Norepinephrine induces transient modulation of the physiological responses of whiteleg shrimp, *Litopenaeus vannamei*

Shinn-Pyng Yeh, Hung-Tien Chiu, Winton Cheng *

Department of Aquaculture, National Pingtung University of Science and Technology, Pingtung 91201, Taiwan

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**Abstract**

Levels of glucose, lactate, Na⁺, K⁺, Cl⁻, protein, and oxyhemocyanin in the hemolymph and its osmolality were measured when the whiteleg shrimp, *Litopenaeus vannamei* (18.3−21.6 g), were individually injected with saline or norepinephrine at 10⁻⁸, 10⁻⁷, or 10⁻⁶ mol shrimp⁻¹. Results showed that elevations of hemolymph glucose and lactate occurred at between 2 and 4 h, increases in hemolymph osmolality, Na⁺, and total protein occurred at 2 h, and a reduction in hemolymph Cl⁻ and K⁺ occurred at 2 h after in shrimp which had received NE at >10⁻⁸, >10⁻⁸, and 10⁻⁶ mol shrimp⁻¹, respectively. All physiological parameters had returned to the control values 2−4 h after receiving the norepinephrine. The injection of norepinephrine at >10⁻⁸ mol shrimp⁻¹ also significantly decreased the oxyhemocyanin/protein ratio of *L. vannamei* at 2 h as a result of elevation of hemolymph protein. These results suggest that stress-inducing norepinephrine causes a transient period of modulation of energy metabolism and osmoregulation, and a respiratory response in *L. vannamei* as it adapts to an environmental stress.

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1. **Introduction**

Whiteleg shrimp (*Litopenaeus vannamei*), which is naturally distributed along Pacific coasts of Central and South America, was introduced to Asia in 1985, and has become a primary specie currently being cultured in Thailand, Taiwan, and China (Lin et al., 1990). For more than 10 years, commercial shrimp farming, mainly based on local species such as the tiger shrimp, *Peneaus monodon* and kuruma shrimp, *Marsupenaeus japonicus*, has decreased due to disease outbreaks associated with bacteria and viruses. Therefore, *L. vannamei* has received much attention, and by 2003, about 3369 ha of ponds had been converted to the culture of this specie in Taiwan.

Several biogenic amines which function mainly as neuroregulators (i.e., neurotransmitters and neuromodulators), including serotonin, dopamine (DA), octopamine, histamine, noradrenaline (norepinephrine, NE), adrenaline (epinephrine), tryptamine, and tyramine have been identified and quantitatively measured in the crayfish, *Pacifastacus leniusculus*, and other decapod crustaceans (Eloffson et al., 1982; Fingerman and Nagabhushnam, 1992; Kuo et al., 1995). They are distributed in the crustacean nervous system and have a diverse array of physiological effects (Kuo and Yang, 1999; Tierney et al., 2003; Chiu et al., in press). NE has been reported to stimulate the hyperglycemic effects in *M. rosenbergii*, and to act on the target tissue and not through the crustacean hyperglycemic hormone (CHH).
(Kuo and Yang, 1999). Studies in marine, freshwater, and terrestrial crustaceans have indicated that biogenic amines are involved in ionic and osmotic regulation, and such studies were reviewed by Morris (2001). Using HPLC with an electrochemical detector, NE was detected in the eyestalk, thoracic ganglia, and brain of *P. monodon* at concentrations of 26.62±4.93 pmol eyestalk⁻¹, 1.54±0.15 μmol (g protein)⁻¹ and 6.71±1.98 μmol (g protein)⁻¹, respectively (Kuo et al., 1995).

Hemocyanin, a respiratory pigment, represents 80~95% of the total protein concentration in the hemolymph of decapod crustaceans (Jeuniaux, 1971). Hemocyanin has been reported to increase under hypoxia in the blue crab, *Callinectes sapidus* (DeFur et al., 1990), and in the shrimp, *Crangon crangon* (Hagerman, 1986), and to increase under hypo-osmotic stress in the shore crab, *Carcinus maenas* (Boone and Schoffeniels, 1979), and in *C. sapidus* (Mason et al., 1983). Hemocyanin and hemolymph protein are affected by salinity in the fleshy prawn, *Penaeus chinensis* (Chen et al., 1993). Environmental factors such as temperature, pH, oxygen, and carbon dioxide are known to influence the oxygen affinity of hemocyanin in the blue crab, *C. sapidus*, which affects oxygen transport (Burnett, 1992).

Several physiological and biochemical responses in crustaceans have been reported to occur under stressful conditions. The release of biogenic amines is believed to be a proto-stress response. In the present paper, we attempted to examine several physiological parameters including oxyhemocyanin, protein, glucose, lactate, Cl⁻, Na⁺, and K⁺ concentrations in the hemolymph and its osmolality of *L. vannamei* following an injection of NE.

### 2. Materials and methods

#### 2.1. *Penaeus vannamei*

*L. vannamei* juveniles (18.3~21.6 g) were obtained from a commercial farm in Pingtung, southern Taiwan, and acclimated in the laboratory for 2 weeks before experimentation. During the acclimation period, shrimp were fed twice daily with a formulated shrimp diet (Shinta Feed Company, Pingtung, Taiwan). For 1 day prior to the experiment, the shrimp were not fed. For the experiment of physiological parameter assays, tests were carried out in 10 replicate test groups consisting of 1 shrimp each in 20-l PVC tanks containing 10 l of an aerated test solution. No significant differences in weight were observed among the treatment groups. During the experiments, the water temperature was maintained at 27±1 °C and pH 7.8~8.2, while salinity was maintained at 20‰.

Among the A, B, C, D₀/D₁ and D₂/D₃ stages of *L. vannamei*, hemolymph osmolality, and Cl⁻, Na⁺, K⁺, protein, and oxyhemocyanin levels were the highest at stage D₀/D₁, and lowest at stage A, respectively. The fact that the hemolymph osmolality, and protein, Cl⁻, Na⁺, and K⁺ levels were lower during the postmolt, and were higher during intermolt and early premolt stages was considered to be associated with water uptake at the time of molting (Cheng et al., 2002). All shrimp used in this study were individually examined to determine molt stages (Robertson et al., 1987), and only those at stage C (intermolt) were used in order to minimize intrinsic variations.

#### 2.2. Test solutions

Norepinephrine hydrochloride (A7256, Sigma Chemical, St. Louis, MO, USA), a neurotransmitter, was used in the study. Norepinephrine hydrochloride was dissolved in sterile saline (0.85% NaCl) to concentrations of 5×10⁻⁴, 5×10⁻³, or 5×10⁻² mol l⁻¹, respectively, before injection (Kuo et al., 1995).

#### 2.3. Effect of norepinephrine on the physiological parameters of *L. vannamei*

*L. vannamei* was individually injected in the ventral sinus of the cephalothorax with either 5×10⁻⁴, 5×10⁻³, or 5×10⁻² mol l⁻¹ of an NE solution (20 μl) to achieve doses of 10⁻⁸, 10⁻⁷, and 10⁻⁶ mol shrimp⁻¹, respectively. The shrimp that received saline (20 μl) served as the control group. There were 4 treatments (saline, 10⁻⁸, 10⁻⁷, and 10⁻⁶ mol NE shrimp⁻¹) with 5 sampling times (2, 4, 8, 16, and 24 h). Ten shrimp for each treatment and time were used for the studies. In addition, 10 shrimp with no treatment were used as the control group.

At the beginning and 2, 4, 8, 16, and 24 h after the injection, hemolymph (100 μl) was withdrawn from the ventral sinus of each shrimp into a 1-ml sterile syringe (25 gauge) containing 0.9 ml of an anticoagulant solution (30 mM trisodium citrate, 0.34 M sodium chloride, 10 mM EDTA; pH 7.55, with the osmolality adjusted with NaCl to 780 mOsm kg⁻¹). The diluted hemolymph was centrifuged at 664 × g at 4 °C for 15 min, the supernatant fluid was collected as the stock solution, and glucose and lactate were immediately analyzed.

Glucose concentrations were measured with a coupled glucose oxidase and peroxidase reaction using a Sigma diagnostics glucose kit (cat. no. 315-100). The optical density at 505 nm was measured using an ELISA plate reader, and glucose concentrations were...
calculated from a standard curve of known glucose concentrations.

Lactate concentrations were measured using the colorimetric lactate oxidase and peroxidase method with a Sigma diagnostics lactate kit (cat. no. 735-10). The optical density at 540 nm was measured using an ELISA plate reader, and lactate concentrations were calculated from a standard curve of known lactate concentrations.

Hemolymph osmolality and medium osmolality were measured by injecting a 20-μl sample into a micro-osmometer (Model 3300; Advance Instruments, Norwood, MA, USA). To determine Na⁺, K⁺, and Cl⁻ levels, 100 μl of a hemolymph sample was immediately injected into an Ion-Selective Electrode Analyzer (Medica EasyLyte PLUS, Bedford, MA, USA).

Hemolymph protein was determined with the Bio-Rad Protein Assay Kit no. 500-0006 (Bio-Rad Laboratories, Richmond, CA, USA) using bovine albumin (with a molecular weight of 66,000) as the standard (Bradford, 1976). To measure oxyhemocyanin, 100 μl of hemolymph was immediately diluted with 900 μl of distilled water, and the absorbance was measured at 335 nm (characteristic of oxyhemocyanin) using a Hitachi (Tokyo, Japan) U-2001 spectrophotometer. The concentration of oxyhemocyanin was calculated based on the methods of Nickerson and Van Holde (1971) and Hagerman (1983). The ratio of oxyhemocyanin to protein was calculated by dividing the concentration of oxyhemocyanin (mmol l⁻¹) by that of protein (mmol l⁻¹), which was converted from mg ml⁻¹ to mmol l⁻¹ by dividing by 66 (Chen and Cheng, 1993).

2.4. Statistical analysis

A multiple-comparison (Tukey’s) test was conducted to compare the significant differences among treatments using the SAS computer software (SAS, 2001). The percentage data of oxyhemocyanin to protein were normalized using arcsine transformation before analysis. For statistically significant differences, it was required that \( p < 0.05 \).

3. Results

At time 0 h, the hemolymph glucose level was 0.85 ± 0.04 mmol l⁻¹. After 2 h, glucose levels had significantly increased to 155.4%, 194.2%, and 238.8% for the shrimp that had received NE at 10⁻⁸, 10⁻⁷, and 10⁻⁶ mol shrimp⁻¹, respectively, as compared to the level of shrimp that had received saline. The increase in the glucose level of shrimp following NE treatment appeared to have been dose dependent. After 4 h, the glucose level had further significantly increased to 128.5%, 133.9%, and 198.8% for the shrimp that had received NE at 10⁻⁸, 10⁻⁷, and 10⁻⁶ mol shrimp⁻¹, respectively, as compared to the level in shrimp that had received saline. However, no significant difference in

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**Fig. 1.** Hemolymph glucose (μmol ml⁻¹) (A) and lactate (μmol ml⁻¹) (B) of Litopenaeus vannamei which received saline or norepinephrine at 10⁻⁸, 10⁻⁷, or 10⁻⁶ mol shrimp⁻¹. Each bar represents the mean value from 10 samples with the standard error. Bars with different letters significantly differ (\( p < 0.05 \)).
hemolymph glucose was observed among the 4 treatments from 8 to 24 h (Fig. 1A).

Hemolymph lactate contents of shrimp which had received NE at $10^{-8}$, $10^{-7}$, and $10^{-6}$ mol shrimp$^{-1}$ were significantly higher than those of prawns which had received the saline control from 2 to 4 h. The increase in lactate contents of shrimp following NE treatment at 2 and 4 h appeared to have been dose dependent. Hemolymph lactate contents after 2 h increased by 160.3%, 217.6% and 269.0%, and after 4 h increased by 178.5%, 231.6% and 265.1% in shrimp that had received NE at $10^{-8}$, $10^{-7}$, and $10^{-6}$ mol shrimp$^{-1}$, respectively, as compared to saline control shrimp. However, no significant difference in lactate contents was observed among the 4 treatments from 8 to 24 h (Fig. 1B).

Hemolymph osmolality and sodium levels of shrimp which had received NE at $10^{-7}$, and $10^{-6}$ mol shrimp$^{-1}$ were significantly higher than those of the saline control shrimp after 2 h. Hemolymph osmolality had increased by 103.5%, 104.8%, and 105.2%, and hemolymph sodium had increased by 102.2%, 103.9%, and 104.1% in shrimp which received saline or norepinephrine at $10^{-8}$, $10^{-7}$, or $10^{-6}$ mol shrimp$^{-1}$. Statistical descriptions are the same as those in Fig. 1.
Fig. 3. Hemolymph protein levels (mg ml$^{-1}$) (A), oxyhemocyanin (µmol ml$^{-1}$) (B), and the ratio of oxyhemocyanin to protein (%) (C) of *Litopenaeus vannamei* which received saline or norepinephrine at 10$^{-8}$, 10$^{-7}$, or 10$^{-6}$ mol shrimp$^{-1}$. Statistical descriptions are the same as those in Fig. 1.
shrimp that had been administrated NE at $10^{-8}$, $10^{-7}$, and $10^{-6}$ mol shrimp$^{-1}$, respectively, compared to the saline control shrimp. However, no significant differences in hemolymph osmolality or sodium were observed among the 4 treatments from 4 to 24 h (Fig. 2A, C).

Hemolymph chloride levels were significantly lower in shrimp which had received NE at $10^{-6}$ mol shrimp$^{-1}$ than in shrimp which had received saline and NE at $10^{-8}$ and $10^{-7}$ mol shrimp$^{-1}$, and had decreased to 95.5% compared to the level of shrimp that had received saline after 2 h. However, no significant differences in hemolymph chloride were observed between the beginning and 2 h after in shrimp which had received NE at $10^{-6}$ mol shrimp$^{-1}$, and among the 4 treatments from 4 to 24 h (Fig. 2B).

Hemolymph potassium of shrimp 2 h after having received NE at $10^{-6}$ mol shrimp$^{-1}$ had significantly decreased compared to shrimp which had received saline and NE at $10^{-8}$ mol shrimp$^{-1}$. Hemolymph potassium had decreased to 82.6% compared to the saline control shrimp. However, hemolymph potassium levels did not significantly differ among the 4 treatments from 4 to 24 h (Fig. 2D).

Although, no significant difference in hemolymph oxyhemocyanin levels was noted in the shrimp 2 h after having received NE at $10^{-6}$ mol shrimp$^{-1}$, the level was slightly higher than those that had received saline and NE at $10^{-8}$, $10^{-7}$ mol shrimp$^{-1}$. However, no significant differences in hemolymph oxyhemocyanin levels were observed among the 4 treatments from 4 to 24 h (Fig. 3A).

Hemolymph protein was significantly higher in shrimp that had received NE at $10^{-6}$ mol shrimp$^{-1}$ than in shrimp which had received NE at $10^{-8}$, $10^{-7}$ mol shrimp$^{-1}$; its level after 2 h was higher than that of shrimp which had received the saline control. Hemolymph protein had increased by 136.6%, 136.8%, and 156.1% in shrimp that had been administrated NE at $10^{-8}$, $10^{-7}$, and $10^{-6}$ mol shrimp$^{-1}$, respectively, compared to the saline control shrimp. However, no significant differences in hemolymph protein were observed among the 4 treatments from 4 to 24 h (Fig. 3B).

The oxyhemocyanin/protein ratio was significantly lower in shrimp that had received NE at $10^{-8}$, $10^{-7}$, and $10^{-6}$ mol shrimp$^{-1}$ than in shrimp which had received saline, and had significantly decreased to 72.6%, 73.5%, and 74.4%, respectively, compared to that of shrimp which had received saline after 2 h. However, no significant differences in the hemolymph oxyhemocyanin/protein ratio were observed among the 4 treatments from 4 to 24 h (Fig. 3C).

4. Discussions

Stress-induced neuroendocrine changes are thought to divert an organism’s energy resources away from physiological functions that do not immediately help the animal overcome a threat and survive to those that do (Maule and Vanderkooi, 1999). To meet the heightened energy demands of stressed animals, glycogen, due to its easy availability for energy production, may be rapidly catabolized resulting in losses of those reserves in tissues; consequently resulting in a significant elevation in blood glucose levels (Ferrando and Andreu, 1991). Hyperglycemia as a secondary stress response has been documented in many species of crustacean in response to a wide range of stresses (Reddy et al., 1996; Kuo and Yang, 1999). It is known that CHH, synthesized and released from the X-organ sinus gland complex in crustaceans, is an important neurohormone involved in glucose metabolism. For hyperglycemic responses to cold shock in the freshwater giant prawn, M. rosenbergii, among the biogenic amines administered, Kuo and Yang (1999) indicated that the hyperglycemic responses induced by injection of NE (norepinephrine) and OA (octopamine) were nearly identical between the intact and eyestalk-ablated prawns. They, therefore, suggested that hyperglycemic effects of NE and OA directly affect the target tissues and are not mediated through the CHH.

Hemolymph glucose levels significantly increased in shrimp L. vannamei, that had received dopamine, and the effects appeared to have been dose dependent (Chiu et al., in press). A similar trend of a hemolymph glucose increase was also observed in shrimp which had received NE in the present study. These facts led us to hypothesize that stresses induce the release of NE in the hemolymph and affect the target tissue in L. vannamei, resulting in a hyperglycemic response. However, the mode of action is unknown and could be achieved in a number of ways yet to be determined.

Increased energetic requirements during stressful concentrations necessitate an increase in ATP through the operation of the reaction sequence normally leading to the formation of excess pyruvate. Under hypoxic conditions, the impaired oxidation of pyruvate in the mitochondria leads to lactic acid formation. A large proportion of lactic acid can diffuse into the blood, thus increasing the concentration of lactic acid in the blood (Karlsson and Jacobs, 1982). In crustaceans, homeostasis of hemolymph glucose is maintained by an interaction between CHH release and carbohydrate metabolites in the hemolymph. During increases in glycolytic flux, lactate may cause the release of CHH.
by a positive feedback mechanism. The hormone then stimulates glycogenolysis, thus increasing glucose availability. The excess glucose released then suppresses CHH release from the X-organ sinus gland complex by negative feedback (Santos and Keller, 1993). For *L. vannamei* which received DA, both the hemolymph glucose and hemolymph lactate levels significantly increased from 2 to 4 h, and then all had returned to the initial level after 8 h (Chiu et al., in press). Similar results were also observed in shrimp which received NE in the present study. The facts suggest that hemolymph glucose and lactate are involved in the regulation of CHH release in *L. vannamei* resulting from an NE release effect on the target tissue under stress. Further research is needed to examine the relationship between CHH and NE variation in the hyperglycemic response of shrimp.

Neuroregulator involvement in osmoregulatory processes in crustaceans was reviewed by Morris (2001) and Tierney et al. (2003). The pericardial organs and, more recently, the sinus gland have both been shown to release osmoregulatory compounds. In *C. sapidus*, Kamamoto and Oyama (1985) demonstrated that extracts from the pericardial organs stimulate Na⁺ uptake by gill tissue. Also, DA and OA elevate cAMP levels, and applications of dibutyryl cAMP elevate Na⁺ uptake in gill tissue, suggesting that the amines act through this second messenger pathway to increase Na⁺ uptake (Lohrmann and Kamamoto, 1987). DA and cAMP acting as first and second messengers, respectively, to increase Na⁺ uptake were supported by subsequent research using gill tissue from *C. maenas* (Sommer and Mantel, 1988), the purple shore crab, *Leptograpsus variegates* (Morris and Edwards, 1995), and the Chinese mitten crab, *Eriocheir sinensis* (Mo et al., 1998). Findings of the above studies suggest that cAMP induces Na⁺ flux by stimulating Na⁺/K⁺-ATPase activity in gill membranes; however, cAMP may also induce Na⁺ flux via V-ATPase (Riestenpatt et al., 1995).

In crustaceans, gills are important organs for respiration as well as for osmoregulation (Mantel and Farmer, 1983; Pequeux, 1995). Morris (2001) indicated that euryhaline marine crabs utilize apical membrane branchial Na⁺/H⁺ and Cl⁻/HCO₃⁻ exchanges powered by basal membrane Na⁺/K⁺-ATPase, but in freshwater crustaceans, an apical V-ATPase provides for the electrogenic uptake of Cl⁻ in exchange for HCO₃⁻. The HCO₃⁻ is provided by carbonic anhydrase which facilitates CO₂ excretion, while NH₄⁺ substitutes for K⁺ in basal ATPase and for H⁺ in the apical exchange. For *C. maenas* transferred to diluted seawater, there was a rapid increase in DA in the hemolymph (Zatta, 1987), and increases in Na⁺/K⁺-ATPase activity and energy metabolism (Sommer and Mantel, 1988). *L. vannamei* receiving 10⁻⁶ mol shrimp⁻¹ DA showed transient elevations in osmolality, and Na⁺ and Cl⁻ levels (Chiu et al., in press). In the present study, however, shrimp receiving 10⁻⁶ mol shrimp⁻¹ NE showed transient elevations in osmolality and Na⁺, and decreases in K⁺ and Cl⁻ levels suggesting that acute modification of osmolality and ions may involve neuroendocrine control in *L. vannamei* under environmental stresses.

The present study indicates that after 2 h, NE administered to *L. vannamei* induced notable protein elevation, oxyhemocyanin/protein ratio reduction, and an invariable oxyhemocyanin concentration in the hemolymph. However, no significant differences in hemolymph protein, oxyhemocyanin, or the oxyhemolymph/protein ratio were observed among the 4 treatments after 4 h. Chiu et al. (in press) indicated that after 2 h, DA administered to *L. vannamei* induced notable protein elevation, oxyhemocyanin/protein ratio reduction, and an invariable oxyhemocyanin concentration in the hemolymph. Those facts suggest that DA and NE promote transient mobilization of protein which diffuses into the hemolymph of shrimp, resulting in a reduction in the oxyhemocyanin/protein ratio.

A rhythmicity of physiological processes under the influences of cyclic changes in the photoperiod regime has often been reported. In the present study, hemolymph lactate concentrations in *L. vannamei* which had received saline were lower after 4 h than those of shrimp at other sampling times. Two hours after an NE injection at 10⁻⁶ mol shrimp⁻¹, *L. vannamei* hemolymph Cl⁻ levels were lower than those of shrimp receiving the saline control and NE at 10⁻⁷ and 10⁻⁸ mol shrimp⁻¹, and did not differ significantly from the control at 0 h. The facts suggest that changes in the rhythmicity of physiological processes do occur, and that NE may inhibit the rhythmic change in Cl⁻ regulation.

In conclusion, the present study documented that *L. vannamei* receiving NE at ≤10⁻⁶ mol shrimp⁻¹ experienced transient elevations in osmolality, and glucose, lactate, Na⁺, and protein levels, as well as reductions in Cl⁻, K⁺, and the oxyhemocyanin/protein ratio levels in the hemolymph. Norepinephrine plays an important role in neurotransmission and causes energy metabolism, osmoregulation, and respiratory response modulation. Further work on NE and other hormones will provide a better understanding of the response of shrimp to stressors in their environment, and in production systems.
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References


