



Short-term storage of lebranche mullet *Mugil liza* (Valenciennes, 1836) semen *in natura* and diluted with CF-HBSS

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ABSTRACT. The present study aimed to evaluate the activation characteristics of the lebranche mullet spermatozoa *in natura* and diluted with CF-HBSS for 96h at $4\pm 2^\circ\text{C}$. The semen was collected from eight wild fish in Florianópolis – SC (Brazil) (27°S) in May, during reproductive migration. Three pools of semen were divided into two treatments: *in natura* and diluted with CF-HBSS 1:3. The semen was activated with seawater (salinity of 34, pH 8.7 and $4\pm 2^\circ\text{C}$) to determine: motility time, motility rate and sperm cell membrane integrity. Dilution with CF-HBSS 1:3 increased motility time of diluted semen (15-20% for 6, 12 and 18h). Motility rate was equal to fresh semen for up to 24h of refrigeration and it was 30% higher than *in natura* semen at 12, 18 and 24h. Cell membrane integrity was maintained in fresh semen up to 6h, and it was 15-20% higher than *in natura* semen from 6 to 48h ($p < 0.05$). The use of CF-HBSS as diluent in the ratio 1:3 is recommended for semen refrigeration of lebranche mullet.

Keywords: gamete conservation; spermatozoa; aquaculture; marine fish farming; near threatened fish.

Armazenamento a curto prazo do sêmen da tainha *Mugil liza* (Valenciennes, 1836) *in natura* e diluído com CF-HBSS

RESUMO. O objetivo do presente estudo foi avaliar em tainha as características de ativação dos espermatozoides *in natura* e diluídos com CF-HBSS mantidos por 96h a $4\pm 2^\circ\text{C}$. O sêmen foi coletado de oito peixes selvagens em Florianópolis – SC (27°S) em maio, durante a migração reprodutiva. Três pool de sêmen foram divididos em dois tratamentos: *in natura* e diluído com CF-HBSS 1:3. O sêmen foi ativado com água oceânica (34 de salinidade, pH 8,7 e $4\pm 2^\circ\text{C}$) para determinar: tempo de motilidade, taxa de ativação espermática e integridade da membrana celular. A diluição com CF-HBSS 1:3 propiciou o aumento no tempo de motilidade do sêmen diluído (15-20% para 6, 12 e 18h); manteve a taxa de motilidade igual ao sêmen fresco por até 24h de refrigeração e foi 30% maior do que em *in natura* em 12, 18 e 24h; manteve também a integridade da membrana celular igual ao sêmen fresco por 6h e 15-20% maior que em sêmen *in natura* ($p < 0.05$) para o período de 6 a 48h. O uso do CF-HBSS como diluidor na proporção 1:3 é recomendado para a refrigeração do sêmen da tainha.

Palavras-chave: conservação de gametas; espermatozoide; aquicultura; piscicultura marinha; peixe quase ameaçado.

Introduction

In Brazil, the lebranche mullet *Mugil liza* (Valenciennes, 1836) is currently categorized as a near threatened (NT) species, according to the International Union for Conservation of Nature and Natural Resources (IUCN). Currently, status of population conservation is being evaluated to define strategies for fisheries management Ministério da Pesca e Aquicultura e Ministério do Meio Ambiente (MPA/MMA, 2015). As a eurythermal, euryhaline and omnivorous species easy to be fed, the mullet has favorable characteristics for aquaculture (Fonseca

Neto & Spach, 1999; Miranda Filho, Tesser, Sampaio, & Godinho, 2010).

Semen conservation is a useful tool for preserving genes of interest, collaborating with artificial propagation reducing costs and risks of transporting live animals, as well as assisting the maintenance and protection of species (Rurangwa, Kime, Ollevier, & Nash, 2004; Suquet, Dreanno, Fauvel, Cosson, & Billard, 2000). The cooling of semen to temperatures close to 4°C is one conservation technique, which prolongs temporal viability of undiluted semen from a few hours to days. This can be explained by reduction of spermatozoa's metabolic activity at temperatures below the physiological range.

The utilization of dilutors and motile immobilizer solutions that mimic ionic composition and osmolality of seminal plasma have been effective in potentiating longevity, without promoting significant changes in semen quality (Peñaranda et al., 2010; Ubilla, Fornari, Figueroa, Effer, & Valdebenito, 2015). Diluents developed for marine species generally contain inorganic salts, small amounts of sugars and protein, with 7.0 to 8.2 pH and osmolality between 205 and 400 mOsMol.kg⁻¹ (Magnotti et al., 2016; Suquet et al., 2000). These solutions have functions of diluting, nourishing and protecting spermatozoa of toxic products of cellular metabolism and sudden temperature variation (Stoss, 1983; Ubilla & Valdebenito, 2011; Valdebenito, Fletcher, Vera, & Fernández, 2009). Therefore, the first step in the development of protocols for semen conservation of a species is to find an appropriate extender medium of dilution (Viveiros, Orfão, Nascimento, Corrêa, & Caneppele, 2012).

The present study aimed to evaluate sperm viability in fresh semen and in semen diluted with CF-HBSS during short-term storage at 4°C.

Material and methods

Semen collection

The biological material used was collected from wild fish caught with trawls by artisanal fishermen from the region of Barra da Lagoa, Florianópolis (27° 34' 26" S, 48° 25' 27" W) on May 23, during the reproductive period of the species (Lemos, Varela, Schwingel, Muelbert, & Vieira, 2014). Eight fish were handled immediately after capture, and total length (cm) and weight (g) were verified. For semen collection, the abdominal and genital areas of each individual were cleaned with deionized water and dried with paper towel. After gentle abdominal pressure, semen was collected with 3 mL syringes and immediately stored in an ice-cold container (without direct contact with the syringes) at 4 ± 2°C protected from light. Samples contaminated with feces or urine (checked by color and viscosity variation) were discarded. Then, the samples were identified according to donor male, and they were individually analyzed for spermatocrit motility. All procedures used in the experiment were previously approved and authorized by the Committee of ethics and animal use of the Federal University of Santa Catarina (CEUA PP00861).

Semen cooling

From the eight individuals collected, semen of seven males presented percentage of mobile cells >

80%, which is considered suitable for refrigeration, and then, the semen was distributed in equal aliquots in three pools. In each pool, equal volumes of semen (100 µL) from selected males were homogenized, stored in 1 mL *Eppendorf* tubes and subsequently distributed into two treatments: semen *in natura* and semen diluted with CF-HBSS. The CF-HBSS diluent (5.26 g L⁻¹NaCl, 0.26 g L⁻¹KCl, 0.33 g L⁻¹ NaHCO₃, 0.04 g L⁻¹ Na₂HPO₄, 0.04 g L⁻¹ KH₂PO₄, 0.13 g L⁻¹ MgSO₄·7H₂O, 0.66 g L⁻¹glucose; 200 mOsMol kg⁻¹) was applied at 1:3 proportion (semen:diluent) and each treatment was performed in triplicates. The semen was kept under refrigeration at 4 ± 2°C without lighting and handled only at the moment of sperm activation analysis (time 0, 6, 12, 18, 24, 36, 48, 72 and 96h).

Sperm motility

The semen was activated in the ratio 1:50 (v:v) (Lanes, Okamoto, Bianchini, Marins, & Sampaio, 2010) with seawater with salinity of 34 and pH 8.7 at 4 ± 2°C. The vessel containing water used as the spermatocrit activator was maintained refrigerated for at least 24 h at 4 ± 2°C, and it was transferred to an ice-cold vessel (without ice direct contact to vessel containing the spermatocrit activator) for temperature maintenance during the analysis period. Immediately after activation, a single evaluator underwent light microscopy (Leica LCC50HD) (200x) analysis, in a field of view chosen at random and unchanged light intensity. Trial was performed in quadruplicates. Motility time was measured with a stopwatch from the moment of activation until the total stoppage of all spermatozoa. To determine motility rate, a graded scale of 0 to 5 was used, as suggested by Fauvel, Savoye, Dreanno, Cosson e Suquet (1999), in which: 0=immotile sperm; 1 = 0-20%; 2 = 20-40%; 3 = 40-60%; 4 = 60-80%; 5 = 80-100% motile sperm. Results of motility rate were expressed as percentage. Statistical analyzes and graphs were performed using the maximum value of percentage referring to each scale as proposed by Fauvel, Savoye, Dreanno, Cosson, and Suquet (1999).

Spermatocrit density

The spermatocrit density was evaluated using a light microscopy (Leica LCC50HD) (400x), by counting sperm cells present in the semen pools, previously diluted (1:1000) in 4% formol buffered citrate solution in Neubauer's hemacytometric chamber (1 mm³). Four counts were performed for each pool of semen. The spermatocrit technique was used to determine sperm concentrations: the semen was placed into hematocrit capillaries, with one end

sealed with plasticine, and centrifuged at 18000 g for 25 min in a centrifuge for capillaries. Rotation and spin time were determined in previous tests. The cell mass of centrifuged samples was measured with a graduated ruler, and the values obtained were expressed as a percentage.

Sperm cell membrane integrity

The semen pools were evaluated by eosin-nigrosin method, performed according to Maria, Azevedo, Santos, Silva, and Carneiro (2010). For this, two glass slides of semen from each replicate were deposited at 1:3 proportion (semen:stain) on a slide, and the smear was performed. At less than 60 s, three random fields of each slide were photographed with a digital camera coupled to the Leica LCC50HD (400x) microscope. The images obtained were analyzed with Leica Application Suite LAS EZ software, by counting 500 cells per slide, differentiating themselves as pigmented and non-pigmented. Values were expressed as percentage of live (non-pigmented) cells.

Statistical analysis

Percent values were transformed to arcsine ($y^{0.5}$) before statistical analysis. Linear regression ($y = a + bx$) and Pearson correlation were performed between motility rate and sperm cell membrane integrity ($p < 0.05$) by the program Graphpad prism 6. Levene test was used to verify homoscedasticity and Shapiro Wilk test was used to verify normality of obtained data. Comparison between treatments in each cooling time was performed by Student's T-test ($p < 0.05$). To compare the treatments along with the refrigeration times, the means were compared by one-way Anova and Tukey's test ($p < 0.05$), through the software Statistica 7.0 (Hill & Lewicki, 2007).

Results

The wild lebranche mullet males (*M. liza*) had a total length of 48.75 ± 2.05 cm and a weight of 1250 ± 163 g. The pool samples of fresh semen obtained $93 \pm 12\%$ of motility rate, 343 ± 6 s of motility time, $76 \pm 2\%$ of sperm cell membrane integrity, $98 \pm 1\%$ of spermocrit and $2.5 \pm 1.5 \times 10^{10}$ cel mL⁻¹ of sperm density. These parameters were considered at the zero time of refrigeration test.

During the 96 h period the spermatozoa of both treatments presented reduction in motility time and rate, as well as in sperm cell membrane integrity. At 6, 12 and 18h of cooling, a significant difference ($p < 0.05$) was observed between the mean motile time of spermatozoa submitted to treatments. From 0 to

18h, in the treatment diluted with CF-HBSS the sperm life span increased from 346 ± 6 s to 415 ± 5 s (20% increase). However, the motility time of both treatments remained the same as that of fresh semen up to 48h of refrigeration (Figure 1A).

A difference ($p < 0.05$) in the motility rate diluted with CF-HBSS was observed, which was higher than that of undiluted semen during 96 h of refrigeration (approximately 30% higher at times 12, 18 and 24h). The semen *in natura* presented loss in this parameter after only 6h of refrigeration, while diluted semen kept activation rate equal to that of fresh semen for up to 24h ($p > 0.05$) (Figure 1B).

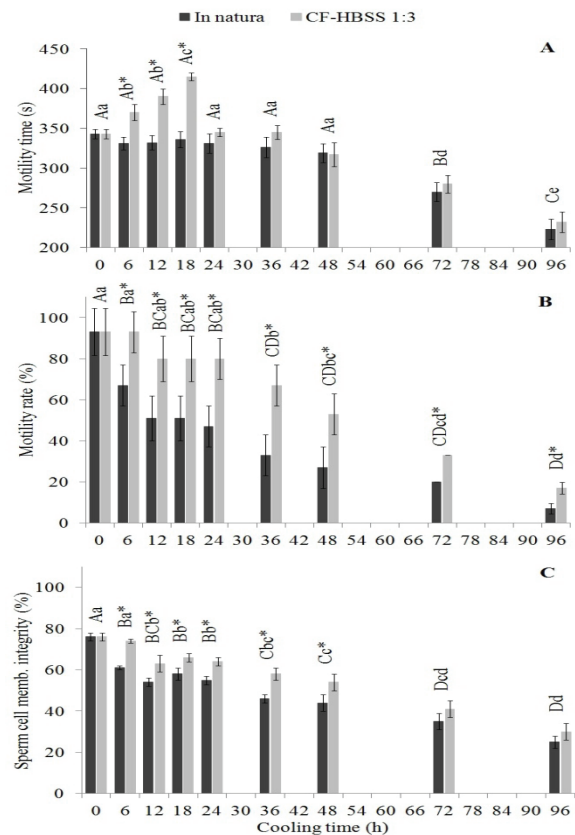


Figure 1. Motility time (A), motility rate (B) and sperm cell membrane integrity (C) of lebranche mullet (*Mugil liza*) spermatozoa stored for 96h at $4 \pm 2^\circ\text{C}$ *in natura* and diluted 1:3 (v:v) with CF-HBSS. Different upper case letters for *in natura* and lowercase for CF-HBSS (1:3) indicate significant difference ($p < 0.05$) verified through one-way Anova and Tukey's test along refrigeration period. *indicates significant difference ($p < 0.05$) verified through Student's T test between treatments in each time analyzed.

The treatment with diluted semen also showed higher rate (15-20% higher) of live spermatozoa from 6 to 48h ($p < 0.05$), matching the treatment without dilution after that period (Figure 1C). The semen *in natura* presented reduction ($p < 0.05$) in cell membrane integrity throughout cooling time,

while the diluted semen had membrane integrity equal to fresh semen up to 6h of refrigeration, with subsequent reduction of quality. Results of sperm cell membrane integrity, and motility rate of treatments *in natura* and diluted with CF-HBSS presented positive Pearson correlation and linear regression ($p < 0.05$) (Figure 2).

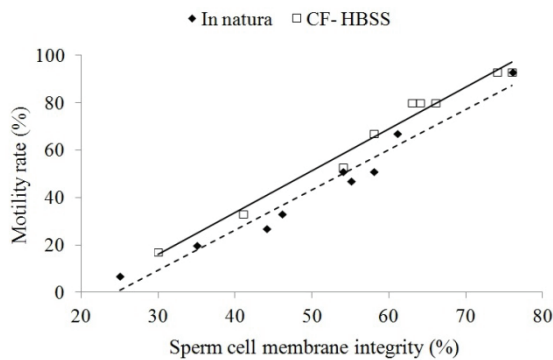


Figure 2. Linear regression between motility rate (%) and sperm cell membrane integrity (%) during the 96h cooling period of lebranche mullet (*Mugil liza*) semen *in natura* (◆) and diluted 1:3 with CF-HBSS (□). Significant linear regression for both treatments ($p < 0.05$), *in natura* $R^2 = 0.96$; CF-HBSS $R^2 = 0.98$. Positive Pearson correlation for both treatments ($n = 9$, $p < 0.05$), *in natura*: $R = 0.98$; CF-HBSS: $R = 0.95$.

Discussion

The semen of *M. liza* presented reduced portion of plasma. The same was observed in other marine fish species, such as: *Mugil cephalus* (Chao, Chen, & Liao, 1975); *Lutjanus synagris* (Sanches & Cerqueira, 2010, 2011; Sanches, Oliveira, Serralheiro, & Cerqueira, 2013; Sanches, Oliveira, Serralheiro, & Cerqueira, 2015) and *Lutjanus analis* (Sanches & Cerqueira, 2011; Sanches et al., 2013). Even with a small amount of plasma and without sperm diluents, the lebranche mullet spermatozoa showed the same motility time of that of fresh semen during 48h cooling. This is an unusual result for refrigeration process *in natura*, which generally exhibits little success and loss of seminal quality in a few hours (Sanches & Cerqueira, 2010). A rapid loss of seminal quality was verified in semen refrigeration of *Centropomus undecimalis* (Tiersch, Wayman, Skapura, Neidig, & Grier, 2004); *Lutjanus synagris* (Sanches & Cerqueira, 2010) and *Lutjanus analis* (Sanches & Cerqueira, 2011), which presented total sperm immobility after only 24h of refrigeration; in *Latris lineata* (Ritar & Campet, 2000) and *Anguilla anguilla* (Peñaranda et al., 2010) total loss of motility was observed after 48h of refrigeration.

For diluted semen, the increase in motility time during semen refrigeration was also confirmed for *Scophthalmus maximus* (Chereguini et al., 1997), *L.*

synagris (Sanches & Cerqueira, 2010) and *L. analis* (Sanches & Cerqueira, 2011). Diluents maintain sperm immobility and are developed for restore continuously osmotic changes in cells during the refrigeration procedures, correcting successive imbalances in ionic concentrations of solutes that accompany changes in temperature. As with lebranche mullet, the use of HBSS formulated with osmolality between 200 and 300 mOsmol kg⁻¹ has been successful for semen refrigeration and cryopreservation of many marine species (Riley, Chesney, & Tiersch, 2008; Tiersch et al., 2004; Wayman & Tiersch, 2000).

The chemical and physical characteristics of lebranche mullet seminal plasma are still unknown and should be studied in order to produce a specific diluent for the species. Seminal plasma presents a unique biochemical composition that favors the normality of sperm functions (Ciereszko, 2008). Some organic and inorganic components (such as saccharides, proteins, triglycerides, phospholipids; and K⁺, Na⁺, Ca⁺², Cl⁻, Mg⁺²) of seminal plasma affect spermatozoa before being activated (Cosson, 2004; Lahnsteiner, Mansour, & Berger, 2004; Lahnsteiner, Patzner, & Weismann, 1993). According to Bezerra (2010), a good diluent is characterized by the species-appropriate osmolality, effective pH value and buffering power, as well as an ionic composition favoring sperm survival. Studies have shown the importance of osmolality and ionic composition of the diluent in preservation of sperm quality (Alavi & Cosson, 2006; Alavi, Jorfi, Hatef, & Saheb, 2010; Wilson-Leedy, Kanuga, & Ingermann, 2009).

The small amount of seminal plasma found in the semen from males used in this experiment may have potentiated the benefits of the sperm diluent, which may have contributed significantly to balance solution with ions and to meet nutrient requirement of spermatozoa. However, to precisely define the maximum storage time of the semen under refrigeration, more studies are necessary in order to evaluate the capacity of fertilize oocytes, which are the end-activity of this procedure.

For semen refrigeration and cryopreservation proposes, semen:diluent proportions normally ranges from 1:1 to 1:20, with positive results at ratios close to 1:3 (Magnotti et al., 2016; Suquet et al., 2000). Despite the positive results observed using the ratio of 1:3, it is recommended to perform more tests with more diluent's compositions and proportions. Peñaranda et al. (2010) obtained positive results with dilutions of 1:50 and 1:100, during cooling of *A. anguilla* semen. The same was verified for other species (Ohta & Izawa, 1996;

Ohta, Kagawa, Tanaka, & Unuma, 2001; Tan-Fermin, Miura, Adachi, & Yamauchi, 1999) and should be considered for future studies. Marine fish release low volume of semen, with high sperm density and viscosity. Therefore, the storage using larger dilutions favors planning fertilization and optimization of biological material.

Conclusion

The use of CF-HBSS at 1:3 proportion of semen:diluent provided better performance in refrigeration of lebranche mullet semen for 96h, when compared with *in natura* semen. The diluent increased sperm motility time by 20% after 18 h of refrigeration, it maintained sperm motility rate for 24 h, and cell membrane integrity for 6 h.

Acknowledgements

The authors are grateful to the team of LAPMAR - UFSC, to the artisanal fishermen community of Florianópolis/ SC/ Brazil, and for the financial support of CAPES (project n° 23038.004323/ 2014-54) for the required material.

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Received on September 19, 2017.

Accepted on December 7, 2017.

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