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Spermatophore quality of the pink shrimp *Farfantepenaeus paulensis* (Decapoda, Dendrobranchiata) broodstock fed with different maturation diets

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ABSTRACT

The objective of the present work was to evaluate the effects of different diets on spermatophore quality and tissue biochemical composition in male pink shrimp *Farfantepenaeus paulensis*. The experiment was carried out with the three following treatments: fresh food, commercial diet and a mix of fresh food and commercial diet. Spermatophore quality was evaluated by spermatophore weight, sperm count, melanization and spermatophore absence rates. Biochemical analyses of glucose, triacylglycerides and protein were completed for hemolymph, hepatopancreas and muscle. Spermatophore weight and sperm count were not significantly different among treatments ($P>0.05$). At the end of the experiment, shrimp fed only with commercial diet showed highest melanization rate and spermatophore absence, 42.85% and 42.85%, respectively. To shrimp fed only with fresh food the values melanization and spermatophore absence were 15.38% and 0%, respectively and the mix 12.51% and 37.50%, respectively. Hemolymph glucose concentration was significantly higher in commercial diet treatment (31.19 mg/dL) than fresh food (12.24 mg/dL) and mix treatments (10.97 mg/dL). *Farfantepenaeus paulensis* broodstocks fed only the commercial diet may present decrease spermatophore quality.

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1. Introduction

The pink shrimp *Farfantepenaeus paulensis* reproductive cycle occurs offshore (40–100 m of depth), and growth in estuarine areas (D'Incao, 1991). The pink shrimp is an important penaeid in fisheries distributed from Ilhéus, Brazil to Mar del Plata, Argentina (D'Incao, 1991). In the last 20 years the pink shrimp was intensely fishery resulting in decline of stocks (D'Incao et al., 2002). Different systems have been tested to rear *F. paulensis* in cages, pens enclosures, ponds (Ballester et al., 2003, 2007; Jensen et al., 2004; Krummenauer et al., 2006; Peixoto et al., 2003b; Poersch et al., 2006; Thompson et al., 2002; Wasielesky et al., 1999, 2001) and these studies represent perpecstive to pink shrimp to Aquaculture.

The production of high quality larvae and post-larvae for use in culture depends on broodstock condition (Racotta et al., 2003). In *F. paulensis* maturation systems, reproductive studies have focused predominantly on female maturation and spawning (Cavalli et al., 1997, 1998; Nakayama et al., 2008a; Peixoto et al., 2003a, 2004a,b). However, the penaeid males broodstocks are also susceptible to fertility problems that could compromise larval production (Alfaro, 1993).

Food is an important factor for the sexual maturation (Browdy, 1992) and males reproductive performance (Meunpol et al., 2005). Shrimp fed with unbalanced or incomplete diets have impaired reproductive performance or which can completely inhibit the reproduction process (Bray and Lawrence, 1992; Wouters et al., 2001). In males, spermatophore quality is a tool to evaluate efficiency of the diet to promote maturation and reproduction (Coman et al., 2007; Meunpol et al., 2005; Perez-Velazquez et al., 2002). Routinely, penaeid maturation success in captivity is obtained using diets that include fresh foods, with or without addition of commercial diet (Browdy, 1992, 1998; Harrison, 1997; Peixoto et al., 2005). The most used food items are molluscs (squid, oyster, mussel), crustaceans (shrimp, crab and artemia), fishes and marine polychaetes (Bray and Lawrence, 1992; Rothlisberg, 1998). However, some natural foods present disadvantages such as price fluctuations, unstable nutritional value, enhanced risks of pathogen transmission, and the potential to cause deterioration of water quality in the maturation systems (Harrison, 1990, 1997). The use of commercial diets is advantageous because they are easy to manage and stock, do not require preparation, and present less danger of contamination (Wouters et al., 2000). Nevertheless, when it only offered the commercial diet during maturation the production results compared with fresh food is unsatisfactory (Wouters et al., 2002).

Biochemical analyses of tissue help in assessing the quality of the foods offered (Racotta et al., 2003). Protein and triacylglycerides

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are involved in reproductive functions such as gamete synthesis and others (Harrison, 1990). For example, in *Litopenaeus vannamei*, biochemical components have performance energetic functions such as biochemical composition of the tissue (Palacios et al., 1999). For this specie, the concentrations of biochemical components have been using as nutritional and reproductive indicators (Mercier et al., 2006).

As previously mentioned, there is a great lack of studies regarding the effects of food on reproductive performance of male broodstocks. Therefore, the present study aimed to evaluate the effects of food on spermatophore quality and the biochemical composition of tissues in male *F. paulensis* broodstocks.

2. Materials and methods

2.1. Animals and experimental design

The experiment to evaluate the effects of diet on the spermatophore quality of pink shrimp lasted 40 days. Three diets were used: fresh food, which consisted squid *Illex argentines*, blue crab *Callinectes sapidus* and fish *Macrodon ancylodon*; only commercial diet (Breed S Inve Aquaculture, Belgium); and a mix of fresh food and commercial diet. In the treatment with fresh food the feed was offered alternately. The method described in AOAC (1995) was used for proximate analysis of squid, fish and crab. Commercial diet composition is approximates values provided by manufacturer. Protein (P), lipids (L), ash (A) and nitrogen-free extract (NFE), consisting of fiber and carbohydrate levels, were provided by food dry weight. NFE was calculated by the following equation: $NFE = 100 - P - L - A$.

Farfantepenaeus paulensis broodstocks ($n = 300$) were collected in Santa Catarina (26°54′S 48°34′W) offshore and transferred to the Marine Station of Aquaculture, Federal University of Rio Grande, Southern Brazil. To each dietary treatment, the animals were acclimated for one week in four circular maturation tanks (3.6 m diameter; 5000 L). In this period, the shrimps were fed four times daily with alternately crab, squid, fish and the commercial diet (Breed S Inve Aquaculture, Belgium). After this period, three shrimps of approximately 22.4 g (±1.1) were randomly stocked in 0.49 m^2, 150 L tanks with aeration. Each dietary treatment had five replicates.

Feed was offered two times daily (900 and 1700 h) *ad libitum*. To fresh food and mix treatments the feed was offered in the sequence to not repeat the last one. Seawater 33 ppt (±1) was renewed twice a week at a 90% rate and maintained the temperature at 27 °C (±1) and the dissolved oxygen at 6.66 mg/L (±0.06). Food remains, feces and exuviae were removed from the tanks twice a week. The artificial photoperiod was 14:10 light:dark.

2.2. Spermatophore quality and biological material sampling

Spermatophore quality was measured by spermatophore weight, sperm count, morphology and color of spermatophores (presence of melanization) and spermatophore absence percentage. All parameters were measured at the beginning (day zero) with all shrimp and at the end (day 40) of the experiment with eight males from each treatment. All males were in intermolt stage to spermatophore extrusion.

Both spermatophores from each male were extruded manually (Petersen et al., 1996) and one was randomly selected and weighed to the nearest 0.001 g. This spermatophore was homogenized in 2 mL of calcium-free saline solution. Sperm counts were estimated by counting cells present in the resulting sperm–saline solution using hemacytometer under a light microscope according to the method described by Leung-Trujillo and Lawrence (1987b). Melanization and spermatophore absence percentages were checked by visual examination of the coxae of the fifth pereiopod pair and the extruded spermatophore.

At the end of the experiment, the hemolymph of eight shrimp randomly selected from each treatment was sampled in the first abdominal segment using a 1 mL syringe. Hepatopancreas and abdominal muscle samples were removed by dissection. All biological material was maintained at −20 °C until biochemical analysis.

2.3. Biochemical analyses

Enzymatic-colorimetric analyses for glucose (GOD-PAP), triacylglycerides (GPO-PAP) and total protein (Biuret protein assay) were carried out in samples of hemolymph, hepatopancreas and muscle. Each hepatopancreas and muscle sub-sample (200 mg) was homogenized in 2 mL of saline solution. Subsequently, these sub-samples and the samples of hemolymph were centrifuged at 6600 × *g* for 10 min at 5 °C.

The supernatant of each sub-sample of hepatopancreas and muscle and sample of hemolymph were used to estimate the total glucose, triacylglycerides and protein concentrations. For estimation of triacylglycerides and protein, the supernatant was used directly for analyses. For estimation of glucose, 200 μL of supernatant was mixed with 500 μL of sodium citrate. This solution was maintained at rest for 5 min at 100 °C and then centrifuged at 12 800 × *g* for 15 min.

Glucose, protein and triacylglycerides absorbencies were read using ELISA microplates (Biotek, Canada). Wavelengths for assessment of glucose, triacylglycerides and total protein were 510, 510, 550 nm, respectively. All analyses were carried out using a commercial reagent kit (Doles Reagents Ltda., Goiânia, GO, Brazil).

2.4. Statistical analyses

Data on survival percentage were arcsine transformed before analyses, but only untransformed values are presented. Previous to analyses, the necessary premises were evaluated analyses of variances (ANOVA) were used to identify significant differences among the mean values of each parameter (spermatophore weight, sperm count, biochemical concentrations of hemolymph, hepatopancreas and muscle). When significant differences were found, ANOVA was followed by Tukey's post-hoc comparison test. All statistical analyses were executed at a level of significance of $P < 0.05$ using STATISTICA (version 7.0) software (StatSoft Inc. 2004, Tulsa, Oklahoma, USA).

3. Results

The protein, lipids, ash and NFE values of the diets composition (squid, crab, fish and commercial diet) are presented in Table 1. Fish and squid had the highest level of protein, followed by commercial diet and crab. The commercial diet contained the highest level of lipids. Crab contained the highest ash levels, followed by commercial diet, fish and squid. Crab and the commercial diet had higher NFE level.

The spermatophore quality data of males fed different diets are summarized in Table 2. Spermatophore weight and sperm count were not significantly different among treatments. However, in all treatments, the means of spermatophore weight were significantly lower at the end of the experimental period. Initially, none shrimp selected showed melanization or spermatophore absence. At the end of the experiment, the highest percentages of melanization and spermatophore absence were observed in shrimp from the commercial diet treatment. No males in the fresh food treatment were found without

Table 1
Proximate composition (%) of the foods (dry weight) offered to the pink shrimp *Farfantepenaeus paulensis* during the experimental period.

	Protein	Lipids	Ash	NFE
Commercial diet*	≥60	≥11	≤11	≤18
Squid	86.23	4.74	5.53	3.5
Fish	89.20	3.39	5.68	1.73
Crab	40.47	4.99	39.30	15.24

* Approximates values provided by manufacturer.

Table 2
Parameters of spermatophore quality and survival (mean values ± standard deviation) of the pink shrimp *Farfantepenaeus paulensis* fed three different diets.

Parameters	Fresh food	Commercial diet	Mix
Spermatophore weight (mg)			
Initial (n = 15)	22 ± 3[a]	24 ± 6[a]	27 ± 8[a]
Final (n = 8)	16 ± 3[b]	15 ± 1[b]	18 ± 5[b]
Sperm count (× 10^6)			
Initial (n = 15)	6.00 ± 4.91[a]	3.37 ± 2.59[a]	5.85 ± 4.29[a]
Final (n = 8)	4.89 ± 1.80[a]	4.26 ± 3.17[a]	3.40 ± 2.2[a]
Melanization (%)			
Initial (n = 15)	0	0	0
Final (n = 8)	15.38	42.85	12.51
Spermatophore absence (%)			
Initial (n = 15)	0	0	0
Final (n = 8)	0	42.85	37.50
Survival (%)			
Final	86.66 ± 18.25[a]	50 ± 28.86[a]	86.66 ± 18.25[a]

Different superscript letters within rows indicate significant differences ($P < 0.05$).

spermatophores. The survival was not different significantly among treatments, but shrimp fed with the commercial diet also showed the lowest average on survival.

Biochemical analysis of the hepatopancreas and muscle did not show significant differences in the concentration of glucose, triacylglycerides and protein among treatments. Hemolymph triacylglycerides and protein concentrations were also not significantly different. On the other hand, in the commercial diet treatment, hemolymph glucose concentration was significantly higher than in shrimp from the fresh food and mix treatments (Table 3).

4. Discussion

The variety of fresh food and the commercial diet has assured the success of penaeid maturation in captivity (Browdy, 1998; Peixoto et al., 2005). However, the effects of diverse diets in maturation system on spermatophore quality of *F. paulensis* currently are unknown. Among organic compounds in penaeid, dietary lipids and protein are involved in gonad maturation process (Coman et al., 2007; Meunpol et al., 2005; Perez-Velazquez et al., 2003; Samuel et al., 1999; Wouters et al., 2002). These compounds are the most abundant in foods, are used in biosynthesis and mobilized during maturation (Harrison, 1990). The difference in lipids and protein reported among food items used in this study (see Table 1) reinforces the variety of foods necessary for the best diet composition used in the laboratory in order to achieve the ideal supplement for gonad maturation.

Table 3
Biochemical compositions (mean values ± standard deviation) of hemolymph, hepatopancreas and muscle of pink shrimp *Farfantepenaeus paulensis* males fed three different diets.

	Fresh food (n = 8)	Commercial diet (n = 8)	Mix (n = 8)
Hemolymph			
Glucose (mg/dL)	12.24 ± 5.18[a]	31.19 ± 14.40[b]	10.97 ± 3.76[a]
Triacylglycerides (mg/dL)	21.46 ± 11.44[a]	34.43 ± 8.79[a]	26.13 ± 23.60[a]
Protein (mg/dL)	53.21 ± 14.02[a]	53.09 ± 12.29[a]	46.49 ± 16.83[a]
Hepatopancreas			
Glucose (mg/g)	2.26 ± 1.51[a]	4.68 ± 1.52[a]	4.19 ± 2.43[a]
Triacylglycerides (mg/g)	20.82 ± 19.89[a]	46.03 ± 19.15[a]	40.08 ± 37.13[a]
Protein (mg/g)	49.02 ± 31.25[a]	109.44 ± 45.86[a]	92.62 ± 112.54[a]
Muscle			
Glucose (mg/g)	0.74 ± 0.38[a]	1.03 ± 0.36[a]	0.51 ± 0.41[a]
Triacylglycerides (mg/g)	1.66 ± 1.21[a]	1.37 ± 1.08[a]	1.25 ± 0.58[a]
Protein (mg/g)	53.57 ± 7.03[a]	68.20 ± 35.33[a]	60.02 ± 22.77[a]

Different superscript letters within rows indicate significant differences ($P < 0.05$).

The spermatophore weight and sperm count were not significantly different among treatments. However, the loss of spermatophore weight ($P < 0.05$) at the end of the experimental period has also been reported by Nakayama et al. (2008b) in *F. paulensis* after 43 days. Some studies have attributed the loss of spermatophore quality to stress, degeneration of the digestive tract, and long time in captivity and/or nutrition due loss nutrient stores when wild animals are brought into captivity (Alfaro, 1993; Leung-Trujillo and Lawrence, 1987a). In the present study, the decrease of spermatophore weight at the end of the experiment probably is due to remaining of wild shrimp in captivity.

Standards for spermatophore quality of *F. paulensis* were reported by some authors (Cavalli *et al.*, 1997; Nakayama *et al.*, 2008b; Peixoto *et al.*, 2004b). For example, for weight class of 20–27 g, the spermatophore weight usually varies of 7 to 18 mg and the sperm count is among 1.2–3.7 million for spermatophore. This weight class is indicated for captivity reproduction (Cavalli *et al.*, 1997) and it was same weight class used in present study. The values of spermatophore weight and sperm count in present study are proximate to standards for *F. paulensis*.

Another parameter of spermatophore quality evaluated in this study was melanization. Alfaro et al. (1993) reported that spermatophore and reproductive tract melanization may be the result of two different syndromes. Male Reproductive System Melanization (MRSM) is an infectious syndrome caused by microorganisms such as opportunistic bacteria, *Vibrio alginolyticus*, *Pseudomonas putrefaciens* and others. Male Reproductive Tract Degenerative Syndrome (MRTDS) is stress believed associated with the effects of captivity such as high temperatures (Pascual et al., 1998, 2003; Perez-Velazquez et al., 2001; Sánchez et al., 2001), unbalanced diet (Goimier et al., 2006) or simply lack of spermatophore ejaculation (Parnes et al., 2006). Complementary, Alfaro and Lozano (1993) described a new condition: spermatophore deterioration, which generates new normal spermatophores after the complete deterioration of the melanized spermatophores.

In crustaceans, melanization has an important role in the immune system. Melanin is a toxic molecule produced by the prophenoloxidase system (proPO) (Alfaro et al., 1993). ProPO is an inactive proenzyme that occurs in hemolymph and is activated to phenoloxidase (PO) when it reacts with bacterial lipopolysaccharide, urea, calcium ions and heat, among others. PO is responsible for the synthesis of melanin (Sarathi et al., 2007), which is deposited near to the invasion of microorganisms in the event of MRSM (Vargas-Albores and Yepiz-Plascencia, 2000). As for MRTDS, Sánchez et al. (2001) proposed the sequence to explain the appearance of the melanization in males shrimp: 1) in captivity, nutritional stress is present and accompanied by other factors (for example, temperature), producing physiologic stress; 2) this stress reduces the immunological capacity through a reduction of hemocytes, altering the mechanisms of regulation of proPO; 3) this alteration produces an increases in activity of the proPO system, which increases melanin production; and 4) an increase of melanin produces cell degeneration and finally male sterilization. The sequence proposed by Sánchez et al. (2001) shows the importance that should be given to the presence of melanization, especially if the animals are destined for post-larvae production in captivity. Care should be considered in the beginning of the maturation system to maintain broodstock quality. Apparently healthy animals without necrosis or melanization on the carapace, reproductive tract, appendices and spermatophores should be selected.

Among treatments no shrimp initially showed melanization. However, at the end of the experiment in all treatments the shrimp showed melanized spermatophores. These results may be consequence of remaining wild shrimp in captivity, consequence of extrusion method, nutritional stress, or immune response system. In

this case the melanization seem effect of different diets and because the highest melanization rates were found in commercial diet treatment.

In this study, the commercial diet treatment showed also the highest rate of spermatophore absence (42.8%). Some authors attribute the spermatophore absence to natural degeneration process (Alfaro and Lozano, 1993) and the fact that spermatophore degeneration is associated with the molt cycle (Heitzmann et al., 1993). For example, Parnes et al. (2006) showed that spermatophores periodically disappeared from the terminal ampoules of males during 24 h premolt and then new spermatophores appeared after the exuvations. Spermatophore renewal is completed every two or three weeks (Pascual et al., 1998). However, this period may be decreased by improper captivity conditions like nutritional factors (Ceballos-Vázquez et al., 2004; Leung-Trujillo and Lawrence, 1987b). At the end of the experiment, spermatophore absence only was reported in commercial diet and mix treatments. These results may have been consequence of remaining in captivity. Nevertheless, food may had worsened the natural spermatophore degeneration because no male was reported spermatophore absence in fresh food treatment.

Glucose is most abundant carbohydrate in hemolymph (Gutiérrez et al., 2007), representing the main energetic font to shrimp metabolism (Hall and van Ham, 1998). In this study, the shrimps fed only with commercial diet (31.19 mg/dL) were hyperglycemic in relation to animals of fresh food (12.24 mg/dL) and mix (10.97 mg/dL) treatments. This result may be consequence of carbohydrate levels in diets. The commercial diet had approximately 18% NFE (fiber and carbohydrate levels). The carbohydrate level is not specified by manufacturer, but the fiber level is approximately 3%, indicating that carbohydrate level is approximately 15%. The use of diet with high carbohydrate level may have caused hyperglycemia in shrimps fed with commercial diet and consequently may compromise the homeostatic process.

Poor water parameters may also affect reproductive quality (Cavalli et al., 1998; Pascual et al., 1998, 2003; Perez-Velazquez et al., 2001). Nevertheless, the water quality for all treatments was considered ideal for the species (Marchiori and Boff, 1983; Peixoto et al., 2005), with temperatures between 24.5 °C to 29 °C and salinity of 33 to 35 ppt. Thus, food probably was important factor for aggrieved the spermatophore degeneration reported in the results of the present study.

Shrimp fed only the commercial diet showed higher melanization and spermatophore absence rates and hyperglycemia in hemolymph. Inclusion of fresh and diverse food items during the maturation of male *F. paulensis* is important to reduce the effects of natural spermatophore degeneration. The mix of fresh food and the commercial diet can be used without reproductive disruption in males. As shown in previous studies with others species of penaeids, the use of the commercial diet only is not recommended in maturation systems for males of the pink shrimp *F. paulensis*.

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