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REPORT

In vitro fertilizations with cryopreserved sperm of *Rhinella marina* (Anura: Bufonidae) in Ecuador

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***Abstract.*—Considering worldwide amphibian population decline, sperm cryopreservation should be a priority for conservation of species in areas of high biodiversity, such as the Neotropics. In this study, we present the results of two cryopreservation experiments involving *Rhinella marina* sperm. Freezing was performed in a -80 °C freezer and dimethyl sulfoxide (DMSO) was used as cryoprotective agent. In the first experiment, the effects of 5%, 10%, and 15% DMSO were evaluated in sperm lysis and fertilization capacity. Samples were incubated for 10 minutes at 4 °C before freezing. For thawing, two procedures were tested: 21 °C thawing to be used immediately and 4 °C thawing, to be used two hours later in *in vitro* fertilizations. The best treatment was 10% DMSO plus thawing at 4 °C, that achieved 20% successful fertilizations. In the second experiment, two solutions were tested: 10% DMSO with and without HEPES. Freezing and post-thawing *in vitro* fertilizations were performed after a two hour incubation period at 4 °C. A considerable improvement in fertilization percentages was obtained in this experiment, with a 75% for DMSO alone, and a 70% for DMSO + HEPES. These results provide good perspectives for future implementation of sperm cryopreservation in Neotropical institutions for local threatened species.**

Keywords. Dimethyl sulfoxide, fertilization percentages, Neotropics, sperm cryopreservation, *in vitro* fertilization, Assisted Reproductive Technologies, toad

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Introduction

The extinction crisis faced by amphibians can be considered as dramatic as that of the Triassic or Cretaceous periods with 31% of species threatened (Kouba et al. 2013). Captive breeding programs (CBP) have been established to ameliorate current amphibian population declines, especially for those species which are faced with poorly understood threats and are rapidly disappearing (Bishop et al. 2012).The aim of dedicated CBP is to maintain *ex situ* populations of target species with high genetic diversity for research and future reintroduction. Assisted reproductive technologies (ART) can be implemented by CBP's when reproduction in captivity is difficult to achieve (Clulow et al. 2014). ART research for amphibians has specialized in gamete collection through hormonal induction, *in vitro* fertilization (IVF), and sperm cryopreservation in several anuran and some caudate species (Bishop et al. 2012). This last technique is very useful because it allows the maintenance of high genetic diversity with a minimum amount of space and resources (Clulow et al. 2014).

Sperm cryopreservation for amphibians still lags behind that of other vertebrate classes (Clulow et al. 2014), though, there are various publications with Pipidae (Sargent and Mohun 2005), Bufonidae (Browne et al. 1998; Beesley et al. 1998), Ranidae (Beesley et al. 1998; Mansour et al. 2010; Mugnano et al. 1998), Eleutherodactyliade (Michael and Jones 2004), Hylidae and Myobatrachidae (Browne et al. 2002) family members. In these studies, testicular sperm is cooled by liquid nitrogen (LN2) quenched in a cooling chamber or by immersion in ethanol/dry ice slurry, and cooling rates determined by a thermocouple. The most commonly reported cryopro-

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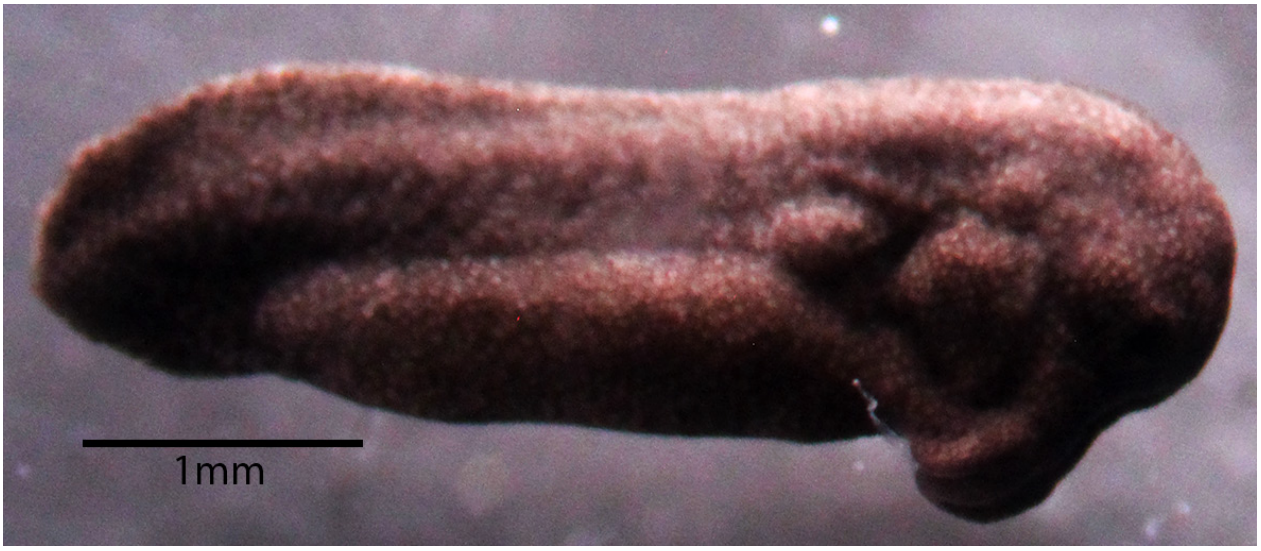

Fig. 1. *Rhinella marina* embryo at 31 Gosner stage from *in vitro* fertilization with cryopreserved sperm.

tective agents (CPA) are dimethyl sulfoxide (DMSO) and glycerol at 5%, 10%, 15%, or 20% v/v diluted in saline or sucrose solutions and high temperatures are employed to achieve a fast thawing. However, the effectiveness of the CPA varies according the species and the cryopreservation protocol.

The standardization of a cryopreservation protocol for a species allows its inclusion into genome resource banks (Clulow et al. 2014). Therefore, there is a need to standardize gamete cryopreservation protocols for neotropical species because they comprise approximately 49% of the world's amphibian species and 60% of all threatened species (Bolaños et al. 2008). Moreover, sperm cryopreservation for conservation purposes in this region has focused mainly on fish (Viveiros and Godinho 2009; Carolsfeld et al. 2003) and mammal (Adams et al. 2009) species. To the authors' knowledge, there are only two research papers describing sperm cryopreservation for anuran neotropical species: one published by Michael and Jones (2004) on *Eleutherodactylus coqui*, and the other by Della Togna (2015) on *Atelopus zeteki*.

Here we present two experiments conducted with *Rhinella marina* sperm. This species is abundant in Ecuador and belongs to the Bufonidae family, which encompasses 53% of the threatened species in the Neotropics (Bolaños et al. 2008). Samples were frozen in a -80 °C freezer in plastic racks and DMSO was used as CPA in both experiments. In the first experiment, DMSO was tested at three different concentrations and with two thawing regiments. The second experiment examined the effects of HEPES buffer incorporation into the isotonic solution. HEPES was used in the isotonic solution of our experiments because it is an effective protector of sperm functionality after short term storage in mammals (Will et al. 2011), and it improved sperm motility after 48 h storage in previous trials (unpublished data). Glycerol, the other common CPA, was not used in these experiments, because, at a 10% concentration, it had lower fertilization percentages (13.43 ± 7.42%) than DMSO 10 % (38.50 ± 6.29%) in a previous experiment under similar experimental procedures (unpublished data).

Materials and Methods

General animal and sperm collection

Rhinella marina male and female adults were collected in Jama, Manabí Province, Ecuador (00°11.160'S 080°17.547'W) during the rainy seasons between late December and late March of 2013 and 2015. Six males and four females were collected in the first field trip, and six males and two females in the second one. In both cases, individuals were transported to Pontificia Universidad Católica del Ecuador (PUCE) in Quito, Pichincha Province, Ecuador, and maintained for two weeks in 56.6 L plastic boxes, provided with two water containers and fed crickets twice a week in accordance with Barnett et al. 2001.

For surgical removal of the testicles, individuals were anaesthetized with a 0.5% w/v solution of MS-222 (Sigma-Aldrich E10521-10G), pH 7, for 15–20 minutes (Wright 2001). A half testicle was used in every freezing treatment, thus whole or half testicle was left in the animal to obtain a control sperm suspension (fresh sperm) when IVF was performed. After testicle removal, animals were sutured with Vycril 3-0, and were placed in individual aquaria for recovery.

The testicles were held on ice in suspension buffer (SB: 104.4 mM NaCl, 2 mM KCl, 6.1 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.4; Beesley et al. 1998) with HEPES (Gibco 15630-080) at a final concentration of 2.5 mM. The testes for each treatment were bisected and weighed to the nearest 0.03 g. Each half was placed in a 1.5 ml microfuge tube with the corresponding experimental

solution. In all cases, except for the DMSO treatment in experiment two, DMSO was diluted to experimental concentrations in SB with HEPES 2.5 mM. Maceration of testicles was performed with Novo Surgical 0250-22 scissors. The tubes were centrifuged briefly, and the supernatant was placed in another 1.5 ml tube. The resulting sperm suspension was distributed, in different volumes in each experiment, in 600 µl microfuge tubes, and placed in plastic racks for freezing in a -80 °C freezer. The sperm concentration was determined by duplicate counts with an improved Neubauer chamber.

For control sperm solutions in both experiments, the remaining testicle in each animal was removed after euthanasia by administration of the same 0.5% MS-222 solution, but for one and a half hours, and the heart was removed to ensure death (Wright 2001). Testicles were macerated in 1.5 ml microfuge tubes containing SB with HEPES, after a brief centrifugation, supernatant was placed in other 1.5 ml tube and held at 4 °C until use.

Experiment one (E1, n = 6 males). The half testicle was macerated in two ml of any of the following solutions: SB + HEPES, 5%, 10%, or 15% DMSO. DMSO sperm solutions were divided in 250 µl aliquots to be frozen. Samples were maintained 10 minutes at 4 °C and one hour at -20 °C before being placed in a -80 °C freezer. One week later, sperm samples were left in their respective plastic racks until ice melted at room temperature (RT, 21 °C) or at 4 °C. For IVF, sperm samples thawed at RT were used immediately, while sperm samples thawed at 4 °C were used after two hours at 4 °C. Embryos that reached gastrula stage (Gosner's 11 stage) were recorded and a gastrula rate was calculated per petri dish. Sperm counts were made only for RT treatments.

Experiment two (E2, n = 6 males). Half testicle was macerated in 500 µl of SB + HEPES; 10% DMSO; or 10% DMSO + 2.5 mM HEPES. DMSO suspensions were divided into 100 µl aliquots and placed in a plastic rack to be held at 4 °C for two hours before freezing at -80 °C for three days. Thawing procedure at 4 °C from E1 was employed. Embryos at second cleavage (Gosner's 4 stage) were recorded and maintained until tail bud stage (Gosner's 17 stage), cleavage and tail bud rates were calculated per petri dish.

In vitro fertilization

For both experiments, ovulation in females was induced by injection of fresh pituitary homogenate from one female of the same species. Twelve hours after hormone administration, females were euthanized as previously described for males. Two females were induced to ovulation in E1, eggs from one female were used for RT thawing treatment and eggs from the other one, for 4 °C thawing treatment. Eggs from only one female were used for all treatments in E2. Eggs were removed from the oviduct and placed in a petri dish for fertilization. Experiment one (E1) used 100µl of sperm solution for 208 ± 20 eggs, while experiment two (E2) used 50 µl of sperm for 116 ± 18 eggs per petri dish. Sperm suspension was pipetted directly from the fresh or thawed sample onto the eggs without any previous CPA wash or dilution. Around two minutes later, the eggs were covered with six ml of filtered tap water, and after 10 minutes, 20 ml of water were added. Embryos were reared to tail bud stage (Gosner's 17 stage) in 10 cm Petri dishes filled with filtered tap water that was changed daily.

Statistics

Two factor ANOVA and Wilcoxon test were performed for E1 and E2, respectively, using SPSS 20. Gastrula rate data of E1 were analyzed by CPA and thawing procedure factors. Cleavage rates within each DMSO treatment of E2 were analyzed by a Wilcoxon test because data size was lower than 30 samples. $\alpha = 0.05$ for both analyses.

Results and Discussion

In both experiments, IVF's with cryopreserved sperm resulted in embryo development that reached tail bud stage, although different embryo survival rates were achieved in each experiment. DMSO 10% + HEPES 2.5 mM treatment was present in both experiments and had 20% embryos in E1, and 54% in E2. These slower embryo rates in E1 could be due to the freezing procedure, which may allowed melting and recrystallization when moving samples from 4 °C to -20 °C and from -20 °C to -80 °C freezers. Besides, it is important to take into consideration factors such as the different sperm concentration, the frozen volume and the pre-freezing DMSO incubation period in E2.

DMSO 10% with 4 °C thawing regiment was the best treatment for E1 (Table 1), and though it was not significantly different from the other DMSO concentrations, it was used in E2 with some modifications. First, assuming a high tolerance of *R. marina* sperm, samples were incubated with DMSO 10% not only after thawing, but before freezing for two h at 4 °C, resulting in high embryo rates, close to control treatment (Table 2). This could indicate that sperm cells needed this amount of time before freezing to allow DMSO to enter the cells and protect them from cryoinjury, and before IVF to restore all their functionality after thawing osmotic stress (Hammerstedt et al. 1990).

Sperm concentration and frozen volume were also modified. A half testicle in two ml of solution in E1 resulted in 1.07; 1.25; and 0.99 × 10^7 sperm/ml for DMSO 5 %, 10 %, and 15 %, respectively. Half a testicle in 500 µl in E2 resulted in 3.41 and 3.23 × 10^7 sperm/ml for DMSO 10 % and DMSO 10 % + HEPES, respectively. Frozen volume in E1 and E2 were 250 µl and 100 µl, respectively. A smaller volume with higher sperm concentration might reduce the volume of water in the extracellular space, making less probable for ice

Table 1. Gastrula and abnormal embryo rates from E1 (n = 6 males).

Treatment	Gastrula rate		Abnormal embryo rate
	(M ± SD %)	Subgroups*	(M ± SD %)
Control	91.28 ± 7.58	a	-
DMSO 5% - RT	03.26 ± 4.00	b	-
DMSO 5% - 4C	19.48 ± 21.73	b	10.99 ± 2.98
DMSO 10% - RT	10.73 ± 13.00	b	-
DMSO 10% - 4C	23.17 ± 27.13	b	10.43 ± 4.64
DMSO 15% - RT	02.44 ± 3.13	b	-
DMSO 15% - 4C	07.90 ± 8.96	b	18.52 ± 10.76

M = mean, SD = standard deviation, RT = Room temperature thawing, 4C = 4 °C thawing.

*Subgroups by DMSO factor ($p < 0.001$, df = 15, F = 93.97) from two factor ANOVA.

to form during the time that the system reaches equilibrium at -80 °C. A reduction in ice nucleation avoids intracellular ice formation, and sperm lesions by ice crystals or hyperosmotic stress during freezing and/or thawing (Rubinsky 2003), thus contributing to protect sperm fertilizing capacity in E2. Sperm lysis can be inferred by the decreased post thawing sperm concentration in E2 (Table 2), but percentage of viable sperm cannot be determined because of the absence of membrane integrity or motility evaluation.

Experiment one (Table 1) showed significant differences in gastrula rates by CPA factor only between control and all DMSO treatments ($p < 0.001$, df = 15, F = 93.97). There were significant differences in gastrula rates for thawing factor, with 4 ºC thawing better than RT ($p < 0.001$, df = 15, F = 20.94). No interaction was found between CPA and thawing factors. Gastrula rates for DMSO concentrations at 4 °C were 19%, 23%, and 7% for DMSO 5%, 10%, and 15%, respectively. While gastrula rates for RT thawing were 3%, 10%, and 2% for DMSO 5%, 10%, and 15%, respectively (Table 1).

It is interesting that a slow thawing at 4°C had a higher gastrula rate than RT thawing considering that fast thawing is recommended to avoid recrystallization or osmotic injuries due to a prolonged exposure to the hyposmotic medium generated during melting (Rubinsky 2003) thus, anuran cryopreservation protocols use thawing temperatures of 21 °C and 30 °C (Browne et al. 1998; Sargent and Mohun 2005). Besides, a prolonged CPA exposure can be considered toxic (Fuller 2004), but in this case, samples used two h later gave higher gastrula rates than samples used immediately. Moreover, tail bud stage was reached by embryos of all DMSO treatments. These gastrula rates could indicate a high tolerance of *R. marina* sperm to prolonged DMSO exposure, as seen for other species like *Rana temporaria* which had been exposed to DMSO for 60 minutes with no detrimental effects on viability or motility (Mansour et al. 2010). Whether it was the temperature or the incubation time that led to higher gastrula rates reached by 4 °C thawing remains to be clarified.

In E2, cleavage rates (Table 2) were 97%, 75%, and 70% for Control, DMSO 10%, and DMSO 10% + H, respectively. Wilcoxon test found no significant differences between Control and DMSO 10% (z = -1.78, p = 0.075), nor between DMSO 10% and DMSO 10% + H (z = -0.52, p = 0.6); but there were significant differences between Control and DMSO 10% + H (z = -2.20, p = 0.028). There was an embryo reduction from second cleavage to tail bud stage in all treatments to 82%, 60%, and 54% tail bud embryos for Control, DMSO 10% and DMSO 10% + H, respectively (Table 2).

Since there were only three ovulating females used in this study, maternal effects could have influenced fertilization rates, so egg condition was revised before IVF. As expected from collection in the same locality during rainy season, only stage VI eggs were found in the oviducts of all females, indicating that they were in a similar reproductive status and the capability of eggs to be fertilized (Rastogi et al. 2011). Oogenetic stage VI is determinant for embryonic development because well differentiated animal and vegetal poles, a maximum size, and a postvitellogenetic condition indicate that oocytes are ready for ovulation (Dumont 1972). Ovulation in these females resulted in high gastrula and cleavage rates in control treatments from E1 (91%) and E2 (97%), both reaching tailbud stage.

Embryo developmental period in cryopreserved sperm treatments from E1 and E2 did not differ with the control treatments; all embryos developed in seven days from fertilization to tail bud stage. However, some abnormalities in tail bud stage were found in all treatments from E1, 4 °C thawing with DMSO 5%, 10%, and 15 % had 11%, 10%, and 18% abnormal embryos (Table 1). There is a 15% embryo reduction from second cleavage to tail bud stages in all treatments from E2. Apparently, it is not unexpected in natural frog populations to exhibit 2% abnormal embryos. Possible causes might be environ-

Table 2. Sperm concentration, cleavage and tail bud rates in control, DMSO 10%, and DMSO 10% + HEPES 2.5 mM treatments from E2 (n = 6 males).

	PF	PT	Cleavage rate		Tail bud rate
	(M ± SD x 10^7 sperm/ml)	(M ± SD x 10^7 sperm/ml)	(M ± SD %)	Subgroups*	(M ± SD %)
Control	2.50 ± 1.26	-	97.38 ± 01.84	a	82.74 ± 8.12
DMSO 10%	3.41 ± 2.38	1.78 ± 1.42	75.67 ± 25.22	a, b	59.99 ± 23.21
DMSO 10% + H	3.23 ± 2.06	1.28 ± 0.93	70.35 ± 19.74	b	54.46 ± 21.14

PF = Pre-freezing sperm concentration, PT = Post-thawing sperm concentration, M = mean, SD = standard deviation, H = HEPES 2.5 mM. *Subgroups from Wilcoxon test.

mental factors, such as UV radiation, extremes in pH, or thermal variations (Pašková et al. 2011). Higher percentages of abnormal embryos (60 %) can be possibly caused by xenobiotics, which interfere with embryo mechanisms for reactive oxygen species (ROS) regulation (Pašková et al. 2011). Captivity rearing conditions could cause ROS regulation to fail, with the consequential embryo abnormalities and mortality seen in E1 and E2, respectively. The presence of higher abnormal embryo percentages in captivity should be considered when planning to perform IVF for captive propagation.

We considered that HEPES could help to protect sperm functionality being one of Good's buffer qualities maintaining adequate pH values in culture media and has been used successfully in mammalian sperm cryopreservation (Will et al. 2011). Moreover, it has been used in a chemotaxis experiment with *Xenopus laevis* sperm (Al-Anzi and Chandler 1998) and we found it to retain sperm motility after a 48 h period at RT and 4 °C (unpublished data). But no improvement in cleavage or tail bud rates were found by the addition of this reactive to cryopreservation solutions (Table 2). The effect of HEPES on the cryopreservation of *R. marina* sperm remains unclear, though, it seems to be unnecessary.

The reported embryo rates in the present study suggest that frozen volume, sperm concentration, and DMSO incubation time can be key elements in improving embryo rates from IVF with cryopreserved sperm. *Rhinella marina* sperm seems to tolerate prolonged DMSO exposures at 4 °C, with favorable effects on sperm response to freezing and thawing. Nevertheless, freezing rates and cell viability or motility tests should be conducted to make possible stronger conclusions about the present data. We hope that this report leads to in-depth studies that can be applied to the conservation of more Neotropical species using ART.

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Belén Proaño graduated in Biological Sciences from Pontificia Universidad Católica del Ecuador (PUCE) in 2013. As an associated researcher at PUCE for two years, her investigations focused on reproductive biology and the application of Assisted Reproductive Technologies in Ecuadorian anurans under captivity conditions. Currently, she is working on personal projects away from the scientific environment, but with the same interest in understanding the wonder of life.

Oscar Pérez was born in Quito Ecuador. He obtained a doctoral degree in 2008 from Pontificia Universidad del Ecuador in collaboration with Duquesne University, Pennsylvania, USA. His advisors were Dr. Richard Elinson and Dr. Eugenia del Pino. Dr. Pérez is interested in the evolutionary comparison of development and the reproductive biology of Ecuadorian vertebrates. His current research focus is in finding new alternative models in developmental biology using the great Ecuadorian mega-diversity country as his playground. More particularly, his interest is in frog oogenesis—oocyte organization can vary between species and these variations can modify the developing pathway of the future embryo. Comparative methodologies are applied to find variations in oogenesis patterns in order to understand how these variations can modify embryogenesis features. These analyses employ a diversity of techniques such as histology, immunohistochemistry, genetic cloning, and bioinformatics tools in order to identify genes of importance for oogenesis and embryogenesis. All these efforts are focused towards shedding light on the reproduction and preservation of Ecuadorian fauna and its unique development features.