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Biology

Carbonic anhydrase and Na/K-ATPase activities during the molt cycle of low salinity-reared white shrimp *Litopenaeus vannamei*

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Abstract Changes in hemolymph osmolality, ion concentrations, and enzymatic activities of carbonic anhydrase (CA) in the gills and epidermal tissue, and Na/K-ATPase in the gills during the molt cycle were investigated in the white shrimp *Litopenaeus vannamei*. Hemolymph osmolality was high in the intermolt and early premolt stages, but started to decrease prior to ecdysis through to postmolt stages A and B. Changes in Na^+ and Cl^- ion concentrations paralleled those in hemolymph osmolality. CA activity levels in the anterior and posterior gills were low at intermolt stage C_0 and premolt stage D_0, and maximum at premolt stage D_3. In the epidermal tissue, activity was relatively high at intermolt stage C_0 and premolt stage D_0, but fluctuated towards premolt stage D_3 and postmolt stage A. On the other hand, Na/K-ATPase activity in the gills decreased between intermolt stage C_0 and premolt stage D_2, but increased at premolt stage D_3 and postmolt stage A. The changes in patterns of CA activity during the molt cycle suggest that CA may be involved in supplying counter-ions for Na^+ and Cl^- uptake during molting. Branchial Na/K-ATPase appears to be involved in producing local osmotic gradients in order to support water influx across the epithelium.

Keywords Carbonic anhydrase · Na/K-ATPase · *Litopenaeus vannamei* · White shrimp · Osmolality · Osmoregulation · Molt cycle

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Introduction

The Pacific white shrimp *Litopenaeus vannamei*, a species native to the eastern Pacific ranging from Mexico to Peru, is a euryhaline species that is capable of tolerating a wide range of ambient salinities from 0.5 to 40 ppt [1]. It is a strong hyper-osmotic regulator in low salinity and an equally strong hypo-osmotic regulator at high salinity [2]. The shrimp has been commercially cultured in low salinities in many countries such as the U.S., Ecuador, and Thailand [3], and in low-salinity inland saline water ranging from 0.5 [4] to 28.3 ppt [5]. Around 30% of commercial shrimp farming in Thailand is conducted using low-salinity water [3], and in Mexico white shrimp has been successfully cultured in freshwater (0 ppt) at intensive densities [6].

The ability of euryhaline invertebrates to respond to changes in ambient salinity is due to their capability to regulate intracellular and extracellular volumes and maintain hemolymph ionic/osmotic balance. Intracellular volume is regulated by adjusting the intracellular pool of inorganic and organic osmolytes in response to changes in hemolymph osmotic concentration, leading to cell swelling or shrinkage. On the other hand, extracellular osmotic balance is maintained by active ion absorption from dilute salinity or ion excretion into high salinity water. Osmoregulatory capacity

has been proposed as a tool to monitor the physiological state of penaeid shrimp in different adverse conditions under artificial rearing conditions [7].

Specific ion transport proteins and transport-related enzymes have been implicated to be involved in the crustacean osmoregulatory system with carbonic anhydrase (CA) and Na/K-ATPase being the two most studied. CA, which reversibly catalyzes the conversion of CO_2 and water to HCO_3^- and H^+, is known to be abundantly present in the gills of brachyuran crabs and crayfish and is salinity-labile [8–11]. Besides its roles in the transport and excretion process, providing H^+ and HCO_3^- as counterions for Na^+ and Cl^- uptake in the osmoregulatory process of fish and crustaceans [12], the enzyme has been reported to be directly involved in calcification in the green crab *Carcinus maenas* [13]. On the other hand, Na/K-ATPase, the plasma membrane-associated enzyme that catalyzes ATP-driven Na^+/K^+ transport, is crucial for ion and water regulation in fish kidneys [14, 15] and has been found at high specific activities in the gills and antennal glands of hyperosmotically regulating crustaceans [16, 17]. Many studies have shown that Na/K-ATPase has a central role in active Na uptake and plays a vital role in both whole body ion regulation and cellular water balance in euryhaline crustaceans [18]. It is the prime mediator of ion transport across cellular membranes [19].

The gills are the sites primarily involved in the active transport of Na^+ and Cl^- between concentrated and dilute media in decapod crustaceans. The posterior gills have been shown to possess higher enzyme activities for purposes of salt transportation, whereas the anterior gills are considered to be more involved in respiratory mechanisms [12]. Both CA and Na/K-ATPase have been shown to be localized in the pillar cells of the gill lamella [20].

Crustaceans have to molt in order to achieve growth, and the molt cycle influences a number of physiological processes. The activities that are related to water and ion permeability and regulation differ and influence animal behavior and its interaction with the environment. Therefore, it is important to examine certain aspects of how the molt cycle affects shrimp physiology. The present study investigated the changes in hemolymph osmolality and ion concentrations, and in CA and Na/K-ATPase activities in the gill and epidermal tissues, in relation to molt stage in *L. vannamei* shrimp that have been cultured in low-salinity water.

Materials and methods

Animals

Pacific white shrimp *L. vannamei* (10–20 g), reared at 28°C in a 5 ppt salinity recirculation system, were obtained from the facilities of International Mariculture Technology (IMT) in Ibaraki, Japan. Five shrimp at each molting stage were sampled for hemolymph, gill, epidermal tissue, hepatopancreas, and carapace. All samples were stored at −80°C until analysis. Molting stage was determined by examination of the changes in the setae of the pleopods [21].

Osmolality and ion concentrations

Hemolymph osmolality was measured using an osmometer (Fiske One-Ten, Fiske, MA, USA). Ion concentrations were determined using an IA-100 analyzer (TOA Electronics, Japan) according to the methods described by Huong et al. [22].

Carbonic anhydrase and Na/K-ATPase assay sample preparation

Gills were differentiated into anterior gills (gills 1–4) and posterior gills (gills 5–8). The gills and the epidermal tissue were rinsed, blotted dry, and weighed before being homogenized in 500–800 µl of cold phosphate-buffered saline (PBS), pH 7.4, for 30 s on ice. Tissue homogenates were centrifuged at 6,500*g*, 4°C for 2 min, and used for the assays as described below.

Carbonic anhydrase assay

Carbonic anhydrase activity was measured by the imidazole-tris technique described by Brion et al. [23] with a minor modification. Briefly, 500 µl of sample was hydrated with CO_2 at 0°C for 30 s, and then 500 µl of imidazole buffer (20 mM imidazole, 5 mM Tris, 0.2 mM *p*-nitrophenol, pH 9.5) was added to the solution. Solution color change from yellow to colorless (pH 5.6) was followed visually, and the time elapsed was taken as the reaction time. Protein concentrations were determined using the Bio-Rad protein assay kit (IgG standard; Bio-Rad, Hercules, CA, USA) and IgG for the standard. CA activity was calculated by the formula:

$$\mathrm{CA(enzyme\ units\ (EU)/mg\ protein)} = \log_{10}(B/S)/(\mathrm{protein})\log_{10}2,$$

where B and S are the times measured for paired boiled inactivated enzyme and active sample, respectively.

Na/K-ATPase assay

The in vitro assay for Na/K-ATPase activity was conducted according to the methods described in detail by Wilder et al. [24] and Huong et al. [25]. Since no significant difference in Na/K-ATPase activity was observed between the

anterior and posterior gills in the preliminary assay, the gills were pooled and used for analysis in this study.

Statistical analyses

Statistical analyses were performed using SPSS 14 software (SPSS, Chicago, IL, USA). Data were analyzed with ANOVA, and post hoc comparison between mean values was tested using Duncan's multiple range test ($P < 0.05$). Results are expressed as the mean ± standard error (SE).

Results

Hemolymph osmolality was 671 mOsm at intermolt stage C_0 and increased slightly at premolt D_0 (693 mOsm) and D_2 (695 mOsm) stages. At premolt D_3 stage, osmolality decreased to 660 mOsm and further decreased significantly ($P < 0.05$) after postmolt stages A (623 mOsm) and B (619 mOsm) (Fig. 1a). Hemolymph Na^+ concentrations were 292.2 ± 5.4 mmol/l at intermolt stage C_0; concentrations showed a marginal decrease and increase at premolt D_0–D_2 stages. However, levels decreased significantly prior to ecdysis to 239.7 ± 7.6 mmol/l and remained low after molting at postmolt stages A (251.7 ± 3.6 mmol/l) and B (251.2 ± 9.5 mmol/l) (Fig. 1b). Hemolymph Cl^- concentrations were highest at premolt stage D_2 (319.7 ± 10.5 mmol/l); however, the levels started to decrease just before ecdysis and remained low during postmolt stages A and B (247.3 ± 5.8 mmol/l), before increasing again as the animal advanced to the intermolt stage C_0 (265.8 ± 6.9 mmol/l) (Fig. 1b). Hemolymph K^+ concentrations were highest at postmolt B stage (12.1 ± 1.2 mmol/l). Levels then showed a steady decrease from intermolt stage C_0 to premolt stage D_2, before starting to increase again at late premolt stage D_3 (Fig. 2a). Hemolymph Ca^{2+} concentrations did not vary significantly during the molt cycle, although levels were lowest (13.1 ± 0.6 mmol/l) at intermolt stage C_0 (Fig. 2a). Hemolymph Mg^{2+} levels were significantly ($P < 0.05$) higher (4.5 ± 0.2 mmol/l) at premolt stage D_0 than at the other molting stages (Fig. 2b).

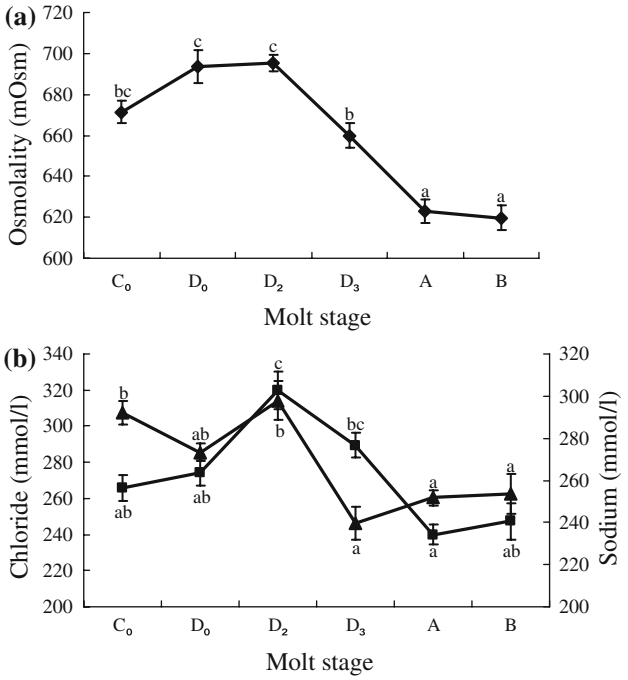

Fig. 1 Hemolymph **a** osmolality (mOsm) (*filled diamonds*) and **b** Na^+ (mmol/l) (*filled triangles*) and Cl^- (mmol/l) (*filled squares*) concentrations in *Litopenaeus vannamei* during the molt cycle. Values are shown as the mean ± SE ($n = 5$). *Bars* with *different letters* are significantly different ($P < 0.05$)

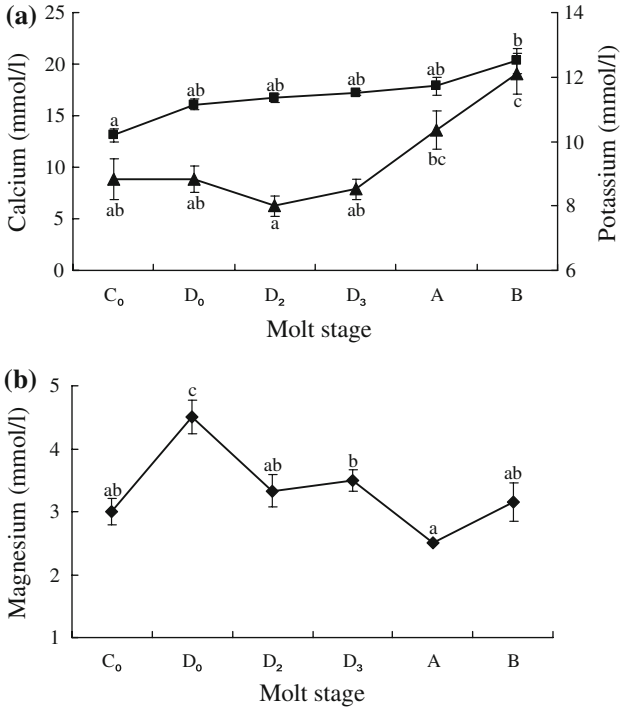

Fig. 2 Hemolymph **a** Ca^{2+} (mmol/l) (*filled squares*) and K^+ (mmol/l) (*filled triangles*) and **b** Mg^{2+} (mmol/l) (*filled diamonds*) concentrations in *Litopenaeus vannamei* during the molt cycle. Values are shown as the mean ± SE ($n = 5$). *Bars* with *different letters* are significantly different ($P < 0.05$)

Carbonic anhydrase activity

Measurements performed with anterior gills of different molting stages (Fig. 3) showed that activity of the enzyme was low (3.64 ± 0.29 EU/mg protein) at intermolt stage C_0 and premolt stage D_0. Activity started to increase significantly at premolt stage D_2 and attained peak levels (6.14 ± 0.28 EU/mg protein) at premolt stage D_3 just before ecdysis. However, after postmolt stage A, activity

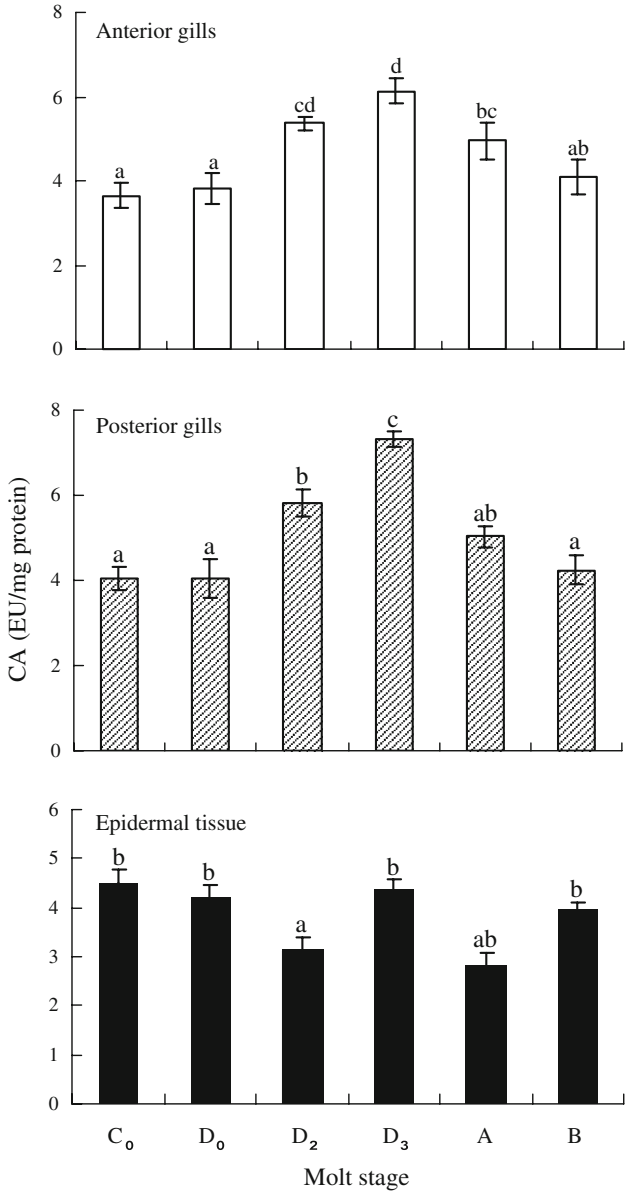

Fig. 3 Carbonic anhydrase activity in the anterior gills, posterior gills, and epidermal tissue of *Litopenaeus vannamei* as a function of molt stage. Values represent the mean ± SE ($n = 5$). *Bars* with *different letters* are significantly different ($P < 0.05$)

decreased until postmolt stage B. CA activity in the posterior gills (Fig. 3) showed a pattern similar to that in the anterior gills, indicating that the enzyme is homogeneously distributed throughout the branchial tissue. In the posterior gills, increased activity (5.82 ± 0.17 EU/mg protein) was seen from premolt stage D_2 towards premolt stage D_3 (7.32 ± 0.25 EU/mg protein) prior to molting. Activity then decreased to a uniform level after postmolt towards premolt stage D_0.

In the epidermal tissue (Fig. 3), activity was relatively high (4.52 ± 0.25 EU/mg protein) at intermolt stage C_0

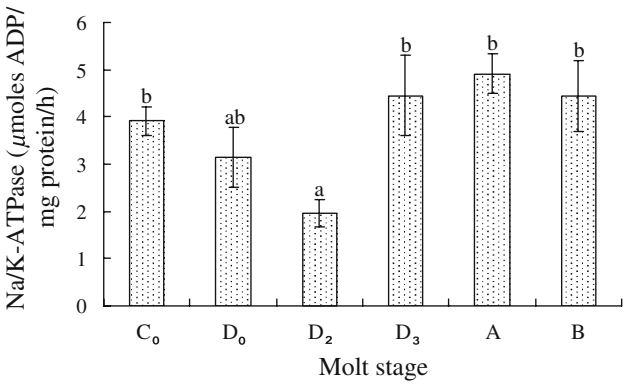

Fig. 4 Na/K-ATPase activity in the gills of *Litopenaeus vannamei* as a function of molt stage. Values represent the mean ± SE ($n = 5$). *Bars* with *different letters* are significantly different ($P < 0.05$)

and premolt stage D_0; however, towards premolt stage D_3 (4.32 ± 0.25 EU/mg protein) and postmolt stages A and B, a pattern of fluctuating activity was observed.

Na/K-ATPase activity

In the pooled gills (Fig. 4), Na/K-ATPase activity decreased from intermolt stage C_0 towards premolt stage D_2, reaching a minimum level (1.96 ± 0.28 EU/mg protein) at stage D_2. However, activity increased significantly to 4.46 ± 0.85 EU/mg protein at premolt stage D_3 prior to molting and remained high at postmolt stages A and B.

Discussion

Litopenaeus vannamei is clearly a hyperosmoregulator in low-salinity rearing water. In 35 ppt salinity (938 mOsm) ambient water, hemolymph osmolality is 774 mOsm (unpublished data), while in 5 ppt (128 mOsm) rearing water, hemolymph osmolality decreases by 15% to a value of 660 mOsm. Decrease in hemolymph osmolality has been observed in several penaeids exposed to lower salinity environments [26, 27]. As osmoregulation is dependent on the active transport of ions by electrogenic pumps located in the cellular membrane that are ATP-dependent, reduced hemolymph osmolality may be seen as an adaptation strategy, in which general energy expenditure for salt uptake can be lowered, and outwardly directed ionic gradients may be reduced [28].

In this study, hemolymph osmolality increased as molt stage advanced from intermolt C_0 to premolt stages D_0 and D_2. In accordance with the fact that crustaceans take up large amounts of water from the environment passively at the time of molting to enable them to shed their old exoskeleton, hemolymph osmolality was observed to decrease prior to ecdysis, and to decrease further at postmolt stages

A and B. Similarly in crayfish, hemolymph is diluted by 40%, Na^+ ion concentrations drop by 36%, and Cl^- ions by 20% at ecdysis [29]. Hyperosmoregulating crustaceans are known to compensate for passive salt loss in a diluted environment by actively absorbing Na^+ and Cl^- across their gill epithelia, which possess Na/K-ATPase activity. Therefore, we consider that hemolymph Na^+ and Cl^- concentrations did not change significantly during the molt cycle in this study because these ions are continuously being absorbed from the medium to maintain a balance between the hemolymph and the ambient water. However, diluted hemolymph osmolality during molting (stage A) observed in the present study is considered to be due mainly to increased water influx from the ambient water through the gill or gut into the body by an increase in permeability. In the hemolymph of *C. maenas*, although considerable water uptake occurs during molt, no significant changes in the concentration of Na^+ and Cl^- or K^+ were observed [30]. On the other hand, increased K^+ levels at the postmolt stages observed in our study could be due to intracellular osmotic pressure and acid-base balance regulation after increased water influx, since potassium is a major cation of the intracellular fluid. Increased Ca^{2+} concentrations observed at postmolt stages A and B may be due to the rapid direct uptake from the external medium. Increased Ca^{2+} may be attributable to the animal's requirements to obtain Ca^{2+} from its rearing water for the purpose of calcification of the exoskeleton. After completion of the molting process, mechanisms that increase hemolymph osmolality against an osmotic gradient, such as reduction in integument permeability, active absorption of ions, or the production of hypoosmotic urine, are considered necessary for the animal to return back to its normal physiological condition. Najafabadi and co-workers [31] revealed that the ionic composition of brown shrimp *Penaeus aztecus* and *L. vannamei* hemolymphs was influenced by water salinity, but not by water pH or temperature.

There was no pronounced difference in CA activity levels observed between anterior and posterior gills of shrimp acclimated to low salinity in the present study. Since our study focused on changes in CA activity as a function of molt stage, and not on low salinity induction, the difference in activity between anterior and posterior gills may not be as pronounced as that seen during salinity induction. Furthermore, Roy et al. [2] reported that the degree of CA induction in gills of shrimp acclimated to low-salinity medium is not as large as that observed in other decapod crustaceans and that differences between anterior and posterior gills were not as pronounced as in crabs [8, 32].

CA activity levels in the gills varied as the molt cycle progressed, suggesting the possibility that branchial CA plays a role in supplying counterions for active uptake of Na^+ and Cl^- ions. During the molting process, a large net influx of water is crucial for increasing the animal's hydrostatic pressure in order to expand the body and break open the old exoskeleton. The acclimation of euryhaline crustaceans to dilute salinity is accompanied by decreased branchial water permeability [33], requiring increased Na/K-ATPase activity to establish an ion gradient for water influx to occur [19, 34]. The significant increases observed in the Na/K-ATPase levels in the late premolt stage and postmolt stage in this study may indicate that Na/K-ATPase is involved in water uptake at molting. Na/K-ATPase creates an ionic gradient by exchanging Na^+ for Cl^-, which in turn induces a water influx. CA, on the other hand, may function to provide counterions through the catalyzed hydration of respiratory CO_2, producing H^+ and HCO_3^- in the intracellular fluid during the molting process. In the blue crab *Carcinus sapidus*, elevated activity of CA during postmolt was accompanied by an increase in Na/K-ATPase activity [35]. The importance of CA in osmoregulation in the gills to enhance the availability of counterions is evidenced in crabs in which a dramatic increase in CA mRNA levels occurs upon transfer from high to low salinity [20]. Na/K-ATPase activity functions in generating a sodium gradient for secondary calcium transport in premolt and early postmolt animals for calcification of the exoskeleton [35–37]. Increased Na/K-ATPase activity in gill and muscle during late premolt and early postmolt stages might correlate with the transport of calcium.

In seawater, where ambient calcium concentrations are about 10 mM and intracellular calcium concentrations are micromolar, uptake of calcium via the calcium channel in the apical membrane is passive down an electrochemical gradient. The low intracellular calcium concentration is controlled by active pumping out of calcium across the basal membrane of the gill ionocyte into the hemolymph, probably involving basal Ca^{2+}ATPase and/or interaction between Na/K-ATPase and CA [37]. In the case of euryhaline crustaceans in a low salinity or freshwater environment, the large increase in net uptake of calcium is through active transport across the gill epithelium [38], although there are possibilities of the existence of calcium transporters such as Ca^{2+}-ATPase [39], Na^+/Ca^{2+} exchange [40], or Ca^{2+}/H^+ exchange by the Na^+/H^+ antiporter [41] in regulating intracellular calcium levels in crustaceans. Meyran and Graf [42] demonstrated that Na^+K-ATPase is involved in an indirect, sodium-dependent mechanism for the transport of calcium, whereas Ca^{2+}ATPase activity in microvilli plays a role in the direct, active extrusion of Ca^{2+}.

The resultant electrochemical gradient allows the active uptake of sodium ions against its chemical gradient in dilute medium. In this study, we found that CA activity

increases during premolt, underlining its role in supplying counterions for increased Na^+ and Cl^- uptake prior to molting. However, CA and Na/K-ATPase levels were low at the intermolt stage due to lowered activity of ion transport and reduced osmotic efforts required by the animal in the postmolt stages.

In most crustacean species, the calcification process is synchronized with the molt cycle. Calcification following ecdysis requires the uptake of calcium from the medium as well as remobilization from storage organs, followed by transport in the hemolymph to the sites of calcification. Several workers [43, 44] have suggested that cuticular epidermal CA possibly functions in maximizing the supply of carbonate ions from respiratory CO_2 by increasing the bicarbonate formation rate at the extracellular site of mineralization. In this regard, based on the CA activity pattern in the epidermal tissues during the molting cycle, it is possible that CA may play a role in calcification via providing the counterions HCO_3^- and H^+ for the ion transferring enzyme, although further studies on the role of CA in calcification are needed to understand the exact role of CA in the formation of the carapace in *L. vannamei.*

In conclusion, hemolymph osmolality decreases prior to ecdysis and just after molting in order to facilitate the molting process. The dilution of the hemolymph prior to ecdysis and during the postmolt stages is considered to be associated with large amounts of water uptake. The activities of CA and Na/K-ATPase fluctuate during the molt cycle of *L. vannamei*. CA is seemingly involved in supplying counterions for ion uptake during molt cycle. Na/K-ATPase may play a role in ionic regulation associated with the molting process.

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