Aquaculture 433 (2014) 313–317

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Spermatophore replacement of pink shrimp *Farfantepenaeus brasiliensis* after manual extrusion: Effect of molting

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ARTICLE INFO

Article history:
Received 6 April 2014
Received in revised form 4 June 2014
Accepted 25 June 2014
Available online 1 July 2014

Keywords:
Shrimp
Farfantepenaeus brasiliensis
Males
Extrusion
Spermatophore replacement
Molting

ABSTRACT

This study aimed to evaluate *Farfantepenaeus brasiliensis* spermatophore replacement after manual extrusion and analyze the effect molting has on this process. Three trials were conducted. In the first trial, the replacement of spermatophores after extrusion was macroscopically evaluated via an analysis of morphological changes in the terminal ampoule during the formation of new spermatophores. In the second trial, the sperm quality in the different stages of spermatophore replacement identified in the first trial was compared. In the third trial, the replacement time of spermatophores after extrusion was determined, with and without molting. In all trials, 30 manually extruded wild males were individually stocked in 0.49 m^2 tanks using different samples of randomly selected animals for each trial. The results were obtained by daily visual examination of the gonopore and coxae regions of the fifth pereiopod pair and sperm quality analyses. In trial 1, three successive macroscopic stages of spermatophores were observed during replacement: unformed, partially formed and formed. In trial 2, the sperm count in formed spermatophores was significantly higher than that in partially formed spermatophores, whereas the spermatophore weight was not significantly different. Therefore, spermatophore replacement most likely comprises the following phases: (1) deposition of a primitive spermatophore with all structural components in each terminal ampoule; (2) gradual deposition of spermatozoa; and (3) stiffening of the spermatophore into typical elongate form. Trial 3 demonstrated that after extrusion, spermatophores are completely formed in 16 days without molting and in 24 h with molting. Spermatophores formed after molting have sperm quality similar to that of gradually formed spermatophores.

1. Introduction

The primary biological functions of penaeid spermatophores are to protect and avoid the loss of spermatozoa in the transfer to females during copulation. Additionally, in closed thelycum species, spermatophores seal the thelycum, preventing the need for replacement of the spermatophore and insemination by other males (Bauer, 1991; Subramoniam, 1991). Penaeids have spermatophores of varying complexity depending on the thelycum type. For example, females with open thelycum, i.e., *Litopenaeus*, receive morphologically complex spermatophores composed of accessory structures, such as wing, germinate body and/or flanges. In the closed thelycum penaeid, males produce simple spermatophores that are divided into a main body and an appendage, which is the structure that internally seals the thelycum (Bauer, 1991; Bauer and Cash, 1991).

Independent of spermatophore morphological complexity, penaeids usually replace their spermatophores after ejaculation via copulation or extrusion (Heitzmann et al., 1993; Malek and Bawab, 1974a,b). When ejaculation does not occur, spermatophores degenerate; melanin is deposited extracellularly and new healthy spermatophores are deposited in the terminal ampoules. This process of deterioration has been described for domesticated *Litopenaeus vannamei*, but there is no evidence that it occurs in wild males (Alfaro and Lozano, 1993; Alfaro-Montoya, 2010; Diamond et al., 2008; Parnes et al., 2006).

Some authors have reported that, when spermatophore is experimentally ejaculated, the replacement process may be affected by population differences, temperature and molting (Heitzmann et al., 1993; Pascual et al., 1998; Rosas et al., 1993). The molting process in crustaceans is divided into four stages: intermolt, premolt, molt and postmolt (Brusca and Brusca, 2003). The following events of spermatophore replacement have been proposed for *L. vannamei* throughout the molting stages: (1) intermolt, when a spermatophore is present in each terminal ampoule; (2) premolt, when the spermatophore is degenerated via degradation of the extracellular matrix and phagocytosis of spermatozoa; (3) molt, when the spermatophores are absent; and (4) postmolt, when two new spermatophores are present, one in each terminal ampoule (Parnes et al., 2006).

Spermatophore replacement time and effect of molting have been evaluated for open thelycum species (Chow et al., 1991; Heitzmann

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http://dx.doi.org/10.1016/j.aquaculture.2014.06.032
0044-8486/

et al., 1993; Rosas et al., 1993). For penaeid with morphologically simpler spermatophores (closed thelycum penaeids), studies on the replacement process have been restricted to *Penaeus kerathurus* (Malek and Bawab, 1974a,b). These studies constitute important morphological research that contributes to the understanding of the replacement process and can be applied to aquaculture in the development of management strategies that lengthen spermatophore quality maintenance.

Some *Farfantepenaeus* species have been considered candidates for culture, such as the pink shrimp *Farfantepenaeus brasiliensis*. This shrimp is distributed from North Carolina, USA to Rio Grande do Sul, Brazil (Pérez-Farfante and Kensley, 1997). The interest in *F. brasiliensis* culture has been motivated by the following characteristics: hardiness and resistance to culture management, e.g., to gonadal induction of females via eyestalk ablation; wide latitudinal distribution; maximum adult size larger than other penaeid; and relatively good growth rates (Braga et al., 2011; Gaxiola et al., 2010).

Morphological studies have been conducted on *Farfantepenaeus* spermatophore. For example, Bauer and Cash (1991) described the spermatophores of *Farfantepenaeus aztecus* and *Farfantepenaeus duorarum* in detail, analyzing the functional significance of the structures. Observations of external morphology of *Farfantepenaeus paulensis* spermatophore are included in Braga et al. (2013). However, the mechanisms of *Farfantepenaeus* spermatophore replacement are still poorly understood. The present study aims to evaluate *F. brasiliensis* spermatophore replacement after manual extrusion, analyzing the effect of molting on this process.

2. Material and methods

2.1. Animals and acclimation

F. brasiliensis broodstocks (n = 300) were collected in Santa Catarina (26°54′S 48°34′W) offshore and transferred to the Marine Station of Aquaculture at the Federal University of Rio Grande, Southern Brazil. In the lab, shrimp were stocked at 7 animals m^{-2} in four 10 m^2 circular maturation tanks (5000 L) and were acclimated to captivity condition during one week. During this period, temperature, salinity and photoperiod were maintained at 27 °C (±1), 33 ppt (±1) and 14:10 light:dark, respectively. Broodstock were fed four times daily with squid *Illex argentinus*, blue crab *Callinectes sapidus*, fish *Macrodon ancylodon* and commercial feed for shrimp maturation (Breed′S, Inve Aquaculture, Baasrode, Belgium) offered ad libitum alternately. The seawater was renewed daily at a 90% rate. Food remains, feces and exuviae were removed daily from the maturation tanks. After the acclimation, three experiments were conducted.

2.2. Trial 1

The aim of this trial was to macroscopically evaluate the *F. brasiliensis* spermatophore replacement process after ejaculation via manual extrusion, analyzing morphological changes in the terminal ampoule during the formation of new spermatophores. After acclimation, 30 intermolt males (26.90 ± 4.45 g) without apparent spermatophore melanization were selected from the maturation tanks. Spermatophores were extruded manually from each male (Nakayama et al., 2008). After extrusion, shrimp were stocked individually into 0.49 m^2 tanks (150 L) with soft aeration. Water quality (e.g., temperature, salinity), photoperiod and feeding parameters similar to those during acclimation were maintained. Gonopore and coxae regions of the fifth pereiopod pair were visually examined each day (800 h), and macroscopic morphological changes in these regions were identified. Different stages of spermatophore replacement were proposed based on these morphological changes. The trial ended once all males showed completely formed spermatophores.

2.3. Trial 2

In the second trial, the sperm quality during the different stages of *F. brasiliensis* spermatophore replacement, identified in trial 1, was compared. This trial was conducted under the same conditions as those in trial 1, though additional 30 intermolt males (27.56 ± 3.61 g) without spermatophores melanization were selected from the maturation tanks. Daily macroscopic observation of the gonopore and coxae regions of the fifth pereiopod pair were conducted for all males after manual extrusion of their spermatophores. Throughout the trial, nine males with spermatophores in incomplete replacement stages were randomly selected. These males were cryoanesthesized, and their spermatophores were collected by dissection. Males with completely replaced spermatophores (n = 17) were manually extruded at the end of the trial. The sperm quality for both groups of males (with completely and incompletely formed spermatophores) was compared. As in trial 1, this trial ended when the last live male had replaced its spermatophores.

2.4. Trial 3

This trial aimed to determine *F. brasiliensis* spermatophore replacement time with and without molting after manual extrusion and evaluate the effect of the molting process on spermatophore replacement. In this trial, the culture conditions of previous trials were maintained. Thirty males (27.90 ± 4.25 g) were collected from the maturation tanks, extruded manually and macroscopically checked daily. When a male presented ecdysis, the replacement stage of that male's spermatophore was classified based on the results of trial 1. All shrimp found in the postmolt period were cryoanesthesized and dissected to obtain their spermatophores. Males that did not molt during the trial were extruded manually when the trial ended. Sperm quality of replaced spermatophores with (formed after molting) and without (formed gradually) molting was compared. The trial ended when all live males had replaced spermatophores.

2.5. Sperm quality

In trials 2 and 3, both male spermatophores were extruded, but only one was randomly selected and weighed to the nearest 0.001 g. Each selected spermatophore was homogenized in a 2 mL calcium-free saline solution with 0.1 mL trypan blue. Sperm were counted using a hemocytometer under a light microscope (Leung-Trujillo and Lawrence, 1987). 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.

2.6. Statistical analysis

Percentage data were arcsine transformed (only untransformed values are showed), and statistical assumptions were evaluated prior to analysis. Student's *t*-test was used to identify significant differences in sperm quality among the spermatophore groups defined in trials 2 and 3 ($P < 0.05$).

3. Results

3.1. Trial 1

Changes in opacity of the coxae of the fifth pereiopod pair and turgidity of the gonopore region were identified throughout the *F. brasiliensis* spermatophore replacement process. Based on these changes, three stages of spermatophore replacement were identified after extrusion: unformed, in which the coxae of the fifth pereiopod pair is transparent and the gonopore region is slightly turgid (Fig. 1A); partially formed, with intermediate opacity and turgidity in comparison with the other stages (Fig. 1B); and formed, in which the coxae of the