FULL ARTICLE

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Acute exposure to hyperosmotic conditions reduces sperm activation by urine in the yellowtail tetra *Astyanax altiparanae*, a freshwater teleost fish

Exposição aguda a condições hipereosmoticas reduz ativação do sêmen por urina em lambari do-rabo-amarelo (Astyanax altiparanae), um teleósteo de água doce

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ABSTRACT

In freshwater fish with external fertilization, sperm sampling can be contaminated with urine, which triggers motility and gives rise to decreased fertilization success. The maintenance of freshwater fish in hyperosmotic conditions may reduce urine production and improve sperm quality. Thus, the aim of this work was to verify if acute exposure to various NaCl concentrations improves sperm quality in the yellowtail tetra *Astyanax altiparanae*. Spermiation was induced using a single dose of carp pituitary gland (5 mg kg^{-1}) and the males were maintained at various NaCl concentrations: NaCl 0.00% (control), NaCl 0.45% (hypoosmotic), NaCl 0.9% (isosmotic) and NaCl 1.0% (hyperosmotic) for 6 h at 26 °C. Sperm was collected and verified for activation by urine and motility traits. At 0.00%, 0.45%, and 0.90%, the sperm was motile just after sampling, indicating activation by urine. Surprisingly, at hyperosmotic conditions, no activation was observed. Other sperm and motility parameters did not show any statistical differences, including sperm viability ($P = 0.7083$), concentration ($P = 0.9030$), total motility ($P = 0.6149$), VCL (curvilinear velocity; $P = 0.1216$), VAP (average path velocity; $P = 0.1231$) and VSL (straight-line velocity; $P = 0.1340$). Our results indicate that acute maintenance at hyperosmotic conditions eliminates sperm activation by urine and maintains sperm quality. Such a new procedure is interesting for both basic and applied sciences, including reproductive practice in fish.

Keywords: Fish. Urospermia. Sperm contamination. Salinity.

RESUMO

Em peixes de água doce com fertilização externa, a amostragem de espermatozoides pode ser contaminada pela urina, o que desencadeia motilidade e gera menor sucesso na fertilização. A manutenção de peixes de água doce em condições hiperosmóticas pode reduzir a produção de urina e melhorar a qualidade do esperma. Assim, o presente trabalho foi delineado para verificar se a exposição aguda a várias concentrações de NaCl melhora a qualidade do esperma no tetra-amarelo *Astyanax altiparanae*. A espermiação foi induzida usando uma dose única de hipófise da carpa (5 mg kg-1) e os machos foram mantidos em várias concentrações de NaCl: NaCl 0,00% (controle), NaCl 0,45% (hipoosmótico), NaCl 0,9% (isosmótico) e NaCl 1,0% (hiperosmótico) por seis horas a 26 °C. O esperma foi colhido e verificado quanto à ativação por urina e traços de motilidade. Em 0,00%, 0,45%, 0,90% os espermatozóides eram móveis logo após a amostragem, indicando ativação pela urina. Surpreendentemente, em condições hiperosmóticas, nenhuma ativação foi observada. Outros parâmetros espermáticos e de motilidade não mostraram diferenças estatísticas, incluindo viabilidade

espermática (P = 0,7083), concentração (P = 0,9030), motilidade total (P = 0,6149), VCL (Velocidade Curvilinear; P = 0,1216), VMD (Velocidade Média de Deslocamento; P = 0,1230) e VLR (Velocidade em linha Reta; P = 0,1340). Nossos resultados indicam que a manutenção aguda em condições hiperosmóticas elimina a ativação do esperma pela urina e mantém a qualidade do esperma. Esse novo procedimento é interessante para as ciências básicas e aplicadas, incluindo a prática reprodutiva em peixes

Palavras-chave: Peixe. Urospermia. Contaminação no sêmen. Salinidade.

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Introduction

Sperm sampling is a critical stage for *in vitro* fertilization in many animal species. In this regard, sperm contamination with urine (urospermia) is a common phenomenon in reproduction practice in species including stallion (Lowe, 2001), bears (Gomes-Alves et al., 2014) and humans (Makler et al., 1981), and is well known to decrease sperm quality (Ellerbrock et al., 2018). In fish, urospermia is also commonly described and such contamination reduces ATP levels and sperm parameters, such as sperm velocity, which can reduce the fertilization capacity. In Nile tilapia (*Oreochromis niloticus*) grown in freshwater environments, high urine production is observed, which has low osmolarity and activates sperm motility due to its low osmotic concentration (Poupard et al., 1998).

To prevent urine contamination, some practical procedures are commonly used in sperm sampling such as fractioned sampling, and its collection after the fish urinates and defecates (Beirão et al., 2019). Urine elimination can also be performed with a slight compression in the peritoneal region, although in many species, semen elimination usually comes with urine, as both are eliminated by a single urogenital pore (Król et al., 2018; Nynca et al., 2012).

The use of specific solutions to treat of urospermic samples is also a strategy to recover sperm quality and increase subsequent successful fertilization. Most protocols focused on the addition of extenders (Galarza et al., 2019), water solutions containing salts and sugars that maintain, temporarily, sperm viability and fertilization capacity, avoiding sperm activation. Other protocols focused on separation using a density gradient for semen fractioning, as used in humans (Ericsson et al., 1973), stallions (Varner et al., 1987), bovines (Arias et al., 2017), and dogs (Barros Mothé et al., 2018).

In freshwater fish with external fertilization, sperm contamination with urine is more critical due to its mechanism of triggering sperm motility (Król et al., 2018). Fish spermatozoa are immobile in the seminal fluid within the testicular lumen, but motility is initiated after release to the external surrounding media (Cosson, 2019; Gallego & Asturiano, 2019), and it lasts for only a few seconds in most fish species. In teleost fish, urine also initiates motility, which leads to decreased sperm quality for subsequent poor fertilization success (Dzyuba et al., 2019). Several strategies were developed to improve sperm quality. A more critical procedure involves euthanasia and subsequent testicular mincing in saline solution, as performed in species that present urine contamination, such as small fish species (Kopeika et al., 2003) and catfish (Linhart et al., 2020). Catheterization of the testicular lumen is also a strategy used to obtain sperm without contamination (Sarosiek et al., 2016), but such a procedure is not viable in some species due to fish size or morphology of the urogenital papilla. Immobilizing solutions are also used in fish, because the addition of a saline solution may re-immobilize the activated sperm (Yasui et al., 2015), although decreased motility parameters and fertilization may occur in such procedure.

A possible technique to prevent urine production and contamination is to manipulate environmental salinity, as the mechanism of osmoregulation and urine production in freshwater species depends on the environmental salinity (McCormick, 2001; Sardella & Brauner, 2008). In hypoosmotic conditions, fish constantly absorb water

from the environment and have to eliminate most parts from the skin and within urine. On the other hand, in hyperosmotic conditions, the absorption of water and urine production is decreased (Baldisserotto et al., 2019). This suggests that the maintenance of a freshwater species under hyperosmotic conditions may reduce urine production and sperm may be released without contamination.

The fish *Astyanax altiparanae* has been used as a model organism for several studies, such as early biology (Santos et al., 2016), toxicology (Fernandes et al., 2019), flow cytometry (Xavier et al., 2017), and chromosome set manipulation (Nascimento et al., 2020, 2017). This species can breed throughout the year and sexual maturity is reached at four months (Porto-Foresti et al., 2005). Consequently, due to its small size, ease of rearing and reproduction, *A. altiparanae* has been used in both basic and applied studies.

Therefore, the aim of the present study was to verify if acute exposure to hyperosmotic conditions may prevent urine contamination in freshwater fish. The yellowtail tetra *Astyanax altiparanae*, a small fish with external fertilization, was used as a model since, in this species, sperm is commonly contaminated with urine.

Material and Methods

Experimental conditions

Adult males of the yellowtail tetra *Astyanax altiparanae* (62.42 ± 1.76 mm; 5.52 ± 0.50 g; n = 16) used in this experiment were previously maintained in 1000 m^2 earthen ponds at the National Center for Research and Conservation of Continental Aquatic Biodiversity/Chico Mendes Institute for Biodiversity Conservation (CEPTA/ICMBio) in Pirassununga, Brazil (21°55'58"S, 47°22'31"W). The animals were fed twice a day with commercial diets (4200 kcal kg^{-1} and 45% crude protein).

Males were selected (n=30) by the presence of bony hooks in the anal fin and maintained in 80-L aquariums with constant aeration and water temperature set at 28 °C for the experimental procedures. Ten fish were separated for the evaluation of serum osmolarity and 20 for induced reproduction and evaluation of different NaCl concentrations (5 for each treatment).

Evaluation of serum osmolarity

Blood samples were obtained from the caudal vein of 10 adult fish (> 40 mm), using a heparinized syringe (3 mL), with a 27-gauge needle. The blood samples were mixed in a 2mL microtube and centrifuged for 20 min at 300 G and a temperature of 15 °C. The supernatant was taken using a cut tip, transferred to 2mL cryotubes, and then maintained in liquid nitrogen (-196 °C) for osmolarity analysis.

The serum (1 mL) was thawed in a water bath at 20 °C for 2 min and the osmolarity was measured using a freezing point osmometer (PZL-1000, Londrina, Brazil), which resulted in osmolality of 272 mOsm.

In order to obtain equivalent NaCl concentrations for the serum osmolarity, firstly NaCl solutions at 0.2%, 0.4%, 0.6% and 0.8% were prepared and the osmolarity was measured, thus creating a linear regression ($y = 30750x-4,4$; $R^2 = 0.9973$, in which y = osmolality and x = NaCl concentration). This equation was used to obtain the equivalent NaCl concentration for serum (0.8989% NaCl), which was then used as the isosmotic concentration during the experiments.

Induced spermiation

Fish were anesthetized with 200 mg L-1 of clove oil (Biodinamica, Ibiporã, Brazil) and induced to spermiation (intraperitoneally) with a single dose of carp pituitary gland (5 mg kg-1). Induced males were then maintained in 30-L aquariums with constant aeration at 26 °C, at various NaCl concentrations (4 males each): NaCl 0.00% (control), NaCl 0.45% (hypoosmotic), NaCl 0.90% (isosmotic), and NaCl 1.00% (hyperosmotic), which were adjusted using a digital refractometer (PAL-03S, Atago, Shiba, Japan). Prior to exposure to NaCl-treatments, the fish were individually maintained in beakers with 1L of freshwater, and the water from each NaCl treatment was slowly added into the beakers. After 10 min of adaptation, the fish were transferred to the aquariums.

After 6 h, the males were anesthetized, the papilla was gently dried using paper, and a small aliquot (~1 µL) of sperm was sampled from the papilla using a 200µL pipette (Eppendorf, Hamburg, Germany) and immediately observed at the microscope at 400x magnification (Nikon Ni, Tokyo, Japan) to evaluate activation by urine. Activation by urine was evaluated by a subjective score in which the criteria was based on high urine contamination (+++), with most spermatozoa presenting motility, with increased velocity; urine contamination (++), with most spermatozoa presenting motility, but with decreased velocity; low urine contamination (+) with few spermatozoa presenting motility, with reduced velocity and non-contaminated spermatozoa (-), with all immobile spermatozoa. This score was used because extremely high concentration did not permit us to visualize the sperm cells individually or by automated analysis (CASA).

The remaining sperm was collected by stripping using a 1000-µm pipette (Eppendorf, Hamburg, Germany) and immediately transferred to a 1.5mL microtubes containing 400 µL of immobilizing Ringers solution (NaCl 128.3 mM, KCl 23.6 mM, $CaCl_2$ 3.6 mM, $MgCl_2$ 2.1 mM) and maintained on ice (~4 °C). Sperm concentration was evaluated by hemocytometer, as in our previous works (Yasui et al., 2012a, 2015).

Sperm quality analysis

Sperm motility was measured using computer assisted sperm analysis (CASA) according to the procedures described for *Danio rerio* (Wilson-Leedy & Ingermann, 2007) and to the studying species *A. altiparanae* (Gonçalves et al., 2018). A small aliquot of sperm (0.5 µL) was pipetted on a Makler chamber (Selfi-Medical Instruments, Haifa, Israel) in which both the chamber and the cover slide were previously coated with a solution of bovine serum albumin at 0.1% in order to prevent sperm attachment to the surfaces. The sperm was activated with 20-fold dilution with distilled water, the cover slide was placed and the motility was visualized on a trinocular microscope (Nikon Ni, Tokyo, Japan) at 200x magnification. Sperm motility was captured using a CCD camera (Nikon DS-Fi, Tokyo, Japan) connected to the microscope and video sequences were recorded at 15 s post activation using the Nis-Ar Elements software (Nikon, Tokyo, Japan). The duration of sperm motility was measured from the video sequences of the sperm activation until the sperm motility became lower than 5% of total motility (see criteria proposed by Yasui et al., 2012b).

The videos were edited through VirtualDub software 1.10.4 generating a sequence of images that were exported to ImageJ® software with the CASA plugin. The parameters of motility were patronized according to Wilson-Leedy & Ingermann (2007). The motility characteristics measured were percent motility (%); curvilinear velocity (VCL); average path velocity (VAP) and straight-line velocity (VSL). Sperm viability was measured by dual staining with SYBR-14 and propidium iodide (PI), using the sperm viability kit (Molecular Probes, Eugene, OR, USA). Stained samples were analyzed by flow cytometer (Accuri C6, BD Biosciences, San Jose, CA, USA) with FL1 (530 ± 15 nm) and FL3 (> 670 nm) filters, respectively. Membrane integrity was evaluated by the percentage of semen stained by SYBR-14 and PI.

Statistics

All data are presented as mean ± standard error. The data were tested for normality using Lilliefors, followed by ANOVA and Tukey's multiple range test. In all cases, the probability was set at 0.05. All the statistical analysis was performed using the software Statistica 10.0 for Windows.

Results

As shown in Table 1, biometric data regarding total weight (*P = 0.4947*) and standard-length (*P = 0.1325*) did not show any statistical differences.

Urine sperm contamination was reduced with increasing NaCl concentrations, as expressed by subjective motility. In freshwater (NaCl 0.0%), all the samples were highly activated with urine (+++). NaCl 0.45% decreased the motility activation (++) and a following decrease was also observed at isosmotic conditions (+), in which few spermatozoa were activated just after sampling. Interestingly, the hyperosmotic treatment (NaCl 1.0%) resulted in no motile spermatozoa after sperm sampling. Sperm viability was not affected by any NaCl concentration tested (*P = 0.7083*), and ranged from 92.53 ± 1.97% (NaCl 0%) to 89.50 ± 1.75% (NaCl 0.9%) (Figure 1). Sperm concentration showed no statistical differences between different NaCl concentrations (*P > 0.05*), with the highest concentration at NaCl 0.45% ($14.70 \pm 3.88 \times 10^7$ spermatozoa mL^{-1}) and the lowest value at NaCl 0.9% ($10.45 \pm 3.11 \times 10^7$ spermatozoa mL^{-1}).

Motility path and motility parameters are shown in Table 2. All the parameters were not affected by NaCl concentrations, including total motility (*P = 0.6149*), VCL (*P = 0.1216*), VAP (*P = 0.1231*) and VSL (*P = 0.1340*).

Table 1 – Biometric data and sperm parameters of *Astyanax altiparanae* males maintained for 6 h at various NaCl concentrations after induced spermiation. Data are shown as mean ± standard error. None of the parameters presented any statistical differences

Treatments		Male parameters		Sperm parameters		
Condition	% NaCl	Total weight (g)	Standard length (mm)	Urine Contamination	Viability (%)	Concentration (spermatozoa mL^{-1})
Hyposmotic (n=5)	0.00%	5.45 ± 1.09	60.86 ± 2.53	+++	92.53 ± 1.97	10.90 ± 3.46 x 10^7
Hyposmotic (n=5)	0.45%	4.25 ± 0.38	56.54 ± 1.87	++	91.93 ± 1.40	14.70 ± 3.88 x 10^7
Isosmotic (n=5)	0.90%	5.95 ± 1.31	64.89 ± 4.31	+	89.50 ± 1.75	10.45 ± 3.11 x 10^7
Hyperosmotic (n=5)	1.00%	6.45 ± 1.07	67.40 ± 3.38	-	90.28 ± 2.81	12.75 ± 6.60 x 10^7

(+++) high urine contamination, with most spermatozoa presenting motility and increased velocity; (++) urine contamination, with most spermatozoa presenting motility but with decreased velocity; (+) low urine contamination, with few spermatozoa presenting motility with reduced velocity; (-) non-urine contaminated spermatozoa, with all immotile spermatozoa.

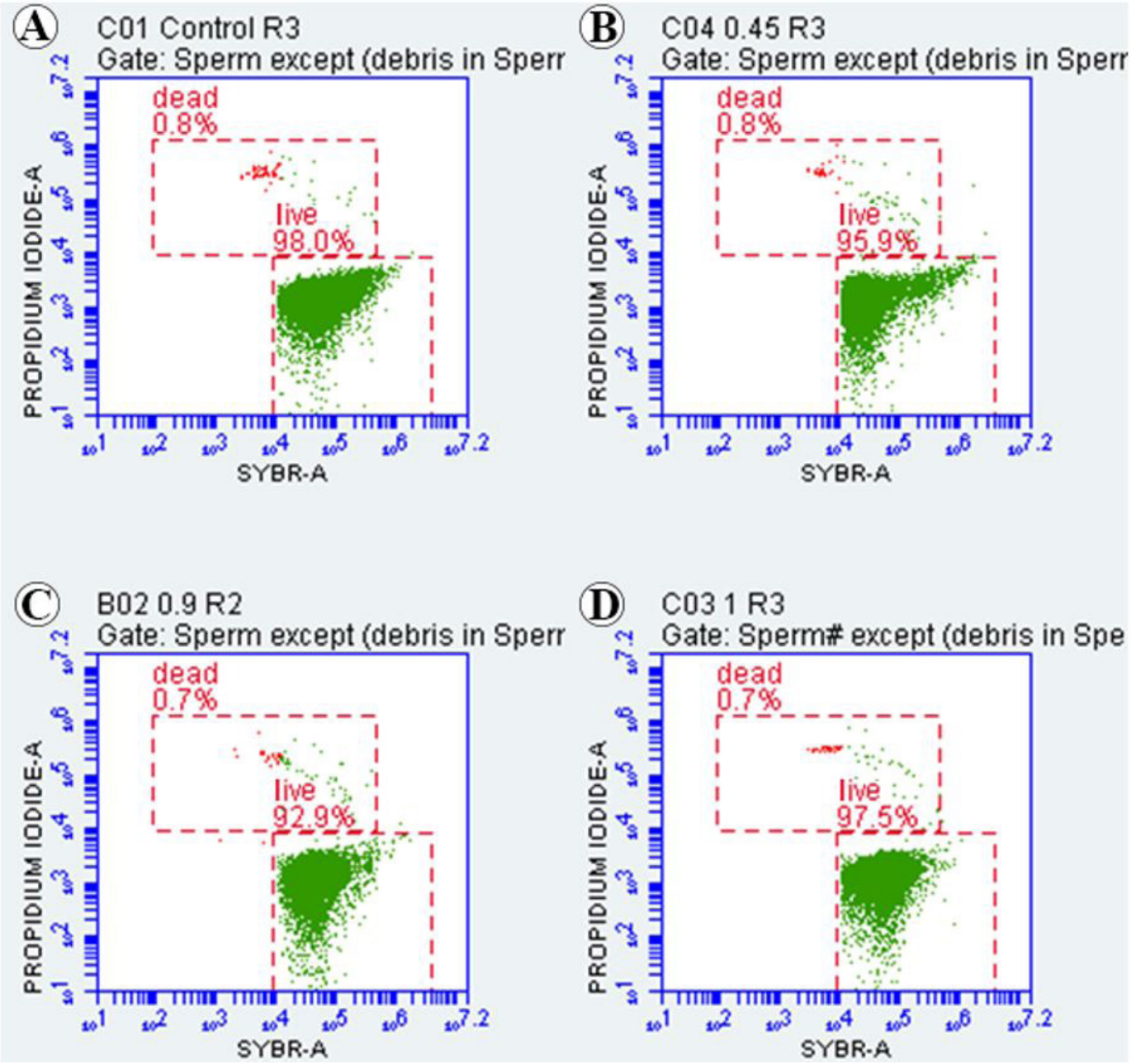

Figure 1 – Flow cytometric histograms showing the sperm viability after dual staining with SYBR-14 and Propidium Iodide and subsequently analyzed by flow cytometry. The detached area indicates the percentages of live and dead cells. (A) Control (0.0% NaCl); (B) Hyposmotic solution (0.45% NaCl); (C) Isosmotic solution (0.9% NaCl); (D) Hyperosmotic. VCL: curvilinear velocity; VAP: average path velocity; VSL: straight-line velocity.

Table 2 – Motility parameters of *Astyanax altiparanae* males maintained for 6 h at various NaCl concentrations after induced spermiation. Data are shown as mean ± standard error. None of the parameters presented any statistical differences

Treatments		Motility parameters			
Condition NaCl		Motility (%)	VCL (μm/s)	VAP (μm/s)	VSL (μm/s)
Hyposmotic	0.00%	62.23 ± 16.27	56.21 ± 7.33	54.21 ± 7.08	45.21 ± 6.23
Hyposmotic	0.45%	54.60 ± 14.82	64.97 ± 10.85	61.97 ± 9.53	48.96 ± 4.67
Isosmotic	0.90%	70.71 ± 15.16	69.18 ± 9.21	63.18 ± 8.69	43.18 ± 6.89
Hyperosmotic	1.00%	43.73 ± 14.71	39.47 ± 1.07	38.47 ± 6.08	43.09 ± 4.27

Discussion

In this work, induced males maintained in hyperosmotic conditions for only 6 h eliminated the sperm activation by urine after sampling, indicating that urine production was reduced. In fact, the maintenance of freshwater fish in a hyperosmotic environment may reduce urine production and also mobilize the water content from the bladder to maintain the osmotic physiological balance (Evans, 2008). This indicates that sperm activation may be reduced by two mechanisms: 1) reduction of urine production and; 2) concentrating the urine solutes. Theoretically, the mobilization of water from urine should increase the sperm concentration, but such increase was not detected by our data. However, possible urine contamination may not be discarded from

sperm sampling, but it did not trigger the sperm motility. This procedure may be applicable in reproduction practice including fish hatcheries and laboratory work. In freshwater fish with external fertilization, urine activation may trigger motility and then reduce the duration of sperm motility during fertilization trials. The general procedure for in vitro fertilization in fish employs the "dry method", in which the immotile sperm is first mixed with the oocytes and, subsequently, the gametes are activated by addition of water. In this procedure, the use of non-contaminated sperm is important to improve motility parameters and subsequent fertilization success. Motility parameters are well known as the main fertilization predictor for most fish species (Rurangwa et al., 2004).

In previous works, the maintenance of freshwater fish in various saline concentrations was described alongside the spermatic quality. In the brown trout *Salmo trutta* f. fario, the sperm quality was higher in males maintained in freshwater when compared with increased salinity (Labbe & Maisse, 2001).

In this work, the manipulation of environmental salinity was possible using the yellow-tail tetra as a model. This species may adapt to increased salinity, such as in the case of this study, in which the salinity was adjusted to 1.0%. Although acute exposure was used in this study, it is important to adapt the fish into the new salinity. The fish were adapted from freshwater into the NaCl treatments within only 10 min, in which water containing NaCl was gently added to the fish. However, for application of NaCl treatments in other fish species, an adjustment of salinity and management adaptation may be necessary, since salinity tolerance and physiological balance are species-specific.

According to Gonzalez (2012), in hyper-saline water, fish may present some adjustments to perform osmoregulation in such conditions, such as 1) reduced branchial water permeability, which limits osmotic water loss and reduces the salt load; 2) increased gut Na^+/K^+ -ATPase (NAK) activity to absorb the salt load, and 3) increased branchial activity to excrete the Na^+ and Cl^-, which occur against a larger gradient. Therefore, besides the fact that *A. altiparanae* was submitted to an acute exposure, such physiological events are probably responsible for the tolerance of the species.

Comparing with other methods to avoid urine contamination, such as fractioned sampling (Beirão et al., 2019), the use of extenders and catheterization (Sarosiek et al., 2016), the use of hyperosmotic conditions has the advantage of being simple, inexpensive and low time-consuming, which can facilitate the routine of fish reproduction in laboratory conditions.

In conclusion, the sperm quality was not affected by acute NaCl treatments as expressed by all motility parameters, sperm viability, and concentration. Such procedure is inexpensive and applicable to in vitro fertilization in fish in both basic and applied sciences.

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethics Statement

This study was conducted according to the Guide for the Care and Use of Laboratory Animals at the Faculty of Veterinary Medicine and Animal Science (FMVZ) from the University of São Paulo (USP), # 9476181219.

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