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Pimelodus maculatus: RASTREAMENTO DE CÉLULAS GERMINATIVAS PRIMORDIAIS E COLETA DE SÊMEN

Mariana Machado Evangelista

Jaboticabal, São Paulo
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RESUMO

Nos últimos anos, *Pimelodus maculatus* vem sendo utilizado como espécie modelo para o desenvolvimento de técnicas de biotecnologia em Siluriformes. Neste estudo foram abordados aspectos da micromanipulação de embriões e obtenção de espermatozoides de *P. maculatus*. Assim, o objetivo do artigo 1 foi identificar e rastrear a rota migratória das Células Germinativa Primordiais (PGCs) em *P. maculatus*. Inicialmente, foram testadas soluções para incubação de embriões decorionados, e a solução de Characin apresentou resultados satisfatórios. Para identificar as PGCs, os embriões decorionados foram microinjetados com mRNA 3'UTR GFP-*nos1*, e o desenvolvimento foi monitorado em estereomicroscópio de fluorescência. Inicialmente, as PGCs foram visualizadas no estágio 6-10 somitos na região medial do embrião. Posteriormente, as PGCs migraram gradativamente na direção anteroposterior e, no estágio 20-24 somitos, localizaram-se próximo à extremidade posterior da região de extensão do vitelo. Nas larvas recém-eclodidas, as PGCs foram encontradas nas cristas genitais. No artigo 2, foi estabelecida uma metodologia não letal para obtenção de espermatozoides de *P. maculatus*, e também descritos aspectos da morfologia dos espermatozoides e da histologia dos testículos. No experimento 1, avaliamos os parâmetros espermáticos de amostras obtidas de peixes submetidos a diferentes protocolos de indução: 1) Solução Fisiológica Salina (controle), 2) Extrato Bruto de Hipófise de Carpa (CCPE) 10 mg kg^{-1} e 3) CCPE 10 mg kg^{-1} + ocitocina 5 UI kg^{-1}. O protocolo que apresentou melhores resultados foi a indução com CCPE 10 mg kg^{-1}, e no experimento 2 foi comparado ao procedimento de indução hormonal seguido de maceração dos testículos. Neste experimento, não houve diferença entre esses procedimentos quanto aos parâmetros avaliados, o que indicou que a indução com 10 mg kg^{-1} de CCPE pode ser satisfatoriamente utilizada para a obtenção do sêmen de *P. maculatus* sem a necessidade de sacrificar os machos para remoção dos testículos. Com relação à morfologia, *P. maculatus* exibiu espermatozóides do tipo *aquasperms*, com características típicas de espécies com fertilização externa. A análise histológica dos testículos revelou diferenças entre as regiões anterior e posterior, onde a primeira mostrou atividade espermatogênica, enquanto a segunda apresentou atividade secretora.

ABSTRACT

In the last few years, *Pimelodus maculatus* has been used as a model species for the development of biotecnology techniques in Siluriformes. In this study were covered aspects of embryo micromanipulation and obtainment of sperm cells of *P. maculatus*. Thus, the aim of article 1 was to identify and trace the migratory route of Primordial Germ Cells (PGCs) in *P. maculatus*. Initialy, it was made a screening of solutions for incubation of dechorionated embryos, and Characin solution showed satisfactory results. To identify the PGCs, dechorionated embryos were microinjected with GFP-*nos1* 3'UTR mRNA, and development was monitored in fluorescence stereomicroscope. Initially, PGCs were visualized at 6-10 somites stage in the medial region of the embryo. Later, PGCs migrated gradually in anteroposterior direction, and in the 20-24 somites stage, were located near the posterior extremity of the yolk extension region. In newly hatched larvae, PGCs were found in the genital ridges. In article 2, we have established a non-lethal methodology to obtain sperm cells from *P. maculatus*, and have also described aspects of spermatozoa morphology and histology of testes. In experiment 1, we assessed the sperm parameters of samples obtained from fish submitted to different induction protocols: 1) Physiological Saline (control), 2) Crude Carp Pituitary Extract (CCPE) 10 mg kg^{-1} and 3) CCPE 10 mg kg^{-1} + Oxytocin 5 UI kg^{-1}. The protocol that showed the best results was induction with CCPE 10 mg kg^{-1}, and in experiment 2 it was compared to the procedure of hormonal induction followed by testes maceration. In this experiment, there was no difference between such procedures concerning the parameters evaluated, which indicated that induction with CCPE 10 mg kg^{-1} may be satisfactorily used to obtain semen from *P. maculatus* without having to kill the fish for testes removal. With regard to its morphology, *P. maculatus* exhibited aquasperm spermatozoa, with some typical characteristics of species with external fertilization. Histological analysis of the testes revealed differences between the anterior and posterior regions, where the former showed spermatogenic activity, while the latter showed secretory activity.

INTRODUÇÃO GERAL

A Ordem Siluriformes é composta por 40 famílias, 490 gêneros e conta com cerca de 3.730 espécies, destas 2.053 se encontram distribuídas ao longo de todo continente americano, predominantemente em água doce [1]. No Brasil, esta Ordem é a segunda mais diversa em relação ao número de espécies de água doce, precedida apenas pelos Characiformes [2]. Além disso, das 353 espécies de Actinopterygii brasileiros ameaçados de extinção 91 são Silurifomes, fazendo desta a segunda Ordem mais ameaçada no Brasil [3]. Tamanha representatividade enfatiza a necessidade de avanços visando buscar informações básicas sobre a biologia das espécies desse grupo, bem como desenvolver técnicas que possam ser utilizadas como ferramentas para conservação. Nesse sentido, é estratégica a utilização de espécies-modelo para alavancar o desenvolvimento de tais tecnologias e, nos últimos anos, *Pimelodus maculatus* vem sendo utilizado como espécie-modelo para Siluriformes no Brasil.

Pimelodus maculatus é popularmente conhecido como mandi, mandi amarelo ou mandi pintado [4], encontra-se amplamente distribuído nas bacias sul-americanas dos Rios Paraná e São Francisco [5], onde realizam migrações de curta distância [6]. Peixes dessa espécie são dioicos, podendo atingir cerca de 50 cm de comprimento, e 2 kg de peso [7]. Economicamente, a importância dessa espécie reside no contexto da pesca artesanal [8,9], além de escassos registros de produção em cativeiro [10]. Independentemente do fim a que se destina o cultivo de exemplares dessa espécie em cativeiro, seja para produção comercial ou manutenção de planteis para pesquisas, é fato que o domínio de técnicas que permitam controlar a reprodução é essencial [11].

Em ambiente natural a reprodução de *P. maculatus* coincide com os meses chuvosos e de temperatura elevada, compreendidos entre novembro e janeiro [12,13]. Ao longo desse período ocorre desova parcelada, com maturação dos oócitos em diferentes grupos [12]. Os ovos produzidos são não-aderentes e de cor amarelada [14] com diâmetro pós-hidratação de 1838.15 ± 61.53 µm e espaço perivitelínico de 323.71 ± 44.28 µm [15]. Em cativeiro, ainda com a utilização da técnica de indução hormonal, a desova não ocorre espontaneamente, sendo necessária massagem abdominal para

liberação dos oócitos [16] e observada fecundidade relativa média de 132,325.00 ± 24,660.00 ovos.kg fêmea^{-1} [15].

No caso dos machos a limitação da reprodução em cativeiro é ainda maior, dado que a indução hormonal seguida de massagem abdominal não garante volume suficiente de sêmen para realização da fertilização *in vitro*, isso pode ser relacionado à morfologia dos testículos nessa espécie, que tem como peculiaridade a presença de projeções digitiformes (“franjas”) [17]. Assim, para a realização da reprodução é comumente utilizada a remoção e maceração dos testículos [18], o que não representa uma prática sustentável. Dessa forma, o desenvolvimento de uma metodologia que permita a obtenção de células espermáticas sem que seja necessário o sacrifício de machos faz-se necessária, ainda mais dentro do contexto de utilização de *P. maculatus* como espécie modelo em estudos de conservação.

Dentre tais trabalhos de conservação tem ganhado destaque aqueles envolvidos na utilização de *P. maculatus* como receptor de células germinativas de espécies de Siluriformes ameaçadas de extinção para produção de quimeras. Nesse sentido, o primeiro passo foi o estudo de aspectos do desenvolvimento embrionário da espécie visando a determinação da temperatura mais adequada, bem como os intervalos de duração das diferentes fases [19]. Posteriormente foi elaborado protocolo para triploidização de embriões de *P. maculatus* e confirmada a efetividade da triploidização na produção de peixes estéreis [20]. Peixes adultos estéreis também já foram utilizados em experimentos como receptores de oogônias e espermatogônias de *Pseudopimelodus mangurus* [21]. No contexto de tais estudos, o próximo passo consiste na micromanipulação de embriões para transferência de células germinativas primordiais (PGCs), o que envolve no primeiro momento a determinação da solução ideal para cultivo de embriões decorionados, além da identificação das PGCs e rastreamento da rota migratória. Uma vez que a rota migratória original é conhecida, é possível compará-la com as de PGCs transplantadas, e assim verificar se estas seguem o mesmo padrão.

OBJETIVOS

OBJETIVO GERAL

- Estabelecer uma solução para cultivo e manutenção de embriões decorionados de *Pimelodus maculatus*, identificar as células germinativas primordiais (PGCs), e rastrear rota migratória destas células.
- Obter uma metodologia não letal para a obtenção de células espermáticas de *P. maculatus*.

OBJETIVOS ESPECÍFICOS

- Determinar a solução de cultivo mais adequada para manutenção de embriões decorionados de *P. maculatus* levando-se em consideração a morfologia da blastoderme, taxa de eclosão e porcentagem de larvas normais;
- Identificar células germinativas primordiais de embriões de *P. maculatus* por meio da injeção de GFP-*nos1* 3'UTR mRNA, e rastrear a rota migratória;
- Testar diferentes protocolos hormonais para obtenção de sêmen de *P. maculatus*, de forma a obter um procedimento não letal;
- Comparar o protocolo com resultados mais satisfatórios com o método de maceração testicular de *P. maculatus*;
- Descrever a morfologia dos espermatozoides e histologia dos testículos de *P. maculatus.*

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ARTIGO 1: Primordial germ cells (PGCs) traceability in *Pimelodus maculatus*

ABSTRACT

Primordial germ cells (PGCs) are precursors of germline, and when transplanted to sterile organisms from other species, it may result in the production of germline chimeras, which are strategic for the formation of gene banks and reconstitution of endangered species. *Pimelodus maculatus* is a potential model for the study and application of chimerism in Siluriformes, once this species is easily reproduced and cultured, and its order is considered the second most endangered in Brazil. Thus, the aim of this study was to identify and trace the migratory route of PGCs in *P. maculatus*. Initialy, it was made a screening of solutions for incubation of dechorionated embryos, and Characin solution showed satisfactory results regarding to blastoderm morphology, hatching rate and percentage of normal larvae. To identify the PGCs, dechorionated embryos were microinjected with GFP-*nos1* 3'UTR mRNA in the blastodisc during the 1-2 cell stage, and development was monitored in fluorescence stereomicroscope. Initially, PGCs were visualized at 6-10 somites stage in the medial region of the embryo. Later, PGCs migrated gradually in anteroposterior direction, and in the 20-24 somites stage, were located near the posterior extremity of the yolk extension region. In newly hatched larvae, PGCs were found in the genital ridges. Knowledge of the PGCs differentiation phase and migration route in *P. maculatus* are fundamental for the use of this species in chimerism of Siluriformes.

Key-words: Chimerism, endangered species, gene banking, micromanipulation, transplantation.

INTRODUCTION

In the last decades, freshwater fish populations have suffered several impacts caused by anthropic activity, especially by the construction of dams, pollution, introduction of exotic species and overfishing [1]. In Brazil, there are 311 species of continental Actinopterygii that are threatened [2], which indicates that actions which contribute to preservation of genetic resources are necessary. Within this scenario, advanced biotechnology tools such as primordial germ cell (PGCs) transplantation are inserted.

PGCs are the embryo cells which give raise to germline [3], thus during embryo development they originate germ cells, wich after gonadal sex differentiation will become either spermatogonia or oogonia [4]. This way, PGCs are the only embryonic cells which have the potential of transmiting genetic information to next generation, preserving both maternal and paternal genome [5]. Thus, PGCs are a valuable resource for the formation of gene banks and reconstitution of endangered species by the production of germline chimera [6]. Germline chimeras are constituted by transplantation of PGCs to sterile recipients that will produce donor gametes [7-9]. Therefore, this methodology can be used in the production of gametes of organisms of endangered species [10].

The first step in the chimera technique using PGCs is to remove the chorion in order to allow the access to embryos. Then, it is necessary to establish an ideal solution for incubation to keep the dechorionated embryos in micromanipulation systems that permit the normal development, and therefore, the analysis of PGCs migration. Describing PGC migration routes in the receptor embryo allows to compare these with the routes of transplanted PGCs in order to evaluate if they follow the same pattern. According to Robles et al. (2016) [6], for the development of basic knowledge, it is strategic the use of model species. In this context, *Pimelodus maculatus* is a potential model for study and application of advanced biotechnologies in Siluriformes. This species is distributed in South America basins of Paraná and São Francisco rivers [11], and is popularly known as catfish, yellow catfish or sppoted catfish [12]. *P. maculatus* females show partial spawning [13], and produce non-adherent eggs which hydration

1838.15 ± 61.53 µm diameter, and have a perivitellin space of 323.71 ± 44.28 µm width [14]. The knowledge of reproduction aspects of this species in captivity [15], embriology [16] and larviculture [17] are reasons that support its utilization as experimental model. In this sense, studies involving chromossomal manipulation on this species have already been iniciated [18], and the next step to the development of biotechnologies is the micromanipulation of embryos. The aim of this study was to stablish an ideal solution for cultivating dechorionated embryos of *P. maculatus* in micromanipulation system, besides identifying PGCs and trace their migratory route by injecting GFP-*nos1* 3'UTR mRNA, a gene related to the migration and survival of PGCs during embryogenesis [22].

MATERIAL AND METHODS

All the procedures described here were approved by the Ethics Committee for Animal Experimentation of CEPTA (CEUA #010/2015). Breeders used on the experiments were collected from October to December 2015 and 2016 (reproductive period) from Mogi Guaçu River, Pirassununga, São Paulo, Brasil (21º55'36,476"S, 47º22'0,836"O, SISBIO #55725-1). Afterwards, fish were taken to Centro Nacional de Pesquisa e Conservação da Biodiversidade Aquática Continental (CEPTA, ICMBio, Pirassununga, São Paulo, Brasil), kept in masonry ponds (8,00 x 8,00 x 1,50 m) and fed twice a day (commercial feed with 55% crude protein).

INDUCED REPRODUCTION

In order to obtain embryos that were used on the experiments, females were selected taking into account external features, such as swollen abdomen and enlarged urogenital papila [19]. Then, induced reproduction was performed with crude carp pituitary extract (CCPE) used in two doses: the first one of 0.5 $mg.kg^{-1}$, and the second one of 5 $mg.kg^{-1}$, applied six hours later. Males received a single dose of 5.0 mg $CCPE.kg^{-1}$ at the time of the second application in the females. After hormonal

induction, males and females were kept together in a circular tank (200 L) with ininterrupt water renovation.

Gametes collect was performed six hours (162 UTA) after the second application. Male was anesthetized using eugenol at 70 $mg.L^{-1}$ [20], euthanized by spinal transection, and the testes were removed and transferred to a recipient containing Minimum Essential Medium Eagle (MEM, M0268, Sigma®), and then were macerated in order to obtain semen [21]. Afterwards, the female was also anesthetized and massaged in ventral region from head to tail for oocytes collection. These were homogenized with semen and it was added water for inducing spermatozoa motility.

EXPERIMENT 1

In this experiment it was evaluated the effect of different solutions in the incubation of dechorionated *P. maculatus* embryos. For this purpose, in each of the four repetitions performed, a sample of 600 embryos was transferred to a petri dish filled with solution of 0.03% protease enzyme Pronase (P8811, Sigma®), diluted in Ringer solution (128.30 mM NaCl, 2.60 mM KCl, 1.80 mM $CaCl_2$) right after hydration. Then, the eggs were observed under stereomicroscope in order to verify the moment when the corion was totally removed from the most part of the zygotes.

After dechorionation (152,25 ± 55,76 s), aliquots of 100 embryos were transferred to petri dishes coated with 1% agar (RM026, Himedia®) and filled with the tested solutions: Characin (35 mOsm; 12 mM NaCl, 1 mM KCl, 1.5 mM $CaCl_2$, 1.5 mM $MgCl_2$), DPBS – Dulbecco's Phosphate Buffered Saline (1.92 mOsm; D5773, Sigma®), Hanks (312.13 mOsm; 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 $NaHCO_3$), Holtfreter (127.24 mOsm; 59.2 mM NaCl, 0.67 mM KCl, 0.9 mM $CaCl_2$, 2.4 mM $NaHCO_3$), MEM – Minimum Essential Medium Eagle (2,27 mOsm; M0268, Sigma®) and Ringer (267,2 mOsm; 128.3 mM NaCl, 2.6 mM KCl, 1.8 mM $CaCl_2$). An aliquot of 100 intact embryos was kept in a petri dish filled with water, and used as control. Development of embryos kept in diferente solutions was monitored in stereomicroscope (SMZ1500, Nikon®, Japan), and abnormalities were photographed (DS-Fi1, Nikon®, Japan). In addition, for each

solution it was also verified survival rate at the stages of cleavage (30 minutes post fertilization - mpf), blastula (1 h 40 mpf), gastrula (4 h 40 mpf), somite (9 hours post fertilization - hpf), hatching (16 hours 30 mpf), as well as the percentage of normal larvae. The solution that showed the best result was chosen to be used in experiment 2.

EXPERIMENT 2

In this experiment, *P. maculatus* embryos were injected with GFP-*nos1* 3'UTR mRNA to evaluate the effects of microinjection in the survival during embryo development, as well as evidence migratory route of primordial germ cells (PGCs).

mRNA SYNTHESIS

For mRNA production, it was used as a template the vector pCS2 + GFP-*nos1* 3'UTR, kindly provided by Prof. Dr. Takafumi Fujimoto (University of Hokkaido, Japan), which contains the 3 'UTR region of the *Danio rerio nos1* gene [22] in fusion with the green fluorescent protein (GFP). Prior to the synthesis, the vector was cleaved with NotI restriction enzyme (IVGN0014, Anza™, Invitrogen™), and for *in vitro* mRNA synthesis it was used the commercial kit mMESSAGE mMACHINE Kit® (AM 1340, Ambion® by Life Technologies™). The mRNA produced was precipitated by LiCl and resuspended in 200 µM KCl, at final concentration of 100 ng.µL^{-1}. The mRNA concentration was mesuared in the NanoDrop spectrophotometer (Thermo Fisher Scientific).

TRACEABILITY OF PRIMORDIAL GERM CELLS

In each one of the three repetitions performed, after the fertilization of *P. maculatus* oocytes, three aliquots of 100 embryos were taken. On the first one, chorion was not removed, and the eggs were transferred to a petri dish filled with water (control with chorion). Embryos from the second and third aliquots were dechorionated as

previously described, transferred to petri dishes coated with 1% agar (RM026, Himedia®) and filled with Characin solution. While dechorionated embryos from the second aliquot were not micromanipulated (control without chorion), those from the third one were injected with GFP-*nos1* 3'UTR mRNA in the region of the blastodisc when they were in the stage of 1-2 cells (up to 30 mpf). Microinjection was performed under stereomicroscope (SMZ18, Nikon®, Japan) using a boron silicate micropipette (10 µm diameter) connected to a microinjector (CellTram vario, Eppendorf, Germany) attached to the micromanipulator (M-152, Narishige, Japan).

Embryos injected with GFP-*nos1* 3'UTR mRNA were observed during embryogenesis in flluorescence stereomicroscope (SMZ18, Nikon®, Japan) to identify GFP-fluorescent PGCs and also photograph them (DS-F2.5, Nikon®, Japan). It was also verified survival of the embryos from the three aliquots in the phases of cleavage, blastula, gastrula, somite, hatching, as well as the percentage of normal larvae. In addition, it was also counted the number of PGCs in the stages of 10 to 24 somites, hatching, and 2 to 5 days after hatching. All the procedures described here were performed in triplicates.

STATISTIC ANALYSIS

All results are presented as the mean ± standard error, and statistic analysis were performed using the software Statistica® 8.0 applying the level of significance (p) of 0.05. Primarily, data were tested for normality (Shapiro-Wilk test) and for homogeneity of variance (Bartlett test). Once these premises were met, analysis of variance (ANOVA) was performed and the means were compared by the Tukey test.

RESULTS

EXPERIMENT 1

The values of cleavage rates were higher than 89% for all solutions tested and none of them were significantly different from the control (Table 1). At the blastula stage, pronounced morphological differences were observed between embryos incubated in different solutions (Fig. 1), although only embryos maintained in DPBS had survival rate significantly lower than control (Table 1). Dechorionated embryos kept in Characin (Fig. 1B) and Holtfreter (Fig. 1F) showed the most regular blastoderm, with semi-spherical shape similar to that observed in the control (Fig. 1A). On the other hand, embryos kept in DPBS (Fig. 1C), Hanks (Fig. 1D-E) and MEM (Fig. 1G) showed numerous blastomeres detached from blastoderm, whereas in those kept in Ringer (Fig. 1H-I) the deformities were remarkable.

In the gastrula stage, embryos kept in MEM and DPBS showed a survival rate significantly lower than the other groups and control, and did not reach somite stage (Table 1), in which there was no difference in the percentage of live embryos kept in Characin, Hanks, Holtfreter and Ringer (Table 1). Regarding to hatching rate, the percentages verified in control and in the embryos kept in Characin, Holtfreter and Ringer (Table 1) were similar. On the other hand, the rate of normal larvae was statistically similar to control in the embryos kept in Characin, Hanks and Holtfreter. Taking into account the morphology of blastoderm, the values verified for hatching rate and the percentage of normal larvae, Characin solution was chosen to be used in the next steps of the work.

Table 1. Survival rates of dechorionated eggs of *Pimelodus maculatus* kept in different solutions of incubation during embryo development, and percentage of normal and abnormal larvae.

Solutions	Cleavage (%)	Blastula (%)	Gastrula (%)	Somite (%)	Hatching (%)	Larvae	
						Normal (%)	Abnormal (%)
Water*	94.37 ± 4.01	92.18 ± 3.49^{a}	87.54 ± 4.14^{a}	68.46 ± 8.94^{a}	55.88 ± 11.05^{a}	96.50 ± 1.27^{a}	3.50 ± 1.27^{bc}
Characin	89.21 ± 5.52	81.77 ± 5.76^{ab}	76.11 ± 4.96^{a}	38.98 ± 9.93^{ab}	33.53 ± 9.98^{ab}	64.01 ± 5.12^{ab}	35.99 ± 5.12^{ab}
DPBS	94.21 ± 2.65	51.12 ± 14.28^{b}	14.26 ± 2.61^{c}	0.00 ± 0.00^{b}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}
Hanks	91.39 ± 3.53	85.01 ± 3.95^{ab}	75.62 ± 1.80^{a}	15.05 ± 6.91^{b}	4.05 ± 3.83^{bc}	80.56 ± 13.75^{ab}	19.44 ± 13.75^{abc}
Holtfreter	92.15 ± 3.28	82.07 ± 7.29^{ab}	76.94 ± 8.05^{a}	40.78 ± 13.60^{ab}	37.37 ± 17.85^{ab}	68.28 ± 8.55^{ab}	31.72 ± 8.55^{abc}
MEM	90.12 ± 3.83	78.39 ± 6.53^{ab}	47.57 ± 8.34^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}
Ringer	90.15 ± 5.07	83.67 ± 6.47^{ab}	79.32 ± 5.91^{a}	27.59 ± 16.55^{ab}	23.46 ± 17.78^{abc}	53.88 ± 13.91^{b}	46.12 ± 13.91^{a}

*Control

Data were obtained in four repetitions, and are presented as mean ± standard error. Means followed by different letters in the same column indicate significant difference ($p<0.05$).

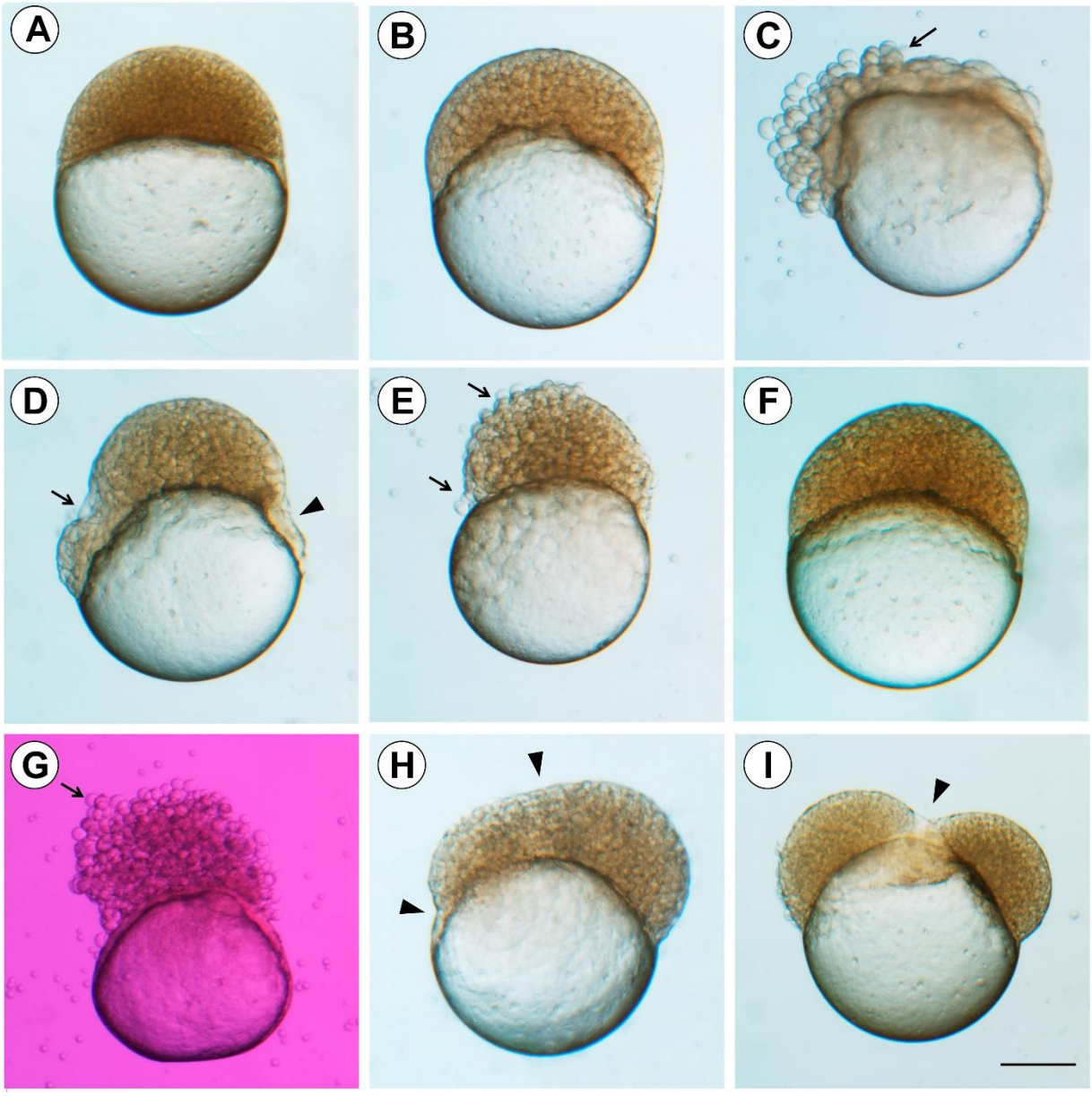

Figure 1. Dechorionated eggs of *Pimelodus maculatus* in blastula stage kept in different incubation solutions. **A.** Water (with chorion). **B.** Characin. **C.** DPBS. **D-E.** Hanks. **F.** Holtfreter. **G.** MEM. **H-I.** Ringer. Arrows indicate blastomers detaching from blastoderm, arrowheads indicate deformities on the blastoderm. Bar: 250 µm.

EXPERIMENT 2

By the injection of GFP-*nos1* 3'UTR mRNA in dechorionated embryos of *P. maculatus* it was possible to evidence the primordial germ cells (PGCs), as well as trace the migratory route of them during embryonic development. A total amount of 148 eggs from three females were injected, in which 132 (89.19%) PGCs were GFP-positive *in vivo* when observed under a fluorescence stereomicroscopic. Regarding to survival at different stages of development, hatching rate and percentage of normal and abnormal larvae, there was no difference between the embryos injected with GFP-*nos1* 3'UTR mRNA, dechorionated embryos (control without chorion) and intact embryos (control with chorion) (Table 2).

The first PGCs were visualized when the embryos were in the somite stage and reached 6 to 10 somites (Fig. 2A); at this stage the number of PGCs ranged from 4 to 18 cells per embryo (mean: 11.5, n = 10). In the previous developmental stages, the GFP expression in somatic cells was still remarkable, making it impossible to identify PGCs. Regarding to the position, in this stage PGCs were located in the medial region of the embryo (Fig. 2B-C) between the first and tenth somites, being present on both sides of the dorsal axis, with rare cases of unilateral distribution.

In sequence, as embryonic development progressed, PGCs gradually migrated anterior-posteriorly and GFP expression in somatic cells decreased gradually. When the embryos reached the 20 to 24 somites stage (Fig. 2D), PGCs were between the eighteenth and twentieth second somites, near the posterior extremity of the yolk extension region (Fig. 2E-F) and varying from 5 to 23 per embryo (mean: 11.9, n = 10). In some embryos we found GFP-positive cells isolated from PGCs groups in ectopic positions, which often remained in the medial region of the embryo or migrated to the region near the head.

In the newly hatched larvae (Fig. 2G) PGCs were found in the upper region of the intestine, reaching the genital ridges (Fig. 2H-I), and ranged from 4 to 18 cells per embryo (mean: 8.9, n = 10). The larvae were monitored until the fifth day post-hatching, and during that period no changes in the position of the PGCs were observed as well as proliferation of these cells. On the second day post-hatch, 1 to 18 PGCs per embryo

were observed (mean: 10.3, n = 10), while on the fifth day we found 3 to 18 PGCs per embryo (mean: 8.8, n = 10).

Table 2. Survival rate of embryos of *Pimelodus maculatus* for control with chorion, control without chorion and dechorionated embryos injected with GFP-*nos1* 3'UTR mRNA during embryo development, and percentage of normal and abnormal larvae.

Groups	Cleavage (%)	Blastula (%)	Gastrula (%)	Somite (%)	Hatching (%)	Larvae	
						Normal (%)	Abnormal (%)
Control with chorion	79.40 ± 10.65	79.14 ± 10.91	78.36 ± 10.98	74.73 ± 12.12	61.85 ± 14.97	79.01 ± 10.31	20.99 ± 10.31
Control without chorion	80.46 ± 7.40	74.74 ± 4.78	69.30 ± 6.31	50.57 ± 5.77	35.25 ± 3.05	83.71 ± 3.35	16.29 ± 3.35
Injected	79.62 ± 10.21	76.82 ± 10.21	68.70 ± 8.94	48.60 ± 10.03	36.35 ± 6.75	72.68 ± 4.34	27.32 ± 4.34

Data were obtained in triplicate, and are presented as mean ± standard error. Means followed by different letters in the same column indicate significant difference ($p<0.05$).

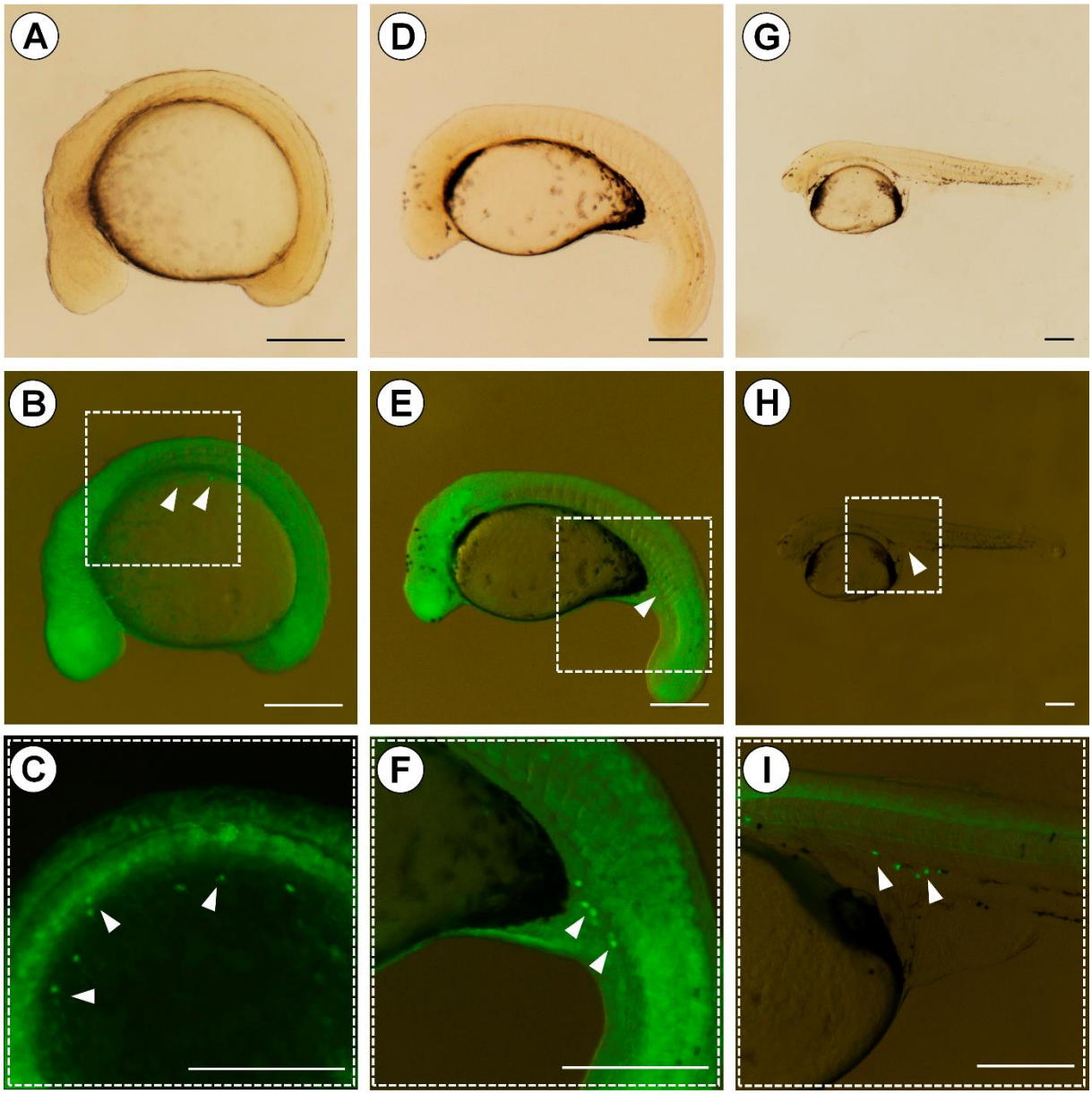

Figure 2. Visualization of primordial germ cells (PGCs) in embryos and larvae of *Pimelodus maculatus* injected with GFP-*nos1* 3'UTR mRNA. **A-B.** Embryo in somite stage (10 somites) with PGCs in the medial region. **C.** Detail of the region highlighted in B. **D-E.** Embryo in somite stage (24 somites) with PGCs near the posterior extremity of the yolk extension region (arrowheads). **F.** Detail of the region highlighted in E. **G-H.** Newly hatched larvae with PGCs on the genital ridges (arrowheads). **I.** Detail of the region highlighted in H. (B, E and H are images captured under fluorescence of A, D and G, respectively). Bar: 250 µm.

DISCUSSION

Micromanipulation procedures of fish embryos have been used in the identification, tracing and isolation of PGCs [23-25], which contributes for stablishing genetic banks of endangered species. However, few studies using such techniques can be found for native species [26]. In order to use these methodologies, the first step consists in the elaboration of protocols for management of embryos in a micromanipulation system, being necessary the dechorionization, which facilitates the manipulation of embryos [27].

Once it is possible to remove the chorion, an acellular structure that surrounds the embryo, protects it against mechanical shocks and acts as a barrier to the entry of substances from external environment [28], the first challenge consists in determine the most suitable solution for incubation of dechorionated eggs. For this, the osmolarity of the incubation solution must be taken into account, since the exposure of embryos to saline solutions that do not match their tolerance range, which is species-specific [29], can affect embryo development reducing hatching rate [30, 31], and increasing the incidence of larvae with deformities [32]. In the present work, the Characin solution presented satisfatory results for incubation of dechorionated embryos of *P. maculatus* when considering blastoderm morphology, hatching rate and percentage of normal larvae. Afterwards, the results obatained using Characin solution showed lower variation than Holtfreter solution, being the most indicated solution for performing micromanipulation procedures, such as the injection of GFP-*nos1* 3'UTR mRNA into newly fertilized eggs.

The results obtained in this study indicate that it is possible to trace PGCs of *P. maculatus* embryos by the injection of from *Danio rerio* GFP-*nos1* 3'UTR mRNA, following the example of Saito et al. (2006) [33] for *Clupea pallasii*, *Danio rerio*, *Danio albolineatus*, *Carassius auratus*, *Misgurnus anguillicaudatus*, *Oryzias latipes* and *Leucopsarion petersii*. In the mentioned research, the authors suggest that the function of *nos1* 3'UTR is quite conserved in teleosts, and according to Köprunner et al. (2001) [22], this gene is related to the migration and survival of PGCs during embryogenesis.

The visualization of the first PGCs in *P. maculatus* occurred at somite stage, and from this phase the expression of GFP in somatic cells became less evident.

This can be explained by the fact that, throughout embryonic development, the 3'UTR portion of *nos1* is degraded in somatic cells, however, it remains stable in germ line cells [34]. On the other hand, in *Tinca tinca [35]*, *Anguilla japonica* and *Danio rerio* [3], PGCs were visualized at the end of gastrula stage. The later observation of PGCs in this work may be related to the fact that the GFP-*nos1* 3'UTR mRNA used was produced from the sequence of another species (*Danio rerio*). Although the *Danio rerio* sequence has already been successfully employed in marking PGCs of *Danio albolineatus*, *Carassius auratus* and *Misgurnus anguillicaudatus* [36], Kawakami et al. (2011) [37] reported that the identification of PGCs of *Cyprinus carpio* was more efficient when using the GFP-*nos1* 3'UTR mRNA produced from the *nos1* gene of the study species. This indicates that, when possible, it is recommended to produce the mRNA specific to the species that will be studied, in order to guarantee better results.

Regarding to the migration pattern of PGCs, Saito et al. (2006) [33] emphasized that although the final location of these cells varies between fish species, as well as migration routes, the latter are limited to the medial region of the embryo and to the yolk extension region. The peculiarities observed between migratory routes of PGCs of different species may be due variations in egg size, shape and developmental characteristics, according to Robles et al. (2016) [6]. In the present work, it was verified that, in the migration of *P. maculatus* PGCs, the cells were firstly identified between the first and the tenth somite, and moved gradually anterior-posteriorly to the region of genital ridges. It was also found that the number of labeled PGCs at different stages of development was similar, confirming the reports by Linhartova et al. (2014) [35] who stated that there is no proliferation of PGCs during migration route.

Although in previous works PGCs have been identified and traced in fishes from Clupeiformes, Cypriniformes, Beloniformes, Perciformes [33, 38] and Salmoniformes orders [39], it is the first time that these cells are traced in Siluriformes. This order comprises 35 families and about 2,867 described species [40], being the second most representative order of freshwater fish in Brazil in relation to the number of species [41]. In addition, from the 311 continental Brazilian Actinopterygii species in danger of extinction, 91 are Siluriformes, being also the second most threatened order in the country [2]. This fact emphasizes the need of

works that contribute to the development of techniques that can be applied in the conservation of species, and in this panorama it is inserted the study of PGCs presented here. PGCs are the only embryonic cells capable of transferring genetic information to the next generation [42], a strategic feature for their use in chimerism. Data obtained in this study on the suitable incubation solution for *P. maculatus* embryos, as well as the elucidation of the visualization phase of PGCs and migration route is an important step towards the advancement of the chimerism technique in this species.

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ARTIGO 2: Non-lethal procedure for collection of *Pimelodus maculatus* semen: morphology of spermatozoa and histology of testes

ABSTRACT

In this work we stablished a non-lethal methodology to obtain spermatozoa of *Pimelodus maculatus*, and described aspects of spermatozoa morphology and testes histology. In experiment 1, we evaluated sperm parameters of samples obtained from fish submitted to different induction protocols: 1) Physiological Solution (control), 2) Crude Carp Pituitary Extract (CCPE) 10 mg kg^{-1} and 3) CCPE 10 mg kg^{-1} + Oxytocin 5 UI kg^{-1}. The protocol that had better results was induction with CCPE 10 mg kg^{-1}, and in experiment 2 this was compared to the procedure of hormonal induction followed by testes maceration. In this experiment there was no difference between both procedures regarding to the parameters evaluated, which indicated that induction with CCPE 10 mg kg^{-1} can be satisfactorily used to obtain semen from *P. maculatus* without being necessary to kill fish for testes removal. In relation to its morphology, *P. maculatus* had aquasperms spermatozoa, with typical characteristics of species with external fertilization. Histological analysis of testes revealed differences between anterior and posterior regions, while the first one had spermatogenic activity, posterior region showed secretory activity.

Key-words: Crude Carp Pituitary Extract, semen release, sperm parameters, spermatic morphology.

INTRODUCTION

Pimelodus maculatus, popularly known in Brazil as *mandi*, *yellow mandi* or *spotted mandi* ([4], is a species of the order Siluriformes which is widely distributed throughout South-American Paraná and São Francisco river basins in Brazil (Lundberg & Littmann, 2003). From an economic point of view, in addition to being important in the context of artisanal fishing (Peixer & Petrere Junior, 2009; Costa et al., 2012), *P. maculatus* is also reared in captivity in the south of Brazil (Baldisserotto, 2008), and the quality of its flesh and absence of intramuscular bones are some characteristics that give it potential for aquaculture (Weingartner & Zaniboni Filho, 2004).

Over the last few years, *P. maculatus* has also been gaining prominence as a model of study for the development of biotechnology applied to conservation in Siluriformes, such as chromosome manipulation (Bertolini et al., 2016), tracking of primordial germ cells (Evangelista et al.), and transplant of oogonia and spermatogonia (Lopez, 2018). Despite extensive knowledge of aspects of reproduction (Arantes et al., 2013), embryology (Arashiro et al., 2018) and larviculture (Luz & Zaniboni Filho, 2002) of this species, difficulties are still encountered with regard to obtaining sperm cells. In *P. maculatus,* even after hormonal induction following traditional methodologies, semen is not released in sufficient amount for fertilization (Sato et al., 1999), and the most commonly used procedure is removal of testes, followed by maceration (Damasceno et al., 2015). Besides the need to sacrifice the males, the adoption of this procedure also requires extra care in order to reduce the risk of contamination by water, environmental fluids and dehydration, and may also compromise the quality of the semen due to the process of autolysis, which quickly initiate the decomposition of internal organs after fish death (Gwo, 2008).

In view of that, the objective of this study was to test different hormonal protocols to obtain semen from *Pimelodus maculatus* in order to find a non-lethal procedure. Furthermore, we intended to compare the protocol with the most satisfactory results to the method of testicular maceration, and also describe the morphology of spermatozoa and histology of testes.

MATERIAL AND METHODS

The *Pimelodus maculatus* broodfish used in this study were collected from the Mogi Guaçu River, in Pirassununga, São Paulo, Brazil (21º55'36,476"S, 47º22'0,836"O, SISBIO #55725-1) during the months of October and November 2016 (reproductive period). Subsequently, the fish were taken to the National Center of Research and Conservation of Continental Aquatic Biodiversity (CEPTA, ICMBio, in Pirassununga, São Paulo, Brazil), where they were kept in brick wall, earthen bottom water ponds (8.00 x 8.00 x 1.50 m), and fed twice a day to apparent satiation (commercial feed 55% CP). All the procedures had been approved by the Animal Experimentation Ethics Committee of CEPTA (CEUA #010/2015).

EXPERIMENT 1

In this experiment, the sperm parameters of *P. maculatus* males submitted to different protocols of hormonal induction and semen collection were assessed. In order to do so, nine males were selected and divided into three groups of three fish each. In each group, the fish were submitted to the following procedures:

- Control: Physiological saline (1 ml kg^{-1});
- Treatment 1: Hormonal induction with crude carp pituitary extract, 10 mg kg^{-1};
- Treatment 2: Hormonal induction with crude carp pituitary extract, 10 mg kg^{-1}, and after 12 hours, application of oxytocin, 5 UI kg^{-1} (Ocitocina Forte, UCBVet), as described by Viveiros et al. (2003) for *Clarias gariepinus*.

SEMEN COLLECTION

Semen was collected twelve hours after the injections in fish from Control and Treatment 1, and one hour after the application of oxytocin in fish from Treatment 2. For that purpose, the fish were anesthetized with eugenol at the concentration recommended by Júnior et al. (2014) (70 mg eugenol, 700 µL absolute alcohol: 1 L water), and then an abdominal massage was performed from head to tail. The material released was collected with micropipettes, and immediately transferred to

cryotubes containing 400 µL of MEM solution – Minimum Essential Medium Eagle (M0268, Sigma®).

SPERM ANALYSIS

After the semen had been collected, the analysis of the sperm parameters concentration, viability and motility was conducted. For the measurement of concentration, the semen was first diluted in fixative solution of buffered formalin (Hancock, 1956) at a proportion of 1 semen: 4 fixative solution, and then the number of sperm cells present in five squares of a Neubauer hematimetric chamber were counted under light microscope (Eclipse Ci, Nikon®, Japão). The concentration was calculated as recommended by the Brazilian College of Animal Reproduction (CBRA - 1998) for mammals, according to the equation:

$$CSPZ = \left(\frac{\sum SPZ}{c.s.}\right) \times \frac{t.s. \times dilution \times 1000}{depth\ of\ the\ camera\ (mm)}$$

where CSPZ = concentration of spermatozoa (cells ml^{-1}), SPZ = number of spermatozoa, c.s. = counted squares, t.s. = total squares, depth of the chamber = 0.10 mm and dilution = dilution factor of semen by fixative solution.

In order to determine sperm viability, membrane integrity was assessed by means of flow cytometry. Whenever possible, the concentration of the cell suspension was adjusted to 3.6×10^6 cells mL^{-1} (Yang et al., 2013). Subsequently, 0.30 µL of SYBR-14 100 nM solution and 0.60 µL of 12 µM propidium iodide were added to 150 µL of the suspension, and the material was incubated for 10 minutes in a place protected from light. After that period, the material was analyzed using a BD Accuri™ C6 flow cytometer (BD Biosciences, USA), and 10,000 events were evaluated at medium speed. Sperm viability was determined considering the percentage of spermatozoa stained with SYBR-14 (intact membrane) out of the total number of spermatozoa (stained with SYBR-14 and Propidium Iodide) using the CFlow software (BD Biosciences, USA).

For the motility analysis, whenever possible, the concentration of the cell suspension was adjusted to 3.6×10^8 cells mL^{-1}. Afterwards, 2.0 µL of that suspension were transferred to a Makler chamber, where 0.1% BSA had previously been applied, and then 18.0 µL of water were added. The samples were observed under light microscope (Eclipse Ci, Nikon®, Japan), and videos were obtained at 100fps (640x480 pixels) (SIGHT

DS-fi1, Nikon® Digital, Japan), and submitted to analysis by the free CASA software (*Computer Assisted Sperm Analysis*), where 1 second of sperm activity was assessed, between 10 and 11 seconds after semen activation. The videos were processed based on the procedure adopted by Wilson-Leedy & Ingermann (2007); however, the configurations were adapted to the species studied, as described by Neumann et al. (2013). Thus, sperm motility (MOT), curvilinear velocity (VCL), average path velocity (VAP), and straight line velocity (VSL) were obtained. In addition, the time after activation when 50% of spermatozoa remained mobile, and the time when all were immobile were determined by a subjective method. The methodology that showed the most satisfactory results regarding sperm parameters was used in experiment 2.

EXPERIMENT 2

In this experiment, semen samples collected by the methodology that provided the most satisfactory results in experiment 1 (Treatment 1) were compared, using the currently most widespread protocol for obtaining *P. maculatus* sperm cells (hormonal induction followed by maceration of testes). Thus, six males were taken and hormonally induced with crude carp pituitary extract (10 mg kg^{-1}). Twelve hours after injection, the males were anesthetized with eugenol at the concentration recommended by Júnior et al. (2014) (70 mg eugenol, 700 µL absolute alcohol: 1 L water), and extrusion was performed in three males. The released material was collected with micropipettes, and immediately transferred to cryotubes containing 400 µL of MEM solution. The other fish were sacrificed by spinal dissection, had their testes removed and weighed, and two fringes were removed, transferred to cryotubes containing 400 µL of MEM solution and macerated. The testes were fixed in 2.5% glutaraldehyde, dehydrated at increasing concentrations of ethanol, included in historesin, sectioned (5.0 µm) in a microtome (RM 2235, LEICA®, Germany) equipped with steel blades, and stained with toluidine blue. The material was then observed under microscope (Eclipse 50i, Nikon®, Japan) attached to a camera (STMPRO-T-LED, BEL®, Milan, Italy). The images were captured by *Bel View* software (BEL®, Italy).

SPERM ANALYSIS

The samples collected were submitted to analysis of sperm parameters concentration, viability and motility according to the methodologies described in experiment 1.

MICROSCOPY AND MORPHOMETRY OF SPERMATOZOA

The samples obtained by the two methodologies tested were observed under light microscope (Eclipse DIC Ni-U, Nikon®, Japan) and photographed (DS-Fi1, Nikon®, Japan). Sub-samples were also fixed in glutaraldehyde solution (2.5%) for 24 hours, transferred to DPBS solution – Dulbecco's Phosphate Buffered Saline (D5773, Sigma®) and later submitted to the routine procedures of scanning electron microscopy, which were carried out at the Laboratory of Electron Microscopy of the *Ribeirão Preto* Medical School (FMRP/USP, in São Paulo, Brazil). Therefore, the samples were included in 2% agar, the blocks were fixed in osmium tetroxide 1% solution for two hours at 4.0 °C, and then rinsed in phosphate buffered solution. After that, the samples were dehydrated in increasing concentrations of acetone and included in resin. Sections of 0.5 µm were cut with microtome and stained with 1% toluidine blue. Selected sections were cut into 70 nm sections using a diamond blade and stained with uranyl acetate and lead citrate, and then photographed in a scanning electron microscope (JSM-6610LV, JEOL®, Japan).

The following parameters were measured from 50 spermatozoa using the images obtained: head length (HL), head width (HW), middle piece length (ML), tail length (TL) and total length of spermatozoa (TLS), using the ImageJ® software.

STATISTICAL ANALYSIS

The results are presented as means ± standard error, and the statistical analysis was carried out with the Statistica® 8.0 software, applying a 0.05 level of significance (p). The data were first tested with regard to normality by the Shapiro-Wilk test, and homogeneity of variance by the Bartlett test. These premises being met, a one-way analysis of variance was performed (one-way ANOVA), and the means were compared by the Duncan test.

RESULTS

EXPERIMENT 1

SPERM ANALYSIS

There was no influence of the treatments on the parameters viability, curvilinear velocity (VCL), average path velocity (VAP), straight line velocity (VSL), sperm motility (MOT) and motility duration (MOT 50, MOT 100) (Table 1). As for sperm cell concentration, both treatments exhibited higher values than control (1.20 ± 0.50 x 10^6 cells.mL^{-1}); however, oxytocin together with carp pituitary extract (treatment 2; 23.77 ± 3.83 x 10^6 cells.mL^{-1}) brought similar results to the ones obtained with hormonal induction performed exclusively with pituitary extract (treatment 1; 53.60 ± 32.11 x 10^6 cells.mL^{-1}). Thus, treatment 1 was considered the most suitable to obtain *P. maculatus* sperm cells, and was therefore chosen to be used in experiment 2.

Table 1: Concentration, viability, curvilinear velocity (VCL), average path velocity (VAP), straight line velocity (VSL), sperm motility (MOT), motility duration (MOT 50: 50% of immobile spermatozoa, MOT 100: 100% of immobile spermatozoa) of semen samples collected from *P. maculatus* submitted to different protocols of hormonal induction.

Group	Concentration ($x10^6$ cells.ml^{-1})	Viability (%)	VCL ($\mu m.s^{-1}$)	VAP (µm.s-1)	VSL (µm.s-1)	MOT (%)	MOT 50 (s)	MOT 100 (s)
Control	1,20 ± 0,5[a]	73,40 ± 6,73	76,96 ± 4,53	80,94 ± 4,89	66,42 ± 5,37	27,78 ± 8,02	10,00 ± 10,00	14,00 ± 14,00
Treatment 1 (Carp Pituitary Extract)	56,30 ± 32,11[b]	71,25 ± 8,26	81,44 ± 5,79	99,31 ± 10,34	76,71 ± 3,66	48,88 ± 12,16	28,00 ± 6,81	95,00 ± 35,13
Treatment 2 (Carp Pituitary Extract + Oxytocin)	23,77 ± 3,83[b]	72,23 ± 10,51	78,85 ± 9,50	89,61 ± 15,54	77,42 ± 11,82	36,12 ± 6,81	22,67 ± 6,12	62,67 ± 16,86

The data were obtained in triplicate and are expressed as means ± standard error. Means followed by different letters in the column show significant difference ($p<0.05$).

EXPERIMENT 2

SPERM ANALYSIS

The data obtained revealed that there were no differences regarding concentration, viability, curvilinear velocity (VCL), average path velocity (VAP), straight line velocity (VSL), sperm motility (MOT), motility duration (MOT 50, MOT 100) between the semen samples obtained by maceration of testes and by extrusion (both treatments were performed after hormonal induction with pituitary extract at a concentration of 10 $mg.kg^{-1}$ – Table 2).

On the other hand, when the sub-samples from different treatments were observed under light microscopy and scanning electron microscopy, some differences became evident. In those obtained after maceration of testes, groups of spermatozoa with intertwined tails were frequently observed, together with fragments of tissue and blood cells (Fig 1A-B), differently from what was observed in samples obtained by extrusion, where such groups were not seen (Fig 1C-D).

Table 2: Concentration, viability, curvilinear velocity (VCL), average path velocity (VAP), straight line velocity (VSL), sperm motility (MOT), motility duration (MOT 50: 50% of immobile spermatozoa, MOT 100: 100% of immobile spermatozoa) of semen samples collected from *P. maculatus* using different methods.

Group	Concentration ($x10^6$ cells.ml^{-1})	Viability (%)	VCL ($\mu m.s^{-1}$)	VAP (µm.s-1)	VSL (µm.s-1)	MOT (%)	MOT 50 (s)	MOT 100 (s)
Extrusion	515,07 ± 329,55	79,54 ± 6,40	96,25 ± 13,86	118,03 ± 20,21	103,40 ± 7,32	60,91 ± 20,58	46,67 ± 5,21	97,67 ± 10,33
Maceration	45,33 ± 12,54	56,95 ± 10,19	105,40 ± 5,55	122,40 ± 7,70	95,12 ± 1,08	55,71 ± 15,50	22,00 ± 10,50	78,00 ± 24,01

The data were obtained in triplicate and are expressed as means ± standard error. Means followed by different letters in the column show significant difference ($p<0.05$).

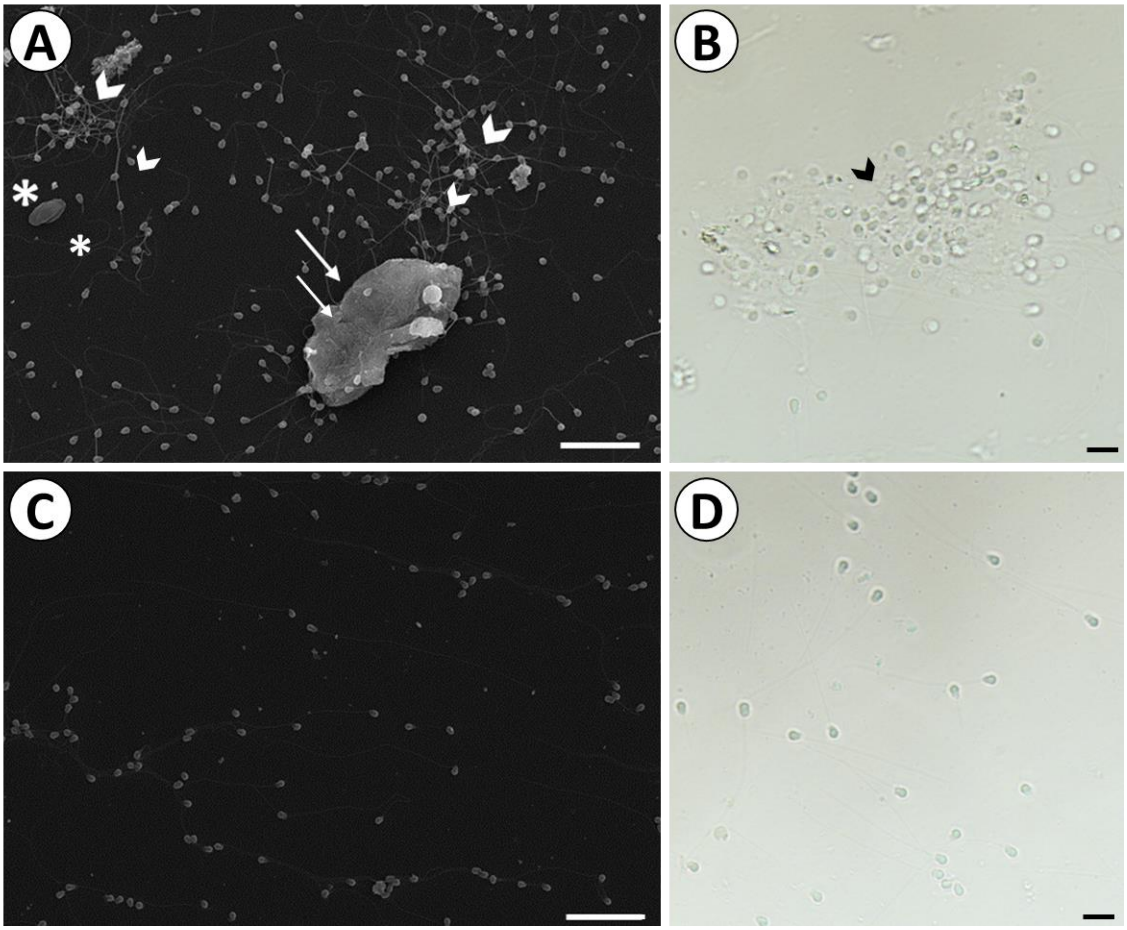

Figure 1. *Pimelodus maculatus* spermatozoa collected by different methods. **A-B.** Spermatozoa obtained by maceration of testes observed by scanning electron microscopy (A) and light microscopy (B); spermatozoa with intertwined tails are clearly seen (arrow head), fragment of tissue (thin arrow) and red blood cell (asterisk). **C-D.** Spermatozoa obtained by extrusion observed in scanning electron microscopy (C) and light microscopy (D). Bar = 20 µm.

MORPHOMETRY OF SPERMATOZOA

The analyzed *P. maculatus* spermatozoa had 42.88 ± 1.74 µm total length, comprising three distinct portions: head, middle piece and a single tail (Fig. 1A). The head was spherical in shape (diameter 1.46 ± 0.02 µm) and its surface had a few irregularities (Fig. 1B). In the middle piece, on the other hand, some villi were observed on the surface, in addition to a slightly conical shape (length 0.87 ± 0.02 µm), with the wider end near the head and the narrower end joining the tail (Fig. 1B); the latter showed mean length of 42.28 ± 1.74 µm.

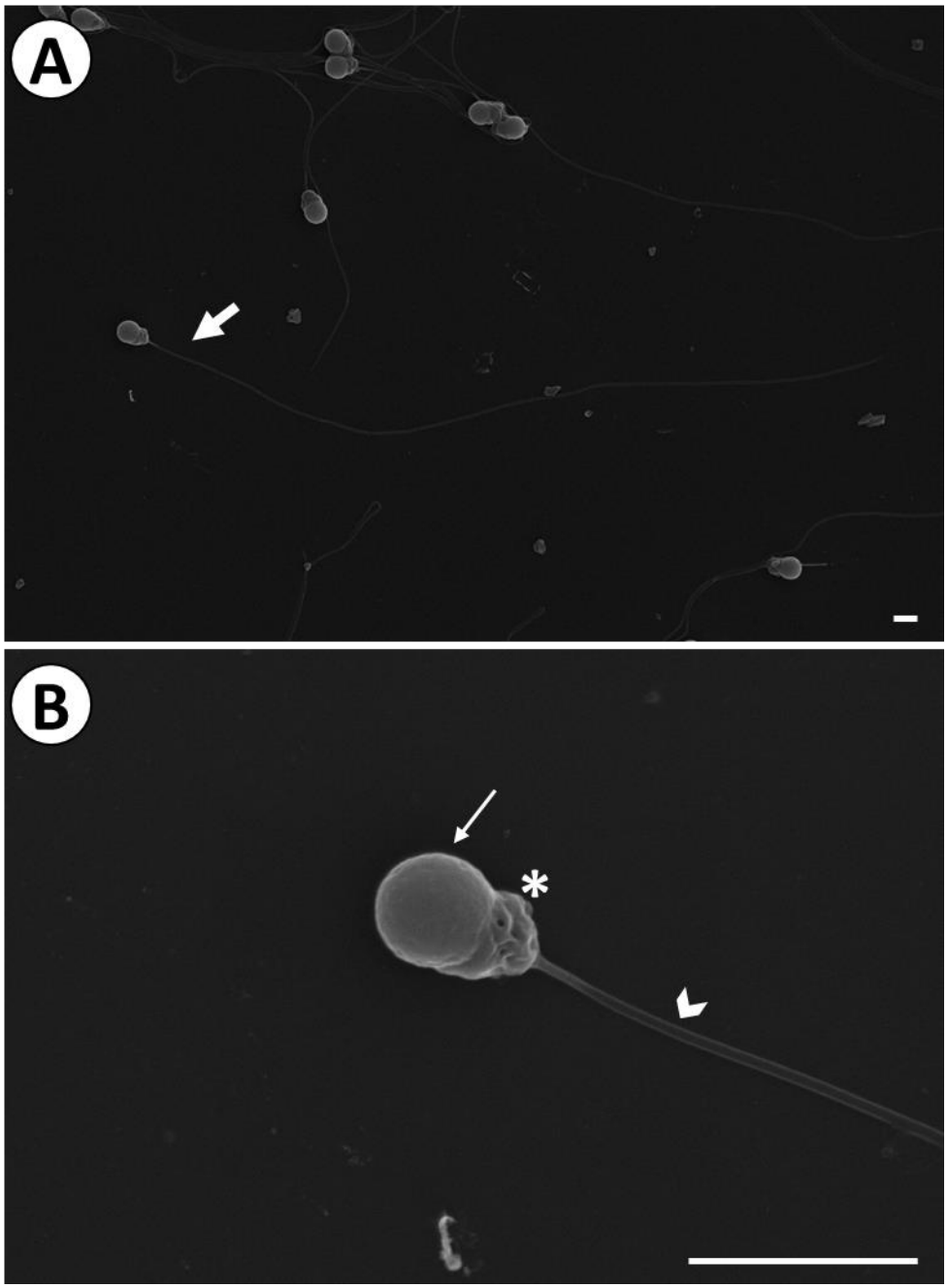

Figure 2. *Pimelodus maculatus* spermatozoa observed by scanning electron microscopy. **A.** General view of the spermatozoon (thick arrow). **B.** Detail of head (thin arrow), middle piece (asterisk) and tail (arrow head). Bar = 3 µm.

HISTOLOGICAL ANALYSIS

Histologically, the evaluated testes of *P. maculatus* were at mature stage, characterized mainly by the presence of spermatozoa in the seminiferous tubules (Fig. 3), as characterized by Cruz & Santos (2004). However, when the anterior, middle and posterior regions of the testes were compared, differences were observed.

In the anterior region of the testes, the lumen of the seminiferous tubules was full of spermatozoa (Fig. 3A). Germ cells at previous stages of development organized in cysts, spermatogonia, spermatocytes and spermatids were also observed (Fig. 3B). The middle portion, on the other hand, exhibited seminiferous tubules with flaccid walls, but still with the presence of spermatozoa (Fig. 3C). In addition, spermatocytes were also observed in this region (Fig. 3D). In the posterior portion, the lumen of the seminiferous tubules was empty (Fig. 3E), and in some cases, with residual spermatozoa (Fig. 3F).

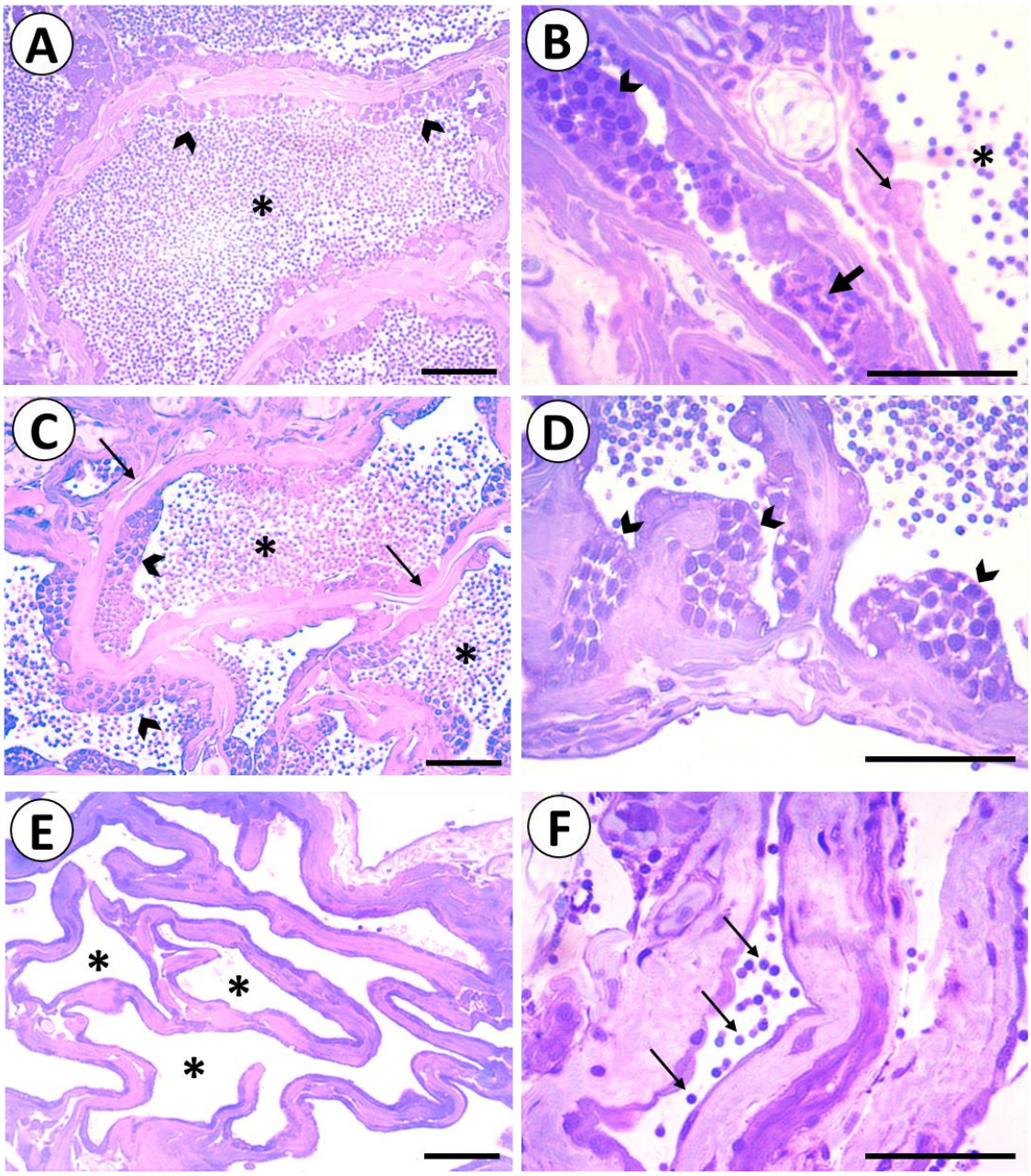

Figure 3. Testes of *Pimelodus maculatus*. **A.** Anterior region with lumen full of spermatozoa (asterisk) and germ cells at previous stages of development (arrow head). **B.** Detail of anterior region, showing a spermatogonium (thin arrow), spermatocytes (arrow head), spermatids (thick arrow) and spermatozoa (asterisk). **C.** Middle region revealing lumen with flaccid walls (thin arrow), but still full of spermatozoa (asterisk) and with germ cells at previous stages of development (arrow head). **D.** Detail of middle portion showing spermatocytes (arrow head). **E.** Posterior region with semi-depleted lumen. **F.** Detail of posterior portion, showing traces of spermatozoa (thin arrow). Bar = 50 µm.

DISCUSSION

As verified in *Pimelodus maculatus* by Sato et al. (1999), the difficulty in obtaining semen is recurrent in Siluriformes, as observed not only in species of the same genus, such as *P. blonchii* (Ramírez et al., 2013), *P. grosskopfii* (Valbuena-Villarreal et al., 2010) and *P. britski (Damasceno et al., 2015)*, but also in *Ictalurus punctatus* (Christensen & Tiersch, 2005), *Clarias macrocephalus* (Tan-Fermin et al., 1999), among other species, impairing reproduction in captivity. In *P. maculatus,* this difficulty may be related to testicular morphology, since in this species the testes are fringed, displaying digitiform projections along their entire length (Arantes et al., 2013). In this context, several strategies have been adopted in order to develop protocols of hormonal induction specifically for these animals, which allow sperm cells to be obtained without sacrificing the males (Viveiros et al., 2002, Damasceno et al., 2015).

When studying *Clarias gariepinus*, a species that due to the morphology of its testes presents limitations for obtaining semen, similarly to what occurs with *P. maculatus*, Viveiros et al, (2003) attested that when testes were incubated in a solution with oxytocin, there was an increase in the concentration of sperm cells in the medium. On the other hand, when oxytocin was administered *in vivo,* there was no difference in semen release in comparison to the control treatments. In mammals, it is known that oxytocin is related to the process of contraction of seminiferous tubules and spermiation, alhough its mechanism of action has not yet been fully elucidated (Thackare et al., 2006). In fish, in turn, the role of this hormone remains poorly understood (Viveiros et al., 2003). In the present study, the administration of oxytocin in *P. maculatus* males after the injection of crude carp pituitary extract (CCPE) brought similar results to the ones obtained with induction performed only with CCPE with regard to concentration of spermatozoa, viability, and other sperm parameters.

Although the effects of oxytocin have not been significant in the results presented here (Experiment 1), the use of CCPE at a dose of 10 $mg.kg^{-1}$ led to a higher concentration of sperm cells than in the control group (fish not hormonally induced). When this protocol was compared to the testes maceration protocol, there was no difference in any of the sperm parameters assessed (Experiment 2). Furthermore, when the samples obtained were observed under light microscopy and scanning electron microscopy, it was verified that there were fragments of tissue and blood cells in the ones obtained by maceration, which may act as a barrier between

the gametes. This reduction of contact between gametes may negatively interfere in the success of fertilization (Suquet et al., 1995). In view of these results, it is concluded that male induction with CCPE at a dose of 10 mg.kg^{-1} followed by extrusion may be used satisfactorily in obtaining semen from *P. maculatus*. Traditionally, lower doses of CCPE are recommended for male induction, as verified for *Leiarius marmuratus* – 2.5 mg.kg^{-1} (Galo et al., 2014), *Pseudoplatystoma fasciatum* – 1.5 mg.kg^{-1} (Leonardo et al., 2004), *Rhamdia quelen* – 2.5 mg.kg^{-1} (Tessaro et al., 2012) and *Steidachneridion parahybae* - 3 mg.kg^{-1} (Caneppele et al., 2015). However, for species in which semen release is difficult, higher doses are more effective, as observed in *P. britskii* – 7.5 mg.kg^{-1} (Damasceno et al., 2015).

With regard to morphological characteristics, *P. maculatus* exhibited typical spermatozoa of species with external fertilization, classified as aquasperms, with spherical head, short middle piece and long tail (Jamieson, 1991). This is the most common type among Siluriformes, but it is also found in *Clarias gariepinus* (Mansour et al., 2002), *Diplomystes mesembrinus (Quagio-Grassiotto et al., 2001)*, *Iheringichthys labrosus* (Santos et al., 2001) and *Pseudoplatystoma corruscans (Velarde, 2013).* Nevertheless, in this order introsperm spermatozoa are also found, such as in *Scoloplax distolothrix (Spadella et al., 2006)* and *Trachelyopterus lucenai* (Burns et al., 2002).

The histological analysis of the *P. maculatus* testes revealed differences in relation to the cell composition of different regions, which indicate that the anterior and middle regions present spermatogenic activity, corroborating the reports made by Cruz & Santos (2004). In addition, it was verified that in *P. maculatus,* the spermatogenesis is cystic, since the differentiation of spermatids into spermatozoa occurs inside cysts. The posterior portion of the testes, on the other hand, was characterized by the presence of seminiferous tubules with empty lumen, exhibiting secretory activity. In this region there is an acidophilic solution, composed of neutral glycoproteins, as well as carboxylic acids and sulfated glycoconjugates ([55].

In short, the results obtained here suggest that hormonal induction with crude carp pituitary extract at a dose of 10 mg.kg^{-1} is a non-lethal alternative to obtain semen from *P. maculatus*. Therefore, by employing this protocol it is possible to collect sperm cells by extrusion, without having to sacrifice the fish to remove their testes. It allows the use of a

smaller number of males for reproduction practices, making the propagation of the species in captivity more viable, both for commercial purposes and for utilization of the species as an experimental model.

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CONSIDERAÇÕES FINAIS

A partir dos resultados obtidos nos dois artigos aqui apresentados foi possível obter avanços no que diz respeito ao desenvolvimento de estudos de conservação com *Pimelodus maculatus*, espécie que vem sendo utilizada como modelo experimental para peixes Siluriformes. No artigo 1 foi estabelecida uma solução de manutenção para embriões decorionados de *P. maculatus*, além de visualizada a rota de migração das células germinativas primordiais (PGCs). Tais informações são de fundamental importância para utilização em futuros trabalhos que envolvam a micromanipulação de embriões de Siluriformes e formação de bancos genéticos.

Por outro lado, no artigo 2 foi estabelecida uma metodologia para a obtenção de células espermáticas de *P. maculatus* sem que haja a necessidade de sacrificar os machos. Assim, tal procedimento torna mais viável a realização da fertilização *in vitro* nessa espécie, que comumente é utilizada em trabalhos de biotecnologia que envolvem a manipulação de gametas e embriões, bem como acompanhamento do desenvolvimento embrionário.