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Functional Characterization of Neuroendocrine Regulation of Branchial Carbonic Anhydrase Induction in the Euryhaline Crab *Callinectes sapidus*

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Abstract. Carbonic anhydrase (CA) plays an essential role as a provider of counterions for Na^+/H^+ and Cl^-/HCO_3^- exchange in branchial ionic uptake processes in euryhaline crustaceans. CA activity and gene expression are low in crabs acclimated to full-strength seawater, with transfer to low salinity resulting in large-scale inductions of mRNA and subsequent enzyme activity in the posterior ion-regulating gills (*e.g.,* G7). In the green crab *Carcinus maenas,* CA has been shown to be under inhibitory neuroendocrine control by a putative hormone in the x-organ-sinus gland complex (XOSG), located in the eyestalk. This study characterizes the neuroendocrine regulation of CA induction in the blue crab *Callinectes sapidus*, a commonly used experimental organism for crustacean osmoregulation. In crabs acclimated to full-strength seawater, eyestalk ligation (ESL) triggered a 1.8- and 100-fold increase in CA activity and mRNA, respectively. Re-injection with eyestalk homogenates abolished increases in CA activity and fractionally reduced CA gene expression. ESL also enhanced CA induction by 33% after 96 h in crabs transferred to 15 ppt salinity. Injection of eyestalk homogenates into intact crabs transferred from 35 to 15 ppt diminished by 43% the CA induction stimulated by low salinity. These results point to the presence of a repressor hormone in the eyestalk. Separate injections of medullary tissue (MT) and sinus gland (SG), two components of the eyestalk, reduced salinity-stimulated CA activity by 22% and 49%, suggesting that the putative repressor is localized to the SG. Crabs injected with SG extract harvested from crabs acclimated to 5 ppt showed no decrease in CA activity, demonstrating that the hormone is down-regulated at low salinity. Our results show the presence in the XOSG of an inhibitory compound that regulates salinity-stimulated CA induction.

Introduction

Carbonic anhydrase (CA) is a central component of osmoregulation in many species of euryhaline crustaceans. CA facilitates ionic uptake by hydrating respiratory CO_2 to H^+ and HCO_3^-, which serve as counterions for Na^+/H^+ and Cl^-/HCO_3^- exchange across the apical gill membrane (Kirschner, 1979; Henry and Cameron, 1983; Henry, 1984, 2001; Henry *et al.,* 2012). The central importance of this enzyme has been shown through experiments in the blue crab *Callinectes sapidus* and the green crab *Carcinus maenas*, where injections of the CA inhibitor acetazolamide (Az) into low-salinity-acclimated crabs disrupt Na^+ and Cl^- influx across the gills and depress hemolymph osmolality and NaCl concentrations (Cameron, 1979; Henry and Cameron, 1983; Henry, 2003). Furthermore, injection of Az into *C. sapidus* during the acute phase of low-salinity acclimation results in 100% mortality (Henry and Cameron, 1982a). Thus, CA is an essential molecular component of ionic uptake and low-salinity tolerance in these animals.

Due to the critical role of CA in osmoregulation, it is not surprising that its levels of activity in the gills are highly sensitive to environmental salinity. With the exception of the two anteriormost pairs of gills, CA activity is evenly divided between the anterior and posterior gills in crabs acclimated to full-strength seawater (~32–35 ppt) (Henry and Cameron, 1982b). After acclimation to low salinity (<26 ppt), CA activity in the posterior, ion-regulating gill pairs increases up to 15-fold, while enzyme activity in the

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Abbreviations: CA, carbonic anhydrase; CHH, crustacean hyperglycemic hormone; ESA, eyestalk ablation; ESL, eyestalk ligation; MT, medullary tissue; SG, sinus gland; XOSG, x-organ–sinus-gland complex.

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anterior respiratory gills remains unchanged (Henry and Watts, 2001; Henry *et al.,* 2002; Serrano *et al.,* 2007; Serrano and Henry, 2008; Mitchell and Henry, 2014). The level of CA induction at low salinity appears to correlate with the degree of environmental dilution. For example, crabs acclimated to 15 ppt will have higher CA activity than crabs acclimated to 25 ppt (Henry and Cameron, 1982a; Henry and Watts, 2001; Henry *et al.,* 2002; Mitchell and Henry, 2014).

Hypoosmotic-stimulated CA induction results from activation of the *CAc* gene and subsequent synthesis of new enzyme. While other CA isoforms, such as a glycophosphotidylinositol (GPI)-linked CA (*CAg*), are expressed in the crustacean gill, it is only *CAc* which contributes to ionic uptake. In *C. sapidus* and *C. maenas*, increases in *CAc* (the cytoplasmic CA isoform functioning in crustacean ionic uptake) gene expression occur rapidly (100- and 10-fold, respectively, after just 6 h at low salinity), with no changes occurring in *CAg* expression (Serrano *et al.,* 2007; Serrano and Henry, 2008). The activation of *CAc* is fast relative to enzyme induction, with initial increases in CA activity occurring at 48–72 h and taking up to 4–7 days to reach new acclimated levels (Serrano *et al.,* 2007; Serrano and Henry, 2008; Mitchell and Henry, 2014). The delay in the onset of enzyme activity is believed to result from limits in the post-transcriptional processes involved in the synthesis of active enzyme (Mitchell and Henry, 2014). Levels of *CAc* mRNA and CA activity then remain elevated above 35 ppt acclimated values throughout the low-salinity exposure. Similar to the induction of CA activity, these gene activation events occur in the posterior ion-regulating gill pairs and are absent from the anterior respiratory gills (Serrano *et al.,* 2007; Serrano and Henry, 2008; Mitchell and Henry, 2014).

Over the past several years, there has been increasing interest in the mechanisms that lead to the activation of the *CAc* gene and resultant induction of CA activity at low salinity. Removal of the eyestalks (eyestalk ablation, ESA) in *C. maenas* results in a doubling of branchial CA activity at full-strength seawater and a 10-fold up-regulation of CA mRNA (Henry, 2006b; Henry and Campoverde, 2006). These results were particularly exciting, as branchial CA induction at full-strength seawater had been previously observed only in molting crabs (Henry and Kormanik, 1985); however, molting-induced changes occur in both anterior and posterior gills, whereas ESA, similar to environmental dilution, produced up-regulation of enzyme activity and gene expression in only the posterior gill pairs (Henry, 2006a; Henry and Campoverde, 2006). Homogenization of the eyestalk and re-injection into ESA green crabs abolishes the effect of ESA (Henry, 2006a, b).

The injection of eyestalk homogenate taken from green crabs acclimated to full-strength seawater into intact crabs transferred from 35 to 15 ppt reduces salinity-stimulated CA activity (Henry, 2006a). When this procedure is repeated with eyestalks taken from donor crabs acclimated to low salinity, there is no inhibitory effect on CA activity (Henry, 2003). Taken together, these data suggest that there is a hormone in the eyestalk that inhibits CA activity and expression in full-strength seawater. When crabs are acclimated to low salinity, this hormone is down-regulated, releasing the inhibitory regulation on CA expression and allowing salinity-stimulated CA induction in the posterior gills to occur.

Within the eyestalk, this hormone is believed to be present primarily in the x-organ–sinus-gland complex (XOSG) (Henry, 2006b), a major endocrine complex in crustaceans (Hopkins, 2012).

An initial biochemical characterization of this substance showed that it is heat- and acid-stable for short periods (2.5 min) but loses potency after 15 min, suggesting that it is a small peptide. This, together with its location, suggests that the putative CA repressor is similar to members of the crustacean hyperglycemic hormone (CHH) family (Mykles, 2001; Henry, 2004). The CHH family consists of several distinct classes of evolutionarily related small neuropeptide hormones with diverse physiological functions ranging from gonadal development to the regulation of energy metabolism (see Chung *et al.,* 2010, for review). These peptides are synthesized in the x-organ (XO) and travel *via* axoplasmic flow to the synaptic terminals of the sinus gland (SG) where they are secreted into the hemolymph (see Hopkins, 2012, for review).

If the putative CA repressor hormone is in fact a CHH neuropeptide, it would not be the first time that this hormone family has been implicated in osmoregulation. However, most previous studies have suggested a stimulatory role on osmoregulation, rather than the inhibitory effects typical of the CHH family. CHH peptides were first implicated in osmoregulation with the observation that SG extracts stimulate Na^+ influx and increases in transepithelial potential in perfused crab gills (Eckhardt *et al.,* 1995; Spanings-Pierrot *et al.,* 2000). In the crayfish *Astacus leptodactylus*, ESA decreased hemolymph osmolality and Na^+ concentration, with these changes being significantly mitigated by injection with CHH (Serrano *et al.,* 2003). In insects, an ortholog of CHH, ion-transport peptide (ITP), is involved in Cl^- transport in the ileum (see Chung *et al.,* 2010, for review). A cDNA homologous to insect ITP (*LvITP*) was found in the shrimp *Litopenaeus vannamei*, and the expression profile of this gene was similar to other genes implicated in osmoregulation, such as *CAc* and the Na^+/K^+-ATPase α-subunit (*NaK*), in that it was expressed most highly in the posterior gills, and mRNA up-regulation occurred following low-salinity transfer (Tiu *et al.,* 2007). Thus, a co-opting of a CHH neuropeptide to regulate CA activity for branchial ion transport in euryhaline crab gills would not be a surprising occurrence.

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Despite the initial characterization of the CA repressor in *C. maenas*, there exists comparatively little evidence of eyestalk regulation in other euryhaline crustaceans. Currently, it is known only that ESA results in up-regulation of CA activity in the posterior gills in *C. sapidus* and that it enhances hypoosmotic-stimulated CA induction (Henry and Borst, 2006), but the mechanism is unknown. This dearth of knowledge is exacerbated by the fact that ESA experiments with *C. sapidus* are quite difficult, as the crab is significantly less durable than *C. maenas* and ESA often results in mortality.

The aim of this study is to present a more complete characterization of neuroendocrine regulation of CA in *C. sapidus*. We report the effects of eyestalk ligation (ESL), a procedure that removes the physiological influence of the eyestalk with much higher survivorship than ESA in *C. sapidus*. We also present the effects of ESL and re-injection of eyestalk homogenate on CA activity and *CAc* expression. Additionally we treated intact crabs that had been transferred from 35 ppt to 15 ppt salinity with whole-eyestalk injections as well as with separate sinus gland and medullary tissue injections in an attempt to demonstrate the capacity of the eyestalk to inhibit CA induction and to determine the anatomical location of the putative hormone. Finally, we report the effects of ESL on branchial expression of cytoplasmic carbonic anhydrase (*CAc*), GPI-linked carbonic anhydrase (*CAg*), and the Na^+/K^+-ATPase α-subunit (*NaK*).

Materials and Methods

Collection and maintenance of animals

Adult male intermolt blue crabs, *Callinectes sapidus* Rathbun 1896, were obtained from local fishermen in East Point, Florida, packed in wet burlap, and placed in 45-l coolers and transported to Auburn University. Crabs were kept in 560-l recirculating aquaria equipped with biological filters (oyster shell and sand) at 24 °C and ambient photoperiod. Crabs were held at 35 ppt salinity for a minimum of 3 weeks to ensure that carbonic anhydrase (CA) activity was at minimum, baseline levels (Henry and Watts, 2001). Blue crabs were fed daily on shrimp, and salinity was monitored with a conductivity meter (YSI 30 1, Yellow Springs, OH) and adjusted with distilled water or concentrated brine made with artificial sea salts (Instant Ocean Reef Crystals, Blacksburg, VA). Water quality was checked by monitoring nitrite concentrations (Dry Tab, Mansfield, MA).

Experimental protocols

Several environmental and physiological manipulations were used in this study. Salinity transfers were conducted by transferring crabs directly from full-strength seawater (35 ppt) to 15 ppt. Transferred crabs were left intact or treated with either eyestalk ablation (ESA) or eyestalk ligation (ESL). For both treatments, crabs were chilled by being packed in crushed ice for 10 min. ESA was accomplished by cutting the eyestalks at their base and then re-packing the crab in ice to prevent excessive bleeding. For ESL, both eyestalks were tied off twice at the base with surgical suture. Crabs were then transferred to their respective treatment salinity or returned to 35 ppt.

For experiments involving injections, three treatments were used: (1) intact controls (no injection), (2) sham injection (injection of crustacean saline), and (3) injection of eyestalk extract. Eyestalk donor crabs were chilled on ice for 10 min to induce anesthesia. The eyestalks were amputated and the crabs were sacrificed by exsanguination. Eyestalks (3 per ESL crab, 2 per intact crab) were placed on ice, and the interior tissue was separated from the chitin and homogenized in crustacean saline (0.8 ml saline/crab/injection) (410 mmol l^{-1} NaCl, 11 mmol l^{-1} $CaCl_2$, 21 mmol l^{-1} $MgSO_4$, 8 mmol l^{-1} KCl, 10 mmol l^{-1} HEPES, 10 mmol l^{-1} $NaHCO_3$ with 10% DTT) for 10 s, and centrifuged at $7500 \times g$ for 10 min. The supernatant (0.5 ml) was injected into the base of the swimming leg with a 22-ga needle. Injections were preformed just after ESL or directly before transfer into low salinity. Following the initial injection, boosters were given at 12 and 24 h post-treatment.

For medullary tissue (MT) and sinus gland (SG) injections, the two tissues were separated by dissection, homogenized, and injected into separate sets of crabs. Injections were performed at 0, 9, 24, and 34 h post-transfer. Each crab received the equivalent of 2 SGs or 2 MTs at each injection. The same protocols were followed for sham injections using saline.

At the end of each experiment, hemolymph was sampled 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 concentrations.

Anterior gills (G3) were used as a non-ion-transporting control tissue, and posterior (G7), ion-transporting gills, were used as the experimental tissue. Both anterior and posterior gills were dissected out of the crabs at the end of each experiment: one pair was 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) for measurement of CA activity; the second pair was placed in 1.5 ml of RNAgents denaturing solution (Promega, Madison, WI) for total RNA extraction.

Analytical procedures

Carbonic anhydrase specific activity. CA activity was measured electrometically by the delta pH method (Henry,

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1991). Anterior (G3) and posterior (G7) gills were homogenized in 5 volumes of cold buffer using an Omni TH-115 hand-held homogenizer and then sonicated at 25 W for 10 s (Heat Systems, Microsonicator, Farmingdale, NY). Homogenates were centrifuged at 10,000 × *g* for 20 min at 4 °C (Sorvall RC5-B, Wilmington, DE), and the supernatant was assayed for CA activity. Briefly, 25–100 μl of the 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_2–saturated water, and the drop in pH was monitored by micro-pH and reference electrodes (World Precision Instruments, Sarasota, FL) and a null-point pH meter. Protein concentrations were measured in the supernatant by Coomassie brilliant blue dye binding (Bio-Rad Laboratories, Hercules, CA), and CA activity was reported as μmol of CO_2 per milligram of protein per minute.

Hemolymph osmolality. Hemolymph and seawater 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).

RNA purification and first-strand cDNA synthesis. Total RNA was extracted from gills using RNAgents Total RNA Isolation System (Promega, Madison, WI) under RNAse-free conditions, with all equipment being treated with RNAse-Zap (Ambion, Austin, TX). RNA purity and quality were assayed using a Bioanalyzer 2100 (Agilent Technologies, Wilmington, DE); no genomic DNA contamination was observed. The total RNA concentration of each sample was determined using a nanodrop spectrophotometer (Thermo Scientific NanoDrop 1000). cDNA was reverse-transcribed with 2 μg of total RNA using Oligo-dT as a primer and SuperScript II reverse transcriptase (Invitrogen, Madison, WI). The samples were normalized to total RNA levels in each preparation (Bustin, 2002). The resulting cDNA samples were stored at −20 °C until they were used as templates for real-time quantitative polymerase chain reaction (qPCR).

Quantitative mRNA expression. The relative abundance of mRNA from each gene of interest was assayed using qPCR with a MiniOpticon real-time PCR detection system using an IQ SYBR Green supermix kit (Bio-Rad Laboratories, Hercules, CA). All PCR reactions were initialized at 95 °C for 3 min followed by 35 cycles of denaturing at 95 °C for 10 s, and annealing-elongation at 55 °C for 60 s. One microliter of each cDNA sample was analyzed in duplicate or triplicate. The cycle number at which the fluorescent signal becomes higher than the minimum assigned value (threshold cycle, Ct) is inversely proportional to the logarithm of transcript abundance contained in the PCR reaction mix. Transcript abundance in experimental samples was then obtained empirically using a standard curve (Ct *vs.* $\log_{10}$ template availability) generated by a dilution series of a reference sample (1, 0.1, 0.01, and 0.001 fold dilution series) of first-strand cDNA prepared from G7 of *C. sapidus* acclimated to 15 ppt salinity for 7 days. Gene-specific primer pairs CsCAcF/CsCAcR and CsCAgF/CsCAgR (Serrano *et al.,* 2007) were used to amplify *CsCAc* (GenBank: EF375490.1) and *CsCAg* (GenBank: EF375491.1), respectively, while the Na^+/K^+-ATPase α-subunit (GenBank: AF327439.1) was amplified with NAKSP12F/NAKSP16R gene-specific primer pair (Li *et al.,* 2006).

Statistics. All statistical tests were performed in R Studio Version 3.0.02 (The R Foundation for Statistical Computing).

Results

Effects of eyestalk ablation on CAc *mRNA expression*

CAc mRNA was at baseline levels in both G3 and G7 gills of crabs acclimated to 35 ppt salinity (0.006 relative to 15 ppt standards) (Fig. 1), similar to the full-strength seawater conditions reported previously for this species (Serrano *et al.,* 2007). ESA resulted in an approximate 30-fold increase in *CAc* mRNA levels in G7 at 24-h post-treatment ($P < 0.05$, one-way ANOVA, Tukey HSD). Mortality in crabs increased at 48 h and longer, so the experiment was not carried past 24 h. There was no significant increase in mRNA expression in G3 as a result of ESA. Transfer of intact crabs to 28 ppt for 24 h did not alter *CAc* mRNA levels in either G7 or G3 (Fig. 1). However, combined ESA and salinity transfer induced a 100-fold increase in *CAc* mRNA at 24-h post-treatment ($P < 0.05$, one-way ANOVA, Tukey HSD). Interestingly, this large increase occurred at a salinity (28 ppt) at which the blue crab is still an osmoconformer and which does not induce CA activity in intact crabs (Henry, 2005). Again, there was no change in *CAc* mRNA in G3 regardless of treatment. CA activity was not measured, because changes in CA induction typically do not occur until 24 h after treatment such as low-salinity exposure (Henry, 2005).

Effect of eyestalk ligation and re-injection on 35 ppt acclimated crabs

Posterior gill (G7) CA activity in intact *C. sapidus* acclimated to 35 ppt was low and not significantly different than anterior gill (G3) levels (287 *vs.* 275 μmol CO_2 mg protein^{-1} min^{-1} (one-way ANOVA, Tukey HSD) (Fig. 2) as previously reported (Henry, 2006b; Henry and Borst, 2006; Serrano *et al.,* 2007), ensuring that crabs were in the high-salinity, osmoconforming state at the start of the experiment. *CAc* (the CA isoform responsible for branchial ionic

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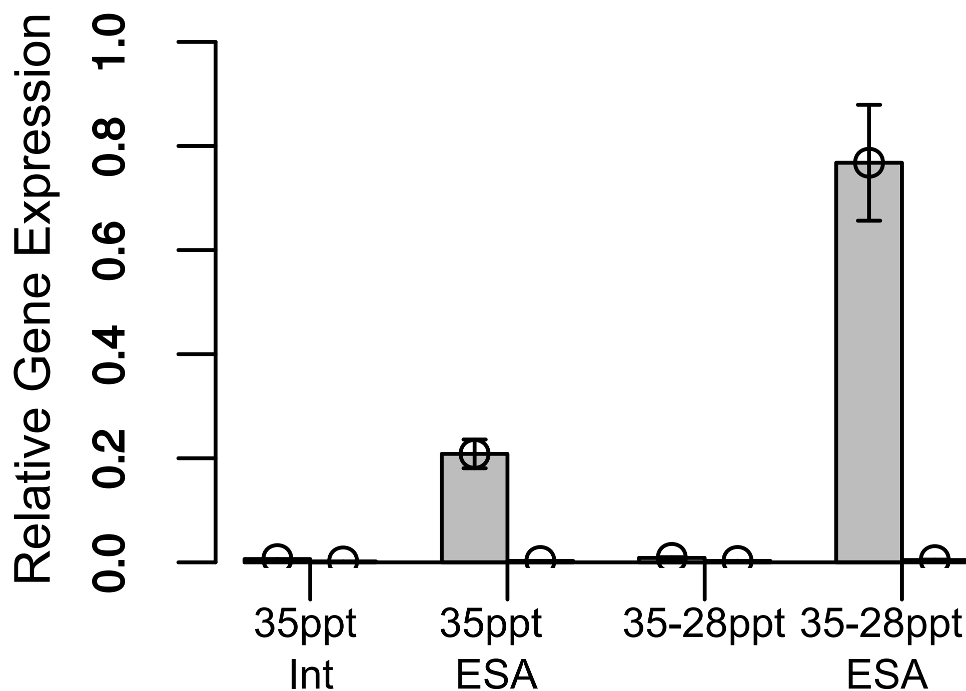

Figure 1. Relative *C*Ac gene expression in the posterior G7 gills (gray bars) and the anterior G3 gills (right-hand bars in which gene expression is so low that the bars are barely visible) of *Callinectes sapdus* specimens at 35 ppt intact, at 35 ppt with eyestalk ablation (ESA), transferred to 28 ppt intact, and transferred to 28 ppt with ESA. Different letters denote significant differences in G7 at the $P = 0.05$ level. Means ± SEM, $n = 6$

uptake) expression in G7 was 0.005 relative to qPCR standards prepared from G7 of 35–15 ppt crabs. *CAg* (a CA isoform functioning in CO_2 excretion) and Na^+/K^+-ATPase α-subunit (*NaK*) expression in G7 at 35 ppt were also low relative to 15 ppt standards (0.081 and 0.048, respectively) (Fig. 3), as previously reported by Serrano *et al.* (2007).

At 48 h after treatment with ESL, CA activity rose by 1.8-fold to 520 μmol CO_2 mg protein^{-1} min^{-1} in G7 ($P < 0.05$, Student's *t*-test) (Fig. 2). CA activity in the anterior gill (control tissue) was unchanged (Fig. 2). Similarly, ESL induced a 100-fold ($P < 0.05$, Student's *t*-test) up-regulation of *CAc* mRNA in G7. *C*Ac expression did increase significantly ($P < 0.05$ Student's *t*-test) in G3, but it was to a much smaller degree than in G7 (7-fold) (Fig. 2). ESL induced a 3-fold up-regulation of *NaK* mRNA ($P < 0.05$, Student's *t*-test), but failed to produce a significant response in *CAg* expression (Fig. 3). There were no significant differences in *NaK* or *CAg* expression in G3 (Student's *t*-test) (Fig. 2).

Treatment of 35-ppt-acclimated ESL crabs with injections of whole eyestalk homogenates administered at 0, 12, and 24 h post-treatment eliminated the aforementioned 1.8-fold ESL-stimulated increase in CA activity (Fig. 2). CA activity in 35-ppt intact crabs and 35-ppt ESL re-injected crabs was of a similar magnitude (287 and 302 μmol CO_2 mg protein^{-1} min^{-1}, respectively), and not significantly different ($P > 0.05$, one-way ANOVA, Tukey HSD) (Fig. 2). A sham injection of crustacean saline failed to diminish the effects of ESL, and CA activity remained significantly above that of 35-ppt intact crabs ($P < 0.05$, one-way ANOVA, Tukey HSD) by 1.7-fold (Fig. 2). Eyestalk re-injection into ESL crabs diminished the degree of ESL-stimulated *CAc* mRNA induction in G7 by 3-fold; however, the level of gene expression was still significantly above that of 35-ppt intact crabs by 33-fold ($P < 0.05$, Student's *t*-test) and not significantly different from that of non-injected ESL crabs (Fig. 2). Sham injection into ESL crabs did not diminish *CAc* mRNA, which was 96-fold above that of 35-ppt intact animals ($P < 0.05$, one-way ANOVA, Tukey HSD) (Fig. 2). There were no differences between G3 *CAc* expression except between sham-injected and 35-ppt animals as well as sham-injected and ESL animals (no injection) (Fig 2).

Hemolymph osmolality in 35-ppt-acclimated intact crabs was approximately equal to that of the surrounding water (1031 *vs.* 1033 mOsm/kg H_2O) (Fig. 4). Treatment with ESL did not alter the osmotic relationship with the surrounding medium, with hemolymph being 1077 and seawater being 1050 mOsm/kg H_2O (Fig. 4).

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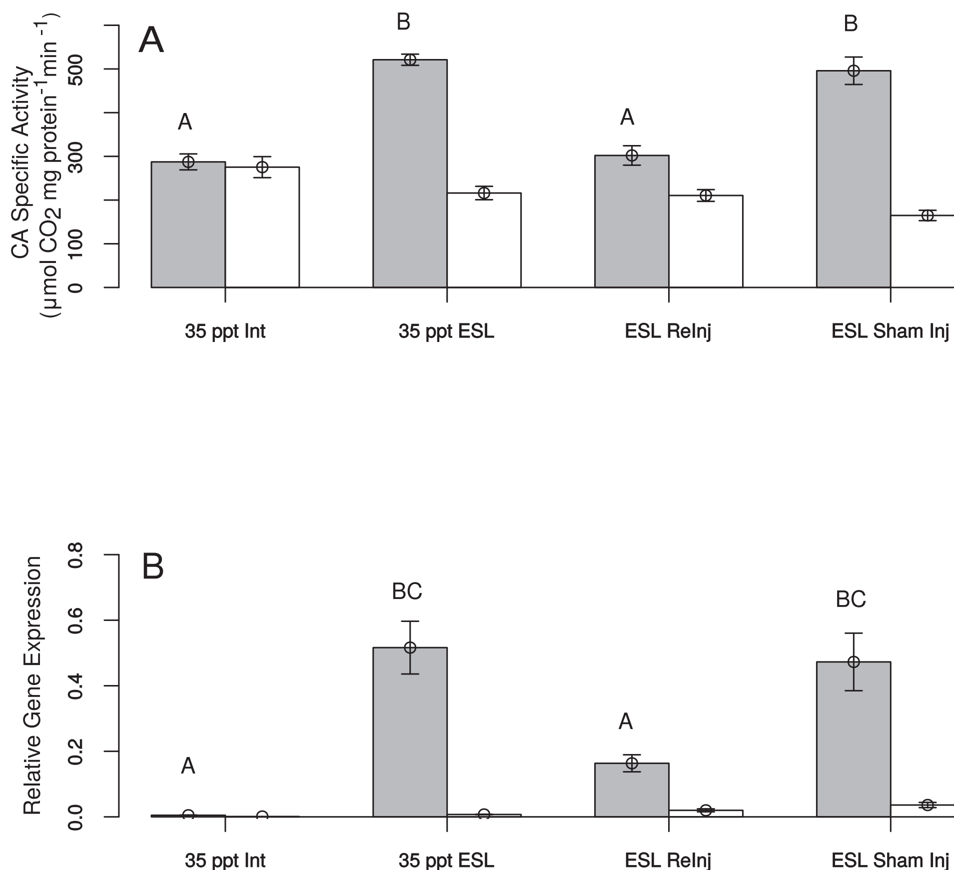

Figure 2. Carbonic anhydrase (CA) activity (A) and *CAc* gene expression (B) in the posterior G7 gills (gray bars) and the anterior G3 gills (white bars) of *Callinectes sapidus* specimens acclimated to 35 ppt. Crabs were left intact (35 Int), treated with eyestalk ligation (35 ESL), treated with ESL with eyestalk homogenate reinjections (Eystalk Reinj), and treated with eyestalk ligations with a sham injection (Sham Inj). Different letters denote significant differences at the $P = 0.05$ level. Means ± SEM, $n = 6$.

Effect of eyestalk ligation on 35–15-ppt transfer

Transfer of intact *C. sapidus* from 35 to 15 ppt salinity elicited a 2.6-fold (287 to 750 μmol CO_2 mg protein^{-1} min^{-1}) increase in CA activity in G7 ($P < 0.05$, one-way ANOVA, Tukey HSD) after 48 h (Fig. 5). CA activity in G3, the control tissue, was unaffected by low-salinity transfer, as has been reported previously (Henry, 2006; Henry and Borst, 2006; Serrano *et al.,* 2007) (Fig. 5). The increase in CA activity in G7 was accompanied by a large (42-fold) up-regulation of *C*Ac mRNA ($P < 0.05$, one-way ANOVA, Tukey HSD) after 48 h (Fig. 5); this was smaller but within the same range of previously reported mRNA up-regulation (Serrano *et al.,* 2007). Additionally, the findings of Mitchell and Henry (2014) that CA activity requires several days to reach maximal levels even after up-regulation of *CAc* mRNA were verified: significant ($P < 0.05$, one-way ANOVA, Tukey HSD) CA induction occurred between 48 and 96 h post-transfer, from 754 to 1056 μmol CO_2 mg protein^{-1} min^{-1}, despite the fact that gene expression was already 42-fold above 35-ppt-acclimated values at 48 h (Fig. 5).

When the 35–15-ppt transfer was repeated with ESL crabs, there was no significant difference in CA activity in either G7 or G3 relative to intact controls after 48 h (Fig. 5). However, *CAc* mRNA was 5.6 fold ($P < 0.05$, one-way ANOVA, Tukey HSD) higher in ESL *versus* intact crabs (Fig. 5). After 96 h at 15 ppt, *CAc* gene expression was not significantly different in ESL *versus* intact crabs, but CA activity was significantly higher ($P < 0.05$, one-way ANOVA, Tukey HSD) by 1.3-fold in ESL crabs (Fig. 5). *CAc* mRNA and CA enzyme activity in G3 were not affected by ESL and/or salinity in any experiment (Fig. 5).

In a second set of intact crabs, 35–15-ppt transfer resulted in a 3.3-fold CA induction after 48 h (Fig. 6). When this transfer was repeated but with crabs that were treated with eyestalk injections prepared from 35-ppt-acclimated ani-

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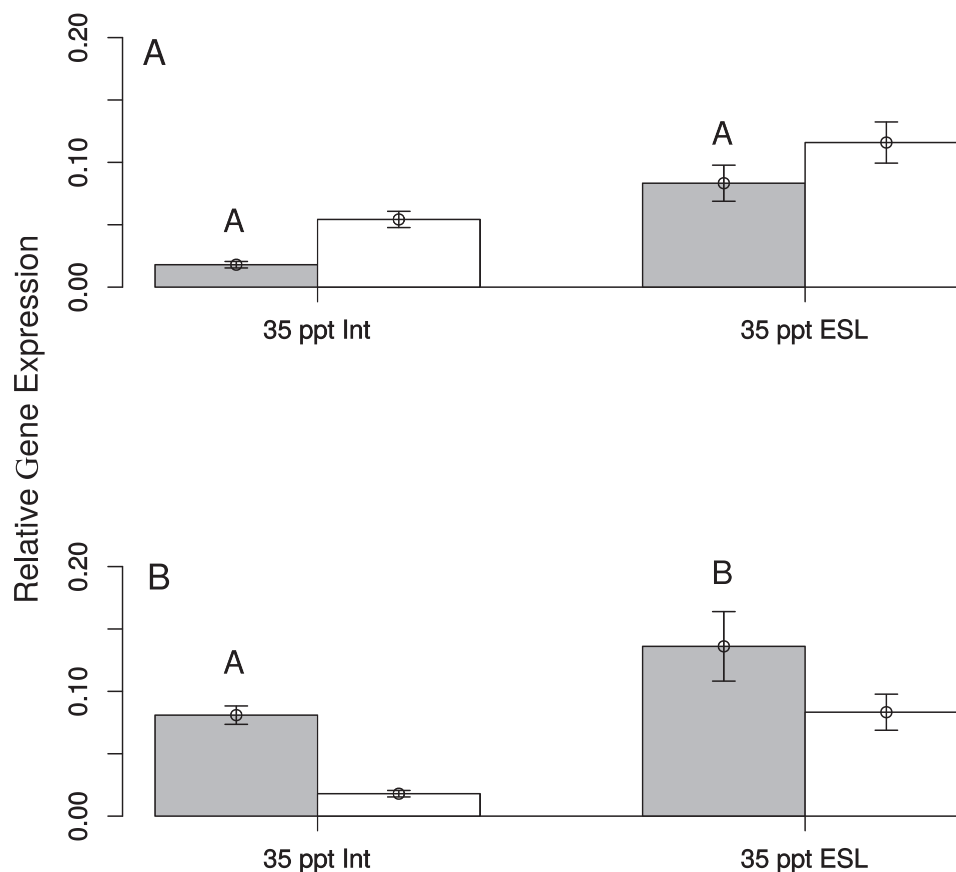

Figure 3. Relative gene expression of *CAg* (A) and *NaK* (B) in the posterior G7 gills (gray bars) and the anterior G3 gills (white bars) of *Callinectes sapidus* specimens acclimated to 35 ppt and left intact (Int) and treated with eyestalk ligation (ESL). Different letters denote significant differences at the $P = 0.05$ level. Means ± SEM, $n = 6$.

mals (administered at 0 h, 12 h, and 24 h) low-salinity-induced CA activity was diminished by 43% relative to intact controls ($P < 0.05$, one-way ANOVA, Tukey HSD) (Fig. 6).

Treatment with ESL did not alter the osmoregulatory ability of *C. sapidus* during low-salinity acclimation. Hemolymph osmolality in intact crabs was 764 mOsm/kg H_2O after 48 h at 15 ppt, while values in ESL-treated crabs were 750 mOsm/kg H_2O, compared to ambient values of 340 and 390 mOsm/kg H_2O, respectively (Fig 7). The same pattern persisted after 96 h, with intact and ESL crabs maintaining hemolymph of 733 and 753 mOsm/kg H_2O, and with ambient osmolality being 370 and 382 mOsm/kg H_2O, respectively (Fig. 7).

Localization of the repressor

In a final set of intact crabs, 35–15-ppt transfer produced a 5-fold increase in G7 CA activity from 132 to 656 μmol CO_2 mg protein^{-1} min^{-1} after 96 h and a concurrent 100-fold up-regulation in *CAc* mRNA (Fig. 8). Injection of isolated sinus gland (SG) homogenate harvested from 35-ppt-acclimated donor crabs (SG-35 ppt) reduced CA induction significantly by 49% ($P < 0.05$, one-way ANOVA, Tukey HSD), while medullary tissue (MT) injections did so nonsignificantly by 22% (Fig. 8). SG homogenate prepared from 5 ppt (SG-5 ppt) did not alter CA induction, with G7 specific activity in injected crabs being only 7% below that of intact controls (Fig. 8). Neither G7 nor G3 *CAc* expression was affected by injection of homogenates (Fig. 8). No SG, MT, or whole-eyestalk injection into intact crabs produced any changes in G3 CA activity relative to control animals (one-way ANOVA) (Fig. 8).

SG and MT injections had little effect on hemolymph osmolality. The 35–15 ppt transfers resulted in new stable hemolymph osmolality values of 658 mOsm/kg H_2O in intact control crabs (Fig. 9). When transfers were repeated with SG-35ppt, MT, and SG-5ppt animals, hemolymph was 681, 639, and 650 compared to respective ambient values of 442, 452, and 390 mOsm/Kg H_2O (Fig. 9).

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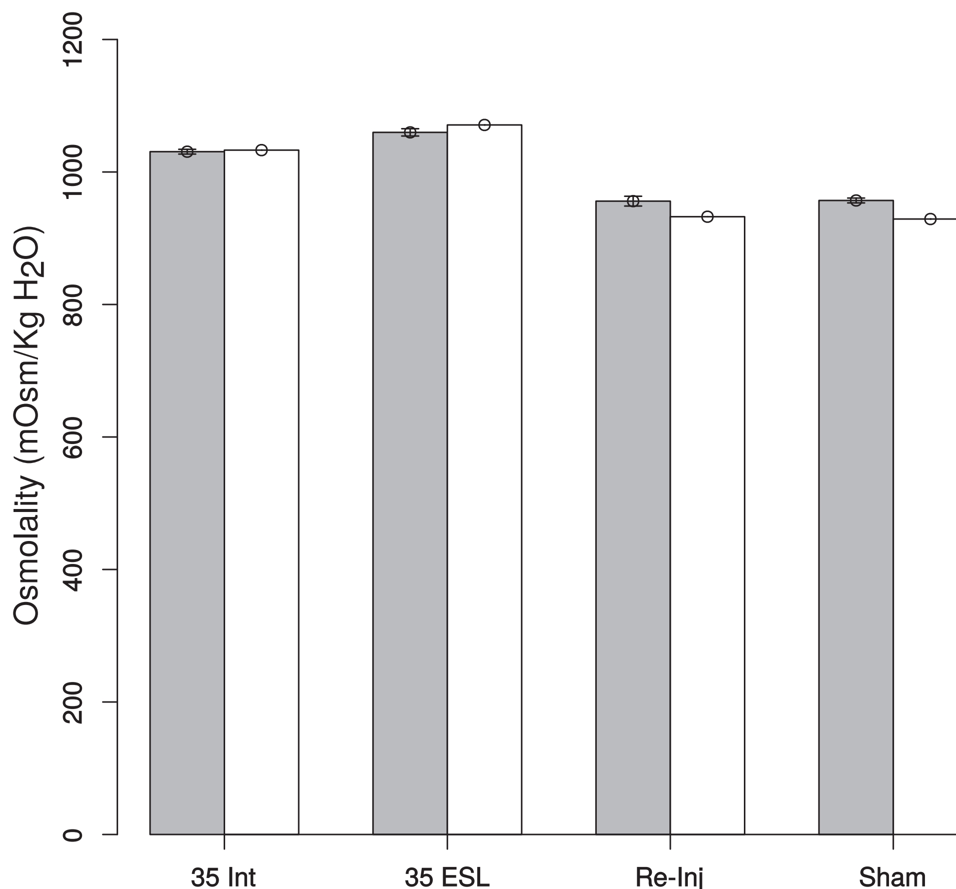

Figure 4. Osmolality of ambient seawater (white bars) and of hemolymph (gray bars) of *Callinectes sapidus* specimens acclimated to 35-ppt, Crabs were left intact (35 Int), treated with eyestalk ligation (35 ESL), or treated with eyestalk ligation followed by eyestalk re-injection (Re-Inj) or sham injections. Means ± SEM, $n = 6$.

Discussion

When crabs were acclimated to full-strength seawater (35 ppt) carbonic anhydrase (CA) activity and *CAc* mRNA were low and evenly distributed between anterior and posterior gills (Figs. 2, 5, 8), as has been reported in previous studies, indicating that all crabs were fully acclimated at both the biochemical and molecular levels to the osmoconforming state, regardless of the initial salinity from which they were collected (Henry, 2006b; Serrano *et al.,* 2007; Mitchell and Henry, 2014). Transfer from 35 to 15 ppt salinity produced the same degree of up-regulation of *CAc* mRNA expression (42-fold) and induction of CA activity (2.6 fold) in the posterior osmoregulatory gill pairs at 48-h post-transfer as has been reported previously for that time period, again validating the physiological responses of intact crabs to the experimental treatment (Figs. 5, 8) (Serrano *et al.,* 2007; Mitchell and Henry, 2014). There were no changes in either CA activity or *CAc* mRNA expression in anterior gills, confirming their use as a control tissue.

This study also confirms that eyestalk ligation (ESL) has the same effect on the CA induction mechanism as eyestalk ablation (ESA), thus validating that ESL functionally cut off the eyestalk from the crab. Both ESA and ESL produced elevations in CA activity exclusively in the posterior, ion-regulating gills of *Callinectes sapidus* (Fig. 2; see also Henry and Borst, 2006). In addition, both ESA and ESL resulted in large increases in *CAc* mRNA at 35 ppt salinity (Figs. 1, 2) and when combined with low-salinity transfer (30–100 fold) (Figs. 1, 5).

The positive effects of eyestalk ablation or ligation on branchial CA activity and *CAc* expression found in this study are similar to but much larger than those reported for eyestalk ablation in *Carcinus maenas* (Henry, 2006b; Henry and Campoverde, 2006).

It should be mentioned that eyestalk ablation induces molting in crabs (Mykles, 2001) and that CA activity increases during the pre-molt phase (Henry and Kormanik, 1985). However, it is unlikely that the stimulatory effects of ESL on CA in these studies are due to the induction of molting, for two reasons. First, molting causes elevation of

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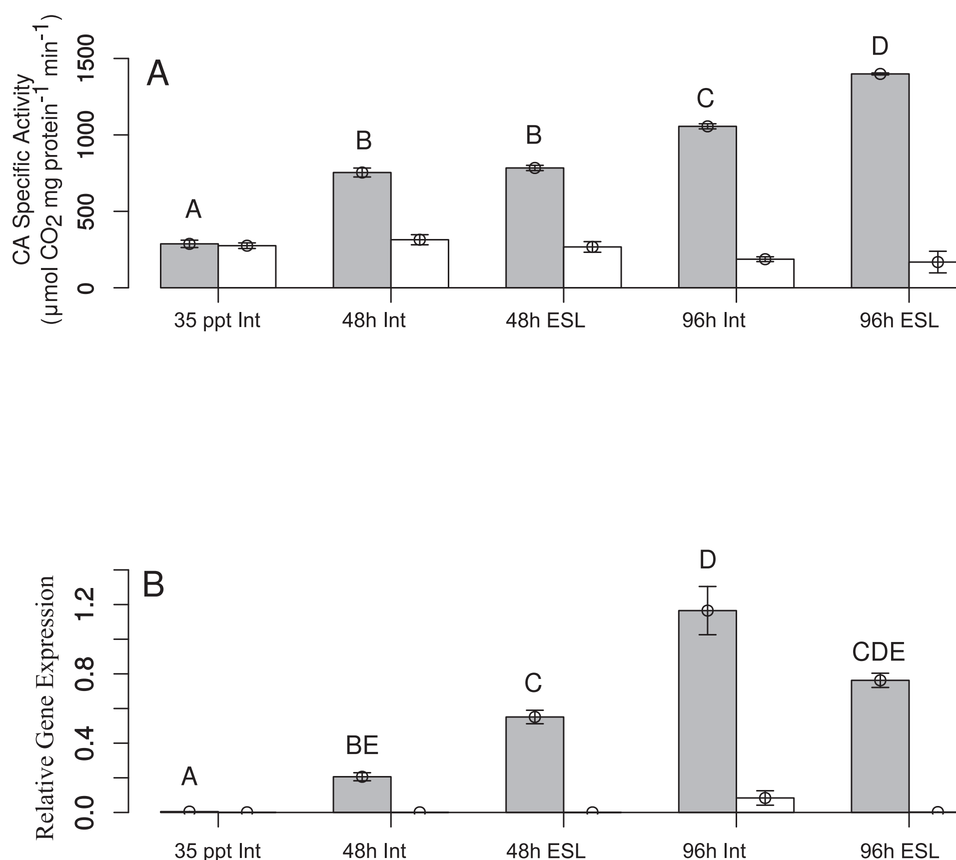

Figure 5. Carbonic anhydrase (CA) activity (A) and *CAc* gene expression (B) in the posterior G7 gills (gray bars) and the anterior G3 gills (white bars) of *Callinectes sapidus* speimens acclimated to 35 ppt (35 Int), and transferred to 15 ppt after 48 and 96 h. Crabs were either left intact (Int) or treated with eyestalk ligation (ESL). Different letters denote significant differences at the $P = 0.05$ level. Means ± SEM, $n = 6$.

CA activity in both the anterior and posterior gills (Henry and Kormanik, 1985), whereas ESL/ESA affects only G7 (Figs. 1, 2) (Henry, 2006b; Henry and Borst, 2006; Henry and Campoverde, 2006). Second, ESA-induced molting takes weeks to occur (Mykles, 2001), but in studies observing CA activity, increases occurred over a much more rapid time scale—48 h after the influence of the eyestalk was removed with ESA/ESL (Fig. 1) (Henry, 2006b; Henry and Borst, 2006; Henry and Campoverde, 2006).

The current experimental evidence shows the presence of a putative repressor substance in the eyestalk that specifically regulates CA expression/induction in the posterior gills. This is supported by (1) ESA/ESL in crabs acclimated to 35 ppt causes an increase in both *CAc* mRNA expression and CA activity even in the absence of a salinity challenge (Fig. 1), and (2) eyestalk re-injection into 35-ppt ESL crabs abolished the ESL-stimulated increases in CA activity and significantly reduced *CAc* mRNA expression (Fig. 2). Reversing the physiological effects of organ removal by the injection of organ extract is a classic method in endocrinology to demonstrate the presence and action of a putative hormone.

Removal of the CA repressor during both 35–28 and 35–15 ppt transfers *via* ESA and ESL, respectively, enhanced salinity-induced *CAc* gene activation (Figs. 1, 5). This demonstrates that ESA/ESL-stimulated CA induction is the result of gene activation, similar to what is believed to occur in *C. maenas*. Despite the fact that ESL resulted in a 5.6-fold increase in *CAc* mRNA in G7 of 35–15-ppt 48-h crabs relative to intact controls, there were no significant increases in CA activity at that time point (Fig. 5). This can be explained by the molecular limitations of salinity-stimulated CA induction present in euryhaline crustaceans. When crabs are transferred to low salinity, it usually takes 48–96 h for significant branchial induction of CA activity to occur, even though *CAc* mRNA up-regulation occurs within a few hours (Serrano *et al.,* 2007; Serrano and Henry, 2008; Mitchell and Henry, 2014). Furthermore, when crabs are acclimated to an intermediate salinity below the isosmotic point to elevate *CAc* mRNA, and are then moved to a lower

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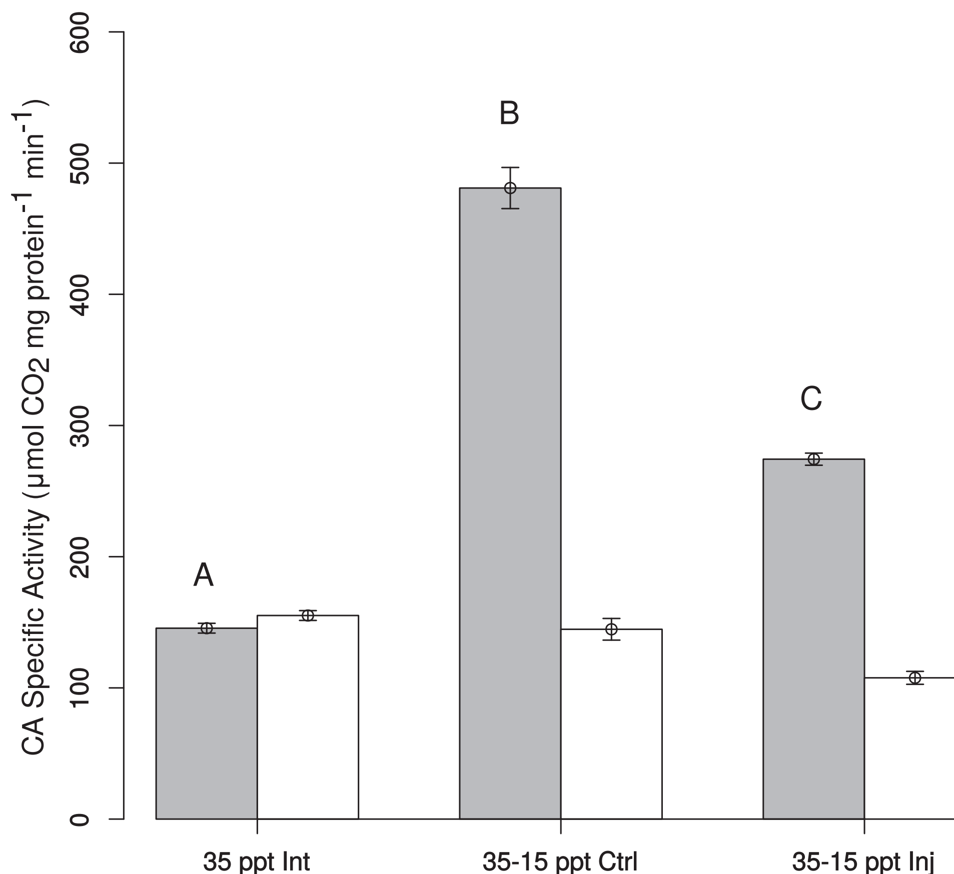

Figure 6. Carbonic anhydrase (CA) activity in the posterior G7 gills (gray bars) and the anterior G3 gills (white bars) of *Callinectes sapidus* specimens acclimated to 35 ppt (35 ppt Int), transferred to 15 ppt for 48 h and left intact (35–15 ppt Ctrl), and transferred to 15 ppt with eyestalk injections (35–15 ppt Inj). Different letters denote significant differences at the $P = 0.05$ level. Means ± SEM, $n = 6$

salinity, subsequent increases in CA activity still take on the order of days (Mitchell and Henry, 2014). Thus, even when there is adequate mRNA template, the onset of CA activity is slow to occur. This may be the result of limits in cellular zinc uptake, a required active site co-factor for CA activity (Mitchell and Henry, 2014). Therefore, in 35–15 ppt ESL crabs, 48 h after eyestalk ligation and transfer to 15 ppt, even though ESL eliminates the secretion of eyestalk hormones into the hemolymph, it may have been biochemically impossible for CA activity levels to increase any faster. This is validated by the fact that ESA enhanced hypoosmotic-stimulated CA induction by 343 μmol CO_2 mg protein^{-1} min^{-1} relative to control in 96-h post-transfer crabs, which presumably was adequate time for increased cellular zinc uptake and incorporation into the CA active site (Fig. 5).

Whole-eyestalk injection into intact crabs decreased the well-documented (Henry and Cameron, 1982a; Henry and Watts, 2001; Henry *et al.,* 2002; Serrano *et al.,* 2007; Mitchell and Henry, 2014) low-salinity-stimulated elevations in CA activity in G7 by 43% (Fig. 6). These data provide strong evidence that an endocrine substance with the capacity to inhibit CA induction exists in the eyestalk of *C. sapidus*. Specifically, this CA repressor hormone is likely to be localized to the sinus gland, as SG injection caused a 49% decrease in salinity-stimulated CA activity while MT injection triggered only a nonsignificant, 22% decrease (Fig. 8). The crustacean SG is the major endocrine complex located in the eyestalk; it acts as a storage and secretory center for many hormones including the crustacean hyperglycemic hormone (CHH) family of neuropeptides (see Christie *et al.,* 2010; Chung *et al.,* 2010; Christie, 2011; and Hopkins, 2012, for reviews). The localization of the repressor to this body, coupled to its inhibitory mechanism of regulation, strongly suggests that it could be a member of the CHH family. It is a possibility that the putative repressor is a member of an SG-localized hormone family besides CHH, such as the pigment dispersing hormones (PDH) or red pigment concentrating hormones (RPCH) (see Hopkins, 2012); however, the sheer number of distinct

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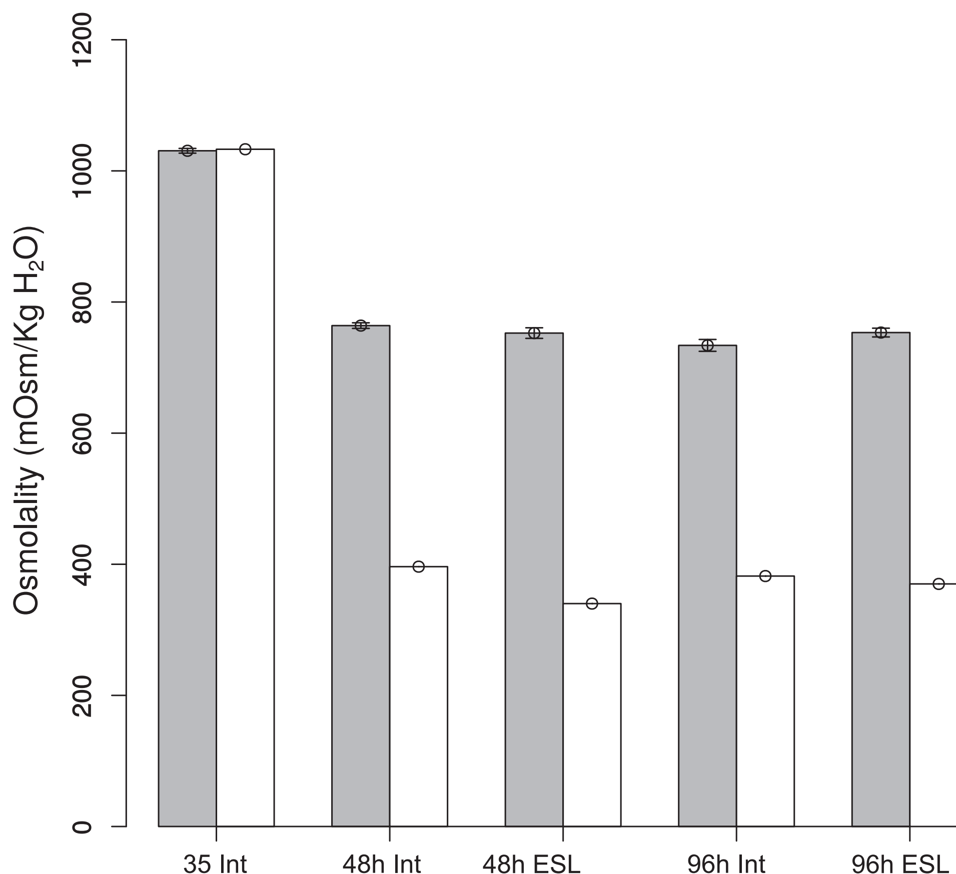

Figure 7. Osmolality of ambient seawater (white bars) and of hemolymph (gray bars) of *Callinectes sapidus* specimens acclimated to 35 ppt (35 Int) and transferred to 15 ppt after 48 and 96 h. Crabs were either left intact (Int) or treated with eyestalk ligation (ESL). Means ± SEM, $n = 6$.

CHH neuropeptides and their physiological diversity make it a more likely candidate. This can be validated when an amino acid sequence is obtained from the repressor.

As SG homogenate prepared from 5-ppt-acclimated crabs failed to inhibit CA induction, it can be concluded that the putative *C. sapidus* repressor hormone is down-regulated within the SG at low salinity, which then allows for salinity-stimulated CA induction to occur.

Despite the fact that medullary tissue (MT) injection resulted in inhibition of low-salinity-stimulated CA induction (Fig. 8), it remains likely that the repressor is localized to the SG instead of being uniformly found in the entire eyestalk. If the repressor were present ubiquitously throughout the eyestalk, one would expect to see more inhibition from MT injections as they contained much more tissue than the SG injections. This, however, was not the case; SG injections reduced salinity-stimulated CA by 49% while MT injections did so by only 22% (Fig. 8). The explanation for the slight CA inhibition caused by MT injections likely lies in the dissection technique. In this study, the SG was carefully excised from the neighboring MT and the two resulting tissues were homogenized separately. However, this would result in the presence in the MT sample of axons running from the x-organ to the SG. If the repressor is synthesized in the x-organ like many other crustacean neuropeptides, then these axons would likely contain significant amounts of the CA repressor hormone, resulting in inhibition of CA activity when injected into 35–15 ppt crabs. In the study by Henry (2006b), which found no inhibitory capacity of the MT in *C. maenas*, the SG was dissected out with some of the surrounding tissue believed to also contain the x-organ, while tissue for MT injection was taken from a more proximal section of the eyestalk which would not have contained any x-organ axons.

Unlike the effects on CA activity, re-injection of eyestalk homogenates into ESL crabs at 35 ppt did not completely eliminate the increased levels of *CAc* expression (Figs. 2, 8). This may be due to the speed and scale at which *CAc* mRNA responds to external stimuli. Environmental dilution results in a 15–100-fold up-regulation of *CAc* mRNA after just 6 h (Serrano *et al.,* 2007; Serrano and Henry, 2008; Mitchell and Henry, 2014). Given the rate at which this

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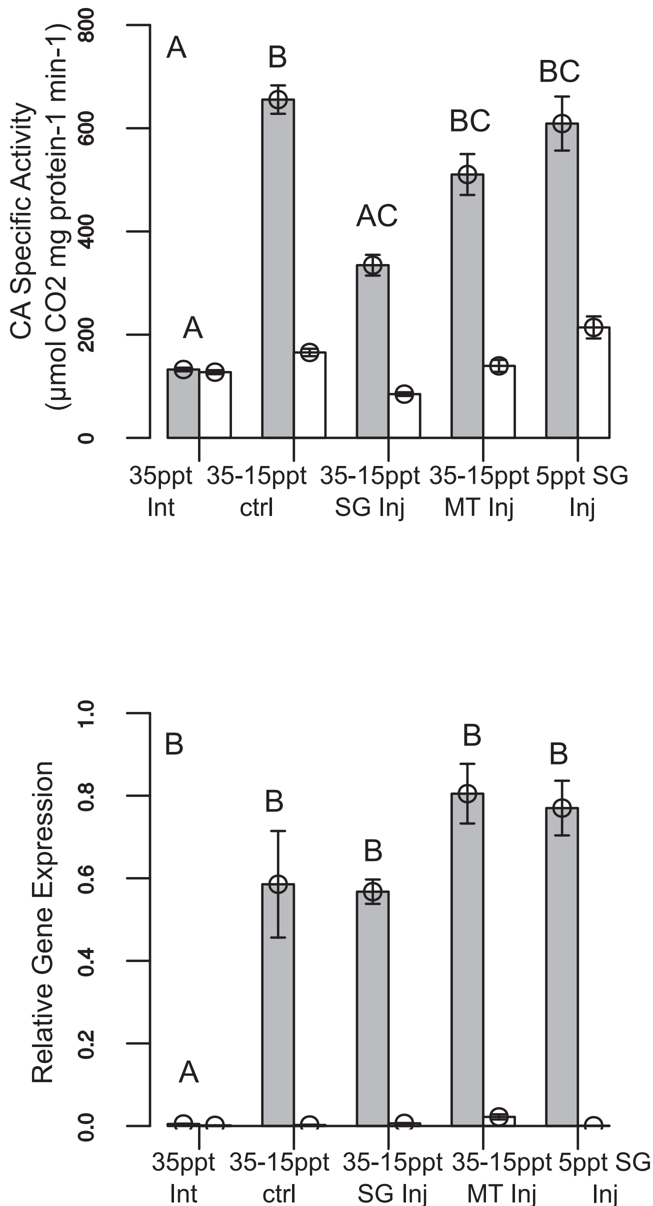

Figure 8. Carbonic anhydrase (CA) activity (A) and *CAc* expression (B) in the posterior G7 gills (gray bars) and the anterior G3 gills (white bars) of *Callinectes sapidus* specimens acclimated to 35 ppt (35 Int), 35–15 ppt intact control (35–15 Ctrl), and 35–15 ppt injections with medullary tissue (MT), and sinus gland (SG) harvested from animals acclimated to 35 and 5 ppt. Different letters denote significant differences at the $P = 0.05$ level. Means ± SEM, $n = 6$.

gene is induced, it may be that there was not enough time for the repressor factors in the eyestalk homogenates to take effect on the gill tissue before the initial rise in *CAc* expression occurred. This would result in the presence of branchial *CAc* in our samples, which was detected by qPCR. The failure of this template mRNA to result in enhanced CA activity may indicate a lack of cellular zinc uptake occurring in the mitochondria-rich cells (see above), or there may be another, as yet undiscovered mechanism of inhibitory regulation involved.

ESL and injection of eyestalk homogenates did not affect hemolymph osmolality in *C. sapidus* specimens acclimated to full-strength seawater or low salinity (Figs. 3, 6, 8). This is possibly a result of incomplete inhibition of CA induction, as the SG injections inhibited salinity-mediated CA induction by only 49% (Fig. 8). Similar results were found in *C. maenas* specimens given daily injections of SG homogenate: CA inhibition did not depress hemolymph osmolality at low salinity (Henry, 2006b). However, hemolymph osmolality decreased by 10% when SG injections were administered twice daily (Henry, 2006b). These experiments have yet to be repeated in *C. sapidus* but will be an important step for future work, as they would not only further demonstrate the critical nature of branchial CA activity for ionic uptake, but would also further characterize the CA repressor by showing dose-dependent effects.

Many studies performed in crustaceans have demonstrated a link between the eyestalk and osmotic regulation. Interestingly, the results of ESL/ESA on these organisms tend to vary in different crustacean taxa. In the Dendrobranchiata, ESA tends to increase hemolymph osmolality at both full-strength seawater and low salinity, suggesting that it turns on an ionic uptake mechanism (Nan *et al.,* 2004). Conversely, in caridean shrimp, ESA decreases hemolymph osmolality and Na^+ influx at low salinity (Nagabhushanam and Jyoti, 1977; McNamara *et al.,* 1990), suggesting that an eyestalk factor is required for ionic uptake. In the Brachyura, there is no effect of ESL/ESA on hemolymph osmolality in either high or low salinity (this study, Henry, 2006b; Henry and Borst, 2006; Henry and Campoverde, 2006); however SG extracts stimulate Na^+ influx and increase transepithelial potential in perfused gills (Eckhardt *et al.,* 1995; Spanings-Pierrot *et al.,* 2000). In marine astacideans, ESA does not affect hemolymph osmolality or ionic concentrations until the animal has molted (Charmantier-Daures *et al.,* 1994), while in freshwater crayfish ESA decreases hemolymph osmolality and Na^+ concentration, with these changes being significantly mitigated by injection with CHH (Serrano *et al.,* 2003). The diversity of the responses of eyestalk manipulation demonstrates the plasticity of the eyestalk neuropeptides in regulating physiological functions, which makes the necessity of a more complete characterization of the CA repressor critical in understanding the evolution of crustacean endocrinology.

In addition to the positive effect on *CAc* gene expression in this study, ESL also increased *NaK* mRNA, which codes for the α-subunit of Na^+/K^+-ATPase, the enzyme that powers active transport in branchial ionic uptake (see Towle, 1984; Towle and Weihrauch, 2001; Henry *et al.,* 2012; and McNamara and Faria, 2012). ESL resulted in a 3-fold up-regulation of *NaK* mRNA in G7 with no change

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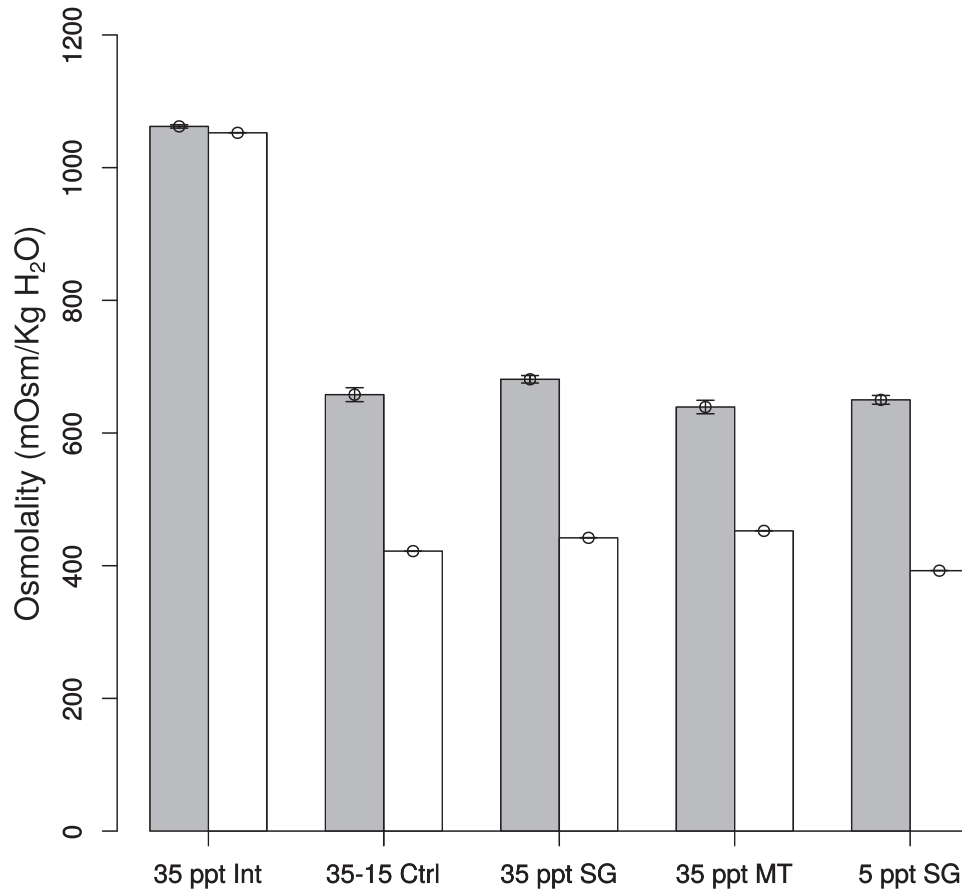

Figure 9. Osmolality of ambient seawater (white bars) and hemolymph (gray bars) of *Callinectes sapidus* specimens acclimated to 35 ppt (35 Int), 35–15 ppt intact control (35–15 Ctrl), and 35–15 ppt injections with medullary tissue (MT), and sinus gland (SG) harvested from animals acclimated to 35 and 5 ppt. Means ± SEM, $n = 6$.

in G3 (Fig. 2). The magnitude of the ESL-stimulated *NaK* and *CAc* gene activation is similar to values reported for hypoosmotic-stimulated expression increase of these genes (Serrano *et al.,* 2007). No significant change in expression was observed in *CAg*, a membrane-bound CA isoform, which is relatively insensitive to salinity changes and is believed to function mainly in CO_2 excretion (Fig. 2) (Serrano *et al.,* 2007; Serrano and Henry, 2008).

The absence of large-scale *CAg* up-regulation demonstrates that ESL-stimulated increases in CA activity are primarily due to the enhanced transcription of only one CA isoform, *CAc*. Furthermore, coupled with the lack of change in gene expression in *NaK* or *CAc* in the anterior gills, the static nature of *CAg* suggests that the putative repressor may only affect genes and tissues involved in ionic uptake. Future studies utilizing whole-transcriptome shotgun sequencing and differential expression analyses to estimate the relative expression of multiple gene clusters in response to ESL/ESA will be helpful to confirm this hypothesis. Additionally, measurement of branchial Na^+/K^+-ATPase activity in response to ESL/ESA could shed some light onto the regulation of Na^+/K^+-ATPase, specifically by the x-organ–sinus-gland complex.

In conclusion, in this study we demonstrate the presence of a carbonic anhydrase repressor hormone localized to the sinus gland of the euryhaline marine crab *Callinectes sapidus*. This substance is down-regulated at low salinity, allowing for *CAc* gene activation and the subsequent induction of CA to occur, forming the molecular basis of active ionic uptake across the gills. The next steps in characterizing neuroendocrine regulation of CA in *C. sapidus* will be to subject eyestalk homogenates to heat, acid, and protease treatments to determine if the repressor hormone is indeed a small peptide like the members of the CHH family. If the hormone is a peptide, then the molecular components of the eyestalk can be separated with high performance liquid chromatography and injected separately to discover the specific molecule(s) responsible for CA regulation in euryhaline crustaceans. Following this, the resulting peptide(s) can be sequenced and the homology to CHH can be investigated.

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