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Functional evidence for the presence of a carbonic anhydrase repressor in the eyestalk of the euryhaline green crab *Carcinus maenas*

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Summary

Carbonic anhydrase (CA) activity and relative expression of CA mRNA were measured in the gills of the euryhaline green crab *Carcinus maenas* in response to eyestalk ablation (ESA), injection of eyestalk extract and exposure to low salinity. For crabs acclimated to 32 p.p.t. salinity, ESA alone resulted in an increase in both CA activity and relative mRNA expression in the posterior, ion-transporting gills, but not in the anterior, respiratory gills. The ESA-stimulated increase in CA activity was abolished by injections of extracts of eyestalks taken from crabs acclimated to 32 p.p.t. salinity. Transfer of intact crabs from 32 to 10 p.p.t. salinity for 7 days resulted in an eightfold increase in CA activity and a sixfold increase in mRNA expression in posterior gills. ESA potentiated the normal low salinity-mediated CA induction by 23%. Daily injections of eyestalk extract reduced low salinity-stimulated CA induction by nearly 50% in intact crabs and by almost 75% in eyestalk ablated crabs. A 4-day transfer to 10 p.p.t. salinity also caused significant increases in both CA activity and mRNA expression in posterior gills, and ESA resulted in a 32% increase in the normal degree of CA induction. Daily injections of eyestalk extracts reduced CA induction in a dose-dependent manner over the 4-day time course. When CA induction was reduced by 66%, hemolymph osmotic regulation was also disrupted. These results are functional evidence for the presence of a CA repressor in the major endocrine complex of the crab, the eyestalk. This compound appears to function in keeping CA expression at low, baseline levels in crabs at high salinity. Exposure to low salinity removes the effects of the putative repressor, allowing CA expression, and thus CA activity, to increase.

Key words: carbonic anhydrase, osmoregulation, crustacean, salinity, green crab, *Carcinus maenas*.

Introduction

The enzyme carbonic anhydrase (CA) has long been believed to play a central role in the mechanism of low salinity adaptation in euryhaline crustaceans. This belief is based on evidence obtained primarily from two experimental model species, the blue crab *Callinectes sapidus* and the green crab *Carcinus maenas*. In both species, CA is found with high activities in the primary organ of ion transport, the gill (Henry and Cameron, 1982a; Bottcher et al., 1990; Henry et al., 2002). The current view of branchial CA function is that the enzyme catalyzes the hydration of respiratory CO_2 as it diffuses through the gills from the hemolymph to the surrounding water, resulting in the production of H^+ and HCO_3^- in the intracellular fluid. These products are then believed to function in ion uptake processes such as Na^+/H^+ and Cl^-/HCO_3^- exchange (Kirschner, 1979; Henry and Cameron, 1983; Henry, 1984; Henry, 2001; Henry et al., 2002). Inhibition of CA activity with the compound acetazolamide disrupts both Na^+ and Cl^- transport and the ability of both crabs to maintain their normally high hemolymph osmotic concentrations in the face of low salinity (Cameron, 1979; Henry and Cameron, 1983; Henry et al., 2003). Furthermore, inhibition of CA activity in blue crabs during the acute phase of low salinity adaption appears to result in death as a result of the breakdown of the osmoregulatory mechanism (Henry and Cameron, 1982b). This body of evidence strongly favors the role of CA in osmoregulation through its function in supporting active ion uptake across the crustacean gill.

Branchial carbonic anhydrase activity in euryhaline crustaceans is highly sensitive to changes in environmental salinity. In blue crabs, there is up to a 14-fold increase in CA activity, and in green crabs, increases of up to tenfold have been reported, depending on the magnitude of decrease in salinity to which the crabs were exposed (Henry and Cameron, 1982a; Henry and Watts, 2001; Henry et al., 2002). The induction process occurs exclusively in the posterior, ion transporting gills of both species, and it is the cytoplasmic fraction of branchial CA, the fraction believed to be involved in ion transport, which is induced (Henry, 1988; Henry et al., 2003). CA induction is also known to occur in more

moderately euryhaline, weaker osmoregulators, such as *Callinectes similis* (Pillar et al., 1995) and in euryhaline freshwater crayfish (*Pacifastacus leniusculus*) (Wheatly and Henry, 1987; Henry and Wheatly, 1988), but not in stenohaline osmoconformers such as *Libinia emarginata* (Henry and Cameron, 1982a).

Early reports on the time course of CA induction after exposure to low salinity have been consistent with the hypothesis that the increase in CA activity was a result of the synthesis of new enzyme. CA induction in both the blue crab and the green crab has been reported to take on the order of 24–48 h to be initiated and 4–7 days to reach new acclimated levels, respectively (Henry and Cameron, 1982b; Henry and Watts, 2001; Henry et al., 2002). Changes in CA activity in other species (e.g. crayfish) are also known to occur over a period of days (Henry and Wheatly, 1988). Recently, CA induction in *C. maenas* was shown to be under transcriptional regulation: CA mRNA increased first, at 24 h after transfer to low salinity, followed by an initial increase in CA activity at 48–72 h post-transfer (Henry et al., 2003).

Preliminary new evidence also suggests that the CA induction process is under neuroendocrine regulation. Eyestalk ablation (ESA) resulted in an increase in CA activity in green crabs acclimated to high salinity even without transfer to low salinity (Henry et al., 2000). These results suggest that CA induction is under inhibitory control by a repressor compound found in the major endocrine organ of the crab, the eyestalk. Perhaps because CA has such a high turnover rate and is typically expressed in excess of what is physiologically needed (e.g. Henry, 2001), its regulation has never been systematically studied, especially in invertebrates. CA and its induction in the crustacean gill, however, represent an ideal system in which to study the regulation of environmentally mediated changes in gene expression. CA activity and expression can be measured in opposing gill pairs of the same individual; the changes are large and easily measured, and the anterior gills serve as a control in which neither activity nor expression change in response to salinity.

This report presents the first functional evidence for the presence of a CA repressor in the eyestalk of the green crab, based on classical endocrinological approaches of eyestalk ablation combined with injections of extracts from the eyestalk and exposure to low salinity.

Materials and methods

Collection and maintenance of animals

Adult, intermolt green crabs *Carcinus maenas* L. were collected by hand from the intertidal zone of Frenchman's Bay, along the property of the Mount Desert Island Biological Laboratory (MDIBL), Salisbury Cove, ME, USA. Crabs were also obtained from the Marine Biological Laboratory (Woods Hole, MA, USA) and from Gulf of Maine Specimen Co. (Pembroke, ME, USA). Only males were used in the experiments. Green crabs were held at MDIBL in 400 liter fiberglass tanks equipped with filtered, running seawater (32–33 p.p.t. salinity and 11–12°C). Crabs were fed a combination of mussels and squid every other day but were starved for a minimum of 48 h prior to use in an experiment. Salinity was checked with a hand-held refractometer, and water quality was monitored by measuring nitrite concentrations of all holding tanks and experimental aquaria (Dry Tab, Mansfield, MA, USA).

Experimental protocol

Green crabs (40–90 g) that were collected from 32 p.p.t. were held at that salinity for 1 week prior to experimentation. For the bulk of the salinity transfers, these crabs were either placed in 80 liter tanks with running seawater of 32 p.p.t. (controls) or transferred to 160 liter recirculating tanks (6–10 crabs per tank) of 10 p.p.t., thermostatted to 12°C, and also equipped with biological filters. Transfer to 10 p.p.t. salinity has been shown to cause an approximate eight- to tenfold induction in CA activity over a 7-day acclimation period (Henry et al., 2002). The time course of low salinity exposure for these experiments was between 4 and 7 days, the time periods that have been shown to be needed for initial and maximal CA induction, respectively, in green crabs (Henry et al., 2002). The crabs were transferred either as untreated (intact) controls, crabs subjected to eyestalk ablation (ESA), or intact or ESA crabs given daily injections of eyestalk extracts.

For the ESA procedure, crabs were chilled on ice for 10 min, and the eyestalks were cut at their base using a dissecting scissors. The crabs were kept on ice for another 10 min to allow the hemolymph to clot, and then they were transferred to the treatment salinity.

For salinity transfer experiments involving injections, three treatments were used: (1) intact controls (no injection), (2) sham injection (injection of filtered seawater), and (3) injection of eyestalk extract. The eyestalk extracts were prepared in the following manner. For a typical sample size of 6 crabs, 12 eyestalks were homogenized in cold, filtered (0.45 μm) seawater using a hand-held homogenizer and stainless steel generator (Omni TH115; Omni Instruments, Warrenton, VA, USA). The homogenate was centrifuged at 10 000 ***g*** for 10 min at 4°C (Sorvall RC5-B, Wilmington, NE, USA), and 400 μl of the supernatant was injected into the hemolymph of each crab. The injection was given through the arthroidial membrane at the base of the walking legs using a 22-gauge needle and 1 ml syringe. Green crabs were given one injection immediately upon transfer to low salinity and one injection daily through the remainder of the time course of the experiment. To avoid potential injury to one area, the injections were given at the base of a different leg each day. A separate set of green crabs was maintained at 32 p.p.t. salinity to serve as 'eyestalk donors'. After ESA, these crabs were returned to Frenchman's Bay. A 7-day experiment, involving daily injections, required the use of a large number of animals (e.g. treatment group of up to 10 crabs plus a donor group of 70). The 4-day experimental time course was validated in order to reduce animal use and increase the number of experiments able to be completed in the same

amount of time, while still being able to show the same treatment effects as in the 7-day time course.

For experiments in which CA activity and CA mRNA expression were measured simultaneously in the same crab, the anterior and posterior gills from the right side of the crab were dissected out and used for total mRNA extraction and analysis, and gills from the left side of the same individual were assayed for CA activity.

For all crabs, at the end of the experiment, hemolymph samples were withdrawn from the infrabranchial sinus at the base of the walking legs, using a 22-gauge needle and 1 ml syringe, transferred to a 1.5 ml centrifuge tube, and stored at –20°C for analysis of osmotic and ionic concentrations.

Anterior gills (e.g. G4), which lack the CA induction response to low salinity exposure (e.g. Henry et al., 2003), were used as a non ion-transporting tissue control, and posterior (e.g. G8), ion-transporting gills were used as the experimental tissue. At the end of each experiment, crabs were immersed in crushed ice for 10 min, and both anterior and posterior gills were dissected out and placed in 5 volumes of cold (4°C) homogenization/assay buffer (225 mmol l^{-1} mannitol, 75 mmol l^{-1} sucrose, 10 mmol l^{-1} Trizma base, adjusted to pH=7.40 with 10% phosphoric acid). Crabs were killed *via* exsanguination.

Analytical procedures

Carbonic anhydrase activity was measured electrometrically by the delta pH method (Henry, 1991). Anterior (G4) and posterior (G8) gills were homogenized in 5 volumes of cold buffer using an Omni 1000 or TH115 hand-held homogenizer (Omni Instruments) and then sonicated at 25 W for 30 s (Heat Systems Microsonicator, Farmingdale, NY, USA). Homogenates were centrifuged at 10 000 ***g*** for 20 min at 4°C (RC5-B, Sorvall Instruments, Wilmington, DE, USA), and the supernatant was assayed for CA activity. Briefly, 50–200 μl of supernatant was added to 6 ml of buffer in a thermostatted reaction vessel (4°C) and stirred rigorously. The reaction was started by the addition of CO_2-saturated water, and the drop in pH (about 0.25 units) was monitored by micro pH and reference electrodes (World Precision Instruments, Sarasota, FL, USA) and a null-point pH meter. Protein concentration was also measured in the supernatant by Coomassie Brilliant Blue dye binding (Bio Rad Laboratories, Hercules, CA, USA), and CA activity was reported as μmol CO_2 mg protein^{-1} min^{-1}.

Total RNA from anterior and posterior gills was isolated under RNAse-free conditions by phenol–chloroform extraction (RNAgents, Promega, Madison, WI, USA). All dissecting equipment and homogenizers were cleaned with RNAse-zap (Ambion, Austin, TX, USA) and rinsed in RNAse-free water. RNA concentrations, purity and DNA contamination were monitored using a Bioanalyzer 2100 lab chip system (Agilent, Wilmington, DE, USA). Single stranded, complementary DNA was then produced from Poly-A mRNA in 2 μg of total RNA by reverse transcription using Superscript II reverse transriptase (Invitrogen, Carlsbad, CA, USA) and oligo dT as primer.

Degenerate primers were designed, and nucleotide sequence for CA was obtained, exactly as previously described (Henry et al., 2003). Using nucleotide sequence data obtained in this manner (data not shown) plus existing sequence data (Henry et al., 2003), gene-specific primers were designed for *C. maenas* branchial CA using Primer Premiere software. These primers are as follows: 168F: 5′ CGC TCA GTT CCA CTT CCA 3′; 398R: 5′ ACA TCT CAG CAT CCG TCA 3′.

These primers were used to amplify the CA cDNA template for quantitative, real time PCR on a real-time PCR instrument (Stratagene MX 4000, Cedar Creek, TX, USA). The sample with the highest measured protein-specific CA activity was used to construct a standard curve consisting of amplification products generated from stepwise tenfold dilutions spanning four orders of magnitude (10^0 to 10^{-3}). Concentrations of amplified CA cDNA were calculated using the standard curve and were reported on the basis of relative amounts.

In most studies of changes in gene expression, a ‘housekeeping’ gene, such as actin, is used as a control. This approach depends on the control gene being expressed constituitively and being unresponsive to the experimental treatment. However, the ion transporting gills in euryhaline species (lower vertebrates and invertebrates) undergo complete molecular and ultrastructural remodeling in response to low salinity, including increases in membrane and cytoskeletal elements (e.g. Perry, 1997; Luquet et al., 2002; Evans et al., 2005), making traditional housekeeping genes inappropriate in these tissues.

The metabolic gene, arginine kinase (AK) was used instead of actin. AK activity in the gills of green crabs was shown not to change with salinity (Kotlyar et al., 2000); and AK mRNA expression, as measured with semi-quantitative PCR, was also shown to be insensitive to low salinity exposure in both *C. maenas* and a closely related species, the blue crab *Callinectes sapidus* (Towle et al., 2001; Henry et al., 2003). The primers used for AK real time PCR in this study were as follows (Towle et al., 2001): AKF51: GCTGAGTCTAAGAAGG-GATT; AKCALLR1: CCCAGGCTTGTCTTCTTGTCC. Real time PCR was performed, using Sybr green, on a Mini-Opticon (Bio Rad, Hercules, CA, USA).

Branchial AK activity in posterior gills of blue crabs is sensitive to low salinity, however, and initial evidence obtained from real time PCR indicates that AK mRNA expression in posterior gills increases substantially in response to low salinity (fivefold; L. Serrano and R.P.H., unpublished data). It is therefore possible that in some species the salinity response of the posterior gills is so extensive that a traditional housekeeping gene may not exist. So in addition to a control gene, a control tissue, anterior gill, a tissue in which the salinity response is absent, was also used to follow both CA activity and mRNA abundance. This was done for two reasons: (1) to show that a gill that lacks induction of CA activity also lacks changes in CA mRNA abundance, and (2) to validate the use of a tissue control for gene expression studies in cases where a traditional housekeeping gene may be absent.

Hemolymph samples were thawed on ice, sonicated, and

centrifuged at 14 000 ***g*** for 1 min to separate out clot material. Osmolality was then measured on 10 μl samples using a vapor pressure osmometer (Wescor 5100C, Logan, UT, USA).

Statistical analysis

Statistical analysis of the data was performed using Sigma Stat 2.0 (SPSS, Chicago, IL, USA) and plotted using Sigma Plot 2001.

Results

Branchial CA activity in *Carcinus maenas* acclimated to 32 p.p.t. salinity was uniformly low and not significantly different between anterior (G4) and posterior (G8) gills (P=0.15, t-test; Fig. 1A), a pattern that is typical of green crabs acclimated to high salinity and that has been reported numerous times in the past (Henry et al., 2002; Henry et al., 2003; Henry, 2005). For crabs acclimated to 32 p.p.t., eyestalk ablation resulted in more than a doubling of CA activity in the posterior gills (P<0.001, t-test) even in the absence of a low salinity stimulus. CA activity in anterior gills was not affected by ESA (P=0.93, t-test; Fig. 1A). A similar pattern was seen for CA mRNA expression, as measured by real time PCR. Relative values of CA mRNA were not different in anterior *vs* posterior gills of 32 p.p.t.-acclimated crabs (P=0.79, t-test; Fig. 1B), but relative expression increased by over fourfold in G8 after 7 days following ESA. These results also confirm previous studies showing that CA activity is a reliable reporter assay for CA gene expression (Henry et al., 2003; Henry et al., 2006).

In a separate experiment, injection of eyestalk extract into green crabs that had been subjected to ESA abolished the increase in CA activity. Again, there was no difference between anterior and posterior gills for crabs acclimated to 32 p.p.t. (P=0.83, t-test; Fig. 2). ESA alone resulted in an approximate 50% increase in CA activity in posterior gills (P<0.005, ANOVA and Tukey's *post hoc* comparison; Fig. 2). This increase was completely abolished by daily injections of extracts of eyestalks taken from crabs also acclimated to 32 p.p.t., as values from injected crabs were not significantly different from those of controls (P=0.10, Tukey's *post hoc* comparison). There was no difference in CA activity in G8 of sham-injected crabs *vs* controls (P=0.27, Tukey's *post hoc* comparison). Interestingly, while there was no difference in CA activity between G4 and G8 in 32 p.p.t.-acclimated crabs, there were slight but significant decreases in activity in G4 in both the sham-operated and eyestalk extract-injected crabs (P<0.01, Kruskall–Wallace ANOVA in ranks and Dunn's *post hoc* comparison).

Hemolymph osmolality in crabs acclimated to 32 p.p.t. was similar to that of ambient seawater and was not affected by any of the treatments (P=0.89, ANOVA, Table 1).

Transfer of green crabs from 32 to 10 p.p.t. salinity for a

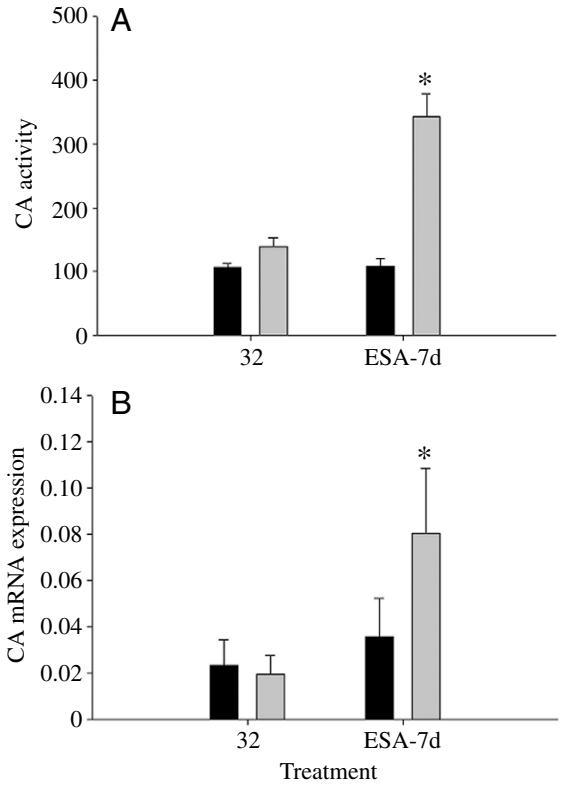

Fig. 1. Carbonic anhydrase activity (A; μmol CO_2 mg protein^{-1} min^{-1}) and mRNA expression (B; relative amounts) in anterior (G4, black bars) and posterior (G8, gray bars) of green crabs acclimated to 32 p.p.t. salinity *vs* eyestalk ablated crabs at 32 p.p.t. for 7 days. Values are means ± s.e.m. (N=6–8). T=12°C. Asterisks indicate statistical differences in G8 from the 32 p.p.t. controls

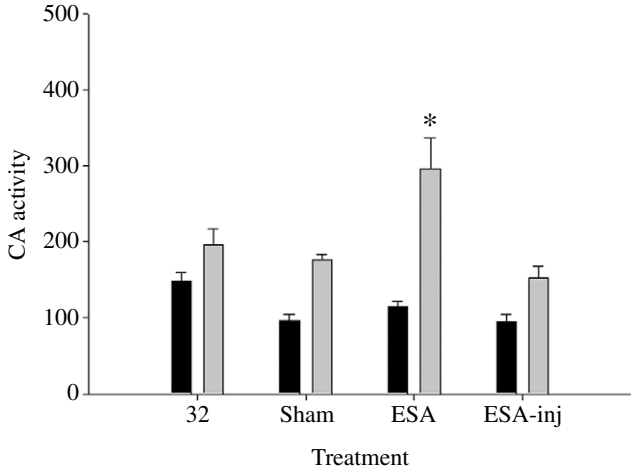

Fig. 2. Carbonic anhydrase activity (μmol CO_2 mg protein^{-1} min^{-1}) in anterior (G4, black bars) and posterior (G8, gray bars) gills of green crabs acclimated to 32 p.p.t. 32, 32 p.p.t. acclimated controls; Sham, intact crabs injected daily with filtered seawater for 7 days; ESA, eyestalk ablated crabs assayed after 7 days; ESA-inj, eyestalk ablated crabs injected daily with eyestalk extract and assayed after 7 days. Values are means ± s.e.m. (N=6–10). T=12°C. Asterisks indicate statistical differences at P<0.05 in G4 and G8 *vs* the corresponding gill in the 32 p.p.t. controls.

Table 1. *Hemolymph osmotic concentration in green crabs acclimated to 32 p.p.t. salinity and given various treatments*

Hemolymph osmotic concentration (mOsm kg H_2O^{-1})			
32 accl	32 sham-inj	32 ESA	32ESA-inj
936±6 (10)	927±8 (10)	924±6 (10)	918±8 (10)

Green crabs were acclimated to 32 p.p.t. (922±3 mOsm kg H_2O^{-1}, *N*=3) and given various treatments over a 7-day period. 32 accl, controls; sham-inj, crabs injections daily with 400 μl of filtered seawater; ESA, eyestalk ablated crabs; ESA-inj, ESA crabs given daily injections of 400 μl of eyestalk extracts from crabs acclimated to 32 p.p.t.

Values are mean ± s.e.m. (*N*). *T*=12°C.

period of 7 days resulted in a greater than tenfold increase in CA activity in posterior gills ($P<0.001$, *t*-test; Fig. 3A). This was accompanied by an approximate sixfold increase in mRNA expression, again only in the posterior gill ($P<0.001$, *t*-test). For both CA activity and mRNA expression, there were no significant differences between values for anterior *vs* posterior gills in crabs acclimated to 32 p.p.t. ($P=0.19$ and 0.77, *t*-test, for activity and mRNA, respectively) or for anterior gills in crabs acclimated to 32 p.p.t. *vs* those transferred to 10 p.p.t. for 7 days ($P=0.90$ and 0.80, *t*-test, for activity and expression, respectively; Fig. 3A,B).

When intact crabs were transferred from 32 to 10 p.p.t., there was a large increase in CA activity in the posterior gills which was inhibited by daily injections of eyestalk extract ($P<0.001$, ANOVA and Tukey's *post hoc* comparison; Fig. 4). In this group of crabs, transfer to 10 p.p.t. for 7 days resulted in an eightfold increase in CA activity in the posterior gills (Fig. 4). There was no significant difference between CA activity in posterior gills of intact *vs* sham-injected crabs ($P=0.38$, Tukey's *post hoc* comparison). However, a daily injection of extract from eyestalks taken from green crabs acclimated to 32 p.p.t. reduced normal CA induction by 60% ($P<0.001$, Tukey's *post hoc* comparison; Fig. 4). The inhibition was not complete, however, as the CA activity in the posterior gills of the injected crabs was still significantly higher (threefold) than that in the 32 p.p.t. acclimated controls ($P=0.003$, Tukey's *post hoc* comparison). In this experiment, while there was no difference between CA activity in anterior *vs* posterior gills in crabs acclimated to 32 p.p.t., there were significant differences among anterior gills as a result of the different experimental treatments ($P<0.001$, ANOVA). Intact crabs transferred to 10 p.p.t. for 7 days, and crabs given daily injections of eyestalk extract both had slightly but significantly lower CA activity in the anterior gills *vs* the 32 p.p.t. controls ($P<0.05$, Tukey's *post hoc* comparison; Fig. 4). This pattern has been reported previously for anterior gills of *C. maenas*

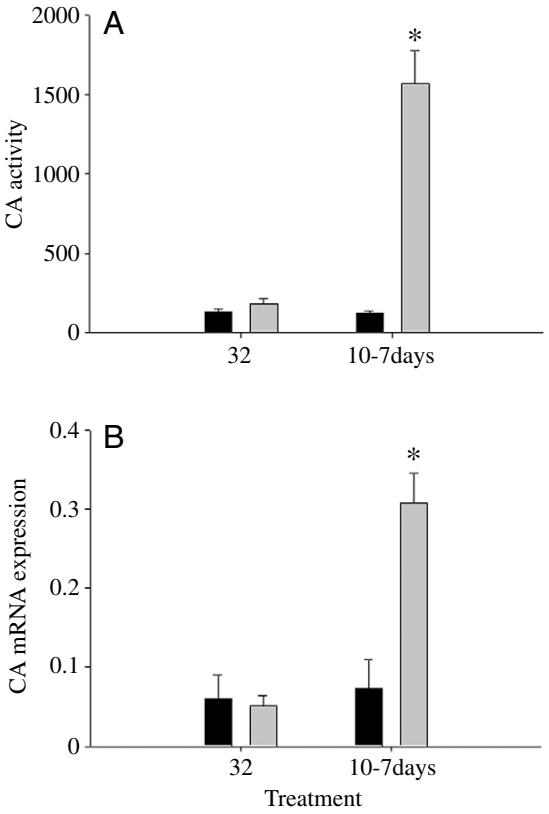

Fig. 3. Carbonic anhydrase activity (A; μmol CO_2 mg protein^{-1} min^{-1}) and mRNA expression (B; relative amounts) in anterior (G4, black bars) and posterior (G8, gray bars) gills of green crabs acclimated to 32 p.p.t. (32) and transferred to 10 p.p.t. for 7 days (10-7days). Values are means ± s.e.m. (*N*=6). *T*=12°C. Asterisks indicate statistical differences in G8 from the 32 p.p.t. controls at $P<0.05$.

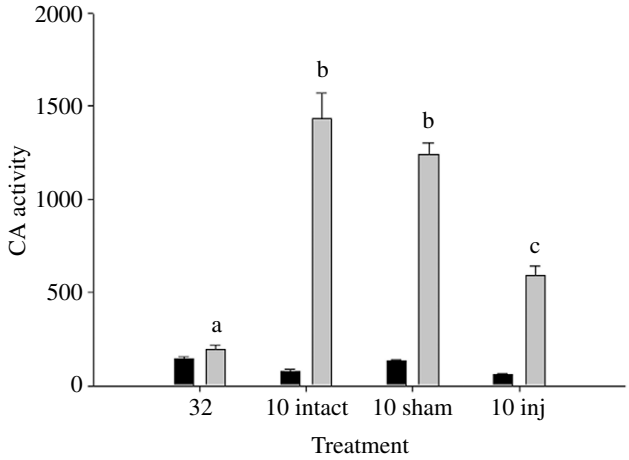

Fig. 4. Carbonic anhydrase activity (μmol CO_2 mg protein^{-1} min^{-1}) in anterior (G4, black bars) and posterior (G8, gray bars) gills of green crabs acclimated to 32 p.p.t. and transferred to 10 p.p.t. 32, 32 p.p.t. acclimated controls; 10 intact, intact crabs transferred to 10 p.p.t. for 7 days; 10 sham, intact crabs transferred to 10 p.p.t. and given daily injections of filtered seawater; 10 inj, intact crabs transferred to 10 p.p.t. and given daily injections of eyestalk extract taken from 'donor' crabs acclimated to 32 p.p.t. Values are means ± s.e.m. (*N*=6–9). *T*=12°C. Asterisks indicate statistical differences in G4 vs 32 p.p.t. controls; different letters indicate statistical differences in G8 across treatments at $P<0.05$.

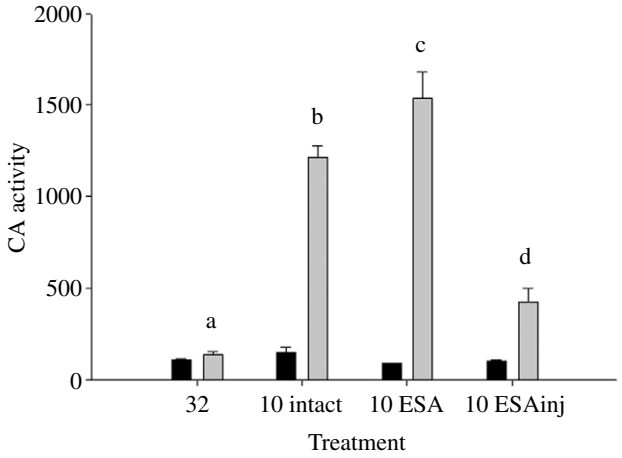

Fig. 5. Carbonic anhydrase activity (μmol CO_2 mg protein^{-1} min^{-1}) in anterior (G4, black bars) and posterior (G8, gray bars) gills of green crabs acclimated to 32 p.p.t. and transferred to 10 p.p.t. for 7 days. 32, 32 p.p.t. acclimated controls; 10 intact, intact crabs transferred to 10 p.p.t. for 7 days; 10 ESA, eyestalk ablated crabs transferred to 10 p.p.t. and assayed after 7 days; 10 ESAinj, eyestalk ablated crabs transferred to 10 p.p.t. and given daily injections of eyestalk extract taken from 'donor' crabs acclimated to 32 p.p.t. Values are means ± s.e.m. (N=6–9). T=12°C. Different letters indicate statistical differences at P<0.05 in G8 across treatments.

(Henry et al., 2003; Henry, 2005), but there has never been an increase in CA activity, as a result of low salinity exposure, in anterior gills.

A similar pattern of CA induction and inhibition was seen for eyestalk ablated crabs either transferred from 32 to 10 p.p.t. without any further treatment or given daily injections of eyestalk extract from high salinity-adapted crabs. For this group of crabs, there were no significant differences in CA activity among anterior gills across treatments (P=0.13, ANOVA, Fig. 5), and there was no significant difference between G4 and G8 for crabs acclimated to 32 p.p.t. (P=0.93, t-test). In posterior gills, however, the treatments produced highly significant changes in CA activity (P<0.001, ANOVA; Fig. 5). Transfer from 32 to 10 p.p.t. for 7 days produced the typically large (ninefold) induction of CA activity (P<0.001, ANOVA and Tukey's *post hoc* comparison; Fig. 5). Eyestalk ablation prior to transfer enhanced the effect of low salinity by 23%. Daily injections of eyestalk extract (again, from crabs acclimated to 32 p.p.t.) into eyestalk ablated crabs reduced the level of CA induction by nearly 75% (P<0.001, Tukey's *post hoc* comparison). This value (396±133 μmol CO_2 mg protein^{-1} min^{-1}) was still significantly higher than that for CA activity in G8 in the 32 p.p.t. acclimated controls (139±16 μmol CO_2 mg protein^{-1} min^{-1}; P=0.012, Tukey's *post hoc* comparison; Fig. 5).

Eyestalk ablation, either by itself or combined with injections of eyestalk extract, resulted in less than 5% mortality in green crabs.

Hemolymph osmolality was slightly but not significantly higher than ambient seawater in crabs acclimated to 32 p.p.t. After 7 days exposure to 10 p.p.t. salinity (307 mOsm kg^{-1}), hemolymph values in intact crabs were significantly higher by about 300 mOsm (P<0.001, t-test, Table 2). These values were not affected by either ESA, a single daily injection of eyestalk extract, or a combination of both treatments (P=0.79, ANOVA, Table 2).

Table 2. *Hemolymph osmotic concentration in green crabs acclimated to 32 p.p.t. salinity and transferred to 10 p.p.t. for 7 days*

Hemolymph osmotic concentration (mOsm kg H_2O^{-1})				
32 accl	32-10 intact	32-10 ESA	32-10 inj	32-10 ESA-inj
928±12 (8)	639±78 (8)	616±18 (10)	624±33 (8)	643±24 (8)

Green crabs were acclimated to 32 p.p.t. salinity (920±8 mOsm kg H_2O^{-1}, N=3) and transferred to 10 p.p.t. (307±12 mOsm kg H_2O^{-1}, N=6) for 7 days. 32 accl, control crabs; 32-10 intact, crabs transferred to 10 p.p.t. with no other treatment; 32-10 ESA: crabs subjected to eyestalk ablation and transferred to 10 p.p.t.; 32-10 inj: intact crabs transferred to 10 p.p.t. and given daily injections of eyestalk extracts; 32-10 ESA-inj: eyestalk ablated crabs transferred to 10 p.p.t. and given daily injections of eyestalk extracts.

Values are mean ± s.e.m. (N). T=12°C.

As mentioned previously, a 7-day time course requires the use of a high number of animals, especially for experiments involving daily injections from groups of 'donor' crabs. Because of this, CA activity and mRNA expression were measured after a 4-day time course in order to determine if the degree of CA induction was large enough, and the degree of subsequent inhibition of CA induction was great enough to be accurately measured by the electrometric pH assay to be used in these experiments. A 4-day exposure to 10 p.p.t. resulted in a 4.5-fold increase in CA activity in posterior gills (P<0.001, Mann–Whitney rank sum test; Fig. 6A) but caused no change in activity in anterior gills (P=0.86, Mann–Whitney). The same pattern was seen for CA mRNA expression. There was no difference between anterior and posterior gills in crabs acclimated to 32 p.p.t. (P=0.77, Mann–Whitney, Fig. 6B), and there was also no difference between anterior gills after 4 days exposure to 10 p.p.t. (P=0.68, Mann–Whitney). There was, however, a large (15-fold) increase in CA mRNA expression in posterior gills in response to a 4-day transfer to 10 p.p.t. (P=0.016, Mann–Whitney, Fig. 6B). The increase in CA activity was, as expected, smaller than that seen after 7 days (Fig. 3A *vs* Fig. 6A), but interestingly, the increase in CA mRNA expression was more than twice as large at 4 *vs* 7 days post-transfer (Fig. 3B *vs* Fig. 6B).

Eyestalk ablation also caused a significant increase in CA activity in posterior gills over that seen in intact crabs, after a 4-day transfer to 10 p.p.t. For intact crabs, there was a fivefold increase in CA activity in G8 at 4 days post-transfer, and ESA potentiated this increase by 32% (P=0.02, Kruskall–Wallace ANOVA in ranks and Dunn's *post hoc* comparison, Fig. 7).

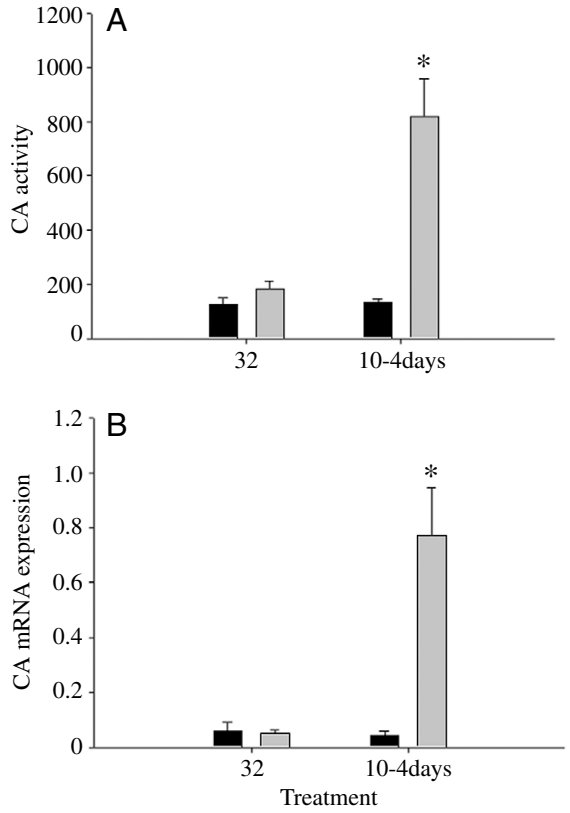

Fig. 6. Carbonic anhydrase activity (A; μmol CO_2 mg protein^{-1} min^{-1}) and mRNA expression (B; relative amounts) in anterior (G4, black bars) and posterior (G8, gray bars) gills of green crabs acclimated to 32 p.p.t. and transferred to 10 p.p.t. for 4 days. Values are means ± s.e.m. (N=6). T=12°C. Asterisks indicate statistical differences in G8 at P<0.05.

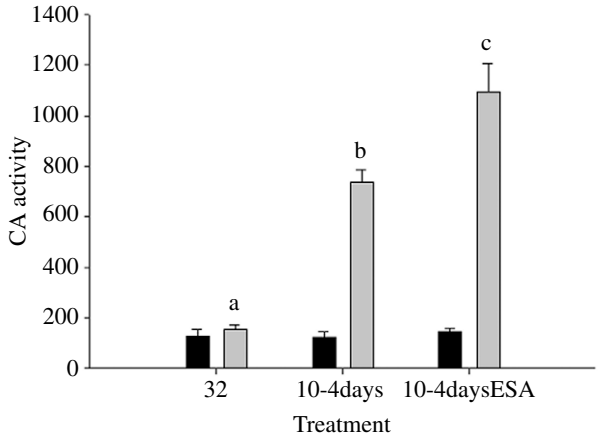

Fig. 7. Carbonic anhydrase activity (μmol CO_2 mg protein^{-1} min^{-1}) in anterior (G4, black bars) and posterior (G8, gray bars) of green crabs acclimated to 32 p.p.t. and transferred to 10 p.p.t. for 4 days. 32, 32 p.p.t. acclimated controls; 10-4days, intact crabs assayed after 4 days at 10 p.p.t.; 10-4daysESA, eyestalk ablated crabs assayed after 4 days at 10 p.p.t.. Values are means ± s.e.m. (N=6). T=12°C. Different letters indicate statistical differences in G8 at P<0.05 across treatments.

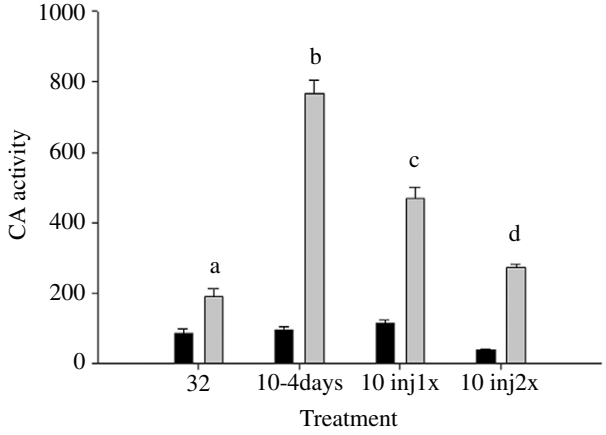

Fig. 8. Carbonic anhydrase activity (μmol CO_2 mg protein^{-1} min^{-1}) in anterior (G4, black bars) and posterior (G8, gray bars) of green crabs acclimated to 32 p.p.t. and transferred to 10 p.p.t. for 4 days. 32, 32 p.p.t. acclimated controls; 10-4days, intact crabs assayed after 4 days at 10 p.p.t.; 10 inj1x, intact crabs given a once daily injection of eyestalk extract from 'donor' crabs acclimated to 32 p.p.t.; 10 inj2x, intact crabs given twice-daily injections of eyestalk extract from 'donor' crabs acclimated to 32 p.p.t. Values are means ± s.e.m. (N=6-7). T=12°C. Asterisk indicates statistical difference at the 0.05 level of significance in G4 from 32 p.p.t. control. Different letters indicate statistical differences at P<0.05 in G8 across treatments.

There were no significant differences in CA activity among anterior gills across treatments (P=0.73, ANOVA).

A 4-day transfer experiment was also sufficient to show significant inhibitory effects of daily injections of eyestalk extracts into intact crabs. In posterior gills, there was a fourfold increase in CA activity after 4 days in 10 p.p.t. (P<0.001, ANOVA and Tukey's *post hoc* comparison, Fig. 8). This level of CA induction was reduced 40% (P<0.001, Tukey's *post hoc* comparison) by a single daily injection of eyestalk extract taken from crabs acclimated to 32 p.p.t. Eyestalk extract appeared to inhibit CA induction in a dose-dependent manner, as two injections of extract, given daily, reduced normal CA induction by 66% (Fig. 8). Even so, CA activity after twice daily injections remained slightly but significantly higher than that in 32 p.p.t. acclimated control crabs (P<0.05, Tukey, Fig. 8). In this experiment, there were also statistically significant differences among anterior gills (P<0.01, ANOVA), but this was due entirely to a significant reduction in CA activity in G4 in the group that received two daily injections of eyestalk extract (P<0.01, Tukey's *post hoc* comparison).

For both the 4-day and 7-day transfers to low salinity, the relative abundance of AK mRNA did not change (Fig. 9). There was no difference in AK mRNA levels between anterior and posterior gills in crabs acclimated to 32 p.p.t. (P=0.87, t-test), and there were no differences either among anterior (P=0.66, ANOVA) or posterior (P=0.72, ANOVA) gills at either 4 or 7 days post-transfer to 15 p.p.t. salinity.

Hemolymph osmolality values for green crabs in the 4-day

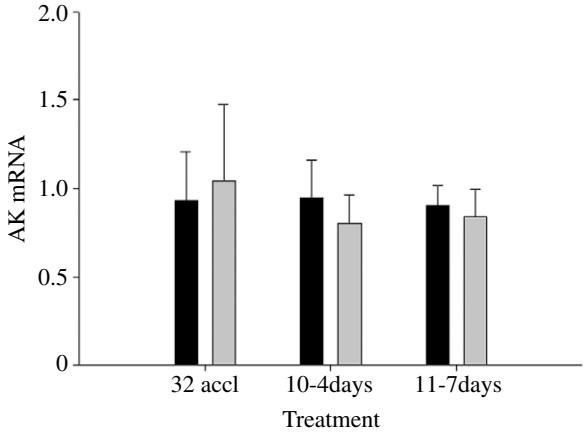

Fig. 9. Arginine kinase mRNA expression (relative amounts) in anterior (G4, black bars) and posterior (G8, gray bars) gills of green crabs acclimated to 32 p.p.t. and transferred to 10 p.p.t. salinity for either 4 (10-4days) or 7 days (11-7days). Values are means ± s.e.m. (*N*=5–6). *T*=12°C.

transfer experiments showed a similar pattern to values from the 7 day transfers: osmolality was slightly higher than ambient at 32 p.p.t. and significantly higher after 4 days exposure to 10 p.p.t. ($P<0.001$, ANOVA; Table 3). The hemolymph–medium difference was also approximately 300 mOsm; and there were no significant differences among intact crabs, eyestalk ablated crabs, or intact crabs given a single daily injection of eyestalk extract ($P>0.05$, Dunn's *post hoc* comparison; Table 3). However, for crabs given two injections of eyestalk extract per day, hemolymph osmolality was 65 mOsm kg H_2O^{-1} lower than for intact crabs ($P<0.05$, Dunn's *post hoc* comparison; Table 3).

Discussion

The results presented here on quantitative changes in CA mRNA expression confirm and expand an earlier study (based on semi-quantitative PCR) reporting that CA induction, in response to low salinity exposure, is under transcriptional regulation (Henry et al., 2003). Semi-quantitative PCR was able to detect an estimated 2.5-fold increase in CA mRNA in posterior gills of green crabs transferred from 32 to 10 p.p.t. salinity for 4 days, which was much less than the corresponding fourfold increase in CA activity over the same time period, and less still than the eight- to tenfold increases in CA activity that occur after 7 days, when acclimation is complete (Henry et al., 2002) (Fig. 3A). Results presented here from real time PCR showed a 15-fold increase in CA mRNA expression after 4 days in low salinity, compared to a 4.5-fold increase in CA activity. This level of mRNA induction is, however, very close to that for acclimated levels of CA activity. These results show that the increase in CA gene activation is much larger than originally believed and is also very similar in magnitude to the induction of CA activity.

In a more detailed study (Henry et al., 2006), increases in both CA activity and CA protein concentrations were shown to result from increases in relative CA mRNA abundance, strongly suggesting that induction of CA activity is a direct result of increased CA gene expression and protein synthesis. As such, measurements of CA activity represent a good reporter system for changes in CA gene expression. The results presented here confirm this relationship and further validate the use of monitoring CA activity as a reporter assay for CA expression.

The patterns of induction of CA mRNA *vs* activity appear to be different. CA mRNA increases within 24 h after low salinity transfer and remains unchanged through 4 days, while CA activity begins to increase at 48–72 h post-transfer and continues to increase through 7 days of low salinity acclimation (Henry et al., 2003). CA activity remains elevated even after 14 days of low salinity exposure (Henry et al., 2002). Quantitative changes in CA mRNA expression were followed through 7 days of low salinity exposure in this study, and a significant decrease was found relative to the value at 4 days (Fig. 3B *vs* Fig. 6B). These differences suggest that the process of CA induction involves a rapid but perhaps transient increase in CA mRNA, followed by a slower but more long lasting increase in the synthesis of new CA protein. This potential relationship deserves a more detailed, systematic investigation.

This study also extends transcriptional regulation of CA activity to other treatments besides salinity. Specifically, the increase in CA activity as a result of eyestalk ablation also appears to be rooted in changes in CA mRNA expression. Furthermore, the increase in CA gene expression persists for at least 7 days after ESA. CA has a very high turnover rate (10^6 molecules s^{-1}) (Maren, 1967), and the enzyme is expressed in large excess of the physiological processes it supports. Consequently, short-term regulation of CA activity is rarely found in animal systems, primarily because it is not believed to be necessary. It will be interesting to see if regulation of CA activity through changes in gene expression represents a more widespread and common mechanism,

Table 3. *Hemolymph osmotic concentration in green crabs acclimated to 32 p.p.t. salinity and transferred to 10 p.p.t. for 4 days*

Hemolymph osmotic concentration (mOsm kg H_2O^{-1})				
32 accl	32-10 intact	32-10 ESA	32-10 inj-1x	32-10 inj-2x
931±11 (8)	649±15 (9)	665±21 (7)	623±17 (8)	584±33 (6)

Green crabs were acclimated to 32 p.p.t. (920±9 mOsm kg H_2O^{-1}, *N*=3) and transferred to 10 p.p.t. (302±6 mOsm kg H_2O^{-1}, *N*=6) for 4 days. 32 acc, 32 p.p.t. acclimated controls; 32-10 intact, crabs transferred to 10 p.p.t. with no other treatment; 32-10 ESA, eyestalk ablated crabs transferred to 10 p.p.t.; 32-10 inj-1x, intact crabs transferred to 10 p.p.t. and given one injection per day of eyestalk extract; 32-10 inj-2x, intact crabs transferred to 10 p.p.t. and given two injections per day of eyestalk extract.

Values are mean ± s.e.m. (*N*). *T*=12°C.

especially in tissues and cell types in which large changes in activity occur.

It should be noted again that a typical control ('housekeeping') gene, such as actin, was not used for comparison to CA in this study. However, the expression of a common metabolic gene, arginine kinase, did not change in either anterior or posterior gills in response to low salinity exposure. Furthermore, there were also no changes in CA mRNA expression in the control tissue, the anterior, respiratory gill (G4). This correlates with the lack of induction of CA activity in this tissue. The posterior, ion-transporting gills in euryhaline crustaceans undergo ultrastructural, biochemical, and molecular changes during the process of low salinity adaptation, and so it is not surprising that the expression of even some of the most commonly used housekeeping genes (e.g. actin) also change (e.g. Lovett et al., 2003). Activity of arginine kinase, which was also used as a control in a previous study of CA expression using semi-quantitative PCR (Henry et al., 2003), does not change in *C. maenas* but doubles in response to low salinity in another euryhaline crab, *Callinectes sapidus*, and also changes in *Chasmagnathus granulatus* (Kotylar et al., 2000; Luquet et al., 2005). Furthermore, AK mRNA expression increases approximately fivefold in posterior gills of *C. sapidus* in response to low salinity exposure (L. Serrano and R.P.H., unpublished data). For salinity studies in crustaceans it may be more accurate to look at changes in expression of the same gene in two different tissues, especially if the two tissues are as closely related physically but as different physiologically as anterior *vs* posterior gills. This approach has also been used successfully in two other species of euryhaline crabs. In *Pachygrapsus marmoratus*, the expression of the $Na^+/K^+/2Cl^-$ cotransporter was shown to increase in response to low salinity in the posterior but not the anterior gills (Spanings-Pierrot and Towle, 2004), and in *Chasmagnathus granulatus*, both the co-transporter and the Na^+/K^+-ATPase were shown to increase in G7 but not G3 in crabs transferred to 10 p.p.t. salinity (Luquet et al., 2003). More recently, Luquet et al. reported (Luquet et al., 2005) that the expression of the Na^+/K^+-ATPase, arginine kinase, the $Na^+/K^+/2Cl^-$ co-transporter and the V-Type ATPase all increased in anterior and posterior gills during the time course of low salinity adaptation in *C. granulatus*. The increases in anterior gills, however, were much less than in posterior gills, and they were not consistent over every time period examined, in contrast to the increases in posterior gills. Nevertheless, all studies on transport protein expression have so far been descriptive; no attempt has been made to identify a mechanism by which expression is regulated.

The results of the eyestalk ablation, injection, and salinity transfer experiments represent the first evidence for the potential mechanism of control of CA expression. For green crabs acclimated to 32 p.p.t., ESA resulted in an approximate doubling of CA activity over a 7-day period, and this increase was due to an increase in CA mRNA expression. The increase reported here is approximately twice as large as that reported earlier from a preliminary study (Henry et al., 2003). The occurrence of CA induction in response to ESA in the absence of a low salinity stimulus suggests that expression is under inhibitory regulation by a compound located in the eyestalk. This compound would appear to function as a repressor, maintaining CA expression (and thus CA activity) at baseline levels in crabs acclimated to high salinity. When the eyestalks are removed, the putative repressor (or its effects) is also removed, allowing CA expression to increase.

Eyestalk ablation is a coarse technique; it removes the entire sinus gland/X-organ endocrine complex as well as neural input from the optical plexus. Therefore, this procedure could potentially have more widespread physiological effects on the crab than just altering CA expression. However, it is doubtful that the resultant CA induction at 32 p.p.t. is a by-product of some other, unrelated, endocrine change in the crab. The most likely artefact, molting, can be ruled out. ESA is known to induce molting in crabs (Mykles, 2001), and branchial CA activity has been shown to increase during pre-molt in a closely related species, *Callinectes sapidus* (Henry and Kormanik, 1985), but these two processes take place on different time scales. ESA-induced molting takes on the order of weeks (Mykles, 2001), but ESA-induced changes in CA expression in green crabs take place within 4–7 days. Furthermore, molting results in an increase in CA activity in both anterior and posterior gills in both *C. sapidus* (Henry and Kormanik, 1985) and *C. maenas* (R.P.H., unpublished data). Typical values for CA activity in anterior gills of intermolt green crabs are in the range of 80–150 μmol CO_2 mg $protein^{-1}$ min^{-1}, and these values more than double in pre-molt crabs. In our experiments, CA activity in anterior gills is used as a diagnostic indicator of the early stages of pre-molt; any crabs that have CA activity greater than 250 μmol CO_2 mg $protein^{-1}$ min^{-1} are considered to be in pre-molt and are excluded from the data set.

One more experimental piece of evidence also suggests that CA induction, as a result of ESA, is the result of an endocrine or neuroendocrine compound found in the eyestalk. Injection of eyestalk extract completely abolished the ESA-induced CA induction in high salinity acclimated crabs. Organ ablation, to induce a treatment effect, coupled with injection of organ extract to abolish that effect, is the classical endocrinological approach to demonstrate the presence of a hormone, and the evidence presented here points to the presence of a CA repressor in the eyestalks of the green crab. Eyestalk ablation in the absence of a low salinity stimulus also results in CA induction in blue crabs (Henry and Borst, 2006), suggesting that inhibitory regulation of CA expression could be a common mechanism in crustaceans. It should be noted that while changes in CA activity are correlated with changes in CA mRNA abundance, it cannot be stated with absolute certainty whether the putative CA repressor acts at the level of transcription or translation. That question is currently under investigation.

In addition to abolishing the ESA-stimulated CA induction at 32 p.p.t., injection of eyestalk extract into intact or eyestalk ablated green crabs inhibits the normal low salinity mediated

CA induction, either over a 4- or 7-day time course. This further strengthens the hypothesis that CA induction is under inhibitory control by a repressor substance found in the eyestalk. From the current evidence, it would appear that CA expression in high salinity acclimated crabs is kept at low, baseline levels by the presence and action of a CA repressor, found in the eyestalk. At the critical low salinity at which the crab makes the transition from osmoconformity to osmoregulation (27 p.p.t.) (Henry, 2005), the repressor, or its effect, is removed, allowing CA induction to occur. Adding this substance back, through the injections of eyestalk extract, prevents that induction.

The majority of studies on the regulation of transport proteins have up until now focused on compounds or regulatory agents that stimulate ion uptake or specific proteins such as the Na^+/K^+-ATPase. Furthermore, these regulatory agents appear to function in the rapid, short-term modulation of activity of existing enzyme, and not in long-term changes in expression (Savage and Robinson, 1983; Sommer and Mantel, 1988; Morohashi et al., 1991; Eckhardt et al., 1995; Lucu and Flik, 1999; Spanings-Pierrot et al., 2000; Serrano et al., 2003). More recent studies have shown, however, that transport protein expression does change in response to low salinity exposure (e.g. Towle et al., 2001; Luquet et al., 2005), but the mechanism by which changes in expression are regulated has not been examined. This is the first report of the upregulation of a transport protein being controlled by inhibition (repression) of expression at high salinity and the removal of that repression, rather than activation of expression, at low salinity. The natural state of CA at high salinity appears to be under repressor control. Rather than being activated, the inhibition of CA expression is released in response to low salinity, allowing CA induction to proceed.

That inhibitory regulation of CA expression should be found is not that surprising. This is a similar mechanism to those found for endocrine control of other physiological processes in crustaceans. Molting, vitellogenesis, and the function of the mandibular gland are all known to be under inhibitory regulation by neuropeptides found in the sinus gland of the eyestalk. These hormones, molt inhibiting hormone (MIH), vitellogenesis inhibiting hormone (VIH) and mandibular organ inhibiting hormone (MOIH), are all inhibitory neuropeptides belonging to the crustacean hyperglycemic hormone (CHH) family (Chang, 2001). From a purely functional standpoint, it is therefore plausible that the putative CA repressor could also be a related inhibitory neuropeptide. This possibility is currently being investigated.

Finally, these results address the fundamental question of the necessity of CA induction during low salinity adaptation. Branchial CA activity, even in crabs acclimated to high salinity, is in excess of branchial ion transport rates in crabs transferred to low salinity (Henry, 2001), yet CA activity is induced to a higher degree than that of other transport proteins. Based solely on turnover rate and transport kinetics, CA induction is theoretically not needed to maintain the supply of counterions for Na^+ and Cl^- uptake. Yet, experimental evidence indicates CA induction is, in fact, necessary. When CA induction was inhibited up to 50% by injection of eyestalk extract, hemolymph osmolality was unaffected. However, when crabs were injected with twice the normal dosage of eyestalk extract, CA induction was reduced by 66% and hemolymph osmolality was significantly depressed as well. This strongly suggests that below a certain level of induction, CA activity cannot support the levels of ion uptake needed to maintain hemolymph osmolality at normal levels above ambient. One potential answer, which is speculative at this point, may be that the ion uptake process, which takes place at the apical branchial membrane, is limited by the intracellular diffusion of H^+ and HCO_3^-. High levels of CA may be necessary to keep the supply of counterions from being limiting in the intracellular boundary layer of the gill. Boundary layers are known to function as separate fluid compartments, especially with regard to diffusive transport (e.g. Gutknecht et al., 1977), and membrane CA is believed to facilitate both CO_2 and NH_3 transport (Henry, 1996). This has not been proven yet, but circumstantial evidence tends to support this idea. Cytoplasmic CA in fish gills appears to be concentrated close to the apical membrane (Conley and Mallatt, 1988), and CA is directly coupled to the band-3 anion exchange protein in the membrane boundary layer of red blood cells to facilitate Cl^-/HCO_3^- exchange (Sterling et al., 2001). It is not implausible to suggest that the high levels of branchial cytoplasmic CA are necessary to supply counterions to transporters located within the gill boundary layer.

In summary, CA induction is a function of changes in CA mRNA expression, and the process appears to be under regulatory control by the major endocrine complex of the crab, the eyestalk. The mechanism of regulation of CA expression is under negative control by a CA repressor, possibly an inhibitory neuropeptide, located in the eyestalk.

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