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The Effect of Dietary Astaxanthin on Physiological Responses of Juvenile White Shrimp *Litopenaeus vannamei* Acclimated to Low-Salinity Water

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Abstract: The effect of dietary astaxanthin supplemented in 0, 40, 80 and 150 mg kg^{-1} (Diet A, B, C and D, respectively) on oxygen consumption, ammonium excretion, O:N atomic ratio and Osmoregulatory Capacity (OC), were evaluated in *Litopenaeus vannamei* acclimated to low-salinity water (3%). Oxygen consumption rate of 1.04 mg $O_2 h^{-1} g^{-1}$ d.w., ammonium excretion rate of 0.04 mg $NH_4^+ h^{-1} g^{-1} d kg^{-1}$, O:N atomic ratio of 26.8 and OC of 240 mOsm kg^{-1} were significantly different in shrimp fed diet C compared with shrimp fed the other diets. These results indicated that dietary supplementation of diet C improved physiological responses in juveniles *L. vannamei*. We recommend the addition of dietary astaxanthin of 80 mg kg^{-1} in the pacific white shrimp cultured in low-salinity water.

Key words: Astaxanthin, oxygen consumption, ammonium excretion, O:N atomic ratio, osmoregulatory capacity, *Litopenaeus vannamei*, low-salinity water

INTRODUCTION

It is well known that crustaceans are unable to biosynthesize carotenoid pigments *de novo* and are complete dependent upon an exogenous source in the diet (Meyers and Latscha, 1990). The antioxidant activities of astaxanthin are 10 times stronger than those of other carotenoids (Miki, 1991) and in cultured crustaceans astaxanthin is more efficiently assimilated than other carotenoid at low energetic expense (Petit *et al*., 1998). Resistance to physical stress has been improved in marine shrimp through dietary astaxanthin's antioxidant properties (Yamada *et al*., 1990; Chien and Jeng, 1992; Dall, 1995; Petit *et al*., 1997; Merchie *et al*., 1998; Darachai *et al*., 1999; Chien *et al*., 2003). Flores *et al*. (2007) reported that metabolic and haematological responses were enhanced in *L. vannamei* fed astaxanthin when acclimated to low-salinity water.

Shrimp farming in inland low-salinity water has become a rapid growing industry recently by the opportunity of farming abundant water inland areas, the risk of disease outbreaks in marine shrimp culture farms and the limited coastal areas in North America (Laramore *et al*., 2001; Lin and Chen, 2003; Gong *et al*., 2004; Wang and Chen, 2005).

Litopenaus vannamei has wide range tolerance from 1-50%, has made the pacific white shrimp an atractive specie for commercial production in low-salinity inland waters in several American countries and is recently the main penaeid specie cultivated in Asia. Actually around 30% of shrimp commercial farming in Thailand is grown in low-salinity water (Menz and Blake, 1980; Bray *et al*., 1994; McGraw *et al*., 2002; Saoud *et al*., 2003; Gong *et al*., 2004).

Among the physiological parameters that can be correlated with environmental changes, metabolic rates are very appropriate because they are strictly related to energy flow in crustaceans to accomplish homeostatic control mechanisms (Rosas *et al*., 2001; Salvato *et al*., 2001). Oxygen consumption has been reported as an useful and valid method to evaluate physiological response in shrimp to environmental variations (Hewitt and Irving, 1990; Taboada *et al*., 1998; Lemos *et al*., 2001; Rosas *et al*., 2001; Re *et al*., 2004; Comoglio *et al*., 2004; Spanopoulos-Hernández *et al*., 2005; Li *et al*., 2007).

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Ammonia excretion has been used as an index to evaluate several environmental factors on shrimp physiology (Chen *et al*, 1993; Chen and Lin, 1995; Jiang *et al*., 2000; Lemos *et al*., 2001; Díaz *et al*., 2001). In crustaceans, nitrogen was mainly excreted as ammonia (60-70%) (Regnault, 1987). Effects of salinity on nitrogen metabolism of shrimp have been investigated by measurements of ammonia excretion as this response seems to be essential in maintaining body fluids concentration in relation to medium salinity variations (Chen and Lin, 1995; Lemos *et al*., 2001; Re *et al*., 2004).

The simultaneous determination of oxygen consumption and ammonia-N excretion rates by marine organisms allows the calculation of O:N atomic ratio, an indicator of the catabolized substrate. (Mayzaud and Conover, 1988). When an organism is oxidizing preferably protein the O: N atomic ratio will be low, less than 7; it will be high when either fat or carbohydrate is oxidized (Conover and Corner, 1968; Barber and Blake 1985; Snow and Williams, 1971).

The Osmoregulatory Capacity (OC) in crustaceans has been used as a convenient sublethal tool to monitor physiological condition and could be used to measure the effect of environmental variations in shrimp and define optimal culture conditions (Lignot *et al*., 1999; Lignot *et al*., 2000; Díaz *et al*., 2004).

Studies focused to improve physiological responses of white shrimp cultured in low-salinity water through diet supplementation are limited. Research has been oriented mainly to post larvae to determine time and rate of acclimation and salinity reduction (Laramore *et al*. 2001; McGraw *et al*., 2002; McGraw and Scarpa, 2004; Saoud *et al*., 2003). The goal of the present study was to determine the effect of three dietary astaxanthin concentrations on oxygen consumption, ammonium excretion, O:N atomic ratio and osmoregulatory capacity in the pacific white shrimp *Litopenaeus vannamei* acclimated to low-salinity water.

MATERIALS AND METHODS

Approximately 3,000 postlarvae (PL_{10}) Pacific white shrimp *L. vannamei* were obtained from Aquapacific S.A. de C.V., a Mexican hatchery located in the state of Sinaloa, México. This study was realized in 2006. The organisms were distributed in 2000 L tanks with constant aeration and continuous sea water flow 35%, maintained at 28±1°C. The PL was fed 40% protein commercial diet (Rangen®), twice daily at 10% wet weight (w.w.) biomass. For salinity reduction 500 shrimps weighting 5.0-6.0 g were randomly selected and placed in two 500 L tanks. Salinity reduction rate was held in 2 phases of 5 days each. In the first phase, salinity was reduced from 35-5% at a constant daily reduction rate of 6%, followed by a constant salinity reduction from 5-3% at a daily reduction rate of 0.4%. The shrimp were then maintained in 3% as an acclimation period for 5 days (McGraw and Scarpa, 2004). During salinity reduction, acclimation and the experimental period salinity was checked twice daily with a temperature/salinity meter (YSI Model Y30, Yellow Spring Instrument).

Three concentrations (40, 80 and 150 mg kg^{-1}) of astaxanthin (Carophyll pink 8%, Nutrabiot Roche) were then added to produce pellets that were subsequently air dried, broken up and stored in darkness at 4°C. Diets were identified as control without astaxanthin (A) and with astaxanthin added in 40, 80 and 150 mg kg^{-1}, (diets B, C and D), respectively. All diets were processed as described by Flores *et al*. (2007).

A 6-week trial was conducted with 200 organisms randomly selected and distributed in 8 of 500 L tanks in groups of 25 placed each shrimp individually in 4 L beakers and fed 1 of the 4 diets (A, B, C, D). Duplicates tanks were used for each of four dietary treatments. During the experimental period, water temperature was 28±1°C, salinity was 3%, dissolved oxygen 6.0±0.5 mg L^{-1}, pH value 8.0±0.3 and hardness 360-380 mg $CaCO_3$ L^{-1}. Shrimps were fed daily at 3% w.w. biomass. Uneaten feed was collected daily with a siphon 3 h after feeding. Feces were removed 4 h after feeding and before the next feed.

Oxygen consumption and ammonium excretion were measured simultaneously in a semi-open respiratory system described by Diaz *et al*. (2007), consisting of 21 chambers of 1000 mL each. Twenty shrimp in intermolt stage were individually introduced into the respiratory chambers 24 h before initiating measurements. The molt cycle stage was identified according to the procedure described by Chan *et al*. (1988). Water flow in the chambers remained open for 2 h; before closing, two water samples were taken to measure the initial concentration of dissolved oxygen with a YSI 52 oxymeter (Yellow Springs Instruments Co.), equipped with a polarographic sensor and the initial concentration of ammonium by the phenolhypochlorite method (Rodier, 1998). The chambers remained closed for 1 ½ h, according to Stern *et al*. (1984) to avoid more than 25-30% dissolved oxygen reduction and consequently respiratory stress to the organisms. Before re-establishing the flow, 2 water samples were taken from each chamber to measure the final concentration of dissolved oxygen and ammonium excreted.

The 21st chamber was used as control to measure oxygen consumption and ammonium production by microorganisms in the water and the necessary corrections were made. Oxygen consumption and ammonium excretion were determined by the difference between the initial and final concentrations of each chamber and were expressed in mg O_2 h^{-1} g^{-1} dry weight (d.w.) and mg NH_4^+ h^{-1} g^{-1} d.w., respectively. To determine dry weight, shrimp were lyophilized for 24 h. and weighted on an Ohaus Explorer balance.

The O:N atomic ratio were calculated by the following formula:

$$O: N \text{ atomic ratio} = \text{Atomic weight } (NH_4^+)/ \text{Atomic weight } (O_2) * [QO_2] / [NH_4^+].$$

Before and after water salinity reduction, 15 shrimps were extracted each time at random to measured shrimp osmolality. A single haemolymph sample of 10 μL was taken from each shrimp with an automatic pipette directly from the thoracic-abdominal membrane (previously dried with absorbent paper). At the end of the experiment, a sample of haemolymph was also taken from each shrimp from the four treatments. Osmolality of haemolymph and medium were measured with a Wescor 5520 vapor osmometer. Osmoregulatory Capacity (OC) was calculated as the difference between osmolalities of haemolymph and the external medium, data was expressed in mOsmol kg^{-1} (Díaz *et al*., 2001).

Oxygen consumption, ammonium excretion and osmoregulatory capacity data were compared using the non-parametric Kruskal-Wallis test. Comparison of the control group with each experimental group was carried out using the Dunnett's test and comparison between groups was done using the Dunn or Tukey tests (Zar, 1999). Data were processed using Sigma Stat version 3.1 and Sigma Plot version 10. Oxygen consumption and ammonium excretion were plotted using parallel box plots (Tukey, 1977).

RESULTS

The lowest oxygen consumption rate of 1.04 mg O_2 h^{-1} g^{-1} d.w. and ammonium excretion rate of 0.04 mg NH_4^+ h^{-1} g^{-1} d.w. were found in *L. vannamei* fed with 80 mg astaxanthin kg^{-1} ($p<0.05$) (Fig. 1 and 2).

Shrimp fed diet C showed the highest O: N atomic ratio of 26.8, compared with lower O:N atomic ratios of shrimp fed diets A, B and D (Fig. 3).

Before salinity reduction, shrimp in sea water had an OC of -187 mOsm kg^{-1} (hypo-osmotic), a change in the OC regulation pattern (hyper-osmotic) was observed in

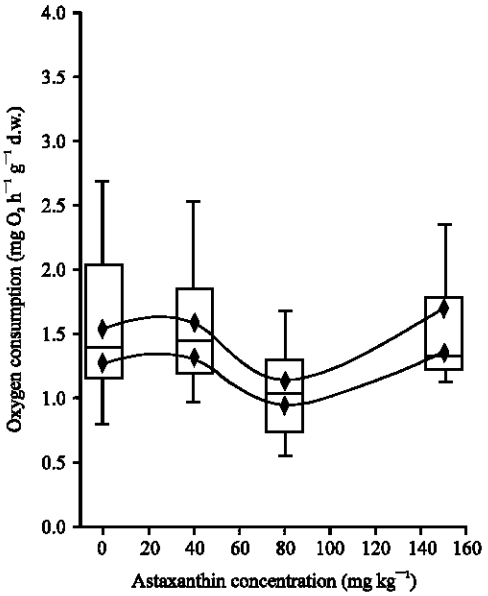

Fig. 1: Oxygen consumption of juveniles *L. vannamei* fed with diet supplemented astaxanthin 0, 40, 80 and 150 mg kg^{-1} (Diet A, B, C and D, respectively). The area bordered by diamonds represents 95% median confidence interval. The bars include 50% of the organism's distribution and the vertical lines represent the quartiles

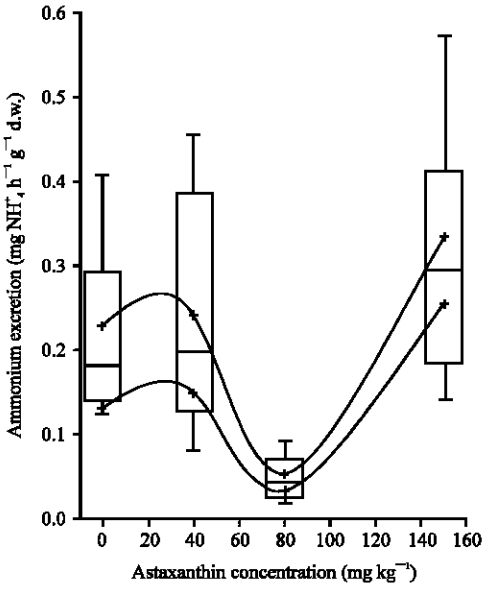

Fig. 2: Ammonium excretion of juveniles *L. vannamei* fed with diet supplemented astaxanthin 0, 40, 80 and 150 mg kg^{-1} (Diet A, B, C and D, respectively). The shaded area bordered by cross represents 95% median confidence interval. The bars include 50% of the organism's distribution and the vertical lines represent the quartiles

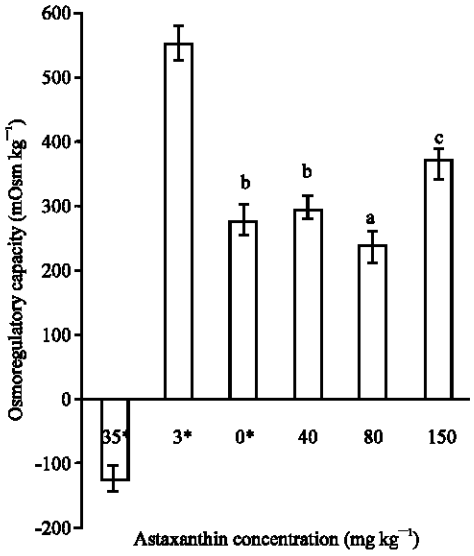

Fig. 3: O:N atomic ratio of juveniles *L. vannamei* fed with diet supplemented astaxanthin 0, 40, 80 and 150 mg kg^{-1} (Diet A, B, C and D, respectively)

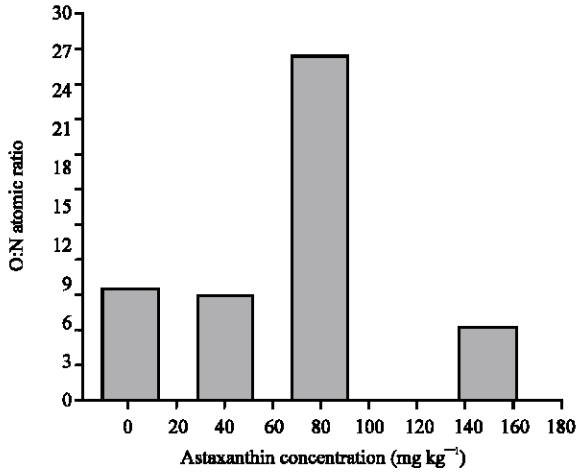

Fig. 4: Osmoregulatory capacity of juveniles *L. vannamei* fed with supplemented astaxanthin 0, 40, 80 and 150 mg kg^{-1} (Diet A, B, C and D, respectively). The first two bars indicate environmental salinity of shrimp fed basic meal without astaxanthin

the organisms after salinity reduction (528 mOsm kg^{-1}). At the end of the experiment, the group of animals fed with diet C had significantly the lowest ($p<0.05$) OC 240 mOsm kg^{-1} (Fig. 4).

DISCUSSION

Oxygen consumption has been used to asses energy utilization in several shrimp species (Hewitt and Irving, 1990) and it can be associated with energy that is used in several adjustment mechanisms that shrimp and other crustaceans have to tolerate salinity changes (Rosas *et al*., 2001). Salinity directly affects oxygen consumption causing an osmoregulatory demand on aquatic organisms (Spanopoulos-Hernández *et al*., 2005). An increased in oxygen consumption was reported in *L. vannamei* exposed to salinity changes by Rosas *et al*. (2001) because shrimp expended more energy to maintain homeostasis. Oxygen consumption increased in *P. chinensis* juvenile under low salinity stress suggesting a decrease in growth (Chen and Lin, 1995). In this study oxygen consumption of shrimp fed diet C was significantly the lowest and within the range value from 1.0-1.31 mg $O_2 h^{-1} g^{-1}$ d.w. reported in *L. vannamei* and *L. setiferus* reared in sea water (30 - 34%) (Taboada *et al*., 1998; Rosas *et al*., 2001; Comoglio *et al*., 2004). In the same way, oxygen consumption in shrimp fed 80 mg astaxanthin kg^{-1} was similar to the values reported in *L. vannamei* and *L. stylirostris* by Rosas *et al*. (2001) and Spanopoulos-Hernández *et al*. (2005) in shrimp found in the state of Sinaloa and other areas on the Pacific coast of México and Central America where the species is cultured in sea water. This indicated that astaxanthin properties supplemented 80 mg kg^{-1} favored the physiological adaptation of *L. vannamei* to the osmotic low-salinity water stress condition. The decreased respiratory metabolism allowed shrimp to channel more energy for growth.

The results obtained in the present study with respect to ammonium excretion of shrimp fed diet C, were similar to the reported for *L. stylirostis* (Taboada *et al*., 1998; Díaz *et al*., 2004; Re *et al*., 2004) and *L. vannamei* (Comoglio *et al*., 2004) in shrimp maintained in sea water (32 - 35%). In crustaceans, when environmental salinity decrease (external concentration) to a level that it can produce an unbalance on the osmoregulatory ability, internal concentration of shrimp is adjusted through catabolism of free amino acids, increasing ammonia production (Chen *et al*., 1993; Lemos *et al*., 2001). For penaeid, an increase in ammonium excretion frequently has been observed as salinity decreases, as shrimp exposed to diluted media are prone to exchange NH_4^+ for the regulation of Na^+ in the haemolymph in the osmoregulatory processes (Spaargaren *et al*., 1977; Regnault, 1987; Jiang *et al*., 2000). The ammonium excretion rate in *L. vannamei* and *L. stylirostris* exposed to different salinities was related to the pattern of osmoregulation (Díaz *et al*., 2001; Re *et al*., 2004). In a similar way ammonium excretion was correlated with osmoregulation in shrimp fed diet C, since in this experimental group both variables were significantly the

lowest. This indicated that supplemented 80 mg astaxanthin kg^{-1} improved the physiological adjustment process minimizing the osmotic stress of shrimp in low-salinity water.

Protein is the most important energetic reserve substrate in shrimp and metabolic energy is obtained mainly from lipids and carbohydrates when shrimp are in suitable health and environmental conditions (Mayzaud and Conover, 1988; Rosas *et al*., 1995). For *L. vannamei* juveniles, a change in the O:N atomic ratio was observed, which indicated a change in the energy substrate used in response to supplemented astaxanthin in the diet. The O:N atomic ratio estimated for the organisms fed with diet C was 3.3 and 4.5 times higher than the ratio obtained when the organisms were fed with diets A, B and D. This indicated that shrimp fed with diet C used preferentially a mixture of lipids and carbohydrates as energy substrate, whereas the organisms fed with diet A, B and D used protein as the main energy substrate. Postlarvae of *F. paulensis* under a range of salinities from 25-34% showed significantly higher O:N atomic ratio compared with PL exposed to lower salinities (Lemos *et al*., 2001). Rosas *et al*. (1995) and Jiang *et al*. (2000) reported that *L. vannamei* exposed to low salinity, had mainly a protein catabolism. In this study the O:N atomic ratio was the highest in juveniles *L. vannamei* fed with diet C, which indicated that 80 mg astaxanthin kg^{-1} in the diet minimized increases in energy consumption in shrimp under osmotic stress and that juveniles had more dietary protein available for growth.

The osmoregulatory capacity of shrimps before salinity reduction was -187 mOsm kg^{-1}, with a hypo-osmotic regulation pattern as the OC reported in *L. stylirostris* (Stimpson)-147 mOsm kg^{-1} reared in salinity of 36% by Lignot *et al*. (1999) and Re *et al*. (2004). The osmoregulatory pattern of shrimp changed to hyper-osmotic when environmental salinity was reduced to 3%. At the end of the experiment we observed that by including 80 mg astaxanthin kg^{-1} in the diet, shrimp exhibited the lowest OC. The OC of shrimps in this study was lower than that reported by Gong *et al*. (2004) in sub adult *L. vannamei* fed a modified diet in ionic composition and lipid content (without astaxanthin) in low salinity (5%) culture in Arizona inland shrimp farming. The difference between studies could be due to life stage of shrimp, because we worked with small juvenile and Gong *et al*. (2004) with sub adult. Gong *et al*. (2004) mentioned that the osmoregulatory ability in shrimp declines naturally when they reach sub adult or adult stages. Chien *et al*. (2003) confirmed that resistance to osmotic stress (salinity reduction) could be improved in *Penaeus monodon* through dietary astaxanthin with similar doses as those used in diet C in this study. The hyper-osmoregulatory ability of juvenile *L. vannamei* was enhanced by effect of astaxanthin in the diet.

Results of this study are in agreement with Flores *et al*. (2007), who demonstrated that the metabolic and haematological responses of juveniles *L. vannamei* improved when the organisms were exposed to low-salinity and fed astaxanthin. On basis in these results, we recommend the addition of dietary astaxanthin of 80 mg kg^{-1} in the pacific white shrimp cultured in low-salinity water.

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