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## Method Article

# Sperm selection of cryopreserved milt of catfish (*Rhamdia quelen*) by density gradient centrifugation with AllGrad® 90%



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## A B S T R A C T

Density gradient centrifugation is a technique used to wash or separate samples of cryopreserved milt, mainly in humans and bovines allowing, for example, reducing the concentration of cryoprotectants or choosing the best portion of sperm. The proposed method seeks to reduce the presence of cryoprotectant in the cryopreserved milt of the *Rhamdia quelen* and to obtain a fraction of better quality sperm. Gradient centrifugation was formed from 90% AllGrad® and different centrifugation times and forces were compared. The separated sperm presented a low increase in motility and decreased head damage and presence of gout, however, it was better compared to the non-separated samples. The speed of  $1000 \times g$  for 10 min, 4 °C, allowed  $22.25 \pm 4.64\%$  of normal spermatozoa, that is, 9.25% more than the non-centrifuged milt ( $p = 0.0013$ ).

- The centrifugation method allows a fraction of spermatozoa morphologically less affected by cryopreservation.
- Density gradient centrifugation with AllGrad® 90% is proposed as a tool of easy adaptation and application for the separation of cryopreserved sperm of *R. quelen*.
- Density gradient centrifugation method at  $1000 \times g$  for 10 min allows obtaining a better fraction of normal sperm.

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## ARTICLE INFO

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## Specifications table

Subject Area:	Environmental Science
More specific subject area:	Fish reproduction, cryopreservation.
Method name:	Sperm selection of cryopreserved milt of fish by density gradient centrifugation
Name and reference of original method:	Sperm selection by density gradient centrifugation. The adaptations were made from other studies performed to separate male reproductive cells from fish (Supplementary Table 1).
Resource availability:	No applicable

## Method details

## Background

Cryopreservation allows the maintenance of viable biological material for indefinite time under temperatures like  $-80\text{ }^{\circ}\text{C}$  or  $-196\text{ }^{\circ}\text{C}$  [13]. The cryopreservation of fish sperm is a useful tool for both the preservation of species and storage of valuable genotypes for animal research and production [13]. To reach success in the cryopreservation techniques it is necessary to use substances named cryoprotectants.

The cryoprotectants are substances that promote cellular dehydration (deplete up to 95% of the water in the cell) during the cryopreservation process, stabilizing membranes [7] and proteins [6]. The dehydration avoid or reduce the formation of ice crystals [13], and depending on the molecular weight of the cryoprotective solution used, it may or may not penetrate the cell membrane [13]. At ideal concentration or exposure time, cryoprotectant solutions are less toxic when at low temperature than at room temperature [6]. In thawing, the sample goes from  $-196\text{ }^{\circ}\text{C}$  to  $30\text{ }^{\circ}\text{C}$  in a few minutes, that is the moment where the greatest number of injuries can happen [9]. Damage occurs, probably due to the cytotoxic activity of the cryoprotectant, mainly at higher temperatures. Therefore, the use of techniques that allow reducing the concentration of cryoprotectants after thawing of the milt samples would be a possible solution to reduce these cytotoxic effects, which can promote injuries on cryopreserved spermatozoa diminishing the sample quality.

The density gradient centrifugation technique has been used for auxiliary tool, in assisted reproduction in mammals [5,12], and lastly in fish [11] for example, with *Salmo salar* [2], allowing a proportion of sperm with greater motility, speed, and fertilization capacity. For the shaping of gradients, silica-based solutions are used, which can be coated with polyvinylpyrrolidone (PVP) [1], or with silane [12]. However, the use of silane-coated silica separation gradients has begun to replace PVP-coated silica for use in humans cells [12], and some species-specific formulations for animals, for example, BoviPure™ for bull, EquiPure™ for stallion and PorciPure™ for sperm boar [8], considering that the silane-coated silica present low levels of endotoxicity [12]. However, quality parameters, such as sperm viability and morphology, can be affected by centrifugation force and time that have not yet been compared [6]. Therefore, this study aims to describe a method that allows identifying the best centrifugation time and force, for the separation of the best portion of sperm from cryopreserved milt, based on the AllGrad® 90% gradient. The *Rhamdia quelen* species was used as an animal model, being the first time it is used for this purpose. The adaptations were made from other studies performed to separate male reproductive cells from fish (Supplementary Table 1).

## Required reagents and equipment

- Fish cryopreserved milt. To the experiment, of the catfish (*R. quelen*).

- AllGrad® 90% (Catalogued Numbers: AG90-050, AG90-100, LifeGlobal Group, Europe).
- Carp pituitary extract (CPE)
- Distilled water.
- Cryopreservation solution (50 g/L fructose [Sigma- Aldrich®], 50 g/L powdered milk [Molico®- Nestlé] and 100 mL/L methanol [Sigma- Aldrich®])
- Saline-buffered formaldehyde (10%).
- Ginsburg extensor solution (123.2 mM NaCl, 3.75 mM KCl, 3.0 mM CaCl<sub>2</sub>, 2.65 mM NaHCO<sub>3</sub> - 300mOsm, pH 7.5).
- Eosin dye solution (3%).
- Nigrosin dye solution (5%).
- Rose Bengal dye solution.
- Neubauer chamber.
- Straws (0.25 mL - Minitube®).
- Micropipettes P10 µL, P100 µL, P200 µL and P1000 µL.
- Eppendorf microcentrifuge tubes of 1.5 mL and 2.0 mL.
- Falcon tube 15 mL.
- Refrigerated centrifuge. For standardizing this method, the Eppendorf Centrifuge 5403 was used.
- Microscope slides.
- Light microscope.

### Animals

All animals were handled in accordance with the regulations of the Ethics Committee in the Use of Animals (CEUA) of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil, with approval protocol No. 38722. The animals, from the Nossa Senhora Aparecida Fish Farm (Rodovia RS 155, S/N Km 6, Ijuí - RS, 98700-000), were acclimated for one month in 500 L plastic tanks, in a recirculation system at AQUAM Laboratory from the same University. A commercial ration was provided, until apparent satiety, twice a day.

### Milt samples

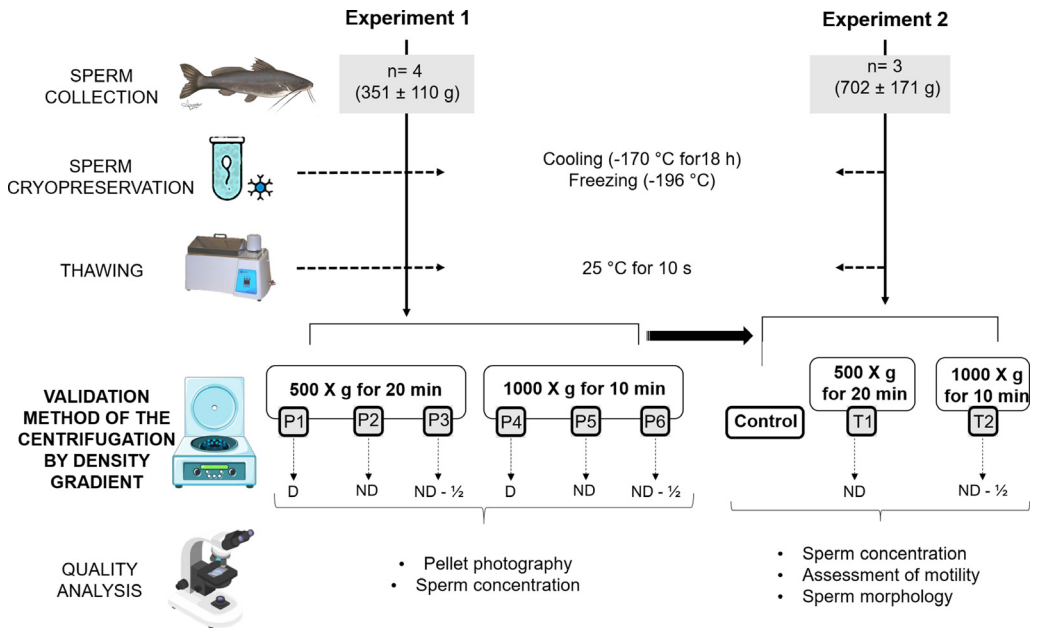
The samples were obtained from seven adult males of the *R. quelen*. The males were randomly divided into two groups and the milt was collected at different times. A pool was forming from each group. The mean  $\pm$  SD weights of the animals in each group were  $702 \pm 171$  g (group [i],  $n = 3$ ) and  $351 \pm 110$  g (group [ii],  $n = 4$ ).

### Milt collection and cryopreservation

Each male was induced intramuscularly with a single dose of CPE 3 mg/kg body weight [3]. Milt collection was carried out after completing 240° h (10 h; water at 24 °C) [3]. For this, the urogenital papilla was dried, followed by a gentle abdominal massage (cephalo-caudal direction). The first portion of the milt was discarded to avoid possible contamination with urine, feces, or blood [13]. The collection was performed in a 15 mL Falcon conical tube. The milt samples were kept at 4 °C until the total of males was collected and previous evaluations were carried out after freezing.

Before forming the milt pool, the motility was assessed subjectively [4]. For sperm activation, an aliquot of 1 µL of milt was diluted at 5 mL of distilled water and the chronometer was immediately started. The motility rate (0 - 100%) was evaluated between 10 and 15 s after activation. The assessment of motility and sperm concentration in each pool was performed with a microscope (Nikon Eclipse E200, Tokyo, Japan), 40 X objective.

The sperm concentration was analyzed with a Neubauer chamber, with previous fixation of the sperm in saline-buffered formaldehyde (10%), in a ratio (v/v) 1: 999 µL [3]. The rate and mean motility time of the fresh milt pool in group (i) was  $80 \pm 5\%$  and  $40 \pm 3$  s. Regarding group (ii), the results were  $68.33 \pm 7.64\%$  and  $48.33 \pm 9.07$  s, respectively. The concentration for each group (number of



**Fig. 1.** Validation method of the density gradient centrifugation with AllGrad® 90% for criopreservation sperm of catfish (*R. quelen*). **D:** dilution sperm. **ND:** no dilution sperm. **ND-½:** no dilution sperm with half the volume of the AllGrad layers. **Control:** milt samples that after thawing were not centrifuged treatment applied in experiment two to compare the quality of the sperm with the treatments where the milt was centrifuged (T1 and T2).

sperm [cells/mL] was  $4.20 \times 10^{11} \pm 6.41 \times 10^{10}$  and  $1.81 \times 10^{11} \pm 8.41 \times 10^9$ , group (i) and (ii), respectively.

The cryoprotective solution was obtained by diluting them in distilled water. The cryopreservation of the pools was carried out by diluting the in the cryopreservation solution (ratio 1: 3). The dilution was placed on 0.25 mL straws (Minitube®) [3]. The straws were conditioned in a canister and kept in liquid nitrogen steam for 18 h (dry-shipper CP300, -170 °C), and finally stored in liquid nitrogen (-196 °C), for a minimum period of seven days [3].

#### Standardization of the density gradient centrifugation procedure

The standardization process of the centrifugation methodology by density gradients with AllGrad® 90% was divided into two validation experiments (Fig. 1). (i) Standardization of centrifugation forces and times, as well as the volume of the layers formed by the gradients, and, (ii) method validation through sperm quality measurement after centrifugation at different speeds and times (that were selected in the first experiment).

In short, each validation experiment consisted of two procedures: (i) Cryopreservation of milt (Milt collection and cryopreservation of this document), (i) milt thawing and centrifugation using density gradient AllGrad® 90%, and, (iii) evaluation of sperm quality parameters. For the first experiment, only the sperm concentration and pellet formation were evaluated, and in the second experiment, in addition to sperm concentration, sperm motility and morphology were measurement.

#### Standardization of centrifugation forces and times

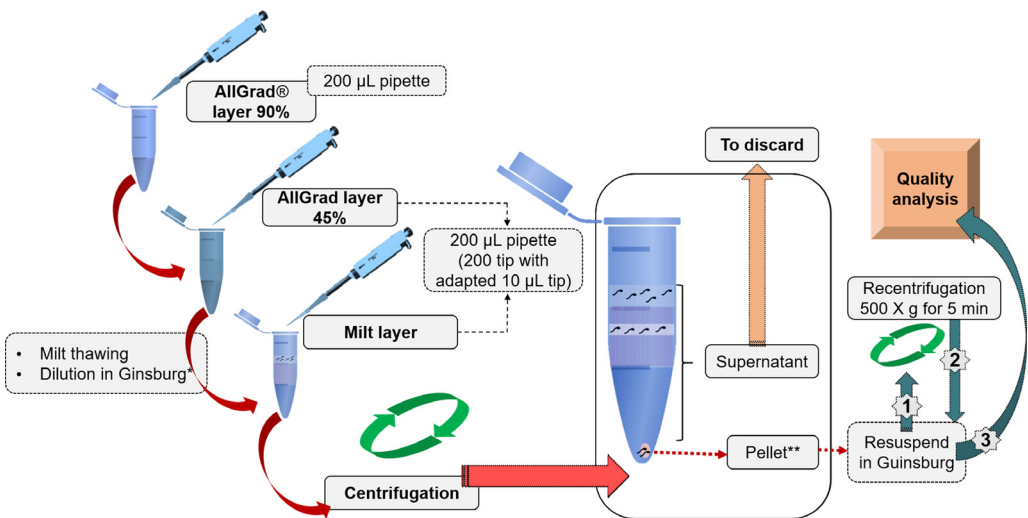
The purpose was to determine the treatments (Table 1) that allow a well-formed pellet and to determine if there is any statistical difference between them when comparing the sperm concentration of each pellet.

**Table 1**

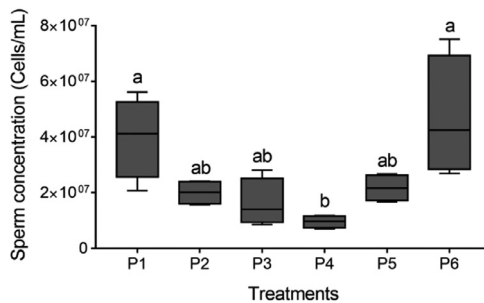
Treatments of the standardization of centrifugation forces and times by density gradients with AllGrad® 90% of cryopreserved fish sperm of the catfish *R. quelen*.

Treatment	Centrifugal Forces (x g)	Time centrifugation (min)	AllGrad layer 90% (µL)	AllGrad layer 45% (µL)	Milt layer (µL)	Milt dilution
P1	500	20	200	200	100	ND
P2	500	20	200	200	100	D
P3	500	20	100	100	100	ND
P4	1000	10	200	200	100	ND
P5	1000	10	200	200	100	D
P6	1000	10	100	100	100	ND

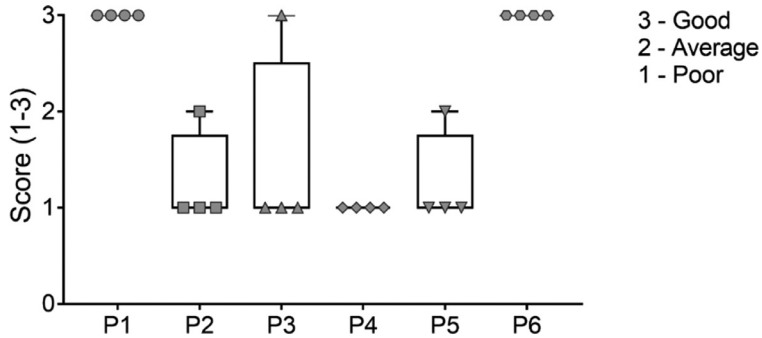
Dilution (D) or no dilution (ND) of the sperm in the Ginsburg solution.



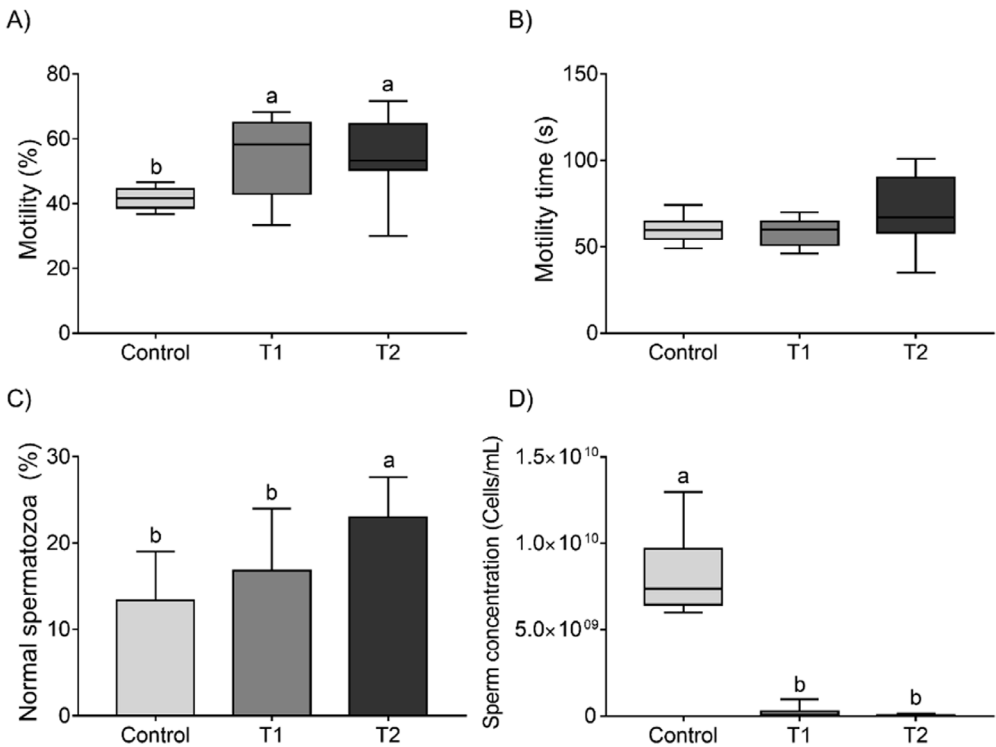
**Fig. 2.** Scheme of the density gradient centrifugation protocol from AllGrad® 90% with cryopreserved milt of the catfish *R. quelen*. \*Milt dilution was only for cases where the procedure was necessary. \*\*For all treatments, the pellet was resuspended in the Ginsburg extender solution, centrifuged again at 500 × g for 5 min. The final pellet was again resuspended in the Ginsburg solution and used to carry out the different quality analyzes.



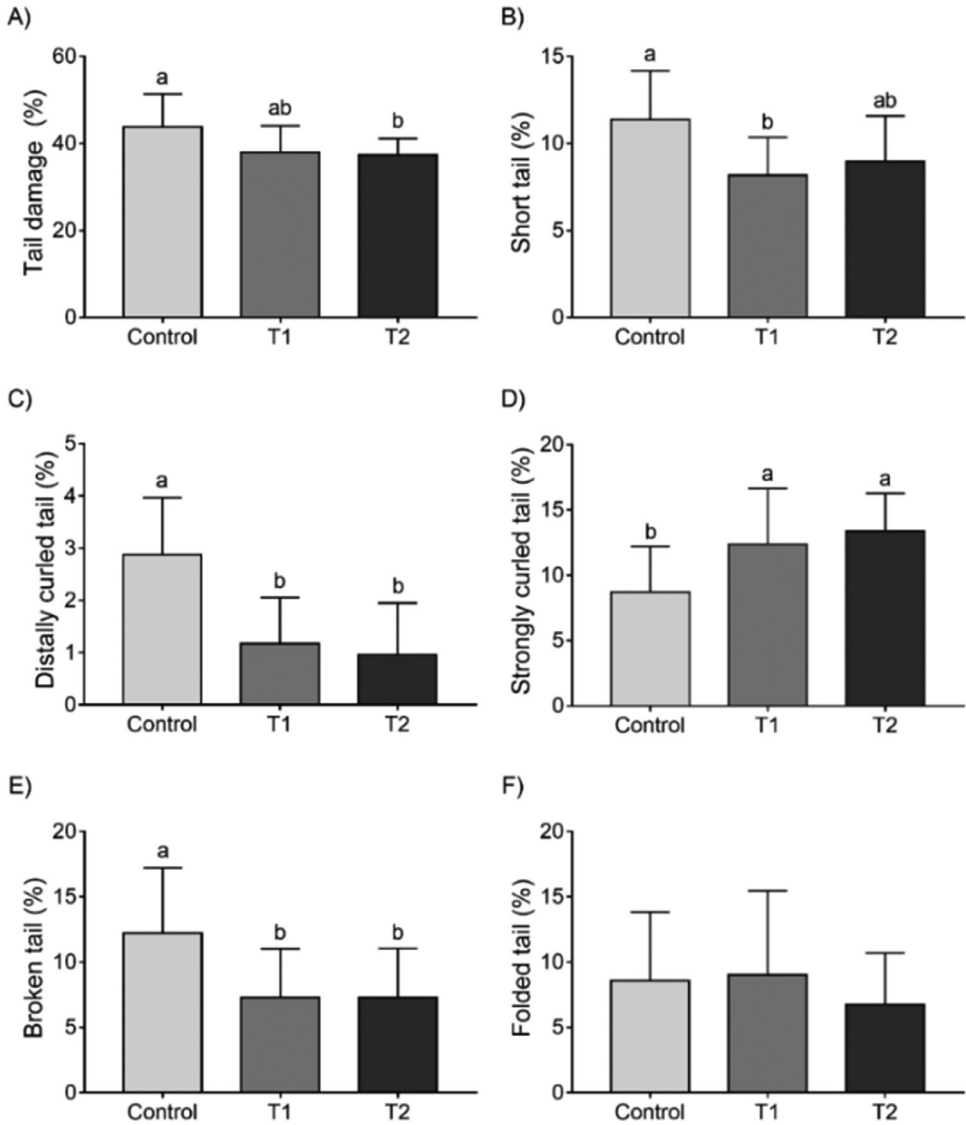
**Fig. 3.** Sperm concentration after application of treatments in milt. Kruskal-Wallis analysis followed by Dunn's test ( $p = 0.0051$ ). Different letters indicate difference between treatments.



**Fig. 4.** Score for pellet formation for each treatment (P). Each treatment included: centrifugation force (x g), centrifugation time (min), AllGrad layer 90% ( $\mu\text{L}$ ), AllGrad layer 45% ( $\mu\text{L}$ ), milt layer ( $\mu\text{L}$ ) and milt dilution in Ginsburg solution. P1 ( $500 \times \text{g}$ , 20 min, 200  $\mu\text{L}$ , 200  $\mu\text{L}$ , 100  $\mu\text{L}$ , ND), P2 ( $500 \times \text{g}$ , 20 min, 200  $\mu\text{L}$ , 200  $\mu\text{L}$ , 100  $\mu\text{L}$ , D), P3 ( $500 \times \text{g}$ , 20 min, 100  $\mu\text{L}$ , 100  $\mu\text{L}$ , 100  $\mu\text{L}$ , ND), P4 ( $1000 \times \text{g}$ , 10 min, 200  $\mu\text{L}$ , 200  $\mu\text{L}$ , 100  $\mu\text{L}$ , ND), P5 ( $1000 \times \text{g}$ , 10 min, 200  $\mu\text{L}$ , 200  $\mu\text{L}$ , 100  $\mu\text{L}$ , D) and P6 ( $1000 \times \text{g}$ , 10 min, 100  $\mu\text{L}$ , 100  $\mu\text{L}$ , 100  $\mu\text{L}$ , ND). The symbols represent the score of the treatments in each repetition, with four repetitions performed. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

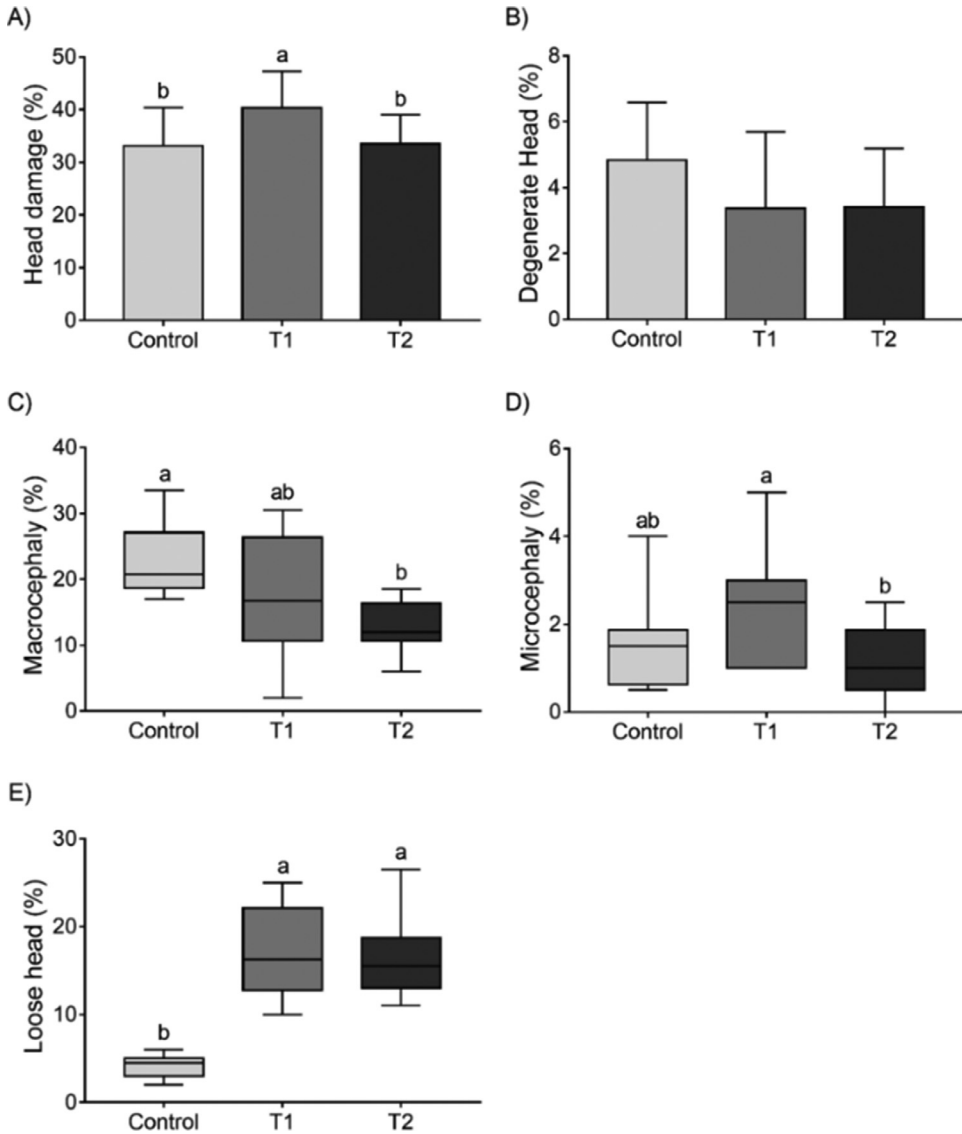


**Fig. 5.** Sperm quality in control treatment (thawed and no centrifuged milt samples), T1 (thawed and centrifuged milt samples -  $500 \times \text{g}$ , 20 min, 200  $\mu\text{L}$  of AllGrad 90%, 200  $\mu\text{L}$  of AllGrad 45%, 100  $\mu\text{L}$  milt) and T2 (thawed and centrifuged milt samples -  $1000 \times \text{g}$ , 10 min, 100  $\mu\text{L}$  of AllGrad 90%, 100  $\mu\text{L}$  of AllGrad 45%, 100  $\mu\text{L}$  of milt). A) Motility ( $p = 0.0020$ ); B) Motility time ( $p = 0.1554$ ); C) Spermatozoa with normal morphology ( $p = 0.0013$ ); D) Sperm concentration ( $p < 0.0001$ ). Kruskal-Wallis analysis followed by Dunn's test (Fig. A, B and D). Analysis of variance followed by Tukey's test (Fig. C). Different letters indicate difference between treatments.



**Fig. 6.** Tail pathologies in control treatment (thawed and no centrifuged milt samples), T1 (thawed and centrifuged milt samples - 500 × g, 20 min, 200 μL of AllGrad 90%, 200 μL of AllGrad 45%, 100 μL milt) and T2 (thawed and centrifuged milt samples - 1000 × g, 10 min, 100 μL of AllGrad 90%, 100 μL of AllGrad 45%, 100 μL of milt). A) Total tail pathologies ( $p = 0.0253$ ); B) Short tail ( $p = 0.0110$ ); C) Tail curled distally ( $p < 0.0001$ ); D) Tail strongly curled ( $p = 0.0080$ ); E) Broken tail ( $p = 0.0091$ ); F) Tail folded ( $p = 0.6061$ ). Analysis of variance followed by Tukey's test. Different letters indicate difference between treatments.

At the end of the AllGrad gradient column a layer of thawed milt (100 μL) was gently placed. The milt dilution, considered in some treatments (P2 and P5), is based on the fourth principle of centrifugation [10]. This indicates that the sedimentation rate increases when the viscosity of a solution decreases. This process was thought-out to obtain a larger pellet or with a difference in sperm concentration in relation to the other treatments. The step-by-step method is described in Sections Thawing and dilution of milt samples and Preparation of density gradients and is schematized in Fig. 2.

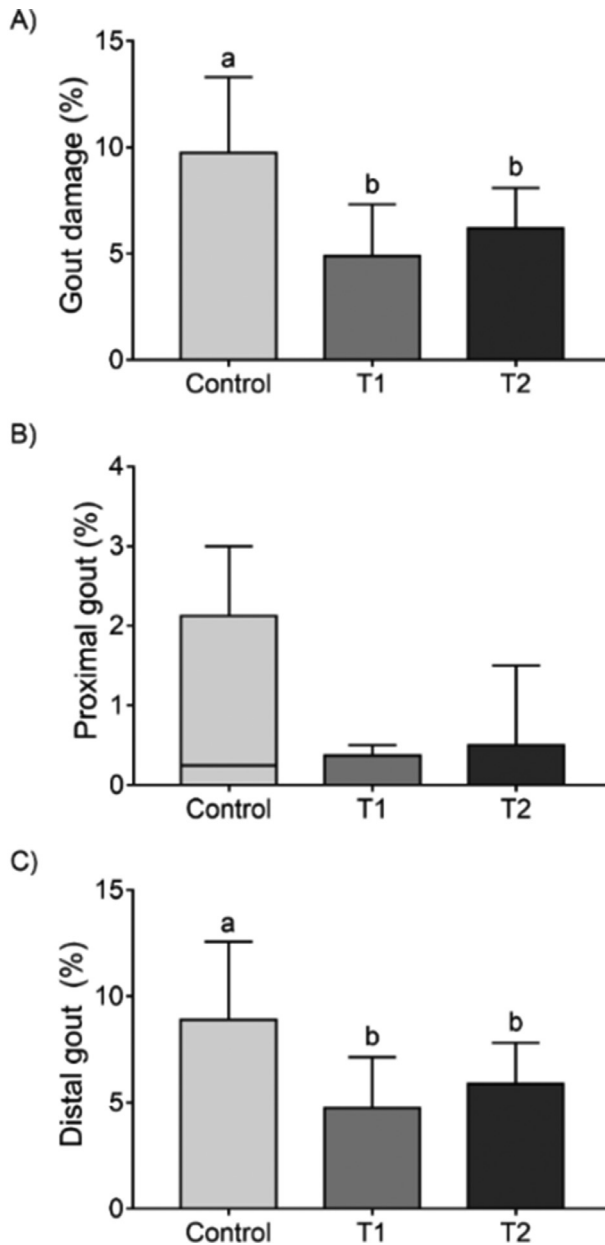


**Fig. 7.** Head pathology in control treatment (thawed and no centrifuged milt samples), T1 (thawed and centrifuged milt samples - 500 × g, 20 min, 200 μL of AllGrad 90%, 200 μL of AllGrad 45%, 100 μL milt) and T2 (thawed and centrifuged milt samples - 1000 × g, 10 min, 100 μL of AllGrad 90%, 100 μL of AllGrad 45%, 100 μL of milt). A) Total head diseases ( $p = 0.0191$ ); B) Degenerate head ( $p = 0.1329$ ); C) Macrocephaly ( $p = 0.0011$ ); D) Microcephaly ( $p = 0.0426$ ); E) Head loose ( $p < 0.0001$ ). Analysis of variance followed by Tukey's test (Fig. A and B). Kruskal-Wallis analysis followed by Dunn's test (Fig. C, D and E). Different letters indicate difference between treatments.

#### Thawing and dilution of milt samples.

- (1) Prior to conformation of density gradients, the cryopreserved milt samples were thawed in a water bath at 25 °C for 10 s.
- (2) Samples that did not need to be diluted were gently placed on the top of the gradient immediately after thawing. In the case of diluted samples, in a 2 mL Eppendorf, three parts





**Fig. 8.** Cytoplasmic gout pathology in control treatment (thawed and no centrifuged milt samples), T1 (thawed and centrifuged milt samples - 500 × g, 20 min, 200 μL of AllGrad 90%, 200 μL of AllGrad 45%, 100 μL milt) and T2 (thawed and centrifuged milt samples - 1000 × g, 10 min, 100 μL of AllGrad 90%, 100 μL of AllGrad 45%, 100 μL of milt).. A) Total cytoplasmic gout pathologies ( $p = 0.0003$ ); B) Proximal drop ( $p = 0.2397$ ); C) Distal drop ( $p = 0.0023$ ). Analysis of variance followed by Tukey's test (Fig. A and C). Kruskal-Wallis analysis followed by Dunn's test (Fig. B). Different letters indicate difference between treatments.

of thawed milt were diluted in four parts of Ginsburg solution and an aliquot was placed at the top of the gradient. In both cases, a milt volume of 100 µL was used.

#### *Preparation of density gradients.*

- (3) The AllGrad 45% solution was built from the commercial AllGrad® 90% stock solution. For this purpose, the AllGrad® 90% was diluted in Ginsburg extensor solution, in a ratio of 1:1 (v:v).
- (4) From AllGrad solutions (45% and 90%), the gradient columns were formed in a 1.5 mL Eppendorf tube. The layers of AllGrad 90% and 45% were placed, respectively, and at the top of the gradient layers, the thawed milt sample was gently applied. To place all layers within the gradient, a 200 µL micropipette and tip was used. However, for the last two layers (AllGrad 45% and milt sample), a 10 µL tip was adapted to the 200 µL tip, allowing it to be poured more smoothly to avoid mixing.
- (5) After shaping the gradients, the samples were centrifuged, at 4 °C, in a refrigerated centrifuge (Eppendorf Centrifuge 5403), following the centrifugation forces and times described for each treatment. The force (X g) was calculated from the following equation:

$$RCF (X g) = 1.12 \times r(mm) \times \left(\frac{RPM}{1000}\right)^2$$

Where, **RCF**: relative centrifugal force or g force (x g), **1.12**: constant value, **r**: radius in millimeters, **RPM**: revolutions per minute and **1000**: constant value.

- (6) Immediately after centrifugation, each conformed pellet was photographed which allowed the analysis of pellet formation and subsequent classification (Supplementary Table 2). This classification was performed using a score that was determined, subjectively, for the purposes of this standardization (Supplementary Table 3).
- (7) The supernatant from each centrifuged sample was discarded using a 1000 µL pipette with a 10 µL tip adapter, to keep the pellet intact.
- (8) Then, each pellet was resuspended in 200 µL of Ginsburg extensor solution.
- (9) Resuspension was again centrifuged at 500 × g for 5 min.
- (10) The supernatant was removed again following the same procedure explained in number seven, and then resuspended in 150 µL of Ginsburg solution. This final sample was used to perform the different quality analyzes required. The samples were kept at 4 °C until the completion of the centrifugation of all samples, and quality analysis.

## **Method validation**

### *Results of standardization of centrifugation forces and times*

Statistically only P1 and P6 (T1 and T2, respectively, for the second method validation experiment) were different from P4 (lower sperm concentration, Fig. 3), they were chosen because they presented a better pellet conformation score (3 – Fig. 4).

### *Method validation through sperm quality measurement*

The treatments for the second experimental phase of the method validation were defined in Table 2. A control treatment was considered (Control: milt samples that after thawing were not centrifuged) to be able to make the comparisons. For all treatments, in addition to sperm concentration, the following quality assessments were also performed: motility and sperm morphology. For these analysis, adaptations were made for the species under study, from test/error, until reaching the most appropriate proportions for each quality assessment (Supplementary Table 4) due to the different forces and centrifugation times applied. On the other hand, it is important to clarify that due to the difference that exists between the species of fish and even within the same species, it will most likely always be necessary to make these adjustments.

**Table 2**

Method validation treatments of the centrifugation by density gradients with AllGrad® 90% of cryopreserved fish sperm of the catfish *R. quelen*.

Treatment	Centrifugal Forces (x g)	Time centrifugation (min)	AllGrad layer 90% (µL)	AllGrad layer 45% (µL)	Milt layer (µL)
Control			Milt samples thawed and not centrifuged		
T1	500	20	200	200	100
T2	1000	10	100	100	100

### Sperm concentration

Sperm concentration was performed with the help of the Neubauer chamber, as previously described.

### Sperm motility

Motility was assessed subjectively [4] with adaptations. Immediately after thawing, videos of each treatment were taken using the camera of a cell phone (Iphone 8, apple) fitted in one of the microscope eyepieces and with a 40 X objective. The aliquots to be evaluated were placed in the Neubauer chamber. In total 12 videos were made per treatment. The videos were evaluated on the computer screen, by a technician with intermediate experience. All videos were randomly numbered before being sent to the evaluator. The quantified variables were motility rate (0–100%) and motility time (s).

### Sperm morphology

The evaluation of sperm morphology was performed using milt previously fixed in buffered formaldehyde solution (10%). An aliquot of 100 µL of fixed milt (same sample used in the sperm concentration) was mixed with 10 µL of the Rose Bengal dye [3]. The adapted methodology includes conformation of the slides per drained drop, placing three drops of 10 µL, each, on a histological slide. The slides were read with the 100 X objective, using immersion oil. The morphology evaluated were [3]: Normal spermatozoa, short tail, distally curled tail, strongly curled tail, broken tail, folded tail, degenerate head, macrocephaly, microcephaly, loose head, proximal gout, distal gout. In total, six repetitions were performed per treatment, counting 200 sperm per slide, in duplicate.

Density gradient centrifugation with AllGrad revealed the notable reduction in sperm concentration after application of the method (Fig. 5D). The centrifuged milt represented a high percentage of motility compared to the control treatment (Fig. 5A). The presence of tail morphological abnormalities was lower in the centrifugation of 1000 × g for 10 min compared to the control treatment (Fig. 6A). In addition, head damage (Fig. 7A) and the presence of gout damage (Fig. 8A) were also significantly lower in separated milt with AllGrad.

The presence of normal cells was better when the thawed milt was centrifuged at a speed of 1000 x g for 10 min, 4 °C (Fig. 5C),  $22.25 \pm 4.64\%$ , which is equivalent to  $8.19 \times 10^6$  normal spermatozoa, this is 9.25% more than the milt that was not centrifuged. The importance of this result is that the good performance of the spermatozoa is related to the integrity of their structures (head, middle piece and tail) and, therefore, in the spermatozoa motility or the fertilization capacity [3].

The centrifugation method with AllGrad density gradients (90– 45%, volume of each layer: 100 uL), at a speed of 1000 × g for 10 min, 4 °C, and with recentrifugation of 500 × g for 5 min, 4 °C, it proved to be a promising technique for obtaining a portion of better quality sperm from cryopreserved milt of the *R. quelen* species however, even with a low number of normal and motile spermatozoa, but that could mark the difference when they are used for *in vitro* fertilization. Considering that this is a first approach to the application of the technique for cryopreserved *R. quelen* milt, improvements could still be made in the protocol. It is also recommended that in the application of the technique for other species or even for the same species, the respective adaptations to the protocol be made considering their physiological differences.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgments

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:[10.1016/j.mex.2021.101422](https://doi.org/10.1016/j.mex.2021.101422).

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