

# Quantitative Changes in Branchial Carbonic Anhydrase Activity and Expression in the Euryhaline Green Crab, *Carcinus maenas*, in Response to Low Salinity Exposure

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**ABSTRACT** Hemolymph osmolality, and changes in gill carbonic anhydrase (CA) activity, relative mRNA expression, and CA protein concentration were measured in the green crab *Carcinus maenas* acclimated to 32 ppt salinity and transferred to 10 ppt. Hemolymph osmolality stabilized at new, acclimated values, by 24 hr after transfer. There was a large increase in CA mRNA concentrations, as measured by quantitative PCR, in the posterior gills by 24 hr post-transfer that remained elevated through 4 days. By 7 days, however, CA mRNA levels began to decline. CA activity, on the other hand, did not begin to increase until 48 hr after transfer to 10 ppt, but it continued to increase through 7 days. CA protein concentration increased by 5-fold in posterior gills in crabs acclimated to 10 ppt. CA activity, mRNA expression, and CA protein concentrations did not change in anterior gills. These results indicate that low salinity-stimulated CA induction is under transcriptional regulation, and that the increase in CA activity is a result of the increase in gene expression and synthesis of new enzyme. Changes in mRNA appear to be transient, but once synthesized, the CA protein appears to persist in the gill for an extended time. In a separate set of experiments, green crabs acclimated to 32 ppt were transferred directly to salinities of 25, 20, 15, and 10 ppt. CA activity and mRNA concentrations increased with decreasing salinity, peaking at 15 ppt but decreasing between 15 and 10 ppt. The decrease may represent a breakdown in the mechanism of transport-related protein induction near the lower salinity limit of this species. *J. Exp. Zool.* 305A:842–850, 2006. © 2006 Wiley-Liss, Inc.

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The enzyme carbonic anhydrase (CA) has long been known to be an important molecular component of the physiological mechanism of low salinity adaptation that allows euryhaline crustaceans to invade stressful habitats such as the estuary (Henry, '84, '88a). The enzyme's distribution, salinity sensitivity, and catalytic function all strongly support a central role for CA in the mechanism of survival of crabs in waters of low and fluctuating salinity (Henry, 2001; Henry et al., 2003).

Euryhalinity in crustaceans is conferred by the ability to maintain the hemolymph osmotic and ionic concentrations above those in the ambient medium as seawater concentrations decrease (reviewed by Mantel and Farmer, '83); and this,

in turn, is a result of the active uptake of the major ions, Na<sup>+</sup> and Cl<sup>-</sup>, from dilute seawater (reviewed by Pequeux, '95). CA is found in the organ responsible for ion uptake, the gill, in higher levels of activity than in other tissues (Henry and Cameron, '82a). Furthermore, branchial CA activity is labile, being induced 6–10-fold in the

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posterior, ion-transporting gills of crabs when they are exposed to low salinity (Henry and Cameron, '82a,b; Pillar et al., '95; Henry et al., 2002), and the initial induction occurs at the critical salinity at which the crabs make the transition from conformity to regulation (26–27 ppt) (Henry, 2005).

The initial step in the mechanism of active  $\text{Na}^+$  and  $\text{Cl}^-$  uptake is believed to involve  $\text{Na}^+/\text{H}^+$  and  $\text{Cl}^-/\text{HCO}_3^-$  exchange at the apical surface of the gill (e.g., Kirschner, '79), and physiological studies on CA function, using the inhibitor acetazolamide (Az), have provided evidence of the enzyme's function in support of both transport processes (Ehrenfeld, '74; Cameron, '79; Henry and Cameron, '83; Henry et al., 2003). Branchial CA is believed to rapidly supply the counterions used in the uptake mechanism through the catalyzed hydration of  $\text{CO}_2$  to  $\text{H}^+$  and  $\text{HCO}_3^-$  (Henry and Cameron, '83; Henry, '88a). It is the cytoplasmic pool of branchial CA that is believed to function in ion uptake, and it is also this pool of CA activity that is highly sensitive to changes in environmental salinity (Henry, '88a,b; Henry et al., 2003).

The process of salinity-mediated CA induction has been described in detail for two species, *Callinectes sapidus* and *Carcinus maenas*, but a number of questions still remain, especially with regard to the regulation and control of the process. Original studies reported that CA induction in *Callinectes sapidus* took place on the order of days after transfer to low salinity, suggesting that synthesis of new enzyme was involved (Henry and Cameron, '82b). More recent studies have shown that the initial increase in CA activity takes place by 24 hr after low salinity exposure, but this is still consistent with the time course of de novo enzyme synthesis (Henry and Watts, 2001). In *C. maenas* the initial increase in CA activity occurs between 48 and 72 hr post-transfer, again, consistent with the time needed for the up-regulation of enzyme synthesis (Henry et al., 2002, 2003). Recently the first evidence was presented suggesting that CA induction was under transcriptional regulation. CA mRNA in *C. maenas*, as measured by semi-quantitative PCR, increased at 24 hr after low salinity exposure, while the initial increase in protein-specific CA activity occurred at 48 hr post-transfer (Henry et al., 2003).

There is a growing body of evidence indicating that regulation of transport protein expression, in response to salinity and other environmental signals, is a widespread mechanism in both invertebrates and lower vertebrates. Immunocalization studies have shown that the expression

of the CFTR anion channel in chloride cells of the goby increases in response to high salinity, the  $\text{V-H}^+$ -ATPase in the gills of the stingray is altered by salinity, and branchial  $\text{Na}^+/\text{K}^+$  ATPase expression is altered in the carp in response to temperature (Piermarini and Evans, 2001; McCormick et al., 2003; Metz et al., 2003). Furthermore, expression appears to be under transcriptional regulation, as measured by semi-quantitative and quantitative changes in mRNA. Changes in mRNA levels for the  $\text{Na}^+$ ,  $\text{K}^+$ ,  $2\text{Cl}^-$  co-transporter in the gills of striped bass precede changes in protein expression, and the same pattern is true for the  $\text{Na}^+/\text{K}^+$ -ATPase in the gills of tilapia (Lin et al., 2004; Tipsmark et al., 2004). Semi-quantitative PCR has shown that a number of transport proteins are expressed in high levels in the posterior, ion-transporting gills of crustaceans (Towle et al., '97; Weihrauch et al., 2004); and preliminary and full reports, based on real-time, quantitative PCR studies, have shown that changes in salinity alter mRNA levels of the  $\text{Na}^+/\text{K}^+$ -ATPase, the  $\text{Na}^+$ ,  $\text{K}^+$ ,  $2\text{Cl}^-$  transporter, and the V-type  $\text{H}^+$ -ATPase in the gills of a number of euryhaline crustaceans (Lovett et al., 2003; Luquet et al., 2003, 2005; Spanings-Pierrot and Towle, 2003, 2004).

CA is an excellent transport-related protein in which to further study this regulatory mechanism. While CA is not directly responsible for trans-epithelial transport of any specific ion, its function is critical in the support of virtually all general cation and anion transporters; thus, it is a central molecular component of the mechanism of low salinity adaptation. Furthermore, this is a tractable and versatile enzyme with which to work, as protein-specific activity, protein concentration, and quantitative mRNA expression can be measured simultaneously in the gills of the same individual. This report represents a study of the time course of CA induction in response to low salinity exposure, and the response of CA expression to step-wise reductions in salinity in the green crab *C. maenas*.

## MATERIALS AND METHODS

### *Collection and maintenance of animals*

Adult, intermolt green crabs, *C. maenas*, were collected by hand from the intertidal zone of Frenchman's Bay, along the property of the Mount Desert Island Biological Laboratory, Salisbury Cove, ME. Crabs were also obtained from the animal supply department of the Marine Biologi-

cal Laboratory, Woods Hole, MA, and from Gulf of Maine Specimen Company, Pembroke, ME. Green crabs were held at MDIBL in 100 gal fiberglass tanks equipped with filtered, running seawater (32–33 ppt salinity and 10–12°C). Crabs were fed a combination of mussels and squid every other day but were starved for a minimum of 48 hr 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).

### **Experimental protocol**

Green crabs (green color morphs, 45–90 gm) were held at 32 ppt salinity for 1 week prior to experimentation. For the experiments involving the time course of low salinity acclimation, crabs were transferred directly to 40 gal recirculating tanks (6–10 crabs per tank) of 10 ppt water (seawater diluted with water made via reverse osmosis), thermostatted to 12°C, and equipped with biological filters. This salinity change has been shown to cause an approximate 10-fold induction in CA activity with new acclimated levels being reached by 7 days after transfer (Henry et al., 2002). In order to follow the time course of CA induction, a subset of crabs was sampled every 24 hr for a period of 7 days after transfer.

A second experiment measured the response of the CA induction mechanism in the gills of *C. maenas* to step-wise reductions in salinity. Crabs acclimated to 32 ppt were directly transferred to 10 gal aquaria (3–4 crabs per aquarium), also equipped with undergravel biological filters, of 25, 20, 15, or 10 ppt salinity. They were allowed to acclimate to the test salinity for 7 days.

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 ga 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) 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 from the left branchial chamber of the crab were dissected out and placed in 5 volumes of cold (4°C) homogenization/assay buffer (225 mM mannitol, 75 mM sucrose, 10 mM Trizma base, ad-

justed to pH = 7.40 with 10% phosphoric acid). These gills were homogenized, centrifuged, and used immediately in the CA assay (see below). The anterior and posterior gills from the right branchial chamber of the crab were dissected out and used for total RNA extraction, reverse transcription, and quantitative PCR (see below). Crabs were killed via exsanguination.

### **Analytical procedures**

CA activity was measured electrometrically by the delta pH method (Henry, '91). Anterior (G4) and posterior (G8) gills were homogenized in 5 volumes of cold buffer using an Omni 1,000 or TH115 hand-held homogenizer and then sonicated at 25 W for 30 sec (Heat Systems Microsonicator, Farmingdale, NY, USA). Homogenates were centrifuged at 10,000g for 20 min at 4°C (Sorvall RC5-B), 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 vigorously. The reaction was started by the addition of CO<sub>2</sub>-saturated water, and the drop in pH (about 0.25 units) was monitored by micro-pH and reference electrodes (World Precision Instruments, Sarasota, FL) 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), and CA activity was reported as µmol CO<sub>2</sub> mg protein<sup>-1</sup> min<sup>-1</sup>.

Total RNA from anterior and posterior gills was isolated under RNase-free conditions by phenol–chloroform extraction (RNAagents, Promega, Madison, WI). All dissecting equipment and homogenizers were cleaned with RNase-zap (Ambion, Austin, TX) and rinsed in RNase-free water. RNA concentrations, purity, and DNA contamination were monitored using a Bioanalyzer 2100 lab chip system (Agilent, Wilmington, DE). Single stranded, complementary DNA was then produced from Poly A mRNA in 2 µg of total RNA by reverse transcription using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and oligo dT as primer. As such, the samples used for quantitative PCR (below) were normalized to total RNA levels in each preparation (Bustin, 2002).

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 (Thomason et al., 2004). These primers are as follows:

168F: 5'/CGC TCA GTT CCA CTT CCA3'  
398R: 5'/ACA TCT CAG CAT CCG TCA3'

These primers, when used in conventional PCR, produced a single band of approximately 230 base-pairs, visualized on a 0.8% agarose gel using ethidium bromide (data not shown). The primers were then used to amplify the CA cDNA template for quantitative PCR (qPCR) on a Stratagene MX 4000 real-time PCR instrument. The sample with the highest measured protein-specific CA activity was used to construct a standard curve consisting of amplification products generated from step-wise 10-fold dilutions spanning four orders of magnitude ( $10^0$ – $10^{-3}$ ). Concentrations of amplified CA cDNA were calculated using the standard curve and were reported on the basis of relative amounts. In this study, instead of using a so-called housekeeping gene, such as actin, as a control, a control tissue was used. During the process of low salinity adaptation, the posterior gills in crustaceans undergo extensive ultrastructural and biochemical remodeling (e.g., Taylor and Taylor, '92), and structural genes such as actin, which are typically expressed in a constitutive manner in other tissues, undergo changes in expression. Instead, anterior gills were used as a "housekeeping tissue", as CA activity has been shown to be insensitive to changes in salinity in this tissue (Henry, '88a,b).

A separate group of crabs were acclimated to 32 and 10 ppt, and anterior (gills 1–6) and posterior (gills 7–9) were dissected out, and each set of gills was pooled to give 2 g of tissue. Gills were homogenized in 4 volumes of Tris-phosphate buffer (see above) and subjected to differential centrifugation modified from Henry ('88b). The crude homogenate was centrifuged at 7,500g for 20 min at 4°C (RC-5B, Sorvall, Wilmington, DE) to separate intact cells, large cell fragments, nuclei, and mitochondria. The resulting supernatant was then centrifuged at 100,000g for 90 min at 4°C (Beckman L8-70M ultracentrifuge) to separate the microsomal fraction from the cytoplasm. The cytoplasmic fraction was assayed for CA activity while being titrated with increasing volumes of a 5 µM stock solution of the CA inhibitor acetazolamide (Az). The data were transformed and graphed as a double reciprocal plot (Easson and Stedman, '37), according to the following relationship:

$$I_0/i = K_i/(1 - i) + E_0$$

where  $E_0$  (the  $y$  intercept of the plot) is the total concentration of free enzyme,  $K_i$  is the inhibition constant, and  $i$  is the fractional inhibition of enzyme activity at an inhibitor concentration of  $I_0$ . This allowed for a measurement of changes in CA protein concentration ( $E_0$ ). CA concentrations from the inhibitor plots were adjusted for differences in sample volumes used in the assay (i.e., 50 µL for posterior gills in 10 ppt-acclimated crabs vs. 100 µL for posterior gills in 32 ppt-acclimated crabs and 100 µL for anterior gills at both salinities).

Hemolymph samples were thawed on ice, sonicated, and centrifuged at 14,000g for 1 min to separate out clot material. Osmolality was then measured on 10 µL samples using a vapor pressure osmometer (Wescor 5100C).

## RESULTS

At high salinity, green crabs are typical osmoconformers; hemolymph osmolality of crabs acclimated to 32 ppt ( $920 \pm 4$  mOsm kg H<sub>2</sub>O<sup>-1</sup>) was not significantly different from that of the ambient seawater ( $P = 0.30$ ,  $t$ -test; Fig. 1). After transfer to 10 ppt ( $312 \pm 2$  mOsm kg H<sub>2</sub>O<sup>-1</sup>), there was a significant decrease in hemolymph osmolality that persisted through the 7-day time course ( $P < 0.01$ , ANOVA). New hemolymph osmolality values stabilized by 24 hr post-transfer and did not change significantly thereafter ( $P > 0.05$  for all pair-wise comparisons, Tukey's post-hoc test). These values were approximately 300 mOsm higher than that for 10 ppt seawater, confirming the

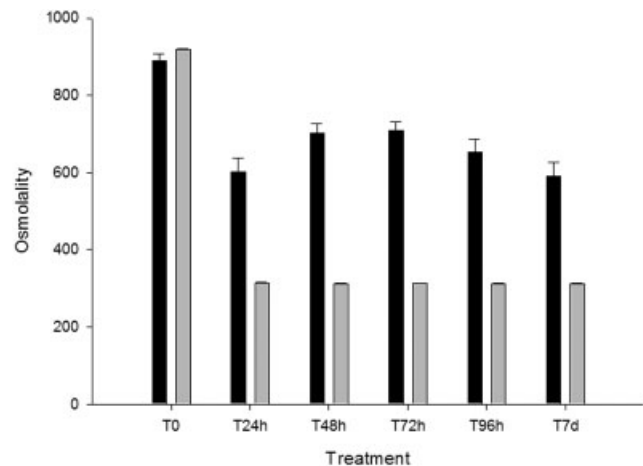


Fig. 1. Hemolymph (black bars) and seawater (gray bars) osmolality (mOsm kg H<sub>2</sub>O<sup>-1</sup>) for green crabs acclimated to 32 ppt (T0) and transferred to 10 ppt for 7 days. Mean ± SEM ( $N = 6$ –10).  $T = 12^\circ\text{C}$ .

ability of *C. maenas* as a moderately strong osmoregulator in low salinity (Zanders, '80; Henry et al., 2003).

Branchial CA activity was uniformly low in crabs acclimated to 32 ppt: CA activity in anterior vs. posterior gills was not significantly different ( $P = 0.187$ , *t*-test; Fig. 2A). CA activity in anterior gills did not change significantly over the time course of low salinity acclimation ( $P = 0.95$ , ANOVA). There was, however, a significant increase in CA activity in the posterior gills (G8) in response to low salinity exposure ( $P < 0.001$ , Kruskal-Wallis ANOVA in ranks). The initial significant increase (from  $185 \pm 32$  to  $319 \pm 34 \mu\text{mol CO}_2 \text{mg protein}^{-1} \text{min}^{-1}$ ;  $P < 0.02$ , Dunn's post-hoc com-

parison) occurred at 48 hr post-transfer (Fig. 2A). CA activity continued to increase significantly ( $P < 0.03$ , Dunn's) at each sample time throughout the time course of low salinity exposure, reaching a new acclimated value of  $1,568 \pm 205 \mu\text{mol CO}_2 \text{mg protein}^{-1} \text{min}^{-1}$  by 7 days post-transfer, an approximate 8-fold increase over values in crabs acclimated to 32 ppt.

CA mRNA expression was not significantly different in anterior vs. posterior gills in crabs acclimated to 32 ppt ( $P = 0.773$ , *t*-test). Expression of CA mRNA in posterior gills was sensitive to low salinity exposure ( $P = 0.005$ , Kruskal-Wallis; Fig. 2B), but the pattern of increase over the experimental time course was different than that seen for CA activity. The initial increase was larger and occurred earlier. There was an 8-fold induction of mRNA levels in posterior gills at 24 hr post-transfer. Furthermore, these elevated mRNA levels remained constant through 96 hr ( $P > 0.05$ , Dunn's). However, CA mRNA concentrations decreased significantly by about 60% ( $P < 0.05$ ) between 96 hr and 7 days post-transfer (Fig. 2B), a time when CA activity was still increasing. There were no changes in mRNA expression in anterior gills in response to low salinity exposure ( $P = 0.802$ , Kruskal-Wallis).

For crabs acclimated to varying degrees of low salinity in a step-wise manner, osmotic regulation occurred at salinities of 25 ppt and below (Fig. 3). Hemolymph osmolality was significantly different across the five experimental salinity treatments ( $P < 0.001$ , ANOVA), with the initial significant decrease occurring at 20 ppt ( $P = 0.021$ , Tukey).

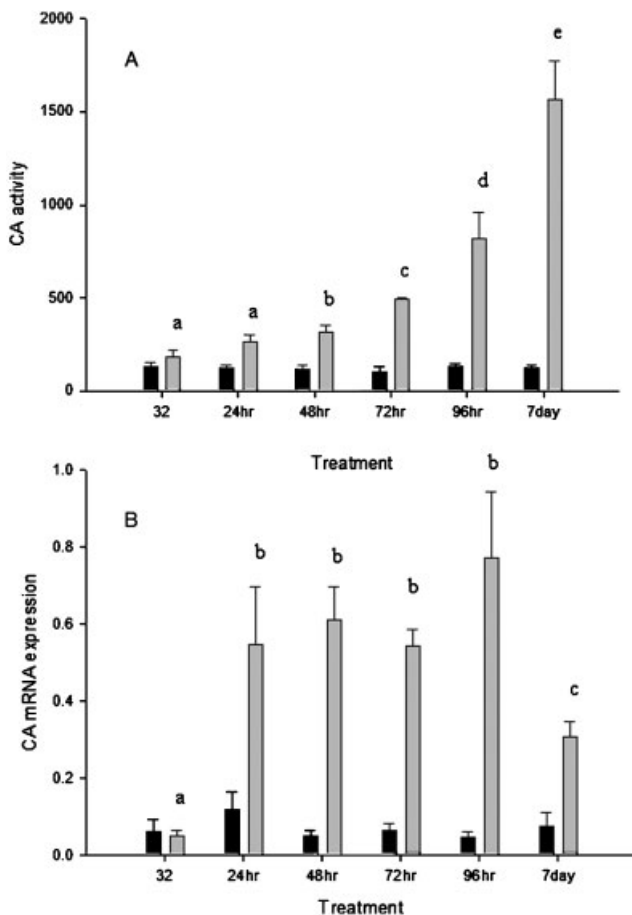


Fig. 2. Carbonic anhydrase activity ( $\mu\text{mol CO}_2 \text{mg protein}^{-1} \text{min}^{-1}$ , panel A), and CA mRNA expression (relative amounts, panel B) in anterior (G4, black bars) and posterior (G8, gray bars) gills of green crabs acclimated to 32 ppt (T0 in the time course) and at various times after being transferred to 10 ppt. Mean  $\pm$  SEM ( $N = 5-8$ );  $T = 12^\circ\text{C}$ . Different letters above bars indicate statistical differences at the 0.05 level of significance in G8 across the time course of low salinity transfer. There were no significant differences among G4.

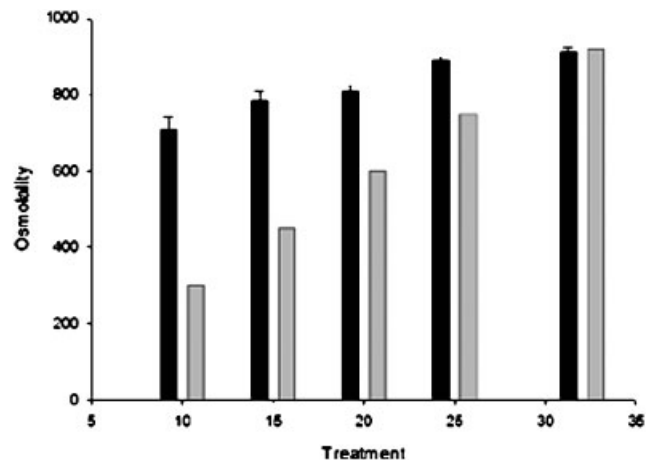


Fig. 3. Hemolymph (black bars) and seawater (gray bars) osmolality (mOsm  $\text{kg H}_2\text{O}^{-1}$ ) for green crabs acclimated to 7 days to 32, 25, 15, and 10 ppt salinity. Mean  $\pm$  SEM ( $N = 6-8$ );  $T = 12^\circ\text{C}$ .

Despite the decrease in the absolute value of hemolymph osmolality, it was always well above the osmotic concentration of the seawater, from 25 to 10 ppt. With each progressive decrease in salinity, the difference in osmolality between the hemolymph and the ambient medium became larger (from 139 mOsm kg H<sub>2</sub>O<sup>-1</sup> at 25 ppt to 408 mOsm kg H<sub>2</sub>O<sup>-1</sup> at 10 ppt).

CA activity in posterior gills increased with decreasing salinity, from 32 to 15 ppt, for crabs subjected to step-wise transfers to dilute media ( $P < .001$ , Kruskal–Wallace). There was a doubling of CA activity in posterior gills of crabs in 25 vs. 32 ppt, and an approximately 4-fold increase for transfer from 32 to 20 ppt (Fig. 4A). The largest increase, over 10-fold, occurred at 15 ppt (Fig. 4A). Interestingly, CA activity in posterior gills of crabs transferred directly to 10 ppt declined

by about 30% compared to crabs transferred to 15 ppt (Fig. 4A). There were no significant changes in CA activity in anterior gills ( $P = 0.74$ , ANOVA).

The pattern of CA mRNA abundance was very similar to that of CA activity. Relative expression of CA mRNA was low and not significantly different in anterior vs. posterior gills in crabs acclimated to 32 ppt ( $P = 0.92$ , *t*-test; Fig. 4B); and there were no significant changes in expression in anterior gills for any of the step-wise dilutions ( $P = 0.86$ , ANOVA). CA mRNA levels in posterior gills, however, were highly sensitive to dilutions in salinity ( $P < 0.01$ , Kruskal–Wallace ANOVA in ranks). Transfer to either 25 or 20 ppt resulted in an approximate doubling of CA mRNA abundance, and transfer to 15 ppt resulted in a near 10-fold increase (Fig. 4B). As with CA activity, the largest increase in CA mRNA was seen for the 15 ppt transfer. For crabs transferred from 32 to 10 ppt, CA mRNA levels remained significantly higher (5-fold,  $P < 0.05$ , Dunn's) than values for crabs transferred to either 25 or 20 ppt; but, compared to crabs transferred to 15 ppt there was a greater than 50% decrease in abundance ( $P < 0.05$ , Dunn's) (Fig. 4B).

Titration of cytoplasmic CA activity with Az resulted in similar slopes for both anterior and posterior gills (Fig. 5). The  $K_i$  values were all near 2 nM and were very close to  $K_i$  values reported previously (Henry et al., 2003). Furthermore, the concentrations of CA ( $E_0$ ) were similar for anterior and posterior gills from crabs acclimated to 32 ppt ( $P = 0.06$ , *t*-test), and for anterior gills from crabs acclimated to either 32 or 10 ppt ( $P = 0.79$ , *t*-test) (Fig. 6). CA concentration in the cytoplasmic fraction of posterior gills, however, increased over 5-fold ( $P < 0.001$ , *t*-test) in crabs acclimated to 10 ppt.

## DISCUSSION

The results of the time course of low salinity adaptation further support the hypothesis that low salinity-mediated CA induction is under transcriptional regulation. The increase in CA activity is a result of an increase in CA mRNA expression, as measured by relative changes in mRNA. The results here cannot distinguish between an increase in mRNA synthesis vs. an increase in retention through decreased mRNA turnover, but it is clear that low salinity does stimulate an increase in CA mRNA abundance. The 8-fold increase in mRNA is rapid and precedes the initial increase in CA activity by 24 hr. New

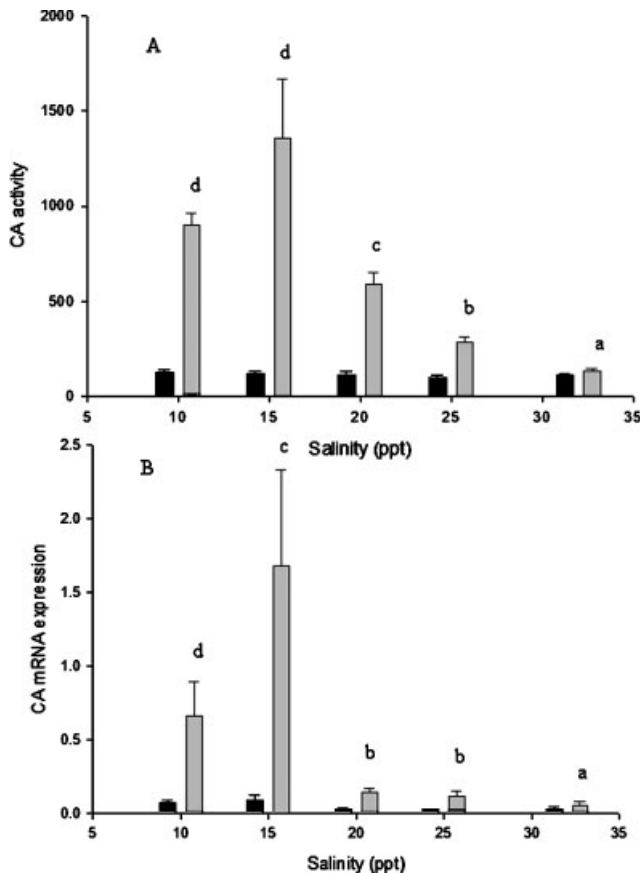


Fig. 4. Carbonic anhydrase activity ( $\mu\text{mol CO}_2 \text{ mg protein}^{-1} \text{ min}^{-1}$ , panel A), and CA mRNA expression (relative amounts, panel B) in anterior (G4, black bars) and posterior (gray bars) gills of green crabs acclimated for 7 days to 32, 25, 20, 15, and 10 ppt salinity. Mean  $\pm$  SEM ( $N = 5-6$ );  $T = 12^\circ\text{C}$ . Different letters above bars indicate differences in G8 at the 0.05 level of significance. There were no significant differences among G4.

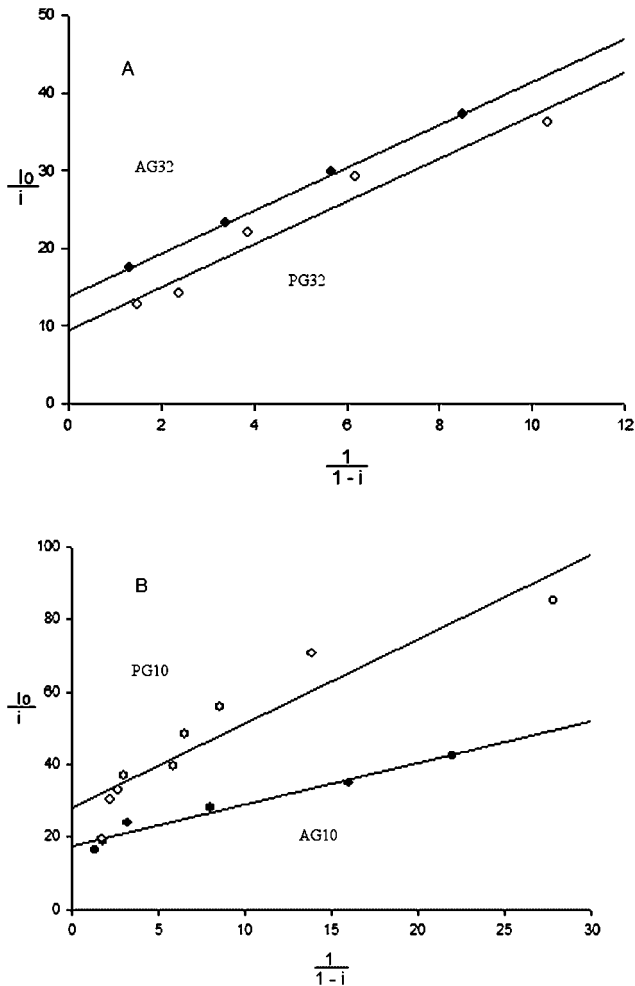


Fig. 5. Double reciprocal inhibitor titration plots of cytoplasmic CA activity vs. acetazolamide from pooled anterior (AG) and posterior (PG) gills of green crabs acclimated to 32 ppt (panel A) and 10 ppt (panel B). Regression lines, as determined by the method of least squares, are as follows: AG32:  $y = 2.76x + 13.90$ ;  $r = 0.94$ . PG32:  $y = 2.76x + 9.50$ ;  $r = 0.98$ . AG10:  $y = 1.16x + 17.37$ ;  $r = 0.96$ . PG10:  $y = 1.88x + 28.51$ ;  $r = 0.95$ . Points are the means of triplicate assays.

acclimated levels of CA activity are reached by 7 days post-transfer and represent an approximate 8-fold increase over values in crabs at 32 ppt. Furthermore, these changes only occur in the posterior, ion-transporting gills. This pattern is similar to that reported by Henry et al. (2003), using semi-quantitative PCR, but the data for quantitative changes in mRNA expression reported here show a much larger increase in CA mRNA expression. The high levels of CA activity measured at 7 days after transfer to 10 ppt are also accompanied by a large (5-fold) increase in the concentration of the CA protein in the cytoplasmic fraction of the posterior gills. It is the cytoplasmic

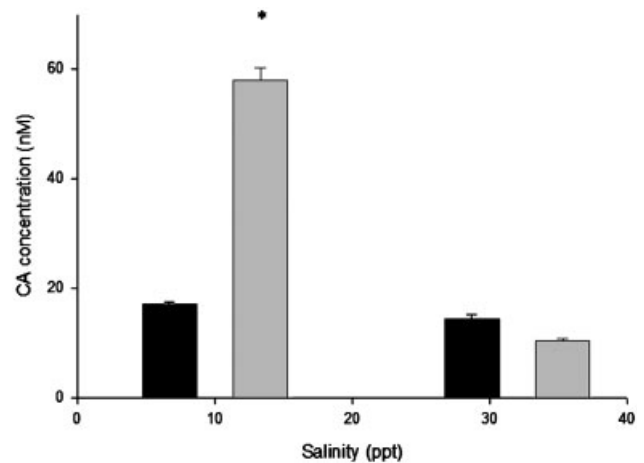


Fig. 6. Carbonic anhydrase concentrations in the cytoplasmic fraction of anterior (G4, black bars) and posterior (G8, gray bars) gills of green crabs acclimated to 32 and 10 ppt. Mean  $\pm$  SEM ( $N = 4$ ). Asterisk represents statistical significance between posterior gills at the 0.05 level.

fraction of branchial CA that is most sensitive to environmental salinity and which is believed to function in support of active ion uptake (Henry, '88a,b). While the measured increases in CA mRNA, protein-specific activity, and protein concentration are not exactly the same, they are close enough to conclude that CA induction is a result of an increase in CA mRNA expression and subsequent increase in CA protein expression.

Interestingly, the maximum level of CA mRNA expression is reached at 24 hr, the minimum time of exposure to low salinity needed by *C. maenas* to commit to the metabolic cost of the synthesis of new CA enzyme (Henry, 2005). Once that commitment is made, the increase in mRNA expression does not appear to be needed over the long-term. Rather, as CA activity is reaching its maximum new acclimated levels at 7 days after transfer to low salinity, relative levels of CA mRNA expression begin to decline. The pattern of low salinity adaptation, therefore, involves a transient increase in CA mRNA expression, but a long-term increase in CA protein induction. Preliminary reports on other transport proteins from the gills of other euryhaline crustaceans also support the idea that these proteins are up-regulated at the transcriptional level. Relative expression of both the  $\text{Na}^+/\text{K}^+$ -ATPase and the  $\text{Na}^+/\text{K}^+/\text{2Cl}^-$  co-transporter increased in the posterior gills of two other euryhaline species within 24 hr after exposure to 10 ppt salinity (Spanings-Pierrot and Towle, 2003, 2004; Luquet et al., 2005).

It should be noted that a control (housekeeping) gene was not used for comparison to CA in this study. Rather, changes in CA mRNA expression were compared between two tissues, with the control tissue being the anterior, respiratory gill (G4) in which CA activity and expression do not change. 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). Arginine kinase, which was 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* (Kotlyar et al., 2000). 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 was also used successfully in two other species of euryhaline crabs. In *Pachygrapsus marmoratus*, the expression of the  $\text{Na}^+/\text{K}^+/\text{2Cl}^-$  co-transporter was shown to increase in response to low salinity in the posterior but not the anterior gills (Spannings-Pierrot and Towle, 2004), and in *Chasmagnathus granulatus*, both the co-transporter and the  $\text{Na}^+/\text{K}^+$ -ATPase were shown to increase in G7 but not G3 in crabs transferred to 10 ppt salinity (Luquet et al., 2003).

The response of both CA activity and mRNA expression to step-wise dilutions of high salinity is very interesting. Maximum values for both measurements occurred at 15 ppt, well above the lower lethal limit of salinity for this species and also well above where this species is found in nature (Zanders, '80). However, the decline in activity and mRNA expression between 15 and 10 ppt may offer a clue as to the physiological basis that sets the lower lethal salinity for a species. The data here suggest that the lower salinity limit may be determined by the failure of the induction mechanism; that there is a salinity below which the induction mechanism becomes refractory, and as a result, low salinity adaptation becomes progressively more difficult due to the lack of adequate transport protein expression. If this is so, one would expect CA activity and expression to decline further at salinities below 10 ppt, and one would also expect to see the activity and expression

of other transport proteins to follow this pattern as well. This is currently under investigation.

In summary, this report represents the first quantitative data for protein-specific activity, protein concentration, and mRNA expression for a transport-related protein that shows an increase in protein-specific activity is a result of an increase in gene expression and protein synthesis. Furthermore, these results suggest that the lower limit of salinity tolerance of a species may be set by the point at which the induction mechanism becomes refractory to further decreases in salinity.

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