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Cryopreservation of mutton snapper (*Lutjanus analis*) sperm

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ABSTRACT

This study aimed to develop a protocol of semen cryopreservation of the mutton snapper *Lutjanus analis*. The interaction between three extenders (pH 6.1; 7.8 and 8.2), two concentrations of dimethyl sulfoxide (DMSO, 5 and 10%) and three cooling rates (-90; -60 and -30°C.min^{-1}) on the sperm motility rate and motility time were analyzed by a factorial experiment. A sample of 30 fishes (1,261 ± 449 g) collected in the nature was kept in floating net cages. The semen was frozen by using cryogenic straws, in nitrogen vapour and transferred, later, to liquid nitrogen. Fertilization test was accomplished to evaluate the viability of the cryopreserved sperm. The highest sperm motility rate and motility time ($P < 0.05$) was achieved by combining extender C (pH 8.2) with DMSO (10%) and cooling rate of -60°C.min^{-1} ($P < 0.05$). The use of cryopreserved sperm presented fertilization rates higher than 59% validating the present protocol for mutton snapper.

Key words: cryoprotector, extender, *Lutjanus analis*, reproduction, sperm.

INTRODUCTION

Interest in the culture of snappers (Lutjanidae family) has been developed throughout the world because of declines in wild stocks combined with a consistent high demand and market value (Watanabe et al. 1998, Benetti et al. 2002, Garcia-Ortega 2009). Some lutjanid species, such as *Lutjanus argentimaculatus*, *Lutjanus johnii*, *Lutjanus russelli* and *Lutjanus sebae*, are currently farmed in Pakistan, China, Singapore, Malaysia, Thailand and Philippines (Hong and Zhang 2002).

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The mutton snapper *Lutjanus analis* (Cuvier 1828) is distributed along the West Atlantic coast. This species is important as commercial food fish due to the quality of meat, high market value and because it is listed as vulnerable by the International Union for Conservation of Nature and Natural Resources (IUCN). Experimental farms in the Caribbean, Colombia and Brazil have shown the potential of this species for marine aquaculture (Benetti et al. 2002, Botero and Ospina 2003, Sanches 2011).

One of the major hurdles to the development of marine fish farming is the breeding of larval stages in species with a potential for commercial culture.

The sperm cryopreservation is an important tool for optimizing reproduction procedures. Utilization of cryopreserved sperm in spawning of mutton snapper allows efforts to be focused on maintaining female broodstock, monitoring ovarian development and increasing efficiency during the strip-spawning process. When properly collected and frozen and after genetic characterization, cryopreserved sperm can be used for restoration programs of endangered species or specific genetic recovering programs to increase growth rates and resistance to diseases or to develop specific desired functional properties (Cabrita et al. 2009).

In Brazil, the sperm cryopreservation has been used in few marine species, including dusky grouper *Epinephelus marginatus* (Sanches et al. 2008), Brazilian flounder *Paralichthys orbignyanus* (Lanes et al. 2008) and fat snook *Centropomus parallelus* (Tiba et al. 2009). However, few studies have focused on the sperm freezing technique of lutjanids. To date, cryopreservation protocols have been developed for only three species: red snapper *Lutjanus campechanus*, grey snapper *Lutjanus griseus* and mangrove red snapper *Lutjanus argentimaculatus* (Riley et al. 2004, 2008, Vuthiphandchai et al. 2009).

The development of an effective sperm cryopreservation protocol depends on knowledge of the factors affecting the process, such as extender composition, sperm:extender ratio, cryoprotectant type and concentration, cooling and thawing rates and equilibrium time. Fertilization tests comparing cryopreserved and fresh sperm are also important. The successful cryopreservation of mutton snapper sperm would expand the sperm bank of endangered marine species (Sanches et al. 2008) and represent a vital tool for the conservation and commercial farming of the species.

This study aimed to develop a species-specific cryopreservation protocol for mutton snapper (*Lutjanus analis*) sperm to improve its reproduction in captivity.

MATERIALS AND METHODS

Fish

The mutton snapper individuals (n = 30), which were originally collected from the coast of Ubatuba/SP, Brazil, by line and hook, during October and November 2007, were kept in 2 m x 2 m x 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% crude protein and 12% ethereal extract).

Sperm Collection

In the early spawning season (January 2008), 30 individuals, fasted for 24 h, were anesthetized with benzocaine (0.1 $g.L^{-1}$), and their length (cm) and weight (g) were measured. 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.

Sperm Characterization

The motility of each sperm sample was estimated by recording the percentage of sperm actively moving forward in the microscope field. 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 200-X 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 parcentages. The correlation between the spermatocrit values and sperm density was determined.

Sperm Cryopreservation

Experiment I – Cryopreservation protocol

The effect of different extenders, cooling rates and dimethyl sulfoxide (DMSO) concentrations on the motility rate and motility time of cryopreserved sperm was analyzed. A factorial design was used in the experiment, with three extender solutions (A, B, C), two cryoprotectant concentrations (DMSO at 5% and 10%) and three cooling rates (-90, -60 and -30°C.min^{-1}), with three replicates for each treatment.

Three extenders that were previously shown to be successful in the cryopreservation of marine fish sperm were used as follows:

Extender A (g.L^{-1}): 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^{-1}): 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^{-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 (Sanches et al. 2008).

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 0.25, 0.50 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). The temperatures evaluated ranged from 26 °C to -196 °C.

Only sperm with a motility higher than 90% were used in the cryopreservation procedure. The sperm cells from 10 individuals were collected, mixed in equal volumes and placed into opaque plastic flasks for use with the respective extenders. The extenders with previously added DMSO at 5% and 10% were slowly added to the sperm up to the desired dilution of 1:4 (v/v). The equilibration time between the initial sperm dilution and initial cooling was 60 seconds. 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.

After 180 days, the straws were thawed in water at 26°C by two minutes for the determination of the sperm motility rate and motility time.

Experiment II – Evaluation of fertilization

Fertility tests were conducted simultaneously with fresh and cryopreserved sperm from the 2008 and 2009 spawning seasons through insemination of oocytes from the same female. LH-RHa 50 µg.kg^{-1} (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 a cooling rate of -60°C.min^{-1}, with an equilibrium time of 1 min and a dilution of 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.

Statistical Analyses

Data were analyzed using two-way ANOVA. Tukey's multiple comparison test was applied to estimate differences among groups. The significant level was set at $P < 0.05$ unless otherwise noted. Results are presented as mean ± SE. Percentage data were arcsine transformed before analysis. SAS software (Statistical Analyses System, SAS/STAT 6.11) (Sas Institute Inc. 1990) was used.

RESULTS

Sperm Characterization

Running milt was available from all individuals (n = 30). The average volume of sperm collected from each fish was 0.90 ± 0.52 mL. Mean density of spermatozoa was $2.6 \pm 0.3 \times 10^9$ cells.mL $^{-1}$ (Table I). There was a positive correlation between the sperm density and spermatocrit value (Fig. 1). The regression equation $y = 0.0428x + 0.7887$ (y = sperm density, x = spermatocrit; $r^2 = 0.90$, $P < 0.05$) was determined from the sperm density and spermatocrit values. Therefore, the sperm density of mutton snapper can be estimated from spermatocrit values.

TABLE I
Morphometric and semen characterization parameters of mutton snapper (n = 30).

Parameter	Mean ± SE
Standart length (cm)	43.7 ± 4.3
Body wet weigth (g)	1,261.11 ± 449.0
Sperm density (x 10^9 cells/mL)	2.6 ± 0.3
Collection volume (mL)	0.90 ± 0.52
Initial sperm motility (%)	100 ± 0
Sperm motility time (s)	148 ± 29
Spermatocrit (%)	78.2 ± 4.6

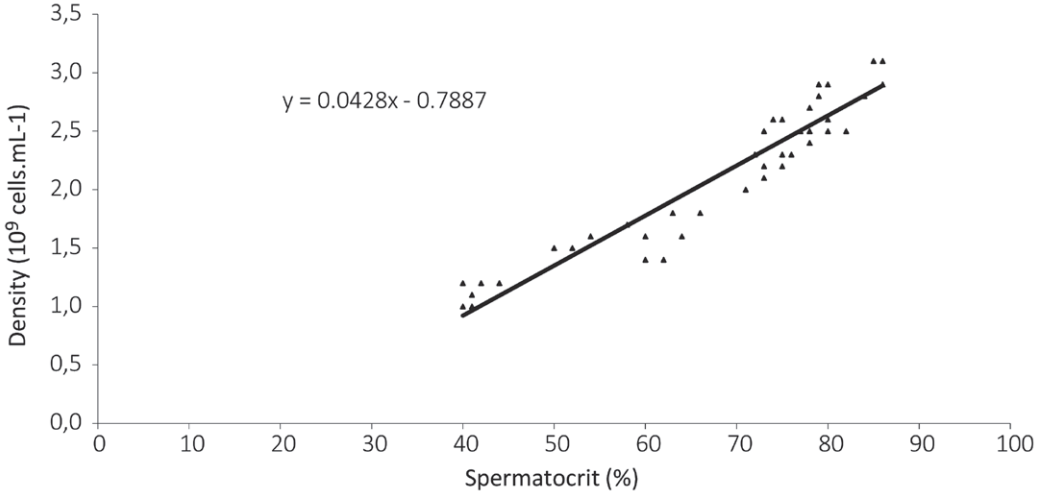

Fig. 1 - Correlation between spermatocrit (%) and spermatozoa density (cells.mL^{-1}) for mutton snapper sperm (n = 30 males, adjusted $R^2 = 0.90$, $P < 0.05$).

SPERM CRYOPRESERVATION

Experiment I – Cryopreservation protocol

There was a significant interaction among the factors influencing sperm motility rate (interaction extender*cooling rate*DMSO, p = 0.018). A Tukey test was conducted to improve the fit among the three factors. The use of extender C (pH 8.2) with 10% DMSO at a cooling rate of -60ºC.min^{-1} resulted in the highest motility (90.1%) (Table II).

Data on the sperm motility time after cryopreservation were submitted to ANOVA. The interaction between the extender solution, DMSO concentration and cooling rate was not significant (p = 0.176). However, the interaction between each two of the factors was statistically significant (P < 0.05).

TABLE II
Sperm motility rate (%, mean ± SE) of mutton snapper sperm (n = 10 males) with different extenders, cooling rates and DMSO concentrations interaction.

Extender	Cooling rate	DMSO	Mean ± SE
A (pH = 6.1) (158 mOsm)	-90°C.min^{-1}	5%	39.3 ± 6.1^{c}
		10%	12.0 ± 0.3^{d}
	-60°C.min^{-1}	5%	61.4 ± 3.2^{b}
		10%	30.1 ± 8.6^{cd}
	-30°C.min^{-1}	5%	72.3 ± 4.2^{b}
		10%	22.0 ± 0.8^{cd}
B (pH = 7.8) (157 mOsm)	-90°C.min^{-1}	5%	29.1 ± 4.1^{cd}
		10%	12.0 ± 0.0^{d}
	-60°C.min^{-1}	5%	29.1 ± 4.1^{cd}
		10%	12.8 ± 0.0^{d}
	-30°C.min^{-1}	5%	34.3 ± 3.3^{cd}
		10%	16.0 ± 0.6^{d}
C (pH = 8.2) (172 mOsm)	-90°C.min^{-1}	5%	40.1 ± 5.4^{c}
		10%	40.1 ± 5.4^{c}
	-60°C.min^{-1}	5%	50.0 ± 0.0^{c}
		10%	90.1 ± 0.6^{a}
	-30°C.min^{-1}	5%	60.2 ± 3.1^{bc}
		10%	75.2 ± 3.3^{ab}

$^{a-d}$ Data are represented as mean ± SE. Values sharing different letters in each colune indicate a significant difference among treatments (P < 0.05).

The sperm motility time was the longest with the use of extender C (pH 8.2) at a cooling rate of -60°C.min^{-1} (Table III). When the extender and DMSO concentration are considered together, the motility time was higher with the use of extender C (pH 8.2) and 10% DMSO (Table IV). For the combination of cooling rate and DMSO concentration, the sperm motility time was higher at a cooling rate of -60°C.min^{-1} with 10% DMSO (Table V).

TABLE III
Sperm motility time (seconds, mean ± SE) of mutton snapper sperm (n=10 males) in three extenders at different cooling rates.

	Cooling rate		
	-90°C.min^{-1}	-60°C.min^{-1}	-30°C.min^{-1}
Extender A	210 ± 20^{c}	300 ± 21^{b}	295 ± 19^{b}
Extender B	140 ± 13^{d}	220 ± 18^{c}	170 ± 12^{d}
Extender C	290 ± 18^{b}	420 ± 26^{a}	330 ± 28^{b}

$^{a-d}$The means followed by different superscripts are statistically different (P < 0.05).

TABLE IV
Sperm motility time (seconds, mean ± SE) of mutton snapper sperm (n = 10 males) in three extenders and two DMSO concentrations.

	Extender A	Extender B	Extender C
5% DMSO	210 ± 12^{cd}	160 ± 22^{d}	360 ± 15^{b}
10% DMSO	290 ± 20^{c}	190 ± 12^{d}	410 ± 28^{a}

$^{a-d}$The means followed by different superscripts are statistically different (P < 0.05).

TABLE V
Sperm motility time (seconds, mean ± standard deviation) of mutton snapper sperm (n = 10 males) at different cooling rates in two DMSO concentrations.

	Cooling rate		
	-90°C.min^{-1}	-60°C.min^{-1}	-30°C.min^{-1}
5% DMSO	180 ± 52^{d}	295 ± 31^{c}	192 ± 35^{d}
10% DMSO	275 ± 20^{cd}	425 ± 29^{a}	338 ± 29^{b}

$^{a-d}$The means followed by different superscripts are statistically different (P < 0.05).

Experiment II – Fertilization tests

The fertilization rate for fresh sperm (81%) was significantly higher ($P < 0.05$) than that of the cryopreserved sperm. There were no significant differences between the fertilization rates for cryopreserved sperm from the 2008 (59%) and 2009 (66%) spawning seasons (Fig. 2).

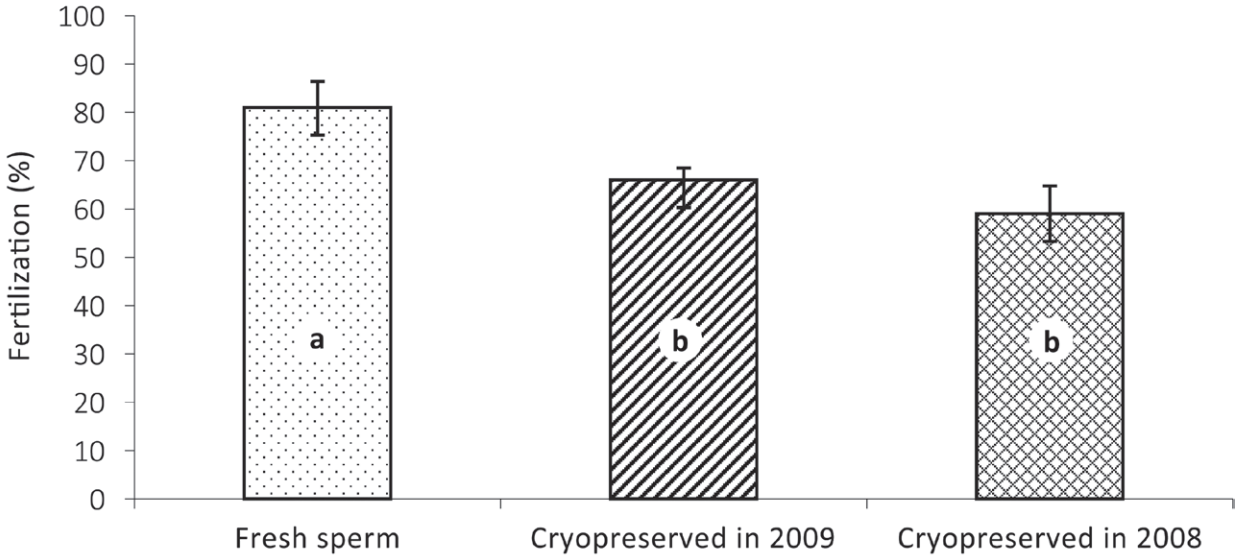

Fig. 2 - Fertilization rates (mean ± SE.) with fresh sperm and cryopreserved sperm of eggs from one mutton snapper female spawned in 2009. Bars sharing different letters indicate significant difference among treatments ($P < 0.05$).

DISCUSSION

The sperm density for mutton snapper are higher than those observed for red snapper (1.0×10^9 cells. mL^{-1}) (Riley et al. 2004) and lane snapper Lutjanus synagris (2.2×10^9 cells.mL^{-1}) (Sanches and Cerqueira 2010) but they are comparable to those of mangrove red snapper, which ranged from 1.3 to 2.8×10^{10} cells. mL^{-1} (Vuthiphandchai et al. 2009). The variation in sperm density between 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).

The best result among the different ionic compositions and pH values tested in this study was achieved in the extender solution at 172 mOsm and pH 8.2. The extender HBSS with an osmolality of 200 mOsm was used for the cryopreservation of red snapper sperm and it was close to the values used in this study, but it was lower than the value of 315 mOsm used for mangrove red snapper sperm (Vuthiphandchai et al. 2009). The cryopreservation of sperm has also been successful in other marine species using extender solutions with osmolalities close to 200 mOsm. For instance, Wayman and Tiersch (1998), studying extender solutions at different osmolalities (200, 300 and 400 mOsm), found that 200 mOsm was the most suitable for the preservation of sperm quality in red drum *Sciaenops ocellatus*. Moreover, an extender with an osmolality of 200 mOsm was successfully used for the cryopreservation of common snook *Centropomus undecimalis* sperm (Tiersch et al. 2004).

Several authors have reported the effect of pH on sperm motility rate. In fact, extenders with different pH values may affect sperm motility (Cosson 2004, Alavi and Cosson 2005). Buffered extenders are used to prevent the accumulation of sperm metabolites, during the cooling process, from changing the sperm pH and damaging the sperm cells. Chen et al. (2004),

using three extenders (202, 335 and 363 mOsm) at different pH values (6.7, 8.2 and 6.5 respectively), had the best results for the cryopreservation of turbot *Scophthalmus maximus* sperm at pH 8.2. The same pH value was successfully used for the cryopreservation of dusky grouper (Sanches et al. 2008), Brazilian flounder (Lanes et al. 2008) and fat snook sperm (Tiba et al. 2009).

The sperm motility time was more positively influenced when extender C (pH 8.2 and 172 mOsm) was used. The average duration of fresh sperm motility was 174 s, and the motility time was 400 s after cryopreservation in extender C; a significant increase even when compared to the semen cryopreserved in other extender solutions. A similar result was achieved with dusky grouper sperm cryopreserved in the same extender (Sanches et al. 2008). The increase in sperm movement, which may result in increased sperm viability, is probably associated with particular features of the extender solution, such as the alkaline pH, higher $NaHCO_3$ concentration and osmolality, that create a new microhabitat for the sperm that is different from that in seminal plasma. Apparently, these constituents were able to interact with the chemical components of the internal structures of the flagella responsible for the initiation of sperm motility (Inaba 2003), changing the pattern of the motility time that was preserved after the freezing process.

Different cooling rates can be obtained in the same freezing straws depending on its maintenance, stabilization time and use frequency. Cooling rates of -45°C.min^{-1} (Carolsfeld et al. 2003) and -35.6°C.min^{-1} (Maria et al. 2006) were recorded for the same container model (CP 300). Moreover, different cooling rates can be achieved more economically than by using controlled-rate programmable freezers by placing straws of different volumes in the same freezing compartment; although controlled-rate programmable freezers are more efficient, they are also high-maintenance and expensive (Richardson et al. 1999, Yasui et al. 2008). When the sperm motility rate and motility time were considered, the best cooling rate for mutton snapper sperm was -60°C.min^{-1}. This value is higher than that used for the cryopreservation of sperm from red snapper (-16°C.min^{-1}; Riley et al. 2004) and mangrove red snapper (-10°C.min^{-1}; Vuthiphandchai et al. 2009). Nevertheless, the cooling rate with the best result in this study is similar to the cooling rates used for the cryopreservation of grouper Epinephelus malabaricus (Chao et al. 1992), kelp grouper *Epinephelus moara* (Miyaki et al. 2005), fat snook (Tiba et al. 2009) and red spotted grouper *Epinephelus akaara* sperm (He et al. 2011).

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 its protection of sperm during cooling due to its capacity for reducing ice formation by lowering the freezing point of intracellular fluid (Peleteiro et al. 1996). In this study, a solution with 10% DMSO was the most efficient cryoprotectant, and a similar concentration was suggested for the cryopreservation of red snapper (Riley et al. 2004) and mangrove red snapper sperm (Vuthiphandchai et al. 2009). In other commercial marine species, satisfactory results were achieved with 10% DMSO for the cryopreservation of cobia *Rachycentron canadum* (Caylor et al. 1994), common snook (Tiersch et al. 2004) and fat snook sperm (Tiba et al. 2009). The beneficial effect with rapid cooling may also rely on the use of DMSO as the cryoprotectant because DMSO can penetrate sperm cells rapidly and prolonged equilibration during slow cooling can exert more cytotoxicity of DMSO on sperm cells (He et al. 2011)

Although sperm motility rate is the key parameter in sperm quality, the cryopreservation process should also be evaluated based on the fertilization capacity of oocytes. However, the sperm:oocyte ratio and contact time may affect fertilization rates, hindering comparisons between different studies (Rurangwa et al. 2004). To ensure maximum fertilization, sperm cells in excess are usually used for the fertilization

of fish oocytes with cryopreserved sperm (Viveiros et al. 2009). However, this procedure may mislead the evaluation of the cryopreservation process. Therefore, to avoid controversy, a sperm:oocyte ratio of 200,000:1 was used in this study. The same ratio was used for cryopreserved sperm in mangrove red snapper (Vuthiphandchai et al. 2009), although Riley et al. (2004) used a ratio of 430,000:1 in the same species.

The fertilization rates observed in this study (81% for fresh sperm and 59% and 66% for cryopreserved sperm) show that a cryopreservation protocol for mutton snapper has been successfully developed. However, the ideal sperm:oocyte ratio for mutton 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 that are 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). 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).

Therefore, the cryopreservation of mutton snapper sperm was the most successful when using an extender solution with pH 8.2, 172 mOsm and 10% DMSO at a cooling rate of -60°C.min^{-1}.

This is the first report on successful cryopreservation of mutton snapper sperm, this procedure should improve broodstock management techniques for this species and consequently augment the potential for its culture.

RESUMO

Este trabalho foi realizado com a finalidade de desenvolver um protocolo de crioconservação do sêmen da cioba *Lutjanus analis*. Em um experimento fatorial foram analisados os efeitos de três diluentes (pH 6,1; 7,8 e 8,2), duas concentrações de dimetilsulfóxido (DMSO, 5 e 10%) e três velocidades de congelamento (-90, -60 e -30°C.min^{-1}) sobre a taxa de motilidade e tempo de motilidade espermáticas do sêmen crioconservado. Uma amostra de 30 exemplares com peso médio de 1.261,11 ± 449,0 g, oriunda da natureza, foi mantida em tanques-rede. O sêmen obtido foi congelado empregando-se palhetas criogênicas em vapor de nitrogênio e, posteriormente, transferido para nitrogênio líquido. Posteriormente um teste de fertilização foi realizado para avaliar a viabilidade do sêmen crioconservado. A combinação que propiciou maior taxa de motilidade e tempo de motilidade espermáticas ($P < 0,05$) foi proporcionada pelo emprego do diluente de pH 8,2 com 10% de DMSO e uma velocidade de congelamento de -60°C.min^{-1} ($P < 0,05$). O sêmen crioconservado apresentou taxa de fertilização superior a 59% validando o presente protocolo para a cioba.

Palavras-chave: crioprotetor, diluidores, *Lutjanus analis*, reprodução, sêmen.

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