Vol. 49, n. 1 : pp. 73-77, January 2006
ISSN 1516-8913 Printed in Brazil

BRAZILIAN ARCHIVES OF
BIOLOGY AND TECHNOLOGY
A N I N T E R N A T I O N A L J O U R N A L

Cryopreservation of Semen From Functional Sex-Reversed Genotypic Females of the Rainbow Trout, *Oncorhynchus mykiss*

Alexandre Ninhaus-Silveira[1*], Fausto Foresti[2], Yara Aiko Tabata[3], Marcos Guilherme Rigolino[3] and Rosicleire Veríssimo-Silveira[2]

[1]Departamento de Biologia e Zootecnia; UNESP; Ilha Solteira - São Paulo - Brasil. [2]Departamento de Morfologia; UNESP; IB; Botucatu - São Paulo - Brazil. [3]Estação Experimental de Salmonicultura; APTA; Campos do Jordão - São Paulo - Brasil

ABSTRACT

Cryopreservation of semen from sex-reversed females of rainbow trout aims at rationalizing the production of stocks composed by 100% females. Semen from normal males (M) and two types of genotypic females (R and G), sex-reversed by the oral administration of 17α-methyltestosterone, were used. R was obtained by the fertilization of normal eggs with semen of sex-reversed females while G via gynogenetic reproduction. Semen was diluted in an extender solution (glucose 5,4 g, egg yolk 10 ml, dimetil sulfoxide 10 ml, water 80 ml) at 1:3 ratio (semen/extender), stored in straws of 0.5 ml and freezed in a dry container Cryopac CP-65, at -180ºC. Thawing was performed with water at 70ºC for 3 seconds. There were no significant fertilization rate differences (P>0.05) among thawed semen groups (M = 73.1±11.5%; R = 67.2±23.6%; G = 64±5.8%), confirming that the freezing methodology used was efficient to cryopreserve semen of all three trout groups.

Key words: *Oncorhynchus mykiss*, Cryopreservation, Sex reversal, Gynogenesis, Semen

INTRODUCTION

Sex maturation of fishes is related to modifications that lead to reduction of growth and decreasing of product quality, because of the high energy demand for gonad development. In salmonids, these problems are more concerned to males, since they usually become mature before reaching the commercial size. Therefore, production of "all female" populations constitutes an useful alternative to improve productivity and quality of commercial stocks (Bye and Lincoln, 1986). The procedure commonly performed to produce stocks composed by 100% of females, comprises sex reversal of females by administration of 17α-methyltestosterone in early development stages, followed by fertilization of eggs from normal females with semen samples from reversed ones (Hunter et al., 1983; Tsumura et al., 1991).

Demand for female monosex lines has been increasing over the last years, requiring the development of specific technologies to standardize this process, aiming to increase the production of sex-reversed females. Cryopreservation of semen from sex-reversed genotypic females with 17α-methyltestosterone (MT) might, thus, contribute to rationalization of the production of all-female rainbow trout stocks,

* Author for correspondence

as it improves semen utilization and reduces hormone treatments, assuring a regular supply of such material.

Studies involving semen freezing of rainbow trout were reported by several authors (Stoss et al., 1978; Stoss, et al., 1983; Baynes and Scott, 1987; Wheeler and Thogaard, 1991; McNiven et al., 1993; Fogli da Silveira et al, 1994; Conget et al., 1996; Cabrita et al., 2001). However, the number of reports related to utilization and application of the semen obtained from reversed females is scarce (Kavamoto et al., 1991; Feist et al., 1995; Tabata and Mizuta, 1997; Robles et al., 2003).

The present work evaluated the fertility of cryopreserved semen from functional sex-reversed genotypic females of the rainbow trout, *Oncorhynchus mykiss*, aiming to form a semen bank to be used in programs of production of monosex female stocks of this species.

MATERIALS AND METHODS

This work was carried out at the Estação Experimental de Salmonicultura, APTA/ Regional do Vale do Paraíba, State of São Paulo, Brazil, located in Campos do Jordão. Milt samples were collected from normal males (M) and from two types of genotypic females, which were sex-reversed by oral administration of 17α-methyltestosterone (MT), at a dose of 250 μg/kg food, during 80 days at a mean temperature of 12.8^{o}C (Accumulated Thermal Units (ATU) = 1024^{o}C/days), starting from the beginning of feeding. (Tabata et al., 2000).

The first type of genotypic female was obtained by fertilizing of normal eggs with semen of reversed females (R). The second type (G) was a result of gynogenetic reproduction, where the eggs were fertilized with spermatozoa previously irradiated with ultraviolet light (UV) and then diploidized by the retention of the second polar body after thermal shock at 28ºC. When two years old (first maturation), the fishes that expelled milt by abdominal pressing were selected as breeders.

Motility of spermatozoa was evaluated on a subjetive scale ranging from 0 to 5, where the lowest value indicated samples without motile spermatozoa and the highest value referred to those samples with up or more than 80% of motility (Fribourg, 1966). Sperm concentration (spermatozoa/mm^{3}) was determined by a Neubauer chamber (1:1000; semen:extender). Motility and concentration of semen samples were analyzed in order to evaluate the cryopreservation procedure. Samples with level 5 of motility were grouped as a pool and further evaluated about their spermatozoa concentration. Four donors were used to compose the sperm pool in each semen producer group. Semen was diluted in a cryoprotectant extender composed by 5.4 g of glucose, 10 ml of chicken egg yolk, 80 ml of water and 10 ml of dimethyl sulfoxide (DMSO). Semen dilution was performed at room temperature (14ºC), in a proportion of 1:3 (semen: extender). After dilution, semen was stored in straws of 0.5 ml and immediately frozen in liquid nitrogen in a dry container (CryoPac / CP-65 - Taylor-Warton) at -180ºC without a previous equilibrium of semen to the cryoprotectant extender. After freezing, straws containing semen samples were transferred to a wet container (Cryometal/DS-34) for cryopreservation (-195ºC).

Fertilization tests were performed 24 hours after semen freezing. About 150 eggs were taken from a pool of eggs from four two years old females and were fertilized with thawed semen (two straws/sample). Fresh semen from three new groups of donors, composed by four normal males (M) and eight sex-reversed females (4R and 4G) were used in the treatment control. For these treatments, 0.2 ml of semen/sample was added. Four replicates were performed for each treatment. Semen was thawed with water at 70ºC for 3s, and immediately mixed to the eggs and activated with a solution of $NaHCO_3$ 1%. After 20 minutes of activation, the eggs were ringed with tap water and kept in incubators. Fertilization rate was determined after 17 days of incubation, with 196^{o}C/days, when the embryos presented pigmented eyes (eyed eggs), and estimated by the ratio between viable eggs and the total number of eggs per sample.

In order to compare the fertility rate of the frozen/thawed semen among the three studied groups (normal males (XY) and the two types of sex-reversed genotypic females (XX)) a multiple way analysis of variance - ANOVA ($\alpha = 0.05$), complemented by the Tuckey's test ($\alpha = 0.05$) was applied. The software SAS version 8.02 was adopted.

RESULTS AND DISCUSSION

Hormonal treatment has been used in fish farming to change the functional sex of specimens. In general, administration of androgens leads to masculine populations, where most of reversed females present incomplete spermatic ducts or lack them, requiring the sacrifice of the fishes to obtain the milt (Tsumura et al., 1991; Geffen and Evans, 2000). Such procedure depends on maintenance of a high number of breeders in fish farms (Robles et al., 2003).

According to Lahnsteiner et al. (1994), the efferent and principal testicular ducts play an important role in storing and nutrition of the spermatozoa, steroids synthesis, seminal fluid ionic composition, auto and heterofagocytic activities, and in the formation of the seminal plasma. Therefore, semen obtained directly from the testis was not completely mature, presents high density, and, probably, several immature cells. It requires, according to Robles et al. (2003), an exogenous maturation prior activation, as the spermatozoa taken directly from the testis were nearly immotile after activation treatment. Thus, production of sex-reversed females of rainbow trout presenting spermatic ducts would avoid surgical extraction of the semen, easing the reproduction management, besides reducing the number of stocked breeders (Feist et al. 1995; Tabata et al., 2000).

Semen produced by sex-reversed females of *Oncorhynchus mykiss* displaying spermatic duct from both stocks showed the same characteristics of semen produced by normal males (low viscosity, milky coloration, spermatic motility at level 5, and high spermatic concentration: M = $15.925x10^6$, R = $12.475x10^6$ and G = $14.20x10^6$ spermatozoa/mm^3. It was also observed that semen provided by sex-reversed females (R; G) bearing spermatic ducts had the same behavior of that from normal males after freezing and thawing, dispensing the development of specific methodologies towards this source of semen (Table 1).

According to Stoss and Holtz (1983), utilization of frozen semen of rainbow trout, without any previous equilibrium period with the cryoprotectant extender, yielded the best and more reliable fertilization rates. Tabata and Mizuta (1997) observed, by tests with semen of reversed rainbow trout, that there was no correlation between equilibrium time (the period from the semen dilution in the extender to the moment of freezing) and fertility. ANOVA values of F = 0.353 and P = 0.712 indicated no significant statistical difference (P<0.05) among the three treatments (M = 73.1±11.5%; R = 67.2±23.6%; G = 64±5.8%) (Fig. 1).

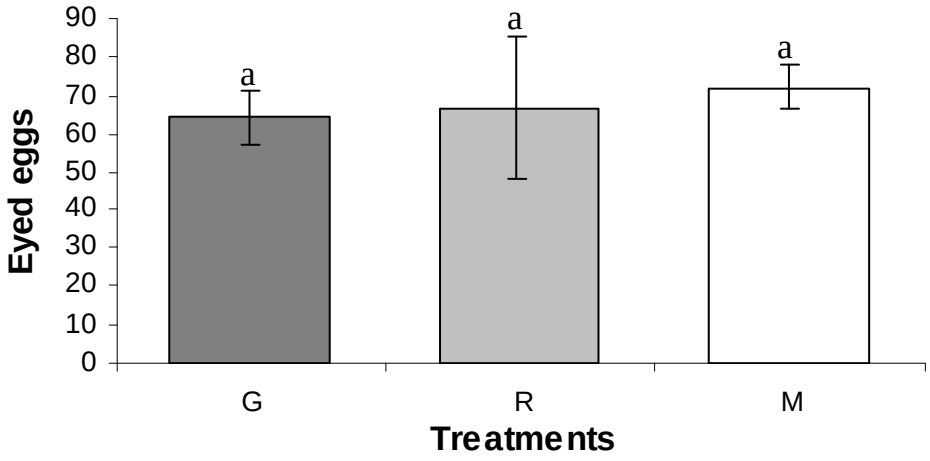

Figure 1 - Relative fertilization rates based on the percentage of eyed eggs for the eggs fertilized with thawed semen from normal males (M) and two types of sex-reversed genotypic females by the oral administration of 17α-methyltestosterone (R = Sex-reversed female; G = Sex-reversed gynogenetic female).

These results confirmed observations by Stoss and Holtz (1983) and Tabata and Mizuta (1997), as in this experiment there was no previous equilibrium period between the suspension of semen in the cryoprotectant extender and its freezing, and the spermatozoa showed to be sufficiently protected, as inferred by the high rates of embryogenesis with the thawed semen in all treatments (Fig. 1).
The equipment used in this work (CryoPac / CP-65 -Taylor-Warton) was easily handled for straw freezing, dispensing the verification of the levels of the liquid nitrogen and conserving a constant temperature, thus maintaining a homogeneous freezing rate. Such variables are considered important issues when the freezing process is performed in cooler boxes (Fogli da Silveira et al., 1994, Lahnsteiner et al., 1995, Tabata and Mizuta, 1997; Robles, et al. 2003), where the air volume is high and evaporation of liquid nitrogen is fast. Besides, this equipment is easy to transport, being useful in laboratory as well as in field works.

Table 1 - Fertilization experiment with thawed semen of rainbow trout from normal males and sex-reversed genotypic females. (Control Group = fresh semen)

Group	Semen Volume (mL)	Sperm/Egg Ratio	Fertilization Mean	Rate (mean±%) Related Mean
Thawed semen				
Male	1.0	2.70×10^7	61.33±5.88	73.11±11.55
Sex-Reversed Female	1.0	2.11×10^7	55.52±18.23	67.16±23.57
Sex-Reversed Gynogenetic female	1.0	2.40×10^7	56.92±7.17	64.03±5.82
Fresh semen				
Male	0.2	2.15×10^7	85.09±12.82	-
Sex-Reversed Female	0.2	1.70×10^7	83.12±4.36	-
Sex-Reversed Gynogenetic female	0.2	1.92×10^7	88.71±4.14	-

**Number of eggs per sample 148.

ACKNOWLEDGEMENTS

We thank the Estação Experimental de Salmonicultura, APTA, Campos do Jordão, SP**,** for the facilities and providing biological material, and to CAPES and CNPq for financial support.

RESUMO

A criopreservação do sêmen de fêmeas masculinizadas de truta arco-íris tem como objetivo a racionalização do processo de produção de estoques 100% femininos. Para tal, foi coletado sêmen de machos normais (**M**) e de dois tipos de fêmeas genotípicas (**R** e **G**), masculinizadas pela administração oral de 17α-metiltestosterona. **R** foi obtido pela fertilização de ovócitos normais com sêmen de fêmeas masculinizadas enquanto **G** foi através de reprodução ginogenética. O sêmen foi diluído em uma solução crioprotetora (glicose 5,4 g, gema de ovo de galinha 10 ml, dimetil sulfóxido 10 ml, água destilada 80 ml) na razão de 1:3 (sêmen/diluidor), envasado em palhetas de 0,5 ml e congelado em um "container" tipo "seco" Cryopac CP-65, à temperatura de -180ºC. A descongelação foi feita em água a 70ºC por 3 segundos. As taxas de fertilização obtidas, não revelaram diferença estatística significativa (P<0.05) entre os três grupos de sêmen descongelados (**M** = 73,1±11,5%; **R** = 67,2±23,6%; **G** = 64±5,8%), indicando que a metodologia de congelação utilizada foi eficaz, tanto na criopreservação do sêmen das trutas normais como para o das masculinizadas.

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Received: June 25, 2004;
Revised: September 22, 2005;
Accepted: June 01, 2005.