

## ADHESIVENESS NEUTRALIZATION IN EGGS OF *Pseudoplatystoma fasciatum* (TELEOSTEI: SILURIFORMES: PIMELODIDAE)

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### ABSTRACT

The aim of this study was to remove the adhesiveness of *Pseudoplatystoma fasciatum* eggs using solutions of urea for 10, 30, 60 and 90 min (treatments T0-T3) or, in urea for 10 min and washed in tannin (T4). In the control group, eggs were kept in water. After two experiments, we observed that eggs of control group presented the best embryo viability rates, even maintaining egg adhesiveness, being better than the other treatments. The T4, had the worst embryo viability rates. We observed that embryos of the T4 treatment presented reduced growth and formed a separate group when analyzing morphological parameters (egg diameter, total egg area, embryo area and yolk sac area) by multivariate analysis. Concluding, the control group, free of chemicals, provided the best results and is considered the best alternative for the immediate conservation and aquaculture production of *P. fasciatum*.

**Keywords:** induced spawning; sticky eggs; urea; tannin

## NEUTRALIZAÇÃO DA ADESIVIDADE EM OVOS DE *Pseudoplatystoma fasciatum* (TELEOSTEI: SILURIFORMES: PIMELODIDAE)

### RESUMO

O objetivo deste estudo foi neutralizar a adesividade de ovos de *Pseudoplatystoma fasciatum* a partir da utilização de soluções de ureia por 10, 30, 60 ou 90 min (tratamentos T0-T3) ou em ureia por 10 min e lavados em tanino (T4). No controle, os ovos foram mantidos apenas em água. Após dois experimentos realizados, no grupo controle foram observadas as melhores taxas de viabilidade embrionária, mesmo mantendo a adesividade dos ovos, sendo melhor que os demais tratamentos. No T4 foram verificadas as piores taxas de viabilidade embrionária. Foi observado que os embriões do tratamento T4 apresentaram crescimento reduzido e formaram um grupo separado após análise dos parâmetros morfológicos (diâmetro do ovo, área total do ovo, área do embrião e área do saco vitelínico) por análise multivariada. Concluiu-se que o grupo controle, livre de produtos químicos, proporcionou os melhores resultados, sendo considerado a melhor alternativa para a conservação imediata e produção aquícola de *P. fasciatum*.

**Palavras-chave:** desova induzida; ovos adesivos; ureia; tanino

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## INTRODUCTION

The *Pseudoplatystoma fasciatum*, *Pseudoplatystoma corruscans* and their hybrids are large Pimelodidae catfishes endemic of the Neotropical region referred as "sorubim" that have excellent flavor, low fat content meat and are among of the most expensive and highly appreciated freshwater fish throughout several of South American countries (HONORATO *et al.*, 2014). The aquaculture production of sorubim has increased by 30.1% in just one year, from 15.71 million kg in 2013 to 20.43 million kg in 2014 (IBGE, 2015).

Despite all their zootechnical qualities, the production of these species presents an obstacle, which is the fry production difficulty due to the high mortality of eggs in incubators. In this concern, it is known that many factors can affect egg and gamete quality, including the reproductive management provided to the breeders throughout the year and also by genetic factors recently explored by transcriptome and proteomic techniques (BOBE and LABBÉ, 2010; ALIX *et al.*, 2015). It is important to know the optimal conditions that allow an adequate embryonic development, because factors, such as time and husbandry practices for spawning induction, gamete handling post striping, egg post ovulatory aging, the range of water physiological temperatures, salinity, oxygen concentrations are known to affect the embryonic development and fish egg quality (WU, 2009; BOBE and LABBÉ, 2010; MYLONAS *et al.*, 2010). For species with adhesive eggs, it is known that adhesiveness must be neutralized to prevent the formation of clusters of eggs that results in high mortality rates caused by suffocation and fungal growth (DOROSHOV *et al.*, 1983). For this purpose the application of an urea solution and then a tannin solution is the most common protocol (KOWTAL *et al.*, 1986; RIBEIRO, 2001; BOUCHARD and ALOISI, 2002; KUJAWA *et al.*, 2010). The adhesiveness neutralization may be obtained using only tannin solution (ZARSKI *et al.*, 2015) or by using specific enzymes (GELA *et al.*, 2003; LINHART *et al.*, 2003a, b; LINHART *et al.*, 2004; CARRAL *et al.*, 2006). However, the combination of urea and tannin is practiced because it seems to prolong the effect of removing the adhesiveness (KOWTAL *et al.*, 1986). The combination of substances and the time of exposure need to be that resulting in

best adhesiveness removal and highest fertility and hatching success. Protocols have been standardized for some fish species during the last decades, in which the exposure to urea (with different concentrations) varies from 15 to 60 min, while the tannin (also in different concentrations) exposure ranges from fast baths up to 5 minutes (SIDDIQUE *et al.*, 2016).

On the other hand, the use of chemical protocols for neutralizing adhesiveness has been reported, in some cases, to impair the embryonic development and reduce fertility and hatching rates (LINHART *et al.*, 2000; DEMSKA-ZAKÉZ *et al.*, 2005; FELEDI *et al.*, 2011). The use of different substances (concentrations and time of exposure) has to be specifically determined for species with adhesive eggs, especially *P. fasciatum* or any species that needs adhesiveness removal so the eggs may be used in hatcheries.

However, mainly because the vast majority of tropical fish of commercial importance are egg scatterers that do not have adhesive eggs, there is no information about these aspects in these species. Concerning *P. fasciatum*, because of the absence of efficient protocols to neutralize the egg adhesiveness, as well as other factors, interspecific breeding is often accomplished using oocytes of *P. corruscans* (which are not adhesive) (RIZZO *et al.*, 2002; RIZZO and GODINHO, 2003) that are fertilized with sperm of *P. fasciatum*. This crossbreeding results in the production of the hybrid fish "pintachara" (PORTO-FORESTI *et al.*, 2008; PRADO *et al.*, 2011) and eliminates the problem of egg adhesiveness. On the other hand, the production of "pintachara" contributes to a problem that has attracted increasing attention: the introduction of fertile hybrids in the wild because of escapes in fish farms. Specifically for the case of hybrids from the Pimelodidae, the major risk is the fertility of these hybrids, which lacks a reliable morphological identification, and may cause atypical crosses with wild fish and generate genetic contamination (HASHIMOTO *et al.*, 2015). Interspecific hybrids of Neotropical fishes are produced for several reasons. In some cases, hybrids can be produced because of the lack of technology for pure species (HASHIMOTO *et al.*, 2015), such as the case of the absence of a protocol for neutralize egg adhesiveness in *P. fasciatum*.

Thus, the aim of this study was to evaluate different protocols (using chemical treatments) for neutralizing the adhesiveness of *P. fasciatum* eggs, to find the most appropriate treatment which should combine both adhesiveness neutralization and promote adequate reproductive rates and thereby contributing to the development of a methodology for the reproduction of this species in captivity for conservation and aquaculture purposes.

## MATERIAL AND METHODS

### *Maintenance and Animal Care*

This study consisted of two experiments conducted separately over two distinct breeding seasons in the Centro de Aquicultura da Universidade Estadual Paulista - UNESP (CAUNESP), Jaboticabal, SP, Brazil (21°15'17"S, 48°19'20"W). The genetic identity of the *P. fasciatum* used was confirmed through this analysis, performed at the Fish Genetics Laboratory of Unesp, campus of Bauru, SP, Brazil. The animals of the breeding stock of CAUNESP were microchipped (ANIMALTAG - Korth RFID Ltda., São Carlos, SP) to ensure the identification of each animal and traceability of fingerlings from the breeding. From each animal a caudal fin fragment was collected and stored in 100% alcohol for molecular analysis. DNA extraction was conducted using the Genomic DNA Purification Kit according to the manufacturer's protocol (Promega). The amount of DNA was assessed with a molecular marker pattern (Invitrogen Low DNA Mass Ladder) by 1% agarose gel electrophoresis. Samples were identified using the Polymerase Chain Reaction (PCR) of regions of the nuclear genes RAG2, Globin, EF1, 18S and sequences of mitochondrial gene 16S, and the Prt microsatellite marker. Data analysis was performed by comparing the bands after PCR using standard samples of the two species analyzed (*P. fasciatum* and *P. corruscans*) previously identified.

Fish were kept in earthen ponds (10 x 20 x 1.5 m) and fed 6 d/week in two portions, equivalent to 5.0% of the total weight in the warm months (September-February) and 1.0% in the cold months (March-August), at 09:00 h and 17:00 h with commercial extruded feed (moisture content

[max.] 8.0%; crude protein [min.] 32.0%; ethereal extract [min.] 6.5%; fibrous matter [max.] 7.0%; ash [max.] 10.0%; calcium [max.] 1.2%; phosphorus [min.] 0.6%; according to the manufacturer). Water parameters measured using a YSI model 55 oximeter and a YSI model 63 multiparameter probe (Yellow Spring Instruments, Yellow Springs, OH, USA) indicated average dissolved oxygen levels of  $3.70 \pm 1.12$  mg L<sup>-1</sup>, temperature of  $23.35 \pm 3.98$  °C, pH of  $7.38 \pm 0.24$ , conductivity of  $84.68 \pm 11.18$  µS cm<sup>-1</sup> and transparency of 0.60 m (measured at 09:00 h using a Secchi disk).

Individuals suited for induced spawning were kept in plastic tanks (1000 L) with constant water circulation, artificial aeration and an average water temperature of  $26.1 \pm 0.4$  °C. In both experiments, males received a single dose of carp pituitary extract (CPE) (2.0 mg kg<sup>-1</sup>), and females received two doses (0.5 and 5.0 mg kg<sup>-1</sup>, with a 12 h interval between doses). Females were selected by external characteristics (swollen abdomen) and males were chosen according to the color and fluidity of the milt extruded after gentle pressure on the abdomen (LEONARDO *et al.*, 2004). Three females and four males were used in both experiments. At the time of ovulation, females were removed from the tank, and the location near the genital papilla was dried with paper towels for dry strip spawning. Just prior to fertilization, a 150 mL oocyte pool was made using 50 mL of oocytes of each female (all three females ovulated in both experiments). Semen was collected from the males ten minutes before fertilization and stored separately in plastic falcon tubes inside the refrigerator at 4.0 °C. Just before fertilization, a 4 mL semen pool was made using 1.0 mL of semen of each male (all four males spermated in both experiments). The insemination dose used for all treatments was  $2.21 \times 10^5$  spz oocyte<sup>-1</sup>, which was based on insemination doses successfully used for other reophilic species in captivity (BOMBARDELLI *et al.*, 2006; SHIMODA *et al.*, 2007; DE SOUZA *et al.*, 2015). In both experiments, the pooled semen was mixed with the pooled oocytes and after that, the gametes were activated according to each treatment, using either the urea solution or hatchery water. Neither of the pooled samples contained urine or fecal contamination.

### Treatments

The treatments were applied during the process of activation of gametes, fertilization and ova hydration (AFH). The solutions used were based on the protocol for carp (*Cyprinus carpio*) described by RIBEIRO (2001), which consists of a modified "Woynarovich solution" (WOYNAROVICH and WOYNAROVICH, 1980) or "urea solution", composed of 40 g of sodium chloride and 30 g of urea (Synth, Diadema, Brazil) dissolved in 10 L of hatchery water (4 g L<sup>-1</sup> or 0.4% of NaCl and 3g L<sup>-1</sup> or 0.3% of urea); and a tannin solution, composed of 5 g of powder tannin or tannic acid (Tanac, Montenegro, Brazil) also dissolved in 10 L of hatchery water (0.5 g L<sup>-1</sup> or

0.05% of tannin). The solutions were prepared 30 min prior to use.

### Experiment 1

The pooled samples of oocytes and semen were divided and subjected to five distinct conditions for AFH in individual plastic bowls, representing four treatments and a control group (Table 1). After applying the treatments, 4 mL of eggs (245 ± 17 eggs in 1 mL of eggs) were placed into each incubator (cone-shaped, with a volume of 2.5 L and constant water flow of 0.5 L min<sup>-1</sup>), with 3 replicates per treatment. The urea solution in the T1, T2 and T3 was slowly renewed at 30 min intervals.

**Table 1.** Control and treatments used in this study for adhesiveness neutralization in eggs of *Pseudoplatystoma fasciatum*. In experiment 2, the oocytes were fertilized in the same manner as in experiment 1.

Treatment identification	Treatment description	
	Experiment 1	Experiment 2
C	AFH* with hatchery water (10 min.)	AFH with hatchery water (10 min.)
T1	AFH with urea solution (30 min.)	AFH with urea solution (30 min.)
T2	AFH with urea solution (60 min.)	AFH with urea solution (60 min.)
T3	AFH with urea solution (90 min.)	AFH with urea solution (90 min.)
T4	AFH with urea solution (10 min.) + washing with tannin solution (15 sec.)	AFH with urea solution (10 min.) + washing with tannin solution (15 sec.)
T0		AFH with urea solution (10 min.)

\*AFH stands for the process of activation, fertilization and hydration of the gametes and eggs.

### Experiment 2

In the Experiment 2, we repeated the same treatments and introduced a new one (T0), in which the urea solution was used during fertilization and hydration for 10 min (instead of water as in C) (Table 1).

### Sampling and reproductive rates

At 5 h and at 15 h (gastrula and almost fully formed embryo respectively) post-fertilization (HPF), 100 eggs from each replicate (300 eggs per treatment) were collected and analyzed under a stereoscopic microscope M50 (Leica Microsystems, Wetzlar, Germany), where viable eggs (with embryonic development) were counted to determine the fertilization (FR) and hatching rates (HR) respectively. Then, the samples were fixed in 2.5% glutaraldehyde in 0.05 M phosphate buffer (pH

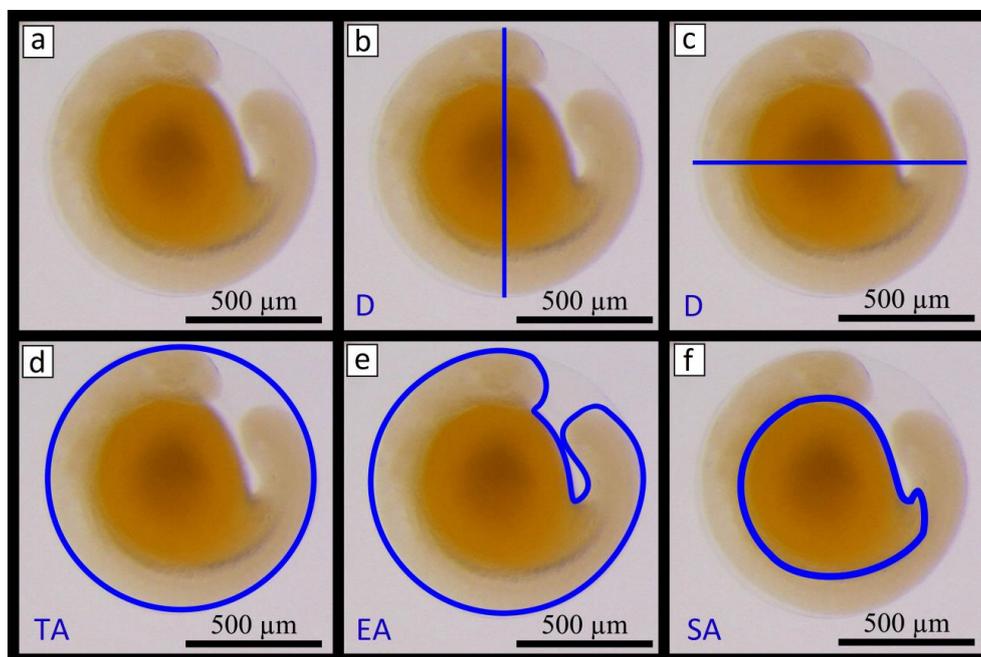
7.2) and refrigerated. The FR and HR were calculated using the following formula: (viable eggs/total eggs) × 100.

### Morphometric analysis

In both experiments, 15 HPF embryonated eggs (previously fixed), randomly selected per treatment, were photographed using a stereoscopic microscope Leica M50 with IC80 HD stereoscopic microscope capture system (Leica Microsystems, Wetzlar, Germany). Analyses were conducted with Leica application suite software 4.3 (Leica Microsystems Limited, Copyright 2003-2013). The average diameter (D), total area of the egg (TA), area of the embryo (EA) and area of the yolk sac (SA) were measured using the freeware program Image J (v 1.46r, Wayne Rasband, National Institutes of Health, USA) (Figure 1). To determine

D, the average horizontal and vertical diameters were considered for determining an average (Figures 1b, c). The areas were automatically calculated by the program (Figures 1d-f). All

measurements were taken in triplicate, and the mean values were used in the analysis. In order to assess the proportion in morphometric traits, three indexes were defined: EA/SA, EA/TA and SA/TA.



**Figure 1.** Step by step morphometric analysis of a *Pseudoplatystoma fasciatum* egg. (a) Contrast adjustment; (b) vertical and (c) horizontal diameter (used to calculate D); (d) total area of the egg (TA); (e) area of the embryo (EA); and (f) area of the yolk sac (SA).

#### *Adhesiveness removal analysis*

The adhesiveness removal was evaluated by visual estimation of the eggs and the arrangement in each treatment (adhered and not adhered eggs). This subjective visual method was used to avoid higher mortality rates by premature manipulation. The results were expressed by the percentage of eggs that remained adhered to the incubator or to other eggs, and the percentage of eggs that were not adhered.

#### *Sperm analysis*

At the time of extrusion of the sperm, part of it was separated to determine the time of sperm motility, membrane integrity and sperm concentration. Only samples that were free of contaminants such as feces and urine were used. In each experiment, freshly stripped sperm was collected and stored at 4.0 °C until the sperm of all males were stripped. The sperm of each male were analyzed separately but the results presented as

mean  $\pm$  standard deviation and the sperm used to fertilize the eggs was a pool from all males used.

#### *Time of motility*

In both experiments the spermatozoa activation was tested using deionized water as a control, and urea solution to test the possibility that the activation solution would interfere in the gamete quality. Observations of sperm motility were conducted at room temperature ( $23.0 \pm 2.0^\circ\text{C}$ ) using three replicates per sample, soon after milt collection, with the aid of a light microscope under 40 times magnification. The duration of sperm motility was subjectively evaluated as the time elapsed from activation until 50.0% of the spermatozoa maintained forward swimming activity using a stopwatch. For this assessment, 1.0  $\mu\text{L}$  of semen from each breeder was applied to a glass slide, proceeded activation using 20.0  $\mu\text{L}$  of deionized water and 20.0  $\mu\text{L}$  of urea solution (in different glass slides), and the stopwatch was

concomitantly started while a cover slip was placed over the solution, similar to the methodology used by DE SOUZA *et al.* (2015) and KURADOMI *et al.* (2016).

#### Membrane integrity

The percentage of live spermatozoa (sperm survival) was evaluated for each male based on the integrity of the membrane determined according to the penetration capabilities of eosin-nigrosin dyes (5.0% eosin and 10.0% nigrosin) in live cells (unstained) and dead cells (pink stained). A total of 200 cells per slide were counted on a microscope under 100 times magnification. The percentage of live cells was calculated as the number of live spermatozoa divided by the number of total cells evaluated multiplied by 100.

#### Concentration

The sperm concentration was estimated according to standard methods (sperm cells mL<sup>-1</sup> milt) using a counting chamber similar to that described by BUYUKHATIPOGL and HOLTZ (1984). For this purpose, a sample of sperm was fixed in buffered formaldehyde saline solution (1:1000).

#### Statistical analysis

The experimental design was completely randomized, with five treatments, three replicates in experiment 1 and six treatments with three replicates in experiment 2. The normality and homoscedasticity of all the variables were confirmed for all variables. For the FR and HR, the data were subjected to arcsin transformation, and one-way ANOVA test, and if found significant, we applied the Fisher least significant difference test. For the morphometric measurements, the one-way ANOVA was also used, and if found significant, we applied the Tukey honestly significant difference test. Principal components analysis (PCA) was performed using morphometric indexes (EA/SA, EA/TA and SA/TA) from all four five groups (C, T1, T2, T3 and T4) of experiment 1. All the results are represented as mean  $\pm$  standard error (SE) and were considered as significantly different when  $p < 0.05$ . All statistical analysis was performed using the software Statistica 7.0 (StatSoft Inc., Tulsa, OK, USA).

## RESULTS

### Adhesiveness

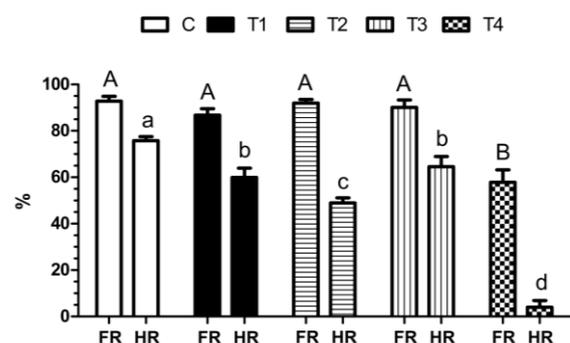
In C, approximately 90.0% of the eggs remained adhered to the walls of the incubator and to other eggs, forming "clumps" or clusters of eggs. In T1, T2, and T3 eggs also adhered to the walls and other eggs but at a lower frequency (approximately 60.0%) than those of C. Eggs of T4 were fully non-adherent and freely dispersed in the incubators. In experiment 2 the arrangement of the eggs in the incubators was similar to that in the respective treatments in experiment 1, and T0 was similar to T1, T2 and T3.

### Reproductive rates

#### Experiment 1

Treatments using only the urea solution (T1, T2 and T3) showed a mean FR ( $86.80 \pm 2.72\%$ ,  $91.97 \pm 1.54\%$  and  $90.17 \pm 3.16\%$ , respectively) similar to that of C ( $92.77 \pm 2.14\%$ ). The mean FR ( $57.90 \pm 5.32\%$ ) of T4 (in which the solution of tannin was used) was lower than that of C (Figure 2).

The T1, T2 and T3 mean HR values ( $59.93 \pm 3.99\%$ ,  $48.97 \pm 2.13\%$  and  $64.53 \pm 4.38\%$ , respectively) were lower than that of C ( $75.80 \pm 1.70\%$ ), and the HR of T2 was lower than the those of T1 and T3. The lowest mean HR was observed in T4 ( $4.03 \pm 2.83\%$ ) (Figure 2).



**Figure 2.** Means  $\pm$  SE of fertilization (FR) and hatching (HR) rates of *Pseudoplatystoma fasciatum* eggs submitted to adhesiveness neutralization in experiment 1. Capital letters indicate significant differences in FR among treatments ( $p < 0.05$ ), and lowercase letters indicate significant differences in HR among treatments ( $p < 0.05$ ).

## Experiment 2

In experiment 1, the use of the urea solution seemed to delay the onset of egg adhesiveness, thereby facilitating egg handling and dispersion. Thus, in experiment 2 we repeated the same treatments and introduced a new one (T0), in which the urea solution was used during fertilization and hydration for 10 min (instead of water as in C). The mean FR values of C and T4 were the highest ( $52.65 \pm 7.19\%$ ) and the lowest ( $3.83 \pm 1.92\%$ ), respectively. The mean FR values of T1, T2, T3 and T0 were similar ( $17.47 \pm 1.06\%$ ,  $19.17 \pm 3.89\%$ ,  $22.20 \pm 2.03\%$  and  $22.30 \pm 5.23\%$ , respectively) (Figure 3).

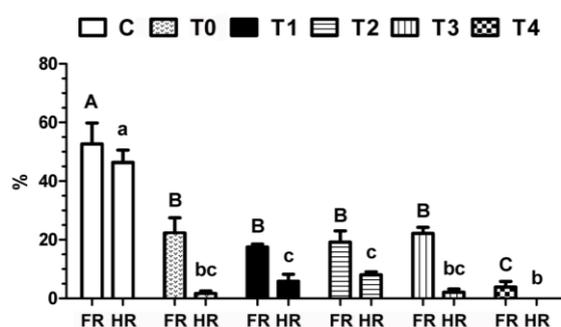


Figure 3. Means  $\pm$  SE of fertilization (FR) and hatching (HR) rates of *Pseudoplatystoma fasciatum*

eggs submitted to adhesiveness neutralization in experiment 2. Capital letters indicate significant differences in FR among treatments ( $p < 0.05$ ), and lowercase letters indicate significant differences in HR among treatments ( $p < 0.05$ ).

Group C showed the highest mean HR ( $46.40 \pm 4.20\%$ ). Among T0, T1, T2 and T3 mean HR values were similar ( $1.67 \pm 0.84\%$ ,  $5.83 \pm 2.40\%$ ,  $8.03 \pm 1.03\%$  and  $2.13 \pm 1.03\%$  respectively). The HR of T4 was 0.00% (Figure 3).

## Morphometric analysis

In both experiments the mean values of D and TA of group T4 were always lower than all other groups (Table 2). The mean values of D of group C was lower than T3 (in experiment 1), but higher than T0 and T2 (in experiment 2). The EA of T4 was lower than all other groups in experiment 1, but could not be determined (because of 0.00% HR) in experiment 2. SA values of T4 were lower than all other groups in experiment 1, but could not be determined (because of 0.00% HR) in experiment 2. In both experiments, except for T4, EA were similar among groups, except for slightly reduced values of T2 in comparison to C in experiment 1 (Table 2).

Table 2. Means ( $\pm$  SE) of the morphometry traits from eggs of *Pseudoplatystoma fasciatum*.

Treatment	D ( $\mu\text{m}$ )	TA ( $\mu\text{m}^2$ )	EA ( $\mu\text{m}^2$ )	SA ( $\mu\text{m}^2$ )
C	1229.46 $\pm$ 5.67 <sup>a</sup>	1098.83 $\pm$ 102.70 <sup>a</sup>	1007.14 $\pm$ 87.58 <sup>a</sup>	695.86 $\pm$ 83.31 <sup>a</sup>
T1	1245.94 $\pm$ 16.25 <sup>ab</sup>	1123.97 $\pm$ 196.69 <sup>a</sup>	982.07 $\pm$ 144.88 <sup>ab</sup>	696.68 $\pm$ 113.98 <sup>a</sup>
T2	1214.69 $\pm$ 10.08 <sup>a</sup>	1090.69 $\pm$ 133.79 <sup>a</sup>	977.70 $\pm$ 136.29 <sup>b</sup>	686.84 $\pm$ 107.51 <sup>a</sup>
T3	1262.91 $\pm$ 11.26 <sup>b</sup>	1121.25 $\pm$ 183.14 <sup>a</sup>	1001.62 $\pm$ 149.17 <sup>ab</sup>	693.33 $\pm$ 104.42 <sup>a</sup>
T4	1053.81 $\pm$ 11.69 <sup>c</sup>	951.49 $\pm$ 143.14 <sup>b</sup>	883.43 $\pm$ 137.67 <sup>c</sup>	633.07 $\pm$ 101.24 <sup>b</sup>
Experiment 2				
C	1216.59 $\pm$ 12.64 <sup>a</sup>	1108.02 $\pm$ 145.97 <sup>a</sup>	1003.12 $\pm$ 141.11 <sup>a</sup>	689.15 $\pm$ 99.23 <sup>ab</sup>
T0	1137.89 $\pm$ 21.01 <sup>b</sup>	1051.03 $\pm$ 189.86 <sup>b</sup>	968.52 $\pm$ 152.80 <sup>a</sup>	695.68 $\pm$ 141.24 <sup>ab</sup>
T1	1182.62 $\pm$ 8.32 <sup>ab</sup>	1070.73 $\pm$ 123.68 <sup>ab</sup>	987.52 $\pm$ 124.05 <sup>a</sup>	716.15 $\pm$ 100.92 <sup>ab</sup>
T2	1159.65 $\pm$ 15.79 <sup>bc</sup>	1051.96 $\pm$ 161.35 <sup>b</sup>	966.10 $\pm$ 152.13 <sup>a</sup>	683.52 $\pm$ 131.28 <sup>a</sup>
T3	1209.28 $\pm$ 13.37 <sup>ac</sup>	1094.96 $\pm$ 131.47 <sup>ab</sup>	966.41 $\pm$ 143.39 <sup>a</sup>	714.86 $\pm$ 117.65 <sup>b</sup>
T4	996.98 $\pm$ 7.56 <sup>d</sup>	890.25 $\pm$ 103.78 <sup>c</sup>	-	-

\*D stands for diameter of the egg; TA – total area of the egg; EA – embryo area and SA – yolk sac area. Different superscript letters in column indicate significant differences among treatments ( $p < 0.05$ ).

### Principal components analysis

Two principal components with eigenvalue above one were identified by PCA, accounting more than 99.0% of variance (Table 3). The first principal component (PC1) was correlated to EA/SA and SA/TA, mainly to SA/TA, while the second principal component (PC2) was correlated to EA/SA and EA/TA, mainly to EA/TA (Table 3). As the principal component evaluation including the 74 samples evaluated and representing four treatments made it possible the identification of two main groups. The first, composed mainly by T4, which tended to have the lowest EA/SA and the highest SA/TA according to PC1 (Figure 4 and Table 3). The first group also had the highest EA/TA, mainly because T4 was the treatment that had eggs with highest EA/TA according to PC2.

The second group was composed mainly by C, which tends to have average EA/SA and SA/TA according to PC1, and it is the group with highest EA/TA according to PC2 (Figure 4 and Table 3). Treatments T1, T2 and T3 did not form a separate group like T4 and C, however, these formed a larger group, that tend to be similar to the group formed by C for EA/SA and SA/TA according to PC1, and showing lower EA/TA compared to the groups formed by C and T4 according to PC2.

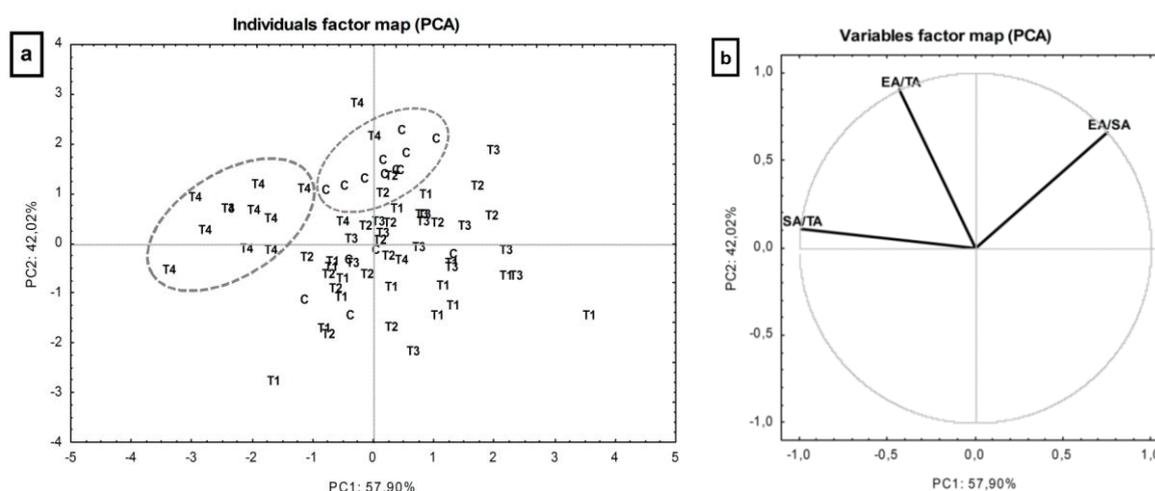
### Sperm analysis

The sperm parameters evaluated in both experiments using either deionized water or urea solution were similar. The sperm used in experiment 2 was overall of better quality compared to the experiment 1 (Table 4).

**Table 3.** Detail of Principal Component Analysis performed on morphometry traits indexes obtained from eggs of *Pseudoplatystoma fasciatum*.

	Principal component 1	Principal component 2
<b>Variance explained % (eigenvalue)</b>	57.90 (1.737)	42.01 (1.260)
<b>Factor loadings*</b>		
EA/SA	0.754	0.656
EA/TA	-0.427	0.904
SA/TA	-0.993	0.110

\* Factor loadings represent correlation between the original variables and the factors. Total area of the egg (TA), area of the embryo (EA) and area of the yolk sac (SA).



**Figure 4.** Distribution of individuals (rows in the database) and direction of morphometric indexes according to principal components 1 (CP1) and 2 (CP2). Principal component analysis was done with. A - Controls (C), treatments using only the urea solution (T1, T2 and T3) and treatment using urea solution in association with tannin solution (T4) is correspond to the number of each observation in the database. B - Morphometric indexes variables as vectors. Principal component 1 (PC1), principal component 2 (PC2). Total area of the egg (TA), area of the embryo (EA) and area of the yolk sac (SA).

**Table 4.** Sperm analysis of *Pseudoplatystoma fasciatum* using urea solution and deionized water for sperm activation.

	Concentration	Membrane Integrity (%)		Time of Motility (sec.)	
		Urea solution	Deionized water	Urea solution	Deionized water
<b>Experiment 1</b>	4.40 ± 2.10 × 10 <sup>10</sup>	98	99	28.20 ± 2.20	29.20 ± 2.80
<b>Experiment 2</b>	6.60 ± 0.90 × 10 <sup>10</sup>	98	99	46.70 ± 1.60	*

\* The data could not be assessed due to the contamination of the sperm by water.

## DISCUSSION

In this study, the neutralization of egg adhesiveness achieved using urea with or without tannin solution showed a negative association with the viability of the eggs. The results of both experiments indicated that the control group (1 egg/2.5 mL), not exposed to any chemical agent, provided the best conditions for egg hatching.

Urea is a polar, highly soluble and low molecular weight molecule that moves through the lipid bilayer of most cells by simple diffusion allowing equilibration of its concentration (KOCHEVAR and SCOTT, 2013; WANG *et al.*, 2014). Urea is used in hatcheries in the form of a solution (the so called "Woynarovich technique") (CABRITA *et al.*, 2008; KUJAWA *et al.*, 2010; SIDDIQUE *et al.*, 2016) for gamete activation and for elimination of egg stickiness. To our knowledge, there are no reports about toxicity to urea when used for neutralization of fish eggs adhesiveness, on the other hand, it has been reported that urea was successfully used in eggs of several species of the *Acipenser* genus (sturgeon) (DOROSHOV *et al.*, 1983; MONACO and DOROSHOV, 1983; KOWTAL *et al.*, 1986), *Spirinchus lanceolatus* (MIZUNO, *et al.*, 2004), *Tinca tinca* (LINHART *et al.*, 2000, GELA *et al.*, 2003; KUJAWA *et al.*, 2010), *Sander lucioperca* (ZARSKI *et al.*, 2015), *Cyprinus carpio* (RIBEIRO, 2001; GELA *et al.*, 2003; LINHART *et al.*, 2003a), *Silurus Glanis* (LINHART *et al.*, 2003a). A large part of studies has been conducted with sturgeon, which eggs are pelagic, presents the cytoplasm filled with higher amounts of reserve nutrients including a large lipid inclusion (DETTLAFF *et al.*, 2012). In this concern it is possible that the relatively less robust structure of South American reophilic species eggs may be related to a higher sensibility and/or toxicity to this substance. This hypothesis came up with our findings and it must be tested in further

studies, but we have to consider that South American reophilic fish (MARQUES *et al.*, 2008; NOGUEIRA *et al.*, 2012; PEREIRA *et al.*, 2013) including *P. fasciatum*, have relatively small demersal eggs (~ ≤1 mm diameter) filled mainly with protein yolk, which are not ovulated in a viscous ovarian fluid. In this concern, our findings reinforce the need to evaluate the toxicity of migratory South American fish eggs to urea since protocols used here were similar or identical to those considered adequate for adhesive eggs of many farming fish.

We also observed that the concomitant use of urea and tannin (T4) showed the best results for the adhesiveness neutralization, but did not provide viable embryos. In T4 egg diameter was 16.55% reduced in comparison to the control group, which led to the formation of a separate group in PCA analysis characterized by the lowest EA/SA and the highest SA/TA and EA/TA. Using conventional statistic evaluation (Tukey test) we observed that T4 eggs had TA, EA and SA means significantly lower than the other treatments. Taken together, the lower embryo area and higher yolk sac indicate that T4 embryos have their development impaired. In this concern, it is known that as a side effect tannin may hardens the wall of the egg and consequently embryos seems cramped inside the egg apparently without enough space for normal development, and hatches with difficulty (BOUCHARD and ALOISI, 2002; DEMSKA-ZAKÉZ *et al.*, 2005; KUJAWA *et al.*, 2010, ZARSKI *et al.*, 2015). In this study, the use of chemical treatments showed a negative association between the neutralization of adhesiveness and viability of eggs. These results associated with the vulnerability of the species indicated that using appropriate storage density of eggs in incubators (control groups used here) free of chemical treatments is temporarily the best alternative to obtain viable *P. fasciatum* embryos.

Another point to be considered was the lower FR and HR of all treatments and controls from the experiment 2 in comparison to their counterparts in experiment 1. We considered that these differences, particularly in control groups, may be associated with variations within the reproductive cycle between years (LEONARDO *et al.*, 2004, BATLOUNI *et al.*, 2005; BATLOUNI *et al.*, 2006), which interferes in reophilic fish with gamete quality (DE SOUZA *et al.*, 2015; KURADOMI *et al.*, 2016) and breeding season (HAINFELLNER *et al.*, 2012; DE SOUZA *et al.*, 2015 KURADOMI *et al.*, 2016). Fertility and HR differences were more pronounced between treatments than between controls, suggesting that the effects of the chemical agents may have been more prejudicial in poorer quality eggs of experiment 2. So it is possible that the worse the quality of the gamete the worse will be the deleterious effects of the chemical treatment, and since it is not possible to evaluate the quality of gametes before spawning (mainly at biochemical level), chemical treatments for neutralizing egg adhesiveness in *P. fasciatum* may be avoided until the establishment of safe protocols.

Unfortunately, there is a large gap between studies on reproductive strategies (ecology) and reproduction applied to aquaculture. Species with adhesive eggs usually have parental care and nesting (RIZZO *et al.*, 2002; JUMAWAN *et al.*, 2014), and in such cases the adhesion of eggs appears to have an important role in natural environment, which must be neutralized when fish are brought into captivity. In that regard, since *P. fasciatum* have no parental care (LEONARDO *et al.*, 2004; BATLOUNI *et al.*, 2005, BATLOUNI *et al.*, 2006), the biological and evolutionary reasons related to egg adhesiveness are unknown, but we must consider that the strategy of laying eggs on a substrate may be important for proper embryonic development. This hypothesis is considered here because *P. fasciatum* eggs not submitted to any treatment (controls) incubated in adequate densities applied in this study aggregated into clumps, but developed properly.

To date there are few experimental methods to quantify the degree of egg adhesiveness (RIZZO *et al.*, 2002; MURRAY *et al.*, 2013), but no standard methods have been defined and most

studies have expressed results of elimination of egg adhesiveness in terms of hatching rate or larval survival (SIDDIQUE *et al.*, 2016). Thus, considering that *P. fasciatum* eggs has a relatively higher sensibility to chemical treatments and the difficulty of obtaining pure *P. fasciatum* wild breeders (HASHIMOTO *et al.*, 2015), testing of new chemical agents does not seem to be the best alternative for *P. fasciatum* immediate propagation. Nevertheless, the best immediate alternative would be to establish the optimal volume of eggs per incubator and/or to increase the available area of the incubators for the adhesion of eggs without neutralizing their natural adhesiveness. Therefore, new strategies for the incubation of adhesive eggs in large quantities should be studied. Due to the high fecundity of *P. fasciatum*, we therefore consider that an effective strategy for the incubation of its eggs would be the introduction of substratum or (for ease of handling) artifacts to increase the area for adhesion of the eggs.

## CONCLUSION

The use of chemicals in the current study did not bring satisfactory results because even the partial or total neutralization of the adhesiveness of the eggs showed deleterious effects over time. The results of both experiments indicated that the control group, in which the eggs were not exposed to any chemical agent, provided the best conditions for egg hatching among the treatments.

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