Research Article

Feeding n-3 HUFA enriched Artemia to the larvae of the pink shrimp Farfantepenaeus paulensis increases stress tolerance and subsequent growth

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ABSTRACT. There has been a considerable amount of work on the effects of Artemia enrichment on shrimp larvae. However, the effects on the subsequent growth performance have not been thoroughly investigated. From mysis I to eight day-old postlarvae (PL8), shrimp (Farfantepenaeus paulensis) were fed either newly hatched Artemia nauplii or Artemia enriched with an emulsion containing high levels of n-3 highly unsaturated fatty acids (n-3 HUFA). Upon reaching PL8, shrimp were exposed to salinity and ammonia stress tests. Growth performance was assessed by further rearing shrimp for 40 days. Shrimp fed the enriched Artemia (named EG) had higher tolerance to both salinity and ammonia than those fed newly hatched Artemia (named CG). Weight of PL from EG was also significantly higher than in CG. Higher levels of n-3 HUFA, especially 20:5 n-3 and 22:6 n-3, were found in the enriched Artemia. In the postlarvae, higher levels of n-6 and n-3 HUFA were observed in EG, while PL from CG presented higher levels of 18:3 n-3 and 18:4 n-3. At the end of the nursery rearing, no differences in survival were observed, but weight was higher in EG (1.08 g) than in CG (0.90 g). The supplementation of n-3 HUFA during larviculture increases the tolerance of F. paulensis postlarvae to salinity and ammonia, and positively affects growth performance after a 40 days long rearing period.

Keywords: Farfantepenaeus paulensis, penaeids, Artemia, nutrition, HUFA, enrichment, tolerance to ammonia and salinity.

INTRODUCTION

It is well known that penaeid shrimp have a limited ability to perform de novo synthesis of highly unsaturated fatty acids (HUFA). This was first shown in Marsupenaeus japonicus (Kanazawa et al., 1979a), Panaeus monodon and Feneropenaeus merguiensis (Kanazawa et al., 1979b). HUFA, particularly eicosapentaenoic acid (20:5 n-3; EPA) and docosahexaenoic acid (22:6 n-3; DHA), are vital in the maintenance of normal cell function; EPA and DHA are essential compounds required for cell membrane formation, osmoregulation, the synthesis of prostaglandin and they also appear to have an activating role in the immune system (Lèger & Sorgeloos, 1992). Therefore, diets for penaeids must contain sufficient supplies of these essential nutrients.

Production of penaeid shrimp postlarvae relies heavily on the brine shrimp Artemia as the main live food organism used in commercial hatcheries (Sorgeloos et al., 1998; Wouters et al., 2009; Cobo et al., 2015). Although from a hatchery management standpoint the use of Artemia presents several advantages, the most important being easiness of storage and use (Lèger & Sorgeloos, 1992), levels of n-3 HUFA in newly hatched Artemia nauplii are either low or greatly variable depending on origin (Lavens & Sorgeloos, 1996; Figueiredo et al., 2009). Since Artemia is a non-selective filter feeding organism, enhancement of n-3 HUFA levels is achieved through enrichment techniques, by which n-3 HUFA rich compounds are provided to Artemia and consequently increase their nutritional value (Sorgeloos et al., 1998; Wouters et al., 2009).

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Previous studies have shown that feeding n-3 HUFA rich diets results in improved survival, growth and/or stress tolerance of several crustacean species (Tackaert et al., 1989; Rees et al., 1994; Cavalli et al., 2000; Chim et al., 2001; Martins et al., 2006; Immanuel et al., 2007; Sui et al., 2007; Jinbo et al., 2013). Nonetheless, few studies have considered the effects of the nutritional quality of larviculture diets on further shrimp performance. In this study, we assessed whether the subsequent growth of *F. paulensis* postlarvae during nursery rearing would be affected by previously feeding n-3 HUFA enriched *Artemia* during the larviculture period.

**MATERIALS AND METHODS**

*Farfantepenaeus paulensis* larvae were obtained from a routine larviculture at the Marine Aquaculture Center, Universidade Federal do Rio Grande, Brazil. Upon reaching mysis I, larvae were individually counted and transferred to triplicate 200 L larviculture tanks. Aeration was provided by four air stones per tank to maintain dissolved oxygen levels above 5.0 mg L\(^{-1}\). Seawater was previously filtered by a 5 μm cartridge filter, and water renewal rate was 80% per day. Temperature was maintained at 26-27°C with submerged thermostatic heaters. Photoperiod regime was natural and the diurnal cycle lasted from sunrise at 06:20 h and sunset at 19:00 h over the course of the experiment. Shrimp were reared at a density of 55 larvae L\(^{-1}\). After their transference to the tanks, larvae were fed solely *Artemia* according to the experimental design. For the control group (CG), larvae were fed newly hatched *Artemia* nauplii, whilst in the enriched group (EG) larvae were fed *Artemia* enriched with a commercial emulsion containing over 400 mg g\(^{-1}\) n-3 HUFA on a dry weight basis (Super SELCO®, INVE Aquaculture, USA).

Enrichment procedures were performed according to Sorgeloos et al. (1998) in 50 L cylindro-conical tanks. Density of *Artemia* during enrichment varied between 100-300 ind L\(^{-1}\), temperature was kept between 25-28°C and strong aeration was provided to guarantee homogeneous distribution of the emulsion and dissolved oxygen levels above 5 mg L\(^{-1}\). *Artemia* were enriched for 24 h with two doses of 300 mg of emulsion per liter added at 12 h intervals. After enrichment, *Artemia* were rinsed with water over a 120 μm sieve to remove any remaining emulsion.

Larvae were fed the *Artemia* diets twice a day (10:00 and 22:00 h). *Artemia* used for the night feeding were stored at 4°C. *Artemia* were offered at an initial density of 2 mL\(^{-1}\), which increased gradually to 8 mL\(^{-1}\) at the end of the trial. Before daily water renewal, the number of remaining *Artemia* was estimated in each tank and increased as needed. These procedures were performed until PL8 (eight day-old postlarvae). At this point groups of PL8 from both groups were exposed to salinity and ammonia stress tests.

The salinity and ammonia stress tests were based on the static toxicity tests proposed by Greenberg et al. (1992). The tolerance to salinity and total ammonia were assessed separately in triplicate sets of plastic beakers containing 1 liter of water that were immersed in a water bath at 27-28°C. For the salinity stress test, thirty PL8 from each group were exposed for 24 h to a control (salinity 30 g L\(^{-1}\)) and to decreasing salinity levels (24, 18, 12, 6 and 0 g L\(^{-1}\)), while for the ammonia test batches of 30 PL8 from each group were transferred to the beakers and exposed during 24 h to increasing concentrations of total ammonia: 0 (control, no added ammonia), 15, 30, 45, 60 and 90 mg L\(^{-1}\). Ammonia concentrations, which were based on previous studies with *F. paulensis* (Ostrensky & Wasielewsky, 1995; Martins et al., 2006), were added to the beakers immediately after stocking the postlarvae. During both stress tests, postlarvae were not fed and no water was exchanged. After 24 h postlarvae not responding to mechanical stimuli were considered dead and counted. Based on the mortality rates, the mean lethal salinity for 50% of the population (LS\(_{50}\)) and the mean lethal ammonia concentration for 50% of the population (LC\(_{50}\)) were estimated with the Trimmed Spearman Karber Method (Hamilton et al., 1977).

PL8 remaining in the larviculture tanks were divided into groups of 300 shrimp and further reared for 40 days. The enrichment group (EG) had three replicated groups, while the control had four replicates. Shrimp were initially reared indoors for 10 days and were gradually acclimated to the salinity 15 using the methodology proposed by Tsuzuki et al. (2000). Upon reaching PL18, they were transferred to PVC-coated polyester cages (bottom area of 1 m\(^2\), mesh size of 1.5 mm) placed outdoors in an estuarine inlet of Patos Lagoon (32°03′55″S, 52°12′30″W), southern Brazil. During both the indoor and outdoor rearing periods and irrespective of experimental treatment, management and feeding procedures were similar among all shrimp groups. Shrimp were fed *ad libitum* once a day in the late afternoon with a commercial nursery diet (CR2 Camaronina, Purina®, Brazil). At least 30 shrimp from each cage were randomly sampled for individual weight measurements on days 25 and 40. At the end of the trial shrimp remaining in each cage were hand counted to determine survival.

Temperature (mercury thermometer, precision ±0.5°C), salinity (Atago® optical refractometer, Japan, ±1.0 g L\(^{-1}\)), pH (Digital pHmeter HandyLab 2BNC
Schott, Germany) and dissolved oxygen (Digital oximeter HandyLab OXI/SET, Schott, Germany) were recorded daily. Every morning, samples were collected to estimate the concentrations of ammonia (UNESCO, 1983) and nitrite (Bendschneider & Robinson, 1952).

Samples of newly hatched Artemia nauplii, enriched Artemia and postlarvae were washed with distilled water and frozen (-20°C) until analysis. After defrosting, samples were again washed and prepared for analysis. Crude lipids were extracted with a mixture of chloroform/methanol (2:1, v/v) as described by Folch et al. (1957). Fatty acids of the Artemia and shrimp were prepared from crude lipid by saponification with KOH (50%). Fatty acid methyl esters (FAME) were prepared by esterification with boron-trifluoride in methanol (7%) according to Metcalfe & Schmitz (1961). FAME were separated by gas-liquid chromatography on Shimadzu GC-15A (Shimadzu Corporation, Japan) equipped with a flame ionization detector (FID) and fitted with a fused silica capillary column Omegawax 320×30×0.32 mm (Supelco Inc., USA). Hydrogen was used as a carrier gas with a flow rate of 4.0 mL min⁻¹. Injector and detector temperatures were programmed to 240 and 250°C, respectively. Column temperature was programmed to be isothermal (205°C). FAME were identified by means of reference standard n-3 PUFA, menhaden fish oil (4-7085-U; Supelco Inc., USA) and quantified with a Shimadzu C-R4A integrator model (Shimadzu Corporation, Japan).

Significant differences (P < 0.05) of survival and final weight at the end of the larviculture and nursery rearing periods were determined using the t-test. Prior to the analysis of survival, data were arcsine-square root transformed. All tests were conducted after the confirmation of homogeneity of variances (Lévêne’s test) and normality distribution of data (Kolmogorov-Smirnov’s test). Differences on the ammonia (LC₅₀) and salinity (LS₅₀) stress tests were graphically determined through the comparison of 95% confidence intervals (Greenberg et al., 1992).

RESULTS

Mean values of temperature, salinity, dissolved oxygen, pH ammonia and nitrite during the larviculture period were 27.0°C, 32 g L⁻¹, 7.7 mg L⁻¹, 8.0 mg L⁻¹, 0.24 mg TAN L⁻¹ and 0.01 mg N-NO₂ L⁻¹, respectively. No significant differences in any of these parameters were detected between EG and CG. Final weight of PL8 was significantly higher in EG (13 ± 7 mg) than in CG (10 ± 4 mg). The LS₅₀ values and their 95% confidence intervals for EG and CG were 18.93 g L⁻¹ (18.25-19.57) and 20.53 g L⁻¹ (19.74-21.26), respectively. Graphical comparison indicates significant differences between groups (Fig. 1). Similarly, mean lethal concentrations of total ammonia (LC₅₀) were also significantly different (Fig. 1).

Water temperature and salinity during nursery rearing in the cages were 28.5°C (range 24-32°C) and 14.7 g L⁻¹ (range 13-17), respectively. After the end of the nursery rearing, mean (±SD) survival of shrimp were 82.5% (±14.8) and 71.5% (±21.5) for EG and CG, respectively, and no significant differences were detected. Differences in weight were highly significant (P = 0.00002) after 15 days of cage rearing (Fig. 2). EG shrimp weighed 0.176 g (±0.03), while those from CG weighed 0.109 g (±0.02). After 40 days, significant differences (P = 0.0001) in shrimp weight persisted, with mean final weights of 1.082 g (±0.118) for EG and 0.896 g (±0.128) for CG, respectively (Fig. 2).

The fatty acid profiles of newly hatched Artemia nauplii and enriched Artemia presented significant differences (Table 1). Although no significant differences were observed for saturated, monounsaturated and n-6 PUFA, the levels of EPA and DHA, and consequently total concentration of n-3 HUFA, were significantly higher (P = 0.000012) in the enriched Artemia than in newly hatched nauplii. As a result the n-3/n-6 relation was significantly higher (P = 0.000001) in the enriched Artemia. In contrast, newly hatched nauplii had higher levels of linolenic (18:3 n-3) and stearidonic (18:4 n-3) acids.

Overall, fatty acid profile of F. paulensis postlarvae (Table 2) reflected the composition of their prey. Postlarvae fed enriched Artemia (EG) presented higher levels of n-6 PUFA and n-3 HUFA than postlarvae fed newly hatched nauplii (CG). Concentrations of arachidonic acid (ARA; 20:4n-6), EPA and DHA were higher in EG, while postlarvae fed newly hatched Artemia had significantly higher levels of linolenic and stearidonic acids.

DISCUSSION

As expected, the fatty acid composition of Artemia was influenced by enrichment, which, in turn, affected the composition of the larvae that fed upon them. Accordingly, higher n-3 HUFA levels, particularly EPA and DHA, were found in the tissues of postlarvae fed enriched Artemia in comparison to those fed newly hatched Artemia. Postlarvae fed enriched Artemia also presented higher weight at the end of the larviculture period. Although this corresponds well with previous findings demonstrating the positive effect of dietary n-3 HUFA in the enhancement of larval growth and development (Lèger & Sorgeloos, 1992; Rees et al.,
One practical advantage of producing larger postlarvae is that they are knowingly more stress tolerant (Charmantier et al., 1988; Dall et al., 1990; Samocha et al., 1998; Tsuzuki et al., 2000). In other words, larger and older postlarvae are better prepared not only to deal with the stress involved in the transference to growout units, but also to cope with possible variations in environmental variables such as low salinity levels (Samocha et al., 1998; Tsuzuki et al., 2000).

A positive effect of the provision of dietary n-3 HUFA to crustaceans is that it often results in higher stress tolerance (Tackaert et al., 1989; Dhert et al., 1992; Rees et al., 1994; Cavalli et al., 2000; Chim et al., 2001; Palacios et al., 2004; Immanuel et al., 2007; Sui et al., 2007). It is therefore recognized that penaeids fed n-3 HUFA deficient diets generally express a low tolerance to stress (Chim et al., 2001; Palacios & Racotta, 2007). In the present study, comparison of the salinity (LS$_{50}$) and ammonia tolerance (LC$_{50}$) of 8 day-old postlarvae fed enriched or newly hatched Artemia indicated pronounced differences. The estimated LS$_{50}$ values for salinity were consistently higher for postlarvae fed enriched Artemia. Similarly, a higher tolerance to total ammonia was observed in postlarvae fed enriched Artemia. All this confirms the superior ability of the animals fed n-3 HUFA enriched Artemia to cope with adverse environmental conditions. Similar to the present findings, Tackaert et al. (1989) and Immanuel et al. (2007) verified that P. monodon postlarvae fed n-3 HUFA enriched Artemia had higher survival when exposed to a range of salinity levels in comparison to postlarvae fed unenriched Artemia, while Chim et al. (2001) demonstrated that feeding higher n-3 HUFA levels increased the cellular membrane...
resistance of Litopenaeus stylirostris which increased the tolerance to abrupt salinity changes. The beneficial effects of n-3 HUFA on tolerance to salinity stress may also be related to changes in the arrangement of fatty acids in the gill membrane and the consequent increase in shrimp osmosis capability (Palacios et al., 2004). Furthermore, shrimp fed n-3 HUFA rich diets have larger gill area (Palacios et al., 2004), which concurs with the more ramified gill structure reported in n-3 HUFA fed P. monodon postlarvae (Rees et al., 1994).

Although stress tests have been commonly used as a tool to compare the quality of crustacean larvae and juveniles in terms of tolerance to a number of physical and chemical parameters (Tackaert et al., 1989; Samocha et al., 1989).
et al., 1998; Cavalli et al., 2000; Chim et al., 2001; Álvarez et al., 2004; Palacios & Racotta, 2004; Martins et al., 2006; Immanuel et al., 2007; Sui et al., 2007), to date few studies have attempted to correlate the results of stress tests with the posterior growth performance of shrimp. In this regard, Bauman & Jamandre (1990) reported that batches of P. monodon postlarvae that responded positively to a salinity stress test presented a comparatively superior growth performance in ponds. Álvarez et al. (2004) found a correlation between stress testing and the survival of Litopenaeus vannamei postlarvae during simulated stocking into growout units. These authors, however, were unable to correlate the results of the stress test to growth or survival during a two-month long growout period. Palacios & Racotta (2007) hypothesized that survival of postlarvae to salinity stress tests would be a short-term phenomenon and thus not necessarily an adequate predictive criterion for longer performance. Results from the present study, however, indicate a possible relationship between survival in the stress tests (for both salinity and ammonia) and posterior growth of shrimp during nursery.

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