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Abstract

The pejerrey, *Odontesthes bonariensis*, is being considered for aquaculture due to its high demand and market price. Reproduction and larviculture studies have demonstrated the feasibility of massive fingerling production, and techniques that prolong life and increase gamete viability can assist in the culture development of this species. In this regard, the main objective of this study was to develop freezing protocols for pejerrey sperm. For this purpose, two extenders: Ex1, a modified Mounib solution (127mM $NaHCO_3$, 159mM Sucrose, 0.025g/ml Reduced Glutathione; pH: 8; osmolality 400mOsm/Kg) and Ex2, a saline based solution (250mM $NaHCO_3$, 100mM trehalose; pH: 8; osmolality 450mOsm/Kg) were developed. Dimethyl sulfoxide (DMSO) and Ethylene glycol, (EG) were added at 10 % as cryoprotectants and two types of containers were used: cryovials (1 ml of volume) and French straws (0.250 ml). Cryopreservation was made without equilibration time using dry ice or liquid nitrogen. The results obtained by freezing pejerrey semen with dry ice showed that all the combinations tested were suitable because high motility indexes (among 4 or 5) and good fertility percentages (between 46–56 %) were obtained after thawing. However, the fertilization percentage obtained with control semen was significantly higher (80 %). No significant statistical differences were observed on the fertilization percentages between experimental combinations or the containers used. In the case of liquid nitrogen as freezing method, it was possible to obtain for all the combinations motility indexes among 3 or 4 after thawing. In the case of fertilization trials, similar percentages (around 80 %) were found for control semen and for the majority of cryopreserved samples. However, statistically lower results (among 44-60%) were found for Ex1DMSO (cryotubes) samples and for Ex1EG samples in both containers. In summary, the feasibility to cryopreserve pejerrey sperm was demonstrated for the first time using simple and practical protocols.

Key words: Cryopreservation, sperm, pejerrey, reproduction.

1. Introduction

The pejerrey *Odontesthes bonarienis* is an eurihaline fish that naturally inhabits the continental waters of the Pampean Region of Argentina (López et al., 2001). Over the last years, decreasing stocks of regional populations associated with overfishing and pollution (Berasain et al., 2005) targeted the pejerrey as a potential species for aquaculture (Miranda and Somoza, 2001; Somoza et al., 2008). Furthermore, the pejerrey is very appreciated by the local fisheries and cuisines, and for this reason, it has been introduced in many water bodies across Argentina and other countries, such as Japan where it's being cultured at a commercial scale (Mituta, 2001).

On this regard, pejerrey intensive culture methods had been succesfully achieved in Argentina (Miranda et al., 2006; Somoza, et al., 2006; Somoza et al., 2008). Despite these advances, many issues still need to be solved in order to establish pejerrey farms in the region (Somoza et al., 2008). For instance, pejerrey breeders present a marked asynchrony between spawning intervals, with a relatively low fecundity ranging from 30 to 40 percent (Strüssmann, 1989; Miranda et al., 2006). In the case of captive pejerrey males, the volume of expressible sperm is very low (100-200µl) from 3 to 4-years-old (Strūssmann et al., 1994) and the sperm concentration ranged between 3.5 to 6.5 x 10^9 cells / ml (Miranda et al., 2005).

Overall, the current scenario, dictates that a large number of broodstock fish is necessary to be maintained, since efficient hatchery operation requires uniformity in larvae size, quantity and quality (Somoza et al., 2008).

Recently, in order to improve the current breeding procedures, some experiments were performed using environmental and hormonal treatments to increase pejerrey sperm volume and synchronize spawning females (Miranda et al., 2001; 2005; 2006). Although this path is pointing in good direction there's a different approach that could result more advantageous and, indeed, complimentary with the pharmacological treatments.

In this context, the other approach was to develop an appropriate sperm cryopreservation protocol, which has been identified as a tool to improve broodstock management and larvae production in more than 200 species of fish (Lanes et al 2008; Yang and Tiersch, 2009). However, the success results of these sperm cryopreservation experiences have been highly variable among species and within methods being necessary to perform in each case a species-specific adaptation to the existent techniques (Billard et al., 2004).

In Summary, the main objective of this study was to develop simple and practical cryopreservation protocols for pejerrey sperm.

2. Materials and Methods

Pejerrey broodstocks were selected from the stock kept at 25.000 l external breeding tanks at the IIB-INTECH aquaculture facilities. Fish of similar size and weight were chosen (SL: 27.97 ± 0.35cm; BW: 301.74 ± 11.36g) and distributed into four 3000 l indoor tanks (20 fish per tank), maintaining a gender proportion of 1:1 in order to obtain more volume of expressible sperm. In all tanks, water quality was kept by means of an open flow system keeping water temperature and salinity around 18°C and 1.5%, respectively all year round. Fish were fed three times a day using pejerrey pelletized food (Shulet SA, Argentina) and were maintained with a natural photoperiod (around 13 h light/11 h dark). The sperm sampling were performed during pejerrey reproductive season (spring time) following Renard et al. (1994) and Miranda et al. (2005) guidelines. Briefly, fish were anesthetized in a 100 ppm benzocaine bath (Droguería Saporiti, Argentina), and semen was sampled by abdominal stripping and collected with 200µl tips adapted for 1ml tuberculin syringes avoiding or discarding urine or fecal contaminated samples. Once filled, each tip was placed inside a 1.5ml tube and kept on ice until motility index was tested. Motility analysis was performed with an optic microscope under a 160x magnification, using the motility index table for

pejerrey (Strüssmann et al., 1994). Sperm motility was characterized in six indexes: 0, corresponds to all immotile spermatozoa; 1, most are immotile and some present lateral vibration; 2, most spermatozoa are vibrating or immotile while some present forward movement; 3, some spermatozoa move rapidly, some move slowly and others are immotile; 4,most spermatozoa move rapidly while some are moving slowly; and 5, most display rapid movements. Immediately after sperm collection, aliquots of each sample were activated with tap water. All the samples with a motility index of 5 were gently pooled (8 fish) and kept on ice until dilution and cryopreservation.

2.1 Extenders

Previously and in order to develop species-specific extenders for *O. Bonariensis*, a sperm plasma ionic composition analysis was performed. Briefly, seminal plasma from a pooled sample of high quality sperm, obtained from 5 fish, was separated by centrifugation (first 200g for 2min followed by 2500g for 10min) and the concentration of some representative cations (Ca^{++}, K^{+}, Mg^{++}, Na^{+}) were determined using a Flame spectrophotometer. Also the osmotic pressure and pH were recorded using a Wescor 5500 Vapor Pressure Osmometer and a Hanna pH 211 phMeter. Considering these findings (see results section) and preliminary results obtained by Lichtenstein and Miranda (2007), two extenders were developed and tested in the present study. Extender 1 (Ex1): A Mounib modified solution (127mM $NaHCO_3$, 159mM Sucrose, 0.025g/ml Reduced Glutathione; pH: 8; osmolality 400mOsm/Kg) and extender 2 (Ex2): A saline based solution (250mM $NaHCO_3$, 100mM trehalose; pH: 8; osmolality 450mOsm/Kg). Combinations of sperm + Ex1 or Ex2 (1:50) were done and aliquots were observed during 10 minutes, then activated with tap water (semen-diluent mixture 1:3 tap water) and motility indexes recorded as described above.

2.2 Cryoprotectants

In order to minimize the risk of cryogenic damage during the freezing stages, two cryoprotectants were tested: Dimethyl sulphoxide (DMSO) and Ethylene glycol (EG) applied in a 10% v/v of the sperm extender solution. Cryoprotectants were added to the sperm extender solution immediately after the beginning of the freezing protocols, thus minimizing the risk of a toxic reaction between sperm and cryoprotectant.

2.3 Containers

Two types of containers were used: cryovials (Minitube, Germany) of 1 ml of volume and French straws of 0.250 ml of volume (Minitube, Germany). Combinations of sperm (3 µl) + Ex1 or Ex2 (147 µl) + DMSO or EG (15 µl). were used to fill the cryovials or the straws (total volume of 165µl). In the case of straw the ends were sealed with clay. All combinations were done by quadruplicate, and mean values and standard errors were used to analyze differences between combinations.

2.4 Cryopreservation

Two freezing methods using dry ice or liquid nitrogen were tested having in consideration, costs and availability of different freezing technologies that can be used in research institutes or working at the field in the coast of Pampasic lagoons.

In the method using dry ice, samples were placed on the top of a dry ice block and kept inside a styrofoam box for 24 h, as described by Aoki et al. (1997). In the case of liquid nitrogen samples were positioned in a floating styrofoam raft for 12 min at 6 cm from the liquid nitrogen surface inside a thermal flask, and immediately after, samples were plunged

into the liquid nitrogen and stored for 24 h, following the methodology described by Rideout et al. (2004).

2.5 Thawing

After 24 h, samples were removed from the liquid nitrogen or the dry ice and thawed in a water bath at 35ºC for 50s for cryovials, and 10s for the French straws. These values were determined from curves of thawing previously performed by Lichtenstein and Miranda (2007) where temperatures between 20 and 40 ºC were tested.

2.6 In vitro fertilization

Immediately after thawing the motility index was analyzed (as described above) and the diluted semen of each sample (total number of sperm around 50,000/egg) was gently mixed with approximately 300 eggs obtained from a pool of eggs stripped from 2 mature females. After the mix, the sperm was activated with tap water, and after 10 min the eggs were then incubated using glass containers at room temperature (18 ºC). Control groups were performed by quadruplicate using undiluted fresh sperm (total number of sperm around 50,000/egg) with a recorded motility index of 5. After 8 h of fertilization morula stage was observed and the % of fertilization was assessed in all cases.

2.7 Statistical Analysis

Analysis of variance (ANOVA) was used to determine significant differences ($P < 0.05$) between groups, and Tukey's multiple comparisons tests were performed for a *posteriori* comparisons using the Graphpad Prims4 software. Data are expressed as mean ± SEM.

3. Results

Pejerrey sperm plasma ionic composition analysis was: 0.2mM [Ca^{++}], 4.82mM [K^{+}], 0.49mM [Mg^{++}], 24.7mM [Na^{+}]. Also, the pH (8) and the osmolality (350mOsm/Kg) were recorded.

The motility results obtained after pejerrey semen dilution in Ex1 and Ex2 demonstrated that it was possible to activate diluted sperm after 10 minutes with high motility indexes (Fig. 1).

3.1 Freezing semen with dry ice or liquid nitrogen

The results obtained freezing pejerrey semen with dry ice showed that all the combinations tested were suitable because high motility indexes (among 4 or 5) and acceptable fertility percentages (between 46–56 %) were obtained after thawing (Figs. 2, 3). However, the fertilization percentages obtained with control semen was higher (around 80 %). No significant statistical differences were observed on motility indexes and fertilization percentages between experimental combinations or the containers used (Figs. 2, 3).

In the case of liquid nitrogen as freezing method, it was possible to obtain for all the combinations, motility indexes among 3 or 4 after thawing **(**Fig. 4). In the case of fertilization trials, similar percentages (around 80 %) were found for control semen and for the majority of the liquid nitrogen cryopreserved samples. However, statistically lower results (among 44-60%) were found for Ex1DMSO (cryotubes) samples and for Ex1EG samples in both containers (Fig. 5**).** Taking in consideration only the types of container, no statistical differences were found in this experiment for each combination (Fig. 5).

4. Discussion

The first step for fish sperm cryopreservation is to know which factors affect sperm motility and develop an appropriate extender to allow the dilution of the sperm with temporary suppression of motility. In pejerrey, osmotic pressure appears to be the main factor regulating sperm motility and that some cations (specially K^+) are able to override the osmotic suppression of motility (Strüssmann et al., 1994). The same authors recommended the dilution of pejerrey semen for cryopreservation with a Modified Mounib Solution (absence of K^+ and presence of Na^+) being this solution isosmotic or slightly hyperosmotic respect pejerrey seminal fluid. It was also reported that pejerrey seminal fluid osmotic pressure was 331mOsm/Kg (Renard et al., 1994) and that motility was unaffected by pH in the range of 5.4-8.3. In our study we characterized the composition of pejerrey seminal plasma finding that Na^+ was the ion at major concentration, having an osmotic pressure of 350mOsm/Kg and with a pH of 8. Then, following Strüssmann et al. (1994) considerations, and taking in account our results, we used two extenders based on saline solutions: a Modified Mounib Solution (400mOsm/Kg) and a new one replacing sucrose by trehalose (Aoki et al., 1997), without reduced Glutathione and with a slightly higher osmotic pressure (450 mOsm/Kg). The dilution of pejerrey semen with these extenders showed excellent results demonstrating that it was possible to activate diluted sperm with high motility indexes.

Despite the fact that DMSO has been referred to as the 'universal cryoprotectant' (Chao and Liao, 2001) and has been used to cryopreserve sperm from numerous marine and freshwater fish species (Mounib, 1978; Withler and Lim, 1982; Kerby, 1983; Dreanno et al., 1997; Yao et al., 2000; Velasco-Santamaría, et al 2006; Lanes et al., 2008; Yang and Tiersch, 2009), others cyioprotectants such as glycerol (GLY), methanol (MET), propylene glycol (PG), dimethylformamide (DMF) and EG were used commonly in fish sperm cryopreservation (Lahnsteiner et al., 1997; Yao et al., 2000; Bergeron et al., 2002;

Glogowski., et al 2002; Zhang et al., 2003; Lanes et al., 2008; Yang and Tiersch, 2009). In a preliminary experiment for pejerrey sperm cryopreservation, DMF, MET, GLY, DMSO and EG were tested at different concentrations. Analyzing only the post thawed motility indexes encouraging results were obtained with DMSO and EG at concentrations between 5-15 % (Lichtenstein and Miranda, 2007). Following these findings, in the present study we used combinations of extenders with DMSO or EG at 10% as cryoprotectants.

In the case of dry ice as freezing method, the motility and fertility of the frozen sperm was seen to decrease respect to control sperm but with acceptable results for all combinations. Using liquid nitrogen, similar fertilization percentages (around 80 %) were found for control semen and for the majority of the cryopreserved combinations. However, statistically lower results were found for Ex1EG samples. It would therefore appear that EX2 and DMSO are the most suitable extender and cryoprotectant for cryopreserving pejerrey sperm.

In most studies, DMSO provides the best results, probable due to its fast penetration into spermatozoa and its interaction with the phospholipids at the sperm membrane (Suquet et al., 2000). But, in same cases, GLY, PG, DMF or EG has also been reported to perform as well as or better than DMSO for the cryoprotection of sperm from other species (Aoki et al., 1997; Richardson et al., 1999; Fabbrocini et al., 2000; Rideout et al., 2004; Zhang et al., 2003; Navarro et al., 2004; Yang et al., 2007; Tian et al., 2008).

As well as the appropriate choice of cryoprotectant and extender used in the cryopreservation process, successful cryopreservation of animal sperm depends on the freezing protocol (Viveiros et al., 2000). In this study, taking in consideration the results obtained, a slow method using the vapor phase of liquid nitrogen and *posterior* plunging into liquid nitrogen was more effective than the dry ice method. Similar results were obtained in *Oryzias latipes* (Aoki et al., 1997), *Melanogrammus aeglefinus, Gadus morhua* (Rideout et al., 2004) and *Paralichthys orbignyanus* (Lanes et al., 2008). However, in the area of pejerrey

distribution, lagoons of the Pampean Region of Argentina (López et al., 2001), it can be difficult to obtain and transport liquid nitrogen to work in the field. For this reason, in spite of the dry ice method being less effective it can be conveniently used in those conditions.

Ours results, showed no differences between the containers used. But, compared to cryovials, the use of French straws has the potential advantage for use with an automated straw filling and sealing equipment.

It was demonstrated that the protocols developed in this study are efficient for the cryopreservation of pejerrey *Odontesthes bonariensis* sperm. The extenders, the cryoprotectants, and the methods for freezing employed resulted in viable fertilization rates for all cases. In summary, this is the first report on successful cryopreservation of *Odontesthes bonariensis* sperm, these procedures should improve broodstock management techniques for this species and consequently augment the potential for its culture.

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References

Aoki, K., Okamoto, M., Tatsumi, K., Ishikawa, Y., 1997. Cryopreservation of Medaka spermatozoa. Zool. Sci. 14 (4), 641-644.

Berasain, G.E., Colautti, D.C., Remes Lenicov, M., Velasco, M., 2005. Variaciones estacionales e históricas de las especies ícticas de la laguna de Chascomús. Biol. Acuat**.** 22, 47-58.

Bergeron, A., Vandenberg, G., Proulx, D., Bailey, J.L., 2002. Comparison of extenders, dilution ratios and theophylline addition on the function of cryopreserved walleye semen. Theriogenology 57, 1061–71.

Billard, R., Cosson, J., Noveiri, S.B., Pourkazemi, M., 2004. Cryopreservation and short-term storage of sturgeon sperm, a review. Aquaculture 236, 1 –9.

Chao, N. H., Liao, I.C., 2001. Cryopreservation of finfish and shellfish gametes and embryos. Aquaculture 197, 161–189.

Dreanno, C., Sequet, M., Quemener, L., Cosson, J., Fierville, F., Normant, Y., Billard, R., 1997. Cryopreservation of turbot (*Scophthalmus maximus*) spermatozoa. Theriogenology 48, 589–603.

Fabbrocini, A., Lubrano Lavadera, S., Rispoli, S., Sansone, G., 2000. Cryopreservation of Seabream (*Sparus aurata*) Spermatozoa. Cryobiology 40, 46–53.

Glogowski, J., Kolman, R., Szczepkowski, M., Horváth, Á., Urbányi, B., Sieczyñski, P., 2002. Fertilization rate of Siberian sturgeon (*Acipenser baeri*, Brandt) milt cryopreserved with methanol. Aquaculture 211, 367– 73.

Kerby, J.H., 1983. Cryogenic preservation of sperm from striped bass. Trans. Am. Fish. Soc. 112**,** 86–94.

Lanes, C.F.C., Okamoto, M., Cavalcanti, P.V., Collares, T., Farias Campos, V., Deschamps, J.C., Robaldo, R.V., Marins, L.F., Sampaio, L.A., 2008. Cryopreservation of Brazilian flounder (*Paralichthys orbignyanus*) sperm. Aquaculture 275, 361–365.

Lahnsteiner, F., Weismann, T., Patzner, R.A., 1997. Methanol as cryoprotectant and the suitability of 1.2 ml and 5 ml straws for cryopreservation of semen from salmonid fishes. Aquacult. Res. 1997, 28:471–9.

Lichtenstein, G., Miranda, L.A., 2007. Desarrollo de diluyentes y evaluación de métodos para la criopreservación de esperma de pejerrey bonaerense *Odontesthes bonariensis.* In: Poceedings of the I Reunión Conjunta de Sociedades de Biología de la República Argentina. Huerta Grande, Córdoba, Argentina. Abstract.

López, H., Baigún, C., Iwaskiw, J., Delfino, R., Padín, O., 2001. La cuenca del Salado: Uso y posibilidades de sus recursos pesqueros. Ed. Univ. de La Plata, pp. 76.

Miranda, L.A., Somoza, G.M., 2001. Biologia reproductiva del pejerrey *Odontesthes bonariensis*: diferenciación sexual y endocrinología de la reproducción, aspectos básicos y su

potencial aplicación en acuicultura. In: Grosman, F. (Ed.), Fundamentos biológicos, económicos y sociales para una correcta gestión del recurso pejerrey. Editorial Astyanax, Buenos Aires, pp. 41-45.

Miranda, L.A., Escaray, R.U., Bustingorry, J.F., Somoza, G.M., 2001. Effects of photoperiod and human chorionic gonadotropin (hCG) administration on spermiation in pejerrey *Odontesthes bonariensis*. Rev. Arg. Prod. Anim. 21, 95-105.

Miranda, L.A., Cassará, M.C., Somoza, G.M., 2005. Increase of milt production by hormonal treatment in the pejerrey fish *Odontesthes bonariensis*. Aquac. Res. 36, 1473-1479.

Miranda, L.A., Berasain, G.E., Velasco, C.A.M., Shirojo, Y., Somoza, G.M., 2006. Natural spawning and intensive culture of pejerrey *Odontesthes bonariensis* juveniles. Biocell 30, 89-95.

Mituta, T., 2001. La historia del pejerrey en Japón. In: Grosman, F. (Ed.), Fundamentos biológicos, económicos y sociales para una correcta gestión del recurso pejerrey. Editorial Astyanax, Buenos Aires, pp. 21-24.

Mounib, M.S., 1978. Cryogenic preservation of fish and mammalian spermatozoa. J. Reprod. Fertil. 53, 13–18.

Navarro, O.J., Velasco-Santamaría, Y.M., Cruz-Casallas, P.E., 2004. Evaluación de cinco protectores para la crioconservación de semen de cachama blanca (*Piaractus brachypomus*). Rev. Col. Cienc. Pec. 17, 53–59.

Renard, P., Strüssmann, C.A., Ling, H., Takashima, F., 1994. Evaluation of extenders for pejerrey *Odontesthes bonariensis* sperm. Fish. Sci. 60**,** 661-666.

Richardson, G.F., Wilson, C.E., Crim, L.W., Yao, Z., 1999. Cryopreservation of yellowtail flounder (*Pleuronectes ferrugineus*) semen in large straws. Aquaculture 174, 89-94.

Rideout, R.M., Trippel, E.A., Litvak, M.K., 2004. The development of haddock and Atlantic cod sperm cryopreservation techniques and the effect of sperm age on cryopreservation success. J. Fish Biol. 65, 299–311.

Somoza, G.M., Miranda, L.A., Guilgur, L.G., Strobl-Mazzulla, P.H., 2006. Characterization of the brain-pituitary axis in pejerrey *Odontesthes bonariensis*. Biocell 30, 89-95.

Somoza, G. M., Miranda, L.A., Berasain, G. E., Colautti, D.C., Remes Lenicov, M., Strüssmann C.A., 2008. Historical aspects, current status and prospects of pejerrey aquaculture in South America. Aquac. Res. 39, 784-793.

Strüssmann C.A.,1989. Basic studies on seed production of pejerrey *Odontesthes bonariensis*. Doctoral Thesis, Tokyo University of Fisheries, Tokyo, Japan, pp. 351 .

Strüssmann, C.A., Renard, P., Ling, H., Takashima, F., 1994. Motility of pejerrey *Odontesthes bonariensis* spermatozoa. Fish. Sci. 60, 9-13.

Suquet, M., Dreanno, C., Fauvel, C., Cosson, J., Billard, R., 2000. Cryopreservation of sperm in marine fish. Aquac. Res. 31, 231–243.

Tian, S.L., Chen, X.S., Ji, J.M., Zhai, L.J., Sun, C., Chen, P.Z., Su, P.Z., 2008. Cryopreservation of spotted halibut (*Verasper variegatus*) sperm. Aquaculture 284, 268–271

Velasco-Santamaría, Y.M., Medina-Robles, V.M., Cruz-Casallas, P.E., 2006. Cryopreservation of yamú (*Brycon amazonicus*) sperm for large scale fertilization. Aquaculture 256, 264–271.

Viveiros, ATM., So, N., Komen, J., 2000. Sperm cryopreservation of African catfish, *Clarias gariepinus*: cryoprotectants, freezing rates and sperm: egg dilution ratio. Theriogenology 54, 1395–1408.

Withler, F.C., Lim, L.C., 1982. Preliminary observations of chilled and deep-frozen storage of grouper (*Epinephelus tauvina*) sperm. Aquaculture 27**,** 389–392.

Yang, H., Carmichael, C., Varga, Z.M., Tiersch, T.R., 2007. Development of a simplified and standardized protocol with potential for high-throughput for sperm cryopreservation in zebrafish Danio rerio. Theriogenology 68, 128–136.

Yang, H., Tiersch, T.R., 2009. Current status of sperm cryopreservation in biomedical research fish models: Zebrafish, medaka, and Xiphophorus. Comparative Biochemistry and Physiology, Part C 149, 224–232.

Yao, Z., Crim, L.W., Richardson, G.F., Emerson, C.J., 2000. Motility, fertility and ultrastructural changes of ocean pout (*Macrozoarces americanus L.*) sperm after cryopreservation. Aquaculture 181, 361–375.

Zhang, Y.Z., Zhang, S.C., Liu, X.Z., Xu, Y.Y., Wang, C.L., Sawant, M.S., Li, J., Chen, S.L., 2003. Cryopreservation of flounder (*Paralichthys olivaceus*) sperm with a practical methodology. Theriogenology 60, 989–996.

Fig. 1. Motility indexes of pejerrey fresh sperm diluted (1:50) in Ex1 or in Ex2. The sperm was activated 10 minutes after the dilution with tap water.

Fig. 2. Motility indexes with fresh and dry ice cryopreserved pejerrey sperm in straws or cryovials. The values correspond to the mean±S.E.M. Bars with different letters above are significantly different ($p < 0.05$), n=4. Ex1D: sperm + extender 1 + dimethyl sulfoxide; Ex1E: sperm + extender 1 + ethylene glycol; Ex2D: sperm + extender 2 + dimethyl sulfoxide; Ex2E: sperm + extender 2 + ethylene glycol.

Fig. 3. Fertilization percentages with fresh and dry ice cryopreserved pejerrey sperm in straws or cryovials. The values correspond to the mean±S.E.M. Bars with different letters are significantly different ($p < 0.05$), n=4. Ex1D: sperm + extender 1 + dimethyl sulfoxide; Ex1E: sperm + extender 1 + ethylene glycol; Ex2D: sperm + extender 2 + dimethyl sulfoxide; Ex2E: sperm + extender 2 + ethylene glycol.

Fig. 4. Motility indexes with fresh and liquid nitrogen cryopreserved pejerrey sperm in straws or cryovials. The values correspond to the mean±S.E.M. Bars with different letters above are significantly different ($p < 0.05$), n=4. Ex1D: sperm + extender 1 + dimethyl sulfoxide; Ex1E: sperm + extender 1 + ethylene glycol; Ex2D: sperm + extender 2 + dimethyl sulfoxide; Ex2E: sperm + extender 2 + ethylene glycol.

Fig. 5**.** Fertilization percentages with fresh and liquid nitrogen cryopreserved pejerrey sperm in straws or cryovials. The values correspond to the mean±S.E.M. Bars with different letters are significantly different ($p < 0.05$), n=4. Ex1D: sperm + extender 1 + dimethyl sulfoxide;

Ex1E: sperm + extender 1 + ethylene glycol; Ex2D: sperm + extender 2 + dimethyl sulfoxide; Ex2E: sperm + extender 2 + ethylene glycol.