UNIVERSIDADE FEDERAL RURAL DE PERNAMBUCO

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA ANIMAL TROPICAL

BIOLOGIA REPRODUTIVA E USO DE BIOTÉCNICAS EM MACHOS DE PENEÍDEOS

THAÍS CASTELO BRANCO CHAVES

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THAÍS CASTELO BRANCO CHAVES

Tese submetida à Coordenação do Curso de Pós-Graduação em Ciência Animal Tropical, como parte dos requisitos para a obtenção do título de Doutorado em Ciência Animal Tropical.
Orientador: Silvio Peixoto
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TESE APROVADA EM 26/02/2015

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SUMÁRIO

Página

Resumo.. 11

Abstract 12

Introdução.. 13

Referência bibliográfica 17

Objetivo geral 20

Objetivos específicos 20

Capítulo I – A review of the scientific literature on penaeid males reproduction 21

Capítulo II - Scanning electron microscopic investigation of spermatophore and spermatozoa of the pink shrimp *Farfantepenaeus subtilis* …………………………………………………….... 46

Capítulo III - Cadaveric sperm viability in the white shrimp *Litopenaeus vannamei* ....….. 54

Capítulo IV - Sperm vitrification in the white shrimp *Litopenaeus vannamei* …………..……… 60

Capítulo V - Artificial insemination with vitrified sperm from the pacific white shrimp *Litopenaeus vannamei* ……………………………………………………………………………. 76

Considerações finais ….…………………………………………………………………….... 90

ANEXO I - Normas do periódico *Aquaculture* 91

ANEXO II - Normas dos periódicos *Invertebrates Reproduction & Development* e *Aquaculture Research* ……………………………………... 107

LISTA DE FIGURAS

Capítulo I - A review of the scientific literature on penaeid males reproduction

Figura 1. Transverse section of *Farfantepenaeus subtilis* spermatophore under scanning electron microscopy ……………………………………………………………………………………31

Figura 2. Penaeid sperm cell ……….……..…………………………………………………… 33

Figura 3. Cryopreservation methods 35

Capítulo II - Scanning electron microscopic investigation of spermatophore and spermatozoa of the pink shrimp *Farfantepenaeus subtilis*

Figura 1. Transverse section of *Farfantepenaeus subtilis* spermatophore 50

Capítulo V - Artificial insemination with vitrified sperm from the pacific white shrimp *Litopenaeus vannamei*.

Figura 1. Fertilized eggs at different stages of embryonic development 3 hours after spawning using artificially inseminated vitrified sperm mass. …………………….………………….... 82

Figura 2. Eggs at different stages of embryonic development and nauplii 15 hours after artificial insemination. ……………………………………………………………………………… 84

LISTA DE TABELAS

Capítulo I - A review of the scientific literature on penaeid males reproduction

Tabela 1. Sperm viability evaluation techniques in crustaceans …………………………….... 34

Capítulo III - Cadaveric sperm viability in the white shrimp *Litopenaeus vannamei*

Tabela 1. Percentage of viable sperm extracted from shrimp *L. vannamei* at 12, 24, 48, 72 and 96 hours post-mortem exposed to temperatures of 26, 4 and -18 ^{o}C…... 57

Capítulo IV - Sperm vitrification in the white shrimp *Litopenaeus vannamei*

Tabela 1. Percentage of apparent sperm viability of *L. vannamei* sperm mass after exposition to the cryoprotectants dimethyl sulfoxide (DMSO), ethylene glycol (EG) and methanol (MeOH) in different concentrations and exposure periods ………………………………………………… 65

Tabela 2. Percentage of apparent sperm viability of *L. vannamei* sperm mass after cryopreservation by vitrification method up to 120 days at different cryoprotective solutions……………………………………………………………………............................... 66

Capítulo V - Artificial insemination with vitrified sperm from the pacific white shrimp *Litopenaeus vannamei*.

Tabela 1. Mean value (± SD) of fertilization (3h), hatching (15h) rates and number of eggs per spawning (n=4 / treatment) after artificial insemination ……..…………………………...… 83

Tabela 2. Mean (± SD) egg diameter (µm) and nauplii lengh (µm) per spawning after artificial insemination ………………………...…………………………………...……………...… 84

RESUMO

CHAVES, Thaís Castelo Branco. **Estudos de biotécnicas reprodutivas em camarões peneídeos** 2015. Tese (Doutorado em Ciência Animal Tropical). Universidade Federal Rural de Pernambuco, Recife, PE, 2015.

O desenvolvimento de tecnologias aplicadas à aquicultura colabora significativamente com a expansão deste segmento. Na carcinicultura as biotécnicas reprodutivas vêm sendo aprimoradas ao longo dos anos, mas ainda apresenta muitas lacunas a serem exploradas. Objetivou-se com o presente trabalho colaborar com o conhecimento das características reprodutivas, além de sugerir uma nova abordagem para a criopreservação do sêmen de camarões peneídeos, com o método de vitrificação. O primeiro capítulo apresenta uma revisão de literatura atualizada abordando os principais tópicos sobre a reprodução de machos de peneídeos. O segundo capítulo traz uma descrição inédita, por microscopia eletrônica de varredura, do espermatóforo e espermatozoide do camarão-rosa *Farfantepenaeus subtilis*, os quais apresentaram características similares aos dos membros do gênero Farfantepenaeus, onde se destaca o posicionamento periférico da massa espermática, próxima à cutícula externa do espermatóforo. O terceiro capítulo teve como objetivo avaliar a viabilidade do sêmen cadavérico em camarões da espécie *Litopenaeus vannamei* armazenados por até 96h em diferentes temperaturas (26, 4 e -18ºC). O sêmen mantido a 4ºC apresentou condições excelentes por até 96h de armazenamento. Enquanto que a pior condição foi encontrada a -18ºC, onde foram observadas altas taxas de mortalidade em todos os períodos testados. No quarto capítulo objetivou-se desenvolver um protocolo de criopreservação do sêmen do camarão *L.vannamei*, por vitrificação. Através de testes de toxicidade foram selecionados os crioprotetores mais adequados à técnica. Metanol e lecitina, crioprotetores intra e extracelulares, respectivamente, foram utilizados combinados ou não. Após vitrificação, o sêmen foi estocado em nitrogênio líquido por até 120 dias. As altas taxas de viabilidade espermática observadas sugerem a eficiência do método de vitrificação para a criopreservação do sêmen do camarão *L.vannamei*. No quinto capítulo, objetivou-se avaliar a eficiência do método de vitrificação através das taxas de fertilização em fêmeas inseminadas artificialmente. Assim como a viabilidade espermática, a taxa de fertilização foi satisfatória em todos os tratamentos. Portanto, diante dos resultados satisfatórios de fertilização, sugere-se que o método de vitrificação pode ser eficiente para criopreservação do sêmen do camarão peneídeo *L. vannamei*.
Palavras chaves: cadavérico, camarão, inseminação artificial, sêmen, vitrificação.

ABSTRACT

CHAVES, Thaís Castelo Branco. **Studies of reproductive biotechnologies in penaeid shrimp 2015.** Thesis (Doctorate in Tropical Animal Science). Universidade Federal Rural de Pernambuco, Recife, PE, 2015.

The development of technologies applied to aquaculture contributes significantly to the expansion of this segment. Reproductive biotechnologies in carcinicultre have been improved over the years, but still have many issues to be explored. This study aimed to collaborate with knowledge of reproductive characteristics and suggests a new approach to sperm cryopreservation of penaeid shrimps with the vitrification method. The first chapter presents an updated literature review comprising the main topics on penaeids males reproduction. The second chapter provides the first description, by scanning electron microscopy, of the spermatophore and spermatozoa of the pink shrimp *Farfantepenaeus subtilis,* which presented similar features of Farfantepenaeus subgenre, highlighting the peripheral positioning of sperm mass, near the outer cuticle of the spermatophore. In the third chapter it was evaluated the cadaveric sperm viability in the white shrimp *Litopenaeus vannamei* stored for up to 96 hours at different temperatures (26, 4 and -18°C). Sperm kept at 4°C showed excellent conditions for up to 96 hours of storage. Meanwhile the worst conditions were found in sperm kept at -18°C that presented high mortality rates for all tested periods. In the fourth chapter we aimed to develop a sperm cryopreservation protocol in the white shrimp *Litopenaeus vannamei* by vitrification method. The most appropriate cryoprotectants to vitrification were selected by toxicity tests. Thus methanol and lecithin, intra and extracellular cryoprotectants respectively, were used singly or in combination. After vitrification the sperm was stored in liquid nitrogen for up to 120 days. The high sperm viability rates observed suggests that vitrification method is efficient for *L.vannamei* sperm cryopreservation. In the fifth chapter, females were inseminated with sperm cryopreserved by vitrification method. The efficiency of the method was evaluated by fertilization and hatching nauplii rates. As sperm viability, the fertilization rate was satisfactory in all treatments. However, only the control group had nauplii hatching. Therefore, by comparing the fertilization and hatching rates, it is suggested that some unknown factor is interrupting embryo development.

Keywords: artificial insemination, cadaveric, shrimp, sperm, vitrification.

1. INTRODUÇÃO

Atualmente a carcinicultura representa uma parcela significativa da aquicultura mundial, atingindo uma produção de mais de 6 milhões de toneladas em 2012. O camarão se destaca por ser a maior commodity em termos de valor, representando aproximadamente 15% do valor total dos produtos aquícolas comercializados mundialmente (FAO, 2014). A família Penaeidae apresenta algumas espécies que se destacam por serem importantes recursos pesqueiros, como por exemplo, o camarão branco do pacífico *Litopenaeus vannamei*, que atualmente é o crustáceo mais cultivado do mundo (PÉREZ FARFANTE; KENSLEY, 1997; FAO, 2014). Em 2012 a produção nacional atingiu aproximadamente 75 mil toneladas de camarão e, seguindo a tendência mundial, a espécie mais cultivada foi do camarão branco do pacífico *L. vannamei* (FAO, 2014).

No entanto, grande parte do camarão disponibilizado no mercado brasileiro é ainda proveniente da pesca de espécies nativas. Na costa brasileira estão distribuídas seis espécies de peneídeos (*Litopenaeus schmitti*, *Xiphopenaeus kroyeri*, *Farfantepenaeus brasiliensis*, *Farfantepenaeus notialis*, *Farfantepenaeus paulensis* e *Farfantepenaeus subtilis*). Alguns estudos já foram feitos para avaliar a viabilidade da produção comercial das espécies nativas e algumas apresentam potencial como alternativa para a carcinicultura nacional, como o camarão rosa *F. subtilis* (NUNES et al., 1997; MAIA; NUNES, 2003; SILVA et al., 2010).

Para a produção comercial destas espécies nativas de camarões peneídeos, é fundamental conhecer as suas características reprodutivas para entender o processo de reprodução e aumentar as chances de sucesso de fertilização. Os camarões peneídeos apresentam dimorfismo sexual e as estruturas morfológicas externas que definem o sexo é o petasma, para os machos, e o télico, para as fêmeas. O télico tem a função de fixar e/ou armazenar a massa espermática depositada pelo macho no momento da cópula (DALL et al., 1990). Além disso, a morfologia do télico das fêmeas nos permite classificar os peneídeos em dois grupos: télico fechado e télico aberto. O télico fechado é uma característica comum às espécies do subgênero Farfantepenaeus. Enquanto

que o télico aberto é característico das espécies do subgênero Litopenaeus (PÉREZ-FARFANTE; KENSLEY, 1997).

Fêmeas de télico fechado apresentam placas laterais, formando um receptáculo seminal onde o espermatóforo é depositado pelo macho e, neste caso, a cópula ocorre logo após a ecdise da fêmea, quando o novo exoesqueleto ainda está se consolidando (DALL et al., 1990). As espécies *Farfantepenaeus chinensis*, *Farfantepenaeus subtilis*, *Farfantepenaeus brasiliensis* e *Farfantepenaeus paulensis* são exemplos de camarões peneídeos de télico fechado. Em espécies de télico aberto, a fêmea não possui placas laterais cobrindo o télico e a cópula só ocorre no período pós-muda (DALL et al., 1990). Portanto, o exoesqueleto deve estar totalmente consolidado, permitindo a total aderência do espermatóforo, para que este não seja perdido. Os camarões peneídeos *L. vannamei, Penaeus setiferus, Peneus stylirostris* e *Litopenaeus schmitti* são exemplos de espécies de télico aberto.

O sistema reprodutor dos machos de peneídeos apresenta externamente dois poros genitais, situados próximos à base do último par de pereiópodos, por onde são exteriorizados os espermatóforos, que são estruturas em forma de saco responsáveis pelo armazenamento dos espermatozoides (DALL et al., 1990). Estudos das características reprodutivas em peneídeos podem colaborar com maiores avanços no desenvolvimento de biotecnologias para reprodução destas espécies. Técnicas como a recuperação de sêmen *post mortem*, criopreservação de sêmen e a inseminação artificial, temas dos quais existem poucos estudos para peneídeos podem ser aplicadas em programas de melhoramento genético e formação de banco de germoplasma de espécies com risco de extinção (PETERSEN; BELTRAME; DERNER, 1996; KOTEESWARAN; PANDIAN, 2002).

O espermatozoide cadavérico (recuperados *post mortem*) é um meio potencial para recuperar gametas de machos que morrem inesperadamente devido a desastres ambientais ou por problemas em instalações aquícolas (KOTEESWARAN; PANDIAN, 2002; THUWANUT et al., 2013). Além disso, a técnica pode auxiliar em estudos de estoques selvagens de peneídeos, onde

não é possível manter uma grande quantidade de indivíduos vivos para posterior análise de sua biologia reprodutiva. Portanto, é importante conhecer os padrões de deterioração dos tecidos e também a metodologia mais adequada aos objetivos específicos de cada estudo.

Os estudos de biotecnologias de reprodução em crustáceos peneídeos vêm sendo realizados há mais de 40 anos, através de pesquisas em criopreservação de sêmen e inseminação artificial (CLARK JR. et al., 1973; CHOW et al., 1985; ANCHORDOGUY et al., 1988). No entanto, as particularidades das diversas espécies de camarões peneídeos abrem um amplo campo de pesquisa que ainda precisa ser explorado.

A técnica de criopreservação permite armazenar o sêmen indefinidamente, permitindo que este esteja viável ao descongelar e ser utilizado na inseminação artificial (VUTHIPHANDCHAI et al., 2007). O desenvolvimento destas técnicas pode promover fontes confiáveis de material genético de alta qualidade, facilitar reservas de curto prazo de espermatóforos e permitir fácil transporte de gametas masculinos em programas de reprodução especializados (NIMRAT et al., 2006).

A criopreservação pode ser feita, basicamente, de duas maneiras: a congelação lenta e a vitrificação. A congelação lenta é realizada através de um resfriamento gradual e muitas vezes dividida em etapas; já a vitrificação consiste na imersão direta no nitrogênio líquido, promovendo um resfriamento ultrarrápido (FAHY et al., 1984). Recentemente a vitrificação vem sendo utilizada com sucesso na criopreservação de sêmen de peixes (CUEVAS-URIBE et al., 2011; MERINO et al., 2012; FIGUEROA et al., 2013). No entanto, não foram encontrados estudos com camarões peneídeos.

Portanto, este trabalho de tese teve como objetivo geral ampliar os conhecimentos na área de biotecnologias reprodutivas aplicadas aos camarões peneídeos, com ênfase nas características dos machos reprodutores. Este trabalho está dividido em cinco capítulos: O primeiro traz uma revisão de literatura atualizada sobre aspectos reprodutivos em machos peneídeos; no segundo capítulo foi realizada uma descrição inédita do espermatóforo e espermatozoide do camarão *F.*

subtilis; o terceiro capítulo explora possibilidades de recuperação de sêmen cadavérico no camarão *L. vannamei*; no quarto capítulo foi desenvolvido um protocolo de vitrificação do sêmen do camarão *L. vannamei*; e no quinto capítulo foi realizada a avaliação da eficiência do método de vitrificação através da inseminação artificial com sêmen vitrificado.

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3. OBJETIVO GERAL

Este trabalho objetivou colaborar com o conhecimento das características reprodutivas masculina, bem como sugerir uma nova abordagem para a criopreservação do sêmen de camarões peneídeos com o método de vitrificação.

3.1. Objetivos específicos

- Apresentar através de uma ampla revisão bibliográfica estudos que abordam a reprodução de peneídeos dando ênfase ao gênero masculino.
- Realizar a primeira descrição estrutural do espermatozoide do camarão rosa *Farfantepenaeus subtilis* através de microscopia eletrônica de varredura (MEV).
- Investigar a qualidade espermática *post mortem* em reprodutores do camarão marinho *Litopenaeus vannamei* em diferentes períodos e temperaturas de armazenamento reproduzindo condições normalmente encontradas em laboratórios e a bordo.
- Avaliar o método de vitrificação como uma opção para criopreservação da massa espermática do camarão branco do pacífico *Litopenaeus vannamei*.
- Avaliar a eficiência da técnica de vitrificação da massa espermática através das taxas de fecundidade e eclosão, utilizando sêmen vitrificado na inseminação artificial do camarão branco do pacífico *Litopenaeus vannamei*.

CAPÍTULO I

Artigo científico a ser submetido ao periódico **Aquaculture**.
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A review of the scientific literature on Penaeid males reproduction

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Abstract

Knowledge of the spermatogenesis and maturation process in penaeids is extremely important to the improvement of domestication and the establishment of breeding programs. However, male reproductive features are little studied and few documents reunite complete information on general structures, sexual differentiation, endocrine regulation, gonadal development, spermatogenesis and techniques to sperm viability evaluation and cryopreservation. In this review we aim to gather the existing literature information around male reproductive biology and biotechnologies to trace the evolution of these topics in penaeid species. This review indicateed the need for further studies on male reproduction which can be significant to improve shrimp participation in world aquaculture production.

1. Introduction

Shrimp farming is a fairly representative activity in global food production, with over 6.0 million tonnes produced every year (FAO, 2014). Penaeid species variety with potential for shrimp farming raised the need for more knowledge about their reproductive characteristics and

ability to produce viable spawnings. However, some of the mechanisms involved in this process are poorly covered in the literature.

Spermatogenesis is the formation of mature spermatozoa from germinal cells in the lumen of testicular acini (Abraham et al., 2007). The reproductive system in penaeid males is a complex physiological unit responsible for hormonal control of sexual maturation, control of sperm production and possibly the release of pheromones (Alfaro-Montoya, 2010). However, studies on male reproduction have received less attention than female maturation and spawning performance.

Knowledge of the spermatogenesis and maturation process in penaeid is extremely important to the improvement of artificial manipulations like feminization and polyploid breeding, as well as for the development of reproductive technologies such as artificial insemination and sperm cryopreservation. Besides, establishing such knowledge would provide the basis to the creation of germplasm banks which may guarantee the conservation the genetic material of such species. Therefore, in the present study we aimed to gather information about male reproductive biology and biotechnologies to trace the evolution of these topics in penaeid species.

2. Male reproductive system in penaeid

Male reproductive system presents two externally gonopore or genital pores that presents an opening at the base of the fifth pair of pereopods. Another external reproductive structure is the petasma, which is located on the first pair of pleopods, near the genital pores, and is responsible for transferring the spermatophore to the female during mating (Guitart et al., 1985).

Internally the testes of penaeid shrimp are composed by 6 pairs of lobes connected by a tissue along its midline. The testis, located dorsally in the posterior thorax, rests directly on the heart (Shigekawa and Clark Jr., 1986) and dorsal to the hepatopancreas (Treece and Yates, 1988; Dall et al., 1990). A pair of vas deferens extends from the edges of the two testicular lobes and continues in a dorsal-ventral direction laterally to the final portion that opens into the genital

pore. Each vas deferens is divided into three parts and presents a thin and short proximal portion which extends to form the second portion, the median ejaculatory vas deferens. The median vas deferens folds in half, dividing into two equal parts forming a double tubular structure (Treece and Yates, 1988). After that begins the distal vas deferens, which is characterized by a rapid decrease in diameter and continues as a long, thin tubule ending in the terminal ampoule (Shigekawa and Clark Jr., 1986; Treece and Yates, 1988; Dall et al., 1990). According to Charniaux-Cotton and Payen (1985), the androgenic gland (AG) in decapods is attached to the subterminal ejaculatory portion of the vas deferens.

Histological descriptions of the vas deferens of *Marsupenaeus japonicus* (Nakamura et al., 1992), *Farfantepenaeus chinensis* (Li and Li, 1993), *Litopenaeus vannamei, Litopenaeus stylirostris* (Alfaro-Montoya, 1994) and *L. vannamei* pre-adult (Campos-Ramos et al., 2006) indicates that the AG attached to the distal part of the middle vas deferens, in the subterminal ejaculatory region, just before the narrowing of the distal vas deferens. The AG is a single, compact cell mass positioned along to the vas deferens, attached to the muscle by a layer of epithelial lining and composed of large oval cells, presenting a vacuolated cytoplasm and a prominent, rounded nucleus.

In *L. vannamei*, thinner cords branch out from both ends of the main cord of the AG, and extend to the middle vas deferens, and to the distal vas deferens, respectively. The AG continues to develop as the shrimp grows, from juvenile to adult, extending along the vas deferens (Campos-Ramos et al., 2006). Li and Xiang (1997) found the AG covering the whole vas deferens in *F. chinensis* pre-adults and adults. The same was observed in *L. stylirostris* adults (Alfaro-Montoya, 1994). The function of the AG is related to several events like the onset of spermatogenesis in *M. japonicus* (Charniaux-Cotton and Payen, 1985), spermatogonial differentiation in *Penaeus indicus* (Mohamed and Diwan, 1991) and petasma development in *F. chinensis* (Li and Xiang, 1997).

3. Sexual differentiation and maturation in penaeids males

Sexual differentiation in penaeid shrimps usually occurs at the same age regardless of size. In *L. vannamei*, it is possible to externally determine the sex in 60 day-old individuals (Campos-Ramos et al., 2006). In *F. chinensis* and *M. japonicus* external sexual differentiation for males and females occur during the second month after the metamorphosis to post-larvae (Charniaux-Cotton and Payen, 1985; Yin et al,. 1986; Nakamura et al., 1992; Li and Xiang, 2002).

Male gonophores and appendix masculine are present respectively, at the base of fifth pair of pereiopods and at the second pair of pleopods, in 58 day-old *L. vannamei*. Petasma appears as a tubular structure in 70 day-old individuals (Campos-Ramos et al., 2006). Vas deferens is formed in about 20 days in *M. japonicus* and probably even before endopodites differentiation. The onset of development of the AG, surrounding the vas deferens, is an evidence of first maturation, since this structure is responsible for spermatogenesis initiation (Nakamura et al., 1992).

In *M. japonicus* AG differentiation occurs in 55 day-old juveniles, during male organogenesis, just before the differentiation of the gonads into testes at 60 days (Charniaux-Cotton and Payen, 1985; Nakamura et al., 1992). In *L. vannamei*, the appearance of AG and testes occurs while the genital pores and petasma are externally being developed, in 60 day-old juveniles (Campos-Ramos et al., 2006).

4. Endocrine regulation of male reproduction

The Penaeidae endocrine system is formed by the neurosecretory system, the Y-organ and the AG (Gabe, 1953, 1956; Carlisle and Knowles, 1959; Bullock and Horridge, 1965; Cooke and Sullivan, 1982; Charniaux-Cotton and Payen, 1985; Skinner, 1985).

The Y-organ, also known as the moulting gland or ecdysial gland, consists of paired organs, with epithelial origin, located beneath the cuticle in the pre-branchial chamber of penaeids. This structure is the source of the moulting hormones, ecdysones, that initiates and sustains the processes of moulting (Bell and Lightner, 1988; Dall et al., 1990; Lachaise et al., 1993).

The AG in penaeid shrimps has been described as a long strip of secretory tissue located along the distal vas deferens (Kulkarni et al., 1984; Charniaux-Cotton and Payen, 1985), with the function of promoting the testis development and male secondary sexual characters (Dall et al., 1990). Information about the consequences of AG manipulation in penaeids would help to elucidate the mechanism of sex determination as well as determine the possible influence of pheromones in reproduction (Campos-Ramos et al., 2006).

The complete sexual differentiation process is particularly complex in Malacostraca, such as in decapods where AG is the organ considered responsible for the maleness. The hormone secreted by this gland seems to control sexual differentiation in the freshwater shrimp (Nagamine et al., 1980; Sagi et al., 1997). Sagi and Cohen (1990) were able to promote sex reversal in *Macrobrachium rosenbergii* by excising the AG, and indicating the influence of androgenic hormones in the mechanism of differentiation of this species. In contrast, the technology of sex reversal in penaeid shrimp received little scientific attention (Campos-Ramos et al., 2006).

The influence of steroid hormones in sexual differentiation by administration of estradiol was tested in *Penaeus penicillatus* (Zhongqing, 1990) larvae and post larvae. However, was observed no influence of the steroid hormones in sexual differentiation of this species, which contrasts with the findings for AG hormones in *M. rosenbergii.*

There is also the possibility of prostaglandins participates of hormonal process in penaeid reproduction and that crustaceans are able to synthesize it. Furthermore, prostaglandins E and F act on muscle contraction of reproductive tract and can possibly influence the spawning in female and spermatophore extrusion for males (Middleditch et al., 1979).

5. Gonadal development in males

On the literature there are few studies of gonadal development in males. The studies of maturation in captivity are based mainly on sperm characterization. Some of these studies focused on the relation between sperm quantity and/or quality with age, size, and grow-out

conditions, such as Ceballos-Vázquez et al. (2003) with *L. vannamei*, Peixoto et al. (2004) with *Farfantepenaeus paulensis*, Jiang et al. (2009) with *Penaeus monodon* and Silva et al. (2015) with captive *L. vannamei* in a commercial reproductive system.

Alfaro-Montoya (1993) proposed a model for the sexual maturation of penaeid males based on three independent levels: maturation of the testis and vas deferens, and synthesis of spermatophores. The testicular maturation is linked to the start of spermatids production followed by the vas deferens, that when fully developed, are responsible to complete the sperm maturation process. At the end occurs the spermatophore synthesis in terminal ampoules. The same model was posteriorly suggested and confirmed by Ceballos-Vazquez et al. (2003), who used it to explain their observations on sperm quality of *L. vannamei* reared in captivity.

Sperm mass is transported along the vas deferens, packed in several tissue layers, and finally stored in the terminal ampoule, forming the spermatophore that will be expelled through the gonopores, but if no reproductive behavior occurs it will be renewed in the next molting cycle. Thus, it is reasonable to infer that the molt cycles can interfere in male reproductive condition (Alfaro-Montoya, 1993, Ceballos-Vazquez et al. 2003).

Each testicular lobe is composed of innumerable testicular acini held together by connective tissue and that in immature testis the acini are completely empty presenting a narrow germinal zone with non-differentiated germ cells. However, mature testis indicates a peripheral germinal zone containing spermatogonial cells, and centrally to the acini is observed spermatocytes in diakinesis. In fully mature males, testes acini become full of cells at advanced maturation stages like spermatids and spermatozoa, where the germinal zone is very restricted (Abraham et al., 2007).

Ceballos-Vázquez et al. (2010) observed significantly decrease on number of spermatogonia in relation to body weight increase; however, secondary spermatocytes and spermatids tended to increase with age. Therefore the authors considered the reproductive male maturation (testicle, vas deferens, terminal ampoule, spermatophore) a sequential process that depends mainly on the

age, although body weight could explain differences caused by different culture conditions. Normal sexual maturation of male penaeid shrimp is characterized by three phases: testes maturation achieved at an age of 6 months, vas deferens maturation and spermatophore synthesis beginning between month 8 and 10, and more accentuated development between month 10 and 12 (Alfaro-Montoya, 2010).

6. Spermatogenesis

In the first report about spermatogenesis in penaeid shrimp, King (1948) studied the reproductive tract of *Penaeus setiferus* and found that the spermatogenesis process begins in the peripheral germinative layer of the testicular tubules when spermatogonia go into the prophase of meiosis. At the same study the author states that the spermatogonia gather at the periphery of the testicular lobe and the lumen is filled with secondary spermatocytes and spermatids.

Several years after this report, Shigekawa and Clark Jr. (1986) argued that testes contain a wide variety of cell types representing various maturation stages of the marine shrimp *Sicyonia ingentis*. They also reported that in Sycioniidae the process of spermatogenesis is not synchronous and begins with the spermatid following the completion of meiosis. Therefore, the authors divided spermatogenesis into 7 stages: Vesicular cytoplasmic stage; Proliferation of the rough endoplasmic reticulum; anterior granule formation; acrosomal vesicle formation; flocculent chromatin stage; membrane pouch formation and subacrosomal development.

Despite the increase in shrimp farming worldwide, few studies about penaeid spermatogenesis was observed until the 90’s, when important information for *P. setiferus, L.vannamei* and *Parapenaeus longirostris* were found out (Chow et al., 1991; Medina, 1994). For the later species, it was observed highly convolute seminiferous tubules showing in a transversal section spermatogonias positioned in the peripheral region, and a lumen mainly occupied by spermatids and the sperm located on the central region from where the sperm cells will continue to the vas deferens.

Spermatogenesis cycle is independent in each seminiferous tubule including two distinguishable phases where sertoli cells (supporting epithelial layer) are responsible for the expansion and contraction of the seminiferous tubules lumen from where starts the new cycle from primary spermatocytes produced by spermatogonia. First occurs the cord phase, where spermatogenesis is supported by multi-functional sertoli cells, generates late spermatids; after that becomes, the lumen phase, and sertoli cells retreats aligning to form a cavity where late spermatids are transferred to the posterior vas deferens, where spermatogenesis continues (Chow et al. 1991).

Spermatogenesis has been described in many ways. In *P. longirostris* this event was described in three main steps: Early spermatids; Mid spermatids; and Late spermatids (Medina, 1994).

Early spermatids are recognized as polarized cells about 5.25 µm in diameter presenting a large acrosomal vesicle. Nucleus appears individualized with an extensive area of condensed chromatin and with numerous small vesicles surrounding it. In the perinuclear region it is also observed endoplasmic reticulum cisternae in close contact with the nucleus or acrosomal vesicle. In this phase can be distinguished three cell membranes at the acrosomal vesicle, from outside-in: the accessory cell plasma membrane, spermatid plasma membrane, and acrosomal vesicle membrane. No Golgi bodies or similar membranous organelles were found, only centrioles are rarely observed.

Mid spermatids are found as elongated cells measuring 6 µm in length by 4.6 µm in width. The acrosomal vesicle becomes slightly concave, the nucleus becomes uncondensed and the perinuclear region reduces drastically. The chromatin presents a granular appearance and the nuclear membrane is ruptured and the nucleoplasm is mixed with the cytoplasm. This pattern is also observed in Sycioniidae (Shigekawa and Clark Jr., 1986). Accumulated on the cell periphery some organelles like mitochondrias begin to appear in the perinuclear cytoplasm, but centrioles are no longer observed in this phase. Inside the acrosome vesicle is formed a cone-like

granulate structure that presents a short apical protuberance on its base. The three membranes observed on the first phase are still distinguishable at the anterior surface of the cell (Medina, 1994). At the late spermatids stage the cells becomes more compressed anterio-posteriorly and its length reduces to 5.6 µm even with the lengthening of the apical protuberance but its width is not changed. The perinuclear membranes observed previously are now aggregated and with some mitochondria form a thin peripheral band that borders the nuclear region. The acrosomal vesicle becomes crescent-shaped with an apical protuberance, which tends to elongate progressively and later form the spike of the sperm. A dense coat covers the plasma membrane on the protuberance region (Medina, 1994). Mature sperm presents 5.5 µm of total length and is located in the vas deferens, with an apical protuberance called spike (Shigekawa and Clark Jr., 1986; Dall et al, 1990). The chromatin presents a fibrillar pattern, usually found in decapod sperm. A dense layer derived from the membrane systems outlines the profile of the nuclear region, but no nuclear envelope is present (Medina, 1994).

In histological sections of testicular lobes of *Metapenaeus Monoceros*, a germinal zone containing spermatogonial cells and nurse cells is apparent. Spermatogenesis always progressed from the periphery of acini to the center and therefore subsequent developmental stages are found towards the center in a graded manner. Spermatogonia pass through a period of quick growth to become primary spermatocytes, which undergo meiosis to form two secondary spermatocytes. The two spermatocytes divide mitotically to form four spermatids from each primary spermatocyte, and the spermatids modified into spermatozoa without further division (Abraham et al., 2007).

7. Sperm and spermatophore characteristics

Penaeids spermatophores can have different structural arrangements (Figure 1), according to the reproductive characteristics of each species (Pérez-Farfante, 1975). Spermatophores of the genus Litopenaeus have a chitinous structure called wing responsible to anchor the

spermatophore to open thelycum females (Bauer and Martin, 1991). On the other hand, in closed thelycum females (all other genus), males insert the sperm mass directly into the thelycum (Bauer and Martin, 1991).

The spermatophores are formed by specific tissue layers that surround the sperm sac, which contains the sperm mass (Pérez-Farfante, 1975). The sperm mass can vary between species in concentration and distribution into the spermatophores (Castelo-Branco et al., 2014b). In the open thelycum species *L. vannamei*, *L. occidentalis*, and *L. schmitti,* the sperm mass is disposed in the center of spermatophore, while in *L. stylirostris* it is concentrated in a round bulge at the base of the wing (Pérez-Farfante, 1975). In closed thelycum *Farfantepenaeus subtilis*, as well as *Farfantepenaeus aztecus*, also have a longitudinal sperm mass, but it is distributed along the spermatophore periphery, near the outer cuticle (Bauer and Cash, 1991; Castelo-Branco et al., 2014b).

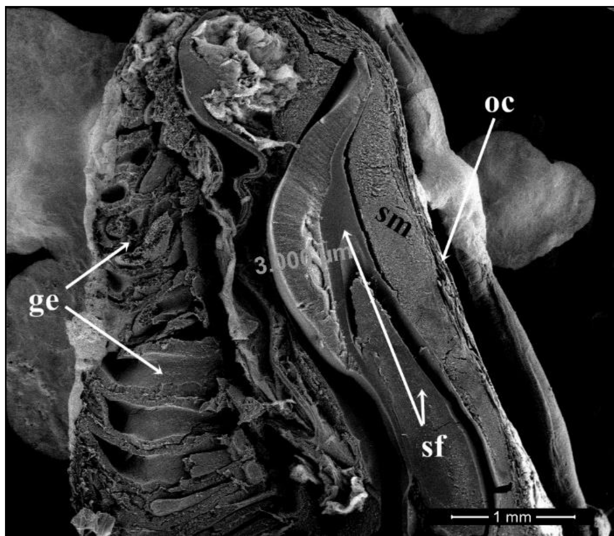

Figure 1. Transverse section of *Farfantepenaeus subtilis* spermatophore under scanning electron microscopy. Glandular epithelium (ge); outer cuticle (oc); sperm mass (sm); sperm-free material (sf).

Decapod crustacean spermatic cells are very unique and different from the sperm of other animals. In general these nonmotile sperm are surrounded by a sperm-free material that can be found in many penaeids such as *F. aztecus*, *P. setiferus* (Bauer and Martin, 1991), *S. ingentis*

(Subramoniam, 1995), *M. monoceros* (Abraham et al., 2007) and *F. subtilis* (Castelo-Branco et al., 2014b).

A great variety of sperm morphology was described among decapods crustaceans (Medina, 1994). This variation is shown into two broad categories according to the different patterns of cellular organization in sperm cells with a single or multiple appendages (Brown et al., 1977; Talbot and Summers, 1978). Sperm cells with single appendage are typical of Dendrobranchiata and Pleocyemata order and the infraorder Caridea. These sperm cells exhibit a single appendage or "spike" that is an extension of the acrossomal structure. Non-caridean pleocyemata sperm cells, presents several appendages of nuclear or cytoplasmic origin (Medina, 1994).

Penaeid spermatic cell is comprised of a main body, containing an uncondensed nucleus, and a variable number of stellate processes that project from the main body (Figure 2). The spike is a single appendage, not continuous with the nucleus and contains microfilaments denoting its acrossomal origin. Internally the cap region is divided into two other regions: acrosomal and subacrossomal (Dall et al., 1990). The first one is distributed peripherally coating the subacrossomal region which is formed by a clear zone and the filamentous meshwork that plays an important role in acrossome maturation. The process of acrosome maturation can occur at the male reproductive system or may continue after mating, with the spermatophore attached to the female thelycum varying among species (Aungsuchawan et al., 2011).

In the main body, the internal structures are the nucleus and cytoplasmic particles, the last one form a narrow hemispherical layer around the nucleus (Alfaro et al., 2007). According to Shigekawa and Clarck Jr. (1986) the determination of cell structures functions involved on sperm activation is an important tool for knowledge about the nature of fertilization.

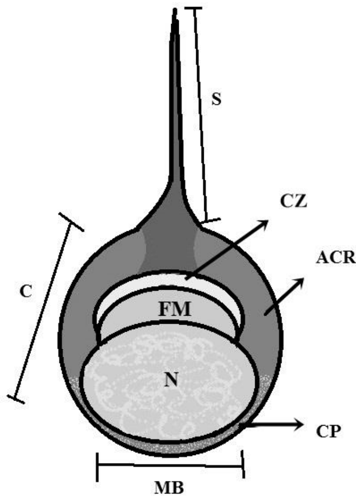

Figure 2. Penaeid sperm cell: Spike (S), cap region (C) and posterior main body (MB). In the cap region is observed the acrosome (ACR) and the subacrosomal region, which is composed of a clear zone (CZ) and the filamentous meshwork (FM). In posterior main body it is observed the nucleus (N) and cytoplasmic particles (CP).

8. Sperm evaluation technics

Sperm counting and its macroscopic evaluation can help to characterize the reproductive potential in penaeids (Wang et al., 1995). Colorimetric techniques using trypan blue and eosin-nigrosin have been widely used to assess the membrane integrity in penaeid sperm (Table 1). However, this approach has been recently questioned as the sperm membrane can apparently be intact, but intracellular damage can compromise the sperm viability (Silva et al., 2015). Fluorescence microscopy technique uses a fluorescent probe propidium iodide (PI) and carboxyfluorescein diacetate (CFDA) to assess the membrane integrity. In intact sperm cells membranes are stained in green due to CFDA binding to esterases. While in damaged sperm cells, membranes are stained in red by PI binding to nuclear DNA (Coleto et al., 2002). The fluorescence microscopy has been used to assess sperm viability in mammals and fish, but in crustaceans sperm it was applied only twice (Castelo-Branco et al., 2015; Silva et al., 2015). This technique may have more specific information on cell viability and should be more explored in studies on crustacean reproduction.

Table 1. Sperm viability evaluation techniques in crustaceans.

Sperm viability evaluation	Technique	Species	References*
Fertilization and hatching	Artificial insemination	*M. rosenbergii*	Chow et al., 1985
In vitro acrossome reaction	Egg water	*S. ingentes*	Anchordoguy et al., 1988
Membrane integrity	Eosin-nigrosin	*Scylla serrata*	Jeyalectumie & Subramoniam, 1989
Membrane integrity	Trypan blue	*P. setiferus*	Rosas et al., 1993
Membrane integrity	Trypan blue	*L. vannamei*	Bray & Lawrence, 1998
Membrane integrity	Trypan blue	*P. setiferus*	Pascual et al., 1998
In vitro acrossome reaction	Egg water	*P. indicus*	Diwan & Joseph, 1999
Abnormal sperm	Light microscopy	*L. vannamei*	Perez-Velazquez et al., 2001
Membrane integrity	Trypan blue	*L. vannamei*	Ceballos-Vázquez et al., 2003
Membrane integrity	Trypan blue	*M. rosenbergii*	Akarasanon et al., 2004
Membrane integrity	Flow cytometry	*L. vannamei*	Lezcano et al., 2004
Membrane integrity	Eosin-nigrosin	*P. monodon*	Nimrat, 2005
Membrane integrity	Eosin-nigrosin	*L. vannamei*	Nimrat et al., 2006
Membrane integrity	Eosin-nigrosin	*P. monodon*	Bart et al., 2006
Membrane integrity	Eosin-nigrosin	*P. monodon*	Vuthiphandchai et al., 2007
Fertilization and hatching	Artificial insemination	*L. vannamei*	Morales-Ueno et al., 2012
Membrane integrity	Light microscopy	*P. merguiensis*	Memon et al., 2012
Abnormal sperm	Light microscopy	*F. paulensis*	Braga et al., 2013
Membrane integrity	Eosin-nigrosin and flow cytometry	*L. vannamei*	Uberti et al., 2014
Membrane integrity	Eosin-nigrosin	*L. schmitti*	Bambozzi et al., 2014
Membrane integrity	Eosin-nigrosin	*L. schmitti*	Castelo-Branco et al., 2014a
Membrane integrity	Fluorescence microscopy	*L. vannamei*	Silva et al., 2015
Membrane integrity	Eosin-nigrosin and fluorescence microscopy	*L. vannamei*	Castelo-Branco et al., 2015

*References are listed chronologically.

As observed in Table 1 the sperm viability diagnostic techniques in penaeids hardly changed in recent decades, based only on colorimetric methods that evaluate superficial characteristics of cell membrane. Therefore, further efforts should be made to develop new techniques and improve existing methods to be more effective in assessing sperm viability.

9. Sperm cryopreservation in penaeids

Sperm cryopreservation is an important method in biotechnologies such as genetic manipulation and gene banks of endangered species (Memon et al., 2012). Cryopreservation of animal tissues has been improving for many decades and can be performed basically in two ways: slow freezing (traditional methods) and fast freezing (vitrification) (Figure 3).

The traditional method consists in cooling steps before immersion in final freezing temperature in liquid nitrogen, dry-ice or freezer (Figure 3). This gradual cooling can be accomplished manually using liquid nitrogen vapor or by programmable cryogenic devices (Chow et al., 1985; Salazar et al., 2008). The cooling rate can vary from one protocol to another, such as the number of steps before reaching the storage temperature (Bambozzi et al., 2014). However, in penaeids sperm studies applying the traditional method presented results below expectations (Castelo-Branco et al., 2015). For all protocols it was observed loss of sperm viability during the storage period, even if stocked in liquid nitrogen (Anchordoguy et al., 1987; Vuthiphandchai et al., 2007; Salazar et al., 2008; Castelo-Branco et al., 2014a).

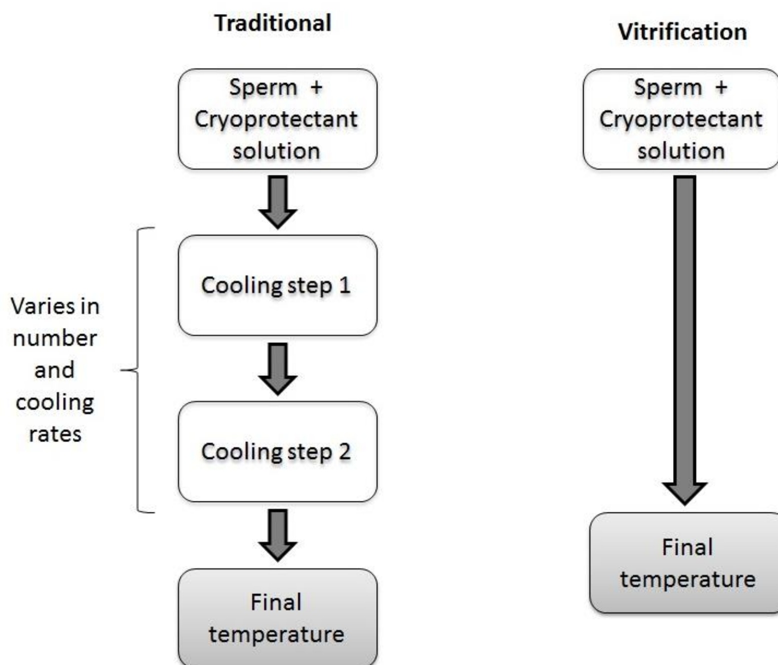

Figure 3. Cryopreservation methods.

Sperm vitrification is a cryopreservation technique that consists in directly immersion in liquid nitrogen and is successfully applied to sperm cryopreservation in fish (Cuevas-Uribe et al., 2011; Merino et al., 2012; Figueroa et al., 2013). Similarly, the vitrification method proved to be efficient for *L. vannamei* sperm mass, providing high and stable sperm viability levels, for up to 120 days (Castelo-Branco et al., 2015).

10. Final considerations

Studies focused on male characteristics are important to outline a new vision for reproductive biotechnologies in penaeids. Many species of penaeid has potential for aquaculture, but researches in reproductive biotechnologies are restricted to the most commercialized species. Thus, knowing the reproductive system, endocrine regulation and sperm characteristics is the first step to improve and develop new biotechnologies such as sperm cryopreservation.

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CAPÍTULO II

Artigo científico publicado em 26 de fevereiro de 2014, no periódico **Invertebrate Reproduction & Development**.

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Todas as normas de redação e citação deste capítulo atendem às estabelecidas pelo periódico (em anexo).

Scanning electron microscopic investigation of spermatophore and spermatozoa of the pink shrimp *Farfantepenaeus subtilis*

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Scanning electron microscopy was used to describe the structure of the spermatozoon and spermatophore of *Farfantepenaeus subtilis*. The spermatophore showed characteristics similar to those of members of the subgenus Farfantepenaeus. This included an extensive glandular epithelium and a lack of a wing. The sperm mass, which was distributed at the periphery of the spermatophore, was surrounded by a large amount of acellular material. The spermatozoon has a spherical main body and a well-defined acrosomal region with a single spike, which was bent in some cells. The immotile sperm cells have an average length of 7.1 ± 0.6 μm. Information on sperm location within the spermatophore will assist in the efficient extraction of the sperm mass during dissection.

Keywords: glandular epithelium; matrix; sperm mass; acrosome; spike

The penaeid shrimp *Farfantepenaeus subtilis* (Farfante 1969) is an important fishery resource in northeastern Brazil. This species is subject to considerable fishing pressure, and despite having great potential for shrimp farming (Nunes et al., 1997) there is little information about their reproduction. This hampers the development of technologies for genebank formation and farming of this species.

The spermatozoa of the penaeidae are aflagellate (Dall et al., 1990) and immotile (Bauer & Martin 1991; Bell & Lightner 1988; Braga et al., 2013) being transferred to females in spermatophores. The spermatophore is formed by specific tissue layers that surround the sperm

sac, which contains the sperm mass. Spermatophores can have different structural arrangements according to the reproductive characteristics of each species (Pérez-Farfante 1975). *F. subtilis* males insert spermatophores into the female thelycum during copulation, which is a typical reproductive behavior of closed thelycum shrimp (Pérez- Farfante 1975). By contrast, the spermatophores of open thelycum penaeids (subgenus Litopenaeus) have an aliform structure (called the wing) distal to it, which is responsible for supporting the spermatophore: anchoring it to the female thelycum (Bauer & Martin 1991).

These different copulatory behaviors among penaeids species lead us to observe the unique characteristics of each species. This study provides new information on the spermatophore and spermatozoal ultrastructure of *F. subtilis*.

The spermatophores of wild-caught *F. subtilis* were obtained by applying gentle pressure with the thumb between the abdomen and the base of the fifth pair of pereopods. Spermatophores were removed with sterile forceps and each one was cut in half to reveal its contents for evaluation of internal structures. The spermatophores were immediately immersed in 2.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.2, at 23 °C, for fixation. The material was post-fixed in 1% osmium tetroxide in 0.1M sodium cacodylate buffer, pH 7.2, for one h at room temperature (23 °C). After three washes in 0.1M sodium cacodylate buffer, material was dehydrated through a graded acetone series, critical point-dried, mounted on copper stubs, and sputter coated with gold. The samples were examined under a scanning electron microscope (QUANTA 200F – FEI) at 20KV, at the Northeast Technology Center (CETENE, Brazil). Three hundred sperm cells (from 10 spermatophores of 10 different males) were measured using the software Image Tool for Windows, version 3.0 (University of Texas Health Science Center, San Antonio).

Cross-sections of the spermatophore show that the sperm mass is concentrated peripherally, near to the spermatophore's outer cuticle (Figure 1A). There is also an extensive area of glandular epithelium (Bauer &Martin 1991) occupying much of the spermatophore

volume (Figure 1A). This is possibly associated with the production of adhesive material responsible for attaching the spermatophore to the female thelycum during copulation (Bauer & Martin 1991). It was also observed that the sperm mass is surrounded by sperm-free material, with dense and uniform characteristics, which may be related to the physical protection of spermatophores from external agents (Figure 1B). The spermatozoa are surrounded by this material forming a sperm matrix (Figure 1 C and D). The sperm cells of F. subtilis have a single appendage, called a spike (Figure 1C and D). Spermatozoa have an average total length of 7.1 ± 0.6 µm and width of 4.9 ± 0.7 µm, and do not have a flagellum (Figure 1D and E). A normal, mature, sperm cell is characterized by a spherical main body and an acrosomal region from which the spike arises (Figure 1 D–F). Some sperm cells had bent spikes (Figure 1 D), which possibly represent a deformity (Dall et al., 1990).

The internal structure of the spermatophores of *F. subtilis* is similar to that of other penaeids being differentiated by the absence of the wing, which is a structure present inside the spermatophore of some species which binds to the sperm mass. The presence of the wing is a common feature in the subgenus Litopenaeus (Malek & Bawab 1974). The wing is associated with the spermatophore binding to the female thelycum during copulation (Pérez-Farfante 1975).

Sperm mass distribution inside the spermatophore varies between species. In *Litopenaeus vannamei*, *L. occidentalis*, and *L. schmitti*, the sperm mass is distributed longitudinally in the center of spermatophore, while in *L. stylirostris* the sperm mass is concentrated in a round bulge at the base of the wing (Pérez-Farfante 1975). *F. subtilis*, as well as *F. aztecus*, and *Litopenaeus setiferus* also have a longitudinal sperm mass, but it is peripheral to the spermatophore body near the outer cuticle (Bauer & Cash 1991). This information can help during spermatophore dissection, to extract the sperm mass quickly and successfully.

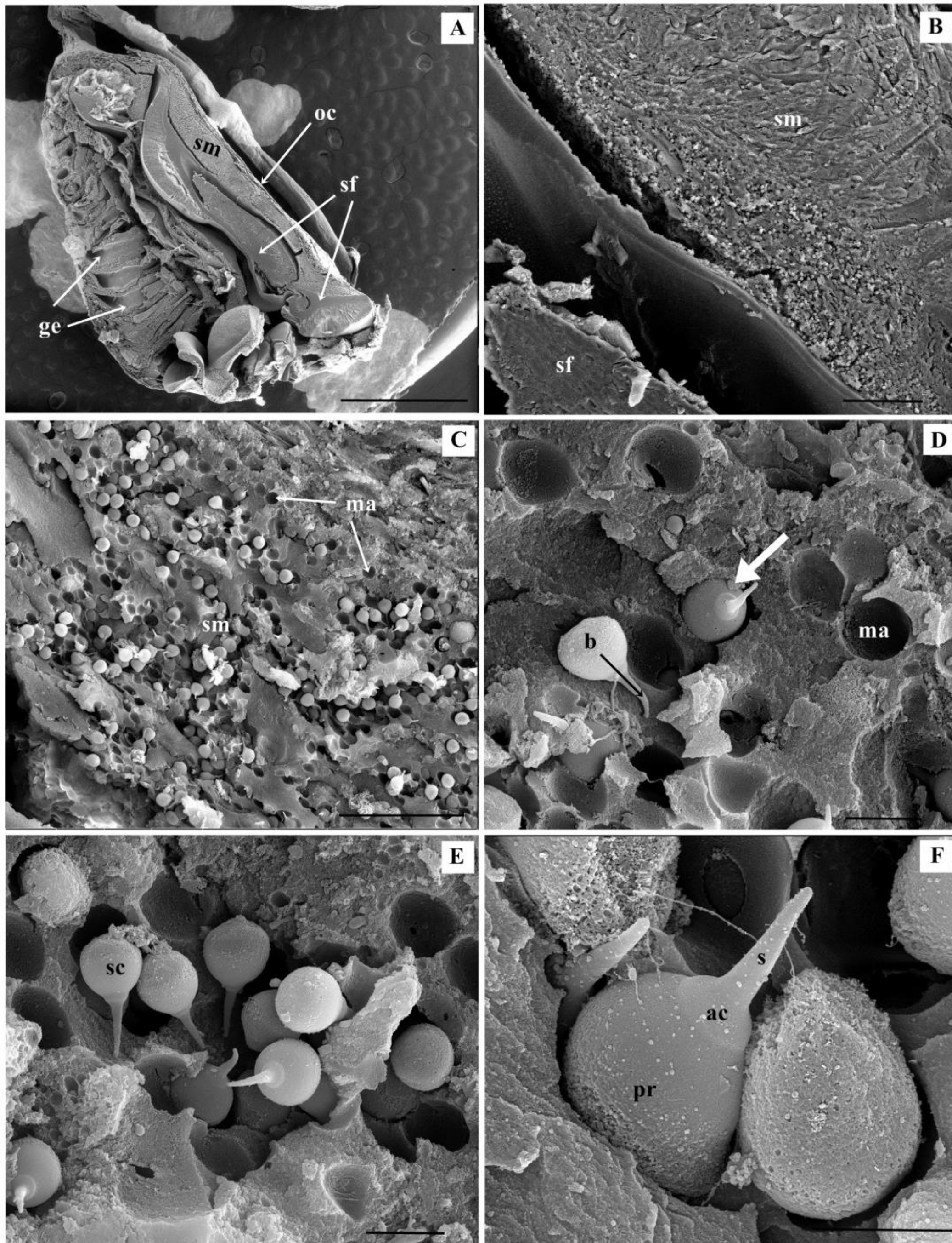

Figure 1. (A)–(C): Transverse section of *Farfantepenaeus subtilis* spermatophore. (A,B): Sperm mass (sm); glandular epithelium (ge); sperm-free material (sf); outer cuticle (oc). (C,D): Sperm matrix (ma). (D): Spermatozoa with bent spike (b); spermatozoon in matrix (white full arrow). (E) Normal sperm cell (sc); (F) Sperm main body posterior region (pr), acrosomal region (ac); spike (s). Scale bar = 2 mm in A; 100 µm in B; 40µm in C; 5 µm in D and E; 3 µm in F.

The sperm mass of F. subtilis is surrounded by sperm-free material (Figure 1(A)–(D)) with dense and uniform characteristics. This material is also found in other penaeids species such as *L. setiferus*, *F. duorarum*, *F. aztecus*, *Trachypenaeus similis*, *Solenocera vioscai* (Bauer and Martin

1991), *Sicyonia ingentis* (Subramoniam 1995), and *Metapenaeus monoceros* (Abraham et al., 2007).

The sperm cells of *F. subtilis* have a structure similar to that found in different penaeid species with a single straight appendage called a spike that originates in the acrosomal region (Medina 1994; Pongtippatee et al., 2007). The spermatozoa of the penaeidae are immotile (Bell & Lightner 1988; Dall et al., 1990; Tudge 2009) and depend on the spermatophore to be stuck to the

female's abdomen during copulation. The spike has to be straight and elongated to fertilize the egg (Dall et al.,1990) and therefore sperm cells with bent or absent spikes may not be able to perform this function (Dall et al., 1990). This deformity is probably due to the packing process of the spermatozoa in the terminal ampulla (Medina 1994). *F. subtilis* spermatozoa share features with the penaeids *Penaeus kerathurus* and *P. japonicas* (Medina et al., 1994), in particular, the spherical main body and well-defined acrosomal region. Additional studies are being undertaken to improve knowledge about the internal characteristics of *F. subtilis* sperm cells and the biochemical profile of the seminal plasma.

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CAPÍTULO III

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Todas as normas de redação e citação deste capítulo atendem às estabelecidas pelo periódico (em anexo).

Cadaveric sperm viability in the white shrimp *Litopenaeus vannamei*

Short communication

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Abstract

This study was carried out to investigate the post-mortem sperm quality of *Litopenaeus vannamei* broodstock under different storage time and temperature under conditions usually found in laboratories and on board. Experimental shrimps were cryo anesthetized and allocated into three experimental groups for up to 96h: Room temperature of 26ºC; cooling temperature of 4ºC; and Freezing temperature of -18ºC. As a control, sperm was extracted from alive shrimp (n= 8) at time 0h. Freezing temperature (-18°C) presented low sperm viability for all tested periods, so it is not recommended for maintenance of *L. vannamei* sperm mass. On the other hand, sperm viability rates remained high (~90%) for 24 and 48h after death when shrimp were kept at room temperature of 26 and 4°C, respectively.

Keywords: cooling, Litopenaeus vannamei, post mortem, sperm

Cadaveric sperm is a potential means to recover gametes of males that die unexpectedly due to environmental disasters (Koteeswaran and Pandian, 2002; Thuwanut et al., 2013). Furthermore, it allows collecting viable spermatophore from wild-caught penaeid for artificial insemination or fisheries research, without the need to keep individuals alive on board. Thus, it is

important to learn about tissues deterioration patterns to adequate methodologies to specific goals.

Many factors influence the rate of cell autolysis after death, such as temperature, pH and dissolved oxygen concentration of water, besides the species and its physiological condition at the death time (Lightner 1973). Additionally, the time interval between death and sperm collection is determinant to cell viability (Kroon et al., 2012). Post-mortem studies on sperm viability have been made on some species such as humans (Kroon et al., 2012), ruminants (Mir et al., 2012) and fish (Koteeswaran & Pandian 2002) showing a constant and irreversible pattern of cell deterioration.

The successful retrieval of cadaveric semen is only possible when the ideal time interval and storage conditions for the dead animals are known. Thus, the present study aimed to investigate the post-mortem sperm quality of *Litopenaeus vannamei* broodstock under different storage time and temperature conditions usually available in aquaculture facilities and fishing vessels.

Mature males of *L. vannamei* (n = 52) with mean body weight of 40 ± 1.27 g and total body length of 19.2 ± 0.15 cm were provided by AQUATEC (Canguaretama/RN - Brazil). Only males presenting no injuries were selected for the tests. Integrity of the antennae, rostrum, pleopods, pereopods and spermatophores was evaluated for selection of individuals. Males were cryo anesthetized by immersion in plastic recipients with ice and were presumed to be dead when they failed to make spontaneous branchial movement and showed no response on being disturbed. Posteriorly, dead males were allocated into three experimental groups for up to 96h: Room temperature (RT) of 26ºC; Cooling temperature (CT) of 4ºC; and Freezing temperature (FT) of -18ºC. As a control, spermatophores (n= 8) were extracted from alive shrimp at time 0h. Spermatophore extrusions from each experimental groups were performed every 12hs by dissection of the terminal ampulla. For the RT group samples were collected only at 12 and 24 h due to the high degree of shrimp deterioration after 48h for spermatophore extrusion.

Immediately after extrusion, the spermatophores were macerated in 1 mL Calcium-free (Ca-F) saline and prepared for sperm viability tests under fluorescence technique (Silva et al., 2015). Spermatophores were subsequently evaluated for apparent sperm viability (ASV) under an epifluorescence microscope (Carl Zeiss, Göttingen, Germany) with DBP 485/20 nm excitation and DBP 580-630 nm emission filters (400 x magnification). To assess ASV, an aliquot of 200 µL of sperm suspension was transferred to 2-mL microtubes, added 5 µL of propidium iodide (PI) (0.5 mgmL-1 in DMSO) and 5 µL of 6-carboxyfluorescein diacetate (CFDA) (0.46 mgmL-1 in DMSO). After that, samples were incubated at 38ºC for ten minutes and an aliquot of 10 µL was placed on a slide and a total of 200 sperm cells were evaluated. The sperm cell was classified as having an intact or damaged membrane when stained green or red, respectively.

Two-way analysis of variance (ANOVA) was applied to evaluate treatments differences using a significance level of $P < 0.05$. Differences among treatments were tested using Tukey´s Honest Significant Difference (HSD) method. Transformed data (arcsine) of viable sperm counts were used for statistical analysis. Data are expressed as means ± S.D, but only untransformed values are presented. The software used was Statistica 7 Software (StatSoft.Inc. Tulsa, OK, USA).

Table 1. Percentage of viable sperm extracted from shrimp *L. vannamei* at 12, 24, 48, 72 and 96 hours post-mortem exposed to temperatures of 26, 4 and -18 °C, compared to *control (time 0h).

	Period post-mortem				
	12 h	24 h	48 h	72 h	96 h
RT (26 °C)	95 ± 3^{Aa}	93 ± 3^{Aa}	-	-	-
CT (4 °C)	96 ± 1^{Aa}	94 ± 2^{Aa}	89 ± 3^{Ab}	80 ± 1^{Bb}	69 ± 2^{Cb}
FT (-18 °C)	9 ± 2^{Ab}	3 ± 1^{Bb}	2 ± 3^{Bc}	2 ± 1^{Bc}	0 ± 1^{Bc}

* Control group. Sperm extracted from alive shrimps at time 0h = 98 ± 1%.
Within the same column (lowercase) and line (uppercase), values without a common superscript are different ($P < 0.05$)

Sperm viability for RT (26°C) and CT (4°C) at 12 and 24h post-mortem did not differ between treatments and control (Table 1). At temperature 4°C sperm viability started to decrease significantly at 48h from control and 72 h when compared to previous time intervals. On the other hand, dead shrimp kept at -18 °C (FT) showed significantly low sperm viability for all tested periods and culminated in total mortality at 96h of storage (Table 1).

The life of testis can be prolonged under ideal conditions of preservation after its removal from alive or recently dead donors (Koteeswaran & Pandian 2002). Histological post-mortem changes were evaluated in Penaeus aztecus juveniles kept at 10, 20 and 30ºC, suggesting that their gonads deteriorated rapidly due to its proximity to the hepathopancreas (Lightner 1973). This could also explain the testicles deterioration at 26ºC observed in the present study, as they became friable with significant changes in color and odor after 24h. Surprisingly, despite of high tissue deterioration, the sperm viability at 26ºC was similar to samples kept at 4ºC and did not differ from control within the initial post-mortem periods (12 and 24h). This method is indicated for maintaining viable cadaveric sperm only for a short time period (up to 24h) when no refrigerator or ice is available.

In conclusion, freezing temperature (-18°C) is not recommended for maintenance of *L. vannamei* sperm mass. On the other hand, cadaveric sperm viability rates remained high (~90%) for 48h after death when shrimp were kept at cooling temperature of 4ºC.

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CAPÍTULO IV

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Todas as normas de redação e citação deste capítulo atendem às estabelecidas pelo periódico (em anexo).

Sperm vitrification in the white shrimp *Litopenaeus vannamei*

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ABSTRACT

This study aimed to evaluate the vitrification method for *Litopenaeus vannamei* sperm cryopreservation by using soy lecithin as cryoprotectant. For the toxicity assay three intracellular cryoprotectants were tested: dimethyl sulfoxide (DMSO), ethylene glycol (EG) and methanol (MeOH), at the final concentrations of 5, 10, or 30% (v/v) using sterile calcium-free saline solution (Ca-F) as the extender medium. After extrusion, the sperm masses were immersed in 0.5 mL of cryoprotectant solutions for 10, 30 and 120 min, posteriorly apparent sperm viability (ASV) was assessed by eosin-nigrosin stain technic. Methanol was selected due to its minimal reduction (< 21%) of apparent sperm viability (ASV) at a concentration of 30 % and an exposure time of 120 min. The soy lecithin was evaluated as extracellular cryoprotectant in the vitrification method, associated or not with methanol. For vitrification sperm masses were immersed in cryoprotectant solutions (1) L-Ca + 0.4M trehalose (base solution), (2) base solution + 30% MeOH, (3) soy lecithin 1 % + base solution + 30% MeOH (4) soy lecithin 2% + base solution + 30 % MeOH (5) soy lecithin 1% + base solution and (6) soy lecithin 2% + base solution. Then the samples were immersed directly in liquid nitrogen (vitrification), stored and assessed after 1, 30, 60 and 120 days by fluorescence microscopy. Soy lecithin did not influence the ASV, as this remained high and stable throughout the 120 days for all tested solutions and with an average of 88 ± 1.6%. Considering the high rates of sperm viability, the vitrification method proved to be efficient for cryopreservation of *L. vannamei* sperm mass.

Key-words: Cryopreservation; Cryoprotectant; *Litopenaeus vannamei*; Sperm mass; Lecithin

1. Introduction

The white shrimp *Litopenaeus vannamei* has been excelling in the global aquaculture (FAO 2012). The development of sperm cryopreservation techniques can promote reliable sources of genetic material of high quality for artificial insemination procedures, as well as the possibility of short-term storage and transportation of spermatophores in specialized breeding programs (Nimrat et al., 2006). Although slow freezing cryopreservation protocols have been developed for *L. vannamei* (Nimrat et al. 2006; Salazar et al. 2008; Uberti et al. 2014) and other penaeids species (Bart et al. 2006; Memon et al. 2012; Castelo-Branco et al. 2014), its achievements are below expectations due to decrease of sperm viability over the stocking period.

The vitrification method consists of direct immersion of the semen in liquid nitrogen and it has been successfully applied to cryopreservation of fish sperm cells (Cuevas-Uribe et al. 2011a; Merino et al. 2012; Figueroa et al. 2013). A classic definition of vitrification is that it is the solidification of a liquid caused not by crystallization, but by an extreme rise in viscosity during cooling. However, due to the sudden reduction of temperature, it is necessary an extra protection to sperm membrane by rising cryoprotectant concentration (Fahy et al. 1984). Nevertheless, the tolerance of spermatozoa to different cryoprotectants and their concentrations used in freezing procedures is decisive for the success of cryopreservation (Vuthiphandchai et al. 2007).

Cryoprotectants are classified into two classes according to its capacity to permeate cell membrane: intracellular and extracellular agents. Glycerol, dimethyl sulfoxide (DMSO) and methanol (MeOH) are the main intracellular agents for sperm cryopreservation of most species, including penaeid shrimp (Curry, 2000). These cryoprotectants can permeate into sperm membrane and prevent injuries caused by the variation of solutes concentration during freezethaw (Holt et al. 1992). Extracellular cryoprotectants adhere to the outer face of the plasma membrane, also preventing damage caused by freeze-thaw events, like ice crystals formation. They are macromolecules, usually sugars such as sucrose, raffinose or lipoproteins and proteins derived from milk, egg yolk and vegetable oils (Moraes et al. 1998). Lecithin is one of the most common extracellular cryoprotectants and can be extracted from egg yolk or soybean. These agents are widely used in cryopreservation germinal cells of mammals, birds and fishes (Brook et al. 1986; Bianchi et al. 2011; Makhafola et al. 2009; Neves et al. 2014), but there is no report for penaeid sperm cryopreservation. This study aimed to develop vitrification protocols for *L. vannamei* sperm cryopreservation and the use of soy lecithin as extracellular cryoprotectant.

2. Material and methods

2.1. Broodstock management

Healthy, mature males of *L. vannamei* (n = 55) with mean body weight of 40 ± 1.27 g and total body length of 19.2 ± 0.15 cm were provided by AQUATEC (Canguaretama/RN - Brazil). Males were allocated in three 350-L tanks (18 shrimps/tank) with seawater salinity 34 in a closed recirculating system. It was provided a natural photoperiod of approximately 12-h light: 12-h dark. Shrimps were fed three times a day (0900, 1400 and 1800 h) with fresh squid (Illex sp.), mussels (Mitilus sp.) and commercial maturation diet (Breed S Fresh, INVE Aquaculture, Belgium) offered alternately at 5% rate of wet body weight. All organic waste was removed every morning from the botton of tanks. Water quality parameters of temperature, pH and dissolved oxygen were monitored daily at 0900 and varied between 26 and 27 ^{o}C, 7.0 and 7.8 and between 5.5 and 6.8 mg/l, respectively.

2.2. Collection of spermatophores

All spermatophores were obtained by gently pressing around the coxas of the fifth pair of pereiopods at the terminal ampoules. Only spermatophores that were not melanized were selected for toxicity and cryopreservation studies. A total of 110 spermatophores were collected and their sperm masses were extracted with forceps and spatula.

2.3. Toxicity tests

Three intracellular cryoprotectants were tested: dimethyl sulfoxide (DMSO), ethylene glycol (EG) and methanol (MeOH) (Sigma Aldrich Chemicals, St. Louis, MO, USA). Cryoprotectant solutions were prepared to final concentrations of 5, 10, or 30% (v/v) using sterile calcium-free saline solution (Ca-F) as the extender medium. The composition of Ca-F is 21.63 g NaCl, 1.12 g KCl, 0.53 g H3BO3, 0.19 g NaOH and 4.93 g MgSO4.7H2O in 1 L sterile distilled water (adjusted to pH 7.4 with HCl) (Vuthiphandchai et al. 2007). After extrusion, the sperm masses were immediately immersed in 0.5 mL of cryoprotectant solutions in 2-mL microtubes (Labware Manufacturing CO, China). The apparent viability of sperm cells was assessed after 10, 30 and 120 min of exposure at 25 ^{o}C. Sperm masses were removed from cryoprotectant solutions rinsed twice with Ca-F saline at a similar temperature and macerated in 1.0 mL Ca-F saline to assess apparent sperm viability (ASV) by eosin-nigrosin stain technique (Jeyalactumie and Subramoniam, 1989). Each cryoprotectant concentration tested had five replicates. No toxicity tests were performed for lecithin because it is considered a non-toxic surfactant of high tolerance by the organism (Brook et al. 1986).

2.4. Development of the freezing protocol

The MeOH was selected as intracellular cryoprotectant on the basis of the toxicity test results. Previously, powder soy lecithin (Sigma Aldrich Chemicals, St. Louis, MO, USA) was diluted in ethanol. After extrusion, sperm masses were immediately immersed in the cryoprotectant solutions, according to the following treatments with ten replicates each: (1) Ca-F saline + trehalose 0.4M (base solution), (2) Base solution + 30% MeOH, (3) Base solution + 30% MeOH+1% Soy lecithin, (4) Base solution + 30% MeOH+2% Soy lecithin, (5) Base solution +1% Soy lecithin and (6) Base solution +2% Soy lecithin. Sperm masses were exposed to cryoprotectant solutions at room temperature (25 °C) for 10 min before being immersed directly in liquid nitrogen for vitrification. Sperm masses were stored in liquid nitrogen and samples (n=60/period of cryopreservation) were thawed after 1, 30, 60 and 120 days for assessment of sperm cell membrane integrity by fluorescence microscopy. Evaluation of total sperm cell count was assessed by homogenizing thawed sperm mass in 1 mL Ca-F saline (sperm suspension) and counting using a hemacytometer under a light microscope. As a control group ten freshly collected sperm masses were immersed in Ca-F saline without cryoprotectant (Day 0).

2.5. Sperm viability assessment by fluorescence microscopy

Thawed spermatophores were subsequently evaluated for ASV under an epifluorescence microscope (Carl Zeiss, Göttingen, Germany) with DBP 485/20 nm excitation and DBP 580-630 nm emission filters (400 x magnification). To assess ASV, an aliquot of 200 µL of sperm suspension was transferred to 2-mL microtubes, added 5 µL of propidium iodide (PI) (0.5 mg/mL in DMSO) and 5 µL of 6-carboxyfluorescein diacetate (CFDA) (0.46 mg/mL in DMSO). After that, samples were incubated at 38ºC for ten minutes and an aliquot of 10 µL was placed on a slide and a total of 200 sperm were evaluated. The sperm cell was classified as having an intact or damaged membrane when stained green or red, respectively. As penaeids spermatozoa are non-motile, tests of motility and vigor are not used for viability assessment.

2.6. Data analysis

Two-way analysis of variance (ANOVA) was applied to evaluate treatments differences using a significance level of $P < 0.05$ in both toxicity and cryopreservation experiments. Differences among treatments were tested using Tukey´s Honest Significant Difference (HSD) method. Transformed data of viable sperm counts on toxicity (arcsine) and vitrification (Rnd values) tests were used for statistical analysis. Data are expressed as means ± S.D, but only untransformed

values are presented. The software used was Statistica 7 Software (StatSoft.Inc. Tulsa, OK, USA).

3. Results

3.1. Toxicity tests

The increase in cryoprotectants concentration caused significant reduction in ASV for most of tested solutions (Table 1). For DMSO treatment, ASV values were significantly lower for all concentrations and exposure periods when compared to control. The DMSO was the most toxic intracellular cryoprotectant agent tested in this study, reaching an average value of 48% ASV for 30% DMSO at 120 min of exposure.

All EG treatment values were significantly lower than the control, but ASV for concentrations of 5% (ASV of 81–88%) and 10% (ASV of 77–87%) were considered satisfactory for all exposure periods. The same was observed for 30% EG at 10 min of exposure, however, at 30 and 120 min ASV was lower ($P < 0.05$) than concentrations of 5 and 10% at the same exposure period (Table 1).

Table1. Percentage of apparent sperm viability of *L. vannamei* sperm mass after exposition to the cryoprotectants dimethyl sulfoxide (DMSO), ethylene glycol (EG) and methanol (MeOH) in different concentrations and exposure periods.

Cryoprotectant (%)	Exposure period (min)		
	10	30	120
DMSO			
5	90.8 ± 1.7 ^{b}	77.3 ± 1.7 ^{c}	67.5 ± 1.7 ^{c}
10	74.3 ± 1.9 ^{c}	67.3 ± 3.1 ^{d}	64.3 ± 1.7 ^{c}
30	71.0 ± 1.8 ^{c}	65.3 ± 1.7 ^{d}	48.8 ± 2.2 ^{d}
EG			
5	88.5 ± 1.3 ^{b}	83.5 ± 1.3 ^{b}	81.8 ± 1.5 ^{b}
10	87.3 ± 2.2 ^{b}	82.5 ± 2.1 ^{b}	77.3 ± 2.5 ^{b}
30	87.5 ± 1.3 ^{b}	78.8 ± 1.7 ^{c}	67.3 ± 1.9 ^{c}
MeOH			
5	96.5 ± 1.3 ^{a}	83.8 ± 1.0 ^{b}	84.3 ± 5.9 ^{b}
10	95.3 ± 1.7 ^{a}	80.0 ± 0.8 ^{b}	78.0 ± 3.6 ^{b}
30	86.0 ± 3.2 ^{b}	81.8 ± 3.4 ^{b}	79.5 ± 1.3 ^{b}
*Control	97.7 ± 1.9 ^{a}	95.4 ± 2.8 ^{a}	94.1 ± 2.9 ^{a}

$^{a\text{-}d}$ Values with different letters within same column are significantly different ($P < 0.05$) among cryoprotectants.
*As a control group freshly collected sperm masses were immersed in Ca-F saline without cryoprotectant.

The ASV at 5 and 10% MeOH at 10 min of exposure period did not differ but were higher ($P < 0.05$) than 30% MeOH at the same period (Table1). However, the ASV for 30%MeOH (79%) at

the longest exposure period tested was significantly higher than DMSO and EG values (Table 1). The MeOH was the least toxic to sperm cells among intracellular cryoprotectants evaluated in this study.

3.2. Vitrification method

Apparent sperm viability did not differ significantly from control in any cryoprotectant solutions tested (Table 2). Treatments containing soy lecithin in concentrations of 1 and 2% did not differ significantly from control or treatments without this agent. Regardless the treatment, the ASV remained high and stable throughout the 120 days of cryopreservation by vitrification in liquid nitrogen, with an overall mean value of 88 ± 1.6%.

Table 2. Percentage of apparent sperm viability of *L. vannamei* sperm mass after cryopreservation by vitrification method up to 120 days at different cryoprotective solutions.

*Cryoprotectant solutions	Cryopreservation period (days)			
	1	30	60	120
S1	92.8 ± 2,4	84.9 ± 5.0	85.6 ± 4.8	90.1 ± 2.4
S2	89.5 ± 5.6	86.5 ± 4.0	90.8 ± 3.5	85.5 ± 6.6
S3	89.6 ± 4.0	85.9 ± 6.3	87.2 ± 4.4	86.1 ± 4.2
S4	89.2 ± 5.0	86.3 ± 5.0	89.8 ± 5.4	91.3 ± 2.3
S5	93.3 ± 3.0	85.3 ± 4.9	86.8 ± 5.4	89.1 ± 4.8
S6	91.8 ± 3.0	84.5 ± 4.5	85.9 ± 4.5	91.1 ± 3.9
Control	93.3± 2.1	93.3± 2.1	93.3± 2.1	93.3± 2.1

* Cryoprotectant solutions: S1= Base solution, S2= Base solution + 30% MeOH, S3= base solution + 30% MeOH +1% Soy lecithin, S4= base solution + 30% MeOH+2% Soy lecithin, S5= Base solution +1% Soy lecithin and S6= Base solution +2% Soy lecithin.
There were no significant differences among values ($P > 0.05$) within same columns and lines.
As a control group ten freshly collected sperm masses were immersed in Ca-F saline without cryoprotectant (Day 0). The control group was not cryopreserved.

4. Discussion

As vitrification method requires high levels of membrane protection, MeOH was selected as the preferred intracellular cryoprotectant on the basis of its minimal reduction (<21%) of ASV for

higher concentration (30%) and longer period (120 min) in the toxicity tests. Methanol was associated with lecithin (extracellular cryoprotectant) as a cryoprotectant solution during vitrification of *L.vannamei* sperm mass. The effectiveness of MeOH as intracellular cryoprotectant has been previously reported for *L. vannamei* sperm cells (Lezcano et al. 2004). However, it was reported to be toxic for *P. monodon* spermatophores (Bart et al. 2006, Vuthiphandchai et al. 2007) and embryos (Vuthiphandchai et al. 2005) even in low concentrations (5 and 10%) and short exposure periods (10 min). Although MeOH showed overall higher results of ASV, the other agents tested did not cause acute toxicity even at 30% concentration and 120 min exposure period.

The most toxic intracellular cryoprotectant in this study was DMSO, probably because of its high membrane permeability that increases the toxicity potential (Anchordoguy et al. 1987). Furthermore, one of the protective mechanisms of DMSO involves a hydrophobic interaction with the bilayer and these interactions are temperature dependent, thus higher concentrations of DMSO could cause destabilization of cell membrane proteins (Arakawa et al. 1990). This could explain the high degree of DMSO toxicity observed in this work at room temperature, mainly at the higher concentration. In accordance, other studies reported low sperm viability for decapods when DMSO was used as intracellular cryoprotectant (Jeyalactumie and Subramoniam, 1989; Lezcano et al. 2004). However, DMSO was indicated for sperm cryopreservation of the marine shrimp Scycionia ingentis (Anchordoguy et al. 1987) and *Penaeus monodon* (Bart et al. 2006, Vuthiphandchai et al. 2007).

The satisfactory sperm viability results of EG for all concentrations and exposure periods in this study, indicated its low toxic effect for *L. vannamei* sperm mass. Although satisfactory results of EG have been observed for embryos and larvae cryopreservation in marine shrimps (Newton et al. 1996). Although satisfactory results of EG have been reported in toxicity tests for *Macrobrachium rosenbergii* (Akarasanon et al. 2004) and *L. vannamei* (Salazar et al. 2008; Lezcano et al. 2004) spermatophores, it was considered toxic to sperm cells of *P. monodon* (Vuthiphandchai et al. 2007) and *Penaeus merguiensis* (Memon et al. 2006) after long periods, even in low concentrations.

Lecithin (phosphatidylcholine) is the main component of the phosphate fraction of the bilayer membrane, and an important structure participating in the maintenance of membrane fluidity, as well as in cellular protection against oxidation (Brook et al. 1986). In the present study, lecithin addition to cryoprotectant solution did not affect the ASV results, which is probably due to the association with trehalose present in the base solution. Previous studies reported that trehalose

was very efficient in preserving membranes and liposomes during freezing procedures (Crowe et al. 1985, 1986). It binds to the phospholipid head group, which causes an expansion of monolayers reducing the transition temperature of liquid crystalline to gel phase. Despite of trehalose not be able to permeate the membrane bilayer it binds externally providing more stability during the freezing process, even in low concentrations (Anchordoguy et al. 1987).

Apparently, this is the first report of decapods sperm cryopreservation using the vitrification method. The results obtained in this study, brings new information on freezing protocols for decapods sperm. Although the sperm viability levels in slow freezing protocols are usually satisfactory for sperm cryopreservation of decapods (Salazar et al. 2008; Vuthiphandchai et al. 2007; Anchordoguy et al. 1987), they are below expectation when compared to results obtained for fishes (Figueroa et al. 2013) and mammals (Sánchez et al. 2011). Moreover, studies with slow freezing protocols have showed significant decrease sperm viability over the stocking period in liquid nitrogen (Diwan and Shoji, 1999; Vuthiphandchai et al. 2007; Salazar et al. 2008; Memon et al. 2012; Castelo-Branco et al. 2014), which contradicts the purpose of cryopreservation that is to maintain the structural integrity and cell viability indefinitely.

Although it has been suggested that fast freezing increases the probability of intracellular ice formation (Vuthiphandchai et al. 2007), in the present study the vitrification method resulted in long term cryopreservation with constant and high sperm survival rates. This may be associated with the presence of trehalose in the base solution, indicating that *L. vannamei* sperm mass can be directly immersed in liquid nitrogen (vitrification) without intracellular cryoprotectants. A similar result was observed for rainbow trout during the spermatozoa vitrification, with high sperm viability rates without an intracellular cryoprotectant using bovine serum albumin and sucrose (Merino et al. 2012). However, sperm vitrification of the ornamental fish *Xiphophorus hellerii* presented better results with the association of glycerol and ethylene glycol as intracellular cryoprotectants (Cuevas-Uribe et al. 2011b). On the other hand, the sperm vitrification of the channel catfish (Ictalurus punctatus) resulted in poor viability using only intracellular cryoprotectants (Cuevas-Uribe et al. 2011a).

5. Conclusions

We can infer that methanol is the least toxic intracellular cryoprotectant to *L. vannamei* sperm cell. All the other solutions tested were suitable for sperm mass cryopreservation. It is suggested that trehalose combined or not with soy lecithin is efficient as extracellular cryoprotectant for vitrification protocol. Thus, considering the sperm viability leves, the vitrification method

proved to be efficient for *L. vannamei* sperm mass cryopreservation for up to 120 days, without intracellular cryoprotectants. It can be associated with the presence of trehalose in the base solution.

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CAPÍTULO V

Artigo científico a ser submetido ao periódico **Aquaculture**.
Todas as normas de redação e citação deste capítulo atendem às estabelecidas pelo periódico (em anexo).

Artificial insemination with vitrified sperm from the pacific white shrimp *Litopenaeus vannamei*

Thaís Castelo-Branco [a,*], Maria Madalena Pessoa Guerra [b]; Roberta Soares [a]; Silvio Peixoto [a].

Abstract

Sperm cryopreservation in penaeids is still a developing technology, but shows promising results. Vitrification is a fast freezing method and apparently has potential for penaeids sperm cryopreservation. Therefore, the aim of this study was to evaluate the efficiency of four cryoprotectant solutions for sperm vitrification. The tested solutions were: 30% methanol; 2% soy lecythin; 30% methanol + 2% soy lecithin; and as a control group, fresh sperm mass. Full mature *L. vannamei* females were artificially inseminated with vitrified sperm, after 3 and 15 hours fertilization and hatching rates were evaluated, respectively. All treatments used for artificial insemination showed high sperm viability after 150 days of cryopreservation. Although fertilization rate was satisfactory (above 50%) for all treatments, nauplii hatching were observed only in control group. Thus the low hatching rates indicates that embryonic development is interrupted at some point. This is the first report of artificial insemination using vitrified sperm in penaeids.

1. Introduction

Given the importance of *Litopenaeus vannamei* to global aquaculture, biotechnology studies applied to the various stages of production have been developed. However, sperm cryopreservation in penaeids is still a developing technology even showing promising results (Vuthiphandchai et al. 2007). Cryopreservation methodologies vary in cooling rate speeds from slow to fast freezing protocols. In penaeids sperm cryopreservation, the slow freezing protocols

has been usually performed, but sperm viability is reduced during storage, even in liquid nitrogen (Nimrat et al. 2006; Salazar et al. 2008).

Recently, our group performed a study of sperm vitrification in *L. vannamei* as a new approach for sperm cryopreservation in penaeids, presenting high rates of sperm viability for up to 120 days (Castelo-Branco et al. 2015). Vitrification is a fast freezing protocol consisting on direct immersion of sperm in liquid nitrogen. This sudden cooling can cause great damage to sperm cell, so it requires high concentrations of cryoprotectants to avoid it (Fahy et al. 1984). Sperm cell tolerance to different cryoprotectants and their concentrations during freezing procedures is determinant for cryopreservation success (Vuthiphandchai et al. 2007). Methanol is an intracellular cryoprotectant and its toxicity was tested in penaeids sperm providing high cell viability, even in high concentrations (Lezcano et al. 2004; Castelo-Branco et al. 2015). Extracellular cryoprotectants have also been used in cryopreservation protocols (Bianchi et al. 2011; Neves et al. 2014). Trehalose and lecithin are widely used as extracellular cryoprotectants and proved to be efficient during vitrification protocol for *L.vannamei* sperm (Castelo-Branco et al. 2015).

Although the viability of sperm vitrification technique has been proved by membrane integrity studies, the next step to attest the efficiency of cryopreservation technique would be the use of this sperm for artificial insemination. Therefore, the aim of this study was to evaluate the efficiency of sperm vitrification technique by using vitrified sperm on artificial insemination in the white shrimp *L. vannamei.*

2. **Materials and methods**

2.1 Broodstock management

2.1.1 Males

Healthy, mature *L. vannamei* males (n = 20) with mean body weight of 40 ± 1.3 g and total body length of 19.2 ± 0.15 cm were provided by AQUATEC (Canguaretama/RN - Brazil). Males

were allocated in 350-L tank with seawater salinity 34 in a closed recirculating system. It was provided a natural photoperiod of approximately 12-h light: 12-h dark. Shrimps were fed three times a day (0900, 1400 and 1800 h) with fresh squid (Illex sp.), mussels (Mitilus sp.) and commercial maturation diet (Breed S Fresh, INVE Aquaculture, Belgium) offered alternately at 5% rate of wet body weight. All organic waste was removed every morning from the botton of tanks. Water quality parameters of temperature, pH and dissolved oxygen were monitored daily at 0900 and varied between 26 and 27 ^{o}C, 7.0 and 7.8 and between 5.5 and 6.8 mg/l, respectively.

2.1.2 Females

Fully mature *L. vannamei* females (n=20) with mean body weight of 43 ± 1.4 g and total body length of 30.2 ± 0.3 cm were selected from the commercial broodstock of GENEARCH Aquacultura Ltda (Rio do fogo/RN - Brazil). Females were allocated in 5000-L tanks with seawater salinity 30. It was provided an artificial photoperiod of 12-h light: 12-h dark. Shrimps were fed three times a day (0900, 1400 and 1800 h) with fresh squid (Illex sp.), mussels (Mitilus sp.) and commercial maturation diet offered alternately at 5% rate of wet body weight. All organic waste was removed every morning from the botton of tanks. Water quality parameters of temperature, pH and dissolved oxygen were monitored daily at 0900 and varied between 29.8 and 30.8 ^{o}C, 7.0 and 7.8 and between 6.5 and 6.8 mg/l, respectively. Females with ripe ovaries were selected to perform artificial insemination.

2.2 Collection of spermatophores and sperm mass

All spermatophores were obtained by gently pressing around the coxas of the fifth pair of pereiopods at the terminal ampoules. Only spermatophores that were not melanized were selected for toxicity and cryopreservation studies. Males during molt or recently molted were not used for spermatophore extrusion. A total of 32 spermatophores were collected and their sperm masses were extracted with forceps and spatula.

2.3 Sperm mass vitrification and thawing

For vitrification, sperm masses were immersed directly in liquid nitrogen according to the protocol suggested by Figueroa et al. (2013). The cryoprotectant solutions used in this study were prepared based on Castelo-Branco et al. (2015) sperm vitrification protocol for penaeids. Cryoprotectant solutions tested are described below:

- M - Base solution + 30% methanol
- L - Base solution +2% soy lecythin
- LM - Base solution + 30% methanol + 2% soy lecythin
- F - Fresh sperm mass, extracted immediately before artificial insemination (Control group).

Sperm masses (n=8/treatment) were immediately immersed in 0.5 mL of cryoprotectant solutions in 2-mL microtubes (Labware Manufacturing CO, China) and cryopreserved in liquid nitrogen for 150 days. Moments before insemination cryopreserved sperm masses (n=5/treatment) were thawed by immersion of microtubes in a 36ºC water bath until complete sample melting (~ 1min).

2.4 Fluorescence microscopy

Previous to artificial insemination, samples (n=3/treatment) of vitrified sperm masses were evaluated for membrane integrity under an epifluorescence microscope (Carl Zeiss, Göttingen, Germany) with DBP 485/20 nm excitation and DBP 580-630 nm emission filters (400× magnification) (Silva et al. 2015). A 200 µL aliquot of sperm suspension was transferred to 2-mL microtubes, to which 5 µL of propidium iodide (PI) (0.5 mg/mL in DMSO) and 5 µL of 6-carboxyfluorescein diacetate (CFDA) (0.46mg/mL in DMSO) were added. Samples were then incubated at 38 °C for 10 min, an aliquot of 10 µL was placed on a slide and a total of 200 sperm cells per sample were evaluated. The sperm cell was classified as having an intact or damaged membrane when stained green or red, respectively.

2.5 Artificial insemination

Artificial insemination was performed by placing the recently thawed sperm mass to the thelycum, between the base of the fifth pair of pereopods perpendicular to the long axis of the body, with forceps. Then the pereopods were returned to their normal position, securing the sperm mass in place. Immediately after insemination females (n=5/treatment) were placed individually in 80-L spawning containers. The water parameters were checked and maintained under suitable conditions for spawning, with an average temperature of 30 °C and salinity 30. Females were checked every hour for spawning detection.

A total of 16 spawnings were observed (Table 1). Three hours after spawning, a 50 mL sample was collected after homogenization from each tank for egg fertilization verification. Then, females were removed from containers and the water drained for egg collection. The eggs were washed and allocated into 30-L conic-cylindric tanks to hatching. After 15 hours all carboys were drained, then eggs and nauplii were collected and fixed for later analysis. Eggs presenting cell divisions were considered fertilized. All samples of eggs (3h) and nauplii (15h) were fixed in 4% formaldehyde solution and analysed by light microscopy connected to a video camera. Images of at least 50 eggs/nauplii per replicate (spawning container) were captured and digitized to determine the fertility rate and size of the eggs/nauplii by using the software ImageTool version 3.0 for Windows (The University of Texas Health Science Center in San Antonio, USA). The total number of eggs/nauplii per treatment was accessed by counting in a hemocytometer. All artificial insemination procedure was performed in the GENEARCH Aquacultura Ltda (Rio do fogo/RN - Brazil) facilities.

2.6 Statistical analysis

Sperm count, viability, fertilization rates and hatch rates were analysed by one-way ANOVA. Differences were considered significant at $P<0.05$. The effects of the cryoprotectants on sperm viability, fertilization and hatch were tested using Tukey's Honest Significant Difference (HSD)

method. Transformed data of viable sperm counts (Rnd values) were used for statistical analysis. Data are expressed as means±S.D., but only untransformed values are presented. The software used was Statistica 7 Software (StatSoft.Inc. Tulsa, OK, USA).

3. Results

3.1 Sperm viability

Sperm viability remained high and stable over the 150 days of cryopreservation for all tested treatments (Table 1).

3.2 Artificial insemination

In the analysis of 3 hours after-spawning samples, all treatments showed eggs with cell division patterns consistent with fertilization (Figure 1). The most common stages were: presence of polar body (Figure 1A) and morula (Figure 1B), followed by advanced stage embryo (Figure 1C), which was less observed.

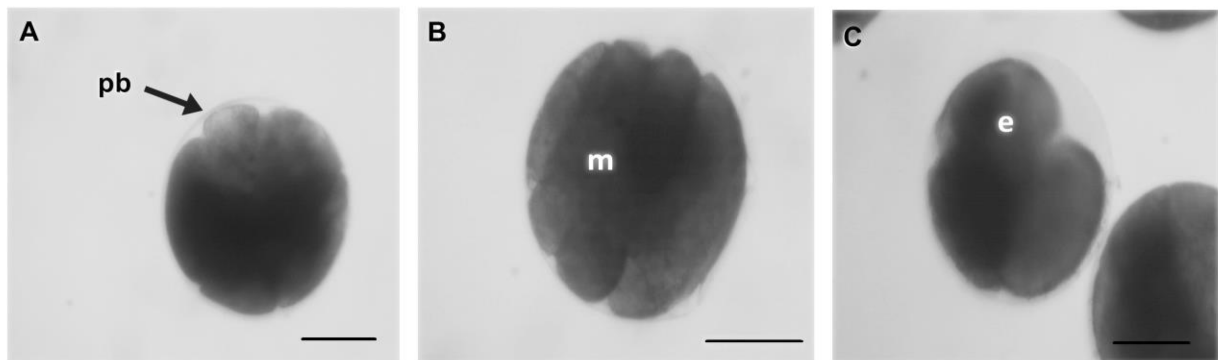

Figure 1. Fertilized eggs at different stages of embryonic development 3 hours after spawning using artificially inseminated vitrified sperm mass. Storage period in liquid nitrogen: 150 days. A. formation of the first polar body (pb); B. morula (m); C. embryo in advanced stages of development (e). The above stages were observed in all treatments in this study. Scale bar = 100μm.

Fertility rates varied significantly among treatments (Table 1), the group of females inseminated with the LM treatment presented the lowest fertilization rate (~ 54%). The M treatment did not differ from the control group or the L treatment, as overall fertilization rates were above 70%. Although all treatments presented fertilization rates above 50%, samples

collected 15h post-spawning showed low hatching rates (~ 12%). Nauplii (Figure 2A) were observed only in 3 of the 4 spawnings in the control group.

Table 1. Mean value (± SD) of fertilization (3h), hatching (15h) rates and number of eggs per spawning (n=4 / treatment) after artificial insemination with vitrified sperm mass kept in liquid nitrogen for 150 days. Methanol (M) and lecithin (L), were used individually or in combination (LM) as cryoprotectants agents with respective mean percentage of sperm viability. As a control group, females were inseminated with fresh sperm mass (F) extracted just before the insemination procedure.

	Sperm viability* (%)	**Fertilization rate (%)**	**Hatching rate (%)**	**Number of eggs**
F (n=4)	-	80.5±5.5^{a}	12.5 ±1.7^{a}	73019 ± 701.8
M (n=3)	89.5 ± 5.6	74.0±3.2 ^{ab}	0 b	91388 ± 1877.8
L (n=4)	91.8 ± 3.0	73.0±2.6 ^{bc}	0 b	68438 ± 1508.4
LM (n=5)	89.2 ± 5.0	54.4±2.6 ^{c}	0 b	65833±1731.4

$^{a-c}$ Values with different letters within the same column differ ($P < 0.05$) between cryoprotectants.
* There was no difference ($P > 0.05$) among the sperm viability values after 150 days of cryopreservation.
** Total number of eggs: average of replicates for each treatment. There was no difference ($P > 0.05$) in number of eggs between treatments.

All the spawns presented eggs at different stages of embryonic development (Figure 2A) or with irregular cell divisions (Figure 2B). This suggests that the embryo development was initiated but stopped at some point. A standard for termination of embryonic development was not found.

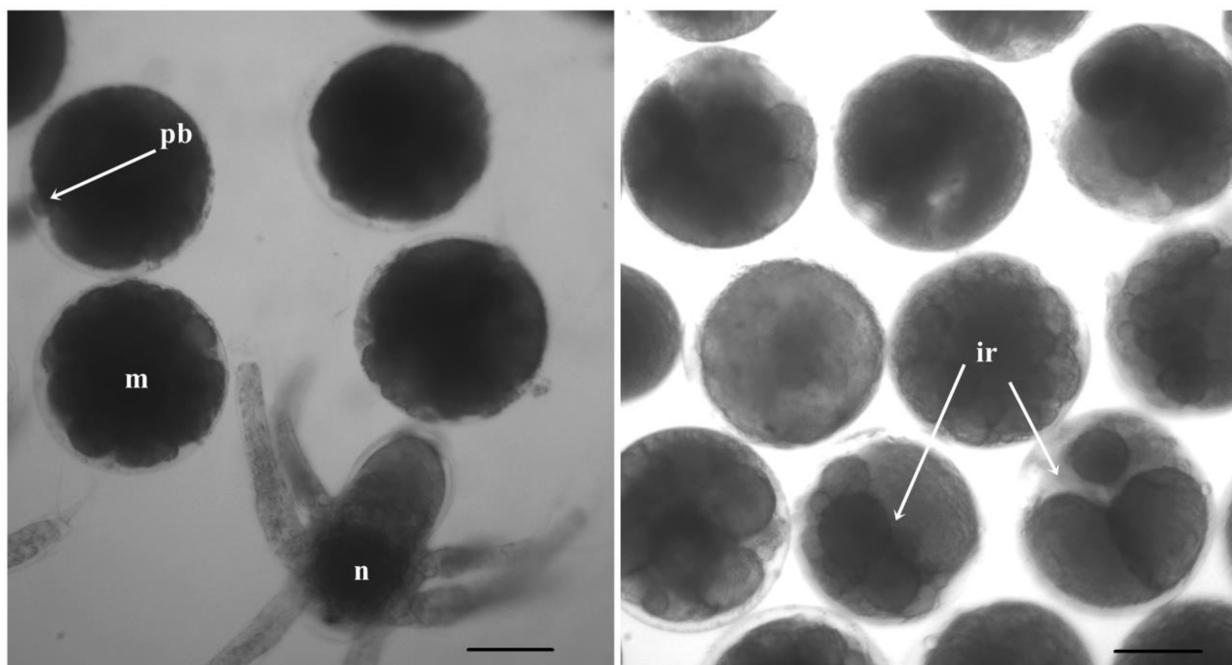

Figure 2. Eggs at different stages of embryonic development and nauplii 15 hours after artificial insemination. Storage period in liquid nitrogen: 150 days. A. polar body (pb); morula (m); nauplius (n). B. irregular cell division (ir). Scale bar = 100µm.

The size of the eggs varies among treatments within both 3 and 15 h post-spawning periods, but the LM treatment showed the highest diameter (Table 2).

Table 2. Mean (± SD) egg diameter (µm) and nauplii lengh (µm) per spawning after artificial insemination with vitrified sperm mass kept in liquid nitrogen for 150 days. Methanol (M) and lecithin (L), were used individually or in combination (LM) as cryoprotectants agents. As a control group, females were inseminated with fresh sperm mass (F) extracted just before the insemination procedure.

	Egg diameter (µm)		Nauplii lengh (µm)
	3 h	15 h	
F	319,0±9,4^{b}	322,9±6,9^{a}	401,3±3,2
M	317,7±8,3^{c}	320,3±8,4 ^{b}	_
L	322,0±9,4^{b}	319,3±5,7 ^{b}	_
LM	325,2±7,9 ^{a}	323,1±5,8 ^{a}	_

a-c Values with different letters within the same column differ (P <0.05) amomg cryoprotectants. There were no differences (P> 0.05) between egg diameter in the same line.

4. Discussion

Sperm viability values (overall average of 88 ± 1.6%) observed in this study suggests that all tested solutions were able to maintain membrane integrity of sperm cells during the vitrification process (Silva et al. 2015). It can also be implied, that the vitrification method was efficient to enable sperm membrane integrity in *L. vannamei* for up to 150 days. The vitrification method has been tested for cryopreservation of primordial cells in many species of mammalians and fishes (Merino et al., 2012; Figueroa et al., 2013). Recently a study of penaeid sperm mass vitrification resulted in long term cryopreservation with constant and high sperm survival rates for up to 120 days (Castelo-Branco et al., 2015).

Lecithin (L) and methanol (M) treatments presented high fertility rates (above 70%), however, when associated (LM) fertility rate decreased significantly (~54%). This may suggest that lecithin and methanol performs better as cryoprotectants in penaeids sperm when used individually. However, performing sperm vitrification in rainbow trout *Oncorhynchus mykiss* authors achieved significantly high rates of membrane integrity and undamaged DNA using a solution combining extra and intracellular cryoprotectants (Figueroa et al., 2013). Nevertheless fertility rates were also significantly lower when compared to fresh sperm control group, as observed in our study.

Studies with sperm cryopreservation using slow freezing protocols of the fresh water shrimp *Macrobrachium rosenbergii* presented high fertility rates when an intracellular cryoprotectant was used (Chow et al., 1985; Akarasanon et al., 2004). However, hatching rates were not satisfactory such as observed in the present study. On the other hand, the artificial insemination with cryopreserved spermatophores of *Penaeus monodon*, resulted in high fertilization and hatching rates which did not differ to fresh sperm control (Bart et al., 2006). In rainbow trout *O. mykiss* sperm vitrification trial fertility rates showed a positive correlation with mitochondrial membrane integrity and a negative correlation with DNA fragmentation (Figueroa et al., 2013).

Cryoprotectant toxicity or cryopreservation processes do not justify the failure on nauplii hatching observed in this study. Given the successful fertilization of the oocytes we can infer that the sperm cell was not structurally damaged. However, studies on DNA fragmentation of sperm cells suggests that this event may be caused during the cryopreservation process and also by the exposition to toxicants in mammals (Evenson and Wixon, 2005; Silva and Gadella, 2006). Apoptosis and reactive oxygen species (ROS) are also potential factors that can cause DNA fragmentation (O'Brien and Zini, 2005). However, in the present study only the sperm cell membrane integrity was evaluated and sperm cell DNA analysis was not performed.

5. Conclusion

Given the high rates of sperm survival and egg fertilization rate we can suggest that the vitrification method was efficient in maintaining sperm membrane integrity of *L. vannamei* for up to 150 days. However, the low hatching rates indicate that embryonic development is interrupted at some point. Therefore, we can infer that more specific studies (e.g. DNA analysis) are required to clarify the low hatching rate found in this study. This is the first report of artificial insemination using vitrified sperm in penaeids.

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CONSIDERAÇÕES FINAIS

Estudos focados em características masculinas são importantes para delinear uma nova visão para biotecnologias reprodutivas em peneídeos. No primeiro capítulo desta tese uma ampla revisão bibliográfica abordou os principais temas referentes às biotécnicas desenvolvidas no campo da reprodução em machos de peneídeos. Pode-se observar que muitas espécies de peneídeos, mesmo com potencial para a aquicultura, são pouco estudadas, sendo as pesquisas focadas nas espécies mais comercializadas. Assim, no segundo capítulo foi apresentada a descrição inédita, por microscopia eletrônica de varredura, do espermatóforo e espermatozoide do camarão *Farfantepenaeus subtilis*. Conhecendo as características do sistema reprodutivo, foi dado um importante passo para melhorar e desenvolver novas biotecnologias para a espécie, como a criopreservação do sêmen.

No terceiro capítulo foram estabelecidas as melhores condições de tempo e temperatura de estocagem para a recuperação do sêmen cadavérico do camarão *L. vannamei*. A técnica permite aproveitar gametas de machos que morrem inesperadamente, além de facilitar a coleta de dados em estudos de biologia populacional de peneídeos selvagens. No quarto e no quinto capítulos o método de vitrificação provou ser eficiente para o sêmen do *L. vannamei*, mantendo altas taxas de viabilidade espermática por até 150 dias. No entanto, as baixas taxas de eclosão indicam que o desenvolvimento embrionário foi interrompido em algum momento, sendo necessário investir em estudos mais específicos (por exemplo, análise de DNA) para maiores esclarecimentos.

ANEXO I

NORMA DO PERIÓDICOAQUACULTURE

DESCRIPTION

Aquaculture is an international journal for the exploration, improvement and management of all freshwater and marine food resources. It publishes novel and innovative research of world-wide interest on farming of aquatic organisms, which includes finfish, mollusks, crustaceans and aquatic plants for human consumption. Research on ornamentals is not a focus of the Journal. Aquaculture only publishes papers with a clear relevance to improving aquaculture practices or a potential application.

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Harrison FW, De Vos L. 1991. Porifera. In: Harrison, FW, editor. Microscopic Anatomy of Invertebrates. 2: Placozoa, Porifera, Cnidaria and Ctenophora, Vol. 2, Wiley-Liss, New York, pp. 28–89.

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