high levels of expression coupled to rapid modulation of activity could account for increases in branchial ion uptake during the initial phase of low-salinity adaptation and the concomitant rapid stabilization of hemolymph ion concentrations after transfer to low salinity, especially since it also appears that CA is present in excess of the ion transport requirements (see above).

This particular response is somewhat different from that seen in the blue crab *C. sapidus*, in which both the Na^+/K^+-ATPase (Towle et al., 1976) and CA are rapidly upregulated in the face of a low-salinity challenge, and the reason may lie in the differences between the ecological lifestyles of the two species. *C. maenas* is primarily intertidal and is more likely to be more exposed to short-term fluctuations in salinity (e.g. the tidal cycle, fresh water runoff and catastrophic dilutions resulting from storms) and, as such, it may need to respond more quickly through rapid activation/deactivation of the ion-uptake mechanism. *C. sapidus*, in contrast, is subtidal and migratory; when it invades waters of low salinity, it is usually on a more long-term basis. Consequently, activities of the two transport-related enzymes are controlled at the level of their expression.

Regardless of species, however, the increase in CA activity appears to be the result of the synthesis of new enzyme in response to selective gene expression. The time course of induction (i.e. hours as opposed to minutes) is consistent with the synthesis of new enzyme, and treatment with cyclohexamide, an inhibitor of protein synthesis, prevents CA

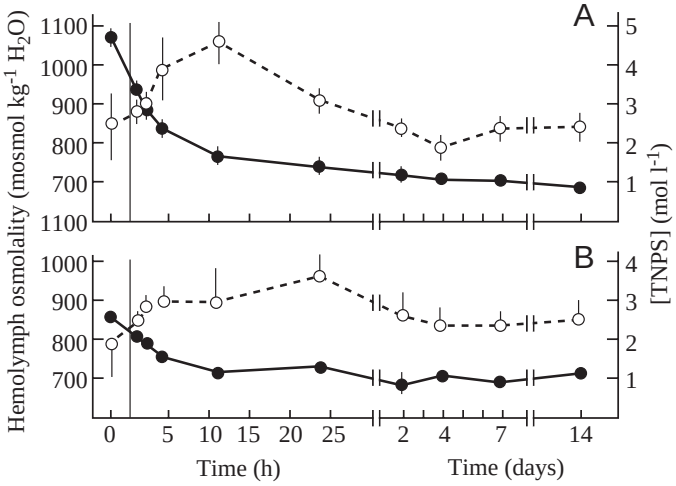

Fig. 7. Time course of hemolymph osmotic concentration (A) (filled circles and solid lines) and total ninhydrin-positive substances (TNPS) (B) (open circles and dashed lines) in blue crabs (*Callinectes sapidus*) acclimated to 35‰ (A) or 28‰ salinity (B) and transferred to 15‰. Values represent means ± S.E.M., *N*=6. (Data redrawn from Henry and Watts, 2001.)

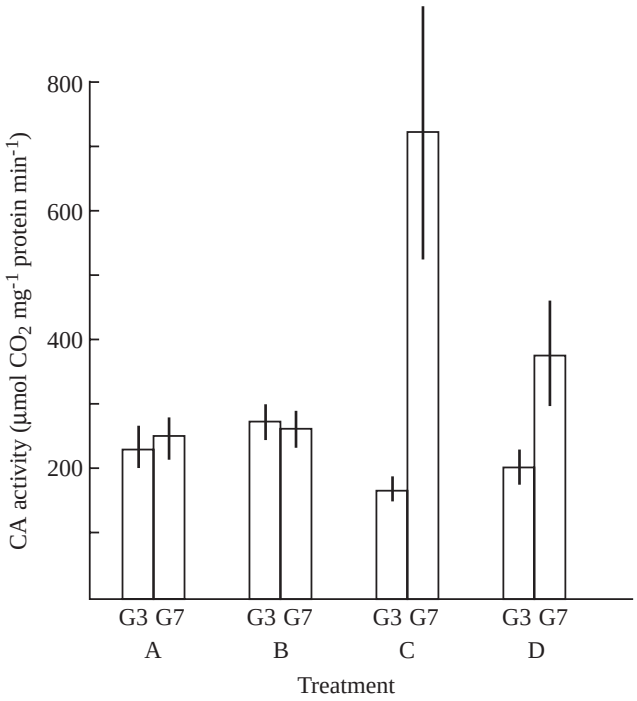

Fig. 8. Carbonic anhydrase activity in anterior (G3) and posterior (G7) gills of blue crabs (*Callinectes sapidus*) acclimated to a salinity of 35‰ for 3 weeks. (A) Control crabs, (B) sham-operated crabs, (C) eyestalk-ablated crabs, 24 h post-treatment, (D) eyestalk-ablated crabs, 96 h post-treatment. Values represent means ± S.E.M., *N*=6–9. (Data from Henry et al., 1999.)

induction (R. P. Henry, unpublished data). Furthermore, in both *C. sapidus* and *C. maenas*, increases in CA activity are preceded by increases in the specific message (mRNA) for the enzyme (Gehnrich et al., 1999; Gehnrich et al., 2000).

Two major remaining questions concerning CA induction involve the nature of the initial signal in the process and the pathway by which that signal is transduced to CA expression. Research into both areas is still in its infancy, especially with regard to euryhaline invertebrates, but there are two promising avenues of investigation. The first, which represents an integration of the two major strategies of salinity adaptation, i.e. cell volume regulation and osmoregulation, may shed light on the initial step in the transduction pathway. As discussed above, all marine organisms, whether osmotic conformers or regulators, undergo an initial dilution of their hemolymph when exposed to low salinity. This results in cell swelling and the initiation of the cell volume regulatory response.

A significant percentage of the total intracellular osmotic concentration in crustacean tissues is made up of small organic molecules, primarily non-protein free amino acids (FAAs; e.g. Henry, 1995). During acclimation to low salinity, cell volume is adjusted by lowering the concentration of the intracellular FAA pool; the amino acids are rapidly released intact into the hemolymph, usually within hours of acute transfer to low salinity (Moran and Pierce, 1984; Harris and Andrews, 1985). This response can be detected indirectly, from the appearance of FAAs in the hemolymph, determined by measuring the concentrations of total ninhydrin-positive substances (TNPSs; mostly FAAs). When this is done in *C. sapidus*, it appears that the crab responds with a typical cell volume regulatory response. There is a large pulse of TNPSs detectable in the hemolymph beginning at 6 h after transfer and essentially being completed by 24 h after transfer from either 35‰ or 28‰ to 15‰ salinity (Fig. 7).

The timing of the pulse is such that it directly precedes the initial CA induction, and the magnitude of the pulse and the degree of CA induction are both correlated with the magnitude of the drop in salinity (Figs 5, 7). Furthermore, both the cell volume response and CA induction take place during the initial phase of acute salinity transfer when hemolymph dilution is occurring and, presumably, cell swelling is at its peak. It is therefore possible that cell swelling could be the first signal in the transduction pathway for salinity-mediated CA induction. While the current evidence is circumstantial, this area deserves more systematic investigation.

The second avenue of research involves the putative hormonal regulation of CA expression. The primary endocrine system of crustaceans resides in the eyestalk (Fingerman, 1987). Hormones from the X-organ/sinus gland complex control physiological processes as diverse as molting and water balance. The possibility that CA expression could also be controlled by this complex was recently tested. Eyestalk ablation was used as an experimental approach (i) to test the effects of the removal of the X-organ/sinus gland complex and (ii) to elevate the production of methyl farnesoate to determine whether either result altered normal CA induction (Henry et al., 1999; Henry et al., 2000). Remarkably, eyestalk ablation caused a significant increase in CA expression in blue crabs

acclimated to 35‰. In posterior gills (G7) only, CA activity increased over threefold within 24 h after eyestalk ablation and remained at approximately double the control value at 96 h post-treatment (Fig. 8). There was no change in branchial CA activity in sham-operated crabs, in which a tip of the walking leg was cut off, and there was no change in CA activity in anterior (G3) gills for any treatment. These data are very preliminary, but they strongly suggest (i) that CA expression is under central hormonal control, possibly through the X-organ/sinus gland complex, and (ii) that the mechanism of expression involves de-repression of CA synthesis. It appears that there is active suppression of the CA gene at high salinity, and that the repressor originates in the eyestalk, because removal of the eyestalks results in CA induction without any external stimulus from a change in salinity. Methyl farnesoate, however, does not play a role in the induction process (Henry et al., 1999b; Henry et al., 2000).

In summary, CA is a central enzyme in the transport processes that are critical to the survival and success of euryhaline marine crustaceans in the estuarine habitat. CA induction in low salinity is a result of selective gene expression, and the study of the initiation and control of this process will shed light on the general mechanisms of environmental signal transduction.

This work was supported by National Science Foundation award IBN 97-–27835, and by funds from the Salsbury Cove Research Foundation of the Mount Desert Island Biological Laboratory.

References

Bottcher, K., Siebers, D. and Becker, W. (1990a). Carbonic anhydrase in branchial tissues of osmoregulating shore crabs, *Carcinus maenas*. *J. Exp. Zool*. **255**, 251–261.

Bottcher, K., Siebers, D. and Becker, W. (1990b). Localization of carbonic anhydrase in the gills of *Carcinus maenas*. *Comp. Biochem. Physiol*. **96**B, 243–246.

Bottcher, K., Siebers, D., Becker, W. and Petrausch, G. (1991). Physiological role of branchial carbonic anhydrase in the shore crab, *Carcinus maenas*. *Mar. Biol*. **110**, 337–342.

Bryan, G. W. (1960). Sodium regulation in the crayfish *Astacus fluviatilis*. I. The normal animal. *J. Exp. Biol*. **37**, 83–89.

Cameron, J. N. (1978a). NaCl balance in blue crabs, *Callinectes sapidus*, in fresh water. *J. Comp. Physiol*. B **123**, 127–135.

Cameron, J. N. (1978b). Effects of hypercapnia on blood acid–base status, NaCl fluxes and trans-gill potential in freshwater blue crabs, *Callinectes sapidus*. *J. Comp. Physiol*. B **123**, 137–141.

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

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

Carriker, M. R. (1967). Ecology of estuarine benthic invertebrates: a perspective. In *Estuaries* (ed. G. H. Lauf). pp. 442–487. Washington, DC: American Association for the Advancement of Science.

Cioffi, M. (1984). Comparative ultrastructure of arthropod transporting epithelia. *Am. Zool*. **24**, 139–156.

Coleman, J. E. (1980). Current concepts of the mechanism of action of carbonic anhydrase. In *Biophysics and Physiology of Carbon Dioxide* (ed. C. Bauer, G. Gros and H. Bartels), pp. 133–150. New York: Springer-Verlag.

Compere, P., Wanson, S., Pequeux, A., Gilles, R. and Goffinet, G. (1989). Ultrastructural changes in the gill epithelium of the green crab *Carcinus maenas* in relation to the external salinity. *Tissue & Cell* **21**, 299–318.

Copeland, D. E. and 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.

Deaton, L. E. and Greenberg, M. J. (1986). There is no horohalinicum. *Estuaries* **9**, 20–30.

Dickson, J. S., Dillaman, R. M., Roer, R. D. and Roye, D. B. (1991). Distribution and characterization of ion transporting and respiratory filaments in the gills of *Procambarus clarkii*. *Biol. Bull*. **180**, 154–160.

Eckhardt, E., Pierrot, C., Thuet, P., Van Herp, F., Charmantier-Daures, M., Trilles, J.-P. and Charmentier, G. (1995). Stimulation of osmoregulatingprocesses in the perfused gill of the crab *Pachygrapsus marmoratus* (Crustacea, Decapoda) by a sinus gland peptide. *Gen. Comp. Endocrinol*. **99**, 169–177.

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

Engel, D. W. (1977). Comparison of the osmoregulatory capabilities of two portunid crabs, *Callinectes sapidus* and *C. similis*. *Mar. Biol*. **41**, 275–279.

Evans, D. H. and Cameron, J. N. (1986). Gill ammonia transport. *J. Exp. Zool*. **239**, 17–23.

Ferguson, J. K. W., Lewis, L. and Smith, J. (1937). The distribution of carbonic anhydrase in certain marine invertebrates. *J. Cell. Comp. Physiol*. **10**, 395–400.

Fingerman, M. (1987). The endocrine mechanisms of crustaceans. *J. Crust. Biol*. **7**, 1–24.

Finol, H. J. and Croghan, P. C. (1983). Ultrastructure of the branchial epithelium of an amphibious brackish-water crab. *Tissue & Cell* **15**, 63–75.

Florkin, M. and Schoffeniels, E. (1969). *Molecular Approaches to Ecology*. New York: Academic Press. 203pp.

Gainey, L. F. and Greenberg, M. J. (1977). The physiological basis of the species abundance–salinity relationship in molluscs: a speculation. *Mar. Biol*. **40**, 41–49.

Gehnrich, S., Brooks, C., Weihrauch, D., Towle, D. and Henry, R. (2000). Alterations in carbonic anhydrase gene expression during low salinity adaptation in the shore crab, *Carcinus maenas*. *Bull. Mt Desert Island Biol. Lab*. **39**, 28–29.

Gehnrich, S., Brooks, C., Weirauch, D., Towle, D. and Henry, R. (1999). Carbonic anhydrase gene expression in the gills of the shore crab during acclimation to low salinity. *Am. Zool*. **39**, 65A.

Gross, M. G. (1972). *Oceanography: A View of the Earth*. Englewood Cliffs, NJ: Prentice Hall. 581pp.

Harris, R. R. and Andrews, M. B. (1985). Total NPS pool and ammonia net efflux rate changes in *Carcinus maenas* during acclimation to low salinity. *Comp. Biochem. Physiol*. **82**A, 301–308.

Harris, R. R. and Micallef, H. (1971). Osmotic and ionic regulation in *Potamon edulis*, a fresh water 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. (1987). Membrane-associated carbonic anhydrase in the gills of the blue crab, *Callinectes sapidus*. *Am. J. Physiol.* **252**, R966–R971.

Henry, R. P. (1988a). Subcellular distribution of carbonic anhydrase activity in the gills of the blue crab, *Callinectes sapidus*. *J. Exp. Zool*. **245**, 1–8.

Henry, R. P. (1988b). Multiple functions of gill carbonic anhydrase. *J. Exp. Zool*. **248**, 19–24.

Henry, R. P. (1991). Techniques for measuring carbonic anhydrase activity *in vitro*: The electrometric delta pH method and the PH stat method. In *The Carbonic Anhydrases: Cellular Physiology and Molecular Genetics* (ed. S. J. Dodgson, R. E. Tashian, G. Gros and N. D. Carter), pp. 119–125. New York: Plenum.

Henry, R. P. (1995). Nitrogen metabolism and excretion for cell volume regulation. In *Nitrogen Metabolism and Excretion* (ed. P. J. Walsh and P. Wright), pp. 63–74. Boca Raton, FL: CRC Press.

Henry, R. P. (1996). Multiple roles of carbonic anhydrase in cellular transport and metabolism. *Annu. Rev. Physiol.* **58**, 523–538.

Henry, R. P. and Cameron, J. N. (1982a). The distribution and partial characterization of carbonic anhydrase in selected aquatic and terrestrial decapod crustaceans. *J. Exp. Zool*. **221**, 309–321.

Henry, R. P. and Cameron, J. N. (1982b). Acid–base balance in the euryhaline blue crab, *Callinectes spidus*, during acclimation from high to low salinity. *J. Exp. Biol*. **101**, 255–264.

Henry, R. P. and Cameron, J. N. (1983). The role of branchial carbonic anhydrase in respiration, ion regulation and acid–base balance in the aquatic crab, *Callinectes sapidus* and the terrestrial crab, *Gecarcinus lateralis*. *J. Exp. Biol*. **103**, 205–223.

Henry, R. P., Campoverde, M. P. and Borst, D. W. (1999b). Effects of eyestalk ablation on salinity-mediated carbonic anhydrase induction in three species of decapod crustaceans. *Am. Zool*. **39**, 65A.

Henry, R. P., Campoverde, M. P. and Borst, D. W. (2000). Effects of eyestalk ablation on carbonic anhydrase activity in the gills of *Carcinus maenas* and *Cancer irroratus*. *Bull. Mt Desert Island Biol. Lab.* **39**, 21–22.

Henry, R. P., Garrelts, E. E., McCarty, M. M. and Towle, D. W. (1998). Transport enzyme induction and low salinity adaptations in *Carcinus maenas*. *Am. Zool*. **38**, 125A.

Henry, R. P., Garrelts, E. E., McCarty, M. M. and Towle, D. W. (1999a). Salinity adaptations in the euryhaline green crab, *Carcinus maenas*. *Bull. Mt Desert Island Biol. Lab.* **38**, 55.

Henry, R. P. and Mangum, C. P. (1980). Salt and water balance in the oligohaline clam, *Rangia cuneata*. I. Anisosomotic extracellular regulation. *J. Exp. Zool*. **211**, 1–10.

Henry, R. P., Perry, H. M., Trigg, C. B., Handley, H. L. and Krarup, A. (1990). Physiology of two species of deep-water crabs, *Chaceon fenneri* and *C. quinquedens*: gill morphology and hemolymph ionic and nitrogen concentrations. *J. Crust. Biol.* **10**, 375–381.

Henry, R. P., Smatresk, N. J. and Cameron, J. N. (1988). The distribution of branchial carbonic anhydrase and the effects of gill and erythrocyte carbonic anhydrase inhibition in the channel catfish *Ictalurus punctatus*. *J. Exp. Biol.* **134**, 201–218.

Henry, R. P., Tufts, B. L. and Boutilier, R. G. (1993). The distribution of carbonic anhydrase type I and II isozymes in lamprey and trout: possible co-evolution with erythrocyte chloride/bicarbonate exchange. *J. Comp. Physiol.* B **163**, 380–388.

Henry, R. P. and Watts, S. A. (2001). Early carbonic anhydrase induction in the gills of the lue crab, *Callinectes sapidus*, during low salinity acclimation is independent of ornithine decarboxylase activity. *J. Exp. Zool*. (in press).

Henry, R. P. and Wheatly, M. G. (1988). Dynamics of salinity adaptations in the euryhaline crayfish *Pacifasticus leniusculus*. *Physiol. Zool.* **61**, 260–271.

Hsueh, P. W. (1992). A comparative study of the population dynamics, life history characteristics and physiological ecology of *Callinectes similis* and *C. sapidus* in estuarine environments of the Northern Gulf of Mexico. PhD thesis, University of Alabama at Birmingham.

Kinne, O. (1971). Salinity: animals. In *Marine Ecology: Environmental Factors*, vol. 1, part 2 (ed. O. Kinne). pp. 821–996. New York: John Wiley & Sons.

Kirschner, L. B. (1979). Control mechanisms in crustaceans and fish. In *Mechanisms of Osmoregulation in Animals* (ed. R. Gilles), pp. 157–222. Chichester: John Wiley.

Lee, K. J. (1992). Developmental regulation of Na^+/K^+-ATPase activity in the brine shrimp *Artemia*: response to salinity and the potential regulatory role of polyamines. MS thesis, University Alabama at Birmingham. 66pp.

Lin, H. and Randall, D. J. (1991). Evidence for the presence of an electrogenic proton pump on the trout gill epithelium. *J. Exp. Biol*. **161**, 119–134.

Lucu, C. and Flik, G. (1999). Na^+/K^+-ATPase and Na^+/Ca^{2+} exchange activities in gills of hyperregulating *Carcinus maenas*. *Am. J. Physiol.* **276**, R490–R499.

Mantel, L. H. and Farmer, L. L. (1983). Osmotic and ionic regulation. In *The Biology of Crustacea*, vol. 5, *Internal Anatomy and Physiological Regulation* (ed. L. H. Mantel), pp. 54–162. New York: Academic Press.

Maren, T. H. (1967). Carbonic anhydrase: chemistry, physiology and inhibition. *Physiol. Rev.* **47**, 595–781.

Moran, W. M. and Pierce, S. K. (1984). The mechanism of crustacean salinity tolerance: cell volume regulation by K^+ and glycine. *Mar. Biol*. **81**, 41–46.

Morohashi, M., Tsuchiya, K., Mita, T. and Kawamura, M. (1991). Identification of (Na,K)ATPase inhibitor in shrine shrimp *Artemia salina*, as long-chain fatty acids. *J. Comp. Physiol.* B **161**, 69–72.

Neufeld, G. J., Holliday, C. W. and Pritchard, J. B. (1980). Salinity adaptation of gill Na/K in the blue crab, *Callinectes sapidus*. *J. Exp. Zool*. **211**, 215–224.

Pequeux, A. (1995). Osmotic regulation in crustaceans. *J. Crust. Biol*. **15**, 1–60.

Pierce, S. K. and Amende, L. M. (1981). Control mechanisms of amino acid-mediated cell volume regulation in salinity-stressed molluscs. *J. Exp. Zool*. **215**, 247–258.

Piller, S. C., Henry, R. P., Doeller, J. E. and Kraus, D. W. (1995). A comparison of the gill physiology of two euryhaline crab species, *Callinectes sapidus* and *Callinectes similis*: energy production, transport-related enzymes and osmo-regulation as a function of acclimation salinity. *J. Exp. Biol*. **198**, 349–358.

Riestenpatt, S., Onken, H. and Siebers, D. (1996). Active absorption of Na^+ and Cl^- across the gill epithelium of the shore crab *Carcinus maenas*: voltage-clamp and ion-flux studies. *J. Exp. Biol*. **199**, 1545–1554.

Savage, J. P. and Robinson, G. D. (1983). Inducement of increased gill Na^+-K^+ ATPase activity by a hemolymph factor in

hyperosmoregulating *Callinectes sapidus*. *Comp. Biochem. Physiol.* **75**A, 65–69.

Sommer, M. J. and Mantel, L. H. (1988). Effect of dopamine, cyclic AMP and pericardial organs on sodium uptake and Na/K-ATPase activity in gills of the green crab *Carcinus maenas* (L.). *J. Exp. Zool.* **248**, 272–277.

Spannings-Pierrot, C., Soyez, D., Van Harp, F. and Charmantier, G. (1999). Involvement of CHH in the control of osmoregulation in a grapsid crab. *Am. Zool.* **39**, 68A.

Tagatz, M. E. (1967). Noncommercial crabs of the genus *Callinectes* in St. John's River, Florida. *Chesapeake Sci.* **8**, 202–203.

Towle, D. W. (1984). Membrane-bound ATPases in arthropod ion-transporting tissues. *Am. Zool*. **24**, 177–185.

Towle, D. W. (1997). Molecular approaches to understanding salinity adaptation of estuarine animals. *Am. Zool*. **37**, 575–584.

Towle, D. W., Palmer, G. and Harris, J. (1976). Role of gill Na^++K^+-dependent ATPase in acclimation of blue crabs (*Callinectes sapidus*) to low salinity. *J. Exp. Zool*. **196**, 315–322.

Towle, D. W., Rushton, M. E., Heidysch, D., Magnani, J. J., Rose, M. J., Amstutz, A., Jordan, M. K., Shearer, D. W. and Wu, W.-S. (1997). Sodium/proton antiporter in the euryhaline crab *Carcinus maenas*: molecular cloning, expression and tissue distribution. *J. Exp. Biol.* **200**, 1003–1014.

Wheatly, M. G. and Henry, R. P. (1987). Branchial and antennal gland Na^+/K^+-dependent ATPase and carbonic anhydrase activity during salinity acclimation of the euryhaline crayfish *Pacifastacus leniusculus*. *J. Exp. Biol.* **133**, 73–86.

Zanders, I. P. (1980). Regulation of blood ions in *Carcinus maenas* (L.). *Comp. Biochem. Physiol.* **65**A, 97–108.