Transcriptional Adaptations to Low Salinity in Euryhaline Crustaceans

by

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Abstract

The transfer of euryhaline crustaceans from full-strength seawater to low salinity is known to induce a myriad of physiology changes corresponding with active ion uptake across the posterior gill pairs. The goal of this thesis will be to answer several specific questions pertaining to the activation of ionic regulation processes in euryhaline crabs. The first chapter of this work contains a literature review of crustacean osmoregulation and a detailed description of the experiments used. The second chapter is a study where I measure the time course of carbonic anhydrase (CA) activity relative to gene expression in the euryhaline crabs *Callinectes sapidus* and *Callinectes sapidus*. A physiological characterization of the neuroendocrine regulation of CA activity in *C. sapidus* composes the third chapter. Finally, in the fourth chapter I present and RNA-seq differential gene expression analysis of *C. sapidus* gills acclimated to high and low salinity.

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Chapter 4

Chapter 1. Review of Literature and Introduction to Thesis

One of the major environment factors affecting the distribution of aquatic organisms is ambient salinity. Since many aquatic animals, especially invertebrates, are quite permeable to the external waters, the concentrations of solutes in the environment have the potential to directly affect the physiology of the animal. In the open ocean, salinity tends to be quite stable, usually ranging from 32 to 37 ppt (Allegra, 2010). Closer to the shore, however, several factors, such as dilute freshwater run-off from land, can result in a much more dynamic salinity environment. If a union between freshwater and ocean water occurs in a partially enclosed area, where the potential for large-scale salinity fluctuations increases, it is known as an estuary (Pritchard,1967).

An estuarine salinity gradient can be quite profound, ranging from less than 0.5 ppt at the head, where freshwater enters, to up to 40 ppt at the mouth, where the estuary contacts the open ocean . Further adding to the variation are the effects of salt and freshwater influxes resulting from tides and increased runoff, respectively, which have the potential to shift the salinity in the entire gradient in one direction or the other.

Despite the chaotic nature of estuaries, they represent some of the most productive ecosystems on the planet. The confluence of water from the land and ocean results in a rich supply of nutrients which bolsters extremely high levels of primary production (Underwood and Kromkamp, 1999). Additionally, estuaries serve as nursery areas for many species of marine birds, fish, and invertebrates (Gillanders et al., 2003). Given the high levels of biomass found in

estuarine systems, it is not surprising that these environments are often utilized by opportunistic feeders such as crustaceans.

Marine crustaceans have two general strategies for coping with salinity stress in dilute environments such as an estuary; osmoconformity and osmoregulation. Osmoconformers passively maintain hemolymph (mixture of blood and interstitial fluid) osmolality and ionic concentrations equal to the surrounding medium while osmoregulators employ physiological pathways to actively maintain hemolymph hypertonic to the environment. These are not strict categories; rather there exists a gradient between the two, with large variations in the magnitude of osmoregulation between different taxa (See Mantel and Farmer, 1983 for review). Additionally, a given species may employ either strategy at different times: for example, almost all marine crustaceans are osmoconformers at full-strength seawater, but at critical salinity levels (usually 25-26 ppt), some species switch to osmoregulation.

The gills of marine crustaceans in general are extremely permeable to the environment; thus hyposaline waters lead to large-scale osmotic influxes and ionic effluxes (Baldwin and Kirschner, 1976; Cameron 1978; Pierrot et al., 1995; Riestenpatt et al., 1996; Onken et al., 2003). This is compounded by the fact that crustacean urine is generally isosmotic with hemolymph; thus excretion at low-salinity results in ion loss. Marine crustaceans produce copious amounts of this urine at low salinity to volumetrically compensate for osmotic water influxes. Thus, urinary excretion contributes up to 43 and 31% of the total Na^+ and Cl^- loss, respectively, in some species (Cameron and Batterton, 1978).

Without compensatory mechanisms, these ion losses through the gill and urine would result in hemolymph dilution and cellular swelling. Marine crustaceans are able to manage cell swelling via the cellular efflux of organic osmolytes to reduce the osmotic gradient between the cell and the dilute hemolymph (See Pierce, 1982; Chamberlain and Strange, 1989 for reviews). Both osmoregulators and osmoconformers are capable of employing this strategy to reduce cell swelling; however, osmoregulators take low-salinity adaptation one step further by utilizing ionic uptake mechanisms to counteract hemolymph dilution.

Crustacean ionic uptake occurs across the gills in specialized mitochondria-rich cells (MRC). It is the concerted action of several proteins in the MRC that facilitates the transport of ions from the environment, across the apical gill membrane, into the cytoplasm, across the basolateral membrane, and finally into the hemolymph (see Henry et al., 2012 for review). Na^+ and Cl^- first enter the cell when they are transported from the water and into the cytoplasm via the action of an apical $Na^+/K^+/2Cl^-$ cotransporter (Ristenpatt et al., 1996). An apical K^+ channel facilitates K^+ efflux across the apical membrane; this establishes a net negative charge inside the cell, driving Cl^- through channels on the basolateral membrane and into the hemolymph (Riestenpatt et al., 1996; Onken et al., 2003; Lucu and Towle, 2010). Na^+ is pumped into the hemolymph via the action of the basolateral membrane-bound Na^+/K^+-ATPase (Lucu and Siebers, 1987; Luquet et al., 2002; Onken et al., 2003; Lucu et al., 2009; Lucu and Towle, 2010). K^+ is recycled through basal K^+ channels. Movement of Cl^- across basal membrane is also believed to passively follow the electrical gradient created by the pumping of Na^+. In addition to this pathway, NaCl uptake also occurs through apical Na^+/H^+ and Cl^-/HCO_3^- exchange proteins (Lucu, 1990; Onken et al.,

2003; Henry, 2001; Henry et al., 2002, 2006). The H^+ and HCO_3^- that serve as counterions for ionic exchange are provided by the hydration of respiratory CO_2 by cytoplasmic carbonic anhydrase (CA).

Interestingly, the degree to which crabs rely on Na^+/H^+ and Cl^-/HCO_3^- exchange seems to vary. For example, in *Chasmagnathus granulata*, treatment with the CA inhibitor acetazolamide eliminates only 10-20% of Na^+ influx in perfused gills (Onken et al., 2003), while in the blue crab *Callinectes sapidus* it completely eliminates net Na^+ and Cl^- influx (Cameron, 1979). Thus, it seems that two different strategies of NaCl uptake have evolved in marine crustaceans; that found in *C. sapidus* which relies primarily on Na^+/H^+ and Cl^-/HCO_3^- exchange and the Na^+/K^+-ATPase, and that found in *C. granulata* which utilizes some Na^+/H^+ and Cl^-/HCO_3^- exchange, but depends mostly on Na^+/K^+-ATPase and the $Na^+/K^+/2Cl^-$ cotransporter (Onken et al., 2003).

In order to overcome the large amount s ionic loss through the gills and urine, marine crustaceans must have even higher levels of ionic uptake across the gill, which requires large increases in the activities of CA and/or Na^+/K^+-ATPase in low-salinity waters. The importance of this was demonstrated in *C. sapidus*, which absorbs the bulk of NaCl through Na^+/H^+ and Cl^-/HCO_3^- exchange, by injecting acetazolamide during the acute phase of low salinity acclimation which resulted in 100% mortality (Henry and Cameron, 1982).

When acclimated to full-strength seawater, branchial ion uptake processes are silent, as the animal is in a state of osmoconformity. It is only upon entering areas of low salinity (generally below 26 ppt) that these cellular mechanisms activate (Henry, 2005). The activation of these processes is best seen by increases in the activities of enzymes directly involved in ionic uptake

across the gills. By far the two best studies enzymes in terms of low salinity induction are the Na^+/K^+-ATPase and CA (Towle et al., 1976; Henry and Cameron, 1982a; Henry, 1984; Holiday, 1985; Wheatly and Henry, 1987; Corratto and Holiday, 1996; Mananes et al., 2000; Henry et al., 2002, 2003; Roy et al., 2007; Tsai et al., 2007).

The transition from basal levels of CA activity in the gills of crabs acclimated to full-strength seawater, to the high levels found in osmoregulating crabs has been closely studied for several decades. In *C. sapidus* acclimated to full-strength seawater, CA activity is evenly distributed among all gill pairs except for the two anterior-most gill pairs, where it is significantly lower (Henry and Cameron, 1982b). Henry (1988a) used density-gradient centrifugation to separate the cellular components of *C. sapidus* gills and found significant CA pools in both the cytoplasm and the microsomes (vesicle-like artifacts formed from the plasma membrane following centrifugation). Interestingly the cytoplasmic fraction was much more responsive to low salinity, increasing by 10-fold following environmental dilution, compared to 1.5-2-fold for microsomal CA (Henry, 1988a). This experiment was later repeated in *C. maenas* with similar results (Henry et al., 2003). Based on this data, it was concluded that within the crustacean gill, there exists at least two pools of CA activity, associated with the cytoplasm and the basal-lateral membrane. The cytoplasmic pool, being salinity-sensitive, is responsible for ionic uptake at low salinity, while the membrane-bound pool catalyzes the dehydration of hemolymph HCO_3^- to CO_2 to facilitate excretion across the gill.

During low-salinity acclimation it is only the posterior 3-4 gill pairs that experience increases in CA activity (Henry, 1984; Towle, 1984; Mananes et al., 2000*)*. These tissues are highly

specialized for ionic uptake, having highly folded basolateral membranes and a dense population of MRC with high Na^+/K^+-ATPase activity (see Henry et al., 2012 for a review).

Salinity-induced CA induction is slow to occur. In *C. sapidus* and *C. maenas* 24-96 hr at low salinity are required for any detectable increases in CA activity, which are usually around 2 or 3-fold (Henry and Cameron, 1982; Henry and Watts, 2001; Henry et al., 2002; Henry et al., 2003, Henry, 2005; Serrano et al., 2007; Serrano and Henry, 2008, Mitchell and Henry, 2014). However, CA activity continues to increase in these crabs until 4-7 days post-transfer, eventually reaching 7-15 fold above full-strength seawater levels (Henry and Watts, 2000; Henry et al., 2002; Serrano et al., 2007; Serrano and Henry, 2008). CA activity has never been observed to decrease in low-salinity acclimated crabs following the initial induction.

Advances in molecular biology over the last two decades have shed additional light on the mechanism of induction of CA. Specifically, the advancement of cloning and sequencing techniques have allowed for the discovery of the genes coding for CA isoforms in multiple species, and the development of the quantitative polymerase chain reaction (qPCR) has permitted the high-resolution study of the expression of these genes during low-salinity acclimation. It is now known that there are at least two different isoforms of CA expressed in the crustacean gill, coined CAc and CAg, which are encoded by two different genes (Serrano et al., 2007; Serrano and Henry, 2008).

The CAg isoform cDNA sequence contains a glycosyl-phosphoinositol (GPI) link, a motif which anchors proteins to the plasma membrane (Ferguson, 1999). The CAc isoform lacks this feature and was highly homologous to cytoplasmic type II CAs in multiple other taxa (Serrano et

al., 2007; Serrano and Henry, 2008). This, coupled with the fact that cellular CA activity is divided between the microsomes and cytoplasm (Henry and Cameron, 1982), suggests that these two reservoirs of enzymes are supplied by the transcription of distinct genes.

While the CAg isoform is more highly expressed at full-strength seawater , it is quickly overtaken by CAc following environmental dilution: 15 ppt transfer elicits a 100-fold increase in CAc mRNA in *C. sapidus*, compared to a 6-fold increase for CAg (Serrano et al., 2007). The pattern of mRNA regulation closely mirrors that of the actual enzymes themselves. Inhibitor titration of microsomal and cytoplasmic gill fractions revealed that free CA enzyme concentration in G7 is higher in the microsomes at 35 ppt, however at 15 ppt free enzyme rises by 66-fold in the cytoplasm compared to only 3-fold in the micrsosomes (Serrano et al., 2007). A similar pattern of CA activity division and sensitivity has also been reported in *C. maenas* (Henry et al., 2003).

The identification of the CA isoform responsible for assisting in ionic uptake (CAc) has allowed for the concurrent observation of CA mRNA and enzyme activity in the same individual. In *C. sapidus*, CAc mRNA increased by 100-fold 12 hr after 35-15 ppt transfer and remained relatively stable (Serrano et al., 2007). In the same sample, CA activity responded more slowly to environmental dilution, with the first significant increases taking 24 hr to occur. Additionally, compared to the rapid maximal up-regulation of CAc mRNA, CA activity takes much longer to reach acclimated levels, increasing again between 24 and 48 hr, and once more after 7 days (Serrano et al., 2007). A similar pattern of rapid maximal CAc gene activation followed by gradual increases in enzyme activity was reported for *C. maenas*, except that mRNA increased

after only 6 hr, and 7 days were required for CA activity to reach maximal levels (Serrano and Henry, 2008). The fact that gene activation precedes increases in enzyme activity provides strong evidence that increases in CA activity in the gills of euryhaline crustaceans results from activation of the CAc gene and subsequent translation of CA enzyme. The observed increases in free CA enzyme concentration in both *C. sapidus* and *C. maenas* support this model.

The identification of the CA isoform responsible for ionic uptake and the measurement of its expression following low salinity transfer was a huge step forward in understanding the role of CA in euryhaline crustaceans; however several critical aspects of CA induction in these animals have yet to be addressed. One such topic is the lag in the onset of salinity-stimulated CA activity relative to CAc gene expression. Serrano et al. (2007) and Serrano and Henry (2008) found that gene activation precedes elevations in CA activity by at least 12 hr, and that 96 hr–7 days are required for acclimated levels of CA activity to be reached. Thus it seems likely that there exists a molecular limitation on the rate at which mature CA enzyme can be produced that is independent of gene expression. It is unlikely that this is simply due to the time required to translate new protein, as some the activity of some salinity-induced enzymes such as ornithine decarboxylase in *Artemia fascia* are capable of doubling in 30 min (Watts et al., 1996). It may be that the delays in CA activity induction are the result of limitations in Zn transport. CA requires a Zn atom to be bound to the active site in order to perform catalysis (McCall et al., 2000). In order to bind Zn to the large cytoplasmic pool of CA, it presumably must be extracted from food in the gut, transported to the gills, and taken into the MRC. Cellular uptake is very likely accomplished via the action of Zip proteins. These proteins have been implicated as the primary mechanism for

zinc transport and regulation in yeast, plants, *Drosophila*, and mammals (Guerinet and Eide, 1999; Liuzzi and Cousins, 2004; Matthews et al., 2005). There are currently no published studies which have examined the possibility of CA limitation via Zn.

Another remaining question in salinity-stimulated CA induction is the cause of CA activity increases following multiple instances of environmental dilution. For example when osmoregulating crabs are acclimated to progressively lower salinities (e.g. 25 then 15 ppt) the degree of CA activity induction is progressively greater in the lower salinities (Henry and Watts 2001, Henry 2005). It is currently not known if these increases in enzyme activity result from enhanced gene expression or if they come about by increased production of CA protien from the same pool of mRNA.

The first goal of this thesis will be to answer these questions in two commonly employed models of crustacean osmoregulation; *C. maenas* and *C. sapidus*. Crabs will be acclimated to salinities below their isomotic point for 1 week to activate ionic uptake processes including the up-regulation of CAc mRNA and the induction of CA activity. CA activity and gene expression will be measured throughout the week, using the delta pH method (Henry, 1991) and qPCR (Serrano et al., 2007; Serrano and Henry, 2008) with particular attention being paid to the first significant increase of CA activity above baseline. Following this, crabs will be transferred to even lower salinities to induce further induction of CA activity. CA activity will again be measured over the course of 1 week to determine if prior elevation of CAc mRNA and priming of the CA induction mechanism reduce the time required for significant increases in CA activity. CAc gene expression will be monitored to see if increased CA activity at low salinity is a result

of increased CAc transcription. Additionally, the expression of the Zn transporter ZIP1 will be measured by qPCR at several timepoints to observe any changes in of the molecular machinery responsible for cellular Zn uptake. This will be helpful in determining if limitations of Zn transport play a possible role in the timecourse of CA induction.

In addition to the relationship between gene activation and enzyme activity following low salinity transfer, this thesis will also address the physiological mechanisms by which CA activity and gene expression are triggered in low salinity. In studies by Henry and Campoverde (2006) and Henry (2006a) the removal of the eyestalks (eyestalk ablation, ESA) in *C. maenas* resulted in a 2-fold induction of CA activity at full-strength seawater and an up-regulation of CAc mRNA in the posterior ion-regulating gills. These results were particularly exciting, as branchial CA induction at full-strength seawater had only been previously observed in molting crabs (Henry and Kormanik, 1985), however molting-induced changes occur in both anterior and posterior gills, while 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 of the homogenate into ESA crabs abolished the effect of ESA, suggesting that the absence of a chemical substance triggered CA activity in ESA crabs (Henry and Campoverde, 2006). Other experiments in *C. maenas* showed that injection with eyestalk homogenate taken from animals acclimated to full-strength seawater into crabs transferred from 32-15 ppt reduced salinity-stimulated CA activity. When this procedure is repeated but the eyestalks are taken from crabs acclimated to low salinity there is no effect on CA activity, suggesting that the endocrine substance is down-regulated at low salinity

(Henry, 2003). Taken together, these data suggest that there is a hormone in the eyestalk that inhibits CA activity and expression at high salinity. When crabs are acclimated to low salinity, this hormone is down-regulated, allowing for the induction of salinity-stimulated CA activity. 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 (Bliss and Welsh, 1952; Carlisle and Passano, 1953). Other experiments have shown this putative eyestalk hormone is heat and acid stable for only a short period of time (2.5 minutes), suggesting that it is a small peptide (Mykles, 2001; Henry, 2004).

An initial biochemical characterization of this putative hormone in *C. maenas* suggests that it is a member of the Crustacean Hyperglycemic Hormone (CHH) family of neuropeptides. These neuropeptides are synthesized in the x-organ (XO) and travel by axoplasmic flow to the sinus gland (SG), where they are released into the hemolymph (see Hopkins, 2012 for review). These proteins play key roles in several physiological processes in crustaceans including metabolism, molting, gonadal development, and osmoregulation (see Chung et al., 2010 for review). Interestingly, like the putative CA repressor hormone, most of the CHH families tend to be inhibitory. For example, molting-inhibiting hormone (MIH) is normally secreted from the XOSG and then binds to receptors on the y-organ (YO) where it inhibits the synthesis of ecdysteroids. MIH is down-regulated during the pre-molt phase, which eliminates the inhibition of the YO and results in ecdysteroid release. These ecdysteroids then promote molting (see Chang and Mykles, 2011 for review). Similarly, crustacean hyperglycemic hormone (CHH), the namesake of this peptide family, is released from the XOSG in times of increased energy demand, binding to

receptors on the hepatopancreas where it activates a signal cascade which inhibits glycogen synthase, thus raising levels of hemolymph glucose (Seidlmeier, 1985).

If the putative CA repressor hormone is in fact a CHH neuropeptide, it would not be the first that this hormone family has been implicated in osmoregulation. However, most previous studies of these peptides have suggested a stimulatory role on osmoregulation, rather than the inhibitory effects of the putative CHH repressor. CHH peptides were first implicated in osmoregulation with the observation that sinus gland (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 resulted in decreases in hemolymph osmolality and Na^+ concentration, and these changes were significantly mitigated by injection with CHH (Serrano et al., 2003).

Members of the CHH family involved in osmoregulation may be expressed in areas other than the XOSG. 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). In a study by Tiu et al., (2007), a cDNA homologous to insect ITP (LvITP) was found in the shrimp *Litopenaeus vannamei.* The expression profile of this gene was similar to other genes implicated in osmoregulation, such as CAc and and the Na^+/K^+-ATPase α-subunit in that it was expressed most highly in the posterior gills, and mRNA up-regulation occurred following low salinity transfer (Tiu et al., 2007).

With the probable role of members of the CHH family in osmoregulation, and the presence of a putative CHH-like repressor of CA in the *C. maenas* SG, it seems likely that the CHH family plays a role in the regulation of CA induction at low salinity in *C. maenas*. Additionally, there

exists evidence that a similar system of regulation exists in *C. sapidus*; in a study by Henry and Borst (2006) eyestalk ablation resulted in significant increases in CA activity in the posterior gills but not in the anterior gills. With this in mind, the second goal of this thesis is to characterize the neuroendorine regulation of CA activity in *C. sapidus*.

The first step to achieving this goal will be to repeat the experiments of Henry and Borst (2006) and observe the effects of ESA on *C. sapidus* acclimated to 35 ppt. Then, ESA crabs will be re-injected with eyestalk homogenate to eliminate the effects of ESA to determine if the absence of a chemical(s) in the eyestalk is responsible for ESA-induced CA activity. Additionally, branchial CAc expression will be measured to determine if any changes are the result of gene activation.

Next, crabs transferred from high to low salinity (35–15 ppt) will be treated with eyestalk injections taken from 35 ppt crabs, to demonstrate that the putative repressor hormone can inhibit CA induction in intact crabs. This will be repeated using eyestalks from 5 ppt acclimated crabs and if it fails to prevent CA induction it will show that this substance is down-regulated at low salinity. This experiment will be repeated again using homogenate made from the SG vs the surrounding eyestalk tissue (medullary tissue) to determine if the repressor is localized to the SG as it is in *C. maenas*.

If my thesis experiments successfully demonstrate the presence of a repressor hormone the next steps will be to observe if the repressor is heat and acid stable for short periods, as it is in *C. maenas* (Henry, 2004), which will determine if the molecule is a small peptide. Eventually,

HPLC can be performed on SG's and different fractions can be injected into 35-15 ppt animal to identify the actual molecule responsible for CA inhibition in euryhaline crabs.

Although CA plays a central role in osmoregulation in crustacean gills, it is important to remember that it is only one component in a complex mechanism of ionic uptake. Therefore, in order to draw a more complete picture of the molecular changes which occur in crustacean gills following environmental dilution, more broad-scale techniques than the measurement of the activity and expression of a single gene must be employed. Such analyses have been performed in several species using DNA microarrays to quantitatively measure changes in the relative abundance of complementary transcripts in cDNA libraries.

The first study of this nature was performed by Towle et al. (2011) on the posterior ion-regulating gills of *C. maenas* transferred from full-strength seawater to low salinity. Unsurprisingly, this study found significant up-regulation of CAc and the Na^+/K^+-ATPase α-subunit, which have been well established in this species (Henry et al., 2006; Serrano and Henry, 2008). Additionally, the expression of a voltage-gated Ca^{2+} channel, a Na^+ glucose co-transporter, and an organic cation transporter increased at low salinity, Surprisingly, mRNAs encoding the $Na^+/K^+/2Cl^-$ co-transporter and the Na^+/H^+ exchange protein which play direct roles in ion uptake across the apical membrane, remained unchanged following low salinity transfer. The authors also examined the expression of Heat Shock Proteins (HSPs) which assist with protein folding and trafficking in times of cellular stress (see De Maio, 1999 for review). Barely any changes in the relative expression of these genes were detected during acclimation from high to low salinity,

suggesting that low salinity exposure is a routine encounter in *C. maenas* to which it has become highly adapted to handle (Towle et al., 2011).

A similar study by Xu and Liu (2011) reported microarray-detected changes in the swimming crab *Portunus trituberculatus*. In contrast to *C. maenas*, environmental dilution in *P. trituberculatus* resulted in significant induction in HSP expression and other genes functioning in cellular stress management. Additionally, there was no significant upregulation of the Na^+/K^+-ATPase α-subunit, however the β-subunit of this protein did significantly increase. Sadly, the microarray employed in this study used very few probes complementary to ion-uptake proteins, so there were no measurements of CAc, the $Na^+/K^+/2Cl$-co-transporter, or the Na^+/H^+-exchanger expression.

Recently, DNA microarrays have fallen out of favor and are rapidly being replaced with whole transcriptome shotgun sequencing, known as RNA-seq. Unlike microarrays, which require specific oligonucleotide probes to analyze a given gene, RNA-seq involves the reverse transcription and subsequent sequencing of every expressed gene in a given sample. Thus with RNA-seq the downstream analysis of any expressed gene of interest in a specific tissue is possible as opposed to DNA microarrays, which are reliant on a-priori knowledge of sequences.

RNA-seq of the *Portunus trituberculatus* ionoregulatory gills would reveal that the expression of CAc, Na^+/H^+-exchanger, a Na^+ glucose co-transporter, and chloride channel protein 2 increased significantly following environmental dilution, by 4.73, 2.56, and 2.8-fold, respectively (Lv et al., 2013). Additionally, contrary to Towle et al. (2009) the expression of HSP 70 increased at low salinity. This agrees with the microarray-based study in *P. trituberculatus* by

Xu and Liu (2011), which found that the mRNAs of several HSP's and other genes related to cellular stress were up-regulated at low salinity. Interestingly, the Na^+/K^+-ATPase β-subunit was down-regulated in dilute waters.

While *P. trituberculatus* is capable of tolerating changes external salinity, it is a comparatively *weak* omsmoregulator (Friere et al., 2008; Lu et al., 2013) when compared to its close relative *C. sapidus*. Given their different osmoregulatory abilities, one would expect to see differences in the transcriptional response of the gills between these two crabs. With this in mind, the final goal of this thesis will be to perform an RNA-seq analysis on the gills of *C. sapidus* in response to low salinity exposure. With these data it will be possible to quantitatively measure the changes in expression of all the genes implicated in crustacean ionic regulation, as well as identify new genes putatively involved in this process. Additionally, the expression of HSPs and cellular stress proteins will be observed if environmental dilution produces a classical stress response in *C. sapidus* like its cousin *P. trituberculatus*, or if its superior osmoregulatory ability is indicative of extreme physiological adaptation to low salinity similar to *C. maenas*. Finally, the transcriptomes of crabs acclimated to high and low salinity will be functionally annotated, with each putative gene being mapped back to specific physiological and cellular pathways. This will allow for the observation of any changes in large-scale physiological trends which occur when crabs travel from high to low salinity waters.

To achieve these goals, RNA will be extracted from anterior and posterior gills of crabs acclimated to 35 ppt and 10 ppt. cDNA libraries will be constructed and subjected to Illumina sequencing using paired-end chemistry. Raw reads will be compiled using Trinity DNA

Assembler (Hass et al., 20013) to generate an assembled transcriptome of both G7 and G3 at high and low salinity. Following this, reads can be mapped back to each contig using RSEM (Li and Dewey, 2011) in order to calculate the relative abundance of each transcript and quantitatively describe the changes in the expression profiles of genes implicated in osmoregulation and cellular stress. Functional annotation will be performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) Automatic Annotation Server (Kanchisa et al., 2010).

The successful completion of this work will be an invaluable contribution to the understanding of how euryhaline crustaceans respond to low salinity environments. Not only will the expression of dozens of genes be compared in the same sample, but the discovery of new genes up-regulated at low salinity has the potential to implicate new unrealized genes in crustacean ionic uptake mechanisms.

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Chapter 2. Carbonic Anhydrase Induction in Euryhaline Crustaceans is Rate-Limited at thePost-Transcriptional Level

Abstract

The transfer of euryhaline crustaceans from full-strength seawater to low salinity results in both a rapid up-regulation of carbonic anhydrase (CA) mRNA and a slow induction of CA activity. There is a delay of several days between the two processes, which is believed to be attributed to the time required to synthesize new enzyme. These delays may also be due to limitations in the cellular uptake of Zn, which is a required post-translational active site modification to CA. To investigate these processes, the euryhaline crabs, *Callinectes sapidus* and *Carcinus maenas*, were acclimated to salinities below their isosmotic points (22.5 and 25 ppt, respectively) for 7 days to activate the physiological and molecular mechanisms of osmoregulation. CA mRNA increased 90-fold in C. *sapidus* and 2-fold in C. *maenas* within 6 hr; whereas it took 48 hr for the initial increases in CA activity (120% and 31%), and 4 to 7 days for new acclimated levels (300% and 100% , respectively). Crabs were then transferred to lower salinities (10 and 15 ppt) to induce further CA activity and to determine if previous increases in CA mRNA reduced the time required for subsequent CA induction. Additionally, the expression

of the Zn transporter ZIP1 was examined in *C. sapidus* at 35 and 22.5 ppt. In both species, prior CA mRNA elevation failed to accelerate the rate of CA induction. Levels of CA mRNA did not change in either crab following transfer from intermediate to low salinity. Taken together, these results show that the timecourse of CA induction at low salinity is not limited by the expression of CA mRNA, but by the synthesis of new enzyme from an existing pool of mRNA. No increases in ZIP1 expression occurred at low salinity, therefore these delays may be the due to the limits of cellular Zn uptake.

Key words: carbonic anhydrase, osmoregulation, salinity, crustaceans

Introduction

Euryhaline marine crustaceans are known to be exceptionally capable of surviving large changes in environmental salinity. These animals are isosmotic when acclimated to full strength seawater (~32-35 ppt), passively maintaining hemolymph osmolality and ionic concentrations equal to those of the surrounding medium. However, at lower salinities, usually around 25-26 ppt, these crustaceans activate ionic regulation processes that allow them to maintain their hemolymph well above ambient osmolality (see Mantel and Farmer, 1983; Pequex 1995; Henry et al., 2012 for reviews)

At low salinity, osmoregulating crustaceans maintain hyperosmotic hemolymph by actively pumping ions (mainly Na^+ and Cl^-) from the environment across the gills and into the hemolymph. The cellular mechanisms by which this is accomplished have been extensively studied (See Pequeux, 1995; Towle and Weihrauch, 2001; Towle, 2011, and Henry et al., 2012; McNamara and Faria, 2012; for reviews).

There are two different classes of mechanisms of ionic uptake that have been observed in crustaceans, one in animals with high gill epithelial conductance and ion transport rates, and one in those with low gill epithelial conductance and transport rates (see Henry et al., 2012, McNamara and Faria, 2012 for reviews). In low conductance crustaceans such as the Chinese crab, *Eriocheir sinensis*, individuals spend the majority of their life cycle in fresh water. Crustaceans with high conductance gills however are typically euryhaline marine species such as the blue crab *Callinectes sapidus* and the green crab *Carcinus maenas.* These species migrate annually between full-strength seawater and the more dilute waters of the estuary (e.g., Henry, 2001; Henry et al, 2012). In the gills of these crustaceans, a $Na^+/K^+/2Cl^-$ co-transporter protein located on the apical membrane transports Na^+ and Cl^- ions from the surrounding water into specialized mitochondria-rich cells (MRC). The presence of K^+ channels on the apical and basolateral membranes results in K^+ efflux across these surfaces, establishing an electrochemical gradient (inside negative) that drives Cl^- through channels on the basolateral membrane and into the hemolymph. The basolateral Na^+/K^+-ATPase pumps Na^+ from the MRC into the hemolymph. NaCl is also absorbed from the surrounding medium via apical Na^+/H^+ and Cl^-/HCO_3^- exchange proteins. In some species this process may be electrogenic, and does not utilize counterions for exchange (Onken et al., 2003). However in other species, such as *C. sapidus* and *C. maenas* this process is electroneutral and utilizes the hydration of respiratory CO_2 by cytoplasmic carbonic anhydrase (CA) to provide H^+ and HCO_3^-,which serve as counterions for Na^+ and Cl^- uptake, respectively, in low salinity (Cameron, 1979; Henry and Cameron, 1982, 1983; Henry et al., 2003). There has been some debate as to whether the presence of the $Na^+/K^+/2Cl^-$ co-transporter

obviates a role for CA in ionic uptake, however a large a body of evidence in both *C.maenas* and *C. sapidus* (localization, salinity-sensitivity, and physiological responses to inhibition of CA – reviewed by Henry et al., 2012), confirms the central role of CA and points to the potential for multiple NaCl uptake mechanisms that operate concurrently

When crustaceans are acclimated to full-strength seawater and are in a state of osmoconformity, the aforementioned processes are physiologically silent, being activated only upon exposure to salinities below 26 ppt (Henry, 2005). This includes the up-regulation of the transport proteins responsible for ion regulation. The Na^+/K^+-ATPase and CA are perhaps the two best-studied molecules regarding the activation of ionic regulatory processes (see Henry, 2001; Towle and Weihrauch, 2001; Henry et al., 2012 for reviews), with increases in specific activity of these enzymes being documented in many different euryhaline marine crustaceans (Towle et al., 1976; Henry and Cameron, 1982; Holiday, 1985; Wheatly and Henry, 1987; Corratto and Holiday, 1996; Mananes et al., 2000; Henry et al., 2002, 2003; Roy et al., 2007; Tsai et al., 2007). These elevations occur in the posterior, ion-regulating gill pairs and are typically absent in the anterior, respiratory gills (e.g., Henry, 1984; Towle, 1984).

Low salinity-stimulated increases in both CA and Na^+/K^+-ATPase activity are preceded by the up-regulation of the mRNAs encoding these proteins (Luquet et al., 2005; Jayasundara et al., 2007; Serrano et al., 2007; Serrano and Henry, 2008). Like salinity-stimulated CA and Na^+/K^+-ATPase activity, these gene activation events occur in the posterior ion-regulating gills and are absent in anterior gills. These data strongly suggest that the increases in activity of these enzymes at low salinity are the direct result of gene activation and subsequent synthesis of new

protein. In most crustacean species studied, the increases in gene expression occur rapidly and are quite dramatic (Luquet et al., 2005; Jayasundara et al., 2007; Serrano et al., 2007; Serrano and Henry, 2008). For example, in the *C. maenas* and *C. sapidus*, the up-regulation of CA mRNA following low salinity transfer increases by 10-fold after 6 hr and 100-fold after 24 hr (Serrano et al., 2007; Serrano and Henry, 2008).

Despite the thoroughness with which CA gene activation and rises in enzyme activity have been studied in the last decade, several unanswered questions still exist pertaining to the molecular mechanism of CA induction. Some evidence suggests that there are molecular limitations that dictate the speed at which CA activity can increase. Despite the rapid onset of CA mRNA upregulation, increases in CA activity in *C. sapidus* and *C. maenas* occur relatively slowly, taking 24 – 96 hr for the initial increase. Furthermore, in both species, CA activity continues to rise from 4-7 days following the initial increase. This is independent of the levels of CA mRNA, as gene expression does not rise further after the initial 6-24 hr upregulation, suggesting that post-transcriptional processes rate-limit CA induction (Henry and Cameron, 1982; Henry and Watts, 2001; Henry et al., 2002; Henry et al., 2003, Henry, 2005; Serrano et al., 2007; Serrano and Henry, 2008). Other proteins have shown the same pattern of rapid mRNA expression and slower increases in activity (e.g., UPC1, a protein used in thermogenesis of mammalian brown adipose tissue) (Nedergaard and Cannon, 2013). However, some proteins show rapid induction: ornithine decarboxylase activity can be up-regulated within minutes (Watts et al., 1996), suggesting that there may be regulatory limits other than protein synthesis. It may be that the delays in CA activity induction are the result of limitations in Zn transport. CA

requires a Zn atom to be bound to the active site in order to perform catalysis (McCall et al., 2000). In order to bind Zn to the large cytoplasmic pool of CA, it presumably must be extracted from food in the gut, transported to the gills, and taken into the MRC. Cellular uptake is very likely accomplished via the action of Zip proteins. These proteins have been implicated as the primary mechanism for zinc transport and regulation in yeast, plants, *Drosophila*, and mammals (Guerinet and Eide, 1999; Liuzzi and Cousins, 2004; Matthews et al., 2005). There are currently no published studies which have examined the possibility of CA limitation via Zn.

Another remaining question in salinity-stimulated CA induction is the cause of CA activity increases following multiple instances of environmental dilution. For example when osmoregulating crabs are acclimated to progressively lower salinities (e.g. 25 then 15 ppt) the degree of CA activity induction is progressively greater in the lower salinities (Henry and Watts 2001, Henry 2005). It is currently not known if these increases in enzyme activity result from enhanced gene expression or if they come about by increased translation of the CA message to new protein from the same pool of mRNA.

In this study we examined these questions by acclimating both *C. sapidus* and *C. maenas* to high salinity and then subjecting them to sequential salinity transfers: first to an intermediate salinity below their isosmotic points in order to activate CA expression and induction, and then to a lower salinity in order to stimulate further CA induction. Our goals were twofold; first, to determine if the delay in CA induction could be reduced by prior activation of the induction mechanism; and second, to determine if the elevations in CA activity at the lowest salinities were accompanied by increases in CA gene expression or if they were simply the result of increased

protein synthesis from the same pool of mRNA. Additionally we investigated the gene expression levels of ZIP1, a member of the Zip Zn-transporting family, in *C. sapidus* at high and low salinity to examine the possible role of Zn as a limiting-factor in CA induction.

Materials and Methods

2:1 Collection and maintenance of animals

Adult, intermolt, blue crabs, *Callenectes sapidus* were obtained from commercial watermen in East Point, FL, USA, packed in wet burlap, placed in 48qt coolers, and transported to Auburn University. Crabs were held in 150 gal recirculating aquaria equipped with biological filters (oyster shell and sand) at 24ºC and ambient photoperiod. Crabs were held at 35 ppt for at least 3 weeks prior to experimentation to ensure that CA activity and expression were at baseline levels (Henry and Watts, 2001; Jillette et al., 2011). Blue crabs were fed daily on shrimp, salinity was monitored with a conductivity meter (YSI 30 1, Yellow Springs, OH, USA) and adjusted with distilled water or concentrated brine made with artificial sea salts (Instant Ocean Reef Crystals, Blacksburg, VA, USA). Water quality was checked by monitoring nitrite concentrations (Dry Tab, Mansfield, MA, USA).

Adult, intermolt green crabs, *Carcinus maenas* L., were collected by hand from the intertidal zone, and by trap from the subtidal zone of Frenchman's Bay along the shoreline of the Mount Desert Island Biological Laboratory (MDIBL), Salisbury Cove, ME, USA. Green crabs were held at MDIBL in 400 liter fiberglass tanks equipped with filtered, running seawater (32-33 ppt salinity and 11-12 ºC). Crabs were fed a combination of mussels and squid every other day

but were starved for a minimum of 48 hr prior to use. 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).

2: 2 Experimental Protocol

C. sapidus and *C. maenas* were acclimated to full strength seawater (35 ppt for *C. sapidus* , 32 ppt for *C. maenas*) for 3 weeks prior to being used in any experiment. For *C. sapidus* one set of crabs was transferred to 22.5 ppt. CA activity and relative gene expression were measured in six crabs at each time point: 0 (35 ppt acclimated), 6, 12, 24, 48, 96 hr, and 7 days following that transfer. To test the effects of previously elevated CAc expression on the induction of CA activity, a second set of crabs was acclimated to 22.5 ppt for 1 week (used as 0 hr) and transferred to 10 ppt, with CA activity and expression being measured at the same time points as done previously. The same protocol was used for *C. maenas*, except that full strength seawater was 32 ppt, and the transfers were 32–25 ppt and 25–15 ppt. ZIP1 expression was measured in 35-22 ppt *C. sapidus* gills at 0, 24, and 96 hr post-transfer.

For all crabs, at the end of the experiment, hemolymph (0.5 ml) was sampled from the infrabranchial sinus at the base of the walking legs using a 22 ga needle and 1ml syringe, transferred to a 1.5 ml centrifuge tube, and stored at -20 ºC for analysis of osmolality.

Anterior gills (G3 for *C. sapidus*, G4 for *C. maenas*) were used as a non-ion transporting tissue control, and posterior (G7 for *C. sapidus*, G8 for *C. maenas*), ion-transporting gills were used as the experimental tissue. Both gills were dissected out of the crabs at the end of each experiment. For each animal, one posterior and one anterior gill, to be used for the measurement

of protein-specific CA activity, was placed in 5 volumes of cold (4ºC) homogenization/ CA assay buffer (225mM mannitol, 75mM sucrose, 10mM Trizma base, adjusted to pH 7.40 with 10% phosphoric acid). Anterior and posterior gills from the other side of the crab, to be used for gene expression or cDNA library preparation, were placed in 3 ml of RNAgents Denaturing Solution (Promega, Madison WI, USA) and chilled on ice until homogenization.

2:3 Measurement of CA Activity

CA activity was measured electrometrically by the delta pH method (Henry, 1991). Anterior (G3/G4) and posterior (G7/G8) gills were homogenized in 5 volumes of cold buffer (described above) using an Omni TH115 hand-held homogenizer and then sonicated at 25W for 30 sec (Heat Systems Microsonicator, Farmingdale, NY, USA). Homogenates were centrifuged at 10,000g for 20 min at 4ºC (Sorvall RC5- B, Wilmington, DE, USA), and the supernatant was assayed for CA activity. Briefly, 25–100 ml of supernatant were added to 6ml of buffer in a thermostatted reaction vessel (4 ºC) and stirred vigorously. 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}.

2: 4 Hemolymph Osmolality

Hemolymph samples were thawed on ice, sonicated for 10 seconds at 15 watts, 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).

2*:5 RNA Purification*

Total RNA was extracted from gills using RNAgents Total RNA Isolation System (Promega, Madison WI, USA) under RNAse-free conditions, all equipment being treated with RNAse-Zap (Ambion, Austin, TX, USA). RNA purity and quality were assayed using a Bioanalyzer 2100 (Agilent Technologies, Wilmington, DE, USA); no genomic DNA contamination was observed. The total RNA concentration of each sample was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2:6 Determination of C. sapidus ZIP1 Sequence

cDNA was synthesized from 1 µg of total RNA extracted from G7 of *C. sapidus* acclimated to 15 ppt for 7 days. First-strand cDNA synthesis was performed using the SMART cDNA library construction kit (Clontech, Mountain View, CA, USA) as per the manufacturer's instructions except that the provided 3' oligo was replaced with the Cap-Trsa-CV oligo as per Matz et al. (2009). Full-length cDNA was then amplified using the Advantage 2 PCR system (Clontech, Mountain View, CA, USA) using the minimum number of PCR cycles possible and sent to HudsonAlpha Institute for Biotechnology (Huntsville, AL, USA) for paired-end (PE) library preparation and Illumina sequencing. Each library was sequenced using approximately one-sixth of an Illumina HiSeq 2000 lane using the 2 X 100 bp PE chemistry.

Raw reads were assembled using Trinity DNA Assembler (Haas et al., 2013). A contig containing an open reading frame which produced highly significant tblastx results against ZIP1 and other putative zinc transporters in multiple taxa was identified (GenBank accession no. **KF827825**). Forward (ZIP1F: 5' TGC TCC ATA GTG AGG ACG AG) and reverse (ZIP1R: 5' AGG AAC AAT TGG ACC TCC TG) PCR primers were designed against a 106 bp region of this sequence using Primer3 (Untergrasser et al., 2012). This primer pair was used to amplify cDNA prepared from G7 of *C. sapidus* acclimated to 15 ppt for 7 days using using a REDTaq™ ReadyMix™ PCR Reaction Mix Kit (Sigma-Aldrich, St Louis, MO, USA). Samples were incubated at 95 ºC for 5 min and held at 65 ºC until the addition of RedTaq polymerase at the beginning of the reaction. Our running conditions were 92 ºC initial denaturation for 5 min, followed by 33 cycles consisting of a 92 ºC 1 min denaturation, 55 ºC 1 min annealing, and a 72 ºC 2 min elongation. The reaction was terminated with a 72 ºC 5 min elongation to extend any short products. This reaction produced a single fragment of approximately 106 bp which was purified using a Qiaquick PCR Purification Kit (Qiagen, Valencia, CA, USA), cloned using a TOPO™ TA™ Cloning Kit (Invitrogen, Madison, WI, USA) and verified by Sanger sequencing at the Auburn University Genomics and Sequencing Center (Auburn, AL, USA).

2:7 cDNA Synthesis and Quantitative Polymerase Chain Reaction

For samples used for gene expression quantification, cDNA was reverse transcribed with 2 µg of total RNA using Oligo-dT as a primer and SuperScript II reverse transcriptase (Invitrogen, Madison, WI, USA). 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).

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, USA). All qPCR 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. 1 µl 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 of cDNA prepared from *C. sapidus* or *C. maenas* gills acclimated to 15 ppt for 7 days (1, 0.1, 0.01 and 0.001 fold dilution series). Two different sets of gene-specific primer pairs were used to amplify CAc cDNA in *C. sapidus* (GenBank accession no. **EF375490.1**) and *C. maenas* (GenBank accession no. **EU273943.1**). For *C. sapidus*, CsCAc was amplified using CsCAcF and CsCAcR (Serrano et al., 2007). The primer pair CamCAcF and CamCAcR was used to amplify CamCAc in *C. maenas* (Serrano et al., 2008). *C. sapidus* ZIP1 was amplified using the sequence-specific ZIP1F and ZIP1R primer pair described above.

2:7 Statistics

All statistical tests were performed in R-studio version 2.15.1 (The R Foundation for Statistical Computing).

Results

3:1 Full-Strength Seawater to Intermediate Salinities

In 35 ppt acclimated *C. sapidus*, CAc mRNA was 0.0020 relative to qPCR standards prepared from gills of crabs acclimated to 15 ppt for 7 days (Fig. 1). Transfer to 22.5 ppt elicited a statistically significant 90-fold increase in CAc expression in posterior gills after 6 hr ($p<0.05$, student's t-test) (Fig. 1). High and somewhat variable levels of CAc mRNA expression were maintained throughout the 7-day experimental period, consistently being at least 100 fold higher than 35 ppt acclimated values ($p<0.05$, one-way ANOVA, Tukey HSD) (Fig. 1). Interestingly, the degree of CAc up-regulation reported here is approximately the same as was reported previously for a much greater salinity change (35–15 ppt)(Serrano et al., 2007). There were no statistically significant changes in the expression of CAc in the anterior respiratory gill pairs (one-way ANOVA), and values were too low to be shown graphically.

At 35 ppt, CA activity in the posterior ion-regulating gills of *C. sapidus* was 356 μmol CO_2 mg protein^{-1} min^{-1} (Fig. 1), which was slightly higher than previously reported baseline values for this enzyme (Henry and Cameron, 1982; Henry and Watts, 2001; Serrano et al., 2007). Whereas gene expression rose 6 hr after low salinity transfer, CA activity increases were slower, taking 48 hr to increase significantly ($p<0.05$, one-way ANOVA, Tukey HSD) by 120% at 22.5 ppt (Fig. 1). CA activity continued to rise, between 48 and 96 hr from 779 to 1444 μmol CO_2 mg protein^{-1} min^{-1} ($p<0.05$, one-way ANOVA, Tukey HSD) (Fig. 1).

When acclimated to full-strength seawater, mean *C. sapidus* hemolymph osmolality was 1064 mosm/Kg H_2O , which was nearly equal to the ambient medium (1030 mosm/Kg H_2O) (Fig 2). Following transfer to 22.5 ppt, osmolality fell to 894 mosm/Kg H_2O, a 16% change, after 6hr, and eventually reached 789 mosm/Kg H_2O after 7 days. These values were well above ambient osmolality at 22.5 ppt, which was 644 mosm/Kg H_2O (Fig 2). These results are consistent with previous studies in *C. sapidus*, which

showed that hemolymph is isosmotic with the environment at full-strength seawater, and with osmolality initially falling at low salinity and eventually establishing a new equilibrium at a concentration well above ambient (Henry and Cameron, 1982; Henry, 2005).

A pattern of CAc mRNA expression and CA induction similar to *C. sapidus* was observed in *C. maenas*. At full-strength seawater (32 ppt) CAc was 0.0003 relative to G8 of 15 ppt acclimated qPCR standards. Following transfer from 32 – 25 ppt CAc expression in the posterior gills rose by 14.5-fold ($p<0.05$, student's t-test) after 12 hr (Fig. 3). As in *C. sapidus*, CAc mRNA levels in *C. maenas* were variable, decreasing slightly from 12 to 24 hr (student's t-test, $p<0.05$) and spiking at 48 hr (Fig. 3). However, gene expression values remained above 32 ppt acclimated levels by over 10-fold from 48 hr through the acclimated state reached at 168 hr (Fig. 3). Unlike *C. sapidus*, the increase in CAc gene expression in *C. maenas* at intermediate salinity was neither as great as previously reported in low salinity (15 ppt) nor did it occur as quickly (Serrano et al., 2008), but the upregulation was significant, persistent, and preceded elevations in CA enzyme activity by 36 hr (Fig. 3).

As with *C. sapidus*, there was a delay between the upregulation of CAc mRNA and the induction of the CA activity. CA activity took 48 hr to significantly increase above baseline by 31% ($p<0.05$, student's t-test) and continued to rise between 48 - 96 hr from 158 to 201 μmol CO_2 mg protein^{-1} min^{-1} ($p<0.05$, student's t-test)(Fig. 3).

C. maenas also had a similar pattern of hemolymph regulation as well: osmoconformity at full strength seawater (32 ppt) and hyperosmotic regulation below 26 ppt (Fig 2). Osmolality dropped to 867 mosm/Kg H_2O 6 hr after transfer to 25 ppt (740 mosm/Kg H_2O) and stabilized thereafter at 807 mosm/Kg H_2O (Fig 2). These results are consistent with previous studies of osmoregulation in *C. maenas* (Zanders, 1980; Henry et al., 2002; Henry, 2005).

3: 2 Intermediate Salinities to Low Salinity

C. sapidus was transferred to 10ppt following 7 days of acclimation at 22.5 ppt. This salinity change resulted in transient increases in CAc mRNA at 12 and 48 hr (one-way ANOVA, Tukey HSD) (Fig. 4). However, these spikes were not consistent for the duration of the 10 ppt acclimation time course; rather, mRNA abundance was found to be similar to values at the intermediary salinity of 22.5 ppt (Fig. 4). Despite the fact that relative gene expression was 65-fold above 35 ppt acclimated values at the beginning of the10 ppt transfer (Fig. 4), there was still a delay in the induction of new CA enzyme activity after transfer to 10 ppt: the first statistically significant rise in CA (63% relative to 0 hr) did not occur until 48 hr post-transfer ($p<0.05$, student's t-test) (Fig. 4). Enzyme activity did not significantly change thereafter (one-way ANOVA).

Similar to *C. sapidus*, *C. maenas* CAc gene expression was well above full-strength seawater levels (17-fold, Fig. 3) at the end of the acclimation period to intermediate salinity (25 ppt for *C. maenas*). Transfer to 15 ppt did not elicit significant increases in CAc gene expression in posterior gills, and expression in anterior gills remained unchanged from 32 ppt (Fig. 5). Elevated CAc mRNA did not reduce the time required for CA induction, and a significant ($p<0.05$, one-way ANOVA, Tukey HSD) elevation in enzyme activity (133%) did not occur until 96 hr post-transfer (Fig. 5). CA activity continued to rise, increasing from 420 to 618 μmol CO_2 mg protein^{-1} min^{-1} between 96 hr and 7 days post-transfer (Fig. 5).

Despite delays in CA induction, crabs were able to maintain hyperosmotic hemolymph following transfer from intermediate to low salinity. After 6 hr at 10 ppt, hemolymph osmolality fell by only 5% in *C. sapidus* and remained well above ambient osmolality by an average of 266 mosm/Kg H_2O for the duration of the experiment (Fig. 6). In *C. maenas* 15 ppt transfer diluted hemolymph by only 2% to 803 mosm/Kg H_2O after 6 hr (Fig 6), with osmolality being 317 mosm/Kg H_2O above ambient after 7 days (Fig. 6).

3:3 ZIP1 Gene Expression

Expression of ZIP1 in G7 of *C. sapidus* acclimated to 35 ppt was 0.1000 relative to control samples of 35-15ppt *C. sapidus* (Fig. 7). ZIP1 mRNA did not significantly increase at either 24 or 96 hr after 22 ppt transfer (one-way ANOVA) (Fig. 7). ZIP1 mRNA was not significantly higher in posterior vs anterior gills except for 24 hr at 22 ppt (student's t-test) (Fig. 7).

4 Discussion

4:1 Initial Delays in CA Induction

The primary goals of this study were to determine if prior elevation of CAc mRNA reduced the time required for large-scale increases in CA induction in euryhaline crustaceans and to observe if progressively higher levels of branchial CA activity require concomitant increases in gene expression. It should be noted that a control gene such as arginine kinase (AK) or actin was not used in this study. Due to the myriad of physiological, biochemical, and ultrastructural changes which occur in the posterior gills of euryhaline crustaceans transferred from high to low salinity (Perry, 1997; Luquet et al., 2005; Evans et al., 2005), even the expression of these housekeeping genes has been known to significantly increase at low salinity (Serrano et al., 2007; Serrano and Henry, 2008). However the anterior gills, which do not function in ionic uptake, have been shown to be unresponsive to changes in environmental salinity, and the expression of CAc, NaK, and AK in anterior gill pairs remains stable in the face of salinity transfer in *C. maenas* and *C. sapidus* (Serrano et al., 2007; Serrano and Henry, 2008). Thus the use of CAc expression in the anterior gills, as was done in this study, serves as a more appropriate qPCR control than a housekeeping gene in the posterior gills.

A rapid (6hr) and profound (90-fold) increase was seen in CAc gene expression in *C. sapidus* following the initial salinity transfer (35-22.5ppt) (Fig. 1). Unlike CAc gene expression, CA enzyme activity induction in transfer from full-strength seawater to intermediate salinity was slow to occur, taking 48 hr (Fig. 1). Similar delays in CA activity induction were observed in *C. maenas*: following 32-25 ppt transfer: CAc mRNA rose by 12.5-fold after 12hr; however, CA activity did not increase significantly until 48 hr post-transfer (Fig. 3). This slow induction of CA activity is consistent with previously published studies in both species (Henry and Cameron,

1982; Henry and Watts, 2001; Henry et al., 2002; Serrano et al., 2007; Serrano and Henry, 2008). The increase in CA activity has been shown to result from the synthesis of new enzyme. Inhibitor titration, which can be used to calculate the concentration of free enzyme, has shown that increases in the CA protein concentration are in line with increases in CA gene expression and activity (Henry et al., 2006; Serrano et al., 2007; Serrano and Henry, 2008).

These data are difficult to compare to other animals, as there are few organisms that have published timecourses for either branchial CA activity or CAc expression, let alone both. One such organism is the tiger prawn *Panaeus monodon*, where CA mRNA increases 24 hr after transfer to low salinity, but significant increases in CA enzyme activity take 3 days (Pongsomboon et al., 2009). Several studies have focused on the timecourse of CA activity without examining gene expression. In general, significant CA induction takes several days to occur. For example, in the euryhaline marine crab *Chasmagnathus granulata*, low salinity-stimulated CA activity does not occur until 3-8 days post-transfer (Mananes et al., 2000). A 2004 study by Palacios et al. found no difference in CA activity in the gills of the white shrimp *Litopenaeus vannamei* within 3 or 24 hr of low salinity transfer; however later studies would show that significant induction occurs after 7 days (Roy et al., 2007). There are no published studies that show branchial CA enzyme activity rising rapidly (<24 hr) after transfer from full strength seawater in any crustacean.

4:2 Effects of Prior Gene Activation

In this study, to determine if prior elevation of CAc mRNA would reduce the 48 hr period needed to increase CA activity in *C. sapidus* and *C. maenas*, crabs were held at intermediate

salinities for one week and then transferred to lower salinities. At the end of intermediate salinity acclimation, CAc mRNA was 65 and 17-fold above full-strength seawater levels, respectively, in *C. sapidus* and *C. maenas* (Figs. 1, 3). After transfer to 10 and 15 ppt, there were no further consistent increases in CAc mRNA in the posterior gills, and CA activity took 48 and 96 hr to significantly increase in *C. sapidus* and *C. maenas*, respectively (Figs 4, 5). Thus in neither species did prior gene activation enhance the rate of increase in CA activity. These data demonstrate that even if the MRC cells in the crustacean gill are in the process of expressing the CA message, there is still a delay in the onset in the production of elevated levels of enzyme following salinity stress.

In a similar experiment by Monserrat et al. (1997), *C. granulata* was held at 20 ppt (below its isosmotic point of 29 ppt) for 10 days, and then transferred to 2.5 ppt. Posterior gill CA activity was monitored and increased significantly after 7 hr. However several points call the results of this study into question. First, the observed increase in enzyme activity was abolished 24 hr post transfer; and this is highly unusual as CA has never before been observed to decrease in osmoregulating crabs following the initial induction. Second, the authors did not utilize an anterior respiratory gill as control tissue. This is an essential component in measuring salinity-mediated branchial CA activity, as elevations of activity anterior and posterior gills occur in premolting crabs regardless of environmental salinity (Henry and Kormanik, 1985); thus increases in anterior gill CA activity can be used as a means to detect and eliminate crabs with hormonally induced CA activity from a sample.

4:3 Acute Verses Chronic Salinity-Stress

It has long been hypothesized that euryhaline crustaceans respond to acute verses chronic low salinity exposure with different physiological mechanisms. The best evidence for this lies in the temporal disparity between the apparent onset of osmoregulation and the enhancement of enzyme activity. In many studies, including this one, animals reach new, steady-state levels of hyperosmotic hemolymph within 24 hr after low salinity transfer (Neufeld et al., 1980; Henry and Cameron, 1982; Henry and Watts, 2001; Lovett et al., 2006a); however, increases in Na^+/K^+-ATPase and CA activity, which are key components of ionic uptake in low salinity take on the order of days (Siebers et al., 1983; Holliday et al., 1990; Lovett et al., 2006 a,b; Serrano et al., 2007; Serrano and Henry, 2008). If crustaceans are able to hyperregulate their hemolymph before the induction of these enzymes, then it seems likely that there exists a short-term, resistance adaptation that prevents excess ion loss (e.g., reduced branchial epithelial permeability).

While it takes up to several days after low salinity exposure to detect increases in the activity of enzymes implicated in long-term survival at low salinity, the process appears to start earlier. Na^+/K^+-ATPase and CAc expression increase within 24 hr of low salinity transfer but activity takes several days to rise (Lovett et al., 2006 a, b; Serrano et al., 2007, Serrano and Henry, 2008). Furthermore, *C. sapidus* transferred to low salinity for 12 hr will synthesize new CA even if transferred back to high salinity (Henry, 2005). This study provides additional evidence of this; when CAc mRNA was elevated rapidly following full-strength seawater to intermediate salinity transfer, CA activity increased slowly (Figs 4, 5). Furthermore during subsequent transfer to lower salinities in both crabs 48-96 hr were required for enhanced CA activity, despite the fact that levels of mRNA were already elevated and were relatively static (Figs 4, 5).

4:4 Possible Explanations for Delays in CA Activity

It is unlikely that increases or delays in enzyme activity are simply due to alterations in the kinetics of pre-existing CA. Carbonic anhydrases have long been known to be incredibly efficient "catalytically perfect" enzymes that will hydrate CO_2 nearly every time it comes into contact with it, being only limited by the diffusion of substrate into the active site (see Herrick et al., 2013 for a review). Since the amount of CO_2-saturated water added to the reaction vessel is constant using the delta pH method (Henry, 1991), increases in the supply of substrate to the enzyme can be ruled out, and therefore the most likely explanation for all observed increases and delays in CA activity is production of mature enzyme.

There are three observations in this study that strongly suggest that synthesis of new enzyme is the rate-limiting step in the induction of CA activity. First, despite the fact that CA activity increased in both crabs following transfer from the intermediate salinity to the lower salinity (Figs. 4, 5), levels of CAc mRNA remained relatively consistent. In *C. maenas*, transfer from 25-15 ppt did not result in any significant changes in CAc mRNA (Fig. 5). In *C. sapidus*, 22.5-10 ppt transfer resulted in CAc elevations at a few time points (12, 48, and 168 hr) (Fig. 5); however, there was no consistent trend of elevated mRNA, which one would expect if enhanced gene expression were responsible for the increases in CA activity from 22.5 to 10 ppt. Thus it appears that the increases in CA activity upon acclimation to lower salinities are due to enhanced translation of protein from the existing pool of CAc mRNA.

Second, there exists a severe disparity between the levels of gene activation and increases in enzyme activity. For example, in this study CAc mRNA in *C. sapidus* transferred from 35-22.5 ppt increased by 64-fold 7d post transfer; however in the same sample CA enzyme activity only rose by 3.4-fold (Fig. 1). This pattern was also seen in *C. maenas*; for 32-25 ppt at 96hr: CAc gene expression was 22-fold above baseline while enzyme activity was elevated 1.6-fold (Fig. 3). From this it appears that only a fraction of the CAc mRNA is used as a template to form mature enzyme. These data also make it unlikely that degradation of CAc mRNA is responsible for any delays in CA induction, as even if 90% of transcribed CAc were degraded, there would still be ample template for CA translation.

Finally, as previously discussed, even though CAc mRNA was elevated 64 and 17-fold above full-strength seawater acclimated levels in *C. sapidus* and *C. maenas* at the onset of transfer to 10 and 15 ppt, CA activity took 48 and 96 hr to significantly increase, respectively (Figs. 4, 5). Therefore even when sufficient mRNA is present, elevations in CA activity following environmental dilution are slow to occur.

CA induction is quite sluggish, especially compared to certain other enzymes. For example, ornithine decarboxylase (ODC) activity doubles within 30 min in *Artemia franciscana* when exposed to 4 ppt (Watts et al., 1996). One reason why elevations in CA activity are so slow may be the post-translational incorporation of Zn atoms into the active site of the enzyme, which is essential for its catalytic activity (see McCall et al., 2000 for review). In order to get the necessary Zn for CA, it presumably must be extracted from food in the gut, or mobilized from other internal stores, transported to the gills, and finally moved into the MRC via the action of

Zip Zn transport proteins. These processes could take considerable time. Therefore it is possible that the CA apo-protein could be present in large quantities in the gills during the early stages of low salinity acclimation, but is not catalytically active due to insufficient Zn, and therefore is not detected by CA activity assays.

We performed a preliminary investigation of the role of Zn in CA induction by measuring the expression of ZIP1 in G7 of *C. sapidus* before and after low-salinity acclimation. Zip proteins are responsible for cellular zinc uptake in eukaryotes from plants to *Drosophila* (Guerinet and Eide, 1999; Liuzzi and Cousins, 2004; Matthews et al., 2005); thus translocation of Zn into crustacean MRC would very likely be a result of the action of these molecules. ZIP1 expression was not elevated relative to 35 ppt after 24 hr at 22.5 ppt, when CA activity was similar to baseline values; nor did ZIP1 expression rise at 96 hr post transfer when CA activity 4-fold above baseline (Figs. 1, 7). Thus increases in ZIP1 expression does not appear to be a critical factor in the timecourse of CA induction.

Due to the potentially damaging effects of high intracellular Zn concentrations, free intracellular Zn concentrations in both prokaryotic and eukaryotic cells tends to be tightly regulated and static, varying only on the nanomolar scale in a given cell even in response to increases in extracellular Zn (Sensi et al., 1997; Maret. 2001; Eide, 2003). Given this highly conserved trend, it is not surprising that a large-scale salinity-stimulated increase in Zn transport machinery is absent in the *C. sapidus* gill. The fact that ZIP1 does not significantly increase at low salinity, coupled with the knowledge that CAc free enzyme increases by 46-fold following low-salinity acclimation (Serrano et al., 2007), makes it quite possible that Zn uptake into MRC

is a limiting-factor in CA activity induction. The delay in CA enzyme induction may be a cellular trade-off in the mechanism of branchial Zn homeostasis that keeps intracellular Zn concentrations below toxic levels. However, this hypothesis should be taken with some caution; as further work will certainly be required to conclusively implicate limitations in Zn transport as a causative agent of delays in CA induction. This will include the measurement of branchial intracellular Zn concentrations at full-strength seawater and throughout the timecourse of low salinity acclimation alongside stoichiometric measurements of both mature CA and apo-protein to determine if it is chemically possible for Zn to be a limiting factor. Additionally, *in vivo* studies of branchial Zn channels will be helpful in determining if these channels are under structural regulation and do indeed increase the amount of Zn uptake at low salinity.

4:5 Conclusions

In conclusion, it appears that the degree of low salinity exposure sets the magnitude of CA induction, but the rate is fixed and independent. Furthermore, several lines of evidence suggest the rate of induction of CA at low salinity in the gills of euryhaline crustaceans is limited by the production of mature enzyme. First, gene activation is rapid, but increases in enzyme activity are much slower. This may be due to limits of Zn transport, however further work will certainly be necessary to confirm this hypothesis. Second, in this study we showed that elevation of CAc mRNA and the priming of the CA induction process does not reduce the time required for increases in CA activity in two species of euryhaline crustaceans. Finally, increases in CAc mRNA tend to be extremely large (10-100-fold) relative to the concomitant increases in CA activity (2-5-fold).

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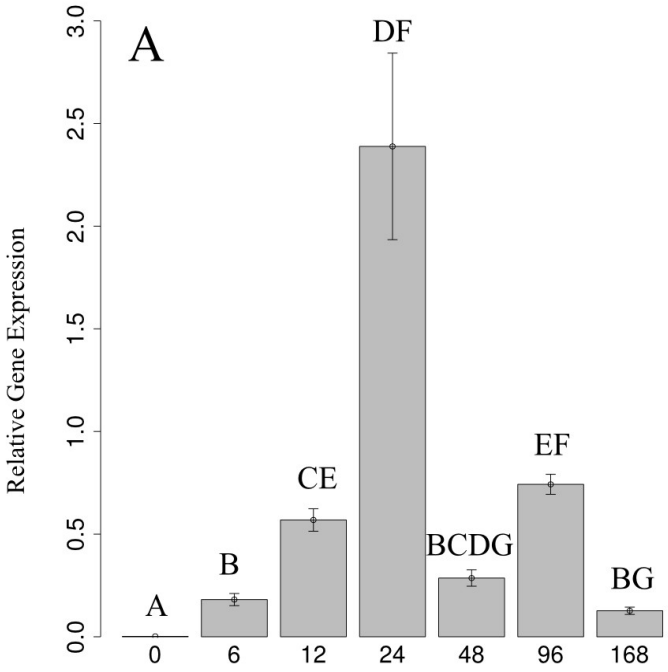
A
Relative Gene Expression
0.0
0.5
1.0
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168

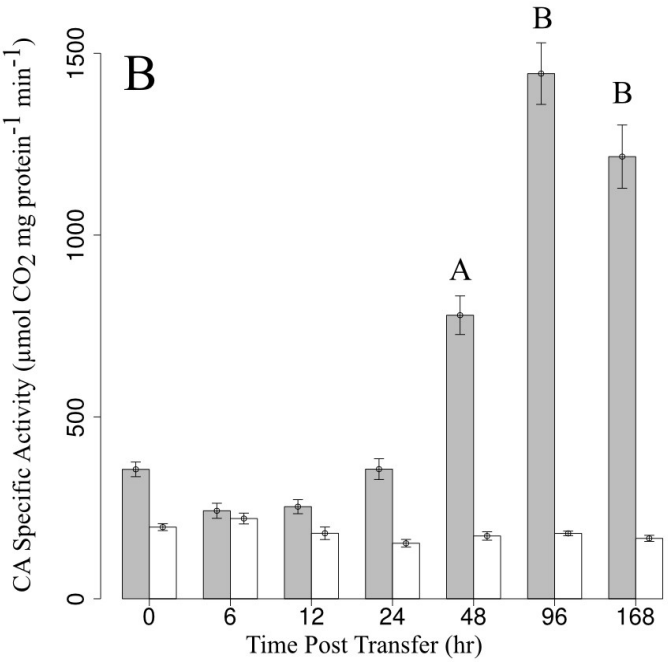
B
CA Specific Activity (µmol CO2 mg protein-1 min-1)
0
500
1000
1500
A
B
B
0
6
12
24
48
96
168
Time Post Transfer (hr)

Fig. 1 CAc mRNA (A) and CA enzyme activity (B) in posterior (G7, gray bars) and anterior (G3, white bars, not pictured for mRNA) gill pairs of *C. sapidus* transferred from 35-22.5 ppt. Means ± SEM, n=6. Different letters denote significant differences between groups at the 0.05 level.

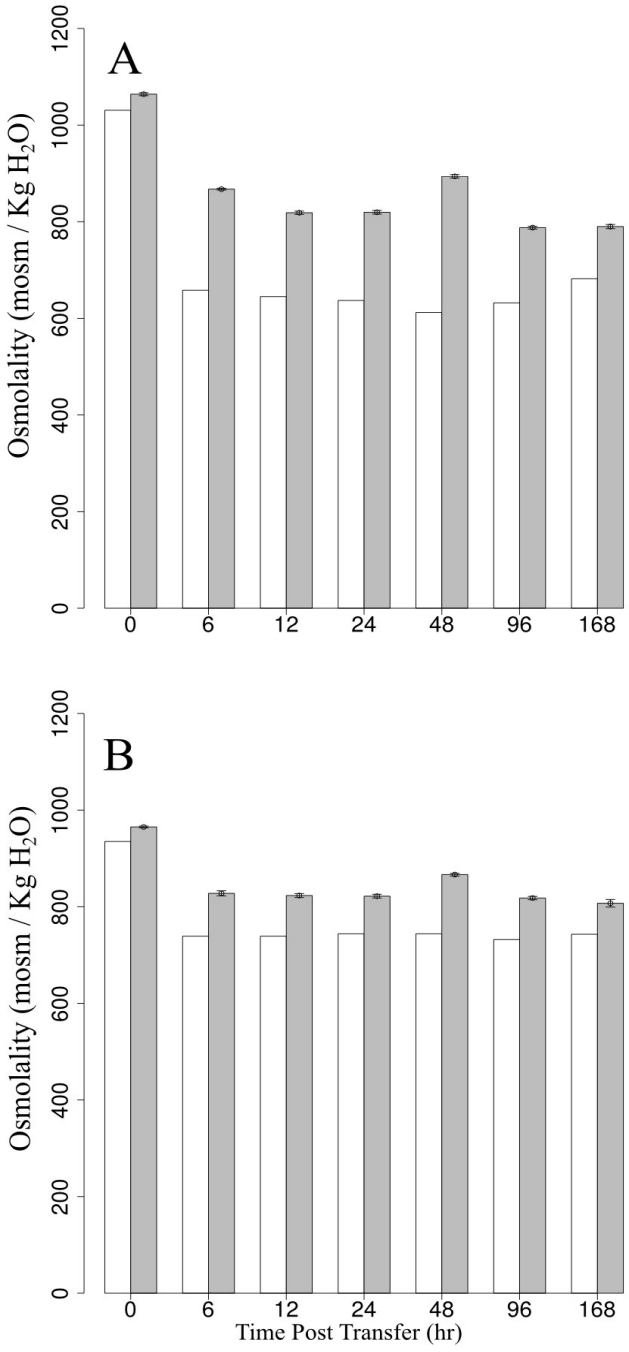

Fig. 2 Hemolymph (gray bars) and ambient (white bars) osmolality in *C. sapidus* transferred from 35 – 22.5 ppt (A) and in *C. maenas* transferred from 32 – 25 ppt (B). Means ± SEM, n=6.

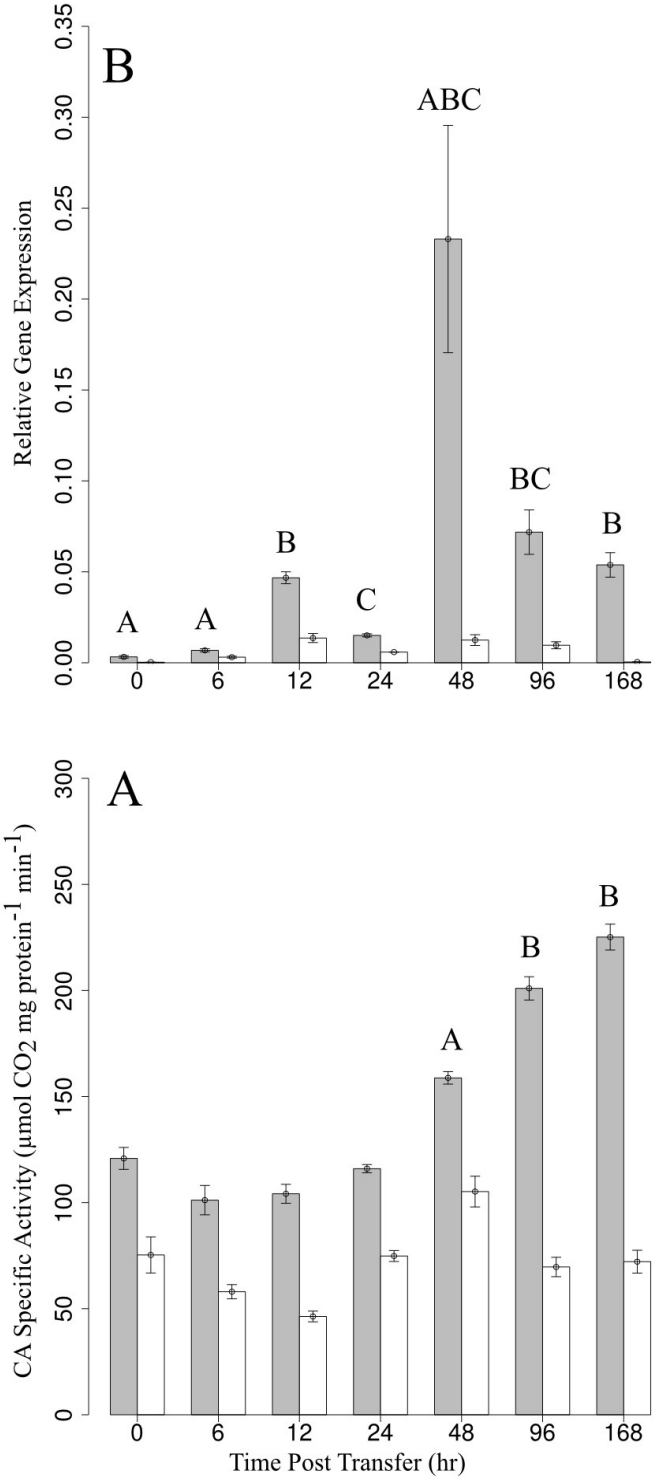

Fig. 3 CAc mRNA (A) and CA enzyme activity (B) in posterior (G8, gray bars) and anterior (G4, white bars) gill pairs of *C. maenas* transferred from 32-25 ppt. Means ±SEM, n=4-6. Different letters denote significant differences between groups at the 0.05 level.

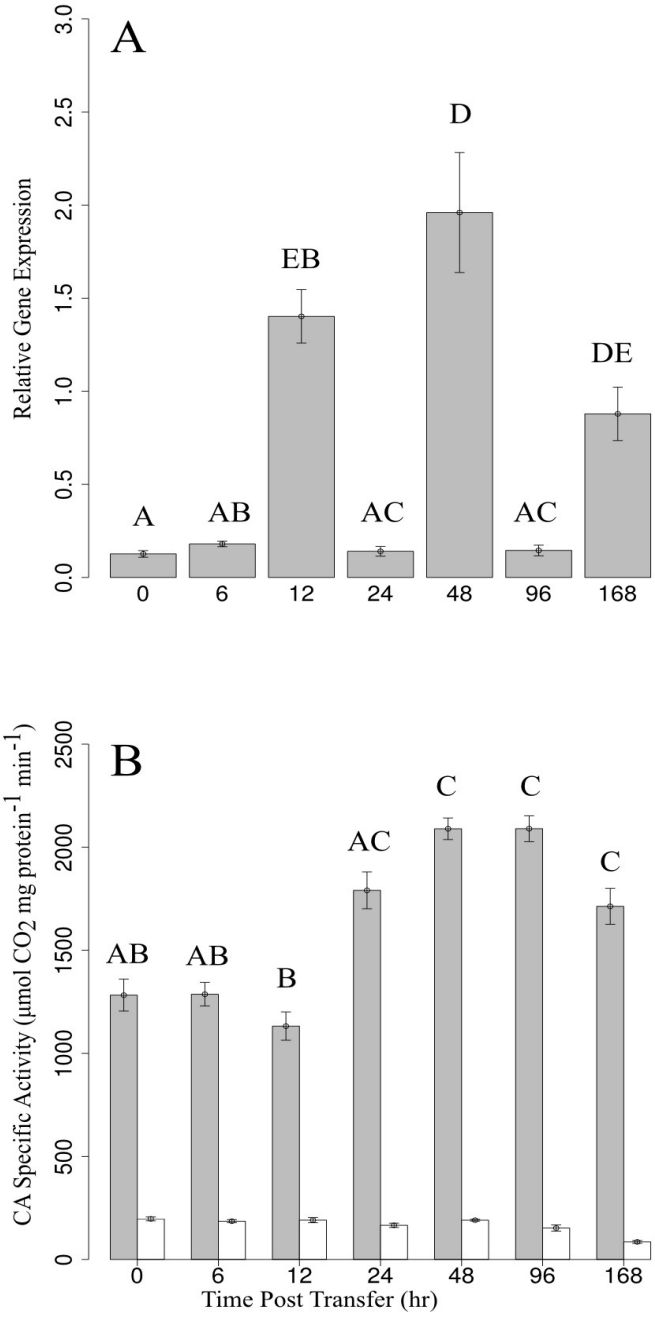

Fig. 4 CAc mRNA (A) and CA enzyme activity (B) in posterior (G7, gray bars) and anterior (G3, white bars, not pictured for mRNA) gill pairs of *C. sapidus* transferred from 22.5-10 ppt. Means ± SEM, n=6. Different letters denote significant differences between groups at the 0.05 level.

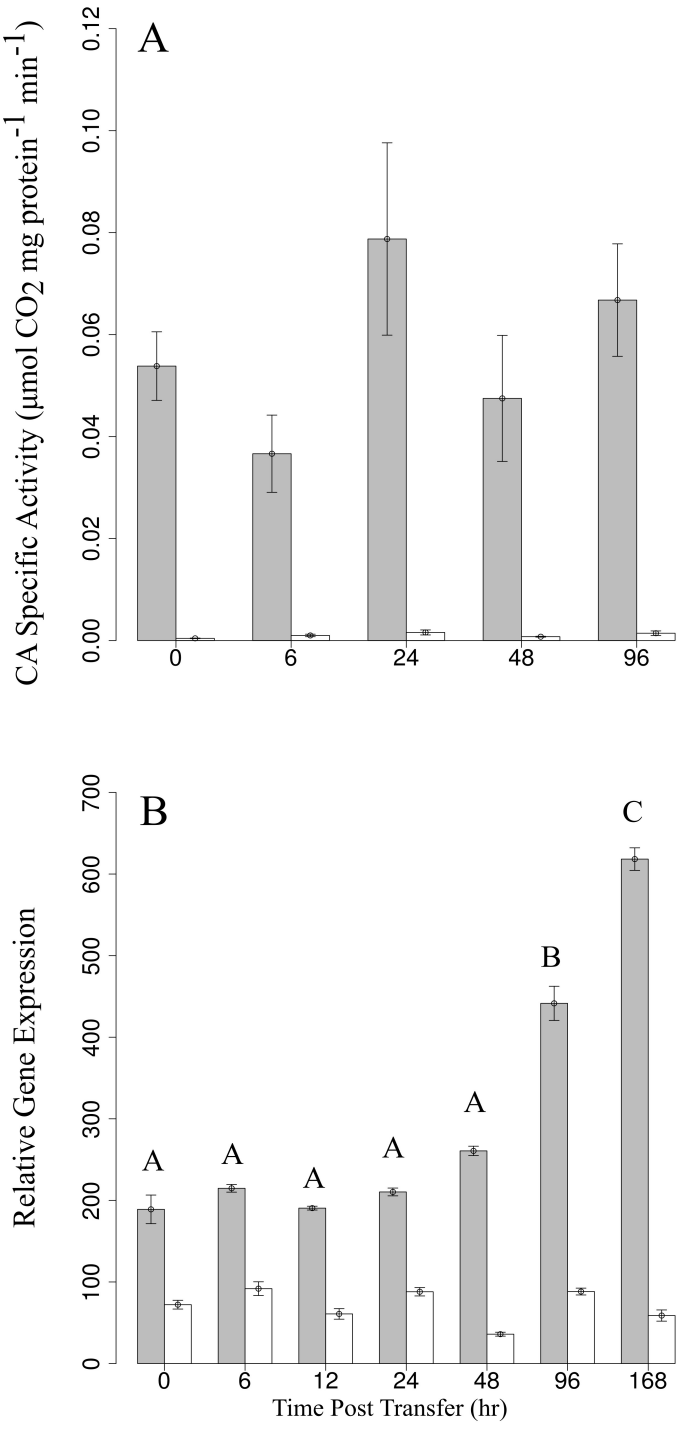

Fig. 5 CAc mRNA (A) and CA enzyme activity (B) in posterior (G8, gray bars) and anterior (G4, white bars) gill pairs of *C. maenas* transferred from 25-15 ppt. Means ±SEM, n=4-6. Different letters denote significant differences between groups at the 0.05 level.

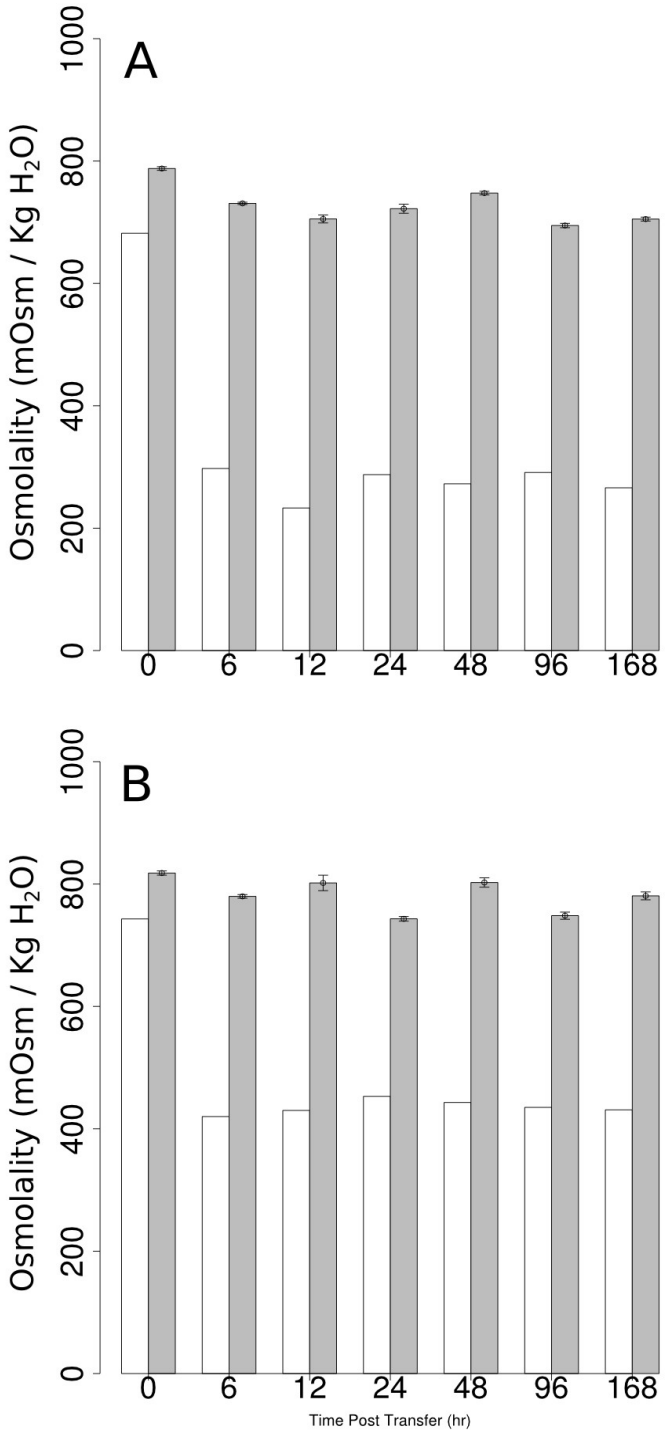

Fig. 6 Hemolymph (gray bars) and ambient (white bars) osmolality in *C. sapidus* transferred from 22.5 – 10 ppt (A) and in *C. maenas* transferred from 25– 15 ppt (B). Means ± SEM, n=6.

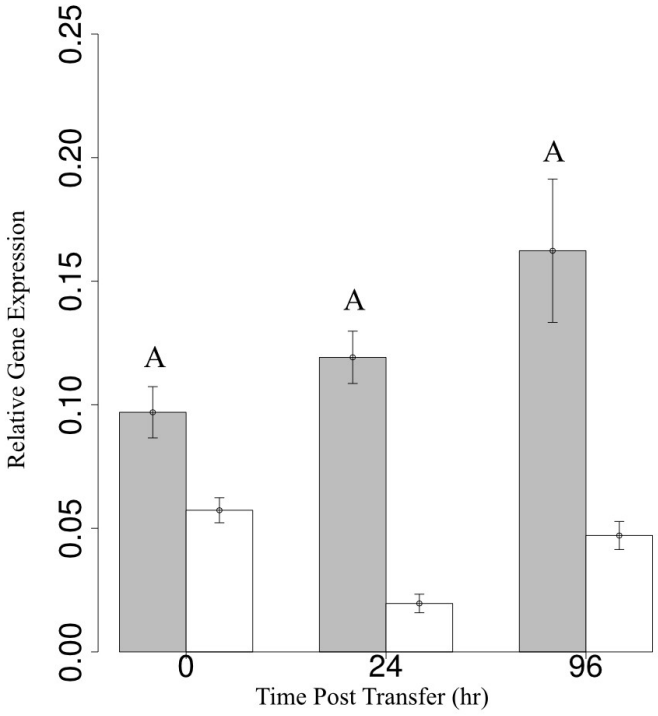

Fig. 7 ZIP1 mRNA in G7 (gray bars) and G3 (white bars) of *C. sapidus* transferred from 35-22.5 ppt. Means ± SM, n=6. Different letters denote significant differences between groups at the 0.05 level.

Chapter 4. Transcriptomic Responses to Low Salinity Acclimation in the Euryhaline Blue Crab *Callinectes sapidus*

Abstract

The transfer of euryhaline crustaceans from high to low salinity is known to induce a myriad of changes in gene expression corresponding with the activation of ionic uptake pathways. In this study, we performed whole-transcriptome shotgun sequencing (RNA-seq) on anterior (G3) and posterior (G7) gills of the marine crab *Callinectes sapidus* acclimated to full-strength seawater (35 ppt) and low salinity (10 ppt). Low salinity acclimation resulted in the significant upregulation of 86 genes in the posterior ion-regulating gills (G7), 6 of which endcoded ion transport proteins. These included the cytoplasmic carbonic anhydrase (CAc) and, $Na^+/K^+/2Cl^-$ co-transporter, which increased by 16 and 10-fold, respectively. Several interesting genes putatively involved in ion uptake at low salinity were found to also increase, including Cl^- channel 2 (CLC-2), an amiloride-sensitive Na^+ channel, a Na^+/HPO_4^- cotransporter, and a NaCl-dependent glycine transporter. Changes in ion transport gene expression were also observed in

the respiratory gills (G3), which may be a relic of the small sample size and several sequencing issues which are discussed. We also measured the expression of cellular stress proteins including heat shock proteins (HSP); no changes were observed in their expression, suggesting that low-salinity does not elicit a classical stress response in *C. sapidus*. Finally, functional annotation was performed on the posterior gills to observe any trends among the usage of specific cellular and physiological pathways at low salinity. The categories showing the highest increases in transcription in response to environmental dilution were digestion (83%), circulation (79%), excretion (79%), ionic uptake (72%), and carbohydrate metabolism (65%). This analysis demonstrates broad-scale gene activation events in cellular and physiological pathways involved in energy metabolism in order to meet the energetic demand of increased active ion transport.

Key words: Euryhaline, RNA-seq, crustacean, osmoregulation

Introduction

The physiological relationships between euryhaline marine crustaceans and the salinity environments they inhabit have been an active area of research for many years. At full-strength seawater (~32-35 ppt), these animals are osmoconformers, passively maintaining hemolymph ion concentrations and osmolality equal to the surrounding water. At low salinity; osmotic influx of water across the gills, the efflux of ions across the gills, and the further loss of ions through the excretion of copious amounts of isosmotic urine results in a significant dilution of hemolymph

osmolality and ionic concentrations (Baldwin and Kirschner, 1976; Cameron 1978; Pierrot et al., 1995; Riestenpatt et al., 1996; Onken et al., 2003).

Thus at low salinity, usually below 26 ppt (Henry, 2005) many species become osmoregulators, employing physiological mechanisms to actively maintain hemolymph osmolality and ionic concentrations at levels well above ambient (see Mantel and Farmer, 1983; Pequex 1995; Henry et al., 2012 for reviews). Due to the aforementioned extensive loss of ions to the environment at low salinity, the most critical aspect of osmoregulation in euryhaline crustaceans is the maintenance of extremely high rates of active ionic uptake from the surrounding medium (see Henry et al., 2012; McNamara and Faria, 2012 for review).

Ionic uptake at low salinity occurs across the posterior gill pairs, with the anterior gills instead functioning in respiration. Within the posterior gills, ion transport occurs in specialized mitochondria-rich cells (MRC). It is the concerted action of several proteins in the MRC that facilitates the transport of ions (mainly Na^+ and Cl^-) from the surrounding medium, across the apical gill membrane, into the cytoplasm, across the basolateral membrane, and finally into the hemolymph. Na^+ and Cl^- first enter the cell when they are transported from the water and into the cytoplasm via the action of an apical $Na^+/K^+/2Cl^-$ cotransporter (Ristenpatt et al., 1996). Apical and basolateral K^+ channels facilitate K^+ efflux across both membranes; establishing a net negative charge inside the cell, driving Cl^- through channels on the basolateral membrane and into the hemolymph (Riestenpatt et al., 1996; Onken et al., 2003; Lucu and Towle, 2010). Intracellular Na^+ is pumped into the hemolymph via the action of the basolateral membrane-bound Na^+/K^+-ATPase (Lucu and Siebers, 1987; Luquet et al., 2002; Onken et al., 2003; Lucu et

al., 2009; Lucu and Towle, 2010). Movement of Cl^- across basolateral membrane is believed to passively follow the electrical gradient created by the pumping of Na^+. In addition to this pathway, NaCl uptake occurs through apical Na^+/H^+ and Cl^-/HCO_3^- exchange proteins (Lucu, 1990; Onken et al., 2003; Henry, 2001; Henry et al., 2002, 2006). The H^+ and HCO_3^- that serve as counterions for ionic exchange are provided by the hydration of respiratory CO_2 by cytoplasmic carbonic anhydrase.

When acclimated to full-strength seawater, these ion uptake processes are physiologically silent, being only activated after exposure to water below 26 ppt (Henry, 2005). The activation of these processes is evident by the presence of inward NaCl-dependent currents in crustacean gills acclimated to low salinity (Riestenpatt et al., 1996; Onken and Riestenpatt 2002, Onken, 2003) and by inductions in the activity of enzymes involved in this pathway (Towle et al., 1976; Henry and Cameron, 1982; Holiday, 1985; Wheatly and Henry, 1987; Corratto and Holiday, 1996; Mananes et al., 2000; Henry et al., 2002, 2003; Roy et al., 2007; Tsai et al., 2007).

Advances in DNA sequencing techniques as well as quantitative polymerase chain reaction (qPCR) have allowed for the activation of ion uptake to be examined at the level of gene expression. Genes which are transcriptionally responsive to environmental dilution include the cytoplasmic carbonic anhydrase (CAc) and the Na^+/K^+-ATPase α-subunit (NaK) (Luquet et al., 2005; Jayasundara et al., 2007; Serrano et al., 2007; Serrano and Henry, 2008; Pongsomboon et al., 2009). In some taxa, the expression of a $Na^+/K^+/2Cl^-$ cotransporter (NaK2Cl) is also induced by low salinity acclimation (Luquet et al., 2005), while in others it is static (Towle et al., 2011).

Although qPCR has been an invaluable tool for the understanding of the molecular responses to low salinity in the crustacean gill, it is somewhat limited in its scope as the evaluation of multiple genes can be a laborious process. The usage of technologies which allow for the simultaneous measurement of hundreds of genes, while still in it's infancy, has the potential to be an extremely useful tool to provide a broad-scale picture of gene activation events in the crustacean gill following salinity transfer.

In a recent study by Towle et al. (2011), the authors examined the salinity-stimulated expression of several dozen genes in the euryhaline green crab *Carcinus maenas* using a DNA microarray. This work found that in addition to CAc and NaK an additional 190 transcripts were strongly upregulated in response to environmental dilution; these included a Na^+/glucose co-transporter, a voltage-gated Ca^{2+} channel, an endomembrane protein, an organic cation transporter, and an anion exchange protein (Towle et al., 2011). Interestingly, this study found that many genes which play an essential role in ionic uptake were not upregulated at low salinity, including the Na^+/H^+-exchanger and NaK2Cl. The authors concluded that a complex pattern of transcriptional regulation exists in the gills of *C. maenas*, whereby some molecular components of ionic uptake mechanisms are expressed at adequate levels at all salinities, while others require significant increases in encoding mRNA at low salinity. Additionally the expression of genes encoding cellular stress proteins including heat shock proteins (HSP) were examined. Low salinity acclimation had virtually no effect on the expression of these genes.

DNA microarray technology has also been used to examine changes in branchial gene expression in the Japanese blue crab *Portunus trituberculatus* in a 2011 study by Xu and Liu. In

contrast to *C. maenas,* environmental dilution in *P. trituberculatus* results in significant induction of HSP90, HSP70, and HSP60 as well as additional genes involved in cellular stress including several proteosome subunits and regulatory proteins (Xu and Liu, 2011). Due to the small number of oligonucleotide probes complimentary to ion transport genes used in this study, the authors did not find any significant increases in ion transport genes except for the Na^+/K^+-ATPase β-subunit.

Recently, DNA microarrays have fallen out of favor and are rapidly being replaced with whole transcriptome shotgun sequencing, also known as RNA-seq. As opposed to microarrays, which can only provide expression data for known sequences that hybridize to predetermined oligonucleotide probes, RNA-seq allows for quantitative downstream analysis of any sequence in a sample without any *a priori* knowledge. RNA-seq was used to confirm and expand upon salinity-stimulated gene expression data in *P. trituberculatus.* RNA-seq also found significant induction in HSPs and other stress genes in this crab, as well as upregulation of transcripts encoding CAc, the apical Na^+/H^+-exchanger, and Chloride Channel Protein 2 (ClC-2) (Lv et al., 2013). When compared with Towle et al. (2011), these data point to the possibility of different molecular and physiological responses to low salinity in different species of euryhaline crustaceans. *C. maenas* is apparently more physiological resilient to changes in environmental salinity than *P. trituberculatus* as evidenced by the lack of induction in HSPs and other stress-related genes in the former. Additionally, there may be differences in the transcriptional responses of genes directly involved in ionic uptake: for example Na^+/H^+-exchanger mRNA increased by 2.5-fold in *P. trituberculatus* but was essentially static in *C. maenas.*

To further explore the transcriptomic responses of euryhaline crustaceans to low salinity, in this study we utilize RNA-seq to perform a characterization of gene expression in response to low-salinity acclimation in the gills of the euryhaline blue crab *Callinectes sapidus*. cDNA libraries from anterior and posterior gills of *C. sapidus* at both full-strength seawater and low salinity acclimated crabs were sequenced in order to observe changes in gene expression in both tissues, with genes involved in ionic uptake and cellular stress being a focal point. Additional attention is paid to the discovery of potentially salinity-sensitive ion transport genes putatively involved in osmoregulation which have not been previously implicated. Finally, we perform a functional annotation of posterior gills at high and low salinity in an attempt to view the trends in usage of various cellular and physiological pathways in the osmoregulatory organs on *C. sapidus* following low salinity acclimation

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Materials and Methods

Collection and Maintenance of Animals

Adult, intermolt, blue crabs, *Callenectes sapidus* were obtained from commercial watermen in East Point, FL, USA, packed in wet burlap, placed in 45 L coolers, and transported to Auburn University. Crabs were held in 150 gal recirculating aquaria equipped with biological filters (oyster shell and sand) at 24^{o}C and ambient photoperiod. Crabs were held at 35 ppt for at least 3 weeks prior to experimentation to ensure acclimation to full-strength salinity (Henry and Watts, 2001; Jillette et al., 2011). Crabs were fed daily on shrimp, salinity was monitored with a

conductivity meter (YSI 30 1, Yellow Springs, OH, USA) and adjusted with distilled water or concentrated brine made with artificial sea salts (Instant Ocean Reef Crystals, Blacksburg, VA, USA). Water quality was checked by monitoring nitrite concentrations (Dry Tab, Mansfield, MA, USA).

Experimental Protocol

Following acclimation to 35 ppt, crabs used for low salinity study were transferred to 10 ppt for one week. At the end of the experimental period, anterior (G3) and posterior (G7) gills were dissected out and immediately placed in 3 ml of RNAgents Denaturing Solution (Promega, Madison WI, USA).

RNA Purification

Total RNA was extracted from gills using RNAgents Total RNA Isolation System (Promega, Madison WI, USA) under RNAse-free conditions, with all equipment being previously treated with RNAse-Zap (Ambion, Austin, TX, USA). RNA purity and quality was assayed using a Bioanalyzer 2100 (Agilent Technologies, Wilmington, DE, USA), no genomic DNA contamination being observed. The total RNA concentration of each sample was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

cDNA Library Synthesis and Sequencing

cDNA was synthesized from 1 μg of total RNA. First-strand cDNA synthesis was performed using the SMART cDNA library construction kit (Clontech, Mountain View, CA, USA) as per the manufacturer's instructions except that the provided 3' oligo was replaced with the Cap-Trsa-CV oligo as per Matz et al. (2009). Full-length cDNA was then amplified using the Advantage 2

PCR system (Clontech, Mountain View, CA, USA) using the minimum number of PCR cycles possible (usually 15-17) and sent to HudsonAlpha Institute for Biotechnology (Huntsville, AL, USA) for paired-end (PE) library preparation and Illumina sequencing. Each library was sequenced using approximately one-sixth of an Illumina HiSeq 2000 lane using the 2 X 100 bp PE chemistry.

Transcriptome Assembly, Differential Expression, and Functional Annotation

Raw reads were assembled using the software packageTrinity with default conditions (Grabherr et al., 2011; Haas et al., 2013). Left and right reads from all cDNA libraries were mapped to contigs in the 10 ppt G7 assembly (the library having the highest number sequencing reads, contigs, and largest N50 statistic) using RSEM (Li and Dewey, 2011). To correct for larger contigs having a bias towards a larger total number of reads, FPKM (fragment per kilobase of exon per million fragments mapped) values for each contig were calculated in the edgeR package of BioConductor (Robinson et al., 2009) with R version 3.0.2. Relative gene expression was reported as FPKM.

To identify specific genes of interest for differential expression analyses, query sequences were obtained from NCBI's GenBank database and aligned against the assembled 10 ppt G7 transcriptome using tblastx (Altschul et al., 1997). Sequences with high scoring hits were aligned against the GenBank database to confirm the gene identity of each contig.

Likely protein coding contigs were extracted from each library using TransDecoder (Haas et al., 2013). Functional annotation was performed on these coding sequences using the Kyoto Encyclopedia of Genes and Genomes (KEGG) Automatic Annotation Server with a bi-

directional best-hit method (Goto et al., 2010). Contigs assigned KEGG Orthology (KO) numbers were grouped into categories of cellular and physiological function in the KEGG database.

Results

Sequencing and Assembly

cDNA libraries were synthesized and sequenced for anterior (G3) and posterior (G7) gills of *C. sapidus* acclimated to full-strength seawater (35 ppt) and low salinity (10 ppt). These libraries are described in Table 1. For G3, a total of 59,571,988 and 47,601,872 PE reads were obtained for the 35 and 10 ppt libraries, respectively. 911,658 and 77,218,958 were produced for 35 and 10 ppt in G7. Following assembly of each library with Trinity (Haas et al., 2013), the G7 10 ppt assembly was found to have the highest amount of contigs and longest mean contig length (Table 1) and was therefore used as the reference transcriptome to measure differential expression in all libraries (Haas et al., 2013).

Differential Expression

Transfer to low salinity resulted in the significant upregulation of 125 contigs in G7 (Table 2). These sequences were cross-referenced with the KO numbers assigned by the KEGG Automatic Annotation Server. 18 of these 125 contigs were matched to reference sequences in the KEGG database (Table 3), and 6 of the 18 were found to be involved in ion transport. There was no trend in the physiological and cellular role of the remaining 12 contigs assigned KO numbers. Genes encoding ion uptake proteins included those previously known to be activated at low salinity in euryhaline crabs such as CAc (Serrano et al., 2007; Serrano and Henry, 2008;

Towle et al., 2011; Mitchell and Henry 2014), which rose by 16-fold (Fig. 1). Interestingly, the relative expression of the NaK2Cl, which in *C. maenas* does not transcriptionally respond to environmental dilution (Towle et al., 2011) increased 9-fold in G7 of *C. sapidus* (Fig. 1). The expression of the NaK; which has been shown to have a strong transcriptional response to environmental dilution in *C. sapidus* (Serrano et al., 2007); did not increase significantly in G7, although expression did rise by 2.7-fold (Fig. 1). This gene was by far the most abundant ion-regulating transcript in the posterior ion-regulating gills at low salinity, followed by CAc, with FPKM values of 7500 and 1100, respectively (Fig. 1).

The remaining four (4) significantly upregulated ionic transport genes have not been previously implicated in ionic uptake mechanisms in euryhaline marine crustaceans. mRNA sequences for these genes were obtained from the NCBI database and aligned against the G7 10ppt transcriptome using tblastx. Highly significant homology in our G7 10 ppt assembly was found for all genes which included a contig homologous to chloride channel 2 (CIC-2) (E = 0.0; *Drosophila pseudoobscura;* XM_003736787.1) (Table 4). This was by far the most highly expressed and salinity-sensitive Cl^- channel transcript found in this study, increasing by 7-fold to 508 FPKM after 10 ppt transfer. Other genes included a amiloride-sensitive Na^+ channel (E = $2e^{-29}$; *Strongylocentrotus purpuratus;* XM_003723292.1),(Fig. 3). a $Na^+/HP0_4^-$ cotransporte^r (E = $1e^{-13}$8 ; *Haplochromis burton*i; EFX74357.1), and an NaCl-dependent glycine transporter (E = $1e^{-173}$; *Apis florea*; XM_003692111.1) (Table 4). Of all the genes measured in this study, the putative amiloride-sensitive Na^+ channel was the most salinity-sensitive; with relative gene expression increasing by 131-fold following 10 ppt acclimation (Fig. 1). Several other genes implicated in

ionic uptake in euryhaline crustaceans did not increase in either gill at 10 ppt; including the V-type ATPase, a voltage-gated Ca^{2+}-ATPase, and the Na^{+}/H^{+} exchanger (Fig. 2).

In addition to ionoregulatory genes, we measured the relative expression of stress related genes, including heat shock proteins (HSPs), which serve as molecular chaperones in response to a wide variety of stress factors in metazoans (reviewed by De Maio, 1999). HSP70 and HSP90 were the most highly expressed stress-related genes in G7 at 35 ppt, with respective FPKM values of 1720 and 625; these did not increase following low salinity transfer, nor did the abundance of lower expressed heat shock transcripts such as HSP10 and HSP40 (Fig. 3). HSP21 was significantly downregulated from 35-10 ppt by 3.8-fold (Fig. 3). Other genes involved in the cellular response to stress such as ubiquitin B, and proteosome α and β subunits did increase at low salinity by 4, 2.4, and 2.2-fold, respectively; however none of these increases were statistically significant (Fig. 3). The only significant increase in expression of a stress-related gene occurred for the proteosome regulatory subunit N9 with an increase of 8.2-fold (Fig. 3).

Low salinity transfer resulted in the significant upregulation of 321 contigs in G3 (Table 2). Surprisingly, these included the ion-regulating transcripts significantly upregulated in G7 also increased in response to environmental dilution in G3 (Fig. 1). In some cases, the magnitude of G3 upregulation was 2-3 times greater than the degree of G7 induction, as was the case for CAc, CLC-2, and the NaCl-dependent glycine transporter (Fig. 1). Additionally, the expression of NaK, which did not significantly increase in G7, did significantly respond to low salinity in G3 by 8-fold (Fig. 1). The expression of heat shock proteins and stress-related genes in G3 was similar to G7: no significant upregulations occurred and HSP70 and HSP90 were the most highly

expressed transcripts examined. A downregulation of HSP21 by 3.2-fold did occur in G3, however this change was not statistically significant (Fig. 3). Increases in expression of ubiquitin B and proteosome α and β subunits were not seen in G3 as they were for G7 (Fig. 4).

Functional Annotation

In order to examine broad-scale changes in transcription in the ion-regulating posterior gills following low salinity acclimation, FPKM values for each contig assigned a Kegg Orthology (KO) number in a specific category were grouped together. In this way, the entire relative expression for genes involved in various physiological and cellular pathways were quantitatively compared at high and low salinity. The largest increases in total expression at low salinity occurred in the genes associated with digestion (83%), circulation (79%), excretion (79%), ionic uptake (72%), and carbohydrate metabolism (65%) (Fig. 5).

Discussion

In this study, a total of 86 contigs were significantly upregulated in the posterior ion-regulating gills (G7) following transfer from full-strength seawater (35 ppt) to low salinity (10 ppt) (Table 1). This is slightly less than expected, as DNA microarrays found 190 and 158 genes significantly upregulated in *C. maenas* and *P. trituberculatus* (Towle et al., 2010; Xiu and Liu, 2011), and RNA-seq in *P. trituberculatus* again found 158 significantly upregulated sequences (Lv et al., 2013). Similarly, the number of downregulated transcripts in this study (39, Table 1) was low compared to microarray data in *C. maenas* (411, Towle et al., 2010) and *P. trituberculatus* (54, Xiu and Liu, 2011), as well as RNA-seq in *P. trituberculatus* (457, Lv et al., 2013). The relatively low number of differentially expressed genes found in *C. sapidus* may be a

result of the small sample size (n=1 for each treatment) and the small amount of sequencing reads recovered from the 35 ppt sample (~1.5% the amount of the other libraries) (Table 1). However, it is quite telling that 30% of the upregulated branchial genes in this study encoded ion transport proteins (Table 3), an observation which is indicative of the importance of enhanced ionic uptake in low salinity adaptation in marine crustaceans. Nevertheless, resequencing of the 35 ppt G7 library and adding several more samples to the dataset will be required before any definitive conclusions can be made from these data.

This is the first study performed on a crustacean utilizing a whole-transcriptome analysis method to compare gene expression in the anterior gills following salinity transfer. Surprisingly, 321 genes were significantly upregulated in G3 despite the fact that the anterior gills are generally believed to be relatively salinity-insensitive (Serrano et al., 2007; Serrano and Henry, 2008). Additionally, several of these upregulated genes are directly implicated in ionic uptake, including CAc, NaK2Cl, and the Na^+/K^+-ATPase (Fig. 1). These results are questionable however, as multiple qPCR studies have shown that the expression of these genes do not increase in the anterior gills of euryhaline crabs following low salinity acclimation (Luquet et al., 2005; Henry, 2006; Jayasundara et al., 2007; Serrano et al., 2007; Serrano et al., 2008; Mitchell and Henry, 2014). Furthermore, an osmoregulatory role of the anterior gills is further obviated by an extensive body of literature which demonstrates that the activity of the enzymes encoded by these genes does not increase in the anterior gills (Henry et al., 2002; Henry et al., 2003; Genovese et al., 2004; Henry, 2005; Henry, 2006; Henry and Borst, 2006; Serrano et al., 2007; Serrano and Henry, 2008; Mitchell and Henry, 2014). Thus the startling results for G3 obtained

in this study should not be taken as conclusive until more samples can be appended to our data set and more studies are published which utilize a whole-transcriptome approach to examine gene expression in the anterior gills.

This work confirms the results of previous studies using qPCR (Serrano et al., 2007; Serrano and Henry, 2008; Pongsomboon et al., 2009; Mitchell and Henry, 2014), DNA microarrays (Towle et al., 2011; Xiu and Lu, 2011) and RNA-seq (Lv et al., 2013), all of which have found substantial upregulations in CAc mRNA following low salinity acclimation in euryhaline crustaceans. Following 10 ppt acclimation, relative CAc expression increased by 16-fold (Fig. 1). In this study, CAc was the second highest expressed ion-regulating gene in the posterior gill pairs, after NaK (Fig. 1). It appears that RNA-seq is less sensitive than qPCR for measuring extremely large changes in gene expression. For CAc, qPCR-detected mRNA induction in *C. sapidus* tend to be on the order of 100-fold in G7 following transfer from full-strength seawater to low salinity (Serrano et al., 2007; Mitchell and Henry, 2014), whereas RNA-seq only detected a 16-fold upregulation (Fig. 1). The same appears to be true for DNA microarrays; Towle et al. (2010) reported a 4-fold increase in CAc expression in *C. maenas*, while qPCR found a 10-fold increase (Serrano and Henry, 2008). This may result from qPCR being a very targeted approach, with only one or a few specific genes being measured in a given analysis. RNA-seq and microarrays on the other hand, are more broad-scale approaches, which may result in a lower signal to noise ratio.

Although expression of the NaK did not significantly change in G7 in this study, it did increase by 2.7-fold (Fig. 1), which is of a similar magnitude to qPCR-reported changes in *C.*

sapidus (Lovett et al., 2006; Serrano et al., 2007). Additionally, Towle et al. (2011) reported significant upregulations of NaK in *C. maenas* using a DNA microarray. The non-significant response of this gene in this study is likely due to the small sample size (n=1) used and the low amount of reads generated in the 35 ppt G7 sample. Interestingly, neither DNA micoarrays nor RNA-seq found any transcriptional response of this gene in *P. trituberculatus* (Xu and Liu, 2011; Lv et al., 2013). The differences in NaK gene activation may reflect the differential osmoregulatory abilities between these crabs. For example, when acclimated to 15 ppt, *C. maenas* and *C. sapidus* were able to maintain hemolymph osmolalities of 317 and 285 mOsm/Kg H_2O above ambient, respectively (Serrano et al., 2007; Mitchell and Henry, 2014). At the same salinity, *P. trituberculatus* was only capable of maintaining hemolymph at194 mOsm/Kg H_2O above ambient (Lu et al., 2013). Thus, the increased induction of Na^+/K^+-ATPase, the active driver of NaCl uptake, may be a deciding factor which results in a superior osmoregulatory ability in *C. sapidus* and *C. maenas* than in *P. trituberculatus*.

This is the first study that has examined the expression of NaK2Cl in the gills of *C. sapidus*. 10 ppt transfer resulted in a 9-fold increase in relative gene expression (Fig. 1), thus it appears that this transporter is quite sensitive to environmental salinity. There seems to be a difference between the salinity-sensitivity of this gene between different species of euryhaline crabs. In *C. maenas,* low salinity acclimation resulted in no changes in NaK2Cl expression, nor did it occur in *P. trituberculatus*. However in *Chasmagnathus granulata* (Luquet et al., 2005), and *C. sapidus* (this study), this gene is one of the most inducible branchial genes measured. The differences in the regulation of this gene, even between closely related species with similar osmoregulatory

abilities such as *C. maenas* and *C. sapidus*, suggests that the evolution of regulatory patterns of ionic uptake may be a relatively plastic process.

Several ion transport-related genes which have not been thoroughly examined for a role in crustacean osmoregulation were significantly upregulated in G7 following 10 ppt acclimation. This included a contig with highly significant homology to *Drosophila pseudoobscura* Cl^- channel 2 (ClC-2) (E = 0.0). Of all the chloride channels expressed in the gills, ClC-2 was by far the most abundant, and the most transcriptionally responsive to low salinity acclimation, increasing 7-fold from 35 to 10 ppt (Fig. 3). The expression of this gene was also observed to increase at low salinity in *P. trituberculatus* (Lv et al., 2013). Interestingly, the opening of this channel and subsequent cellular Cl^- efflux is known to be stimulated by hyposmotic stress (Gyömorey et al., 2000). The high branchial expression value of this gene, coupled with its transcriptional and structural sensitivity to low-salinity, suggests that this may be the channel by which the bulk Cl^- flows across the basolateral membrane and into the hemolymph at low salinity. In the future, measuring the Cl^- current in perfused gills treated with selective Cl^- channel blockers such as 5-nitro-2-(3-phenylpropyl-amino) benzoic acid (NPPB) and 4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) may be helpful in implicating the role of this protein in crustacean ionic uptake.

Of all ion transport genes examined, the amiloride-sensitive Na^+ channel was the most induced by low salinity transfer, increasing by 131-fold in G7 (Fig. 1). The protein encoded by this gene has been implicated in ionic uptake in low conductance freshwater crustacean gills (Zeiske et al., 1992), where it facilitates active Na^+ absorption in concert with the basolateral

Na^+/K^+-ATPase (see Onken and Riestenpatt, 1998; Henry et al., 2012; McNamara and Faria, 2012 for reviews). Evidence of an apical epithelial Na^+ channel in marine crustacean gills (such as *C. sapidus* and *C. maenas*) is absent; however, due to the extreme salinity sensitivity of this gene in *C. sapidus,* it seems likely that it is a component of ionic uptake.

Another interesting upregulated branchial gene that was upregulated by environmental dilution was a NaCl-dependent glycine transporter, which increased by 15-fold (Fig. 1). This protein moves glycine into the cell against a concentration gradient by coupling its transport to the energy built up by transmembrane Na^+ gradient (see Kristensen et al., 2011 for review). The protein encoded by this gene is capable of transporting trimethylglycine, a critical osmolyte involved in cell volume regulation (Lang et al., 1998; Anas et al., 2007). Cell volume regulation is a critical component of salinity tolerance in euryhaline crustaceans, however it is unlikely that this protein functions in volume regulation in dilute environments as amino-acid excretion, as opposed to uptake, is responsible for altering the osmotic gradients between the cell and surrounding medium at low salinity (see Chamberlin and Strange, 1989 for review). Rather it is more likely that this gene facilitates Na^+ uptake across the apical membrane independent of glycine transport (Perez-Stiles, 2012). Thus, this transporter may function similarly to the amiloride-sensitive Na^+ transporter by allowing Na^+ to enter the cell by following the inward Na^+ gradient established by the Na^+/K^+-ATPase.

A Na^+/HPO_4^{-2} cotransporter was trancriptionally enhanced by 11-fold following 10 ppt acclimation. This protein facilitates the electrogenic cellular uptake of 3 Na^+ coupled with an HPO_4^{-2} ion (see Murer et al., 2004 for review). The presence of this transporter in ion-regulating

crustacean tissue is an interesting case, as it (along with the previously discussed amiloride-sensitive Na^+ channel and NaCl-dependant glycine transporter) would move the charge of the MRC towards the positive, which apparently presents a problem, as an inside negative charge is required to drive Cl through channels on the basolateral membrane (see Henry et al., 2012 for review). However, this could be easily overcome by the action of the Na^+/K^+-ATPase, which rapidly eliminates intracellular Na^+. For example, in this study, the relative expression of the Na^+/K^+-ATPase in G7 was 50 and 100-fold higher than the amiloride-sensitive Na^+ channel and the Na^+/HPO_4^{-2} cotransporter, respectively (Fig. 1). Thus, despite the presence of these proteins, the maintenance of a net negative charge to drive Cl^- transport should still be possible. Additionally, it should be noted that the Na^+/HPO_4^{-2} cotransporter, the NaCl-dependent glycine transporter, and the amiloride-sensitive Na^+ channel probably contribute to a relatively small amount of Na^+ influx, due to their low expression values compared to other Na^+ transporters such as NaK2Cl and the Na^+/H^+ exchanger (Fig. 1).

Beyond the expression of ion transport genes, a further goal of this study was to determine if low salinity acclimation in the *C. sapidus*, an extremely capable osmoregulator, induced signs of physiological and cellular stress. To this end, we measured the relative expression of genes encoding HSPs and other proteins involved in stress tolerance. Nearly no transcripts encoding stress-related proteins were significantly upregulated at low salinity in this study, with the exception of the proteosome regulatory subunit N9 (Fig. 3), that by itself does not seem likely to indicate large increases in proteosome activity. Thus, it appears that low salinity acclimation does not produce a classical cellular stress response in *C. sapidus*. Similar results were found in *C.*

maenas by Towle et al. (2011) using DNA microarrays. Conversely, in *P. trituberculatus* salinity stress resulted in significant upregulation of several HSPs and other stress-related proteins including proteosome subunits and regulatory factors (Lv et al., 2013). The difference in the stress response between these animals is likely a reflection of the physiological adaptation of each species to their respective niches. Both *C. sapidus* and *C. maenas* routinely encounter environmental dilution, and thus over evolutionary time, have likely developed physiological mechanisms in managing salinity stress (see Henry, 2001 for review). *P. trituberculatus*, on the other hand, is a less capable osmoregulator (Lu et al., 2013). Thus the inductions of HSPs, proteosome subunits, and several proteosome regulatory cofactors at low salinity in *P. trituberculatus* may be a reflection of an inability to dilute environments relative to *C. sapidus* and *C. maenas*.

The final aim of this study was to observe broad-scale branchial transcriptional changes in of *C. sapidus* posterior gills by mapping the relative expression of transcripts in both 35 and 10 ppt acclimated gills back to a functionally annotated G7 transcriptome. Once this was done, the relative expression of every gene within each functional KEGG category was combined to calculate the relative transcriptional response of various cellular physiological pathways. Among physiological pathways, environmental dilution produced the largest responses in genes implicated in digestion, circulation, and excretion (Fig. 5). For cellular processes, ionic uptake, carbohydrate metabolism, and energy metabolism had the largest responses. Aside from ionic uptake genes, which as previously discussed, are transcriptionally active due to the increased demands of ion transport at low salinity, the most responsive cellular and physiological pathways

in the gills seem to be involved in energy uptake, metabolism, circulation of hemolymph, and management of waste products. This is not surprising due to the increased energetic demands of gills engaged in ionic transport against a concentration gradient. Supporting these findings, multiple studies have shown increases in metabolic rate (Sabourin, 1985; Piller et al., 1995; Pillai and Dewan, 2002; Biachini et al., 2008) and carbohydrate metabolism (Nery and Santos, 1993) in crustaceans following salinity transfer.

The finding that amino acid metabolism was one of the most static cellular pathways, increasing only 11% at low salinity, is unexpected. *C. sapidus* gills are known to require increases in amino acid oxidation at low salinity for both energy metabolism and cell volume regulation (Gilles, 1973; Pressley and Graves, 1983; Walsh and Henry, 1992). It may be that the enzymes involved in the induction of amino acid metabolism are under allosteric as opposed to transcriptional regulation and thus increases in amino acid oxidation can be seen without mRNA upregulation. Nevertheless, this unexpected finding warrants future investigation into the transcriptional response of amino acid metabolic pathways to salinity stress.

In summary, we present three major findings pertinent to low salinity acclimation in the euryhaline crab *C. sapidus*. Firstly, the expression of some, but not all, genes involved in branchial ion transport are upregulated following low salinity acclimation; this includes genes which have not been implicated in marine crustacean osmoregulation including an amiloride-sensitive Na^+ channel, a Na^+/PO_4^- cotransporter, and ClC-2, which is likely the source of passage of Cl^- across the basolateral membrane. Secondly, unlike *P. trituberculatus*, low salinity exposure does not elicit a cellular stress response in the ionoregulatory organs of *C. sapidus*, which can be

attributed to their relative adaptabilities to hyposmotic environments. Thirdly, among broad categories of physiological systems and cellular pathways, the most transcriptionally responsive to low salinity exposure within the gill are those involved in metabolism, circulation, and management of waste products. The surprising exception to this is amino-acid metabolism, which is known to be a critical component of energetics and volume regulation in the crustacean gill. However, re-sequencing the 35 ppt G7 library and appending more samples to the dataset will be required before any finite conclusions can be drawn from this study.

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