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Hemocytic melanization in shrimp spermatophores

André Braga[a,*], Diogo Lopes[b], Vitalina Magalhães[a], Marta C. Klosterhoff[a], Luis A. Romano[a], Luís H. Poersch[a], Wilson Wasielesky[a]

[a] Estação Marinha de Aquacultura, Universidade Federal do Rio Grande, C.P. 474, Rio Grande (RS), 96201-900, Brazil
[b] Departamento de Zootecnia, Universidade do Estado de Santa Catarina, Chapecó, SC 89815-630, Brazil

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ABSTRACT

The melanization of shrimp spermatophores caused by a hemocytic infiltration was demonstrated in this study. A 30-day trial was carried out using wild *Farfantepenaeus brasiliensis*. On days 0, 15 and 30, the spermatophores were manually extruded and used for sperm quality and histological analyses. Survival decreased after the extrusion events, whereas spermatophore melanization percentage increased. An inflammatory process characterized by the presence of hemocytes around and embedded in the connective tissue was identified as the trigger to the spermatophore melanization. The hemocytic infiltration was not observed in the sperm mass, but sperm quality was negatively affected.

1. Introduction

Melanization is a process characterized by extracellular deposition of melanin on crustacean tissues. Melanin is produced by the prophenoloxidase system (proPO) (Alfaro et al., 1993). Prophenoloxidase is an inactive pro-enzyme present in hemolymph, which is activated to form phenoloxidase (PO) when it responds to factors such as bacteria lipopolysaccharides, urea, calcium ions and heat. The action of PO is responsible for the production and deposition of melanin (Sarathi et al., 2007).

Melanization on shrimp spermatophores may result in reduction of the sperm quality due to a progressive reduction of sperm count and an increase in abnormal and dead sperm rates (Díaz et al., 2001). Alfaro et al. (1993) have suggested that the melanization of spermatophores and the reproductive tract may be caused by two syndromes. Male reproductive system melanization (MRSM) is a syndrome caused by pathogens such as *Vibrio alginolyticus* and *Pseudomonas putrefaciens*. This infection is characterized by the swelling and melanization of different regions of the reproductive tract. Male reproductive tract degenerative syndrome (MRTDS) is associated with stress conditions in captivity, such as high temperatures (Pascual et al., 2003; Perez-Velazquez et al., 2001; Sánchez et al., 2001) and inappropriate diets (Braga et al., 2010; Goimier et al., 2006).

Some authors have demonstrated a third cause for spermatophore melanization in shrimp. The spermatophore degeneration occurs when old spermatophores degenerate via melanin deposition when there is no ejaculation (Alfaro and Lozano, 1993; Diamond et al., 2008; Heitzmann and Diter, 1993). After the complete degeneration of old spermatophores, melanization-free spermatophores are produced (Parnes et al., 2006).

Most efforts of the shrimp culture industry are currently aimed at strategies to improve animal growth. On the contrary, reproductive aspects are usually neglected because the larvae supply has been guaranteed by a small number of large commercial hatcheries around the world, resulting in a misleading feeling that the techniques for reproduction in captivity are well-dominated. Unfortunately, the research follows this trend. The production and maintenance of broodstocks to obtain high-performance larvae are expensive. In addition, the techniques used in maturation laboratories, e.g., eyestalk ablation and spermatophore extrusion, do not care about principles of animal ethics and, consequently, reduce the life time and quality of the broodstocks. Studies demonstrating that the currently applied methods decrease the reproductive quality highlight the need for more ethic and efficient methods in aquaculture. This study is the first histological report demonstrating the association of the spermatophore extrusion method with the melanization and reduction of sperm quality.

2. Material and methods

2.1. Animal origin and acclimation

Farfantepenaeus brasiliensis broodstock (n = 300) were collected offshore in Santa Catarina and transferred to the Marine Station of Aquaculture of the Federal University of Rio Grande (Southern Brazil).

* Corresponding author.
E-mail address: andrebraga_pa@yahoo.com.br (A. Braga).

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Shrimp were stocked at 7 animals m^{-2} in four 10 m^2 circular maturation tanks (5000 L). During the one-week acclimation, the temperature, salinity and photoperiod were maintained at 27 °C (± 1), 33 ppt (± 1) and 14:10 h light:dark, respectively. Shrimp were fed squid (*Illex argentines*), blue crab (*Callinectes sapidus*), fish (*Macrodon ancylodon*) and commercial feed specially designed for broodstock (Breed'S, Inve Aquaculture®, Baasrode, Belgium) offered ad libitum and alternately four times daily. The water exchange rate was 100% day^{-1}. Food remains, feces, mortalities and exuviae were removed from the tanks daily.

2.2. Experimental design

Fifty males (28.54 ± 4.94 g) in the intermolt period and with no melanization were selected and stocked in 25 0.49 m^2 tanks with constant aeration. Feeding management, daily checking, photoperiod and selected water quality parameters (temperature and salinity) were the same as those applied during acclimation.

The trial lasted 30 days. On days 0, 15 and 30, spermatophores were manually extruded. These events of spermatophore sampling were named extrusions 1, 2 and 3. The periodicity among the extrusions was based on previous findings concerning *F. brasiliensis* spermatophore replacement presented by Braga et al. (2014). In extrusion 1, spermatophores were extruded from every male. From the following extrusion events, melanization was recorded by visual examination, and the spermatophores of live males in the intermolt period were collected.

2.3. Sperm quality and histology

The number of spermatophores used for sperm quality and histological analyses are shown in Table 1. Males showing both spermatophores completely formed had one of them randomly collected to analyze the sperm quality, whereas the second was selected to analyze the histology. This criterion was standardized in all extrusion events to avoid using spermatophores from one male for the same analysis.

Extruded spermatophores selected for sperm quality analysis were weighed to the nearest 0.001 g and homogenized in a 2 mL calcium-free saline solution and 0.1 mL of trypan blue. Sperm were counted using a hemocytometer under a light microscope. Spermatozoa with malformations of the main body and/or spike (broken, bent or absent) were identified and classified as abnormal, whereas blue stained spermatozoa were classified as dead.

Spermatophores selected for histology were fixed with a 10% formalin solution, processed in automatic equipment (LUPE PT05, LupeTec®, São Carlos, Brazil) and embedded with paraplast. Transverse and longitudinal histological sections of 3 μm thickness were obtained with the aid of a microtome (LUPE MRP03, LupeTec®, São Carlos, Brazil) and stained with hematoxylin-eosin and Fontana-Masson for melanin. Melanin was removed in some of the sections with 0.25% potassium permanganate and 5% oxalic acid for better observation of the tissue injury. The sections were examined under a light microscope (Olympus BX 45, Olympus Corporation, Tokyo, Japan) coupled to a digital camera (Olympus DP 72, Olympus Corporation, Tokyo, Japan) for image acquisition.

Table 1
Number of the *Farfantepenaeus brasiliensis* spermatophores used to analyze the sperm quality and histology in extrusion 1, 2 and 3.

	Extrusion 1	Extrusion 2	Extrusion 3
Live males	50	42	34
Males in post-molt period	0	2	1
Males with 1 melanized spermatophore	0	3	3
Males with 2 melanized spermatophore	0	2	6
Total of melanized spermatophores	0	7	15
Spermatophores used to analyze the sperm quality	15	4	9
Spermatophores used to analyze the histology	5	3	6

2.4. Statistical analysis

The percentage data were arcsine-transformed, but only untransformed values are shown. The spermatophores collected in extrusions 2 and 3 were classified and statistically analyzed as non-melanized and melanized. A non-parametric Kruskal-Wallis test (Sokal and Rohlf, 1995) was used to identify significant differences in the sperm quality parameters among the extrusion events.

3. Results

Survival decreased after the extrusion events (84% and 68% in extrusions 2 and 3, respectively), whereas melanization increased (Table 2). Melanization was restricted to the spermatophore in all analyzed males. The weight of the melanized spermatophores collected in extrusion 3 was significantly lower than those non-melanized from extrusion 1. Sperm count and normal sperm rate significantly diminished in melanized spermatophores collected in extrusions 2 and 3 compared with the non-melanized spermatophores collected in extrusions 1, 2 and 3 (Table 2).

Histological sections of melanized spermatophores collected in extrusions 2 and 3 are shown in Fig. 1 and Fig. 2. The sperm mass of a melanized spermatophore is shown in the Fig. 1. Spermatozoa were basophilic and most of them showed deformed main body and/or absent spike.

Fig. 2A shows a heavy hemocytic infiltration around the connective tissue of the melanized spermatophores (extrusions 2 and 3). Fig. 2B shows that hemocytes also infiltrated in the connective tissue of these spermatophores. These cells were basophilic, with strongly basophilic nuclei. Hemocytes were not infiltrated in the sperm masses of the analyzed spermatophores (including non-melanized and melanized). Hemocytic infiltration was not observed in the non-melanized spermatophores collected in extrusions 1, 2 and 3 (Fig. 3).

4. Discussion

According to the results of the histological analysis, melanized spermatophores showed a heavy hemocytic infiltration. It is known that a tissue injury may be a trigger to the melanin production by the proPO system in invertebrates (Cerenius and Söderhäll, 2004). It is possible to relate the hemocytic infiltration observed in this study with the method of manual compression for extruding the spermatophores. This hypothesis is supported by the progressive increase in the melanized spermatophore rate after extrusion events. On the other hand, the hypothesis of spermatophore melanization being an MRSM cannot be totally refuted because analyses to evaluate the presence of pathogens were not performed. However, the spermatophore-restricted melanization is a clear indication that this condition was not caused by an infection.

The effect of the extrusion methods on spermatophore melanization was previously considered in the literature. Chamberlain et al. (1983) commented on the possibility of the melanization of the *Litopenaeus vannamei*, *Litopenaeus stylirostris* and *Litopenaeus setiferus* reproductive tracts observed in their study being related to electrical or manual extrusion. Harris and Sandifer (1986) also hypothesized on the relationship between *Macrobrachium rosenbergii* spermatophore melanization and the periodicity of electrical extrusion. However, the effects of the extrusion methods on shrimp spermatophore melanization have not been histologically demonstrated.

Hemocytic melanization differs from the other pathways of spermatophore melanization previously demonstrated in the literature by the occurrence of hemocytes around or embedded to the connective