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Branchial carbonic anhydrase activity and ninhydrin positive substances in the Pacific white shrimp, *Litopenaeus vannamei*, acclimated to low and high salinities

Luke A. Roy^{a,*}, D. Allen Davis^a, I. Patrick Saoud^b, Raymond P. Henry^c

^a Department of Fisheries and Allied Aquacultures, 203 Swingle Hall, Auburn University, Auburn, AL 36849, USA

^b Department of Biology, American University of Beirut, Beirut, Lebanon

^c Department of Biological Sciences, 101 Life Science Building, Auburn University, Auburn, AL 36849, USA

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Abstract

The Pacific white shrimp, *Litopenaeus vannamei*, acclimated to 30 ppt salinity, was transferred to either low (15 and 5 ppt), or high (45 ppt) salinity for 7 days. Hemolymph osmolality, branchial carbonic anhydrase activity, and total ninhydrin-positive substances (TNPS) in abdominal muscle were then measured for each condition. Hemolymph osmotic concentration was regulated slightly below ambient water osmolality in shrimp acclimated to 30 ppt. At 15 and 5 ppt, shrimp were strong hyper-osmotic regulators, maintaining hemolymph osmolality between 200 and 400 mOsm above ambient. Shrimp acclimated to 30 ppt and transferred to 45 ppt salinity were strong hypo-osmotic and hypo-ionic regulators, maintaining hemolymph osmolality over 400 mOsm below ambient. Branchial carbonic anhydrase (CA) activity was low ($\sim 100 \mu\text{mol CO}_2 \text{ mg protein}^{-1} \text{ min}^{-1}$) and uniform across all 8 gills in shrimp acclimated to 30 ppt, but CA activity increased in all gills after exposure to both low and high salinities. Anterior gills had the largest increases in CA activity, and levels of increase were approximately the same for low and high salinity exposure. Branchial CA induction appears to be functionally important in both hyper- and hypo-osmotic regulations of hemolymph osmotic concentrations. Abdominal muscle TNPS made up between 19 and 38% of the total intracellular osmotic concentration in shrimp acclimated to 5, 15, and 30 ppt. TNPS levels did not change across this salinity range, over which hemolymph osmotic concentrations were tightly regulated. At 45 ppt, hemolymph osmolality increased, and muscle TNPS also increased, presumably to counteract intracellular water loss and restore cell volume. *L. vannamei* appears to employ mechanisms of both extracellular osmoregulation and intracellular volume regulation as the basis of its euryhalinity.

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Keywords: Carbonic anhydrase; Osmoregulation; Volume regulation; Pacific white shrimp; Salinity

1. Introduction

The Pacific white shrimp, *Litopenaeus vannamei*, which is found on the Pacific coast of the Americas from northern Mexico to Northern Peru, is a euryhaline species capable of tolerating a wide range of environmental salinity (0.5 to 40 ppt) (Menz and Blake, 1980; Bray et al., 1994). *L. vannamei* is commonly found in offshore waters at depths of up to 72 m (Bailey-Brock and Moss, 1992), so adults typically experience stable salinities circa 35 ppt. The penaeid life cycle, however,

involves a variety of stages that occur in a number of different habitats and salinities. While adults generally inhabit open ocean waters, larvae migrate to low salinity estuaries where they remain until the juvenile stage and then migrate back to oceanic waters. Therefore, adults are primarily exposed to low and fluctuating salinity environments during their reproductive migrations into estuaries.

L. vannamei is also economically important, being the most widely cultured species of shrimp in the western hemisphere. Furthermore, since much of the culture occurs in low salinity environments (ponds and embankments), various researchers have studied the osmotic tolerance of this species (e.g., Gong et al., 2004; Palacios et al., 2004a). In general, penaeid shrimp are

* Corresponding author. Tel.: +1 334 332 8754; fax: +1 334 624 4050.
E-mail address: royluke@auburn.edu (L.A. Roy).

osmotic and ionic conformers in the range of 24–26 ppt (700–780 mOsm kg H₂O) (Castille and Lawrence, 1981). Below that range they are hyper-osmotic regulators, maintaining hemolymph osmotic concentrations as much as 500 mOsm above that of the ambient medium; and above that range they are hypo-osmotic regulators, maintaining hemolymph osmotic concentrations as much as 300 mOsm below ambient (Bray et al., 1994; Castille and Lawrence, 1981; Gong et al., 2004). Despite these studies, little is known about the actual physiological mechanisms of salinity adaptation in *L. vannamei*.

Euryhaline marine invertebrates respond to changes in salinity using intracellular volume regulation and hemolymph ionic/osmotic regulation. Volume regulation, in response to altered hemolymph osmotic concentration and subsequent cell swelling or shrinking, is typically accomplished by adjusting the size of the intracellular pool of inorganic (e.g., K⁺ for short-term adjustment) and organic osmolytes (e.g., free amino acids and/or quaternary ammonium compounds for longer-term adjustment) (Pierce and Amende, 1981; Henry, 1995). Extracellular osmotic regulation, on the other hand, is a result of active ion uptake from dilute seawater, or ion excretion into concentrated seawater (Pequeux, 1995). These two mechanisms are linked: in organisms that are strong osmotic regulators, hemolymph osmotic concentrations change very little despite large changes in external salinity, and as a result, the need for intracellular volume adjustment is minimal. Most euryhaline shrimp are believed to fall into this category.

The mechanism of ion uptake in shrimp has not been systematically studied, but in other groups of decapod crustaceans (e.g., crabs, lobsters and crayfish) specific ion transport proteins and transport-related enzymes have been identified to play a role in the active uptake of the major ions, Na⁺ and Cl⁻ (Mantel and Farmer, 1983). One of the central proteins in this mechanism is believed to be the transport-related enzyme carbonic anhydrase (CA). CA is known to be present in high levels of activity in the organ of ion transport, the gill, and its activity is sensitive to changes in environmental salinity (Henry, 1984, 1988a,b). For brachyuran crabs, only the posterior 3 pairs of gills are involved in ion transport and have high, salinity-sensitive levels of CA activity. In fresh water crustaceans, such as crayfish, all gills have high CA activity (Wheatly and Henry, 1987). The distribution and salinity-sensitivity of branchial CA has not been examined in a hyper/hypo regulating species such as *L. vannamei*. One study reported no difference in CA activity in either anterior or posterior gills of *L. vannamei* in 35 vs. 10 ppt (Palacios et al., 2004a,b), but the period of low salinity exposures (3 h and 24 h) could have been too short for low salinity mediated CA induction to occur. While initial CA induction was shown to occur at 24 h in the blue crab, *Callinectes sapidus* (Henry and Watts, 2001), it took 72 h to occur in the green crab, *Carcinus maenas* (Henry et al., 2002). Bouaricha et al. (1991) reported no differences in CA activity between larval and postlarval stages of *Penaeus japonicus*.

The present study reports on CA activity in individual gills in shrimp fully acclimated to salinities in which they are isosmotic, hyper-regulating, and hypo-regulating. Furthermore, we present data on the relationship between the size of the intracellular pool

of organic osmolytes, acclimation salinity, and hemolymph osmotic concentrations.

2. Materials and methods

2.1. Experimental system

The following study was conducted at the North Auburn Fisheries Research Station in Auburn, Alabama. Juvenile shrimp were obtained from Harlingen shrimp farm (Bayview, TX, USA) and were held at 30 ppt for 4 weeks prior to commencement of experiments. Shrimp were maintained in a 220 L polyethylene nursery tank connected to a biological filter and were offered a commercially prepared feed four times per day (Rangen 35% protein, Buhl, ID, USA). The experimental system consisted of a series of 150 L tanks, each equipped with an airlift biofilter, air stone, and submersible heater to maintain constant temperature of 28.0 °C. Seawater was prepared by adding artificial sea salt (Reef Crystals) to natural pond water. Salinity was measured with a hand-held salinity meter and checked daily. Final values were also checked for osmolality using a vapor pressure osmometer (Wescor 5100C). Throughout the course of each experiment, shrimp were fed Rangen 35% protein feed four times per day using an automatic feeder. Light control was set at 16 h day and 8 h night. Dissolved oxygen (DO), pH, salinity, and temperature were measured daily, whereas ammonia (Solorzano, 1969) and nitrite (Parsons et al., 1985) were measured twice weekly. In the CA experiment dissolved oxygen (5.96±0.72 mg L⁻¹), temperature (26.6±2.4 °C), pH (7.8±0.2), total ammonia nitrogen (0.077±0.10 mg L⁻¹), and nitrite nitrogen (0.24±0.21 mg L⁻¹) remained within acceptable limits. Likewise, in the FAA experiment dissolved oxygen (6.03±0.29 mg L⁻¹), temperature (27.6±0.30 EC), pH (8.0±0.2), total ammonia nitrogen (0.13±0.06 mg L⁻¹), and nitrite nitrogen (0.16±0.12 mg L⁻¹) also remained within acceptable limits.

2.2. Carbonic anhydrase

For the carbonic anhydrase experiment, four 150 L tanks were filled with reconstituted seawater of the following salinities: 5.0, 15.0, 30.0, 45.0 ppt. Shrimp were transferred directly from the 30.0 ppt nursery system into the target salinity water and allowed to acclimate for 7 days prior to CA activity evaluation. At the 7th day 6 postmolt and intermolt shrimp (mean weight: 9.6±1.8 g) were selected from each salinity, and their gills were excised under a dissecting microscope. In order to differentiate among gills, a classification system was devised (Table 1). The gill excision method utilized by Palacios et al. (2004a,b) grouped all gills from the 3rd maxilliped to the 2nd pereopod and designated them as “anterior”, while all other gills were also pooled and designated as “posterior”. Our classification scheme delineates clear excision of individual gill pairs and allows comparison of enzyme activity on an individual gill-pair basis. Individual gill pairs (left and right) from the same animal were identified, dissected out, and immediately homogenized in 2 mL of tris-phosphate buffer (225 mM

Table 1
Gill number classification code and their corresponding location

Gill number	Description
1 (G1)	Anterior arthrobranch, corresponding to the third maxilliped
2 (G2)	Posterior arthrobranch, corresponding to the soma of the first pair of pereopods
3 (G3)	Pleurobranch, corresponding to soma of the second pair of pereopods
4–8 (G4–G8)	The remaining gills were excised in succession from the 3rd to the 5th pereopod.

The classification system begins with the anterior gills located at the anterior end of the gill chamber (closest to mouth) and runs backward to posterior gills.

mannitol, 75 mM sucrose, 10 mM Tris base, adjusted to pH 7.40 with 10% H_2PO_4) using an Omni TH113 homogenizer (Waterbury, CT, USA). The homogenate was centrifuged at $10,000 \times g$ for 20 min at 4 °C, and the CA activity in the supernatant was assayed electrometrically using the delta pH method described by Henry (1991). Either 50 or 100 μL of supernatant (depending on gill size and acclimation salinity) was added to 6 mL homogenization buffer in a vigorously stirred, thermostated (4 °C) reaction vessel. The reaction was initiated by adding 100 μL of CO_2 -saturated water, and the subsequent change in pH was monitored via micro pH and reference electrodes (World Precision Instruments, Sarasota, FL, USA) which were connected to a null point pH meter and linear chart recorder. Typically, a change in pH of around 0.2 unit was utilized to measure the initial velocity of the hydration reaction. Protein concentrations were determined utilizing the Coomassie blue dye binding method (Bio Rad Laboratories, Hercules, CA, USA) and CA activity was reported as $\mu\text{mol CO}_2 \text{ mg protein}^{-1} \text{ min}^{-1}$ (Henry, 2005).

2.3. Total ninhydrin positive substances

For the TNPS experiment a separate group of 10 shrimp were transferred directly from the nursery system to salinities of 2.5, 5.0, 15.0, 30.0, 45.0 ppt and allowed to acclimate for 14 days. At the end of this period, shrimp were dried by blotting on filter paper, and hemolymph was withdrawn from the pericardial cavity using a 23 ga needle and 1 cc syringe inserted beneath the carapace at the cephalothorax–abdominal junction. Following hemolymph extraction, approximately 1 g of abdominal muscle was excised from each shrimp using a scalpel. Hemolymph samples were stored at -20 °C. Muscle samples were minced and placed in 5 mL of 80% ethanol at 4 °C for 48 h to extract intracellular NPS. Muscle tissue was then removed, dried, and weighed; and the ethanol extracts were stored at 4 °C until being assayed. Total ninhydrin positive compounds were assayed colorimetrically utilizing the method of Lee and Takahashi (1966) using a norleucine standard. For TNPS assays with hemolymph, 100 μL of hemolymph was deproteinized in 400 μL of 80% ethanol and centrifuged at $10,000 \times g$ for 60 s. A sub-sample of the supernatant (100 μL) was then used in the assay. The abdominal muscle extracts were then diluted 1:100 and TNPS concentration assayed, while TNPS in hemolymph

samples were assayed directly with no dilution. NPS in hemolymph were reported as mmol L^{-1} , and as $\text{mmol g dry mass}^{-1}$ for muscle.

For analysis of individual amino acid composition, a sample (4 individual samples pooled into one composite sample) of both the muscle tissue extracted in 80% ethanol and hemolymph (non-extracted) from shrimp exposed to each salinity (2.5, 5.0, 15.0, 30.0, 45.0 ppt) was sent off for analysis of physiological free amino acids by the Protein Chemistry, Laboratory, Texas A and M University, Corpus Christi, TX, USA. Free amino acids were extracted and quantified with reverse phase HPLC using UV absorbance with a diode array detector.

2.4. Hemolymph osmotic and ionic measurements

In order to determine osmotic and ionic concentrations, hemolymph samples were thawed on ice and then sonicated (25 W, 30 s, Microson, Heat Systems, Farmingdale, NY, USA) to disrupt the clot (Henry et al., 2003). The samples were then centrifuged at $10,000 \times g$ for 60 s to separate the clot from serum. Total osmolality was then measured using 10 μL of serum by dew point depression (Wescor 5100C vapor pressure osmometer). Hemolymph chloride ion concentration was determined by Ag titration (Labcon Co. digital chloridometer, Petaluma, CA, USA). Finally, hemolymph Na^+ and K^+ concentrations were measured by flame photometry (Cole Parmer digital flame photometer, Model 2655-00, Vernon Hills, IL, USA).

2.5. Statistics

Statistical analyses were performed using SAS (version 8.2, SAS Institute, Cary, NC, USA). Data from both experiments were analyzed using one-way analysis of variance to determine if significant differences ($P \leq 0.05$) existed among treatment means. Student–Newman–Keuls multiple comparison test (Steel and Torrie, 1980) was utilized to determine differences among treatment means in the TNPS experiment, while Dunnett's test was utilized to assess differences in carbonic anhydrase at the test salinities from activity at 30 ppt.

3. Results

3.1. Hemolymph ion regulation

Shrimp acclimated to 30 ppt salinity maintained their hemolymph hypo-osmotic to that of the ambient seawater by about 100 mOsm (Fig. 1). At the lower salinities (15 and 5 ppt), shrimp were hyper-osmotic regulators with hemolymph osmolality being maintained at 274 mOsm above ambient at 15 ppt (682 vs. 408 mOsm $\text{kg H}_2\text{O}^{-1}$ for hemolymph and water, respectively, Fig. 1) and 518 mOsm above ambient at 5 ppt (645 vs. 127 mOsm $\text{kg H}_2\text{O}^{-1}$ for hemolymph and water, respectively, Fig. 1). At 45 ppt, however, shrimp were hypo-osmotic regulators. Hemolymph osmolality was maintained 436 mOsm below that in the medium (838 vs. 1274 mOsm $\text{kg H}_2\text{O}^{-1}$ for hemolymph and water, respectively, Fig. 1). Similar patterns were observed for Na^+ and Cl^- (Fig. 1). K^+

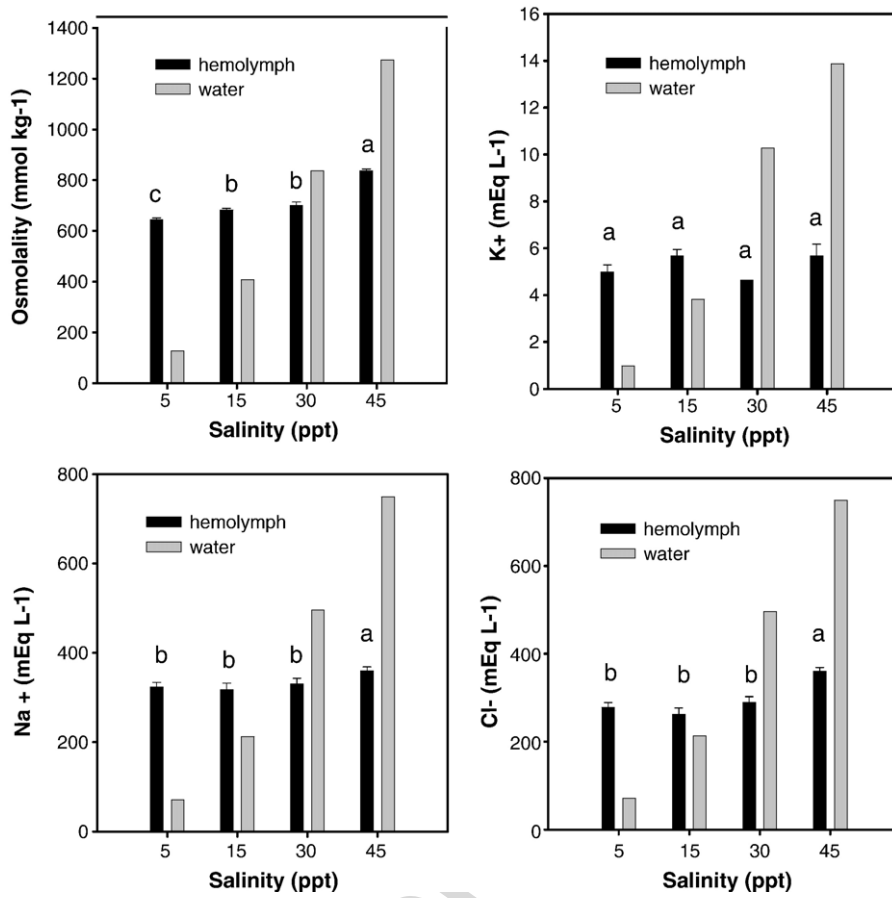


Fig. 1. Hemolymph osmotic and ionic concentrations in Pacific white shrimp reared in 30 ppt salinity and acclimated to three different salinities for 7 days. Values are Mean ± SEM ($n=3-6$). Bars with the same letter are not significantly different ($P<0.05$). Bars for the seawater values represent single measurements.

concentrations were held relatively constant at between 4 and 6 mM across the range of experimental salinities, and as such, this ion was strongly hyper-regulated at 5 ppt and strongly hypo-regulated at 30 and 45 ppt (Fig. 2).

3.2. Carbonic anhydrase

For shrimp acclimated to 30 ppt salinity, CA activity was uniformly low (approximately 100 $\mu\text{mol CO}_2 \text{ mg protein}^{-1}$

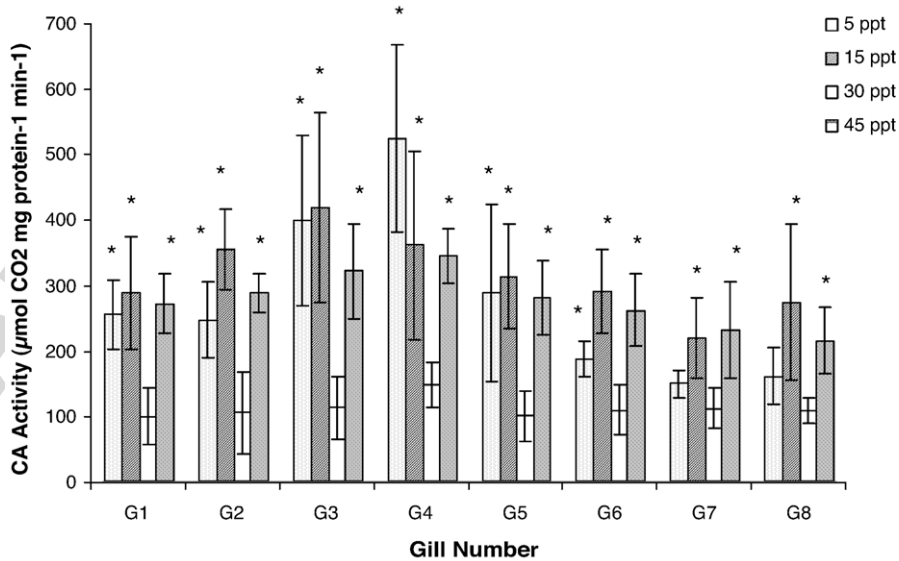


Fig. 2. Carbonic anhydrase activity in gills 1–8 of Pacific white shrimp acclimated to 30 ppt salinity and exposed to low (15 and 5 ppt) and high (45 ppt) salinities for 14 days. Values represent Mean ± SEM ($n=5-6$). Asterisks denote significant differences from 30 ppt ($P<0.05$).

Table 2

Total ninhydrin positive compounds (TNPS) in the tail muscle and hemolymph of *L. vannamei* exposed to low and high salinities for 14 days

Salinity	Tail muscle (mmol g ⁻¹ dry tissue)	Hemolymph (mmol L ⁻¹)	Water osmolality (mmol kg ⁻¹)	Hemolymph osmolality (mmol kg ⁻¹)
2.5	169.9±41.1 ^b	0.291±0.099 ^a	80	632.2C±17.8 ^d
5	132.9±45.1 ^{bc}	0.133±0.053 ^b	139	660.8±12.9 ^d
15	91.0±35.4 ^c	0.127±0.068 ^b	427	702.0±28.2 ^c
30	117.6±21.6 ^{bc}	0.068±0.036 ^b	889	759.9±22.9 ^b
45	473.5±69.8 ^a	0.149±0.040 ^b	1355	854.1±57.7 ^a
<i>P</i> -value	<0.0001	<0.0001	–	<0.001
PSE*	16.8	0.024	–	11.5

*Pooled Standard Error.

– Only one water sample was taken.

Values represent the mean±standard deviation (*n*=4–10). Values with different letters are significantly different from each other (*P*<0.05).

min⁻¹) across the 8 gills (Fig. 2). CA activity increased in all gills in shrimp exposed to both low and high salinity. CA activity in anterior gills (G1–G6) were induced 2–4-fold, depending on salinity (*P*<0.05, Dunnett's post-hoc comparison)(Fig. 2). The magnitude of CA induction was approximately equal in 15 and 45 ppt. The changes in CA activity in posterior gills (G6–G8) were much less. The maximum CA induction was 2-fold, occurring at 15 and 45 ppt; but remarkably, CA activity in posterior gills was not different in shrimp acclimated to 30 ppt vs. 5 ppt (Fig. 2).

3.3. Total ninhydrin positive substances

In general, concentrations of TNPS in abdominal muscle were correlated with hemolymph osmotic concentration. While there were statistically significant differences in hemolymph osmotic concentrations for shrimp acclimated to 2.5 to 30 ppt, the changes were small and the values were relatively stable, varying no more than 120 mOsm kg H₂O⁻¹ (Table 2). The concentrations of muscle TNPS, while also significantly different at some salinities, were also relatively stable across this range of salinity, varying between 90 and 170 mmol g dry weight⁻¹ (Table 2). Assuming that wet muscle tissue has a water content of 70% total weight, TNPS made up between 18 and 38% of the total intracellular osmotic concentration of muscle, depending on acclimation salinity. Shrimp acclimated to 45 ppt were hypo-osmotic to the ambient seawater by 500 mOsm but still had elevated hemolymph osmolality compared to all other acclimation salinities (Table 2). Also, at 45 ppt, abdominal muscle has the highest concentration of TNPS, a 3–5-fold increase over values seen at the lower salinities (Table 2). TNPS made up 79% of the total intracellular osmotic concentration of abdominal muscle at 45 ppt. Hemolymph concentrations of TNPS were low and constant for all salinities except 2.5 ppt, at which there was a 2-fold increase (Table 2).

HPLC analysis of pooled abdominal muscle samples revealed that glycine (44.5–53.4%), alanine (8.8–12.0%), arginine (2.9–9.1%), and glutamine (3.2–6.2%) were the most abundant free amino acids regardless of salinity. The pooled hemolymph samples analyzed by HPLC revealed that proline (11.1–25.6%), taurine (11.0–20.0%), glutamine (12.1–16.2%), alanine (9.7–12.0%) and glycine (7.6–14.7%) were the predominant FAA.

4. Discussion

The present study confirms that *L. vannamei* is a strong hyper-osmotic regulator in low salinity and an equally strong hypo-osmotic regulator at high salinity. The isosmotic point for this species has been reported to be approximately 718 mOsm kg H₂O⁻¹ (24 ppt)(Castille and Lawrence, 1981). Since the baseline salinity used in this study, 30 ppt, was slightly above that point, hemolymph osmolality was predictably slightly hypo-osmotic. That *L. vannamei* is a strong regulator is evidenced by the fact that at the two extremes of acclimation salinity, 5 and 45 ppt, hemolymph was regulated approximately 400–500 mOsm above and below ambient values, respectively. This magnitude of difference between the hemolymph and the ambient medium at 5 ppt is as large as that of some species of decapod crustaceans considered to be examples of the strongest osmotic regulators (e.g., the blue crab, *Callinectes sapidus*) (Henry, 2001; Henry and Watts, 2001). The same is true for hypo-osmotic regulation: *L. vannamei* can maintain its hemolymph osmotic concentration below that of a hypersaline medium to the same extent as a number of fiddler crab species (e.g., Rabalais and Cameron, 1985).

Hemolymph osmotic regulation was achieved by regulating the concentrations of the major ions, Na⁺ and Cl⁻. These ions make up over 90% of the total osmotic concentration of the hemolymph, and their concentrations changed a little when the salinity was reduced from 30 to either 15 or 5 ppt. Shrimp acclimated to 45 ppt had a slight but significant increase in both hemolymph Na⁺ and Cl⁻ levels; however, ionic concentrations in the hemolymph were still regulated significantly below those in the ambient medium. This is typical of the mechanism of hyper- and hypo-osmotic regulation in other species of shrimp (McFarland and Lee, 1963), and in the more well-studied decapod crustaceans (e.g. fiddler crabs). A similar pattern was also seen for K⁺, although this ion was more tightly regulated. Regulation of hemolymph K⁺ within a relatively narrow range of concentrations appears to be a consistent trait in penaeid shrimp. Dall and Smith (1981) reported that *Penaeus plebejus*, *P. esculentus*, and *P. merguensis* regulated their K⁺ levels within the range of 2–7 mEq L⁻¹.

The ability of euryhaline crustaceans to regulate hemolymph osmotic and ionic concentrations is believed to depend on the presence of a number of ion-transport proteins and transport-related enzymes in the gills (Towle, 1984, 1997; Henry, 2001).

The two most important transport-related enzymes are the Na^+/K^+ ATPase and carbonic anhydrase (CA). When a euryhaline crustacean makes the transition from osmotic conformity to osmotic regulation, as a result of low salinity exposure, the expression and activity of these proteins are strongly up-regulated (Towle et al., 2001; Henry et al., 2002, 2003). Most previous studies have focused on the transition from conformity to hyper-regulation, for which CA is up-regulated by as much as 15-fold in decapod crustaceans, depending on species and salinity (Henry et al., 2003). This report is the first to show that branchial CA is up-regulated in response to both hyper- and hypo-osmotic regulation in a euryhaline marine organism. The degree of CA induction reported here is not as large as that observed in other decapod crustaceans, but the data here are most likely a conservative measure of the changes in CA activity. The baseline acclimation salinity used in this study was 30 ppt, a salinity that is slightly above the isosmotic point of the shrimp (Castille and Lawrence, 1981). CA induction in crustaceans has been shown to be highly sensitive to even small changes in salinity, doubling at the point of transition between conformity and regulation (Henry, 2005). CA activity in Pacific white shrimp acclimated to 30 ppt, therefore, was probably already above the baseline levels at that species' isosmotic point, and this most likely resulted in an underestimation of the degree of induction at either 5 or 45 ppt. Even so, it is interesting to note that the degree of CA induction observed here was equal for the transition to both hyper- and hypo-osmotic regulation, indicating that CA plays a role in both the active uptake and excretion of ions. In this case, the pattern of CA activity is similar to that of the Na^+/K^+ ATPase, whose activity is induced in the gills of fiddler crabs acclimated to hypersaline conditions in which they are hypo-regulators (D'Orazio and Holliday, 1985). Preliminary results using fiddler crabs indicate that CA activity is induced when the crabs are actively regulating their hemolymph either above or below ambient seawater (L. Serrano and R. Henry, unpublished data).

Overall, *L. vannamei* displayed a pattern similar to that of freshwater crayfish (*Pacifastacus leniusculus*), in which CA induction occurred in both anterior and posterior gills in response to low salinity exposure (Wheatly and Henry, 1987). Branchial CA activity was uniformly low in all gills near the isosmotic point (750 mOsm), and activity was induced in gills 2–7 following a 3 week acclimation to low salinity (450 mOsm) (Wheatly and Henry, 1987). Although the distribution of CA activity across shrimp gills was not as homogeneous as in crayfish, differences between anterior and posterior gills were not as pronounced as in crabs (Henry, 1984, 2001). The major difference between euryhaline crayfish and shrimp appears to be that crayfish become osmotic conformers at salinities above their hemolymph-medium isosmotic point, while shrimp become hypo-osmotic regulators. The response of branchial CA is different as well; it is down-regulated in response to high salinity in crayfish but up-regulated in shrimp. While CA activity in euryhaline marine crabs in low salinity is greatest in posterior gills, in our study *L. vannamei* displayed slightly higher CA activity in the anterior gills. CA induction in anterior

and posterior gills may be an adaptation of some species to low and high extremes of salinity, where ion uptake or excretion must occur at very high rates in order to ensure hemolymph osmotic regulation. This idea deserves more systematic investigation.

While the dynamics of CA activity have not been extensively examined in euryhaline shrimp, previous reports had indicated no differences in CA activity in gills of post-larval (PL₂₀) *L. vannamei* exposed to a 3 h salinity challenge in which salinity was dropped from 35 ppt to 10 ppt (Palacios et al., 2004a). Palacios et al. (2004b) compared CA activity in shrimp acclimated from 35 to 10 ppt over 3 and 24 h periods in fed and starved post-larval shrimp and reported no differences in CA activity between anterior and posterior gills. However, in both of these studies insufficient time was allowed for salinity mediated induction of CA activity which generally takes several days to reach peak levels (Henry and Watts, 2001; Henry et al., 2002). Bouaricha et al. (1991) examined CA activity in larval (nauplii, zoea, mysis) and postlarval (PL₁, PL₂, PL₄, PL₅, PL₁₆) *P. japonicus* held in seawater and found no statistical differences in CA activity. In that study, however, CA activity was assayed on whole animal homogenates. CA induction occurs exclusively in the gills (e.g., Henry and Cameron, 1982), and thus, remaining tissue of adult or larval organisms would dilute changes in branchial CA activity to the point where they would not be measurable.

The induction of branchial CA activity in response to low salinity exposure has been studied in a number of crustacean species, and it has been linked to the process of branchial ion uptake that underlies hemolymph osmoregulation. Branchial cytoplasmic CA is believed to function in hydrating respiratory CO_2 to H^+ and HCO_3^- , which serve as counterions for general cation and anion uptake, respectively (Henry, 1988b). This is probably also the case for *L. vannamei* in low salinity.

Specifying a function for CA in the gills of hypo-regulating shrimp, however, is more difficult, given the paucity of data on the mechanism of hypo-regulation. First of all, the pattern of CA activity is not the same as that of the Na/K -ATPase, a central transport enzyme of the gill. In marine crustaceans exposed to low salinity, both CA and the Na/K -ATPase activities are induced, but for species that hypo-regulate in hypersaline conditions, Na/K -ATPase activity does not increase compared to levels in normal strength seawater. In the fiddler crabs, *Uca pugilator* and *Uca pugnax*, Na/K -ATPase activity increased only in response to low salinity (Holliday, 1985; D'Orazio and Holliday, 1985).

Hypo-osmotic/hypo-ionic regulation is no doubt a result of the active transport of Na^+ and Cl^- across the gill, from the hemolymph to the ambient seawater, but the mechanism by which that is accomplished is virtually unknown in crustaceans. However, the situation may be physiologically similar to that of teleost fish, which hypo-regulate their blood in seawater. CA has been reported as being more abundant in the gills of fish acclimated to seawater (Dimberg et al., 1981; Zbanyszek and Smith, 1984; Flugel et al., 1991; Kultz et al., 1992), but these reports were based on histochemical staining or crude assays. Based on this evidence, branchial CA may be physiologically

important in supporting active Na^+ and Cl^- secretion in fish, and by extrapolation, in hypo-regulating crabs. Other studies, however, reported no difference in branchial CA between freshwater- and seawater-adapted fish (Lacy, 1983; Sender et al., 1999), using essentially the same techniques. The physiological role of CA in the active secretion of NaCl , especially across the crustacean gill, requires more systematic investigation.

Despite the high degree of extracellular osmotic regulation, the hemolymph of *L. vannamei* does become more concentrated by about 150 mOsm when shrimp are acclimated to 45 ppt. At that point it appears the shrimp employ a mechanism common among invertebrates for adjusting intracellular volume: increasing the intracellular pool of organic osmolytes as measured by changes in muscle tissue TNPS. As extracellular fluid becomes more concentrated, the osmotic equilibrium with the intracellular fluid is disrupted, water is lost from the intracellular compartment, and cell shrinkage results (Pierce and Amende, 1981). Large changes in cell volume can disrupt normal cell function and lead to cell death (Deaton and Pierce, 1994). Many invertebrates adjust intracellular osmotic concentration (and therefore cell volume) by regulating the size of an intracellular pool of amino acids and quaternary ammonium compounds. This pool is increased in response to an increase in salinity in order to retain osmotically obligated water and thus restore cell volume. In *L. vannamei*, the increase in the muscle TNPS pool coincided with the increase in hemolymph osmolality. The major components of the intracellular pool of organic osmolytes were glycine and alanine, and this is typical of crustacean tissue (Bishop et al., 1994) as well as muscle tissue in other invertebrate groups (Henry, 1995).

In summary, the Pacific white shrimp, *L. vannamei*, is able to withstand a wide range of salinity fluctuations by maintaining hemolymph osmotic and ionic concentrations within narrow levels. The enzyme carbonic anhydrase appears to be important in both hyper- and hypo-osmotic regulation, because it is induced in response to exposure to both low and high salinities. Furthermore, when hemolymph osmotic concentrations vary due to variations in environmental salinities, intracellular volume is adjusted by changing the size of the intracellular pool of organic osmolytes.

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