

## Effect of salinity on the metabolism and osmoregulation of selected ontogenetic stages of an amazon population of *Macrobrachium amazonicum* shrimp (Decapoda, Palaemonidae)

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### Abstract

Probably as a function of their wide geographical distribution, the different population of *Macrobrachium amazonicum* shrimp may present distinct physiological, biochemical, reproductive, behavioral, and ecological patterns. These differences are so accentuated that the existence of allopatric speciation has been suggested, although initial studies indicate that the genetic variability of populations happen at an intraspecific level. Among the biological responses described for *M. amazonicum* populations, those regarding osmoregulation and metabolism play a key role for being related to the occupation of diverse habitats. To this effect, we investigated osmoregulation through the role of free amino acids in cell volume control and metabolism, through oxygen consumption in larvae (zoeae I, II, V and IX) and/or post-larvae of a *M. amazonicum* population from Amazon, kept in aquaculture fish hatcheries in the state of São Paulo. The results add information regarding the existence of distinct physiological responses among *M. amazonicum* populations and suggest that possible adjustments to metabolism and to the use of free amino acids as osmolytes of the regulation of the larvae and post-larvae cell volume depend on the appearance of structures responsible for hemolymph osmoregulation like, for example, the gills. In this respect, we verified that zoeae I do not alter their metabolism due to the exposition to fresh or brackish water, but they reduce intracellular concentration of free amino acids when exposed to fresh water, what may suggest the inexistence or inefficient performance of the structures responsible for volume regulation and hemolymph composition. On the other hand, in zoeae II and V exposed to fresh and brackish water, metabolism alterations were not followed by changes in free amino acids concentration. Thus it is possible, as the structures responsible for osmoregulation and ionic regulation become functional, that the role of free amino acids gets diminished and oxygen consumption elevated, probably due to greater energy expenditure with the active transportation of salts through epithelial membranes. Osmotic challenges also seem to alter throughout development, given that in zoeae II oxygen consumption is elevated on brackish water of 18, but in zoeae V it happens in fresh water. After *M. amazonicum* metamorphosis, free amino acids begin to play an important role as intracellular osmolytes, because we verified an increase of up to 40% in post-larvae exposed to brackish water of 18. The main free amino acids involved in cell volume regulation of ontogenetic stages evaluated were the non essential ones: glutamic acid, glycine, alanine, arginine, and proline. Interestingly, larvae from estuarine population studied here survived until the zoeae V stage in fresh water, but in some populations far from the sea, zoeae die right after eclosion in fresh water or they do not reach zoeae III stage. In addition, given that in favorable conditions caridean shrimp larvae shorten their development, we may infer that the cultivation environment, in which larvae developed in the present work, was appropriate, because almost all zoeae VIII kept on brackish water underwent metamorphosis directly to post-larvae and did not go through zoeae IX stage.

**Keywords:** *Macrobrachium*, osmorregulation, Crustacea, metabolism, physiology.

### Efeito da salinidade sobre o metabolismo e a osmorregulação de estágios ontogenéticos selecionados de uma população amazônica do camarão *Macrobrachium amazonicum* (Decapoda, Palaemonidae)

### Resumo

Provavelmente como função da sua ampla distribuição geográfica, as diferentes populações do camarão *Macrobrachium amazonicum* podem apresentar distintos padrões fisiológicos, bioquímicos, reprodutivos, comportamentais e ecológicos. Essas diferenças são tão acentuadas que tem sido sugerido a existência de especiação alopatrica embora estudos iniciais

indiquem que a variabilidade genética das populações ocorre ao nível intraespecífico. Dentre as respostas biológicas descritas para as populações de *M. amazonicum*, aquelas relacionadas à osmorregulação e metabolismo têm papel central por estarem relacionadas à ocupação dos diversos habitats. Nesse sentido, investigou-se a osmorregulação, por meio do papel dos aminoácidos livres no controle do volume celular e o metabolismo, por meio do consumo de oxigênio, em larvas (zoeas I, II, V e IX) e/ou pós-larvas de uma população de *M. amazonicum* oriunda da Amazônia e mantida em viveiros de aquicultura no estado de São Paulo. Os resultados adicionam informações a respeito da existência de respostas fisiológicas distintas entre as populações de *M. amazonicum* e sugerem que possíveis ajustes no metabolismo e no uso de aminoácidos livres como osmólitos da regulação do volume celular das larvas e pós-larvas dependem do surgimento de estruturas responsáveis pela osmorregulação da hemolinfa como, por exemplo, as brânquias. Nesse sentido, verificou-se que as zoeas I não alteram seu metabolismo em função da exposição à água doce ou salobra, mas reduzem a concentração intracelular de aminoácidos livres quando expostas à água doce, o que pode sugerir a inexistência ou um desempenho ineficiente das estruturas responsáveis pela regulação do volume e composição da hemolinfa. Por outro lado, nas zoeas II e V expostas à água doce ou salobra alterações no metabolismo não foram acompanhadas por mudanças na concentração dos aminoácidos livres. Assim é possível que à medida que estruturas responsáveis pela osmo e ionorregulação tornam-se funcionais, o papel dos aminoácidos livres se torne reduzido e o consumo de oxigênio elevado, provavelmente em função do maior gasto energético com o transporte ativo de sais através das membranas epiteliais. Os desafios osmóticos também parecem se alterar ao longo do desenvolvimento visto que em zoeas II o consumo de oxigênio é elevado em água salobra de 18 mas em zoeas V essa resposta ocorre em água doce. Após a metamorfose de *M. amazonicum*, os aminoácidos livres passam a ter papel importante como osmólitos intracelulares, pois se verificou um aumento de até 40% nas pós-larvas expostas à água salobra de 18. Os principais aminoácidos livres envolvidos na regulação do volume celular dos estágios ontogenéticos avaliados foram os não essenciais ácido glutâmico, glicina, alanina, arginina e prolina. Interessantemente, as larvas da população estuarina aqui estudada sobrevivem até o estágio de zoea V em água doce mas em algumas populações distantes do mar as zoeas morrem logo após a eclosão em água doce ou não chegam ao estágio de zoea III. Adicionalmente, visto que em condições favoráveis as larvas de camarões carídeos abreviam o seu desenvolvimento pode ser inferido que o meio de cultivo em que as larvas se desenvolveram no presente trabalho foi adequado, pois quase todas as zoeas VIII mantidas em água salobra sofreram diretamente a metamorfose para pós-larvas e não passaram pelo estágio de zoeas IX.

*Palavras-chave:* *Macrobrachium*, osmorregulação, Crustacea, metabolismo, fisiologia.

## 1. Introduction

The *M. amazonicum* shrimp has a wide geographical distribution that goes from Caribbean and Atlantic coasts of South America to northern Argentina and Paraguay and the eastern slopes of Andes in Ecuador, Bolivia and Peru to the Atlantic coasts of northeastern Brazil (Maciel and Valenti, 2009). There is a geographical separation and, in consequence, a genetic isolation among *M. amazonicum* populations of the northern region (also including the Atlantic and Caribbean coasts and bays of Amazon and Orinoco) and southern region (La Plata System) of Brazil. This wide geographical distribution has as consequence the existence of distinct physiological, reproductive, behavioral, and ecological patterns among many populations. In relation to reproduction, Urzúa and Anger (2011) verified differences in biomass and chemical composition of larvae of *M. amazonicum* populations from Pantanal (state of Mato Grosso, Brazil) and Belém (state of Pará, Brazil). In relation to physiology, it is observed, among the different *M. amazonicum* populations, a curious pattern of brackish water dependence, in which there are either populations that complete their life cycle in fresh water (Zanders and Rodriguez, 1992; Charmantier and Anger, 2011) and those in which larvae die when kept in this salinity (Augusto et al., 2007a). Charmantier and Anger (2011) compared two *M. amazonicum* populations

hydrologically and genetically separated and verified distinct osmoregulation and tolerance to salinity patterns among them. Urzúa and Anger (2011) suggested that the existing biological diversity among *M. amazonicum* populations may happen due to the limited genetic exchange among them, a case of allopatric speciation. Although the studies about the genetics of different populations are still incipient, Vergamini et al. (2011) verified that the genetic variability of coast and countryside *M. amazonicum* populations happen at an intraespecific level.

The wide distribution of *M. amazonicum* followed by the diversity of biological responses makes this specie a very interesting object to be studied. Among physiological mechanisms which can be investigated, osmoregulation and metabolism have a notable importance for being related to the distribution of the species in different environments. In this sense, osmotic and ionic regulation in crustaceans is composed of two processes, extracellular anisomotic regulation, responsible for the maintenance of the osmolality and performed by the action epithelial enzymes like  $\text{Na}^+/\text{K}^+$ -ATPase, V-ATPase,  $\text{HCO}_3^-$ -ATPase, carbonic anhydrase (Freire et al., 2008; Garçon et al., 2013) and intracellular isosmotic regulation, responsible for the maintenance of intracellular media through the adjustment of the concentration intracellular osmolytes, mainly free amino acids (Péqueux, 1995; McNamara et al., 2004; Augusto et al., 2007a, b; Faria et al., 2011). As

more efficient the mechanisms of osmolality regulation of hemolymph, lower is the necessity of adjustment to the cellular volume through osmolytes. Both mechanisms involve active transportation through membranes and, however, adjustment to the respiratory metabolism.

Given that some authors have showed a broad diversity of biological responses among *M. amazonicum* populations and that this knowledge is still fragmented, the present work has the objective of adding data on the physiology of an estuarine population of *M. amazonicum* from Amazon, maintained in fish hatcheries for 10 years in the Aquaculture Centre of UNESP, in Jaboticabal, state of São Paulo. We evaluated the effect of *M. amazonicum* larvae (zoeae I, II, V, IX) and/or post-larvae exposure to different salinities (fresh water, 6, 12 or 18) about the metabolism and/or intracellular isosmotic regulation. The metabolism was assessed by oxygen consumption and intracellular isosmotic regulation through the identification and quantification of body free amino acids.

## 2. Material and Methods

### 2.1. Collection, maintenance of the animals in laboratory and larviculture

*Macrobrachium amazonicum* was obtained from the CAUNESP (UNESP Aquaculture Centre, Jaboticabal) of the State of São Paulo, Brazil. The broodstock originated from an estuarine population near Belém in the Amazon Delta (01°14'30"S and 48°19'52"W). Oviparous females were collected in reproductive fish hatcheries and kept in a tank of larvae eclosion, in water with a salinity of 5. After eclosion, larvae were transferred to a polypropylene tank containing 60 L of fresh water (salinity  $\leq 0.5$ ) or brackish water of 6, 12 or 18 in the density of 100 ind/L. After metamorphosis, post-larvae were transferred to tanks with capacity of 1000 L to a density of 5 post-larvae/L. The water temperature in all the experiments was kept at 30°C, photoperiod of 12h:12h of light/dark. Larvae were fed with *Artemia sp* nauplii beginning on the second day of cultivation due to the existence yolk in zoeae I bodies. From the 6<sup>th</sup> day, humid diet was added to larvae diet twice a day (Maciel and Valenti, 2009; Maciel et al., 2012). Salinity was daily checked using a refractometer (Atago S/Mill-E), as well as siphonage of the tanks.

All the ontogenetic stages here studied were selected for the evaluation of the physiological parameters 24h after the change of stage. Thus, all the stages remained at least 24h in experimental salinities (fresh water, 6, 12 or 18). After this period, larvae and post-larvae were withdrawn from the tanks and metabolism and concentration of free amino acids in the body were determined. It was not possible to evaluate the concentration of free amino acids in *M. amazonicum* zoeae IX because most of the zoeae VIII of our experiments underwent metamorphosis directly to post-larvae; it was possible to obtain enough material for analyses. It was not possible either the determination of oxygen consumption in post-larvae because their elevated oxygen consumption makes the oxygen level

within the chambers get to below 70% of saturation, a value that could alter physiological functions and could not be representative.

### 2.2. Evaluation of the oxygen consumption

Oxygen consumption in zoeae and post-larvae was assessed using a high precision respirometry system for aquatic animals (Strathkelvin Instruments). This system is formed by digital monitor (Mod. 782), respirometric chamber (Mod. MT200), and electrode with micro cathode sensitive to variations in the oxygen dissolved in water, with precision of 0.01  $\mu\text{g/mL}$  (Mod. 1302). The temperature within the respirometric chamber was kept at 30°C with help from a water flow, around the chamber, from thermostatic bath equipment (Tecnal).

The number of larvae transferred to the respirometric chamber in each analysis was defined after a series of preliminary tests using the respirometry system and varied from five (zoeae I) to one (zoeae V, IX) individuals. All the evaluated animals had empty digestive tube. Larvae were acclimated to the respirometric chamber for 30 minutes. After this period, the value of oxygen concentration within the chamber was registered, 60 minutes after a new measure was registered. The difference among values was used in the calculation of individual oxygen consumption. Readings in respirometric chambers with no animals inside were performed, following the same experimental conditions, which were used as control. Variations in oxygen concentration observed in the control chambers were subtracted from the values obtained in respiratory measures of the animals.

After the determination of the oxygen consumption, the animals were killed by freezing, dried at 60°C for 48h (Nova Ética, 400-6ND-200C) and weighted (dry mass). All weight measurement was performed in analytical scale with precision of 1  $\mu\text{g}$  (Mettler). Oxygen consumption is expressed according to the dry mass ( $\mu\text{gO}_2 \text{ mgMS}^{-1} \text{ h}^{-1}$ ).

### 2.3. Quantification and identification of the free amino acids by hplc

For FAA analyses, the dried tissue samples were homogenized in distilled water, protein being precipitated with 80% ethanol (v/v). An internal standard of 6.24 nmol  $\alpha$ -aminobutyric acid was added and the samples were derivatized with triethylamine and phenylisothiocyanate, forming FAA/phenylthiocarbamil derivatives (Bidlingmeyer et al., 1987). The individual FAA were identified and quantified by HPLC (Milton Roy) using a Picotag C18 Column (Waters Corporation) according to Augusto et al. (2007a, b).

### 2.4. Statistical analysis

The effects of exposure to the different salinities on oxygen consumption or FAA concentration were evaluated using 1-way ANOVA followed by the Student-Newman-Keuls multiple means test to locate statistically significant groups. All statistical analyses were performed after determining normality of distribution and equality of variance using Sigma Stat 2.03, employing a minimum

significance level of  $P = 0.05$ . Data are expressed in the text as mean  $\pm$  SE.

### 3. Results

#### 3.1. Survival and duration of larval stages

The exposure of the *M. amazonicum* larval stages to different salinities revealed differences in what concerns to the survival and duration of the larval development. Whereas in brackish water of 6, 12 and 18 occurred ecdysis in larvae and change the stage until metamorphosis to post-larvae, in fresh water (salinity  $\leq 0.5$ ), does not occur ecdysis in the zoea V to zoeae VI. In relation to larval development, we observed an accentuated suppression of zoeae IX stages in all salinities (6, 12 and 18), given that the majority of zoeae VIII underwent metamorphosis directly to post-larvae. The duration of larval development until reaching metamorphosis also varied according to salinity, once on brackish water of 12, larvae suffered metamorphosis in only 17 days, but it took 18 days in brackish water of 6 and 20 days in the salinity of 18.

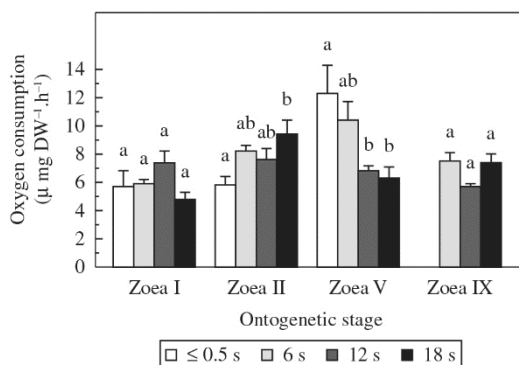
The accentuated suppression of larval IX stage did not enable the evaluation of the concentration of free amino acids in this *M. amazonicum* stage due to the reduced quantity of available material. However, it was possible to successfully measure the metabolism of zoeae IX because it is only necessary one individual of this stage in the respirometric chamber to assess the oxygen consumption.

#### 3.2. Metabolism of the selected ontogenetic stages of *M. amazonicum*

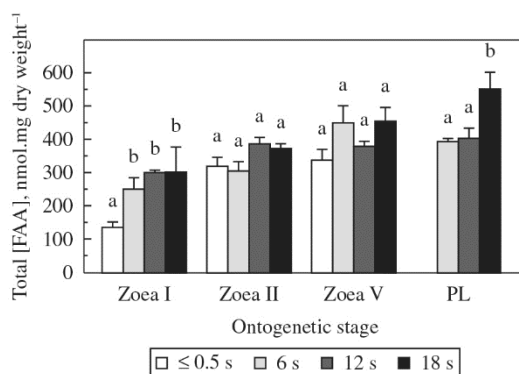
Oxygen consumption of *M. amazonicum* zoeae I, II, V and IX is shown in Figure 1. In zoeae I, oxygen consumption did not alter due to the exposure salinity. In zoeae II, oxygen consumption increased in the salinity of 18 in comparison to the larvae kept in fresh water. In contrast, in zoeae V, oxygen consumption was higher in zoeae kept in fresh water in comparison to the ones kept in brackish water of 12 or 18. Oxygen consumption was also measured in few larvae that went through the zoea IX stage and we observed that there are no differences in the metabolism of larvae kept in brackish water of 6, 12 or 18. In this stage, there are no data in fresh water because from the zoeae VI stage, all larvae die when kept in this salinity.

#### 3.3. Quantification and identification of free amino acids of the selected ontogenetic stages of *M. amazonicum*

The total concentration of free amino acids presented variation only in zoeae I and post-larvae (Figure 2). In zoeae I, the total concentration of free amino acids increased from  $139.0 \pm 15.7$  nmol/mg dry mass in fresh water to about 300 nmol/mg dry mass in brackish water of 6, 12 or 18 S. In zoeae II and V, the concentration of free amino acids remained unaltered after the exposure to different salinities and varied from about 320 nmol/mg dry mass in zoeae II in fresh water until about 460 nmol/mg dry mass in zoeae V exposed to brackish water of 18. In post-larvae, the total concentration of free amino



**Figure 1.** Effect of exposure to freshwater or dilute seawater (6, 12 or 18) on oxygen consumption ( $\mu\text{g} \cdot \text{mg dry weight}^{-1} \cdot \text{h}^{-1}$ ) in zoea I, II, V and IX of the shrimp *Macrobrachium amazonicum*. Means in each stage followed by different letters differ statistically. ( $X \pm \text{SEM}$ ,  $N = 4$ ).



**Figure 2.** Effect of exposure to freshwater or dilute seawater (6, 12 or 18) on total free amino acid concentration (nmol/mg dry weight) in zoea I, II, V and post larvae of the shrimp *Macrobrachium amazonicum*. Means in each stage followed by different letters differ statistically. ( $X \pm \text{SEM}$ ,  $N = 4$ ).

acids increased about 40% after exposure to salinity of 18 ( $553.4 \pm 51.6$  nmol/mg dry mass) in comparison to those exposed to brackish water of 6 or 12 (respectively,  $395.6 \pm 9.5$  and  $405.4 \pm 30.7$  nmol/mg dry mass).

In all evaluated ontogenetic stages, the most concentrated free amino acids were glutamic acid, glycine, alanine, arginine, and proline together they correspond to about 40% out of the total (Table 1). The exposure of zoeae I to brackish water caused increases in glycine concentration (about 40%), alanine (about 250%), and proline (about 500%) in relation to zoeae kept in fresh water. In zoeae II, the main free amino acids did not have their concentration altered. In zoeae V, proline is the main free amino acid that had its concentration altered, increasing about 200% in larvae exposed to brackish water of 6, 12 and 18 in relation to those kept in fresh water. In post-larvae, glycine concentration reduced about 40% and proline increased about 70% in the individuals exposed to brackish water of 12 in relation to other salinities.

**Table 1.** Individual and total free amino acid concentrations (nmoles/mg dry weight) in zoeae I, II, V and post larvae of the shrimp *Macrobrachium amazonicum* in fresh water or exposed to saline media (6, 12 or 18). Means in each amino acid followed by different letters differ statistically from same stage. (X ± SEM, N = 4).

	Salinity	Glutamic Acid	Glycine	Arginine	Alaline	Proline	Others	Total
Zoea I	≤ 0.5	<sup>a</sup> 10.8 ± 3.5	<sup>b</sup> 47.2 ± 4.6	<sup>a</sup> 27.9 ± 3.3	<sup>b</sup> 14.5 ± 1.3	<sup>b</sup> 4.8 ± 0.8	33.9	<sup>a</sup> 139.0 ± 15.7
	6	<sup>a</sup> 11.9 ± 0.9	<sup>a</sup> 63.4 ± 2.5	<sup>a</sup> 32.1 ± 2.5	<sup>a</sup> 42.7 ± 3.7	<sup>a</sup> 25.2 ± 2.1	76.8	<sup>b</sup> 252.1 ± 33.1
	12	<sup>a</sup> 12.3 ± 0.8	<sup>a</sup> 78.8 ± 6.0	<sup>a</sup> 36.2 ± 3.2	<sup>a</sup> 54.7 ± 4.4	<sup>a</sup> 32.6 ± 1.5	88.1	<sup>b</sup> 302.3 ± 10.3
	18	<sup>a</sup> 11.7 ± 1.8	<sup>a</sup> 66.7 ± 3.9	<sup>a</sup> 44.7 ± 14.5	<sup>a</sup> 48.1 ± 8.4	<sup>a</sup> 34.0 ± 5.3	98.2	<sup>b</sup> 303.3 ± 73.7
Zoeae II	≤ 0.5	<sup>a</sup> 15.5 ± 1.2	<sup>a</sup> 80.4 ± 5.5	<sup>a</sup> 46.4 ± 3.0	<sup>a</sup> 56.4 ± 5.0	<sup>a</sup> 56.4 ± 5.0	65.4	<sup>a</sup> 320.5 ± 26.5
	6	<sup>a</sup> 19.4 ± 1.0	<sup>a</sup> 86.7 ± 6.0	<sup>a</sup> 38.9 ± 2.2	<sup>a</sup> 52.7 ± 5.5	<sup>a</sup> 52.7 ± 5.5	76.0	<sup>a</sup> 307.4 ± 28.7
	12	<sup>a</sup> 21.1 ± 5.3	<sup>a</sup> 92.0 ± 20.9	<sup>b</sup> 26.5 ± 1.6	<sup>a</sup> 48.7 ± 12.0	<sup>a</sup> 48.7 ± 11.9	152.3	<sup>a</sup> 389.3 ± 19.2
	18	<sup>a</sup> 16.4 ± 0.3	<sup>a</sup> 81.2 ± 2.5	<sup>a</sup> 41.7 ± 6.0	<sup>a</sup> 62.1 ± 3.0	<sup>a</sup> 62.1 ± 3.0	110.5	<sup>a</sup> 374.1 ± 15.5
Zoea V	≤ 0.5	<sup>ab</sup> 12.7 ± 0.1	<sup>a</sup> 87.8 ± 7.2	<sup>a</sup> 45.2 ± 3.0	<sup>a</sup> 60.1 ± 4.6	<sup>a</sup> 15.4 ± 3.2	118.0	<sup>a</sup> 339.2 ± 33.9
	6	<sup>a</sup> 16.0 ± 1.2	<sup>a</sup> 98.1 ± 6.4	<sup>a</sup> 50.5 ± 10.2	<sup>a</sup> 74.5 ± 9.8	<sup>b</sup> 44.5 ± 7.1	168.5	<sup>a</sup> 452.0 ± 51.6
	12	<sup>ab</sup> 14.2 ± 1.0	<sup>a</sup> 88.3 ± 4.7	<sup>a</sup> 60.7 ± 9.4	<sup>a</sup> 59.2 ± 2.5	<sup>b</sup> 47.7 ± 6.9	111.1	<sup>a</sup> 381.0 ± 15.2
	18	<sup>b</sup> 11.1 ± 0.8	<sup>a</sup> 77.1 ± 6.7	<sup>a</sup> 50.4 ± 5.6	<sup>a</sup> 68.6 ± 5.1	<sup>b</sup> 61.3 ± 4.8	188.8	<sup>a</sup> 457.2 ± 40.7
Post larvae	6	<sup>a</sup> 11.7 ± 2.1	<sup>ab</sup> 91.1 ± 6.6	<sup>a</sup> 62.4 ± 13.8	<sup>a</sup> 67.6 ± 3.1	<sup>a</sup> 38.2 ± 2.5	124.5	<sup>a</sup> 395.6 ± 9.5
	12	<sup>a</sup> 11.5 ± 1.9	<sup>a</sup> 119.0 ± 7.2	<sup>a</sup> 76.5 ± 3.1	<sup>a</sup> 65.3 ± 4.4	<sup>b</sup> 24.2 ± 2.6	107.8	<sup>a</sup> 405.4 ± 30.7
	18	<sup>a</sup> 13.8 ± 3.4	<sup>b</sup> 63.9 ± 18.1	<sup>a</sup> 74.0 ± 3.1	<sup>a</sup> 81.7 ± 12.6	<sup>a</sup> 47.6 ± 4.1	272.6	<sup>b</sup> 553.4 ± 51.6

Values with different superscript letters within the same column are significantly different.

#### 4. Discussion

The results here presented that come from a population from the estuary of the state of Pará added information concerning to the existence of distinct physiological responses among *M. amazonicum* shrimp populations. The majority of the populations of this caridean shrimp cannot complete their life cycle in fresh water, independent on the population coming from the Plata bay or northern region (Vega Perez, 1984; Zanders et al., 1992; Augusto et al., 2007a; Charmantier and Anger, 2011). Interestingly, when maintained in the laboratory, estuarine population here studied survives until the zoeae V stage in fresh water, whereas in some distinct marine populations, zoeae die right after eclosion in fresh water (Augusto et al., 2007a) or do not get to the zoeae III stage if kept in this salinity (McNamara et al., 1983; Gamba, 1984). Apparently, from the zoea V stage, *M. amazonicum* larvae of the population here studied lose the capacity to hyperosmoregulate in fresh water, not being able to deal with the water influx and salts efflux. Charmantier and Anger (2011) verified that all the post-embryonic stages of the population here studied are able to hyperosmoregulate until the salinity of 17, although in their experiments only zoeae I had survived in fresh water. It is possible that different methodological details used in larvae culture might have caused this imbalance among the results here presented and the ones from Charmantier and Anger (2011) regarding zoeae survival in fresh water.

*Macrobrachium amazonicum* have between 9 and 11 larval stages under laboratorial conditions in which salinity and temperature are kept, respectively, around at 10 and 29°C (Guest and Durocher, 1979; Vega Perez, 1984; Anger and Hayd, 2009). We observed in the present work an abbreviation of the larval stage once almost all

zoeae VIII kept on brackish water of 6, 12 or 18 underwent metamorphosis directly to post-larvae. Diverse works have shown that caridean shrimps under unfavorable conditions, for example, during osmotic or nutritional stress, high temperatures, tend to increase the number of stages or prolonged the duration of larval development (Criales and Anger, 1986; Mascetti and Wehrtmann, 1996). Low salinity may cause a reduction of the average rate of feeding or growth efficiency due to metabolic disadjustments caused by osmotic stress. However, it can be inferred that the cultivation media in which larvae developed in the present work as salinity, temperature, and feeding was appropriate to the studied population and it is reflected in the reduction of the number of larval stages.

Data presented here suggest that possible adjustments in the respiratory metabolism and in the use of free amino acids in the regulation of larval cellular volume are dependent on the appearance of structures responsible for osmoregulation and its functioning. It is known that as more efficient the regulation mechanisms of volume and composition of extracellular fluid lower the necessity of osmotic effectors in the regulation of cellular volume. We verified that *M. amazonicum* zoeae I did not alter their respiratory metabolism due to exposure to fresh or brackish water, but reduce the intracellular concentration of free amino acids when exposed to fresh water, what may suggest the inexistence or an inefficient performance of the structures responsible for volume regulation and hemolymph composition. It is even possible that it may be due to a higher permeability of larvae than other ontogenetic stages to salts and water. The reduction in the concentration of free amino acids up against the exposure to a diluted media is related in various crustaceans as

*Palaemon northropi* (Augusto et al., 2009) and *Litopenaeus vannamei* (Shinji et al., 2012) and it is responsible for the maintenance of isosmotic intracellular media with surrounding extracellular media. This adjustment in the free amino acids concentration may occur through the reduction of the synthesis rate or increase in the oxidation of these compounds, increase of amino acid efflux, increase in the synthesis of proteins or reduction of their catabolism (Mantel and Farmer, 1983). Hemolymph amino acids increase on exposure to dilute media and may be stored like hemocyanin, which may increase the amount of oxygen available to cells (Gilles and Péqueux, 1981; Mantel and Farmer, 1983). Shinji et al. (2012) and Shinji and Wilder (2012) verified still that in adult *L. vannamei* amino acids were consumed as an energy source when animals were exposed to low salinity.

In *M. amazonicum* zoeae II and V, the alterations in the metabolism during exposure to fresh or brackish water were not followed by alterations in free amino acids concentration. Thus it is possible that as the structures responsible for osmoregulation and ionic regulation become effectively functional in larvae, the role of free amino acids gets diminished and oxygen consumption more elevated, probably because of the highest energy expenditure with the active transportation of salts through epithelial membranes. It is possible that gills or other structure present in initial phases of the development of crustaceans as branchiostegites, pleurae or dorsal organ are performing osmoregulatory functions, what would reduce the role of free amino acids in the intracellular volume regulation. The participation of these structures in the osmotic regulation is cited during the development of diverse crustaceans as *Callinassa jamaicensis* (Felder et al., 1986) and *Carcinus maenas* (Cieluch et al., 2004).

The osmotic challenges seem to alter throughout the development given that in zoeae II oxygen consumption is elevated in brackish water of 18, but in zoeae V this increase happens in fresh water. In addition, from zoeae VI stage, larvae become incapable of surviving in fresh water and zoeae IX keep unaltered metabolism on brackish water of 6, 12 or 18. In a *M. amazonicum* population from Sertãozinho, Augusto et al. (2007a) verified that only *M. amazonicum* zoeae I alter their concentration of free amino acids after exposure to brackish water; the authors attribute it to the development of structure responsible for the regulation of extracellular fluid in zoeae II, which would make the osmolality regulation of hemolymph more efficient, reducing the role of the mechanisms of intracellular isosmotic regulation in this and in the next phases of development. In *M. olfersii* diadromous shrimp, free amino acids concentration did not alter either in zoeae II, although zoeae I present an increase after exposure to brackish water (Augusto et al., 2007a).

After metamorphosis of *M. amazonicum*, free amino acids begin to play an important role as intracellular osmolytes because we verified an increase of up to 40% in post-larvae of the population here studied exposed to

brackish water of 18. These data are similar to the ones presented by the *M. olfersii* shrimp post-larvae that present an increase of 28% in the concentration of free amino acids after the exposure to brackish water (Augusto et al., 2007a). Diadromous shrimp post-larvae of the *Macrobrachium* gender constitute the phase of the life cycle that marks the return to fresh water after larval development in brackish water. Given that *M. amazonicum* possess from estuarian to freshwater populations distant from the sea, the pattern of dependence on brackish water and migration of the specie also seem to be dependent on their geographical localization.

The main free amino acids involved in the regulation of cellular volume of the ontogenetic stages of the *M. amazonicum* population here studied were the non essential glutamic acid, glycine, alanine, arginine, and proline. These amino acids together constitute about 60% of the total of free amino acids, independent on the ontogenetic stage evaluated. The results obtained here are in agreement with the literature that shows that the main amino acids which take part in the osmoregulatory process of crustaceans are the non-essential ones, that means, those obtained through a biosynthetic via and not through feeding (McNamara et al., 2004; Augusto et al., 2007a, b; Augusto et al., 2009; Faria et al., 2011; Prymaczk et al., 2012; Shinji et al., 2012).

The results presented here broaden the range of physiological responses described for *M. amazonicum* populations, because larvae can survive until zoeae V stage in fresh water and only some stages possess dependence on free amino acids in the cellular volume regulation and adjust their metabolism according to exposure to brackish water. This pattern is different from the one found by some *M. amazonicum* populations where larvae die in fresh water right after eclosion or in other initial stages of the life cycle. Additionally, data add useful information to aquaculture of *M. amazonicum*, considered promising specie for commercial cultivation (New et al., 2010; Bentes et al., 2011). The fact that zoeae II canalize greater quantity of energy for osmoregulatory processes when kept in brackish water of 18 may negatively influence their growth and development. Taking into account only metabolic parameters, it would be interesting that the larvae cultivated for commercial purposes were kept in fresh water or brackish water of up to 12 until zoeae II stage.

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