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PHYSIOLOGICAL AND BIOCHEMICAL VARIATIONS DURING THE MOLT CYCLE IN JUVENILE *LITOPENAEUS VANNAMEI* UNDER LABORATORY CONDITIONS

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ABSTRACT

Biochemical changes, specifically in hemocyanin and glucose concentrations in hemolymph, glycogen in the digestive gland, were studied throughout the molt cycle in juveniles of *Litopenaeus vannamei* in a high salinity condition (36), and related to variations in the osmotic capacity of organisms. Increasing hemolymph volume before molting modifies circulating glucose and hemocyanin concentrations acting on the osmotic capacity. Variations in glucose concentrations are indicators of differential energy requirements throughout the molt cycle. Hemocyanin was used as an indirect indicator of the energy consumed by shrimp in various molt processes that affect the osmotic capacity. The co-variability of these metabolites is the result of the biochemical adaptations displayed by shrimp to maintain homeostasis. Their relation to changes in the osmotic capacity is given as a model to understand and predict events associated with molting under hypo-osmotic conditions.

KEY WORDS: biochemistry, hypo-osmotic conditions, *Litopenaeus vannamei*, molt cycle

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INTRODUCTION

Crustaceans have to face the pervasive influence of the molt cycle on their internal environment during their entire life cycle (Passano, 1960; Bliss, 1985; García, 1988; Aiken and Waddy, 1992; Franco et al., 2006). Molting represents a physiological crisis and many events condition its success. Under culture conditions, abiotic factors, such as salinity within the species' preferences, and biotic factors, such as a practical diet and a proper rhythm of feed distribution (Dall, 1986; Chan et al., 1988), should be set accordingly.

Molting success can be assessed by measuring cyclical variations in metabolites such as glycogen in the hepatopancreas, and glucose and hemocyanin in the hemolymph. Osmotic pressure indicators (Lignot et al., 1999) add to the comprehension of mechanisms that take place during a molt cycle (Skinner, 1985; Chang, 1995). Variations of osmoregulatory capacity and blood glucose concentrations have also been studied to detect physiological stress in crustaceans, including *Litopenaeus vannamei* (Boone, 1931) (Hall and van Ham, 1998; Charmantier et al., 1994; Lignot et al., 2000). It has been shown that the crustacean hyperglycemic hormone (CHH) plays an important role in the regulation of glycemia by increasing D-glucose levels in the hemolymph through the mobilization of D-glucose from hepatopancreas and muscle glycogen reserves (Verry et al., 2001). According to Cheng et al. (2002), molting is one of the main intrinsic factors affecting hemocyanin levels in *L. vannamei* juveniles maintained under isosmotic conditions (salinity at ~25). Their hemocyanin levels were higher during the premolt stage and lower during the postmolt stage.

This study was performed to evaluate variations in blood glucose and hemocyanin in high salinity conditions (36) during the complete molt cycle of *L. vannamei*. The relationship among these parameters to changes in the osmotic capacity is given as a model to understand and predict events associated with molting in hypo-osmotic conditions. The changes in blood glucose during the molt cycle were related to glycogen variations in the hepatopancreas.

MATERIAL AND METHODS

Experiments were carried out with organisms spawned by a single female. Starting with 50-day-old postlarvae (PL50), shrimp were acclimated to temperature (29 ± 0.5°C) and salinity (36) conditions that were maintained for the duration of the study. Shrimp were fed a diet composed of 40% animal protein (Table 1). Six 40 L tanks with 12 divisions, one of which was used for aeration, were used to individually monitor the molt stages of 68 *L. vannamei* (8-12 g) through the daily observation of the setal development of the uropod (Drach and Tchernigovtzeff, 1967; Aquacop et al., 1975). Individuals found in late premolt stages (D_1'''-D_2) were monitored during periods of up to 48 h and sacrificed immediately after ecdysis (< 3 min). All other organisms were placed in containers with chilled (24°C) and aerated water to reduce metabolic activity and to decrease the effects of manipulation during sampling. Previous studies demonstrated that in this way the stress caused by sampling is markedly reduced (Rosas et al., 2000). After 5 to 10 min, organisms were blotted dry to eliminate excess water. Hemolymph (100 µl) was sampled with a new syringe previously cleaned with an anticoagulant solution (SIC-EDTA, at 2-8°C). The solution was discarded immediately before puncturing the ventrolateral sinus of the first pleomere to obtain hemolymph (Vargas-Albores et al., 1993). Hemolymph was placed on a piece of parafilm® located over a cooling gel to keep the sample cold. Sub-samples were taken to assess blood metabolites (glucose) and hemocyanin as the main supply of amino acids for osmoregulation.

Table 1. Diet formulation.

	%
Fishmeal	34
Squid meal	5
Dried whey	12
Cpsp[70]	4
Starch	20
Lecithin	2
Fish oil	3.7
Cholesterol	0.5
Vit premix	1
Robimix C	0.5
Carophyll red	0.05
Booster DSM	0.5
Na-alginate	1
Filler (talc)	16
TOTAL	100

Premix = DSM; Robimix = stay-C; Booster = DSM.

Individuals were weighed (± 0.01 g), measured (total length, TL ± 0.1 cm), and the molt stage of each individual was determined. Once the organisms had been sacrificed, the hepatopancreas was removed through an incision practiced on the back of the cephalothorax and weighed. A portion (0.02-0.06 g) of the digestive gland was placed in a 1.5 ml Eppendorf tube and preserved in liquid nitrogen. Samples were maintained at −40°C until further analysis.

Immediately after obtaining the hemolymph, osmotic pressure was measured with a micro osmometer (American Advanced Instruments). Osmotic capacity (OC, mOsm kg^{-1}) was calculated as the difference between the osmotic pressure in hemolymph and the external medium (Charmantier et al., 1989; Lignot et al., 2000). Hemocyanin concentrations were determined from 10 µl of hemolymph diluted in 990 µl of distilled water in a quartz cuvette. Absorbance at 350 nm was obtained using a spectrophotometer, (Bio-Rad), manually calibrated with distilled water.

To obtain hemolymph glucose concentrations, plasma was extracted from hemolymph, which had been previously diluted in an isotonic solution with a complex to avoid coagulation of the hemolymph (SIC-EDTA at 2-8°C; Vargas-Albores et al., 1993) considering a hemolymph-anticoagulant ratio equal to 1:2. The hemolymph diluted in anticoagulant was centrifuged at 2500 rpm for 3 min at 4°C and the supernatant was collected in Eppendorf tubes. Glucose determinations were made in plasma aliquots of 20 µl with 200 µl of the reactive solution (Kit Bayer-Sera-Pak Plus B01 4509-01; Hall and van Ham, 1998), which were placed in microplates and read in an ELISA lector (BIO-RAD model 550) at 540 nm. SIC-EDTA was used as a blank. The metabolite concentration was calculated using a calibration curve, in which the standard was the substrate that acts as the reagent in the kit.

Glycogen in the digestive gland was extracted in the presence of sulphuric acid and phenol (Dubois et al., 1965). To do so, the digestive gland was first homogenized in 5% trichloroacetic acid for 2 min at 3340 g. The supernatant was quantified and 200 µl were pipetted into a tube and mixed with 5 volumes of 95% ethanol. Tubes were placed in an oven at 37-40°C for 3 h. After precipitation, the tubes were centrifuged at 7000 *g* for 15 min. The glycogen pellets were then dissolved by adding 0.5 ml of boiling water. One ml of concentrated sulphuric acid and phenol (5%) was added and mixed. Tube contents were transferred to microplates in duplicate and read at 490 nm with a microplate reader (Bio-Rad 550).

Differences in osmotic capacity, hemocyanin, glucose, and glycogen among molt stages were assessed with a one-way ANOVA. Normality and homoscedasticity were checked by Hartley, Cochran, and Bartlett tests. Hemocyanin, glucose, and glycogen data were $\log_{10}(X + 1)$ transformed to achieve normal distributions (Zar, 1999). Multiple post hoc comparisons were carried out using Tukey's honestly significant difference (HSD) test for different sample sizes.

To further identify the relationship between osmotic capacity, hemocyanin, glucose, and glycogen, and to condense these data into a smaller series of dimensions (or factors), a Q-mode factor analysis was used to establish the factional composition of cases (organisms) based on the cross correlations of variables to group individuals with similar patterns. Two factors with eigenvalues > 1 were extracted (Hair et al., 2000), explaining 69% of the variability in the original data.

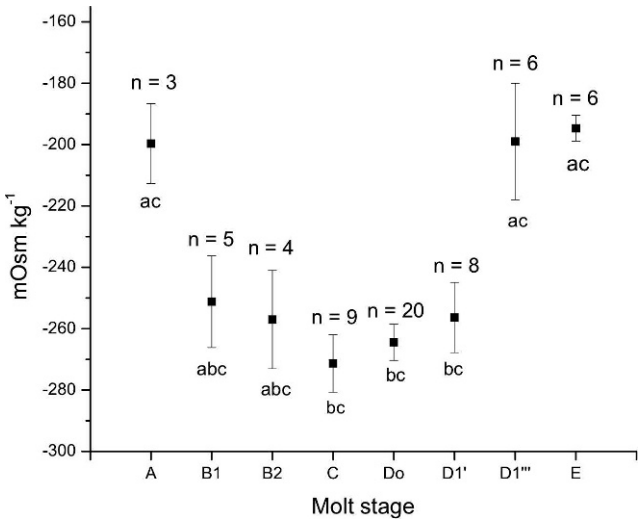

Fig. 1. Mean values (± standard error) of the osmotic capacity (mOsm·kg^{-1}) of juvenile *L. vannamei* in relation to molt stage. Different letters denote significant differences ($P < 0.05$).

Results

Osmotic Capacity

Significant differences were obtained ($P < 0.05$), evidencing variation in osmotic capacity among molt stages. Relatively high and homogeneous values (200 mOsm × kg^{-1}, at salinity of 36) were found in late premolt (D_1'''), ecdysis (E), and early postmolt (A) stages. Osmotic capacity of shrimp diminished in intermolt stages (Fig. 1). HSD test allowed for the separation of two statistically different groups, one is formed by organisms in stages C, D_0 y D_1'; while, organisms in D_1''', E, and A form the other.

Hemocyanin

Hemocyanin values varied among molt stages ($P < 0.05$). Organisms in early postmolt (A) to intermolt stage (C) had lower hemocyanin concentrations than individuals in premolt stages (D) to molt (E). The HSD test performed on transformed data, grouped two homogeneous assemblages. One is formed by organisms in molt stages B_1 and C, and the other is composed of individuals in molt stages D_0, D_1', D_1''', and E (Fig. 2).

Glucose

Glucose values varied throughout molt stages ($P < 0.05$). Relatively low values were obtained while organisms were in intermolt (C) and early premolt (D_0 and D_1') stages (Fig. 3). The HSD test grouped two homogeneous assemblages, one formed by organisms in molt stage A and the other, by organisms in molt stages D_0 and D_1'.

Glycogen in the Hepatopancreas

Significant differences ($P < 0.05$) were found between values obtained amongst molt stages but a clear pattern was

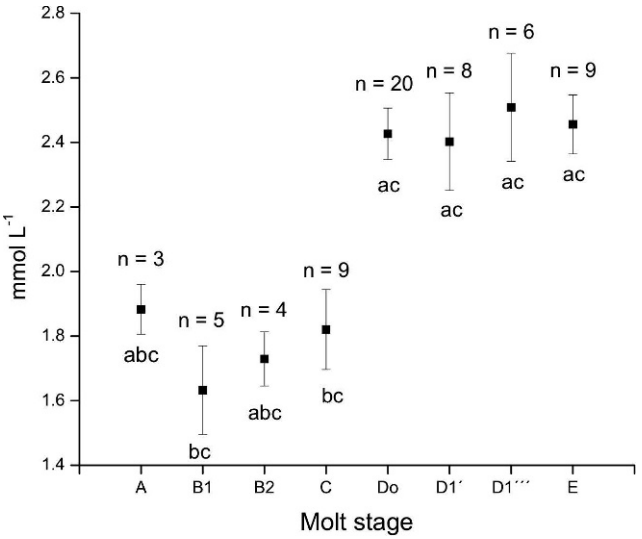

Fig. 2. Mean values (± standard error) of hemocyanin concentrations in the hemolymph (mmoles·L^{-1}) of juvenile *L. vannamei* in relation to molt stage. Different letters denote significant differences ($P < 0.05$).

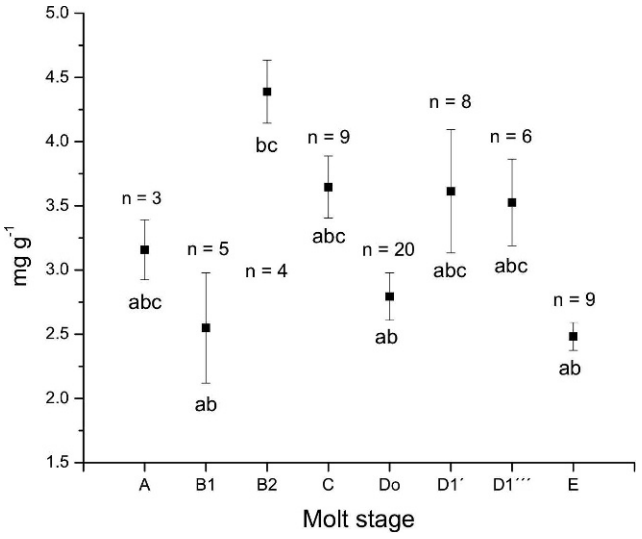

Fig. 4. Mean values (± standard error) of hepatopancreas glycogen concentrations (mg·g^{-1}) in juvenile *L. vannamei* in relation to molt stage. Different letters denote significant differences ($P < 0.05$).

difficult to observe due to the dispersion of data (Fig. 4). The HSD test broke up the homogeneous group formed by organisms of stages B_1, D_0, and E from the other group (stages A, C, D_1', D_1''').

Factorial Analysis

Factorial charges are shown in Table 2. Osmotic capacity and glucose concentration are significant for the first factor (charge value > 0.5), hence explaining the variability associated with this factor. Significant variables for factor 2 were glycogen and hemocyanin. Communalities, or the proportional variance of the extracted factors explained by each variable (Hair et al., 2000), are shown in Table 3. Osmotic capacity, hemocyanin, and glucose explained 82%, 72%, and 71% of the variance respectively. Glycogen's contribution to the variance of extracted factors is less than 50%. The scatter plot of factors 1 and 2 showed clusters of data points grouping molt stages in four categories (Fig. 5).

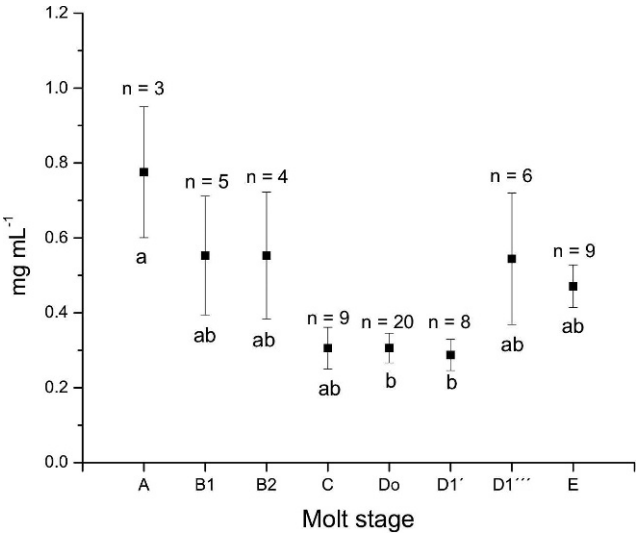

Fig. 3. Mean values (± standard error) of circulating glucose concentrations in the hemolymph (mg·ml^{-1}) of juvenile *L. vannamei* in relation to molt stage. Different letters denote significant differences ($P < 0.05$).

Discussion

Osmotic Capacity

Osmoregulation is an important mechanism of environmental adaptation in aquatic species, particularly in crustaceans (Péqueux, 1995). Values of osmotic pressure in hemolymph and consequently in osmoregulatory capacity of *L. vannamei* varied during the molt cycle. Low osmotic capacity values were observed in B_1, B_2, D_0, and D_1'. The lowest value was observed in stage C while highest values were found in D_1''', E, and A. These results coincide with those reported by Charmantier et al. (1994), which obtained similar results for *L. vannamei*, and those reported by Mugnier and Justou (2004) and Lignot et al. (1999) for *L. stylirostris* (Stimpson, 1874). Data indicate that a permeability decrease in the integument occurs (Hunter and Uglow, 1993) allowing water intake to increase body volume in late premolt and early postmolt stages (Ferraris et al., 1987), causing a reduction in the osmotic capacity. Charmantier et al. (1994) suggest that in late premolt and early postmolt stages, changes in tegument permeability and activity of Na^++K^+-ATPase pump take

Table 2. Explained variance by each extracted factor (%), eigenvalues, and cumulative variance (%).

Factor	Eigenvalues	% of variance	Cumulative variance (%)
1	1.68	42	42
2	1.05	26	68
3	0.83	21	89
4	0.42	11	100

Table 3. Matrix of factorial weights obtained after the varimax rotation and estimated communalities for the osmotic capacity (OC), blood glucose and hemocyanin glycogen, and hepatopancreatic glycogen concentrations during the molt cycle of *L. vannamei*.

	Factor 1	Factor 2	Estimated communalities
OC	0.898724	−0.101065	0.82
Glycogen	0.047471	0.697227	0.49
Glucose	0.750601	0.388047	0.71
Hemocyanin	−0.096018	−0.842774	0.72

place in the posterior gills allowing water and ions to enter tissues, resulting in an increase in body volume. In crustaceans, CHH induces this pump activity (Lucu and Towle, 2003). From stage D_1''' to A, hyporegulation increases as a response to water and ions absorption before molting.

The energy expenditure for osmoregulation in *L. vannamei* is usually higher at high salinity than in isosmotic conditions (Cheng et al., 2002). At a salinity of 36, penaeid shrimp tend to hyporegulate since hemolymph is isosmotic at a salinity of ~25. Variation of the osmotic capacity is a unique adaptation for molting, and regulation of glucose and hemocyanin levels contribute to the maintenance of internal homeostasis in shrimp during this critical stage.

Hemocyanin

Hemocyanin is a multifunctional protein of great importance to crustaceans. Through the molt cycle, we found hemocyanin concentrations within the range 1.63-2.51 mmol L^{-1}. At a salinity of 25, Cheng et al. (2002) found a similar tendency than the one showed in this work, but hemocyanin concentrations were lower at each molt stage (1.03-1.74 mmol L^{-1}). *L. vannamei* and *Homarus vulgaris* (Milne Edwards, 1837) maintained in isosmotic conditions showed a two- or three-fold decrease in circulating hemocyanin levels (Glynn, 1968; Cheng et al., 2002).

Molting is one of the intrinsic factors that also affected hemocyanin levels in *Marsupenaeus japonicus* (Bate, 1888) (Chen and Cheng, 1993) and in *Farfantepenaeus duorarum* (Burkenroad, 1939) (Bursey and Lane, 1971). The significantly lower levels of circulating hemocyanin after molt observed in this study may be the result of hemolymph dilution due to water uptake.

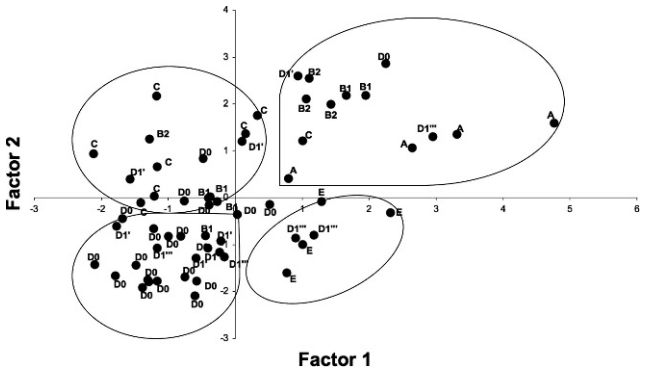

Fig. 5. Definition of four conglomerates in relation to molt stage for juvenile *L. vannamei* (Factor 1: osmotic capacity and glucose needed for homeostasis; factor 2: represents the turnover in hepatopancreas reserves, mainly glycogen and hemocyanin).

The low protein values in hemolymph of *L. vannamei* that were observed during postmolt and intermolt are related to a change in the function performed by hepatopancreatic F-cells because they produce digestive enzymes during these stages (Lehnert and Johnson, 2002).

Hemocyanin plays an important role as an oxygen transporter, and its values observed during premolt (0.55 mmoles L^{-1}), which are higher than those obtained during postmolt, might be associated with the energy demand for ecdysis, when shrimp use the aerobic route. When a deficit occurs, energy is supplied by the anaerobic route, in which lactic acid is formed from the incomplete oxidation of glucose (Waterman, 1961; Chang and O'Connor, 1983; Santos and Keller, 1993a). It has been reported that hemocyanin levels in *L. vannamei* are among the highest within Penaeidae, fact that seems to be related to the activity patterns of species. Species with a closed-thelycum are less active than those with an open-thelycum, including juveniles of *L. vannamei* (Rosas et al., 2007). Since the metabolism of this species is high, our data support the idea that hemocyanin concentrations reflect a physiological adaptation of crustaceans related to satisfy varying energetic demands.

Circulating Glucose

Glucose is a molecule that has a major role in the energy metabolism of crustaceans. Variations in glucose levels measured in the hemolymph are related to the quantity and quality of carbohydrates contained in the diet (Rosas et al., 2001). Through the molt cycle of *L. vannamei*, we found great variability in glucose concentrations (0.1-0.3 mg ml^{-1}), and significant differences were found between stages D_1 and A ($P < 0.05$). In contrast with the information available for *Penaeus monodon* Fabricius, 1798 (Ferraris et al., 1987) and *L. vannamei* (Cheng et al., 2002) maintained in isosmotic conditions, showing that no significant differences of glucose concentrations are found throughout the molt cycle, our results demonstrate that there is a combined effect of high salinity and molt stage that account for changes in the glucose levels in the hemolymph of *L. vannamei*.

A correspondence between the hepatopancreatic glycogen reserves and circulating glucose during late premolt and early postmolt stages was also noted. The higher mean value of circulating glucose observed in stage A coincided with lower hepatopancreatic glycogen concentration during D_1''', suggesting that CHH is acting on the glycogen reserves just before molt. The glycolytic route is active in this stage, while the hexose monophosphate cycle (HMP) is active when shrimp are in intermolt (Santos and Keller, 1993b). This strategy was selected for in shrimp to overcome the energy debt left after the ecdysis and in preparation for protein synthesis (Whiteley et al., 2001). The evidence of CHH action was reported by Chung et al. (1999) and Webster et al. (2000) in relation to the presence of a precursor peptide and the CHH in gut endocrine cells of the crab *Carcinus maenas* (Linnaeus, 1758) during premolt. It is suggested that CHH and its precursor were

secreted during ecdysis, further linking this mechanism with water uptake.

High circulating glucose levels were maintained until late postmolt stage (B_2), when shrimp reached normal feeding rates allowing glucose to be obtained from their diet, especially from starch hydrolysis by α-amylase, α-glucosidase, and hexoquinase enzymes (Gaxiola et al., 2005). Synthesis of glycogen would occur from dietary glucose, whereas circulating glucose comes from amino acids through the gluconeogenic route (Dall, 1965; Verry et al., 2001). This can explain a lower level of circulating glucose during intermolt and premolt periods, in spite of the fact that shrimp actively feed to replace water by body tissue, as reported for *L. vannamei* (Chan et al., 1988).

Hepatopancreatic Glycogen Reserves

Glycogen reserves are the source of glucose for the synthesis of N-acetyl glucosamine, which is the oligomer of chitin (Gwinn and Stevenson, 1973). Large variations of glycogen concentrations occurred along the molt cycle. In premolt (D_1' to D_1'''), the hepatopancreatic glycogen reserves increased, an outcome that can be related to the synthesis of a new cuticle (Loret, 1993; Cuzon et al., 2003). From D_1''' until A, the hepatopancreatic glycogen concentration decreased and was followed by a release of glucose in preparation for ecdysis (Chan et al., 1988). During late postmolt, a peak of glycogen reserves, related to the increment of the feeding activity of shrimp, was observed. During intermolt, hepatopancreatic glycogen concentration decreased because it was used for glucosamine formation, which was then transported to the hypodermis (Santos and Keller, 1993a).

Multivariate Analysis

Factor analysis showed an interdependence of all variables throughout the molt cycle (Fig. 5). Factor 1 is the result of the covariation of glucose concentrations and osmotic capacity indicating a biochemical adaptation to regulate homeostasis. Water uptake through the gills and the intestine during premolt favors the detachment of the cuticle during ecdysis and dilutes the hemolymph. Osmotic capacity values increase resulting in high osmotic work where energy (through ATP) is required. Homeostasis is an efficient mechanism of glucose regulation in hemolymph mediated by the action of CHH. It has been shown that CHH acting on the Na^++K^+-ATPase pump controls water absorption before ecdysis (Charmantier et al., 1994). The increment in circulating glucose during premolt may be a strategy used by shrimp to save energy.

A high load due to hemocyanin and glycogen values defines factor 2. Glycogen reserves tend to accumulate during D_1' to handle the energy demand for ecdysis (Waterman, 1961; Santos and Keller, 1993a, b). During this molt stage, F-cells in hepatopancreas begin the synthesis of hemocyanin that is then liberated into the hemolymph (Lehnert and Johnson, 2002). The energy requirements needed for ecdysis establishes a relationship between the increment in circulating hemocyanin and an increase of glycogen reserves in the hepatopancreas. During premolt stages, these changes are necessary to meet oxygen and glucose demands for ecdysis. Our results indicate that factor 2 is defined as a result of a turnover in hepatopancreatic reserves.

In conclusion, increasing hemolymph volume before molt modifies circulating glucose and hemocyanin concentrations acting on the osmotic capacity. The activation of the physiological process regulating water absorption is hormonally regulated by the 20-OH-ecdysone, which acts on the hypodermis modifying the tegument permeability, and by CHH, which acts on the Na^++K^+-ATPase pump allowing water and ions to enter tissues.

Hemocyanin, as a respiratory pigment, is a good indirect energy indicator for ecdysis and showed a typical pattern along the molt cycle. Glucose is a substrate for energy reserves, and its concentration is linked with glycogen reserves during late premolt and early postmolt stages. During these stages, glycogen reserves decreased as circulating glucose increased, evidencing CHH action on the hepatopancreas.

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