Sperm cryopreservation of lane snapper *Lutjanus synagris* (Linnaeus, 1758)

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Abstract

This study aims developing and evaluate a protocol of semen cryopreservation of the lane snapper *Lutjanus synagris*. Firstly, sperm motility rate, motility time, density and spermatocrit were appraised to characterize the sperm quality of the lane snapper. The effect of three extenders with distinct ionic compositions and pH values combined with seven concentrations of cryoprotector dimethylsulfoxide (0; 2.5; 5.0; 7.5; 10.0; 12.5 e 15.0%), five cooling rates (110, 90, 60, 45 e 30°C $^{-min}$), nine equilibration time (1; 2,5; 5; 10; 15; 20; 25; 30 e 60 minutes) e five dilutions ratio (1:1; 1:3; 1:6; 1:10 e 1:20) on the sperm motility rate and motility time were analyzed. Fertilization test was accomplished to evaluate the viability of the cryopreserved sperm. The higher sperm motility rate and motility time ($P<0.05$) was achieved by combining extender with pH 8.2 with 10% concentration of dimethylsulfoxide and cooling rate 60°C $^{-min}$, 1 minute of equilibration time and 1:3 (v/v) dilution ratio. The use of cryopreserved sperm presented fertilization rates >60% validating the present protocol for lane snapper. The cryoconserved sperm of lane snapper is a viable alternative, being possible to maintain appropriate sperm viability.

Keywords: extender, cryoprotector, *Lutjanus synagris*, reproduction, mariculture.

Criopreservação do sêmen do ariocó *Lutjanus synagris* (Linnaeus, 1758)

Resumo

Este estudo teve a finalidade de desenvolver e avaliar um protocolo de crioconservação do sêmen do ariocó *Lutjanus synagris*. Para caracterizar o sêmen foram avaliados a taxa de motilidade, a duração da motilidade, a concentração espermática e o espermatócrito. Em seis experimentos foram analisados os efeitos de três diluentes, com distintas composições iônicas e valores de pH distintos, combinados com sete concentrações de dimetilsulfóxido (0; 2,5; 5,0; 7,5; 10,0; 12,5 e 15,0%), cinco velocidades de congelamento (–110, –90, –60, –45 e –30°C/min), nove tempos de equilíbrio (1; 2,5; 5; 10; 15; 20; 25; 30 e 60 minutos) e cinco proporções de sêmen:diluente (1:1; 1:3; 1:6; 1:10 e 1:20) sobre a taxa de motilidade e a duração da motilidade espermáticas. Posteriormente um teste de fertilização foi realizado para avaliar a viabilidade do sêmen crioconservado. O tratamento que propiciou maior taxa de motilidade e duração da motilidade espermáticas ($P<0,05$) foi aquele proporcionado pelo emprego do diluente com pH 8,2 com dimetilsulfóxido a 10%, em uma velocidade de congelamento de –60°C/min, com tempo de equilíbrio de 1 minuto e na proporção de 1:3 (v/v). O sêmen crioconservado apresentou taxa de fertilização superior a 69% validando o presente protocolo para o ariocó. A crioconservação do sêmen do ariocó é uma alternativa viável, sendo possível manter uma apropriada qualidade espermática.

Palavras-chave: diluente, crioprotetor, *Lutjanus synagris*, reprodução, maricultura.

1. Introduction

The sperm cryoconservation is an efficient technique storage viable cells for long periods and can provide during the year a supplement of male gametes (Zhang et al., 2003). Currently it is estimated that more than 200 species with experimentally determined cryoconservation protocols only 40 protocols are from marine species (Thirumala et al., 2006), and in Brazil had only four marine species protocols for sperm cryoconservation determined: Brazilian flounder *Paralichthys orbignyanus* (Valenciennes, 1839) (Lanes et al., 2008), fat snook *Centropomus parallelus* (Poey, 1860)

(Tiba et al., 2009), dusky grouper *Epinephelus marginatus* (Lowe, 1834) (Sanches et al., 2009), and mutton snapper *Lutjanus analis* (Cuvier, 1828) (Sanches et al., 2013). This technique is considered as an essential tool to protect endangered species or distortion, allowing the storage of their gametes in germoplasm banks. Also, allows synchronization of the availability of gametes with the demand of production systems, better utilization of the volume of semen available especially in species whose sperm is difficult to be obtained or that release small amounts, reducing costs of maintaining the reproducers breeding stock and facilitate the transport of gametes especially when they are obtained in different locations (Suquet et al., 2000; Chao and Liao, 2001).

For the definition of cryoconservation protocols becomes necessary to know several factors: sperm characteristics of the species, extender composition, sperm:extender ratio, cryoprotectant and dosage, cooling rate and thawing, equilibrium time and fertilization tests.

The lane snapper *Lutjanus synagris* (Linnaeus, 1758) belongs to the family Lutjanidae and inhabits rocky bottoms from the surface to about 120 m depth, from North Carolina (USA) to southeastern Brazil. Reaches up to 60 cm reaching 3.8 kg with sexual maturity between 15-18 cm (Claro and Lindeman, 2008). Depending on the quality of meat, high market value and be an important fishery resource, this species, along with the red snapper *L. analis*, have been identified as candidates for cultivation (Sanches and Cerqueira, 2010).

Although sperm cryoconservation is an important strategy for the control of reproduction, few studies have focused on these techniques for species of lutjanidae, with emphasis results obtained with *Lutjanus campechanus* (Poey, 1860), with *Lutjanus argentimaculatus* (Forsskål, 1775) (Riley et al., 2004; Vuthiphandchai et al., 2009) and the mutton snapper *L. analis* (Sanches et al., 2013).

The goal of our study were to evaluate the effect of different factors in *L. synagris* cryopreserved sperm, and to propose a protocol for the cryopreservation of lane snapper sperm that could be applied to marine aquaculture.

2. Material and Methods

2.1. Fish

The lane snapper (n=50) were originally collected from Ubatuba coast/SP, Brazil, by line and hook, during October and November 2007, were kept in 2 m × 2 m × 2 m (8 m^3) net cages at a density of 2 individuals/m^3. The net cages were set up in the coastal area at Itaguá Beach, Ubatuba/SP and fish were fed daily with a commercial feed for marine fish (45% C.P. and 12% E.E.).

2.2. Sperm collection

In the early spawning season (January 2008), 25 individuals, fasted for 24 h, were anesthetized with benzocaine (0.1 g/L), and their length (cm) and weight (g) were measured. Mature lane snapper males were identified by the presence of flowing milt upon palpation of the abdomen and an extended urogenital papilla. The sperm was collected (without hormonal induction) in plastic graduated syringes (1 mL) that were placed on the urogenital papilla while applying gentle abdominal pressure until the first sign of blood. The syringes were wrapped in foil paper to avoid exposure to light and the volumes were registered.

2.3. Sperm characterization

The motility of each sperm sample was estimated by recording the percentage of sperm actively moving forward in the microscope field. Per cent motility was defined as the percentage of progressively motile sperm within each activated sample. Sperm that vibrated in place without forward movement were not considered to be motile. The duration of the motility was timed from the initiation of motility until the end. The analyses of sperm motility rate and motility time were performed simultaneously in the same preparation by a single technician on a single, randomly chosen focal field. The sperm density was determined by counting the sperm cells under a microscope at 200X magnification in a sperm sample that was previously diluted with 5% buffered formalin and prepared in a Neubauer hematimetric chamber (1 mm^3). The spermatocrit technique was used for the determination of the sperm density. The sperm cells were transferred into microhematocrit capillaries, with one tip sealed with plastiline, and centrifuged for 15 min in a microcentrifuge at 7,000 rpm (18,000 g). These settings were optimized in a previous experiment. After centrifugation, the cell mass was determined with a graduated ruler, and the values are expressed in percentage. The correlation between the spermatocrit values and sperm density was determined.

2.4. Sperm cryopreservation

The sperm cells from 10 individuals were collected, mixed in equal volumes and placed into opaque plastic flasks for use with the respective experiments. With the aim to evaluate the effect of different factors (extenders, dilution ratio, concentration of cryoprotectant, cooling rate, equilibration time and fertilization rate) were performed six experiments of cryoconservation as described below:

2.4.1. Experiment I – extenders

The effect of different extenders on the motility rate and motility time of cryopreserved sperm was analyzed. Three extenders that were previously shown to be successful in the cryopreservation of marine fish sperm were used as follows:

Extender A (g/L): NaCl, 7.89; KCl, 1.19; $CaCl_2$, 0.2; $MgCl_2$, 0.4266; pH 6.1; 158 mOsm (Chao et al., 1975); Extender B (g/L): NaCl, 6.5; KCl, 3.0; $CaCl_2$, 0.3; $NaHCO_3$, 0.2; pH 7.8; 157 mOsm (Peleteiro et al., 1996); Extender C (g/L): NaCl, 7.89; KCl, 1.19; $CaCl_2$, 0.22; $MgCl_2$, 0.72531; NaH_2PO_4, 0.0805; $NaHCO_3$, 0.84; pH 8.2; 172 mOsm (Sanches et al., 2009).

Only sperm with motility higher than 90% were used in the cryopreservation procedure. The concentration of the cryoprotectants dimethylsulfoxide (DMSO) was

10%, dilution ratio 1:3 (v/v) and cooling rate 60°C $^{-min}$. The equilibration time between the start of dilution of sperm and the start of cooling was 1 minute, with three replicates for each treatment. The straws are 0.5 mL. A cryogenic container with nitrogen steam at –196°C (CP 100 Taylor-Wharton - Harsco Corp., Theodore, AL, USA) was used for cooling the sperm samples. After 24 h, the straws were transferred to a storage container (Cryometal, model DS-34) with liquid nitrogen.

2.4.2. Experiment II - cryoprotectant

Different concentrations of cryoprotectant dimethylsulfoxide (DMSO) (0, 2.5, 5.0, 7.5, 10.0, 12.5 and 15%) was used extender C (pH 8.2) with dilution ratio 1:3, cooling rate 60°C $^{-min}$ and equilibration time 1 minute, with three replicates for each treatment. The straws are 0.5 mL.

2.4.3. Experiment III – cooling rate

This experiment tested different cooling rates. To achieve different cooling rates, straws were manually constructed from cryogenic plastic tubes with an internal diameter of 4 mm to contain final volumes of 0.15, 0.25, 0.50, 0.75 and 1.00 mL. The tubes were cut to different lengths to store the different volumes of diluted sperm so that the cooling rates selected in this study could be achieved during cooling. Cooling rates were previously determined in sperm samples with extenders in test straws, using thermo electrical pair (Ethics Scientific Equipment, 521-200). Cooling rates were 110, 90, 60, 45 and 30°C $^{-min}$ respectively. The temperatures evaluated ranged from 26 °C to –196 °C. For this experiment semen was diluted with extender C (pH 8.2). Dilution ratio was 1:3 and the concentration of DMSO was 10%. The equilibration time was 1 minute, with three replicates for each treatment.

2.4.4. Experiment IV - Equilibration time

Different equilibration times (1, 2.5, 5, 10, 15, 20, 25, 30 and 60 minutes) were evaluated. The sperm was diluted with extender C (pH 8.2). Dilution ratio was 1:3 and DMSO 10%. The cooling rate was 60°C $^{-min}$. For each treatment were frozen three replicates. The straws are 0.5 mL.

2.4.5. Experiment V – dilution ratio

Differents dilution rations (1:1; 1:3; 1:6; 1:10; 1:20) were analyzed. For this experiment was used extender C (pH 8.2). The concentration of DMSO was fixed at 10% and cooling rate 60°C $^{-min}$. The equilibration time was 1 minute. For each treatment were frozen three replicates. The straws are 0.5 mL.

After 180 days, the straws of the all experiments were thawed in water at 26°C by two minutes for measuring the sperm motility rate and motility time that were used as parameters to evaluate the freezing protocol.

2.4.6. Experiment VI – evaluation of fertilization

Fertility tests were conducted simultaneously with fresh and cryopreserved sperm of 2008 and 2009 spawning seasons through insemination of oocytes from the same female. LH-RHa 50 µg/kg (SIGMA, USA) was used to induce the female to spawn. The sperm used in this experiment were cryopreserved with extender C (pH 8.2) and 10% DMSO at cooling rate of 60°C $^{-min}$, with equilibrium time 1 min and dilution rate 1:3 (v/v).

The release of mature oocytes began approximately 36 h after induction, and the extrusion for the dry fertilization tests was performed. The oocytes were collected in plastic trays and separated into 30 aliquots, with approximately 1,000 oocytes each. The oocytes were placed into 50-mL plastic containers for the simultaneous fertilization of 10 aliquots of fresh sperm and 10 aliquots of each of cryopreserved sperm from the 2008 and 2009 spawning seasons. Before being mixed, the fresh sperm was previously diluted in the same extender at the same sperm:extender ratio that had been used for the cryopreserved sperm.

After the mixture of sperm and oocytes using 0.05 mL of sperm per 1,000 oocytes for a sperm:oocyte ratio of 200,000:1, 20 mL of seawater (35 ppt) were added to activate the sperm and initiate fertilization. After 5 min, each aliquot was placed in an individual incubator (1 L) and kept in a tank with a continuous circulation of seawater at 28°C. The fertilization rates, based on the relationship between the number of fertilized eggs and total number of eggs, were calculated 4 h after the fertilization.

2.4.7. Statistical analyses

Percentage values were arc-sine transformed before analysis. One-way ANOVA was used to determine whether the mean values of the different treatments were significantly different. Tukey's test was used as a post hoc test. Linear regression analysis was carried out in order to determine the correlation between spermatocrit and spermatozoa density. Data are presented as mean ± SE. SAS software (Statistical Analyses System, SAS/STAT 6.11) (Sas Institute Inc., 1990) was used.

3. Results

Fresh sperm was available from all individuals (n=25). The average volume of sperm collected from each fish was 0.61±0.35 mL. Mean density of spermatozoa was $2.2\pm0.2\times10^9$ cells mL $^{-1}$ (Table 1). There was a positive correlation between the sperm density and spermatocrit value (Figure 1). The regression equation $y = 0.0219x + 0.5979$ (y = sperm density, x = spermatocrit; $r^2 = 0.75$, $P < 0.05$) was determined from the sperm density and spermatocrit values. Therefore, the sperm density of lane snapper can be estimated from spermatocrit values.

3.1. Experiment I – extenders

The sperm motility rate and motility time of lane snapper with the use of extender C (pH 8.2) were significantly higher ($P < 0.05$) in comparison with other extenders (Table 2). Compared to fresh sperm was possible to observe an increase in the duration of motility, more meaningful with this extender, from 137 to 267 seconds.

Table 1. Morphometric and fresh sperm characterization parameters of lane snapper *Lutjanus synagris* (n=25).

Parameter	Mean ± SE
Standart length (cm)	27.4 ± 4.6
Body wet weigth (g)	336.3 ± 171.4
Sperm density (x 10^9 cells/mL)	2.2 ± 0.2
Collection volume (mL)	0.61 ± 0.35
Initial sperm motility (%)	100 ± 0
Sperm motility time (s)	137 ± 20
Spermatocrit (%)	73.4 ± 4.8

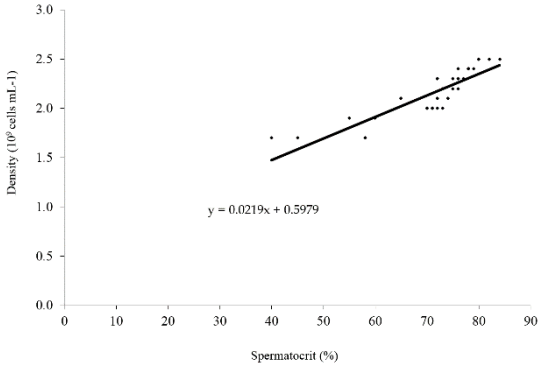

Figure 1. Correlation between spermatocrit (%) and spermatozoa density (cells mL^{-1}) for lane snapper *Lutjanus synagris* sperm (n = 25, adjusted $r^2 = 0.75$, $P < 0.05$).

Table 2. Motility rate (%) and motility time (s) of lane snapper *Lutjanus synagris* cryopreserved sperm (n=10 males) in different extenders, dilution ratio 1:3 (v/v), DMSO 10%, cooling rate 60°C $^{-min}$ and equilibrium time 1 minute. Date are represented as mean ± SE(1).

Extender(2)	Motility rate (%)	Motility time (s)
A	72 ± 8 ^{b}	194 ± 28 ^{B}
B	82 ± 8 ^{b}	228 ± 29 ^{B}
C	98 ± 3 ^{a}	267 ± 16 ^{A}

(1)Values sharing different letters in each colune indicate a significant difference among treatments ($P < 0.05$). (2)Extender A (g L^{-1}): NaCl, 7.89; KCl, 1.19; $CaCl_2$, 0.2; $MgCl_2$, 0.4266; pH 6.1; 158 mOsm kg^{-1}. Extender B (g L^{-1}): NaCl, 6.5; KCl, 3.0; $CaCl_2$, 0.3; $NaHCO_3$, 0.2; pH 7.8; 157 mOsm kg^{-1}. Extender C (g L^{-1}): NaCl, 7.89; KCl, 1.19; $CaCl_2$, 0.22; $MgCl_2$, 0.72531; NaH_2PO_4, 0.0805; $NaHCO_3$, 0.84; pH 8.2; 172 mOsm kg^{-1}.

3.2. Experiment II - cryoprotectant

The best perform was DMSO 10% concentration in cryoconservation of lane snapper sperm in terms of motility rate and motility time, with significant differences ($P < 0.05$), compared to other concentrations (Table 3).

3.3. Experiment III – cooling rate

The cooling rate more effective in cryoconservation of lane snapper sperm was 60°C $^{-min}$, provided by the use of straws 0.50 mL, with significant differences ($P < 0.05$) compared with the others tested (Table 4).

Table 3. Motility rate (%) and motility time (s) of lane snapper *Lutjanus synagris* cryopreserved sperm (n=10 males) in extender C, dilution ratio 1:3 (v/v), different DMSO concentrations, cooling rate 60°C $^{-min}$ and equilibrium time 1 minute. Date are represented as mean ± SE(1).

DMSO (%)	Motility rate (%)	Motility time (s)
0.0	0 ± 0 ^{c}	0 ± 0 ^{C}
2.5	55 ± 17 ^{b}	217 ± 49 ^{B}
5.0	79 ± 6 ^{b}	276 ± 11 ^{B}
7.5	80 ± 8 ^{b}	292 ± 51 ^{B}
10.0	98 ± 3 ^{a}	371 ± 10 ^{A}
12.5	88 ± 3 ^{b}	344 ± 12 ^{A}
15.0	73 ± 5 ^{b}	232 ± 8 ^{B}

(1)Values sharing different letters in each colune indicate a significant difference among treatments ($P < 0.05$).

Table 4. Motility rate (%) and motility time (s) of lane snapper *Lutjanus synagris* cryopreserved sperm (n=10 males) in extender C, dilution ratio 1:3 (v/v), DMSO 10%, different cooling rates and equilibrium time 1 minute. Date are represented as mean ± SE(1).

Volume (mL)	Cooling rate (°C $^{-min}$)	Motility rate (%)	Motility time (s)
0.15	110	77 ± 6 ^{a}	277 ± 24 ^{B}
0.25	90	80 ± 5 ^{a}	315 ± 30 ^{B}
0.50	60	92 ± 6 ^{a}	409 ± 45 ^{A}
0.75	45	62 ± 3 ^{b}	249 ± 12 ^{B}
1.00	30	53 ± 6 ^{b}	155 ± 38 ^{C}

(1)Values sharing different letters in each colune indicate a significant difference among treatments ($P < 0.05$).

3.4. Experiment IV - equilibration time

It was possible to observe an inverse relation between sperm viability (motility rate and motility time) and equilibration times evaluated, and the lowest equilibration time showed the best motility rate and motility time for lane snapper sperm with significant differences ($P < 0.05$), compared with other times tested (Table 5).

3.5. Experiment V – dilution ratio

The results showed no significant differences ($P > 0.05$) between dilution ratio 1:1 and 1:3, which, however, differed significantly ($P < 0.05$) others ratios evaluated (Table 6).

3.6. Experiment VI – evaluation of fertilization

The fertilization rate for fresh sperm (90%) was significantly higher ($P < 0.05$) than that cryoconserved sperm. There were no significant differences between the fertilization rates for cryopreserved sperm of 2008 (69%) and 2009 (75%) spawning seasons (Figure 2**).**

4. Discussion

The sperm motility rate and motility time have been correlated with fertility from the beginning of fertilization techniques of fish (Chereguini et al., 2001). The sperm of lane snapper examined after cryoconservation process,

Table 5. Motility rate (%) and motility time (s) of lane snapper *Lutjanus synagris* cryopreserved sperm (n = 10 males) in extender C, dilution ratio 1:3 (v/v), DMSO 10%, cooling rates 60°C $^{-min}$ and different equilibrium time. Date are represented as mean ± SE[(1)].

Equilibration time (min)	Motility rate (%)	Motility time (s)
1.0	78 ± 8 ^{a}	132 ± 16 ^{A}
2.5	22 ± 4 ^{b}	101 ± 17 ^{B}
5.0	18 ± 3 ^{b}	84 ± 8 ^{B}
10.0	16 ± 8 ^{b}	83 ± 15 ^{B}
15.0	13 ± 4 ^{bc}	77 ± 11 ^{BC}
20.0	13 ± 4 ^{bc}	78 ± 7 ^{BC}
25.0	10 ± 0 ^{c}	59 ± 6 ^{C}
30.0	6 ± 2 ^{d}	28 ± 7 ^{D}
60.0	0 ± 0 ^{e}	0 ± 0 ^{E}

[(1)]Values sharing different letters in each colune indicate a significant difference among treatments (P<0.05).

Table 6. Motility rte (%) and motility time (s) of lane snapper *Lutjanus synagris* cryopreserved sperm (n = 10 males) in extender C, different diluition ratio, DMSO 10%, cooling rates 60°C $^{-min}$ and equilibrium time 1 minute. Date are represented as mean ± SE[(1)].

Sperm: Extender	Motility rate (%)	Motility time (s)
1:1	90 ± 0 ^{a}	266 ± 33 ^{A}
1:3	89 ± 3 ^{a}	213 ± 40 ^{A}
1:6	80 ± 6 ^{a}	145 ± 26 ^{B}
1:10	70 ± 8 ^{b}	128 ± 29 ^{B}
1:20	68 ± 10 ^{b}	101 ± 20 ^{B}

[(1)] Values sharing different letters in each colune indicate a significant difference among treatments (P<0.05).

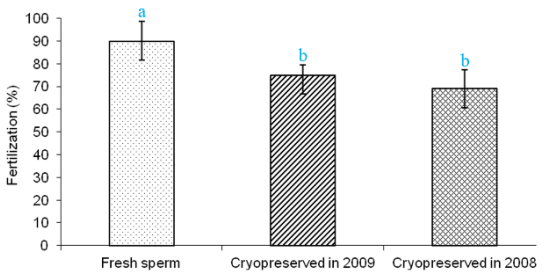

Figure 2. Fertilization rates (mean ± SE.) with fresh sperm and cryopreserved sperm of eggs from one lane snapper female spawned in 2009. Bars sharing different letters indicate significant difference among treatments (P<0.05).

showed a high percentage of motile cells (> 90%), indicating that the techniques employed in this study were suitable in preserving sperm quality. Comparatively, Sanches and Cerqueira (2011) obtained for the fresh sperm of mutton snapper of 100% motility rate and motility time of 174 ± 29 s. Our results for the fresh sperm were similar to those obtained by Vettorazzi et al. (2010) that using lane snapper the Northeast of Brazil, had rates of up to of 100% sperm motility rate and motility time between 62 and 135 s. All these findings conflict with those obtained by Gaitán-Espitia et al. (2013) for lane snapper when reporting a sperm motility time of 592 s. Different methodological techniques can be the cause of these higher values.

In this study lane snapper sperm density was 2.2×10^9 cells mL^{-1}. This value was higher than that obtained for *L. campechanus* 1.0×10^9 cells mL^{-1} (Riley et al., 2004), but in contrast to that reported for other species of lutjanidae (*L. argentimaculatus*) from 1.3 to 2.8×10^{10} (Vuthiphandchai et al., 2009). For lane snapper the Colombian coast, sperm density reported was 1.3×10^9 cells mL^{-1} (Gaitán-Espitia et al., 2013). The variation in sperm density among different species of the same genus may be caused by differences in the geographic range or by sampling at different periods during the spawning season (Lanes et al., 2010).

Significant correlation ($r^2 = 0.75$, $P < 0.05$) found between sperm concentration and spermatocrit value enables the use of this technique to estimate the concentration of sperm *L. synagris* in a practical way. This technique had been used to estimate sperm concentration of the flounder *P. orbignyanus* (Lanes et al., 2010).

The first step for the sperm cryopreservation from a fish species found in the development of a suitable extender (Lichtenstein et al., 2010). The best performance for sperm motility rate and motility time for lane snapper were obtained with the use of extender C (osmolality 172 mOsm, pH 8.2). Extenders pH adjusted from 7.8 to 8.5 (alkaline) suitably buffered and have showed improve performance in preserving the viability of the spermatozoa, unlike extenders without buffering capacity and pH near neutral or acid (Peleteiro et al., 1996). Chereguini et al. (2001) reported a significant correlation between the pH of the seminal plasma and with sperm motility, suggesting that pH may be an important feature of seminal plasma which influences sperm motility. The use of extender at pH 8.1 showed better results in sperm cryopreservation of *Pseudopleuronectes americanus* (Walbaum, 1792) (Rideout et al., 2003). Similar results were obtained by other authors using extenders with a pH around 8.0 to sperm cryoconservation of dusky grouper sperm (Sanches et al., 2009) and fat snook sperm (Tiba et al., 2009).

The osmolarity of extender effect on motility and velocity of spermatozoids there is a close connection between osmolality and ionic composition on the activation or inhibition of sperm motility (Wilson-Leedy et al., 2009; Alavi et al., 2010). Wayman et al. (1998) studied extenders with different osmolalities (200, 300 and 400 mOsm/kg) concluded that 200 mOsm/kg was the most suitable for preservation of *Sciaenops ocellatus* (Linnaeus, 1766) sperm. Extender with osmolarity of 200 mOsm already been used successfully in sperm cryoconservation of *L. campechanus* (Riley et al., 2004). This value is very close to the values of the osmolarity of the extender in this study, but contrast with the extender with osmolarity of 315 mOsm used in sperm cryoconservation of

L. argentimaculatus (Vuthiphandchai et al., 2009). These results demonstrate that only the osmolarity alone can not be used to evaluate a extender.

DMSO has been considered to be the most efficient cryoprotectant for use in the cryopreservation of marine fish sperm because of its low toxicity and protection of sperm during cooling due to its capacity for reducing ice formation by lowering the freezing point of intracellular fluid (Chao and Liao, 2001). While the action is not completely understood, it is known that DMSO interacts with structural phospholipids of the cell membrane, sperm transport property while maintaining the water at temperatures below 0°C (Thirumala et al., 2006). In this study the concentration of 10% DMSO was more effective in preserving the quality of lane snapper sperm. This same concentration was considered adequate for the sperm cryopreservation of *L. campechanus* and *L. argentimaculatus* (Riley et al., 2004; Vuthiphandchai et al., 2009).

Alongside the selection of cryoprotectant and diluent, the success of cryoconservation depends on using an appropriate cooling rate (Viveiros et al., 2000). The use of straws with different volumes, using the same apparatus freezer, provides different cooling rates (Richardson et al., 1999). The use of this artifice is very interesting for the high cost of the use of freezers with programmable speed, more efficient but with high financial cost and difficult maintenance (Yasui et al., 2008). The results of this experiment indicated that for sperm cryoconservation of lane snapper, the cooling rate of 60°C $^{-min}$ showed a better performance, with a significant difference ($P < 0.05$) of all velocities tested. This rate is considerably higher than that used in the sperm cryoconservation of *L. campechanus* 16 °C $^{-min}$ (0.5 mL vanes) (Riley et al., 2004), used in the sperm cryopreservation of *L. argentimaculatus* that employing 0.25 ml straws and different cooling rates (3, 5, 10 and 12 °C $^{-min}$) obtained the best results with 10 °C $^{-min}$ (Vuthiphandchai et al., 2009) and used to lane snapper sperm 15 °C $^{-min}$ (Gaitan-Espitia et al., 2013). However, the results obtained in our study agreed with those obtained by Chao et al. (1992) who studied the sperm cryoconservation of *Epinephelus malabaricus* (Block and Schneider, 1801) obtained the best results using 60 °C $^{-min}$ and with those obtained by Miyaki et al. (2005) who used this same cooling rate on sperm cryopreservation of *Epinephelus moara* (Block, 1793).

The best result for the equilibrium time in sperm cryoconservation of lane snapper was 1 minute, in contrast to the result for 20 minutes *L. campechanus* (Riley et al., 2004). Evaluating different equilibration times in sperm cryoconservation of *L. argentimaculatus* (10, 20, 30, 40, 50, 60, 90 and 120 minutes) it was observed a decrease in motility rate from 10 minutes (Vuthiphandchai et al., 2009). The results of our study agree with those obtained by Tiba et al. (2009) to determine the equilibrium time most suitable for the sperm cryopreservation of fat snook. These authors found no significant differences between the periods of 30 to 120 seconds, and the decreasing quality of sperm from the elevation of this time. In sperm cryoconservation of common snook using equilibration time of 30 min was observed a low sperm motility rate, indicating the importance of using an appropriate equilibration time (Tiersch et al., 2004). For sperm cryoconservation of European seabass *Dicentrarchus labrax* (Linnaeus, 1758) the equilibrium time is minimal, and sperm to be diluted immediately envased in straws and cooling (Zilli et al., 2005).

The sperm dilution before cooling has been recommended to optimize the viability of the spermatozoa after thawing. The dilution ratio affects the sperm motility rate after cryoconservation (Viveiros et al., 2009). For lane snapper sperm were no significant differences between the proportions of 1:1 and 1:3 ($P > 0.05$), while for the other ratios evaluated motility rate and motility time decreased significantly ($P < .05$). These results agree with those obtained by Riley et al. (2004) using dilution ratio of 1:3 for sperm cryoconservation of *L. campechanus* and with the obtained by Vuthiphandchai et al. (2009) using 1:1 to *L. argentimaculatus* sperm.

The fertilization rates observed in this study (90% for fresh sperm and 69% and 75% for cryopreserved sperm) show that a cryopreservation protocol for lane snapper has been successfully developed. However, the ideal sperm:oocyte ratio for lane snapper was not determined and should be addressed in future studies. The use of the ideal ratio may result in fertilization rates for cryopreserved sperm similar to those for fresh sperm. Red snapper cryopreserved sperm yielded fertilization rates ranging from 11% to 85%, and this variation was attributed to differences in oocyte quality (Riley et al., 2004). Moreover, high fertilization rates (90%) were observed in mangrove red snapper using cryopreserved sperm (Vuthiphandchai et al., 2009) and mutton snapper (Sanches et al., 2013). Sperm refrigerated for 48 h yielded fertilization rates greater than 50% in lane snapper (Sanches and Cerqueira, 2010). Although cryopreservation protocols are designed to yield similar fertilization rates, differences between fresh and cryopreserved sperm are common. For instance, no significant differences in fertilization rates between fresh and cryopreserved sperm (54% and 41%, respectively) were observed in common snook (Tiersch et al., 2004). Nevertheless, fertilization rates in fat snook were higher for fresh (84%) than for cryopreserved (74%) sperm (Tiba et al., 2009).

To our knowledge this is the first published successful fertilization of lane snapper ovocytes with sperm cryoconserved. This procedure should help to improve the development of protocols for lane snapper marine aquaculture.

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