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Improvement of gamete quality and its short-term storage: an approach for biotechnology in laboratory fish

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In fish, in vitro *fertilization is an important reproductive tool used as first step for application of others biotechniques as chromosome and embryo manipulation. In this study, we aimed to optimize gamete quality and their short-term storage from the yellowtail tetra* Astyanax altiparanae*, for future application in laboratory studies. Working with sperm, we evaluated the effects of spawning inducers (carp pituitary gland and Ovopel® [(D-Ala6, Pro9-NEt) – mGnRH + metoclopramide]) and the presence of female on sperm motility. Additionally, we developed new procedures for short-term storage of sperm and oocytes. Briefly, sperm motility was higher when male fish were treated with carp pituitary gland (73.1 ± 4.0%) or Ovopel® (79.5 ± 5.5%) when compared with the control group treated with 0.9% NaCl (55.6 ± 27.2%;* P *= 0.1598). Maintenance of male fish with an ovulating female fish also improved sperm motility (74.4 ± 7.4%) when compared with untreated male fish (42.1 ± 26.1%;* P *= 0.0018). Storage of sperm was optimized in modified Ringer solution, in which the sperm was kept motile for 18 days at 2.5°C. The addition of antibiotics or oxygen decreased sperm motility, but partial change of supernatant and the combination of those conditions improve storage ability of sperm. Fertilization ability of oocytes decreased significantly after storage for 30, 60 90 and 120 min at 5, 10, 15 and 20°C when compared with fresh oocytes (*P *= 0.0471), but considering only the stored samples, the optimum temperature was 15°C. Those data describe new approaches to improve semen quality and gametes short-term storage in yellowtail tetra* A. altiparanae *and open new possibilities* in vitro *fertilization.*

Keywords: cryopreservation, sperm, oocyte, fish, yellowtail tetra

Implications

The yellowtail tetra *Astyanax altiparanae* is a characin fish largely distributed in Neotropical regions. As a small-sized intertidal spawner, this species is a promising candidate for laboratory and aquaculture technologies including polyploidy, chimerism and nuclear transplantation. However, the first step for such kind of studies is to establish a protocol for *in vitro* fertilization (IVF) at laboratory conditions. In this work several factors affecting gamete quality were evaluated, and the findings will collaborate for an effective protocol for IVF in this species.

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Introduction

In vitro fertilization (IVF) is a key factor for biotechnology in fish. Studies regarding chromosome-set manipulation, embryology, transgenesis, nuclear transplantation and chimerism requires a precise fertilization timing for successful manipulation (Tanaka *et al*., 2009; Yasui *et al*., 2010, 2011). The timing for gamete activation is necessary for those techniques because manipulation must take place within a few minutes after fertilization (Nam *et al*., 2004; Yasui *et al*., 2010). For induction of polyploid fish, temperature or pressure shocks must be applied before the first cell cleavage few minutes after gamete activation (Piferrer *et al*., 2009). Techniques that requires embryo (i.e. embryology, chimerism) or gamete manipulation (intracytoplasmic sperm

injection, cryopreservation, nuclear transplantation, transgenesis) must also be performed in an adequate time post-fertilization (Lin *et al.*, 1992; Poleo *et al.*, 2001; Lee *et al.*, 2002; Liu *et al.*, 2002; Sun *et al.*, 2005). In this regard, sperm sampling is the first critical stage to develop such biotechniques. Fish sperm is well known to be immotile in the seminal plasma but the ionic content of the external media triggers its motility assuring subsequent fertilization (Coward *et al.*, 2002; Cosson, 2004). In small fish (i.e. laboratory fish), sperm is generally sampled with urine contamination that also initiates motility. Such a problem is critical for IVF because the duration of sperm motility is short for most species (1 to 2 min) and then sperm quality decreases rapidly in urine contaminated samples (Dreanno *et al.*, 1998; Perchec-Poupard *et al.*, 1998). Additionally, if the initiation of sperm motility is not controlled, the fertilization timing and consequently the biotechniques cannot be developed successfully. Then, it is necessary to develop physiological media for sperm sampling in order to re-immobilize the contaminated sperm. Later, the immobilized sperm may be activated properly during IVF. In addition, short-term preservation (i.e. refrigerated storage) is another procedure necessary for later use of the gametes at adequate moment for fertilization.

In this study, we used the yellowtail tetra (*Astyanax altiparanae*) as a model fish. This species presents small size (8 to 14 cm) and may be bred through the year (intertidal spawner). Such characteristics are interesting for laboratory (aquarium) systems. Though the yellowtail tetra is used for aquaculture purposes, fingerlings are generally produced by natural (spontaneous) or semi-natural spawning (induced maturation followed by spontaneous spawning), and IVF is not fully developed.

Considering the aspects above, the aim of this work was to establish laboratory procedures to improve semen quality and gametes short-term storage of the yellowtail tetra, including: (1) gamete sampling, (2) optimizing sperm quality and (3) gamete storage for future application in chromosome and embryo manipulation for this species.

Material and methods

All the procedures were performed in line with the Guide for the Care and Use of Laboratory Animals in Sao Paulo University.

Induced maturation, gamete sampling and fertilization

Adult broodstock of yellowtail tetra *A. altiparanae* were obtained at Chico Mendes Institute of Biodiversity Conservation, Sao Paulo State, Pirassununga, Brazil. These fish were previously sampled at Mogi Guassu river (21.925706 S, 47.369496 W) and then maintained in earthen ponds or aquariums. Fish were induced to spawn using a single dose of Ovopel® (Interfish Ltd., Budapest, Hungary) [(D-Ala^6, Pro^9-NEt) − mGnRH + metoclopramide] (3 mg/kg for male and female fish) or carp pituitary gland (3 mg/kg for male and female fish) and then maintained in a 200 l-tank at 25°C to 30°C. Gametes were sampled 8 to 12 h afterwards, depending on the temperature and ovary development. Sperm was collected at 7 to 12 h depending on water temperature. Fish were anesthetized with menthol at 0.1 mg/l and the genital area was carefully dried. Sperm sampling was achieved by stripping and the sperm was immediately collected in the papilla using a 1000 μl-Eppendorf micropipette (Eppendorf, Hamburg, Germany). The sampled sperm was transferred to a 1.5 ml tube containing 300 μl of modified Ringer solution (128.3 mM NaCl, 23.6 mM KCl, 3.6 mM $CaCl_2$, 2.1 mM $MgCl_2$) and homogenized by gently pipetting. This solution, chosen based on our previous observation, maintain the sperm of *A. altiparanae* motile up to 3 days at 2°C to 3°C. After each sampling, we measured immediately the sperm motility and the sperm concentration using hemocytometer. For evaluation of sperm motility, 1 μl of diluted sperm was pipetted on a microscope slide previously coated with 0.1% bovine serum albumin and the sperm was activated by a 30-fold dilution with distilled water. Then, we evaluated the total motility, progressive motility and duration of progressive motility using the same criteria of our previous studies (Fujimoto *et al.*, 2008; Yasui *et al.*, 2008, 2009, 2010).

Egg sampling and gamete activation followed the previous procedures with the loach (see Fujimoto *et al.*, 2006). Oocytes were stripped on a plastic Petri dish (90 mm diameter) covered by a polyvinylidene chloride film (saran wrap). For fertilization, 50 to 100 μl of sperm was pipetted on the oocytes and homogenized by gentle hand mixing. Gamete activation was performed by addition of hatchery water (~5 ml/10 000 oocytes). After 2 min (period of duration of sperm motility), the eggs were transferred to another Petri dish without the plastic film containing ~50 ml of water for incubation. Incubation was performed in biochemical oxygen demand (BOD) incubators set at 26°C. After gamete activation, fertilization rates at blastula stage (~3 hpf), hatching rates and percentage of normal and abnormal larvae were measured (~16 hpf).

Effects of maturation inducers on sperm quality

In this experiment, we evaluated the effects of two spawning inducers on sperm quality. Adult male fish ($n = 4$) were injected with Ovopel® [(D-Ala^6, Pro^9-NEt) − mGnRH + metoclopramide] (3 mg/kg) or carp pituitary gland (3 mg/kg). Both hormones were diluted in a 0.9% NaCl solution. As control groups, fish were injected only with a 0.9% NaCl solution. After injection with Ovopel®, carp pituitary gland and 0.9% NaCl, the male fish were placed in a 125-l aquarium with temperature set at 27°C at dark conditions. Sperm was sampled 10 h afterwards and parameters of motility and sperm concentration were evaluated. For this experiment we used four male fish for each treatment.

Presence of female and hormonal treatment on sperm quality

Here we evaluated the effects of carp pituitary gland (3 mg/kg) and the presence of the female upon the sperm quality. We used seven treatments as shown in Table 1. We used the same stock density at six fish per tank for all treatments, including male and female fish. Fish were maintained in

Table 1 *Treatments for evaluation of induced spawning and the presence of female fish on sperm quality*

Treatments	Male treatment	*n*	Female treatment	*n*	Total fish
Treatment 1	None (untreated)	6	–	0	6
Treatment 2	0.9% NaCl	6	–	0	6
Treatment 3	Pituitary gland	6	–	0	6
Treatment 4	0.9% NaCl	4	0.9% NaCl	2	6
Treatment 5	Pituitary gland	4	0.9% NaCl	2	6
Treatment 6	0.9% NaCl	4	Pituitary gland	2	6
Treatment 7	Pituitary gland	4	Pituitary gland	2	6

In treatment 1, we used untreated male fish (not injected). For other treatments, we injected both physiological medium (0.9% NaCl) or pituitary gland (3 mg/kg for female fish dissolved in 0.9% NaCl). Sperm from four male fish from each treatment was analyzed.

a fiberglass tank with a constant water flow at 28°C. After 8 h, male fish from treatments T6 and T7 started spawning behavior (male fish following the female fish across the tank). Then, all the female fish from treatments T4, T5, T6 and T7 were removed to avoid natural spawning. At that moment, the female fish from treatments T6 and T7 were ovulating and promptly released oocytes after gently stripping. For each treatment, we evaluated the sperm concentration and motility parameters as shown above. For this experiment we used four male fish for each treatment.

Short-term storage of sperm

First, we evaluated six different kind of semen extenders to immobilize and maintain the sampled sperm. Sperm from eight male fish presenting progressive motility above 85% were mixed, and diluted 10-fold in the six extenders, as follow: modified Ringer solution (128.3 mM NaCl, 23.6 mM KCl, 3.6 mM $CaCl_2$, 2.1 mM $MgCl_2$), Kurokura's solution (128.4 mM NaCl, 2.7 mM KCl, 1.4 mM $CaCl_2$, 2.4 mM $NaHCO_3$, Kurokura *et al.*, 1984), Cyprinid extender (76 mM NaCl, 70.4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 2.4 mM $NaHCO_3$ – modified from Lahnsteiner *et al.*, 2003), Dulbecco's Phosphate Buffer solution calcium and magnesium-free (Sigma, St. Louis, USA), Eagle's-MEM solution (Sigma), and Hank's saline solution (137 mM NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1 mM $MgSO_4$, 4.2 mM $NaHCO_3$). After dilution, the samples were stored at 2.5°C and the sperm motility was measured daily as described above.

The best extender was selected based on the experiment above. To improve gamete storage ability, we evaluated five treatments: treatment 1 (control): extender; treatment 2: extender with antibiotics (100 μg/ml of streptomycin and 100 IU of penicillin); treatment 3: replacement of supernatant extender (50%) each 4 days and re-suspension of sperm; treatment 4: addition of oxygen to the tube (daily); treatment 5: combination of all treatments above (addition of antibiotics, replacement of supernatant each 4 days and addition of oxygen). Storage ability was evaluated based on sperm parameters as in the previous experiment.

Short-term storage of eggs

Different temperatures were evaluated for short-term storage of eggs *in vitro*. Eggs from three female fish were stripped on a plastic Petri dish (90 mm diameter) covered by a polyvinylidene chloride film (saran wrap). The oocytes from each female were divided into four Petri dishes (all containing polyvinylidene chloride film) and the egg mass was covered by a small piece (~50 × 50 mm) of plastic film in order to prevent dehydration. The Petri dishes were stored at 5°C, 10°C, 15°C and 20°C in BOD incubators. Aliquots of about 120 eggs were fertilized at 0, 30, 60, 90 and 120 min of storage, and then checked the fertilization at blastula stage (~3 hpf) and hatching rates (~16 hpf). The number of normal and abnormal larvae was also counted as percentages of hatched larvae.

Statistics

Data are shown as mean ± s.d. All experiments were performed in triplicates using different gamete sources. Data were checked for normality using the Liliefor test, and then compared using ANOVA followed by Tukey's multiple range test. All statistical analysis were performed using the software Statistica v10 and the significance was set at $P \leqslant 0.05$.

Results

Effects of maturation inducers on sperm quality

Induced spermiation did not increase the progressive motility of the sperm (Figures 1, $P = 0.1598$). The use of carp pituitary gland and Ovopel gave rise to motility levels of 73.1 ± 4.0% and 79.5 ± 5.5%, respectively. Control groups injected with 0.9% NaCl showed a motility of 55.6 ± 27.2%. Sperm concentration was not affected by induced spermiation ($P = 0.5160$). In control group sperm concentration was $1.0 \times 10^{10} \pm 7.0 \times 10^{8}$ spermatozoa/ml. The same parameter was $6.2 \times 10^{8} \pm 3.9 \times 10^{8}$ spermatozoa/ml for fish treated with pituitary gland and $6.3 \times 10^{8} \pm 2.1 \times 10^{8}$ spermatozoa/ml for Ovopel. The duration of sperm motility was shorter (54.0 ± 7.7 s) in control when compared with carp pituitary gland (92.8 ± 15.5 s, $P = 0.0031$) and Ovopel (86.5 ± 10.6 s, $P = 0.0090$).

Presence of female and hormonal treatment on sperm quality

As shown in Figure 2, the progressive sperm motility was significantly lower ($P = 0.0018$) within untreated male fish (T1 = 42.13 ± 26.1%), male fish injected with 0.9% NaCl (T2 = 55.9 ± 14.5%), and when male fish and female fish

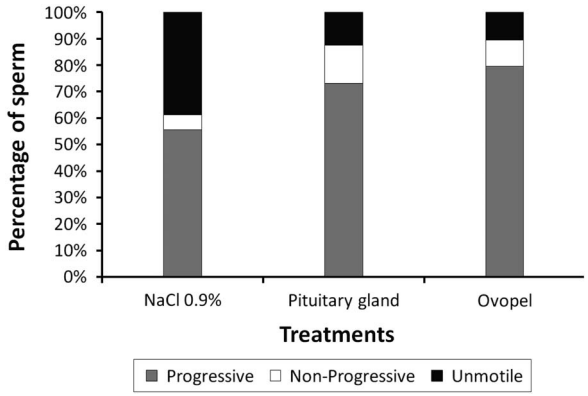

Figure 1 Effect of induced spermiation upon the sperm motility of *Astyanax altiparanae* using Ovopel (3 mg/kg) and carp pituitary gland (3 mg/kg) in comparison with 0.9% NaCl (control). Sperm motility are expressed as progressive motility, non-progressive motility and immotile spermatozoa. Progressive motility had no significant differences within treatments ($P < 0.1598$).

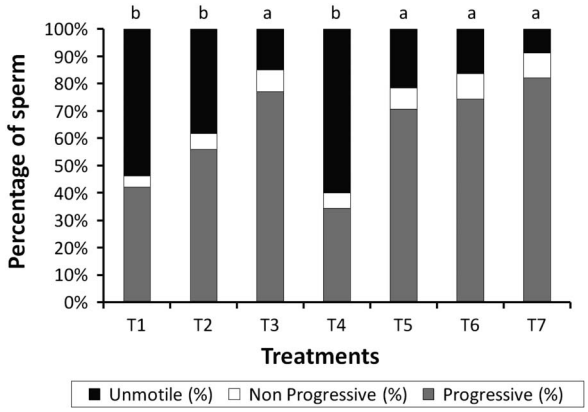

Figure 2 Effect of hormonal treatment and the presence of female fish upon the sperm motility of *Astyanax altiparanae* (see Table 1). Sperm motility are expressed as progressive motility, non-progressive motility and immotile. Asterisks indicates significant differences within treatments by the Tukey multiple range test ($P < 0.05$).

were injected with 0.9% NaCl (T4 = 34.3 ± 12.4%). Hormonal treatment increased significantly the sperm motility in all conditions, with the male fish kept separately (T3 = 77.1 ± 15.1%), with non-induced female fish (T5 = 70.6 ± 15.1%) or induced female fish (T7 = 82.1 ± 4.3%). Interestingly, male fish injected with 0.9% stored with induced female fish presented increased sperm motility at 74.4 ± 7.4% (T6).

The duration of progressive motility was lower ($P = 0.0365$) in the treatments without hormonal treatment (T1 = 55.2 ± 9.5 s, T2 = 43.6 ± 11.5 s, T4 = 42.3 ± 3.2 s), but when an induced female was kept together, the duration of progressive motility was increased (T6 = 82.0 ± 15.2 s). Induced male fish presented increased progressive motility when kept separately (T3 = 105.6 ± 19.9 s) or with non-induced (T5 = 68.3 ± 13.0 s) or induced female fish (T7 = 98.0 ± 14.0 s). Sperm concentration did not show a clear correlation with treatments ($P = 0.1573$, data not shown).

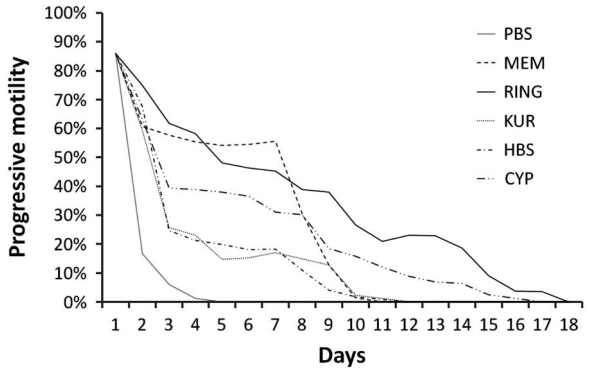

Figure 3 Progressive sperm motility of yellowtail tetra *Astyanax altiparanae* after refrigerated storage. Sperm samples were diluted 10-fold using six extenders and then maintained at 2.5°C. Motility parameters were then measured daily. Our results demonstrate that sperm of yellowtail tetra presented motility until 18 days when diluted in modified Ringer solution.

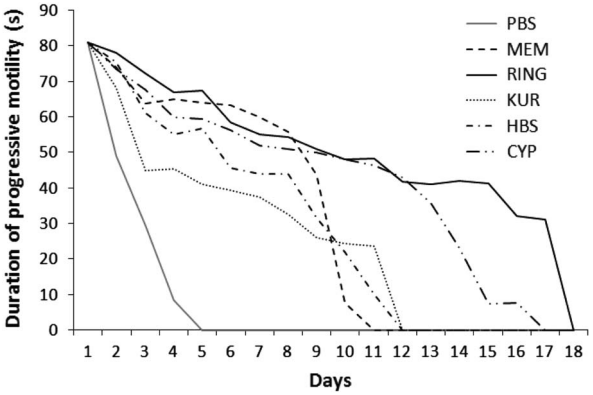

Figure 4 Duration of progressive sperm motility of yellowtail tetra *Astyanax altiparanae* after refrigerated storage. Sperm samples were diluted 10-fold using six extenders and then maintained at 2.5°C. Motility parameters were then measured daily. Our results demonstrate that sperm of yellowtail tetra presented motility until 18 days when diluted in modified Ringer solution.

Short-term storage of sperm

The extenders used to dilute the sperm presented different storage ability (Figure 3). Sperm motility decreased rapidly with 2 days of storage in Dulbecco's PBS, and the sperm remained motile for only 4 days. Hank's saline, Kurokura's solution, cyprinid extender decreased rapidly the sperm motility during the first 3 days of storage, but the sperm was still viable if stored from 10 to 16 days. Eagle's MEM kept the sperm motility in good conditions until 7 days of storage, and from this point the motility decreased rapidly until 10 days of storage. Sperm motility presented a linear decrease in Ringer solution and the storage ability remained until 18 days. Duration of progressive motility was also decreased when the sperm motility was decreased (Figure 4). Based on the results above, Ringer solution was chosen for other experiments with sperm.

Optimization of sperm storage

As observed on Figure 5, the use of oxygen and antibiotics decreased the storage ability of sperm when compared with control group in which only Ringer solution was used.

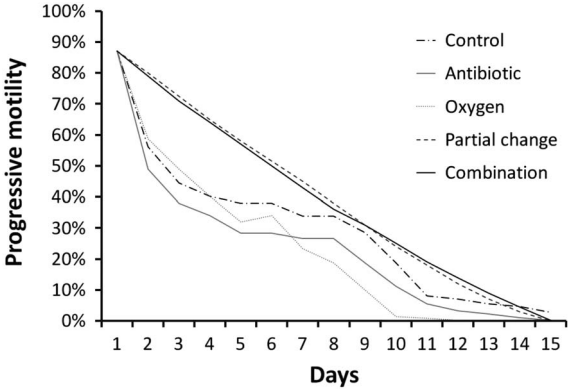

Figure 5 Optimization of refrigerated storage of sperm of yellowtail tetra *Astyanax altiparanae*. Sperm samples were diluted 10-fold using Ringer solution and stored at 2.5°C. For evaluation of storage conditions, additives as oxygen, antibiotics, partial change of the supernatant and a combination thereafter (oxygen + antibiotics + partial changes) were evaluated.

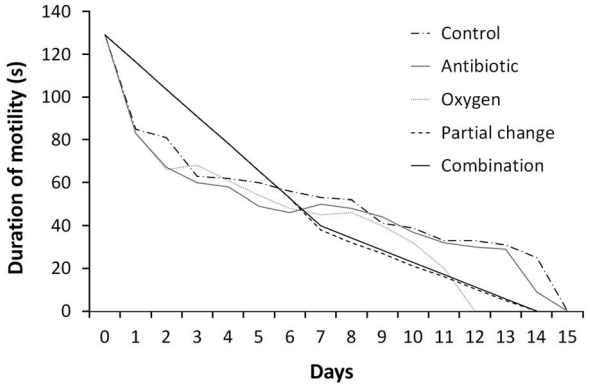

Figure 6 Duration of progressive sperm motility of yellowtail tetra *Astyanax altiparanae* after short-term storage. Sperm samples were diluted 10-fold using Ringer solution and stored at 2.5°C. Additives as oxygen, antibiotics, partial change of the supernatant and a combination thereafter (oxygen + antibiotics + partial changes) were evaluated.

Partial change of the extender and a combination of all treatments maintained the sperm motility better than control group, although the duration of storage was similar in those treatments (about 15 days).

The duration of progressive motility was also decreased with the storage conditions (Figure 6).

Short-term storage of eggs

Storage of the oocytes decreased the fertilization ability in all conditions when compared with controls, which were fertilized just after gamete sampling (Figure 7). However, no significant differences were found within the stored samples, and ranged from 4.1 ± 5.3% (120 min at 5°C) to 50.6 ± 36.2%.

Considerable variation was found in results from each treatment. The percentages of normal and abnormal larvae did not show a clear relationship within treatments.

Discussion

Semen quality and gametes short-term storage of yellowtail tetra was optimized in the present study. Regarding the

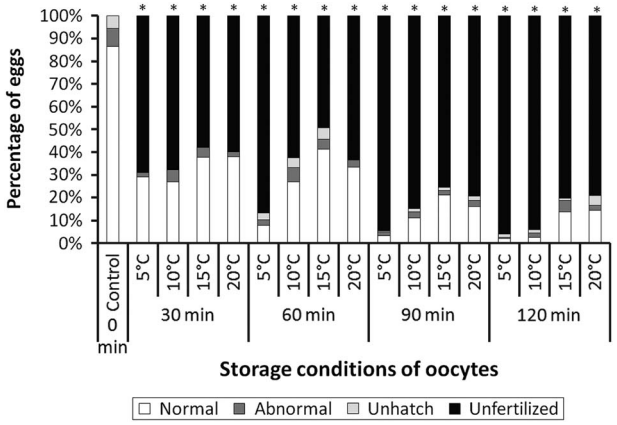

Figure 7 Development of *Astyanax altiparanae* after short-term storage of oocytes. Oocytes were incubated at 5, 10, 15 and 20°C and aliquots (120–150 oocytes) were fertilized at 30, 60, 90 and 120 min. Control group was fertilized immediately after oocyte sampling (no storage). After fertilization, the embryos were incubated at room temperature (24–26°C). Fertilization was measured at blastula stage (3 hpf), and the percentages of normal and abnormal larvae were measured after hatching (16 hpf). Fertilization rates decreased significantly in all treatments when compared with control groups.

sperm, hormonal treatments and the presence of female fish improved the sperm quality. We observed that induced spermiation is suggested in this species because of increase on sperm motility, although the sperm concentration is decreased. Interestingly, we found that ovulating female or hormonal treatments are both efficient to induce spermiation in this species. In laboratory studies, hormonal treatments is suggested to increase sperm motility. After sampling, the sperm of the yellowtail tetra was kept under refrigerated storage for several days when diluted in Ringer solution. Additionally, partial change of the extender was effective to improve the storage ability. Theoretically, extenders dilute the sperm, reducing the viscosity, maintain the pH, increase the oxygen availability and improve the accuracy for measuring the sperm motility (Billard and Cosson, 1992). However, dilution of sperm using extenders may improve (Billard *et al.*, 2004; Fauvel *et al.*, 2012) or even decrease (Jing *et al.*, 2009; He *et al.*, 2012) the maintenance of sperm, suggesting a species-specific relationship. Although the addition of oxygen and antibiotics improved the storage ability in some species (Brown and Mims, 1995; Bencic *et al.*, 2000), and in the yellowtail tetra the combination of both treatments improved the sperm storage. For most of aquaculture species, undiluted sperm is used for fertilization. On the other hand, in the yellowtail tetra the utilization of extenders is essential for sperm sampling and storage due to very low volume and urine contamination. It suggests that our procedures of gamete sampling and storage are essential for artificial fertilization in this species. Considering those aspects, the best extender selected in our work will have an important role in this species including (1) gamete sampling as an immobilizing media, (2) increase of accuracy during measuring the sperm motility, (3) improvement of sperm usage under refrigerated storage, and (4) standardization of the timing for fertilization.

In all treatments, we observed highly variable results. For instance, the duration of sperm motility, concentration and other sperm parameters were different in our experiments, even when we used the same procedure (sperm sampling and storage in Ringer solution). Similarly, the storage ability of the sperm using Ringer solution varied from 16 to 18 days. Those results indicate that sperm quality is highly variable between individuals of this species.

Regarding the storage of the oocytes, only changing storage temperatures gave rise to highly variable and poor results. Such data did not result in applicable procedures for hatchery or laboratory studies. In general, warm-water species presents low storage ability after ovulation (Rizzo *et al*., 2003; Nguenga *et al*., 2004). Better storage are found in some cold-water species including salmonids (Barrett, 1951; Withler and Morley, 1968; Poon and Johnson, 1970; Jensen and Alderdice, 1984; Babiak and Dabrowski, 2003; Niksirat *et al*., 2007), sturgeons (Billard *et al*., 2004; Sohrabnezhad *et al*., 2006), flatfishes (Suquet *et al*., 1999) and walleye (Dietrich *et al*., 2012) although it is dependent on species and storage conditions (Rothbard *et al*., 1996; Linhart *et al*., 2001; Yasui *et al*., 2010). Based on our results, we conclude that oocytes from yellowtail tetra cannot be stored successfully, although other procedures may be used in future works including solutions with additives that may improve storage ability as was observed in the other warm-water fish such as the tilapia (Harvey and Kelley, 1984).

Considering our data of storage of oocytes and sperm, we also conclude that sperm may be stored for several days under refrigeration although the viability of oocytes decreases rapidly after ovulation. The sperm may be sampled and stored several hours before the oocyte sampling, and this procedure is important because the timing for ovulation varies according to the female and environmental conditions. In addition, oocyte viability decreases quickly after sampling, and then it is important to collect the sperm before the oocytes. Such information is essential to develop all procedures for artificial insemination in the yellowtail tetra by optimizing gamete handling including gamete sampling and storage. Several important information are necessary for fish biotechnology and those information are not fully understood even in well studied fish like the zebrafish in which a protocol for sperm sampling and artificial fertilization was improved recently (Hagedorn and Carter, 2011). This study is the first step for future works involving IVF in yelowtail tetra.

In conclusion, the gamete quality was optimized using the following procedures: induced spermiation with carp pituitary gland or Ovopel® and dilution and storage of sperm using modified Ringer solution. Such procedures may facilitate the application of IVF in the yellowtail tetra under laboratory conditions.

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