TRYPAN BLUE STAINING IS NOT EFFICIENT IN DETERMINING OOCYTE VIABILITY IN *Colossoma macropomum* AND *Brycon amazonicus**

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ABSTRACT

Currently, there is no effective technique to evaluate the quality of oocytes in fish farming in a practical and affordable way. The cell membrane integrity test with the vital dye trypan blue (TB) could be an option. In this study, *Colossoma macropomum* and *Brycon amazonicus* oocytes were exposed to different TB concentrations seeking to verify a possible relationship between the results of staining tests and reproductive rates. Oocytes were exposed to concentrations of 0.05, 0.04, 0.03, 0.02 and 0.01% TB for 1 minute and subsequently were evaluated under a stereomicroscope. The percentage of unstained (viable) oocytes from each sample was correlated with fertilization and hatching rates using a linear regression (P>0.05). We observed a weak correlation between the results of the staining tests and the fertilization and hatching rates in both species. TB integrity tests were not effective in predicting spawning viability in *C. macropomum* and *B. amazonicus*.

Keywords: fish gametes; vital stain; membrane integrity; Amazonian fish

COLORAÇÃO COM AZUL DE TRIPAN NÃO É EFICIENTE NA DETERMINAÇÃO DA VIABILIDADE DE OÓCITOS EM *Colossoma macropomum* E *Brycon amazonicus*

RESUMO

Atualmente não existem técnicas efetivas para avaliar a qualidade de oócitos na piscicultura de forma prática e acessível. O teste de integridade da membrana celular com corante vital azul de tripan (AT) surge como uma alternativa. Nesse estudo, oócitos de *Colossoma macropomum* e *Brycon amazonicus* foram expostos a diferentes concentrações de AT, buscando verificar uma possível relação entre os resultados dos testes de coloração e as taxas reprodutivas. Oócitos foram expostos a concentrações de 0,05; 0,04; 0,03; 0,02 e 0,01% de AT durante 1 minuto e, posteriormente avaliados em estereomicroscópio. A porcentagem de oócitos não corados (intactos) de cada amostra foi correlacionada com as taxas de fertilização e eclosão utilizando análise de regressão linear (P>0,05). Fraca correlação entre resultados dos tratamentos e as taxas de fertilização e eclosão nas duas espécies foi observada. Os testes de integridade com AT foram ineficazes em predizer o sucesso da desova em *C. macropomum* e *B. amazonicus*.

Palavras-chave: gameta de peixe; corante vital; integridade de membrana; peixe amazônico

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INTRODUCTION

The economic success of fish farming is based on several factors that go beyond the nutritional quality of its products and the high demand for fish globally. It also depends on a quick and dynamic production cycle. High fecundity and short embryonic development are characteristics of many teleosts, which increase their economic potential for farming.

Despite the potential for expansion, Brazilian fish larviculture still presents low productivity rates, which is mainly due to low control of the main agents reducing the quality of the gametes. The factors that define gamete quality during reproduction are related to external characteristics such as water quality, the adopted reproductive management practices, and the internal characteristics of the broodstock such as genetics, yolk composition, and gamete morphology (LAHNSTEINER *et al*., 2009).

Oocytes are keys to reproductive success by providing the amount of yolk required for embryo development and the mitochondrial DNA (BOBE and LABBÉ, 2010). The quality of the female gamete will directly affect fertilization and hatching rates and the development of larvae with greater chances of survival. The direct relationship between the quality of the gametes and reproductive success prompted the development of tests to evaluate this quality such as analyses of membrane integrity, motility and morphology, which are already used for quality assessment of semen (GARCIA *et al*., 2016). Despite the importance of female gametes, techniques for evaluating the viability of oocytes in fish are still scarce and limited to the experimental stage.

Chemical substances called vital dyes are widely used in basic and applied research to evaluate specific characteristics of a cell or tissue and have become an alternative method for quality assessment of female gametes in fish. Vital dyes are defined as chemical compounds that bind to tissues or cells, regardless of whether they are alive or not (RODRIGUES *et al*., 2009). Comparing vital staining with other techniques such as flow cytometry or fluorescence microscopy, the use of vital dyes allows a quicker, more objective and less expensive cellular evaluation (OMNES *et al*., 1999).

Given the lack of oocyte viability tests for fish and other aquatic organisms, in conjunction with the results achieved in the evaluation of semen quality in mammals and the advantages of its use motivated the development of experiments such as those carried out by RAMIREZ *et al*. (1999) and OMNES *et al*. (1999), which assessed the viability of Pacific oyster (*Crassostrea gigas*) and turbot (*Psetta maxima*) oocytes using the vital dye trypan blue (TB). TB is a synthetic organic dye widely used for the assessment of cell membrane integrity (TRAN *et al*., 2011). TB exclusion test is based on the integrity of a cell membrane where an intact membrane will not allow penetration of the dye, whereas a cell with a damaged membrane will allow dye penetration (STROBER, 1997).

Fish farming in Northern Brazil is characterized by rearing of native species. Among them, *Colossoma macropomum* has shown a growing production over the last years, and currently is the most reared native species in the country, representing 24% of the 574 thousand t produced in 2015 (IBGE, 2016). Another native species that has gained attention from farmers is *Brycon amazonicus*, with a production of 11.4 thousand t (IBGE, 2016). The species has been considered a promising candidate for farming in the Amazon region (MONTOYA *et al*., 2017). Both species are migratory, total spawner fish (VAZZOLER, 1996) and reach sexual maturity between two and three years old. Despite the importance of both species for Brazilian fish farming, cases of failed spawning with low fertilization rates are usual (GUERREIRO *et al*., 2015) and most of the time the causes are unknown.

The applicability of a technique that enables analyzing oocyte quality and quantitatively predicting spawning viability would be useful not only in research labs but also in fish fry farms that address reproduction from an economic point of view. In this study, we exposed *C. macropomum* and *B. amazonicus* oocytes to different concentrations of TB seeking to verify a possible relationship between the results of staining tests and fertilization and hatching rates.

MATERIALS AND METHODS

The experiments were carried out in December 2015 and have been approved by the animal ethics committee of Nilton Lins University, under Protocol No. 017/2014. The animals used were from lots of broodfish raised in the Amazonas state (North Brazil), which were kept in ponds and fed a diet with 30% crude protein offered once a day, corresponding to 1% biomass.

For the experiments, three females of each of the following species were used: *C. macropomum* (8.0 ± 0.0 kg) and *B. amazonicus* (2.3 ± 0.16 kg). Similarly, three males of each species were selected: *C. macropomum* (5.58 ± 0.58 kg) and *B. amazonicus* (1.83 ± 0.16 kg). The criteria for the selection of the females were based on exterior characteristics such as distended abdomen and reddish genital papilla. The criteria used for the selection of the males were the release of semen after abdominal massage in the craniocaudal direction.

Reproduction was induced by hormonal treatment with carp pituitary extract (CPH), using doses of 0.5 and 5.0 mg CPH kg^{-1} for the females and 0.5 and 1.0 mg CPH kg^{-1} for the males. The interval between applications was 12 h for both sexes, being males and females kept separately in 3.2 m^3 indoor tanks supplied by an open flow system with water temperature at 28.30 ± 0.07 °C and 7.30 ± 0.45 mg L^{-1} of dissolved oxygen. Water parameters were monitored throughout experimental period by an YSI™ Professional Plus multiparameter.

The extrusion of oocytes for each female occurred 6 h after the second hormone application. During extrusion, the female was carefully secured on a padded workbench with the aid of wet towels, and the oocytes were extruded using a soft abdominal massage in the craniocaudal direction. The oocytes were collected in a clean and dry beaker and weighted. Females' *C. macropomum* yielded 607 ± 212 g of oocytes and females' *B. amazonicus* yielded 170 ± 18 g of oocytes. For semen collection, the males were secured as detailed above. To avoid contamination of the semen and consequent activation of motility, the ventral region was dried with paper towels, and the semen of each animal was collected with a disposable 10 mL syringe.

Trypan blue membrane integrity tests

In order to determine the TB concentrations to be assessed we first carried out a pilot test using *C. macropomum* oocytes. The starting concentration was 0.2%, which has already been standardized (ZAMPOLLA *et al.*, 2008) for zebrafish oocytes, followed by a lowering to 0.1, 0.05 and 0.025% and two exposure times: 1 and 5 min. Five min was considered a long exposure time as all oocytes were stained at any concentration tested. When exposed for 1 min, it was possible to see some oocytes with unstained membrane at concentrations ≤0.05%. As a result, five concentrations of TB were evaluated in this study: 0.05%; 0.04%; 0.03%; 0.02% and 0.01%. The test solutions were prepared from diluting 0.4% TB stock solution (Sigma Aldrich™) with distilled water.

The procedure consisted of adding 1 ml of TB test solution to each well of a 6-well culture plate, considering each well as an experimental unit. Then, with the aid of a Pasteur pipette, an aliquot (± 40 oocytes) was immersed in the test solution, and the timer was started to ensure the exact TB exposure time. After 40 sec, the sample was rinsed to remove the TB solution and then was washed with distilled water; this procedure was repeated three times so that the oocytes would no longer be in contact with the staining solution when the 1-min period was over. Unstained oocytes were considered viable (intact membrane), whereas those partially or completely stained blue were considered unviable (damaged membrane). The assessments were conducted using a Zeiss stereomicroscope (model Stemi 2000).

The viability of the oocytes in terms of membrane integrity after staining with TB was calculated according to the following formula:

$$Membrane\ integrity\ (\%) = \frac{number\ of\ unstained\ oocytes}{total\ number\ of\ oocytes}\ X\ 100.$$

Semen quality assessment

The semen samples were evaluated to identify any possible contamination by substances that could cause the activation of the sperm (water, urine, or blood). An aliquot of semen from each male was evaluated using an optical microscope

immediately after collection, and the samples that showed motile sperm were discarded. The samples classified by this test were then subjected to motility activation and sperm concentration tests.

- Sperm motility: subjective estimate of the percentage of motile cells in the optical field. An aliquot of semen was placed on a slide and then activated with a 0.29% NaCl solution at a ratio of 1:5 (semen:activator) (VIVEIROS *et al*., 2009). The assessment was conducted under an optical microscope with 40X objective, and only those samples with motility equal to or greater than 80% were selected for the experiment.
- Sperm concentration: the methodology was conducted based on methods described by STREIT JR. *et al*. (2003), in which each semen sample was diluted in a buffered formaldehyde saline solution at a ratio of 1:1000 (semen:solution). An aliquot of the diluted semen was placed in a Neubauer chamber for sperm count. The sperm concentration was determined using the following equation:

$$\text{CSP (SP/mL)} = \left(\frac{\sum SP}{10\ s.c.}\right) \times \left(\frac{25\ s.t. \times dilution \times 1000}{chamber\ depth\ (mm)}\right);$$

where SP = sperm; $\sum SP$ = total number of sperm counted; s.c. = number of squares counted; s.t. = total number of squares; chamber depth = 0.10 mm; and dilution = dilution factor of semen by fixative.

Egg fertilization and incubation

Among the oocytes extruded from each *C. macropomum* and *B. amazonicus* female, three samples were intended for fertilization and incubation. Fertilization was performed with a pool of semen from the selected broodfish in order to avoid the effect of the male (semen quality). For *C. macropomum,* the ratio of sperm to oocyte (insemination dose) was 100,000:1 (sperm:oocyte), which was based on LEITE *et al*. (2013). For *B. amazonicus*, the ratio used was of 65,000:1 (sperm:oocyte), which was based on the findings by OLIVEIRA (2015). The eggs were incubated in 60-liter conical incubators with continuous water flow. The incubators used for *C. macropomum* received a density of 1.5 g of eggs L^{-1}, whereas those used for *B. amazonicus* received 1.3 g L^{-1}. Dissolved oxygen concentration in the water during incubation was kept at 5.08 ± 0.30 mg L^{-1} and water temperature kept at 31.60 ± 0.09 °C for *C. macropomum* eggs and at 29.20 ± 0.20 °C for *B. amazonicus* eggs.

- Fertilization rate: was evaluated at the time of blastopore closing, 6 h after fertilization, using five egg samples (± 100 each) from each incubator;
- Hatching rate: determined 12 h after fertilization by evaluating five samples (± 100 each) from each incubator.

The rates were determined according to equations (FORNARI *et al*., 2014):

$$Fertilization\ rate\ (\%) = \frac{number\ of\ viable\ eggs}{total\ number\ of\ eggs\ counted}\ X\ 100;$$

$$Hatching\ rate\ (\%) = \frac{number\ of\ viable\ hatched\ larvae}{total\ number\ of\ the\ sample}\ X\ 100.$$

Experimental design

The data were subjected to linear regression analyses at a significance level of 5% to assess the relationship between the results of the viability tests using the vital dye and the fertilization and hatching rates, considering the membrane integrity percentage as the dependent variable (y) and the fertilization and hatching rates as the explanatory variables (x). The analyses were conducted with STATISTICA® 10 software. The following assumptions inherent to the linear regression model were tested: linear relationship between the variables X and Y, normal distribution between the variables, homoscedasticity between X and Y, and normal distribution among errors. The residues were analyzed by normality tests, homoscedasticity tests of Breusch-Pagan and Goldfeld-Quandt, independence of the residues by the Durbin-Watson test, and verification of the model's linearity was performed by the lack-of-fit test (MONTGOMERY *et al.*, 2012). The tests of formal analysis of residues were carried out using the software Action Stat version 3.1.

RESULTS

Trypan blue membrane integrity test

There was a distinct difference between species for the different concentrations of TB tested (Figure 1).

In *C. macropomum,* the viability of the oocytes decreased as the concentration of TB increased. In contrast, the *B. amazonicus* oocytes presented high viability regardless of the dye concentration tested (Figure 1).

For both species, was observed that the dye penetrated the zona radiata (ZR) of all the samples. However, for the intact gametes, the dye was not able to cross the oolemma membrane, which was not seen in the damaged oocytes, whose interior was partially or fully stained (Figure 2).

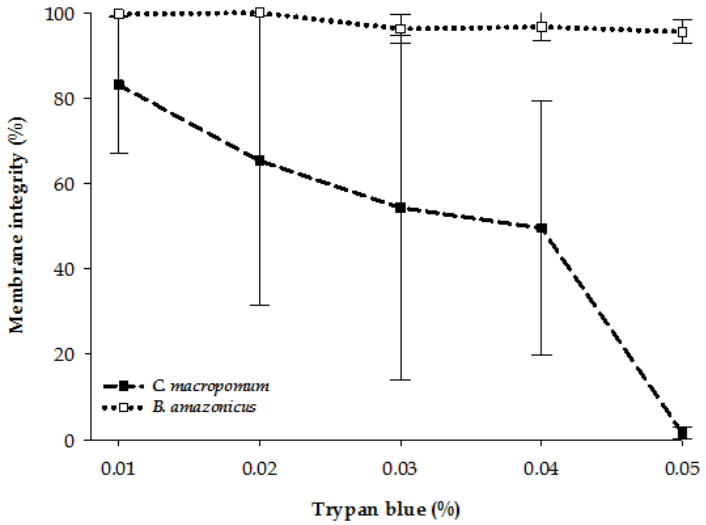

Figure 1. Membrane integrity of *Colossoma macropomum* and *Brycon amazonicus* oocytes after exposure to different concentrations of trypan blue (TB) dye.

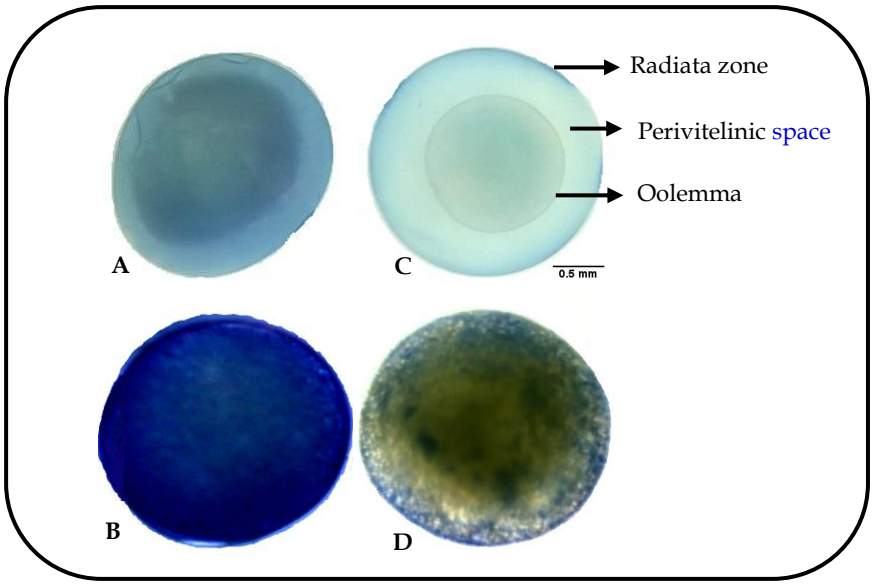

Figure 2. Oocytes exposed to 0.03% trypan blue (TB) dye. (A) Stained *Brycon amazonicus* oocyte; (B) Stained *Colossoma macropomum* oocyte; (C) Unstained *Brycon amazonicus* oocyte; and (D) Unstained *Colossoma macropomum* oocyte.

Semen quality assessment

The semen pools for *C. macropomum* and *B. amazonicus* presented excellent quality in terms of motility (97.50 ± 3.54 and 98.51 ± 3.38%, respectively) and sperm concentration (8.95 ± 3.92 and 10.7 ± 6.76 x 10^9 mL^{-1}, respectively).

Fertilization and hatching rates

The spawning of females' *C. macropomum* was characterized by fertilization and hatching rates of 61.22 ± 37.98% and 61.95 ± 43.74%, respectively. The females' *B. amazonicus* presented higher fertilization (83.14 ± 10.24%) and hatching (75.17 ± 11.49%) rates.

Regression analysis

Table 1 shows the degree of correlation between the spawning variables and oocyte membrane integrity tested for the two species as assessed using the adjusted coefficient of determination (R^2 adjusted).

The highest concentrations of TB presented negative adjusted R^2 values for the two independent variables, whereas the 0.03% concentration showed the strongest relationship between the treatments tested (Table 1).

Table 1. Adjusted coefficient of determination (R^2) from the linear regression analyses between the spawning variables and trypan blue (TB) concentrations.

TB Concentration (‰)	Adjusted R^2 (Fertilization)	Adjusted R^2 (Hatching)
Colossoma macropomum		
0.05	-0.02	-0.08
0.04	-0.11	-0.07
0.03	0.30	0.23
0.02	0.21	0.17
0.01	0.17	0.16
Brycon amazonicus		
0.05	-0.02	-0.01
0.04	-0.01	0.03
0.03	0.20	0.11
0.02	-	-
0.01	0.01	-0.02

The R^2 values of the tests with *B. amazonicus* showed a lower relationship between the variables subjected to the regression analysis than did the results obtained in *C. macropomum.* We did not conduct regression analyses for the fertilization and hatching rates for the 0.02% treatment in *B. amazonicus* because the data for all females had no variance in this treatment, with a standard deviation equal to zero.

DISCUSSION

We observed greater selectivity of oolemma compared to ZR regarding the permeability of the dye. FINN and CERDÀ (2011) claimed that teleosts in general have a wide variety of aquaporins, which permeate water and other solutes and are distributed according to their role in the different tissues. The passage of the TB through the ZR of *C. macropomum* and *B. amazonicus* oocytes shows an apparent permeability of aquaporins for the dye molecule, despite its large molecular weight.

OMNES *et al*. (1999) assessed the use of TB for evaluating the quality of *P. maxima* oocytes and, using scanning electron microscopy, observed the presence of regular pores on the surface of the ZR that allowed for the passage of TB. The oocytes surface in *C. macropomum* and *B. amazonicus* is characterized by a smooth zona radiata with visible pore-canals (RIZZO *et al.*, 2002). The

diameter and number of the pore-canals increase towards the micropyle at the animal pole, whereas at the vegetative pole the pore-canals show a similar diameter and are uniformly distributed. This pattern of oocyte surface seems to be present in majority of the species within Characidae family (RIZZO *et al.,* 2002; ROMAGOSA *et al*., 2002; ALEXANDRE *et al*., 2010; ISAÚ *et al.,* 2013; HONORATO-SAMPAIO *et al.,* 2015) and the no selectivity of ZR pore-canals to TB molecules may be one of the factors for ineffective results of the staining tests.

The oolemma in the two species studied was more resistant to the passage of water and the action of the dye. This difference in permeability to the dye between the ZR and oolemma indicates greater selectivity to the passage of solutes between the two membranes, with the lower permeability of the oolemma to TB being a potential differentiating factor between an oocyte considered viable (unstained) and one considered unviable (stained). When comparing the results for *C. macropomum,* where the integrity of the gametes was inversely proportional to the concentration of TB tested, to the results for *B. amazonicus,* which showed high membrane integrity for all TB concentrations, it was clear that the *C. macropomum* oocytes were more sensitive to the dye.

MELO *et al*. (2011) described that in most freshwater fish species, as was also observed in marine species by KUNZ (2004), pelagic oocytes usually have a thinner ZR when compared to demersal oocytes, with the thickness of the ZR being proportional to the adverse conditions of the environment where the oocytes are released. Such differences were observed (MELO *et al*., 2011) when studying the relationship between the morphology and reproductive strategies of six neotropical Siluriformes, where pelagic spawning species present oocytes with a thin ZR compared to demersal spawning species whose oocytes have a thick ZR.

The weak relationship between the results of the membrane integrity tests and the fertilization and hatching rates may be a result of the effect of external and internal factors on reproductive management. OMNES *et al*. (1999) analyzed the TB membrane integrity test results in *P. maxima* oocytes and showed that the environmental conditions during embryonic development could influence the success of the evaluation. When evaluating the statistical correlation between the TB integrity tests and the success of hatching in *C. gigas,* RAMIREZ *et al*. (1999) proposed that membrane integrity is not the main cause of development failure; however, it is a significant component of it. The interval between the membrane integrity test and the quantification of the fertilization and hatching rates subjects the viability of eggs to other factors related to incubation and embryonic development that can hinder the comparison and/or correlation of these characteristics.

The gametes represent a biological and financial cost that has not been taken into consideration in fish farming. The improvement of reproductive indexes lies not only on good management practices of the broodstock, but also in developing refined techniques to allow assessment of gamete quality and higher control of the artificial reproduction, seeking the establishment of a professional and competitive fish farming business.

In this study, we observed a weak correlation between the membrane integrity of oocytes and the fertilization and hatching rates. The trypan blue staining test is not efficient in predicting the spawning viability in *C. macropomum* and *B. amazonicus* and cannot be used to this aim.

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