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Cold storage of the sperm of the endemic trout *Oncorhynchus mykiss nelsoni*: a strategy for short-term germplasm conservation of endemic species

Almacenamiento en frío del esperma de la trucha endémica *Oncorhynchus mykiss nelsoni*: una estrategia para la conservación a corto plazo del germoplasma de especies endémicas

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Abstract. Aiming to determine an appropriate protocol for short-term storage of the sperm of San Pedro Mártir trout, *Oncorhynchus mykiss nelsoni*, wild fish previously matured in captivity were anesthetized and the semen, obtained by abdominal pressure, was suspended in Erdhal and Graham extender at 1:3, 1:6 and 1:9 dilution ratios and kept at 4° C for 9 days with an undiluted sample as control. The percentages of sperm motility and membrane integrity were assessed daily. The results indicated that sperm suspended at a dilution of 1:9 can be stored at 4° C with ≥ 80% motility and ≥ 65% membrane integrity for 7 and 6 days respectively. In addition, the results show that membrane integrity decreased more rapidly than motility, which suggests that it may be used as an early indicator of sperm damage during short-term cold storage. The results obtained are important to support future conservation programs of this trout.

Key words: sperm storage, viability, extender solution.

Resumen. Con el objetivo de determinar un protocolo apropiado para el almacenamiento a corto plazo de los espermatozoides de trucha de San Pedro Mártir, *Oncorhynchus mykiss nelsoni*, se anestesiaron peces silvestres previamente madurados en cautiverio y el esperma, obtenido por presión abdominal, fue suspendido en una solución extender de Erdhal y Graham a tasas de dilución de 1:3, 1:6 y 1:9 y mantenido a 4° C durante 9 días con una muestra control sin diluir. Los porcentajes de movilidad e integridad de membrana fueron evaluados diariamente. Los resultados indicaron que los espermatozoides suspendidos en una dilución de 1:9 se pueden almacenar a 4° C con movilidad ≥ 80% e integridad de membrana ≥ 65% por 7 y 6 días respectivamente. Además, los resultados muestran que la movilidad disminuye más lentamente que la integridad de la membrana, lo que sugiere que ésta puede utilizarse como un indicador temprano del daño al esperma durante el almacenamiento a corto plazo en bajas temperaturas. Los resultados obtenidos son importantes para apoyar los programas de conservación de esta trucha.

Palabras clave: almacenamiento del esperma, viabilidad, solución extender.

Introduction

The anthropogenic pressures on marine and terrestrial ecosystems, accompanied by depletion of natural stocks have aggravated the degradation of some native and threatened fish species. The strategies for the conservation of their genetic resources should include the application of reproductive strategies, since it would allow continuous fry production throughout the year. However, continuous breeding may be impossible due to lack of spermiating males or asynchronous maturation of male and female broodstock (Bozkurt and Seçer, 2005; Mañanós et al., 2009). Thus, appropriate short-term storage techniques to maintain viable sperm for hours or days, with no alteration in fertilizing ability, may be of great importance to enhance fish production (Scott and Baynes, 1980; Hatipoðlu and Akçay, 2010).

Short-term storage has been considered a useful strategy for preservation of threatened or endangered fish species (Basavaraja and Hegde, 2005; Maria et al., 2006a; Hatipoðlu and Akçay, 2010). Therefore, it may be a worthy strategy for the conservation of the endemic San Pedro Mártir trout (SPMT), *Oncorhynchus mykiss nelsoni*, which inhabits the streams of the western slopes of Sierra San Pedro Mártir (Ruiz-Campos and Pister,

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1995) and is considered the southernmost coastal rainbow trout in North America (Behnke, 2002). It is resistant to high temperatures during summer and has a non-migratory nature, which are bioecological attributes different from those of other rainbow trout (Ruiz-Campos, 1993).

Due to its restricted distribution and low abundance, SPMT is subject to special protection (Jelks et al., 2008; Semarnat, 2010). Therefore, conservation programs are required but efforts to maintain it in captivity for conservation and restocking have been difficult, mainly due to the difficulty of synchronizing male maturity and female spawning under controlled conditions (Ruiz-Campos, 1993; Aguilar-Juárez, 2010).

Short-term storage of SPMT sperm until eggs become available for reproduction in captivity may enhance programs for species conservation. This would also allow transportation of sperm to hatcheries with fertile female broodstock, or when difficulties arise in obtaining synchronization of male-female maturity. In addition, due to the high genetic diversity of this species (Camarena-Rosales et al., 2008), short-term sperm conservation could solve the problems of inbreeding of other native species currently held in captivity (De los Santos-Camarillo, 2008).

In this paper we describe a protocol for short-term storage of SPMT sperm at low temperatures (4° C), and assess the effect of this procedure on sperm motility and membrane integrity.

Materials and methods

Capture and maintenance of fish. In March 2007, 11 wild SPMT males were captured and maintained at the Banco Periférico de Germoplasma del Noroeste of the Centro de Investigación Científica y de Educación Superior de Ensenada (CICESE, BPGN). The organisms were kept under artificial photoperiod during 7.5 months in a 250-L fiberglass tank connected to a recirculating system until maturation (Aguilar-Juárez et al., 2011).

Fish were fed initially with a daily ration of frozen mysids (Ocean Aquatics, Kelowna, British Columbia) equivalent to 3% of their wet weight, which was replaced gradually with commercial food (42% protein and 15% lipid, 3.5 mm, floating, El Pedregal Silver Cup, Toluca, Mexico). When fish were fully acclimated, the total feed ration was equivalent to 5% of their wet weight supplied in 2 equal parts at 08:00 and 18:00 hours.

Sperm collection. Eight of the 11 males matured. These were starved for 2 d to avoid feces production during milt collection and anesthetized in a water bath with 100 µL/L of a clove oil/ethanol (1:9 v/v) mixture during 10-15 min (Keene and Noakes, 1998), after which the genital opening of each male was cleaned, dried with paper towels and the semen was obtained by applying gentle abdominal pressure. The first sample was discarded to avoid contamination with urine and feces, and the clean milt samples were collected in 1.8-mL plastic pipettes, placed on crushed ice and transported immediately (~2 min) to the BPGN.

Evaluation of motility. Immediately after collection, triplicate 0.5 µL subsamples of each sample were placed on a clean microscope slide and mixed with 20 µL of activation medium DIA 532 prepared with NaCl 0.009 M, glycine 0.05 M and 7-9 Sigma tris 0.02 M and 87.40 mOsmol/kg (Herráez et al., 2009). The percentage of rapid, vigorous, and forward-moving spermatozoa was evaluated at 400*X* using dark-field microscopy (Nikon H600L, Eclipse 80i). All samples had ≥ 80% motility, and were pooled and used for the experiment.

Evaluation of sperm concentration. After determination of volume (µL), the sperm concentration of each sample fixed with a 1% lugol solution was evaluated on triplicate subsamples under a microscope at 400*X* (Nikon H600L, Eclipse 80i, Nikon Corporation, Tokio, Japan) with a hematocytometer (0.1 mm, Brightline, Hausser Scientific, Horsham, Pennsylvania, USA).

Dilution and short-term storage. Increasing dilutions (1:3, 1:6, or 1:9) of the pooled sperm suspended in Erdhal and Graham's (EG) antibiotic-free extender solution ($CaCl_2$•$2H_2O$ 0.001 M, $MgCl_2$•$6H_2O$ 0.001 M, Na_2HPO_4 0.002 M, KCl 0.034 M, citric acid 0.001 M, glucose 0.055 M, 10mL of a 0.002 M KOH solution, 20 mL of a bicine 0.006 M solution, 323 mOsmol/kg and pH 7.4) were prepared for short-term storage. All chemicals were analytical grade (Sigma Chemical Co., St. Louis Missouri, USA).

Triplicate sperm samples (100 µL) were mixed with 300, 600 and 900 µL of EG solution (dilution 1:3, 1:6 and 1:9 respectively), placed in 0.6-mL (control sample and 1:3 dilution) or 1.5-mL (1:6 and 1:9 dilutions) conical Eppendorf tubes hermetically sealed to avoid evaporation, and kept in a conventional refrigerator at 4° C for 9 days, during which the percentages of sperm motility and membrane integrity were assessed daily. Three undiluted sperm samples (250 µL in 0.6-mL tubes) were used as control.

Motility was estimated as described previously, using 0.5 µL aliquots of all samples. Membrane integrity was evaluated with the live/dead sperm viability kit (L-7011 Molecular Probes, Inc., Eugene, Oregon, USA), according to the Molecular Probes (2005) protocol. For this, 10 µL aliquots of each sperm dilution were mixed with 0.05 µL of SYBR-14 (20 nM final concentration) and incubated in the dark at ~19 C for 10 min. After addition of 0.50 µL propidium iodide (PI, 12 µM final concentration),