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Research Article

Neuroendocrine and metabolic responses of Pacific whiteleg shrimp *Penaeus vannamei* exposed to hypoxia stress

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ABSTRACT. Neuroendocrine mechanisms involved in crustacean stress response are different and less understood than those of vertebrates. Although indirect evidence suggests the participation of catecholamines (CA), few studies have analyzed their levels during stress response in crustaceans. This study examined CA levels in hemolymph and tissue of whiteleg shrimp *Penaeus vannamei* besides other biochemical indicators in response to hypoxia (0.8-1.0 mg L^{-1}). Shrimp were stressed by hypoxia exposure, and their responses were analyzed at several intervals (10-480 min). Hemolymph glucose levels decreased gradually due to a higher demand not compensated by the gluconeogenesis process from hepatopancreas or muscle after 10 min. Lactate levels in hemolymph showed a 5-fold increase after 10 min, and a correlation with CA levels in hemolymph was observed suggesting its possible role in signaling catecholaminergic activation from eyestalk in a period from 10 to 120 min. Protein levels in muscle and hepatopancreas increased gradually throughout the trial indicating the possible use of another energetic substrate as arginine phosphate. A decrease in triacylglyceride levels denotes its preferential utilization as an immediate energy source in the scape response during the first minutes of hypoxia exposure. Heart adrenaline and noradrenaline levels increased rapidly at the beginning of the trial showing a correlation with the use of triacylglycerides and carbohydrates in hepatopancreas and muscle ($r = 0.89$ and $r = 0.93$, $P < 0.05$, respectively). This manuscript reports evidence of CA participation in response to stress due to hypoxia and discusses possible adaptation mechanisms.

Keywords: *Penaeus vannamei*, catecholamines, glucose, lactate, eyestalk, hemolymph, aquaculture.

INTRODUCTION

Shrimp farming has been one of the fastest growing sectors in Asia and Latin America, and lately in Africa, but it has also been one of the most controversial due to some negative environmental and social impacts of this activity (FAO, 2006). In recent years, this increase in shrimp culture has been accompanied by different problems that arise as a result of the usual and inherent practices commonly used in aquaculture systems, which are exposed to variations of multiple environmental factors. Among these problems, the main ones affecting culture success in a greater degree are the conditions that commonly produce stress in the organism.

Stress response can be considered as a set of the organism's adaptive mechanisms in an attempt to reestablish homeostasis (Chrousos, 1997; Barton, 2002) although under conditions of chronic stress, the adaptive value is lost and several negative effects could be present at both immunological and growth and reproduction levels (Wendelaar-Bonga, 1997; Ross & Ross, 1999). Based on the above, stress has also been considered as an alteration in the physiological stable state of an individual making it more vulnerable to subsequent environmental adverse conditions. As part of this stress response, researchers have reported the role of catecholamines (CA), which have been observed to participate in the control of ventilation (Rajashekhar & Wilkens, 1992), cardiac output (Guirguis

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& Wilkens, 1995; Wilkens *et al*., 1996), immune response (Cheng *et al*., 2005), osmoregulation (Zatta, 1987; Péqueux *et al*., 2002; Liu *et al*., 2008), and glucose regulation (Lüschen *et al*., 1993; Kuo & Yang, 1999).

Probably the most studied effect of these hormones has been on glucose levels, and secondarily, on lactate levels in hemolymph. To cite some examples, the well-known increase in glucose and lactate levels in hemolymph could be considered after exposure to atmospheric air in *Cancer pagurus* (Webster, 1996), *Carcinus maenas*, and *Orconectes limosus* (Santos & Keller, 1993), as well as in *Cambarus robustus* (Telford, 1974). In addition to glucose and lactate, other physiological stress responses, such as hemolymph triacylglyceride levels (Racotta & Palacios, 1998); protein and hemocyanin in hemolymph (Racotta & Palacios, 1998; Perazzolo *et al*., 2002; Mercier *et al*., 2006); the use of glycogen in tissues (Racotta & Hernández-Herrera 2000; Racotta *et al*., 2003), as well as osmotic pressure in hemolymph represent the capacity of osmoregulation (Lignot *et al*., 2000; Chim *et al*., 2003; Pascual *et al*., 2003). Although dopamine, norepinephrine, and epinephrine have been histochemically identified and quantitatively measured in the crustacean nervous system (for review, see Fingerman *et al*., 1994), only have a few studies reported CA levels in response to a particular stressor.

For example, several studies reported changes in hemolymph and gill levels of dopamine or norepinephrine in response to salinity changes (Zatta, 1987; Péqueux *et al*., 2002), but they seem to be more related to an osmoregulatory response, especially because they were analyzed in response to a long-term effect (days) (Péqueux *et al*., 2002; Liu *et al*., 2008). Analyses of norepinephrine levels in thoracic ganglia, eyestalk, and hemolymph have indicated that CA could mediate hyperglycemia induced by cold shock stress in the giant prawn *Macrobrachium rosenbergii* (Hsieh *et al*., 2006). Clearer evidence of CA release during stress in invertebrates is supported by studies in mollusks in response to mechanical disturbance (Lacoste *et al*., 2001) or air exposure. For all of the above, the stress generated by intensive production conditions requires being minimized to avoid a decrease in growth and massive disease mortalities (Dhert *et al*., 1992). Since information on neuroendocrine control of stress response is scarce in crustaceans in general and particularly in peneids, as well as on their metabolic and immunological consequences, it is necessary to deepen in basic knowledge of this problem. Therefore, the objective of this work was to analyze the influence of hypoxia conditions on the metabolic response associated with stress, as well as on the circulating and tissue levels of CA as possible neuroendocrine modulators of this response.

MATERIALS AND METHODS

Experimental animals

Juvenile whiteleg shrimp (23 ± 0.26 g), obtained from earthen ponds, were transferred to 1.4 m^2 outdoor concrete tanks under the following conditions: 30 shrimp/tank; natural photoperiod; seawater at 39 salinity; pH 7.8; dissolved oxygen 5.1 ± 0.5 mg L^{-1}; total ammonia concentration 0.3 ± 0.2 mg L^{-1}; and controlled temperature at 25 ± 1°C. Shrimp were fed a commercial shrimp diet (25% protein) at a daily rate of 5% of total biomass. Water was exchanged at a rate of 50% per day to remove feces and food that was not ingested. Animals were acclimated for one week. Shrimp were carefully caught, placed in individual 50 L tanks, and acclimated for 24 h (including a 24 h fast) at 28°C and 7 ± 0.5 mg L^{-1} of dissolved oxygen. At the end of the acclimation period, nitrogen gas bubbling was started until an oxygen concentration of 0.8 to 1.2 mg L^{-1} using a multiparameter probe (YSI Environmental 556) was achieved; then the water surface was covered by a plastic film to avoid gas exchange between atmospheric air and water. The shrimp were exposed to different hypoxia periods and sampled at 10, 30, 60, 120, 240, and 480 min (n = 13 for each time point) after the stress event. Shrimp in the control group had the same handling but without nitrogen bubbling. Oxygen levels used in this experiment were based on previous works about hypoxic conditions from 1.0-1.7 mg L^{-1} and where the authors (Zenteno-Savín *et al*., 2006; Abe *et al*., 2007; Mugnier *et al*., 2008) observed an evident response in variables other than those analyzed in this work.

Sample collection and biochemical analyses

Hemolymph (approximately 400 µL) was collected from the ventral sinus at the base of the first abdominal segment, using a 1 mL syringe rinsed with 5% sodium oxalate in cooled isotonic saline anticoagulant solution (Mercier *et al*., 2006). After hemolymph sampling, shrimp were weighed, frozen in liquid nitrogen, and stored at -70°C for further tissue analysis. Hemolymph was centrifuged at 1350 g at-4°C for 10 min; plasma was separated from precipitated cells for biochemical analyses. For hemocyanin determination, hemolymph samples were diluted 1:20 with an isotonic saline solution; absorbance was recorded at 335 nm; and the concentration was calculated using an extinction coefficient of $E^{1\%}$ = 2.83 for shrimp hemocyanin (Hagerman, 1986). Plasma was diluted to 1:100 with the isotonic saline solution for protein determination

according to Bradford (1976) and using a commercial chromogen reagent (Bio-Rad, California, USA) and bovine serum albumin (Sigma, Missouri, USA) as standard. Commercial kits were used for determination of glucose (Randox, GOD-PAP, County Antrim, UK), lactate (Randox, PAP, County Antrim, UK), and triacylglycerides (Randox, GPO-PAP, County Antrim, UK). Methods were adapted to a microplate, using 20 µL plasma and 200 µL enzyme chromogen reagent (Palacios *et al*., 2000). Absorbance was recorded at 490 nm for triacylglycerides and glucose and at 540 nm for lactate on a microplate reader, and concentrations were calculated from a standard substrate solution. The hepatopancreas and the first abdominal segment muscle were dissected from the frozen shrimp, and weight was recorded. Samples of hepatopancreas and muscle were then homogenized in 5 mL cold 10% trichloroacetic acid and centrifuged at 3000 g at 5°C for 15 min. The resulting deproteinized supernatant was used for total carbohydrate determination by the anthrone colorimetric reaction (Roe, 1955) and for lactate determination by the same kit as for plasma. Another sample was homogenized in saline solution for determination of total lipids by the sulphophosphovanillin method of Barnes & Blackstock (1973); total proteins were determined according to Bradford (1976) and triacylglycerides by the same kit as for plasma.

Catecholamines (CA) analyses

The same shrimp than those sampled for biochemical analysis were used for CA analysis and with the same sampling procedure and post-stress time intervals (n = 13 for each time) to obtain plasma and tissues that were stored at -70°C till further analyses. CA were analyzed by HPLC coupled to electrochemical detection, briefly summarized as follows: CA were extracted from 400 µL plasma and added to columns containing 40 mg alumina; 1.5 mL 0.5 M Trizma buffer at pH 8.6, 200 µL EDTA (5%); and 100 µL 3,4-dihydroxybenzylamine hydrobromide (DHBA, 100 ng mL^{-1}) as internal standard. After CA adsorption, alumina was washed twice with de-ionized water; then CA was eluted with 0.1 N perchloric acid (PCA). Eluted samples were filtered (Millipore 0.45 µm) and injected into the HPLC system. Tissue samples were weighed and homogenized in two mL of 0.4 N PCA and centrifuged at 3000 g for 10 min; the supernatant was collected, and CA from 500 µL was adsorbed on alumina, as described for plasma. CA was analyzed by reversed phase (column C-18, 15×4.6 mm) HPLC coupled to an electrochemical detector (ESA, Bedford, MA). The first detector potential was set at -200 mV and the second one at +200 mV. The mobile phase was composed of 1 mM heptanosulfonic acid sodium salt, 1 mM EDTA, 0.15 M $NaH_2PO_4 \cdot H_2O$, and 6% methanol at pH 3.6, adjusted with phosphoric acid and at a flow rate of 0.4 mL min^{-1}. CA was identified by comparison of retention times to those of known standards and quantified by using the peak area ratio related to DHBA, the internal standard to correct for incomplete recovery. Data are presented as means ± standard error (SE). Significant differences between groups were assessed by one-way ANOVA followed by the Tukey HSD *post-hoc* test for mean comparisons ($P < 0.05$).

RESULTS

Circulating fuels and tissue reserves

The results of the metabolic variables analyzed in hemolymph and tissues that have reported no significant differences ($P < 0.05$) are shown in Table 1. Subsequently, the rest of the results are particularly described.

Hemolymph

The biochemical variables analyzed in hemolymph did not show significant differences ($P < 0.05$) among the different hypoxia periods to which the organisms were subjected (Table 1). Nonetheless, it is important to highlight that a non-significant lactate increase was observed 15 min after hypoxia exposure. Due to the great variation among the organisms subjected to hypoxia (coefficient of variation up to 130%), the differences did not reach statistical significance.

Hepatopancreas

Triacylglyceride levels showed a significant decrease ($P < 0.05$) after 10 min of hypoxia exposure, and they were partially restored at 30 min, after which they maintained without changes until the end of the analyzed period (Fig. 1a). On the other hand, the proteins of this tissue showed a significant increase ($P < 0.05$) after 10 min of hypoxia exposure; subsequently, the levels were maintained without significant changes (Fig. 1b). Neither lipid and carbohydrate concentrations of this tissue showed significant differences ($P < 0.05$) among the different hypoxia periods to which the organisms were subjected (Table 1).

Muscle

Protein levels in muscle showed an increasing trend, which was only significant ($P < 0.05$) with respect to the control group after 480 min of hypoxia exposure (Fig. 2). The analyzed lipid, triacylglyceride, carbohydrate, and lactate levels in this tissue did not show significant differences ($P < 0.05$) related to the different hypoxia periods to which the organisms were subjected (Table 1).

Table 1. Metabolic variables (mean ± SE) analyzed in stress and control groups of *Penaeus vannamei* after different hypoxia exposure periods. n = 13 shrimp for each time point.

	Control group	Time exposed to hypoxia (min)					
		10	30	60	120	240	480
Hemolymph							
Lactate (mg dL^{-1})	0.97 ± 0.1	5.04 ± 1.5	6.11 ± 2.1	1.87 ± 0.3	3.78 ± 2.2	5.56 ± 2.4	7.81 ± 3.2
Glucose (mg dL^{-1})	36.6 ± 9.8	32.76 ± 6.3	26.8 ± 4.3	27.0 ± 3.6	29.5 ± 4.6	21.4 ± 3.8	17.8 ± 4.7
Hemocyanin (mg mL^{-1})	89.3 ± 5.3	78.7 ± 11	88.4 ± 6.9	90.9 ± 8.3	81.4 ± 5.2	72.3 ± 8.2	71.6 ± 9.3
Protein (mg mL^{-1})	160.7 ± 18	157.4 ± 21	121.8 ± 5	137.7 ± 14	108.6 ± 9	117.4 ± 12	120 ± 8.6
Lipid (mg dL^{-1})	2.18 ± 0.3	2.03 ± 0.4	2.62 ± 0.5	2.63 ± 0.2	3.50 ± 0.3	1.88 ± 0.4	2.72 ± 0.7
Tryacylgliceride (mg dL^{-1})	60.8 ± 7.5	50.8 ± 8.8	68.4 ± 14	64.7 ± 7.4	75.2 ±10	54.4 ± 7.7	66.4 ± 16
Hepatopancreas							
Lipid (mg g^{-1})	52.2 ± 5.1	32.3 ± 7.7	52.3 ± 9.4	62.4 ± 9.1	59.8 ± 8.9	49.6 ± 11	44.4 ± 10
Carbohydrate (mg g^{-1})	9.4 ± 0.8	7.4 ± 0.8	9.2 ± 0.4	10.1 ± 0.6	8.6 ± 0.6	9.6 ± 1.0	8.3 ± 0.7
Muscle							
Lipid (mg g^{-1})	10.9 ± 0.5	9.2 ± 0.7	8.7 ± 0.4	9.2 ± 1.2	11.8 ± 0.6	9.2 ± 0.6	9.5 ± 0.6
Tryacylgliceride (mg g^{-1})	2.2 ± 0.1	1.9 ± 0.1	2.1 ± 0.1	2.9 ± 0.2	2.7 ± 0.1	2.5 ± 0.5	2.3 ± 0.2
Carbohydrate (mg g^{-1})	7.07 ± 0.9	6.32 ± 1.5	7.57 ± 1.3	11.4 ± 1.4	10.5 ± 1.3	7.9 ± 1.6	7.4 ± 1.7
Lactate (mg g^{-1})	0.99 ± 0.0	0.98 ± 0.13	1.01 ± 0.0	1.13 ± 0.0	1.30 ± 0.1	1.03 ± 0.1	1.01 ± 0.0

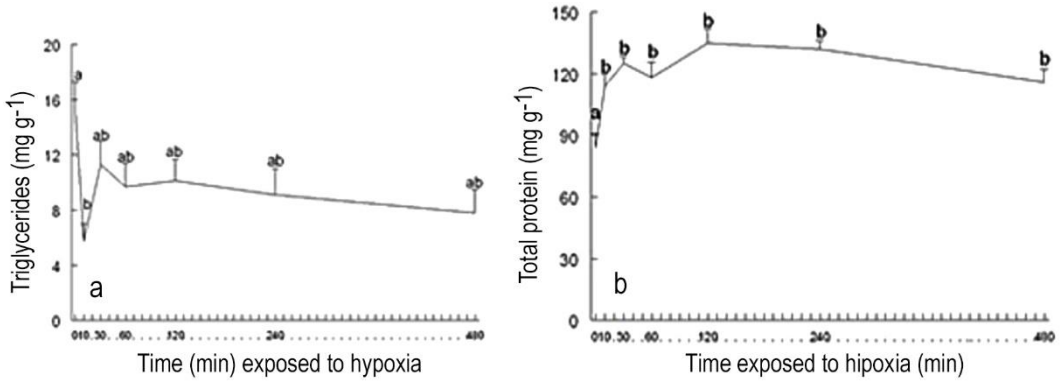

Figure 1. *Penaeus vannamei* a) Levels of triacylglyceride and b) protein in hepatopancreas in before (0) and after hypoxia stress. Means ± SE with different letters are significantly different at $P < 0.05$; n = 13 shrimp for each time point.

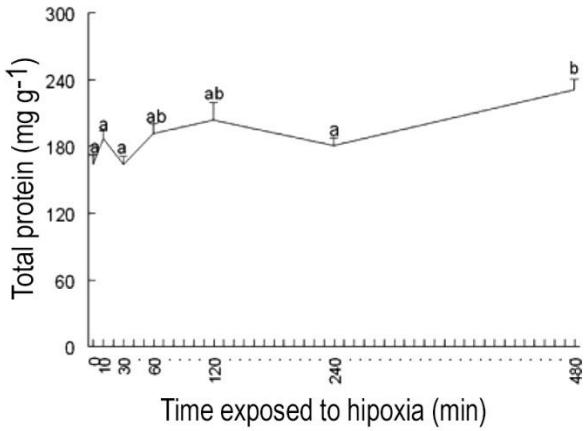

Figure 2. Levels of protein in muscle in *Penaeus vannamei* before (0 min) and after hypoxia stress. Means ± SE with different letters are significantly different at $P < 0.05$; n = 13 shrimp for each time point.

Catecholamines

Hemolymph

Dopamine concentration showed a decreasing trend at 20 min and later showed an increase at 120 min although these changes were not statistically significant (Fig. 3a). Norepinephrine concentration also showed a non-significant increase at 120 min after hypoxia exposure (Fig. 3b). Epinephrine levels showed a similar variation pattern to that of dopamine although in this case, changes were significantly different ($P < 0.05$) since a decrease was observed at 60 min to return to basal levels at 120 min. Subsequently, a non-significant gradual decrease was observed until 480 min (Fig. 3c).

Eyestalk

Dopamine levels showed a significant increase ($P < 0.05$) at 30 min to decrease significantly after 60 min of

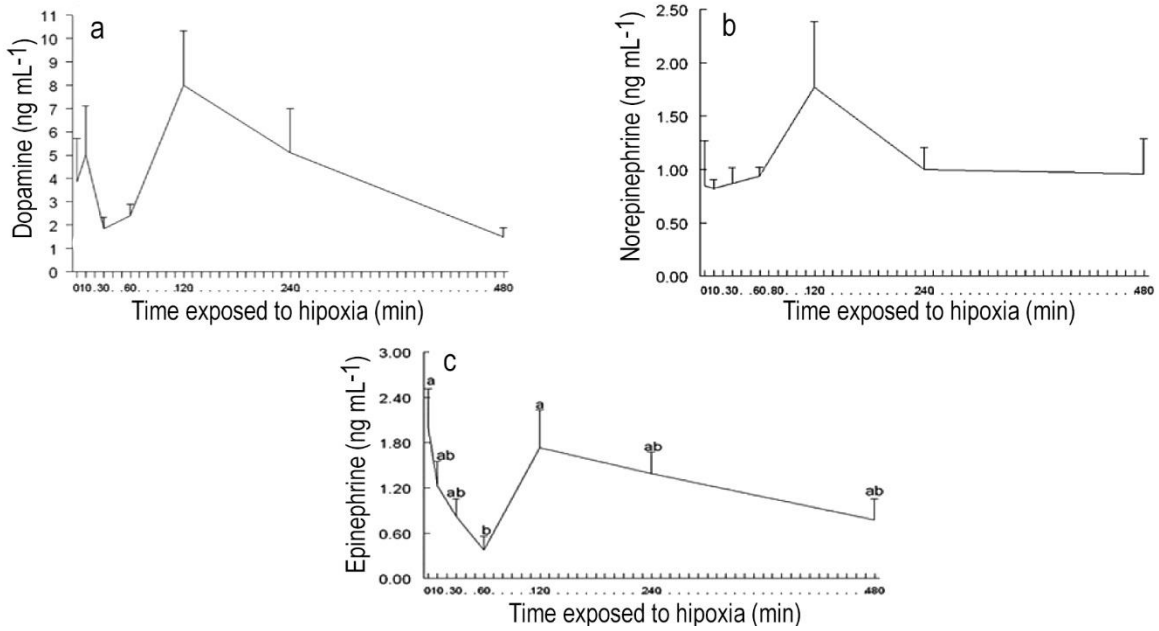

Figure 3. a) Levels of dopamine, b) norepinephrine, and c) epinephrine in hemolymph in *Penaeus vannamei* before (0 min) and after hypoxia stress. Means ± SE with different letters are significantly different at $P < 0.05$; n = 13 shrimp for each time point.

hypoxia exposure; subsequently, they showed an increasing trend reaching a second peak at 480 min (Fig. 4a). Norepinephrine levels showed a gradual decrease from 30 to 120 min, from which they gradually increased up to levels similar to basal (Fig. 4b). Epinephrine concentration showed two significant increases ($P < 0.05$) at 30 and 240 min of hypoxia exposure to later decrease significantly to values similar to basal at 480 min (Fig. 4c).

Hepatopancreas

Dopamine values in this tissue maintained relatively constant up to 60 min with an increase starting from 120 min, which became significant ($P < 0.05$) at 240 min with maximum levels. Subsequently, a significant decrease ($P < 0.05$) was observed to basal levels after 480 min of hypoxia exposure (Fig. 5a). Norepinephrine levels showed a non-significant variation along the hypoxia exposure period (Fig. 5b). Epinephrine levels showed an increasing trend after 60 min to decrease at 120 min although these changes were not significant (Fig. 5c).

Heart

Dopamine levels showed a significant increase ($P < 0.05$) at 120 min of hypoxia exposure; subsequently, they tended to decrease although basal levels were not recovered (Fig. 6a). Norepinephrine concentration increased significantly after 10 min to later decrease significantly from 30 to 60 min. Subsequently, a gradual not significant increase was observed (Fig. 6b). Epinephrine concentration tended to increase after 30 min of hypoxia exposure and decreased significantly ($P < 0.05$) after 120 min to later increase and decrease significantly at 240 and 480 min, respectively (Fig. 6c).

DISCUSSION

Before analyzing the hypoxia effect on a series of metabolic variables, it was necessary to compare the protocol employed in this work as to experimental conditions, levels, and hypoxia length, and why it was considered to represent a stress situation for shrimp. Firstly, it was necessary to place the organisms in individual tanks to control precise timing of hypoxia induction and individual sampling without disturbing other organisms in the experimental unit. Possible stress caused by transferred of organisms the day before could be ruled out based on a parallel work where no differences were observed in glucose levels among the organisms transferred to experimental units 12 h before and organisms without transfer (Carreño-Mejía, 2009). Oxygen decrease was performed by bubbling nitrogen same as in other works (Racotta *et al*., 2002; Pérez-Rostro *et al*., 2004; Zenteno-Savín *et al*., 2006;

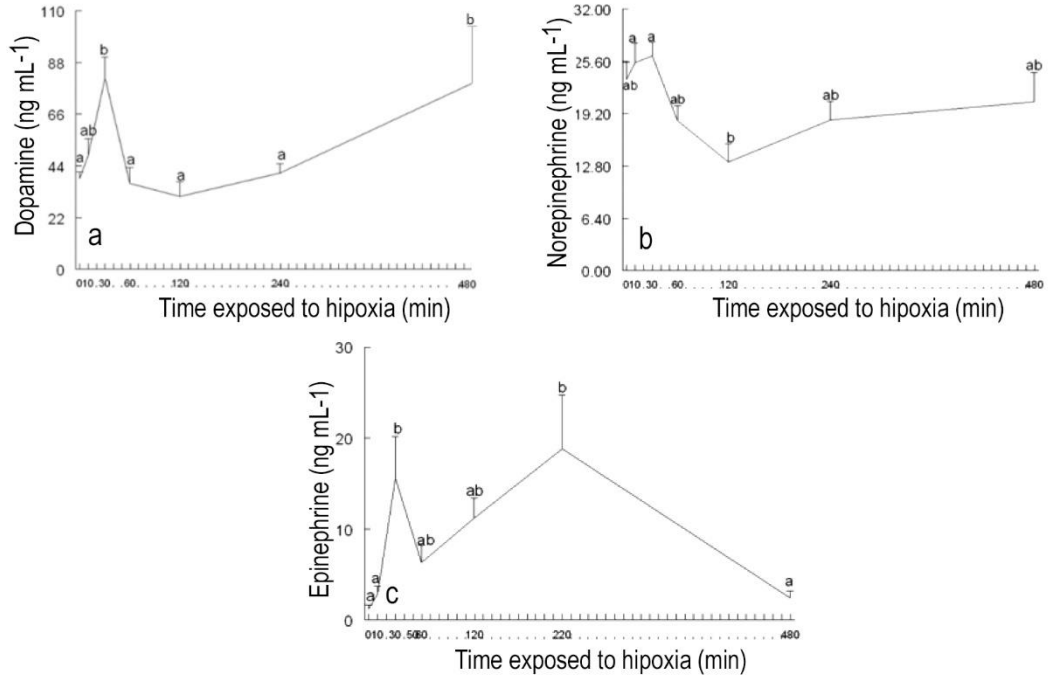

Figure 4. a) Levels of dopamine, b) norepinephrine and c) epinephrine in eyestalk in *Penaeus vannamei* before (0 min) and after hypoxia stress. Means ± SE with different letters are significantly different at $P < 0.05$; n = 13 shrimp for each time point.

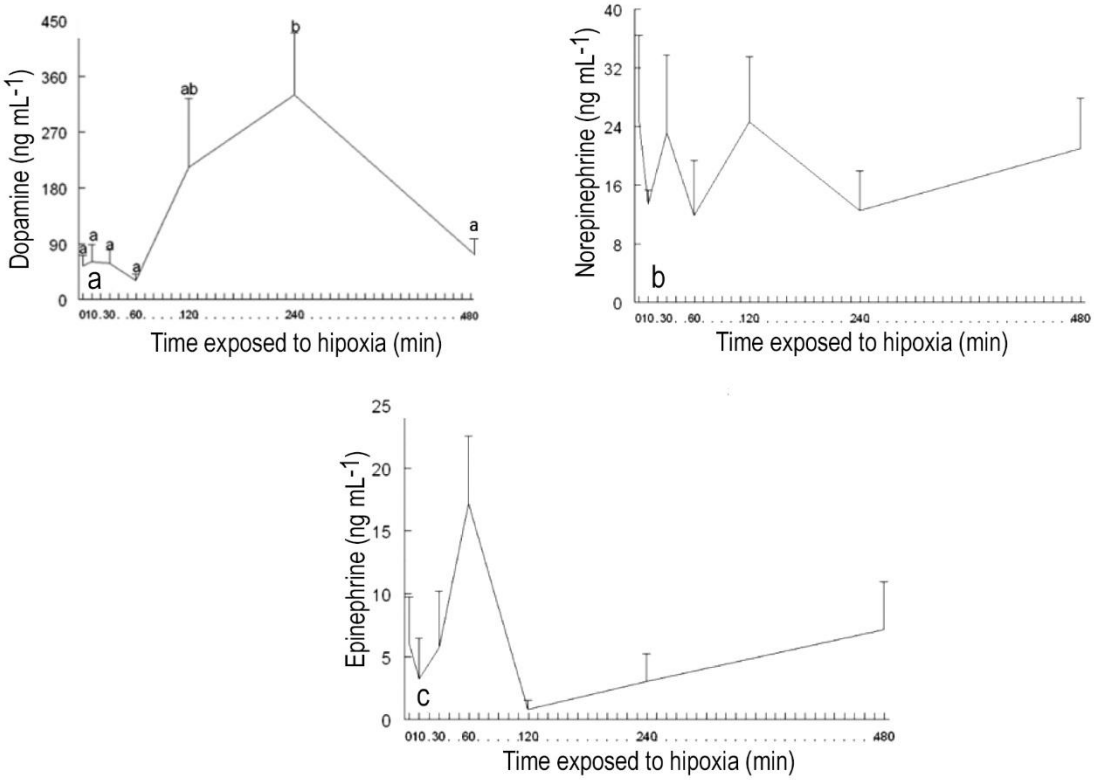

Figure 5. Levels of a) dopamine, b) norepinephrine, and c) epinephrine in hepatopancreas in *Penaeus vannamei* before (0 min) and after hypoxia stress. Means ± SE with different letters are significantly different at $P < 0.05$; n = 13 shrimp for each time point.

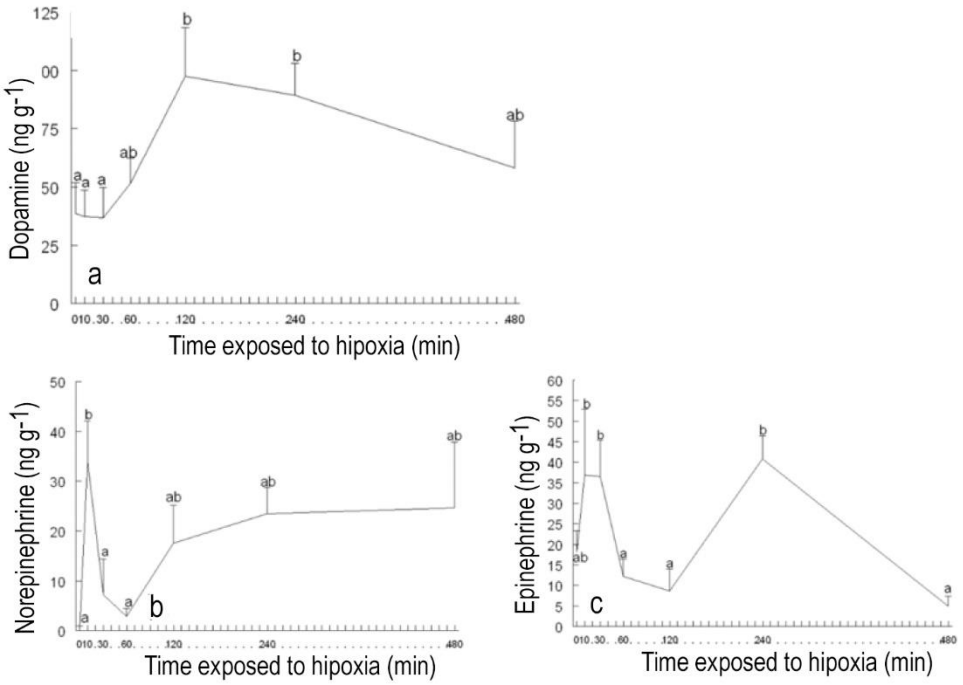

Figure 6. Levels of a) dopamine, b) norepinephrine, and c) epinephrine in *Penaeus vannamei* before (0 min) and after hypoxia stress. Means ± SE with different letters are significantly different at $P < 0.05$; n = 13 shrimp for each time point.

Mugnier *et al*., 2008), which implied an intense bubbling and hose movement inside the tank to homogenize water and oxygen measurement, procedure that took around 15 min. Given that this procedure could have caused certain disturbance to the organisms (stress), it was also applied in tanks of the control group but only with air instead of nitrogen.

In a series of previous works of hypoxia response in crustaceans, an increase of glucose and lactate in hemolymph was reported although exposure time and magnitude were very variable. For example, in *Eriocheir sinensis* (Zou *et al*., 1996), under hypoxia conditions by gradual decrease from 6 to 1 mg L^{-1} in 15 h, an increase from 2 to 9 mg L^{-1} d^{-1} was observed in circulating glucose levels after 12 h, followed by a lactate increase even greater from undetectable levels up to 13.2 mg mL^{-1} after 19 h of exposure to such conditions. In the long term, Racotta *et al*. (2002) observed glucose increase from 13 to 45 and 57 mg L^{-1} d^{-1} when specimens of *P. vannamei* were exposed to hypoxia conditions from 2-2.5 mg L^{-1} at two days or two weeks, respectively. Changes in lactate concentrations in hemolymph in the same work increased from 8-30 to 34 mg L^{-1} d^{-1}, respectively. The increase in glucose levels represents a physiological adaptation given that it constitutes the movement of an energetic substrate from a possible storage site toward circulation to be used by tissues that require it.

In contrast to previous information, glucose levels in hemolymph did not show any increase during the hypoxia exposure period even though a non-significant gradual increase was observed in the 8 h period (from 36.6 to 17.8 mg L^{-1} d^{-1}). Similarly, neither did Ocampo *et al*. (2003) observe in *Panulirus interruptus* an increase in circulating glucose under similar hypoxia conditions. On the other hand, in similar conditions to those of our work (exposure at 1.5 mg L^{-1} for 24 h), Mugnier *et al*. (2008) observed glucose increase in *Penaeus stylirostris* only in late premolt (D2 stage) while the effect was not observed in intermolt (C stage). It could explain our results, given that 60% of the organisms were in intermolt or early premolt stages, that is, less sensitive stages to environmental changes compared to postmolt and late premolt. On the other hand and as it will be discussed, for the lactate effect in tissues the hypoxia level might not have been enough to induce an evident metabolic response. Another explanation could be that glucose use was greater than movement from a reserve site; thus, levels even tended to decrease. In other stress conditions as hypothermia in the short or long term, glucose decrease was observed, which was also interpreted as a greater demand for glucose not compensated by production (Carreño-Mejía, 2009). In addition, the organisms in the control group of this experiment might have already been partially stressed, due to the relatively high values

reported (36 mg L^{-1} d^{-1}), therefore an additional stress did not produce a second increase but a decrease for its possible utilization. On the other hand, after 10 min of having subjected the organisms to hypoxia conditions (1.0 mg L^{-1}), lactate levels in hemolymph increased from 5 to 6 fold (7.81 mg L^{-1} d^{-1}) when compared with basal levels (0.97 mg L^{-1} d^{-1}), though not significantly due to the great variation among organisms (coefficient of variation up to 130%). Likewise, levels decreased at 60 min and a gradual increase started until reaching 7 mg d^{-1} L^{-1} at 8 h of hypoxia exposure, which indicated an accumulation in hemolymph. In the same manner, exposure of *Penaeus japonicus* to hypoxia only induced a slight elevation in lactate levels, which were maintained below 4.5 mg L^{-1} d^{-1} (Lallier & Truchot, 1989) same as with *Palaemonetes varians* (Nielsen & Hagerman, 1998); whereas *Homarus vulgaris* did not show significant increase in lactate levels when exposed to hypoxia (Butler *et al*., 1978), including in *Triops longicaudatus*, after being exposed to hypoxia conditions for 12 h, no increase in lactate and in hemocyanin were recorded (Harper & Reiber, 2006). One possible stress condition in the control group with a subsequent lactate increase did not seem to be the case given that the levels in the control group were very low (<1 mg L^{-1} d^{-1}). If such initial stress had occurred, as the high glucose values suggest, lactate levels might have recovered before those of glucose. Nonetheless, in such case, it was not possible to determine in what measurement this initial stress could have influenced the subsequent one. In this respect, only two possible speculations could be brought forward 1) a second stress response could be less than the first one although it does not seem to be the case in several crustaceans exposed to two consecutive periods of intense physical activity (Henry *et al*., 1994), and 2) the mechanisms activated to remove lactate by the first stressor stimulus already analyzed for handling influence (Aparicio-Simón *et al*., 2010) were more efficient during the second response.

Besides the causes previously mentioned in an isolated manner for the lack of hypoxia effect on glucose, lactate, and hemocyanin levels as typical hypoxia responses in crustaceans, it could be due to other types of adaptation mechanisms in front of lower O_2 levels that could occur in natural habitat or aquaculture systems for this species. To this respect, it should be mentioned that maintaining a constant heart rate together with a constant cardiac output allows the organism regulating its metabolic rate as the external environment turns out to be more hypoxic. Nonetheless, pauses in ventilation activity could take place, allowing energy savings during quiescence, which has also been observed in *Cancer magister*, together with bradycardia processes and a greater preferential irrigation of hemolymph toward nervous and/or vital structures (McGaw, 2005).

No changes were observed in carbohydrate values or in any other variable analyzed in muscle, except for a gradual but significant increase in protein levels after 480 min of hypoxia exposure (230 mg g^{-1}), compared with basal levels (164 mg g^{-1}). This increase was also observed in protein levels in hepatopancreas and the results agree with that reported by Pérez-Rostro *et al*. (2004) in *Penaeus vannamei* exposed to very sharp hypoxia conditions (0.1 mg L^{-1}) in a short-term (1 h). Such increase in proteins has also been observed in the long term (15 d) although at moderate hypoxia levels (one to 2.5 mg L^{-1}) in *P. vannamei* (Racotta *et al*., 2002). Nevertheless, no explanation seems to clarify this increase during hypoxia through short periods of exposure, both degradation, and protein synthesis, are known to decrease in response to lack of oxygen (Hochachka *et al*., 1996). On the contrary at least in the long term (60 d) and in moderate hypoxia conditions (3-4 mg L^{-1}), the relationship O:N decreased in *Penaeus setiferus*, which indicates that protein use increases under these hypoxia conditions (Rosas *et al*., 1999).

As opposed to the increasing trend of lactate in hemolymph, no variation was observed in lactate levels in muscle, contrasting with a series of previous works on hypoxia exposure in crustaceans in general (Gäde, 1984; Fujimori & Abe, 2002). In the case of penaeid shrimp, *Marsupenaeus japonicus* exposed to similar hypoxia conditions of 1.3-1.7 mg L^{-1} after 6 h showed an increase in lactate from 0.2 to 0.43 mg g^{-1}; even for *P. vannamei* in more severe hypoxia conditions of 0.4 mg L^{-1} for one hour lactate levels increased from 0.77 to 0.96 mg g^{-1} (Pérez-Rostro *et al*., 2004). Considering the exposure time and hypoxia level used in this work, these comparisons reinforce that the hypoxia level used was not enough to obtain a clear metabolic response in the case of *P. vannamei* although it was observed it did not tolerate levels of 0.8 mg L^{-1} in preliminary assays.

Regarding carbohydrate levels in muscle or in hepatopancreas, no significant differences were found in contrast with other works that have reported a moderate but significant decrease of glycogen in *Marsupenaeus japonicus* in both organs (Abe *et al*., 2007) or a significant decrease of total carbohydrates in hepatopancreas but not in muscle of *P. vannamei* (Pérez-Rostro *et al*., 2004). It is important to mention that besides the possible use of glycogen as energetic substrate source, the use of arginine phosphate has been observed in muscle during the first hours of hypoxia exposure; in the case of *Marsupenaeus japonicus* the levels decreased drastically during the first 4 h of hypoxia, showing that arginine phosphate is preferen-

tially used during the beginning of the period and subsequently, glycogen is used as source of energy during the recovery process (Abe *et al*., 2007). On the other hand, triacylglyceride levels in hepatopancreas in the beginning of the hypoxia period (10 min) decreased significantly, suggesting its uptake and use by tissues during the initial stress produced by oxygen decrease.

Few works are available on CA levels in crustaceans in different stress situations, including hypoxia. Thus, the results reported in this work should be analyzed mainly based on works on the effect of CA on a series of variables related to hypoxia response, as well as to the correlations between CA levels and variable metabolic levels in this work.

It seems that in this work, despite showing an increase of dopamine in the eyestalk at 30 min of exposure, no increase in circulating glucose levels was observed mediated by crustacean hyperglycemic hormone activation, as it has been proposed by several authors (Sarojini *et al*., 1995; Zou *et al*., 2003) and whose effect has been shown in *Orconectes limosus* and *Carcinus maenas* (Lüschen *et al*., 1993), in *Penaeus monodon* (Kuo *et al*., 1995) and *Macrobrachium rosenbergii* (Kuo & Yang, 1999). Particularly in the case of *Penaeus monodon*, Chang *et al*. (2007) observed an increase in lactate and glucose concentrations in hemolymph 2 h after administering dopamine to the organism. Nonetheless, a significant correlation ($r = 0.88$, $P < 0.05$) was observed between dopamine and glucose levels in hemolymph after 10 min suggesting that the organisms that responded with certain dopamine increase also did it with certain glucose movement although none of the two variables were significantly different with respect to control. Another interesting correlation was between lactate and epinephrine, norepinephrine, and dopamine levels in hemolymph ($r = 0.91$, $P < 0.05$) but up to 120 min. Such relationship could have been due to the role of lactate as a signal for catecholaminergic activation as it has been observed for epinephrine in the crab *Carcinus maenas* (De Wacheter *et al*., 1997).

In the case of the eyestalk, it should be pointed out that the three CA presented an initial increase, although not significant for norepinephrine, followed by different oscillating patterns depending on the particular CA.

The relationship between synthesis and CA secretion is complex, which is why it is commonly necessary to measure the rate of CA re-change more than the levels as such (Racotta *et al.*, 1994). In any case, variations in CA levels in the eyestalk were considerably lower in magnitude than in other experiments (Aparicio-Simón *et al*., 2010), which is why the magnitude of secretion processes and compensatory synthesis might have been different in response to hypoxia. On the other hand and given that the three CA could be detected in hemolymph in this experiment, a decrease observed in the eyestalk for the three CA at 60 min agrees with a hemolymph increase at 120 min. These results partially agree with the reciprocal changes in crustacean hyperglycemic hormone or serotonin levels between the eyestalk and hemolymph starting from which the release of these neurohormones took place from the eyestalk toward hemolymph (Lorenzon *et al*., 2004, 2005). Although lactate levels did not vary significantly, the highest values were obtained at 15 and 30 min, followed by a decrease at 60 min, which agrees partially with the temporal pattern of epinephrine and dopamine in the eyestalk. This result could have been due to the role of lactate "signaling" (De Wachter *et al*., 1997) where lactate produced from 0 to 30 min would show an increase of CA synthesis (and maybe overcompensated secretion by synthesis). In turn, CA releases from 30 to 60 min can stimulate gluco-neogenesis, thus explaining lactate decrease from 30 to 60 min. Subsequently and given that the hypoxia status continued, a second lactate "cycle" -eyestalk- gluco-neogenesis might appear as it increased lactate again at 120 min jointly with the eyestalk CA. Despite the fact that it is merely speculation, more precise protocols should be explored, including a possible evaluation of CA turnover rate, expression of crustacean hyperglycemic hormone metabolic enzyme activities (*e.g.*, gluconeogenesis), and certainly, exposure to a more severe hypoxia at shorter time intervals to appreciate metabolic and neuroendocrine changes with a higher precision.

Hepatopancreas changes were only significant for dopamine; an important variation was observed though not significant for epinephrine from 30 to 120 min. Even though it agrees with the same critical time period, no association pattern was observed with metabolic changes in this organ given that the changes were in triacylglycerides and proteins during the first 10 min, and no changes were observed in carbohydrate levels.

Finally, changes in the heart are very clear as to the increase of epinephrine and norepinephrine from the start of hypoxia exposure. Seemingly, as to the correlation analyses performed ($r = 0.86$, $P < 0.05$), heart CA concentrations play an important role in this work on the use of energetic reserves in muscle and hepatopancreas, particularly triacylglycerides and carbohydrates in both tissues. Although it is known that the eyestalk releases CA, the heart could be and additional source of circulating CA that could play a role a more specific and local role. Several neurohormones have been identified in decapod precardiac organs, which are ideally located to release their

products in hemolymph on its bypass to the heart (Alexandrowicz, 1953), which include serotonin, dopamine, and octopamine (Beltz & Kravitz, 1983); this last one relaxes the anterior cardio-arterial muscle and causes contraction of the posterior valve in lobster, and it changes hemolymph distribution toward the anterior arterial system in crabs (Airries & McMahon, 1994). On the other hand, a differential response has been observed in the anterior and posterior cardiarterial valves of hearts isolated from *Panulirus japonicus* toward neurohormones or peptides (Kuramoto & Ebara, 1984) suggesting the presence of a control mechanism of hemolymph distribution in crustacean as *Cancer magister* and in response to hypoxia for *Homarus americanus* (Reiber *et al*., 1992). This possible control of blood distribution at heart level is a mechanism that performs an analog function to peripheral tissue infusion control in vertebrates suggesting a greater complexity of decapod crustacean cardiovascular system (Airries & McMahon, 1994). This preferential distribution of hemolymph could be occurring to ensure a better O_2 supply to vital organs or tissues during hypoxic situations.

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