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Short communication

Infertility of biofloc-reared *Litopenaeus vannamei* males associated with a spermatophore mycobacterial infection: Description of the pathological condition and implications for the broodstock management and larval production

André Braga[a,*], Diogo Lopes[b], Vitalina Magalhães[a], Marta Klosterhoff[a], Luis Romano[a], Luís 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

This study describes a mycobacteriosis in spermatophores of reared *Litopenaeus vannamei*, which had relevant implications for the management of broodstock and larval production. The broodstock was reared in mixed-sex biofloc-dominated systems composed of two grow-out phases. Melanization was observed in males during the grow-out II phase. The affected males showed melanization restricted to the spermatophores, varying in color from brown to black and showing swelling in the later. The histological analysis showed the presence of acid-alcohol fastness bacilli in black spermatophores. In addition to the histology, the sperm quality was evaluated in non-melanized and melanized. Based on the results on the sperm count and abnormal and dead sperm rates, it was possible to infer that the males were most likely experiencing a progressive process of spermatophore degeneration related to stressful culture condition, which then resulted in the bacterial infection. The animals that had the spermatophores extruded at the advanced stage did not show the capacity to produce the structure again or died. No fertilized spawning was obtained when males with non-melanized spermatophores were transferred to the maturation laboratory.

1. Introduction

The production of close-life broodstock is primarily stimulated for factors such as increases in the demand for postlarvae as a consequence of the intensification of aquaculture, the transmission of diseases from wild broodstock, and the sustainability of the shrimp farming industry through more ecological and economical production methods compared with the traditional systems (Preston et al., 1999; Regunathan, 2008). The advantages of the use of closed systems based on biofloc technology (BFT) for production, maintenance and pre-maturation of broodstocks have been demonstrated in the literature (Braga et al., 2015; Emerenciano et al., 2012, 2014; Magaña-Gallegos et al., 2018a). For example, Emerenciano et al. (2013) reported better reproductive performance from females reared in BFT system during the pre-maturation in comparison with animals kept under clear water conditions. Although there is indication that biofloc may improve the physiological status of reared animals (Becerra-Dorame et al., 2013), it is important keeping monitoring for diseases in broodstock that could compromise the larval production.

The production of broodstock to obtain high-performance larvae is expensive and requires time. During this period, the penaeid males broodstock are susceptible to fertility problems that could compromise larval production (Alfaro, 1993), representing negative cost impacts for commercial hatcheries. The main issue associated to captive conditions is the spermatophore melanization (Alfaro-Montoya, 2010). Melanin biosynthesis in arthropods is the final product of a complex process that comprises cascade chemical reactions named prophenoloxidase (proPO) system (Nappi and Vass, 1993). This system is recognized as one of the major immunoeffector responses of crustacean and is composed of protease zymogens, inactive proenzyme proPO and associated pattern-recognition proteins. The proPO is activated to phenoloxidase (PO) when it reacts with lipopolysaccharides bacteria, urea, calcium ions, heat and other (Sarathi et al., 2007).

Four pathways of shrimp spermatophore melanization have been previously suggested in the literature: (1) natural degeneration, spermatophores degeneration via melanin deposition; (2) Male

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

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Reproductive System Melanization (MRSM), a pathogens-caused infection; (3) Male Reproductive Tract Degenerative Syndrome (MRTDS), which is associated to the reduction of the immunological capacity as result of stress in captivity; (4) hemocytic melanization associated to the method of the manual extrusion of the spermatophore (Alfaro, 1993; Alfaro-Montoya, 2010; Braga et al., 2018).

The association between bacterial infection and spermatophore melanization have been previously suggested in the literature (Alfaro et al., 1993; Brown et al., 1980; Chamberlain et al., 1983; Talbot et al., 1989). Mycobacteria-caused muscular infections in *Litopenaeus vannamei* have been already reported in the literature (Pedrosa et al., 2017), but there is no previous record of the occurrence of mycobacteria in the male reproductive tract. Thus, this study includes the first description of a mycobacteriosis in spermatophore of reared *L. vannamei*, which had relevant implications for the management of broodstock and larval production.

2. Material and methods

L. vannamei postlarvae (PL) were obtained from a commercial hatchery (Aquatec, Canguaretama, RN, Brazil) and were reared at the Marine Station of Aquaculture of the Federal University of Rio Grande (FURG), where the broodstock production is a strategy adopted annually to obtain females and males for research purposes. This study does not include the carrying out of trials, the described condition was recorded during a crop of the annual broodstock production and the data were obtained from the corresponding production control sheets. The production was based on the BFT and is divided in two phases. The grow-out I was conducted in nine 600-m^{-2} ponds lined with high-density polyethylene (HDPE), where shrimp (0.08 ± 0.02 g) were stocked at 180 animals m^{-2} and harvested (10 ± 2 g) after 120 days. In the grow-out II, shrimp were stocked (40 animals m^{-2}) in two 50-m^3 rectangular tanks lined with HPDE and sheltered in an enclosed greenhouse. During both phases, shrimp were reared in mixed-sex systems and fed a commercial 38% CP feed (Potimar Active 38, Guabi, Campinas, Brazil) twice daily. A commercial probiotic (PRO-Sanilife W® INVE, Salt Lake City, UT, USA) was added in the water weekly according to manufacturer's instructions for use. The water quality parameters were monitored and maintained within the adequate range for development of penaeids (Van Wyk and Scarpa, 1999).

During the grow-out II, black spermatophores were manually extruded when observed. Some of these spermatophores ($n = 10$) were fixed with a Davidson's solution and processed in automatic equipment (LUPE PT05, LupeTec®, São Carlos, Brazil) and were 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 Fite-Faraco method. In the later, the sections were deparaffinized with a xylene-peanut oil mixture, differentiated with 10% sulfuric acid, cleared in xylene and mounted in Canada balsam. 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.

Sperm quality analysis was conducted in non-melanized and melanized spermatophores collected from males randomly selected from the grow-out II tanks. The extruded spermatophores were classified according to their color. These spermatophores 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. A non-parametric Kruskal-Wallis test (Sokal and Rohlf, 1995) was used to identify significant differences in the sperm quality parameters among the spermatophore color categories ($P < 0.05$).

For larval production, only broodstock without melanization were selected (37.52 ± 5.22 g for males and 46.00 ± 2.38 g for females). The selected animals were stocked at 7 animals m^{-2} in two 10 m^2 circular maturation tanks (5000 L). During one-week acclimation, the temperature, salinity and photoperiod were maintained at 27 °C (± 1), 33 ppt (± 1) and 14:10 light:dark, respectively. Shrimp were fed squid (*Illex argentinus*), 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.

After acclimation, females were unilaterally eyestalk ablated and maintained under the same acclimation conditions. The development of gonads was examined daily using a flashlight. Mature females were transferred to 120 L tanks, where they were kept overnight. After spawning, the females were returned to their original maturation tank. The number of eggs per spawning was estimated with three samples of 250 mL. The fertilization rates were determined by examining the eggs under a microscope. As criterion to evaluate the viability of the spawning, those composed of 50% or more of viable eggs were classified as fertilized, whereas unfertilized spawning showed fertilization rate below 50% of the total of produced eggs.

3. Results and discussion

Melanization was observed only in two years-old males during the grow-out II phase. Males without melanization were also present in the tanks, showing white spermatophores. The affected males did not show melanization on the body, which was restricted to the spermatophores. There were individuals showing only one or both spermatophores melanized. These spermatophores varied from brown to black. The later had hard consistency and showed swelling, making difficult their extrusion (Fig. 1). The histological analysis of the black spermatophores via Fite-Faraco method showed the presence of acid-alcohol fastness bacilli (Fig. 2).

Chamberlain et al. (1983) also reported that melanized spermatophore within the terminal ampoules ranged in color from “tan” (brown) to black, classifying the later as the advanced melanization stage. The classification made by these authors is supported by the results of the

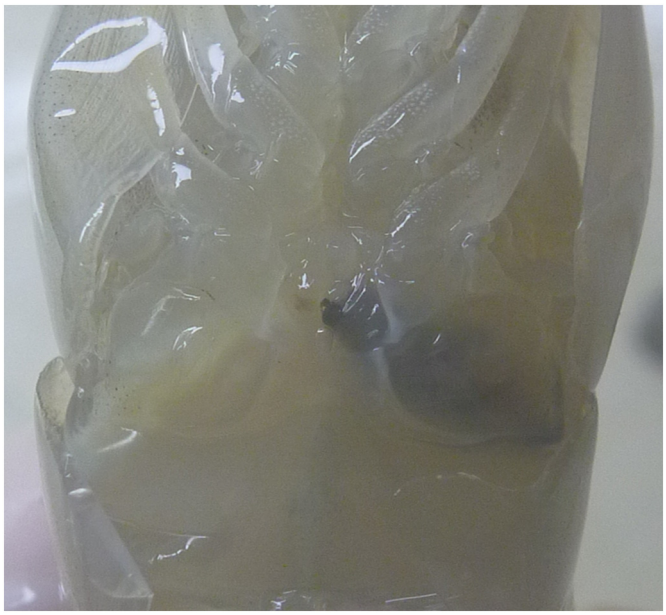

Fig. 1. Region of the terminal ampoule of a *Litopenaeus vannamei* male produced in mixed-sex biofloc-dominated systems, showing one of its spermatophores completely black as result of the advanced stage of melanization.