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UNIVERSIDADE ESTADUAL PAULISTA
“Júlio de Mesquita Filho”
INSTITUTO DE BIOCÊNCIAS DE BOTUCATU

Pós-graduação em Ciências Biológicas - Área de Concentração: Zoologia

Dissertação Mestrado

FERRAMENTAS PARA A CONSERVAÇÃO DO BAGRE SAPO
***Pseudopimelodus mangurus*: DOMESTICAÇÃO E APLICAÇÃO**
DE BIOTECNOLOGIAS

GUSTAVO FONSECA SHIGUEMOTO

GEORGE SHIGEKI YASUI

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DE BIOTECNOLOGIAS

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RESUMO GERAL

Ações voltadas para a conservação da biodiversidade aquática incluem medidas que visam assegurar a reconstituição de espécies em risco em seus ambientes naturais. Neste trabalho foram investigadas ferramentas para a conservação e exploração do bagre sapo *Pseudopimelodus mangurus*, espécie listada no Livro Vermelho da Fauna Ameaçada do Estado de São Paulo, focando em aspectos de domesticação para o cultivo inédito da espécie em ambientes artificiais, incluindo o domínio dos manejos alimentar e reprodutivo, bem como a aplicação de biotecnologias reprodutivas e constituição de banco genético *in vivo* e *ex situ*. Esse trabalho está dividido em dois capítulos. No primeiro capítulo a domesticação de *P. mangurus* foi conduzida em três experimentos: o primeiro relacionado ao manejo nutricional e manutenção em cativeiro de espécimes selvagens adultos que foram mantidos em ambiente artificial entre as estações reprodutivas; o segundo avaliando a capacidade reprodutiva e eficiência do manejo reprodutivo entre animais selvagens e animais mantidos em ambiente artificial entre as estações reprodutivas; e o terceiro, treinamento alimentar em juvenis de *P. mangurus*. No segundo capítulo, a rastreabilidade de células germinativas primordiais (CGPs) no desenvolvimento embrionário inicial de bagre sapo *P. mangurus*, utilizando o método de hibridização *in situ* por montagem total (WISH), é descrita. Essa biotécnica permite que sequências específicas de nucleotídeos sejam detectadas em organismos morfologicamente preservados, possibilitando, entre outras finalidades, identificar e elucidar o padrão espaço temporal de migração de CGPs no desenvolvimento embrionário em *P. mangurus*. As CGPs possuem relevante papel no processo reprodutivo de inúmeras espécies e vem sendo alvo de notáveis esforços de compreensão que viabilizam sua aplicação em diversas biotecnologias reprodutivas, como quimerismo germinativo e criopreservação, que podem ser utilizadas na conservação da diversidade biológica.

Palavras chave: banco genético, propagação mediada, treinamento alimentar, Siluriforme, WISH.

INTRODUÇÃO GERAL

Alterações nos cursos naturais dos rios, poluição de mananciais, desmatamento de vegetação marginal, introdução de espécies exóticas, sobrepesca, o lixiviamento de produtos fitossanitários provenientes da indústria agrária (SLANINOVA et al., 2009; SOBJAK et al., 2017), desastres ambientais decorrentes da exploração de minérios (ESPINOLA et al., 2016), entre muitos outros fatores (VERONES et al., 2010), impactam sobre o bioma aquático resultando no acelerado declínio do número de espécies da ictiofauna aquática continental (DUDGEON et al., 2006).

Face à iminente transformação do ambiente natural advinda de ações antrópicas, muitos esforços vêm sendo empreendidos buscando a conservação da diversidade biológica aquática continental (BARLETTA et al., 2010; ICMBIO, 2018). São diversos os interesses pelo uso da biodiversidade aquática, cuja quantidade e qualidade são fundamentais para a manutenção da dinâmica destes ecossistemas (ALLAN DANIEL J; S, 1993; DIANA, 2009).

A atividade de domesticação de peixes remonta dos primórdios da civilização humana (JOBILING, 2011), e pode ser empregada como estratégia de conservação da biodiversidade (DAS AND ZAMAL 2000; DIANA, 2009), no cultivo de espécies que se encontrem perigosamente susceptíveis aos efeitos da atividade antrópica. Juntamente com a consolidação de bancos genéticos *in vivo* e *ex situ* (Gorda, Bakos, et al. 1995; Mijkherjee, Praharaj, et al. 2002), o cultivo em ambientes artificiais de espécimes ameaçados permite a realização de estudos de outra forma impraticáveis, ao mesmo tempo reduzindo custos econômicos e ambientais envolvidos nos esforços para a obtenção dos animais, que muitas vezes possuem populações reduzidas habitando locais remotos de difícil acesso.

Outra abordagem de grande interesse para a conservação da diversidade biológica é a aplicação de biotecnologias reprodutivas, que possam assegurar a reconstituição de uma espécie em vias de extinção ou até mesmo extinta no ambiente natural. É o caso de bancos genéticos constituídos a partir de material criopreservado e do transplante interespecífico de células germinativas ou quimera germinativa (OKUTSU et al., 2006; SAITO et al., 2019). Ambos intrinsecamente relacionados as células reprodutivas. Neste contexto, a técnica de hibridização *in situ* por montagem total (WISH) permite que

sequências específicas de nucleotídeos sejam detectadas em organismos morfológicamente preservados, possibilitando, entre outras finalidades, identificar e elucidar o padrão espaço temporal de migração de células primordiais germinativas (CGPs) durante o desenvolvimento embrionário do bagre sapo *P. mangurus* (THISSE; THISSE, 2008). As CGPs possuem relevante papel no processo reprodutivo de inúmeras espécies e vem sendo alvo de notáveis esforços de compreensão que viabilizam sua aplicação em diversas biotecnologias reprodutivas que podem ser utilizadas na conservação da diversidade biológica.

O *P. mangurus*, popularmente conhecido como bagre sapo, foi listado no Livro Vermelho da Fauna Ameaçada no Estado de São Paulo (ALESP, 2014). Esta espécie pertence à ordem dos siluriformes, família Pseudopimelodidae, gênero *Pseudopimelodus*, com distribuição nas bacias dos rios Uruguai, Paraná, Paraguai e La Plata, podendo atingir mais de 8 Kg (FROESE; PAULY, 2010), e é uma espécie pouco estudada dentre os peixes continentais neotropicais (MARTINEZ; OLIVEIRA; FORESTI, 2004). Entretanto essa espécie piscívora, possui potencial relevância no equilíbrio dos ecossistemas aos quais pertence, atuando na dinâmica populacional como predador nos ambientes em que se insere, além de representar significativo subsídio na dieta das populações ribeirinhas, possuindo atributos de interesse para o cultivo como ausência de ossos intramusculares (SMERMAN et al., 2002).

OBJETIVO

Gerais:

- Obter ferramentas para constituição de bancos genéticos e aplicação de biotecnologias para espécies siluriformes nativas, com vista à conservação e reposição de estoques em ambiente natural.
- Gerar subsídios para futuros trabalhos voltados à conservação da diversidade biológica

Específicos:

- Domesticação para o cultivo em ambientes artificiais, treinamento alimentar e reprodução de *Pseudopimelodus mangurus*.
- Rastrear células primordiais de linhagem germinativa no desenvolvimento embrionário inicial de *P. mangurus* por meio da técnica Whole-Mount *In Situ* Hybridization (WISH).
- Estabelecer protocolo de WISH aplicável às espécies de peixes siluriformes nativos ameaçados de extinção.

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CAPÍTULO I - Domestication of the catfish species *Pseudopimelodus mangurus* Valenciennes, 1835 (Siluriformes: Pseudopimelodidae)

ABSTRACT

Fish domestication can be considered a strategic approach to biodiversity conservation, supporting studies and reducing economic and environmental costs. Here, was evaluated, in three experiments, domestication management aspects for the neotropical catfish *Pseudopimelodus mangurus*, including their artificial reproduction in captivity, the species feeding management, and the establishment of an *ex situ* genetic bank. The first and second experiments were successful at the maintenance and artificial reproduction of wild-caught *P. mangurus* kept in tanks in between the reproductive seasons. The results demonstrate that the reproductive performance of animals kept in captivity (initial relative fertility-IRF = $609,25 \pm 36,6$ eggs/g) was near ($p > 0,05$) to the wild individuals (IRF = $679,21 \pm 45,66$ eggs/g). Feed training of *P. mangurus* juveniles (experiment III) was also conducted, using the gradual feed ingredient transition method, evaluating three feeding treatments with different concentrations of bovine heart and ration. At the end of the experiment, the treatment containing half bovine heart and half ration generated the highest values of weight gain ($0,10 \pm 0,16$ g), specific growth rate ($0,37 \pm 0,11$ mm), highest length ($47,78 \pm 2,35$ mm) and growth ($2,15 \pm 2,27$ mm), suggesting reasonable acceptability to artificial diets in the cultivation of this species.

Key-words: artificial propagation, feed training, genetic bank, marbled catfish, Siluriform.

RESUMO

A domesticação de peixes pode ser considerada uma abordagem estratégica para a conservação da biodiversidade, apoiando estudos e reduzindo custos econômicos e ambientais. Aqui, foram avaliados, em três experimentos, aspectos de manejo para a domesticação do peixe neotropical *Pseudopimelodus mangurus*, sua reprodução artificial em cativeiro, seu manejo alimentar e o estabelecimento de um banco genético *ex situ*. O primeiro e segundo experimento obtiveram sucesso na manutenção e reprodução artificial de *P. mangurus* mantidos em tanques entre as estações reprodutivas. Os resultados demonstram que o desempenho reprodutivo dos animais mantidos em cativeiro (fertilidade inicial relativa-FIR = $609,25 \pm 36,6$ ovos / g) foi próximo ($p > 0,05$) ao dos indivíduos selvagens (FIR = $679,21 \pm 45,66$ ovos / g). O treinamento alimentar de *P. mangurus* juvenis (experimento III) também foi realizado utilizando o método de transição alimentar gradual, avaliando-se três tratamentos alimentares com diferentes concentrações de coração bovino e ração. Ao final do experimento, o tratamento contendo metade coração bovino e metade ração gerou os maiores valores de ganho de peso ($0,10 \pm 0,16$ g), taxa de crescimento específico ($0,37 \pm 0,11$ mm), comprimento ($47,78 \pm 2,35$ mm) e crescimento ($2,15 \pm 2,27$ mm), sugerindo razoável aceitabilidade para dietas artificiais no cultivo desta espécie.

Palavras-chave: bagre sapo, banco genético, propagação artificial, Siluriforme, treinamento alimentar.

INTRODUCTION

Anthropogenic activities such as changes in the natural courses of rivers, pollution, deforestation, introduction of alien species and overfishing (Sobjak, Romão, et al. 2017) are deleterious to natural fish populations. Moreover, environmental disasters, such as occurred in the Brazilian cities of Mariana (2015) and Brumadinho (2019), leads to almost instantaneous death of hundreds of thousands of fish and other life forms, strongly affecting the natural fish stocks and, consequently, the regional socioeconomic activities (Carvalho, Ribeiro, et al. 2017; Rudorff, Rudorff, et al. 2018; Lopes, de Freitas, et al. 2019; Pereira, Cruz, et al. 2019).

In this regard, according with the IUCN Red List of Endangered Species (ICMBio 2018), only in the Neotropical region, 410 species are considered endangered. It suggests conservation efforts in order to attenuate the status of such fish species, including *ex-situ* genebanks (Comizzoli and Holt 2014). In fish, the most effective procedure for genebanking is to combine *in vivo* and *in vitro* genebanks (Froese and Pauly 2010) and in this regard the maintenance of live fish in artificial ponds and the cryobanking into liquid nitrogen are the most reliable procedures. However, before the establishment of such a conservation strategy, the first bottleneck that arise regards in the capture and domestication of wild specimens. In fact, the maintenance per se does not ensure the conservation of a given species, because the fish may be lost by simple death or poor traceability. Then it is necessary to establish procedures of artificial propagation and long-term genebanks (Gorda, Bakos, et al. 1995; Mijkherjee, Praharaj, et al. 2002).

Under this view, the maintenance of *in vivo* and *ex situ* genetic banks and the artificial propagation of endangered species are of great importance to their conservation making the domestication of wild fish an essential stage in both cases. To establish an *ex-situ* genebank, fish are captured in natural environments and then maintained in artificial tanks. Due to stress caused by capturing, transportation and the maintenance in the new environment, wild-caught fishes may present diseases and frequently die few days after capturing. In addition, wild-caught fish generally does not accept artificial diets such as commercial pellets giving rise to low survival. To feed the fish with natural food items is difficult to proceed in large scale and are both, time consuming and economically impracticable. In the case of carnivorous species, the administration of live fish also does

not give satisfactory results because the fish does not eat for long periods after capturing, period in which survival is critical.

In this context, the marbled catfish *Pseudopimelodus mangurus* Valenciennes, 1835 is an endangered fish species from the Neotropical region, recently listed in the Red Book of Endangered Fauna in the State of São Paulo (Bressan, Kierulff, et al. 2009; ALESP 2014). The marbled catfish is a carnivorous catfish from the order of Siluriformes, distributed in the basins of the rivers Uruguay, Paraná, Paraguay, and La Plata, and can reach more than 8 kg. Although unexploited in aquaculture, this species is part of the subsistence of thousands of fisherman families, presenting good meat quality and no intramuscular bones (Smerman, Díaz Castro, et al. 2002; Schuingues, De Lima, et al. 2013), making a potential candidate species for freshwater aquaculture. Moreover, *P. mangurus* plays an important role in the ecosystems where are endemic, acting in the population dynamics as predator and serving as food for birds and reptiles. However, there is not a procedure to capture and maintain the fish in artificial condition, what is critical for establishment of genebanks.

In order to develop conservation strategies for the marbled catfish, the present study was divided into three experiments: the first and second experiments aimed to domesticate and breed, respectively, wild-caught *P. mangurus* and, the third experiment aimed to determine a protocol of feed transition for the juveniles, evaluating the productive parameters and survival. The results obtained in such procedures are innovative and applicable in conservation and aquaculture. Therefore, this study aimed the use of domestication for the establishment of a management package that enables the cultivation and the establishment of a genetic bank for this neotropical catfish species, in order to ensure its reconstitution in the natural environment, and at the same time, serving as a starting point for other studies that may contribute to the exploration and conservation of this biological resource.

MATERIAL AND METHODS

Ethical Standards

All the procedures were performed according with the standards of the Ethics Committee on Animal Use (CEUA/CEPTA #010/2015) of the National Center for Research and Conservation of Continental Aquatic Biodiversity - ICMBio - CEPTA. Fish sampling were conducted with the permission (Sisbio #60.383-1).

Experiment I: Domestication of wild-caught adult *P. mangurus*

During the first semester of 2017, thirty wild-caught individuals of *P. mangurus* (0.670 g and 1.224 g 9 males and 21 females) were collected in the Mojiguassu river (21°55'36.476 "S, 47°22'0.836" W), of Pirassununga city, São Paulo State, Brazil, using line and hook (> 5 cm) and dead yellowtail tetra *Astyanax altiparanae* as a baitfish. Those fish were transported to the Laboratory of Fish Biotechnology, ICMBio – CEPTA, anesthetized with 100 mg L⁻¹ clove oil (Biodinâmica[®], Ibitiporã-PR, Brazil) and obtained the parameters of total length (TL), standard length (SL) and weight (W). Each fish was tagged with microtransponder for later identification.

The tagged fish were divided into three groups of ten individuals in 3000-L fiberglass tanks with constant water flow. Each tank was covered with a 1-mm nylon mesh in order to reduce light incidence, and PVC tubes (2.5 x 0.15 m) were provided for shelter.

Each month, gavaging (forced feeding) and prophylactic treatments were provided. For gavaging, a powdered commercial ration containing 55% of crude protein was hydrated (3 L of water for 1 kg of ration) and loaded in a 50-mL syringe. Feeding was achieved by administration using a silicone cannula (inner diameter: 6.4 mm; outer diameter: 8.0 mm) directly in the stomach (20 - 50 mL, depending on the fish size). The prophylactic treatments consisted on the application of mixture of enrofloxacin 5% (10 mg kg⁻¹) (Vencofarma[®], Londrina-PR, Brazil), levamisole 7.5% (8 mg kg⁻¹) (Ripercol[®], Campinas-SP, Brazil) and vitamin complex (0.1 mL kg⁻¹) (Potenay[®], Campinas-SP, Brazil). Such a procedure of forced feeding and prophylactic treatments was repeated each 10 days until four months of feed training. Feed training was achieved using three diets containing: 1) dead yellowtail tetra (*Astyanax altiparanae*); 2) bovine heart (25-g strips) and 3) frozen sardine fillet (25 g-pieces). Those food items fixed at the bottom of the tanks using a wire. Food consumption, and mortality of the fishes were checked twice

a day with replacement of the food items. Each 30 days, parameters of length and weight were measured in order to obtain growth rates.

Experiment II: *P. mangurus* Reproduction.

After the 1st experiment, the resultant 21 females were fed daily *ad libitum* or until apparent satiety, with *A. altiparanae* diet and spared of any handling during two months. After this period, the fish were collected, anesthetized and induced to spawn. Nineteen females were selected for spawning trials, based on external characteristics (ventral bulging and hyperemia in the urogenital region). Fish were induced to spawn using the protocol proposed by Arashiro et al. (2018), by the “dry” method. Females were injected with carp pituitary extract (CPE) with 0.6 mg kg⁻¹ followed by a second dose of 6 mg kg⁻¹ six hours afterwards. As control group, 19 wild-caught females were collected and immediately induced to spawn using the same procedures. Males were induced by a single dose of 2 mg kg⁻¹ at the same time of the second dose of the females. For sperm sampling, males were euthanized 5-h after injection with an overdose of clove oil (500 mg L⁻¹) (Biodinâmica®, Iporã-PR, Brazil), the testis were removed and minced in Eagle’s MEM solution pH 7.8 (Sigma # M0268, St. Louis, USA) and the debris were removed in order to keep only the cell suspension. Oocytes were obtained by stripping after 156 hourgrades (approximately 6 h after the second dose of CPE at 26°C), and then estimated the Relative Initial Fertility (RIF), where FR = number of viable eggs / 100 eggs fertilized, adapted from Godinho (2007). The oocyte mass was inseminated and the gametes were activated using hatchery water. Incubation was achieved in a 60-L jars and the early development was observed at the stereomicroscope (Nikon SMZ 18, Tokyo, Japan) and measured the fertilization rate (FR) by the percentage of viable embryos at the blastopore stage.

Experiment III: *P. mangurus* Juvenile Feed Training

From the spawning resulted from the experiment 2, three batches of post-fry were used to composed a pool. Those larvae were hatched at the same day, with differences of few hours of hatching. The juveniles were maintained in a 300-L fiberglass tank with

constant water flow. From the third day, those juveniles were fed four to six times a day with *Artemia nauplii* and *Prochilodus lineatus* larvae until 30-days-age.

The resultant juveniles were divided onto 12 fiberglass tanks (10 L each) at a density of seven fishes per tank, with constant water flow and were covered by a 1-mm nylon mesh to reduce light intensity as recommended by several authors (Appelbaum and McGeer 1998; Cestarolli 2005; Feiden, Hayashi, et al. 2006). Four feed treatments were conducted: minced bovine heart (treat.1); minced bovine heart with gradual ration increments until reaching 50% bovine heart and 50% ration (treat. 2); bovine heart with gradual increases of ration until reaching 100% ration (treat. 3). The gradual increase of the feed in treatments 2 and 3 was equivalent to 10% every week.

At the beginning of the experiment and each 7 days, weight and length were determined until the period of 10 weeks. The animals were observed three times a day for counting, removal of the dead fish to determine the survival rate.

Parameters of weight gain (WG=Final Weight - Initial Weight), Growth (Final Length - Initial Length), Specific Growth Rate (SGR: $[\ln(\text{Final Weight}) - \ln(\text{Initial Weight})] / n \text{ } ^\circ \text{ of days} * 100$) were calculated from each treatment.

Statistics

All data were evaluated for normality and homoscedasticity using the Cramer-Von Mises and Levene tests, respectively. For TF and FIR, a t-test was used. For the other parameters, a two-way ANOVA was performed and the means were compared with Tukey's multiple range test ($\alpha = 5\%$). Analysis was performed using the software STATISTICA v. 10.0 (Statsoft, Tulsa, U.S.A.) and significance was set at $P < 0.05$.

RESULTS

Experiment I: Domestication of wild-caught *P. mangurus*

The food consumption of wild-caught *P. mangurus* keep in tanks for four months was initially higher in the treatment that used yellowtail tetra (treat 1), lower in the bovine heart treatment (treat 2) and had a gradual increase in the sardine treatment

(Fig. 1). Consumption was not observed in any group during the first month of experimentation. In the last month of feed training period, all the food offered (175g per treatment, daily) was consumed in all three treatments.

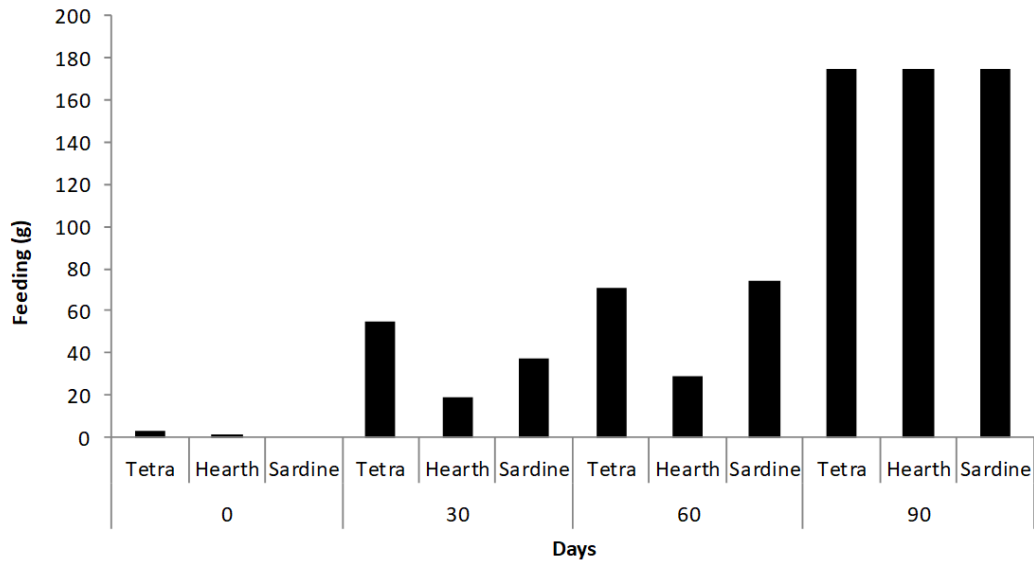


Figure 1: Feed consumption of wild-caught *Pseudopimelodus mangurus* kept in tanks and fed with tetra fish, bovine heart and sardine for three months (n = 30).

The total weight of the females did not present statistical differences within all the treatments during the four months of experimental period (Fig. 2).

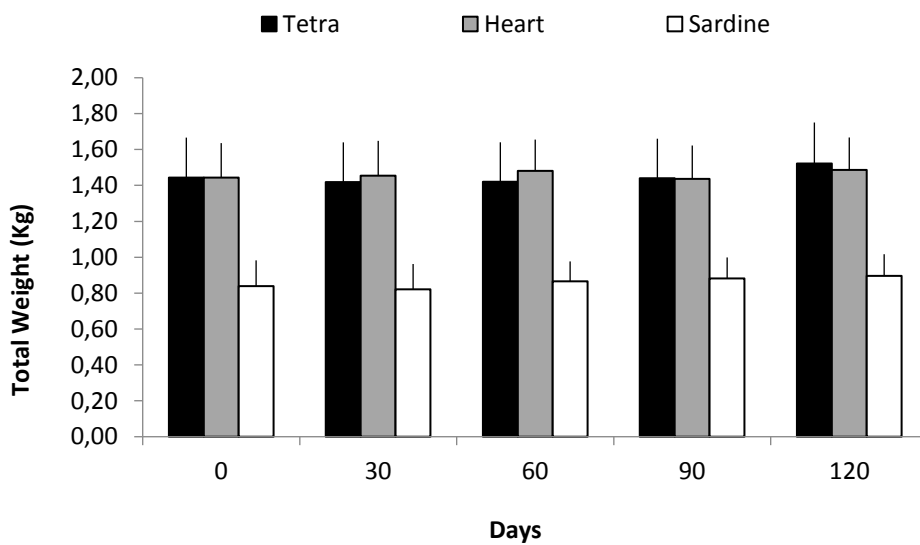


Figure 2: Total weight means of wild-caught *P. mangurus* females kept in tanks and fed with tetra fish, bovine heart and sardine for four months (n = 21).

Similarly, as occurred with the total weight, the *P. mangurus* female's standard length did not present differences during the four months of experimental period (Fig. 3).

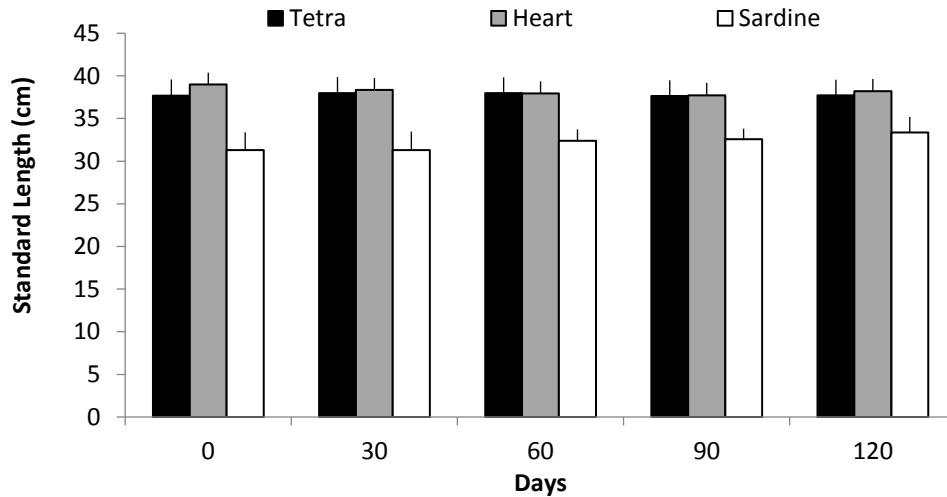


Figure 3: Standard length means of wild-caught *P. mangurus* females kept in tanks and fed with tetra fish, bovine heart and sardine for four months (n = 21).

The nine conditioned wild-caught males of *P. mangurus* gave similar results to those females, maintaining a constant standard length (Fig. 4) and total weight means (Fig. 5), which obtained a progressive slight increase with a small oscillation during the experiment with no significant differences.

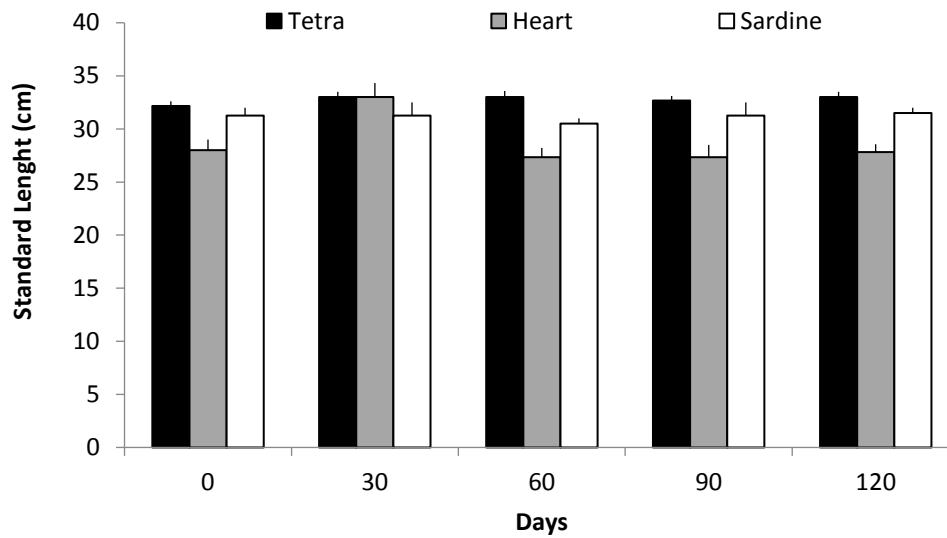


Figure 4: Standard length of wild-caught *P. mangurus* males kept in tanks and fed with tetra fish, bovine heart and sardine for four months (n = 9).

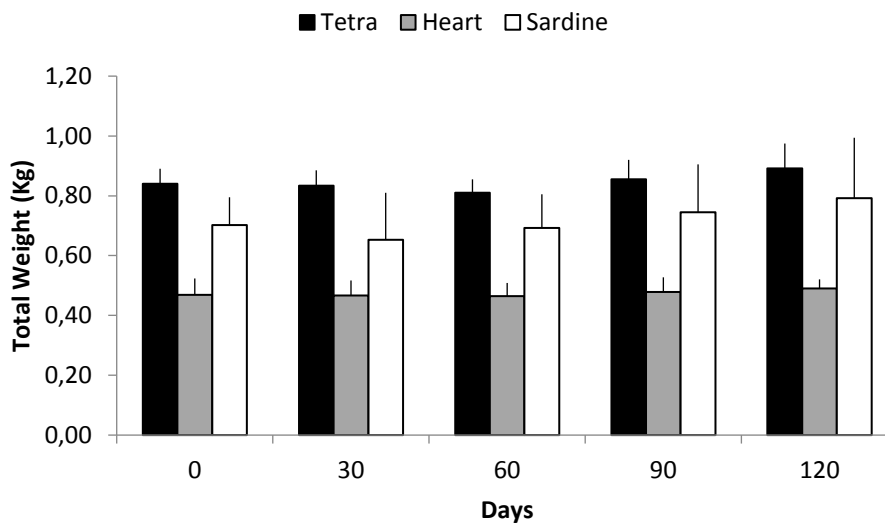


Figure 5: Total weight of *P. mangurus* wild-caught males kept in tanks and fed with tetra fish, bovine heart and sardine for four months (n = 9).

Based on the results above, in which the natural food item yellowtail tetra as food source gave rise to good results, this was the treatment chosen to feed the animals until the spawning period and for experiment 2.

Experiment II: *P. mangurus* Reproduction.

Among the wild-caught *P. mangurus* females, keep in tanks for seven months and feed for two months with *ad libitum* yellow tail tetra diet, 19 of 21 captive females have a positive response to hypophysation method. The analyzed reproductive parameters, fertilization rate and relative initial fertility, showed a relative proximity (Relative initial fertility: wild 679.21 ± 45.66 eggs.g⁻¹; captivity 609.25 ± 36.00 eggs.g⁻¹; *P*-value = 0.2660; Fertilization Rate: wild $91.6 \pm 2.5\%$; captivity $82.4 \pm 3.1\%$; *P*-value = 0.0415) between the captive and wild animals evaluated.

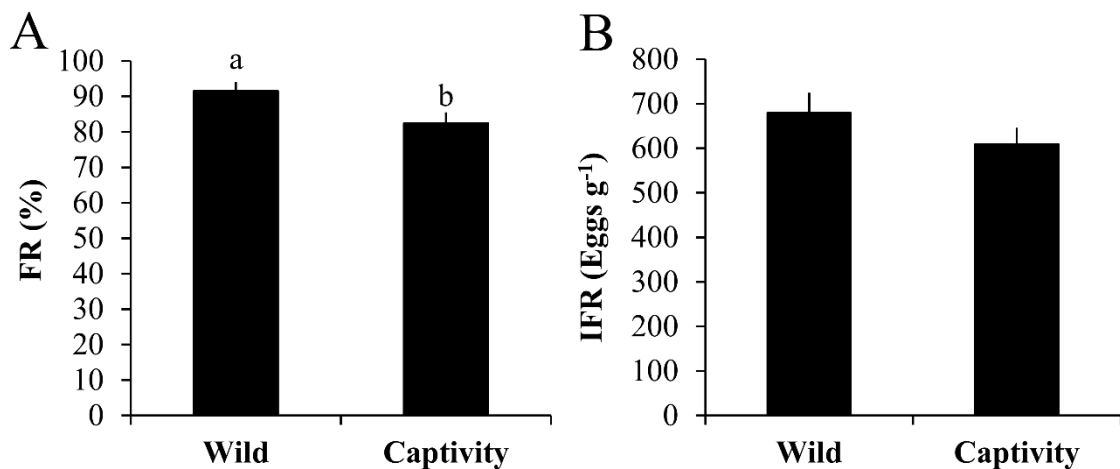


Figure 6: Fertilization rate (A) and relative initial fertility (B) of captive and wild-caught *P. mangurus*. (*P*-value: A= 0,0415; B = 0,266).

Experiment III: Juvenile Feed Training

The performance of *P. mangurus* juveniles submitted to three different diets for 10 weeks is shown on Fig. 6. Although it was observed an increase in standard length and live weight and no significant effects were found on any parameter evaluated. The mean live weight, which in the first week was 0.73 ± 0.04 g, reached 1.23 ± 0.26 in the tenth week (fig. 7).

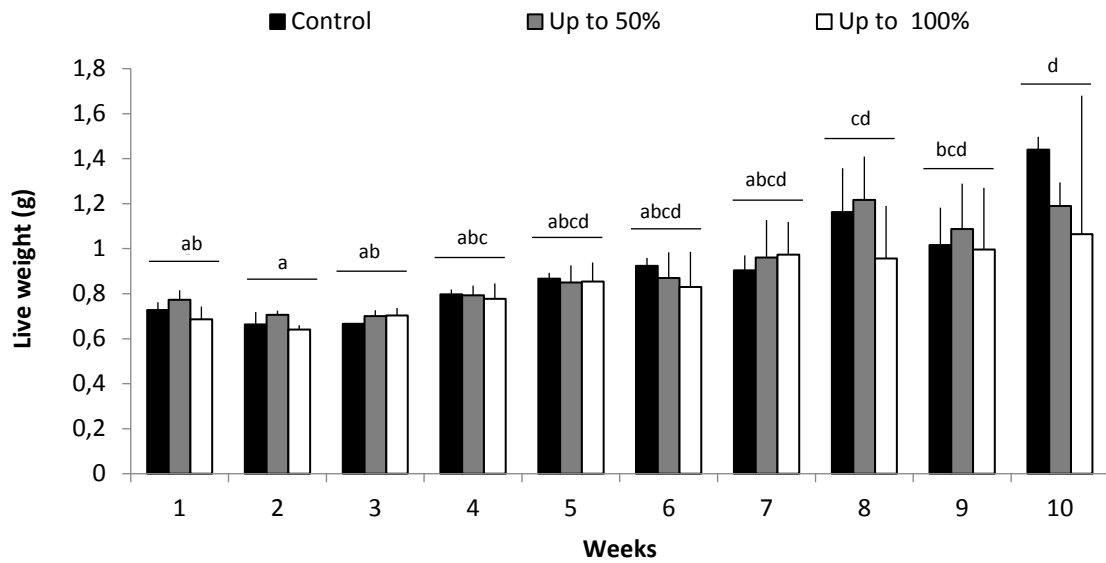


Figure 7: Live weight means of *P. mangurus* juveniles fed with the three experimental diets (treat 1: bovine heart; treat 2: bovine heart substituted with up to 50% of artificial feed; treat 3: bovine heart substituted with up to 100%) in triplicate for ten weeks. Different letters indicate a significant difference according to Tukey's multiple range test ($\alpha = 0.05$).

The zootechnical parameters varied strongly during the experimental period. The total weight gain was 0.50 ± 0.20 g and the mean weight gain was 0.02 ± 0.09 g.week⁻¹ (minimum -0.08 at the eighth week and a maximum of 0.17 g.week⁻¹ at the ninth and tenth weeks) (fig. 8).

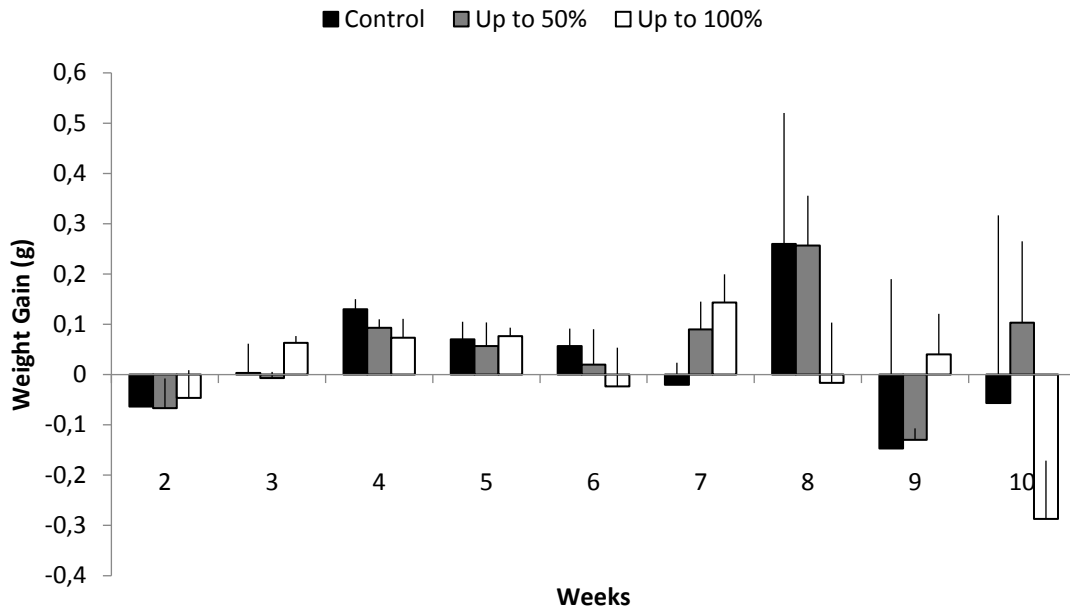


Figure 8: Weight gain of *P. mangurus* juveniles fed with the three experimental diets (treat 1: bovine heart; treat 2: bovine heart substituted with up to 50% of artificial feed; treat 3: bovine heart substituted with up to 100%) in triplicate for ten weeks ($P = 0.0601$).

Mean standard length, which was 37.14 ± 0.68 mm in the first week, was 46.30 ± 1.87 mm in the tenth week (fig. 9).

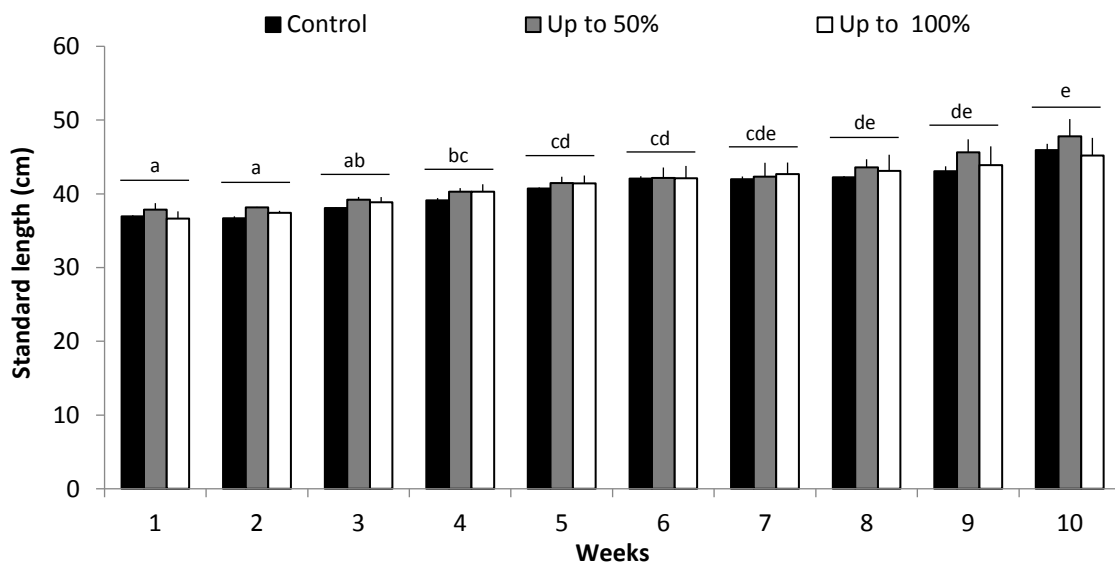


Figure 9: Standard length means of *P. mangurus* juveniles fed with the three experimental diets (treat 1: bovine heart; treat 2: bovine heart substituted with up to 50% of artificial feed; treat 3: bovine heart substituted with up to 100%) in triplicate for ten weeks. Different letters indicate a significant difference according to Tukey's test ($\alpha = 5\%$).

Total growth was 9.16 ± 1.19 mm and mean growth was 0.93 ± 0.61 mm.week⁻¹ (minimum of 0.21 in the seventh week and maximum of 1.31 in the fifth week) (Fig. 10).

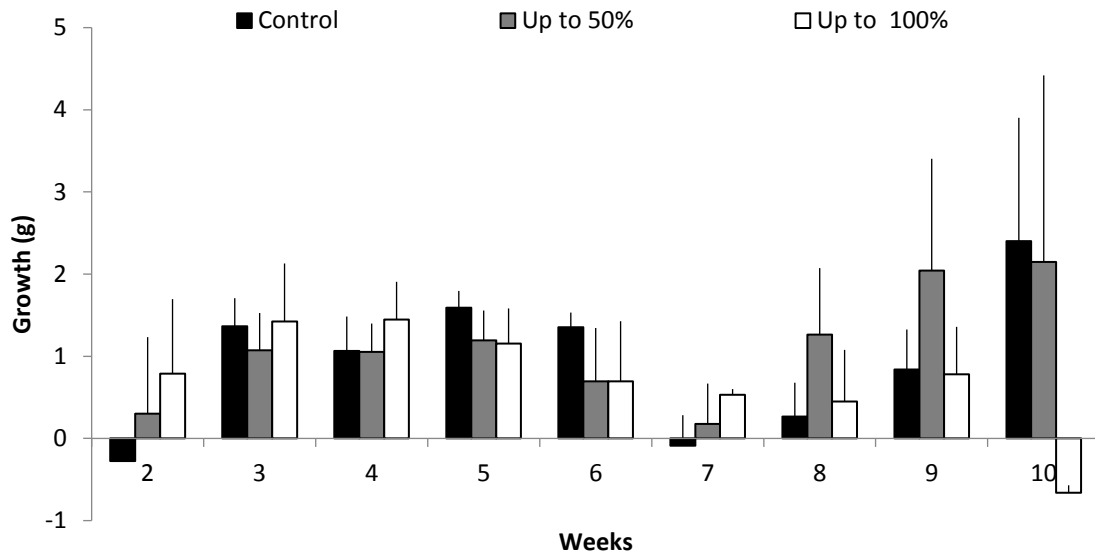


Figure 10: Growth of *P. mangurus* juveniles fed with the three experimental diets (treat 1: bovine heart; treat 2: bovine heart substituted with up to 50% of artificial feed; treat 3: bovine heart substituted with up to 100%) in triplicate for ten weeks (P-value = 0.6142).

Although no significant differences were found between the treatments proposed, the treatment with half bovine heart and half ration at the end of the experiment (treat. 2) was the one that generated higher values of weight gain (0.10 ± 0.16 g) specific growth rate (0.37 ± 0.11 mm), and also the highest length (47.78 ± 2.35 mm) and growth (2.15 ± 2.27 mm) values observed in the tenth week (fig. 11).

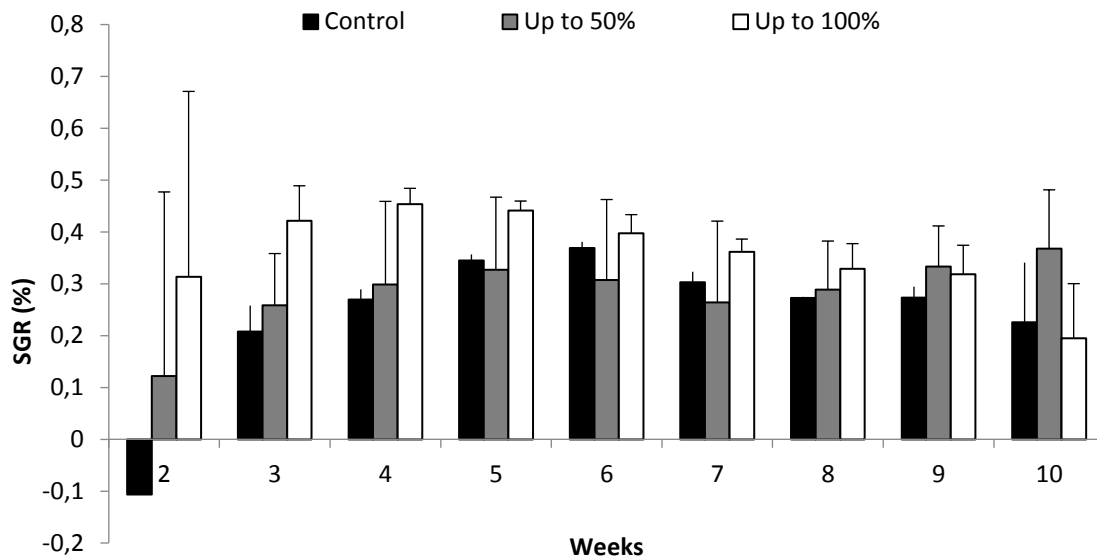


Figure 11: Specific growth rate (SGR) of *P. mangurus* juveniles fed with the three experimental diets (treat 1: bovine heart; treat 2: bovine heart substituted with up to 50% of artificial feed; treat 3: bovine heart substituted with up to 100%) in triplicate for ten weeks (P -value = 0,05961).

The survival of juveniles of *P. mangurus* also showed no significant differences within treatments. Survival, however, decreased approximately 5% per week until the seventh week. After the eighth week the survival reached 50% ($49.2 \pm 7.3\%$), and decreased more than 8% per week until reaching a mean of $24.4 \pm 7.3\%$ at the end of the experiment (fig. 11).

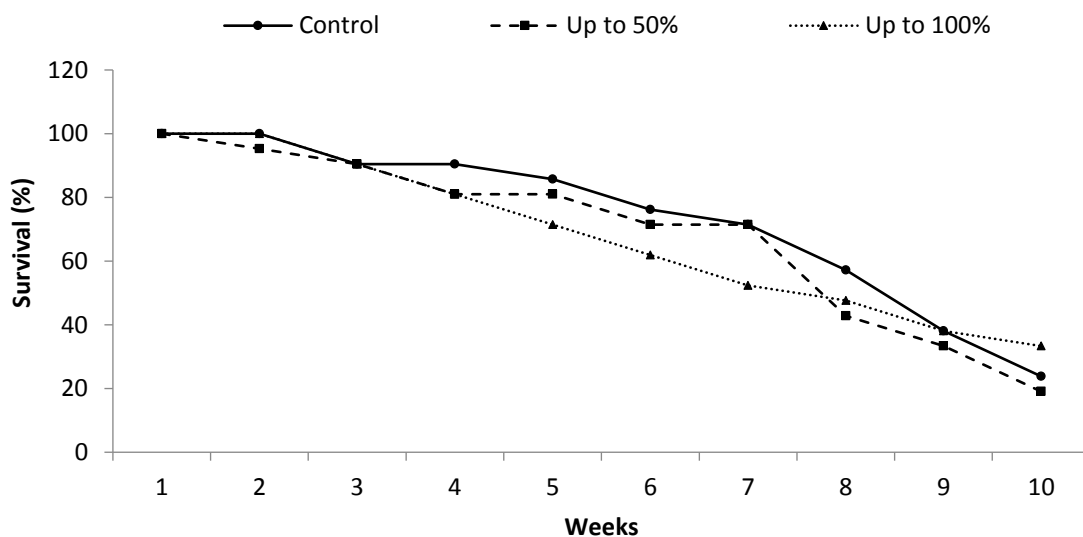


Figure 62: Survival of *P. mangurus* juveniles fed with the three experimental diets (treat 1: bovine heart; treat 2: bovine heart substituted with up to 50% of artificial feed; treat 3: bovine heart substituted with up to 100%) in triplicate for ten weeks.

DISCUSSION

The domestication of a fish species is not based only in the capture and maintenance of the specimens in captivity, but also in the control of reproduction, being considered fully domesticated after the achievement of total independence of natural stocks (Teletchea and Fontaine 2014). Feeding and reproductive factors is a critical point in the production of aquatic organisms (Izquierdo 2001). Moreover, the domestication of carnivorous species may be more difficult because of their predatory and competition behavior resulting in cannibalism (Ruzzante 1994). Therefore, it is important to determine the feeding procedure and provide a prophylactic protocol to avoid diseases until reaching a satisfactory reproductive performance.

Feeding management in Siluriformes is still challenging for many species (Robinson, Li, et al. 2001; Soares 2008.) as in the case of *P. mangurus* in which this study is the first report for the species. In addition, after the acceptance of artificial feeding, the fish were able to spawn and presents similar reproductive performance with wild specimens collected during the spawning season which were then presumably in good conditions

The analysis performed in this experiment showed no preference among the provided feeds, however, the fact that *A. altiparanae* presented a quicker acceptance and that it is a part of the natural nutrition of *P. mangurus*, as observed in pre-experimental necropsies, resulted in this food to be the chosen. The consumption data of the three treatments at the *ad libitum* feeding period showed a high capacity of food ingestion in this species, reaching intake indices higher than thirty percent of the live weight per meal observed in some female individuals, which may potentially influence on an appropriate feeding frequency for the specimens' cultivation.

For Arashiro *et al.* (2018), *P. mangurus* can be considered a rheophilic neotropical fish, which performs reproductive migration. According to Costa *et al.* (2012), rheophilic fish need to be displaced against the stream to spawn and are stimulated by rainfall and flooding, which alter the availability of various water substrates necessary for sexual maturation and spawning induction. For Almeida (2013), in Brazilian rheophilic species, the lack of current in fish tanks is the main obstacle to reproduction, and the use of hormones for the practice of artificial reproduction is mandatory. The increase of the food supply and interruption of the managements aiming at the minimization of the stress due to the handling (Schreck et al., 2001), intended to provide

physiological conditions for sexual maturation, since the reproductive hormone induction using crude pituitary extract acts directly on the gonads of the fish, being necessary that these are functionally mature for the success of the artificial reproduction (Mylonas et al., 2010; Hainfellner et al., 2012). However, the feed management with ad libitum *A. altiparanae* diet and the environment were effective to provide the conditions for *P. mangurus* spawning. It indicates that the management provided gonadal maturation, with a similar egg weight and size and a complete oocyte vitellogenesis, which are important for reproductive success (Wallace and Selman 1981). The reproduction in captivity is one of the most important indicators of success for domestication of wild animals (Bilio 2007; Fabrice 2018).

The fertilization rates observed here (82,4%), is similar with other neotropical Siluriformes species: 59% in *Lophosilurus alexandri* (Santos, Sampaio, et al. 2013), 60% in *Trachelyopterus galeatus* (Santos, Arantes, et al. 2013), 69.5% in *Steindachneridion parahybae* (Honji, Tolussi, et al. 2012), 72.4% in *Rhinelepis aspera* (da Rocha Perini, Sato, et al. 2010).

The reproduction per se is not the final step for species maintenance. The survival of juveniles is also a critical stage in which will provide the juveniles for restocking programs. In this study, we successfully established a procedure of artificial feeding of the juveniles. Diemer, Neu, et al. (2010), working with *Pimelodus britskii* feeding using nauplii of artemia + feed, found the much higher weight and length gains, with a mean survival of 94% of the lots. Luz, Salaro, et al. (2002), also obtained success in feeding giant trahira juveniles (*Hoplias cf. lacerdae*), providing a diet similar to the present experiment, obtaining a satisfactory weight gain and survival of 96%. Luz, Santos, et al. (2011), obtained similar survival rates with the present study, providing a dry diet for juvenile *Lophosilurus alexandri*, a species belonging to the same family as *P. mangurus* (Pseudopimelodidae). Feiden, Ferrari, et al. (2008), carried out black bass (*Micropterus salmoides*) feeding training using formulated diet mixture, achieving better results when compared to formulated diet mixture and bovine heart. Salaro, Junior, et al. (2012), verified interesting results using gelatin as a moist ingredient in the feed training of giant trahira *Hoplias cf. lacerdae*, which may be an alternative to the training of *P. mangurus* juveniles.

In conclusion, the present study was successful in the domestication of wild-caught *P. mangurus*. Furthermore, the results presented here demonstrate that the reproductive performance was similar with wild-caught species during the spawning

reason. It was also established in this study a feeding strategy to rear the obtained potentially applicable in both, based and applied sciences.

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CAPÍTULO II – Traceability of primordial germ cells (PGCs) in the early embryonic development of marbled catfish *Pseudopimelodus mangurus* (Teleostei: Siluriformes: Pseudopimelodidae) using whole-mount *in situ* hybridization.

ABSTRACT

The primordial germ cells (PGCs) in the early embryonic development of marbled catfish *Pseudopimelodus mangurus* are tracked by the whole mount *in situ* hybridization (WISH) method. The WISH technic allows specific nucleotides sequences to be detected in morphologically preserved organisms and, among other purposes, can be used to identify the PGCs, as well as to elucidate the spatial and temporal migration pattern of PGCs and formation of the reproductive tissues during the embryonic development. WISH was performance using a gen-cell-specific-marker *P. mangurus nanos 3* RNA antisense probe to determinate the localization of PGCs. The present study aims to trace primordial germ cells in the early embryonic development of marbled catfish *Pseudopimelodus mangurus*, through the method of *in situ* hybridization by whole mount and to establish a protocol of biotechnology tools applicable to native Siluriformes species, in order to provide subsidies for conservation efforts.

Key words: biotechnology, conservation, mediated propagation, Siluriform

RESUMO

Neste trabalho são rastreadas as células germinativas primordiais (CGPs) no desenvolvimento embrionário inicial de bagre sapo *Pseudopimelodus mangurus*, utilizando a o método de hibridização *in situ* por montagem total (WISH). A técnica de WISH permite que sequências específicas de nucleotídeos sejam detectadas em organismos morfológicamente preservados e, entre outras finalidades, pode ser empregada na identificação de CGPs, bem como elucidar o padrão espaço temporal de migração de CGPs e a formação dos tecidos reprodutivos durante o desenvolvimento embrionário. A técnica de WISH foi conduzida utilizando sonda de RNA antisense com marcador celular específico *P. mangurus nanos-3* para determinar a localização de CGPs. O presente estudo objetiva o rastreamento de CGPs e a determinação de seu padrão migratório durante o desenvolvimento embrionário inicial de *P. mangurus*, por meio de hibridização *in situ* por montagem total, e estabelecer um protocolo para o uso de ferramentas biotecnológicas aplicáveis em espécies Siluriformes nativas, afim de gerar subsídios para esforços de conservação.

Palavras chave: biotecnologia, conservação, propagação mediada, Siluriformes.

INTRODUCTION

Primordial germ cells (PGCs) are the precursor cells of the germ line cells in teleosts and are the only cells in developing embryos that can transmit genetic information to the next generation. PGCs are the cells that will give rise to the reproductive tissues and initiate the differentiation from the somatic-cell lineage early in embryogenesis, migrating to the gonadal ridge during embryonic and larval development, and then differentiate into gametes in the gonads (RAZ, 2003; WYLIE, 1999)

Currently, PGCs has played a significant role in teleost biotechnology, including genetic modification, cryopreservation, and mediated propagation. Interspecific transplantation, induced polyploidy and clonal reproduction in the *P. mangurus* are suitable for experiments using PGCs (KOBAYASHI et al., 2007; OKUTSU et al., 2006; YAMAHA et al., 2007). Therefore, PGC have a considerable potential value for biodiversity conservation efforts such as gene banking and cryopreservation, particularly through the production of donor gametes using germ-line chimeras to obtain donor-derived offspring, within and between species (KAWAKAMI et al., 2010; SAITO et al., 2019). Such complex procedures demand deep knowledge to be effective, including the origin and the migration pattern of the PGCs, with have species-specific particularities.

In recent years, were identified in several species genes that are specifically expressed in germline cells, for example, vasa-related genes, which can be employed as marker genes of PGCs (GEIJSEN et al., 2004; MOLYNEAUX; WYLIE, 2004). Relating microscopic topological information to gene activity at the DNA or mRNA level, the whole mount in situ hybridization (WISH) is a method widely used to describe the expression pattern of developmentally regulated genes. WISH allows specific nucleotide sequences to be detected in morphologically preserved embryos and, among other purposes, can be used to identify the PGCs, as well as to elucidate the spatial and temporal migration pattern of PGCs and formation of the reproductive tissues during the embryonic development (THISSE; THISSE, 2008).

Having a varied popular denominations where it is endemic, such as “Bagre Sapo, Jaú Sapo, Peixe Sapo, Brecumbucu, Manguriú, Piacoruru, Pacamão, Piracururu”, the marbled catfish *Pseudopimelodus mangurus* (CUVIER; VALENCIENNES, 1835), is a carnivorous neotropical catfish from the order of Siluriformes, Pseudopimelodidae

family, distributed in the basins of the rivers Uruguay, Paraná, Paraguay, and La Plata, and can reach more than 8 kg, being the largest representative of the *Pseudopimelodus* genus (FROESE; PAULY, 2010). Recently, this species was listed in the Red Book of Endangered Fauna in the State of São Paulo (ALESP, 2014; BRESSAN; KIERULFF; SUGIEDA, 2009) and, for this reason, has been the focus of research aimed at its conservation (ARASHIRO et al., 2018; MARTINEZ; OLIVEIRA; FORESTI, 2004).

However, there is no previous studies on the germ cell lineage in the *P. mangurus*. Here, we have tracked, using whole-mount *in situ* hybridization method, with a gen-cell-specific-marker *P. mangurus vasa* RNA antisense probe to determinate the localization of PGCs in the early embryonic development of marbled catfish *Pseudopimelodus mangurus*, in order to provide subsidies for conservation efforts of neotropical siluriform species, with the use of developmental engineering by means of PGCs. The knowledge of the molecular PGC aspects as well the migration route formation is essential for future biotechnology applications for conservation species.

MATERIALS AND METHODS

Ethical Standard

All the procedures performed in the animals used in the present study are in compliance with the standards of the Ethics Committee on Animal Use (CEUA / CEPTA #010/2015) of the National Center for Research and Conservation of Continental Aquatic Biodiversity - ICMBio - CEPTA.

Origin of *P. mangurus* embryos

Mature *P. mangurus* wild-caught specimens were collected from the Mojiguassu River, downstream of the Emas Waterfall (21°55'36.476 "S, 47°22'0.836" W), in the city of Pirassununga - SP, using line (0.4 mm) and hook (> 5 cm), licensed in according to the Brazilian law (Sisbio #60.383-1). To obtain the fertilized gametes, artificial reproduction

was performed by hypophysation using crude carp pituitary extract (CPE), as previously described in Arashiro *et al.*, 2018.

After fertilization, the embryos were incubated in Petri dishes in aliquots of approximately 150 eggs, which were kept in BOD (Biological Oxygen Demand) incubators at 26 ° C. Embryonic development was followed and samples were collected periodically, from the first cleavage to hatching stage, using a trinocular stereomicroscope (Nikon SMZ 1500, Japan).

Determination of primordial germ cells

The identification of *P. mangurus* primordial germ cells during embryogenesis was determined by detection of *nanos 3* mRNA related gene expression using whole mount *in situ* hybridization (WISH) method. WISH was performed by immunohistochemistry using an alkaline phosphatase-conjugated antibody against digoxigenin and a chromogenic substrate, as previously described in zebrafish (Thisse and Thisse, 2010), with adjustments.

Expression of *nanos-3* was detected with a digoxigenin-labeled antisense *nanos-3* RNA probe transcribed from 576 pb fragment of the coding region of *P. mangurus*. The gene sequence was obtained from egg transcriptome of *P. mangurus* and used as a template for the synthesis of antisense RNA probe, labeled with digoxigenin-linked nucleotides. For gene sequencing, the total RNA from *P. mangurus* eggs was isolated using Trizol (Invitrogen®) and sent for sequencing at the São Paulo University - USP Genome Center and genes assembled by bioinformatics tools, and bioinformatics analysis was performed by the Center for Bioinformatics of Campinas State University - UNICAMP. The *nanos-3* sequence from *P. mangurus* was not deposited at Gen Bank yet, and it can be observed below.

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ATGGTGTTTTCTCTGCTGCACTACGTCCTGTCGGCTCACGGCTCCATGCAGT  
CCCGAGAGCAGCGGCACTTTCAGCCGTGGCGTGATTACATGGGTCTGGCGG  
ACACGGTGCAGAGATGCGAGATTCCCGGCTCACGGAAAGCTCCGGTCCCG  
AACCTCACCAGGAGGTTCGGTTTTACGCGTGAGGTCTCTCCTCACAGTCACGA  
TGGCGAGCTCCAGGCGTTGCCCGAGACCGAGGTGTCCGAGGTAGGAGGAAG  
AGCGAAGGTGCGACTGAAATGCGGTCGTTCCAGGACTACTGGGTATCATCC  
ACCTCCACCTTACCTCCATCTCCATTACCACCATCACCACCATCACCTCCA
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GCGGTCGGTCCTCGCTCTGGGGAGAAAGTCTGCACCTTCTGCAAACACAAC
GGCGAGTCTGAAAACGTGTTTCATGTTCGCACCGCCTGAAAGGCCGCGGGCGGA
GAGGTGGTGTGTCCTTACCTGCGCCGGTACGTGTGTCCGCAGTGCGGGGCG
ACGGGCGCCCGCGCGCACACTAAGCGCTTCTGCCCGCTTGTGGACAACACG
TACAGCTCCGTGTACACCCGGGATCCCCGGTAATGA

For gene amplification, the primers were drawn for *nanos-3* gene (Forward- 5'-GAATTCTTTTCTCTGCTGCACTACG-3' and reverse 5'-AAGCTTGGTGTACACGGAGCTGTA-3', with EcoRI and HindIII restriction site, respectively). cDNA from egg total RNA was used as template, and the cDNA was obtained using the SuperScript III First-Strand kit (Invitrogen®). The amplified region of approximately 576 bp, was performed, visualized in 2% agarose gel and recovered using E.Z.N.A Gel Extraction (Omega®). The sequence amplified was cloned into pGEM vector (Promega®) and subcloned into the pSP64 vector (Promega®), using the EcoRI and HindIII restriction enzymes. For the *in vitro* synthesis of antisense RNA were used the DIG RNA Labeling Mix (Roche®) and mMMESSAGE mMACHINE SP6 kit (Invitrogen®). Antisense RNA probes were used in the WISH technique, in accordance as recommended in Thisse and Thisse, (2010).

For WISH, dechorionated *P. mangurus* embryos at every stage were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS), pH 5.5, for 20 to 30 h at 4°C. Fixed embryos were treated with hydrogen peroxide (3% H₂O₂/0.5% KOH) for 60 min at room temperature, dehydrated with a graded methyl-alcohol series (25% (vol/vol), 50% (vol/vol) and 75% (vol/vol) methanol in PBS) and stored in 100% methanol at -20°C until use.

The embryos were gradually rehydrated by successive dilutions of methanol in PBS (75% (vol/vol), 50% (vol/vol) and 25% (vol/vol) methanol in PBS), and washed four times, for 5 min each, with PBS containing 0.1% Tween 20 (PBT), at room temperature. The embryos were then treated with Proteinase K (10 µg/mL in PBS) at room temperature, for 1 min for the blastula to gastrula period, for 2 min for 1-somite to 8-somite stage embryos, for 6 min for 9- somite to 18 somites, and for 20 min for embryos older than the 18-somite stage. Embryos were postfixed in 4% paraformaldehyde in PBS for 20 min and then washed four times with PBT for 5 min per wash.

The embryos were prehybridized for 5 hours at 70°C in hybridization mix (50% deionized formamide, 5× standard sodium citrate (SSC), 50 µg/mL of heparin adjusted to

pH 6.0) and hybridized in 200 mL of the same buffer containing antisense DIG-labeled RNA probes (approximately 0.1 µg/mL) overnight at 70°C. The embryos were transferred to hybridization mix (HM), without RNase-free tRNA and heparin, and gradually changed from HM to 2×SSC through four series of 10 min (75% HM, 50% HM, 25% HM and 100% 2×SSC) at 70°C.

The embryos were washed twice with 2×SSC at 70°C for 30 min, then the 2×SSC was progressively replaced per PBT through four series of 10 min at room temperature in horizontal shaker (40 r.p.m.). The embryos were incubated in blocking buffer (1×PBT with 2% goat serum (vol/vol) and 2 mg/mL BSA (bovine serum albumin) for 4 hours at room temperature and hybridized overnight in blocking buffer containing 1/10,000 anti-dioxigenin-AP Fab fragments (Roche) at 4°C with gentle agitation (40 rpm on a horizontal shaker).

The embryos were then washed six times, 15 min per wash, in PBT and three times, 5 min per wash, in alkaline Tris buffer (100 mM HCl, pH 9.5, 50 mM MgCl₂, 100 mM NaCl and 0.1% Tween 20 (vol/vol)) at room temperature with gentle agitation.

The embryos were replaced with 0.7 mL staining solution (NBT/BCIP), fresh prepared and kept in the dark to prevent background staining, and periodically monitored until the color develops to the desired extent: 1 hour for the cleavage stage; 2-4 hours for the blastula to gastrula period; 4-8 hours for segmentation.

When the desired staining intensity was reached, they were washed 3 times for 15 min with stop solution (1×PBS, pH 5.5, 1mM EDTA, 0.1% Tween 20 (vol/vol)). They were then incubated in 1×PBS, pH 3.0, and observed with a trinocular stereomicroscope (Nikon SMZ 1500, Japan), being taken digital images through a CCD camera (Nikon DSFI1, Japan) coupled to the microscope. Digital images were obtained by Nis-Ar Elements software, (Nikon, Tokyo, Japan).

RESULTS

The vector pSP64-*nanos-3* used for probe synthesis was evaluated by map restriction, using the restriction enzymes EcoRI and HindIII, and the sizes of the cleavage products were confirmed in 2% agarose gel (Fig. 1).

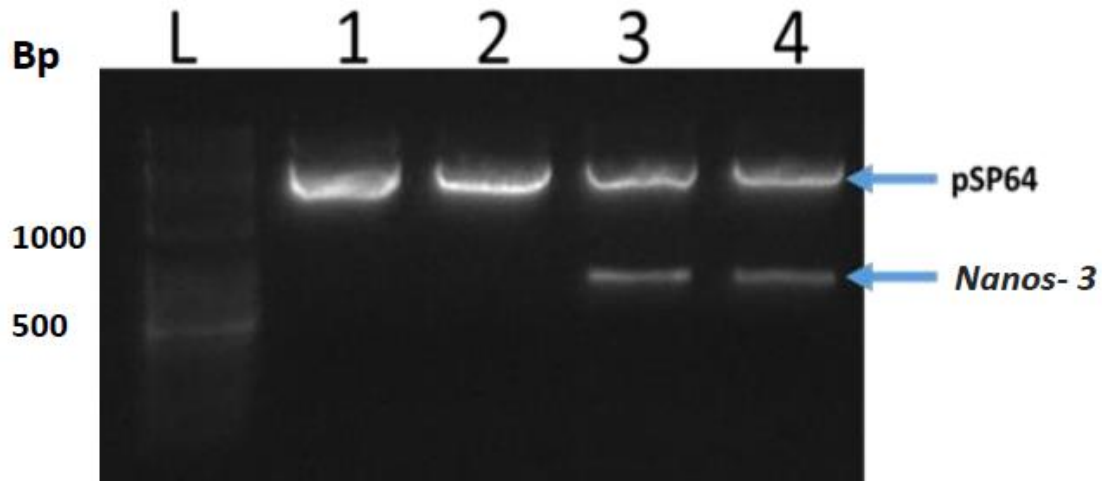


Fig. 1: vector restriction map constructed for WISH technique. L-ladder. 1 and 2 - pSP64-nanos3 vectors.

To trace primordial germ cells in the early embryonic development of marbled catfish *P. mangurus*, the *nanos-3* transcripts signals was observed by WISH using *P. mangurus* antisense *nanos-3* RNA probes. At the early cleavage period, 2-cell, 4-cell and 8-cell stages, *nanos-3* mRNA strongly aggregated at both ends of cleavage furrows (Fig. 2a, b.). Signals of *vasa* transcripts were also observed at one or both ends of the third cleavage furrows at the 8-cell stage (Fig. 2c).

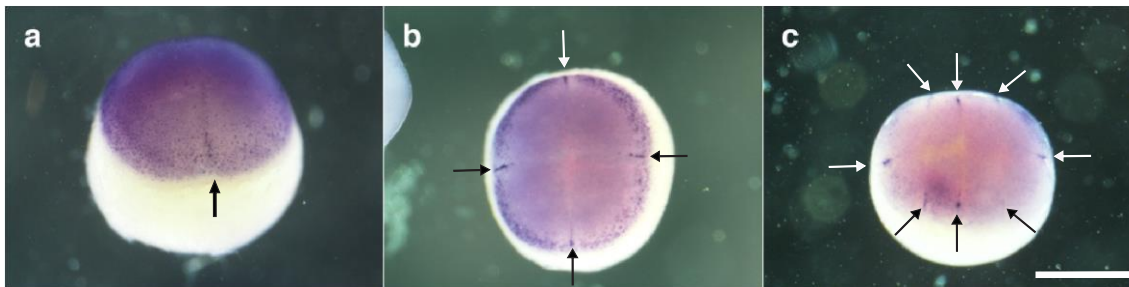


Fig. 2: Expression of *nanos-3* during early cleavage embryonic stages of the marbled catfish *P. mangurus*. Embryos were hybridized with a *P. mangurus nanos-3* antisense probe. **a**, 2-cell stage; **b**, 4-cell stage; **c**, 8-cell stage. Arrowheads indicate *nanos-3* signals. Scale bar indicates 500 μ m.

Nanos-3 transcripts signals were observed distributed on cell boundaries in the 16-cell stage, though weakly compared with those in the earlier cleavage furrows (Fig. 3a). From the 64-cell stage, the transcripts signals began to differed among embryos and to aggregate in clusters on blastomere boundaries (Fig. 3b).

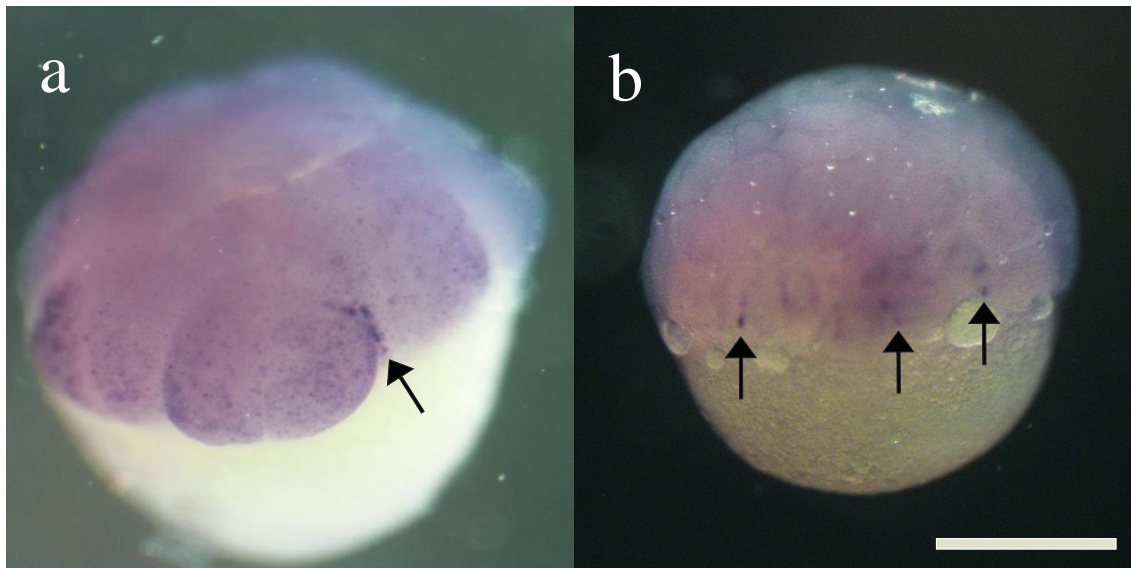


Fig. 3: Expression of *nanos-3* during cleavage embryonic stages of the marbled catfish *P. mangurus*. Embryos were hybridized with a *P. mangurus nanos-3* antisense probe. **a**, 16-cell stage; **b**, 64-cell stage. Arrowheads indicate *nanos-3* signals. Scale bar indicates 500 μ m

At the blastula stages, *nanos-3* transcript signals were observed in small spots distributed on blastoderm marginal part, some of them in pairs (Fig. 4a, b). The clusters of proliferated *nanos-3*-positive cells were present in the limit between the blastoderm and the yolk region (Fig. 4c).

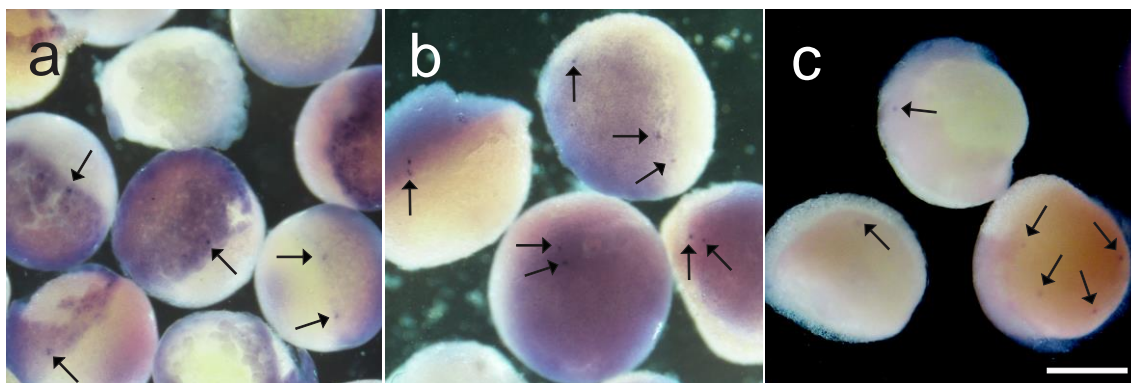


Fig. 4: Expression of *nanos-3* during the blastula embryonic stages of the marbled catfish *P. mangurus*. Embryos were hybridized with a *P. mangurus nanos-3* antisense probe. **a**, 128-cell stage; **b**, 1k-cell stage; **c**, spherical stage. Arrowheads indicate *nanos-3* signals. Scale bar indicates 500 μ m.

At the early gastrula stages, *nanos-3*-positive cells were observed frequently located in the marginal region of the blastoderm. From the late gastrula stages, some *nanos-3*-positive cells clusters began to rearrange and rally on the embryonic shield (Fig. 5a). At the 90% epiboly stage, were observed *nanos-3*-positive cells located between the dorsal equatorial region and the ventral posterior region (Fig. 5a).

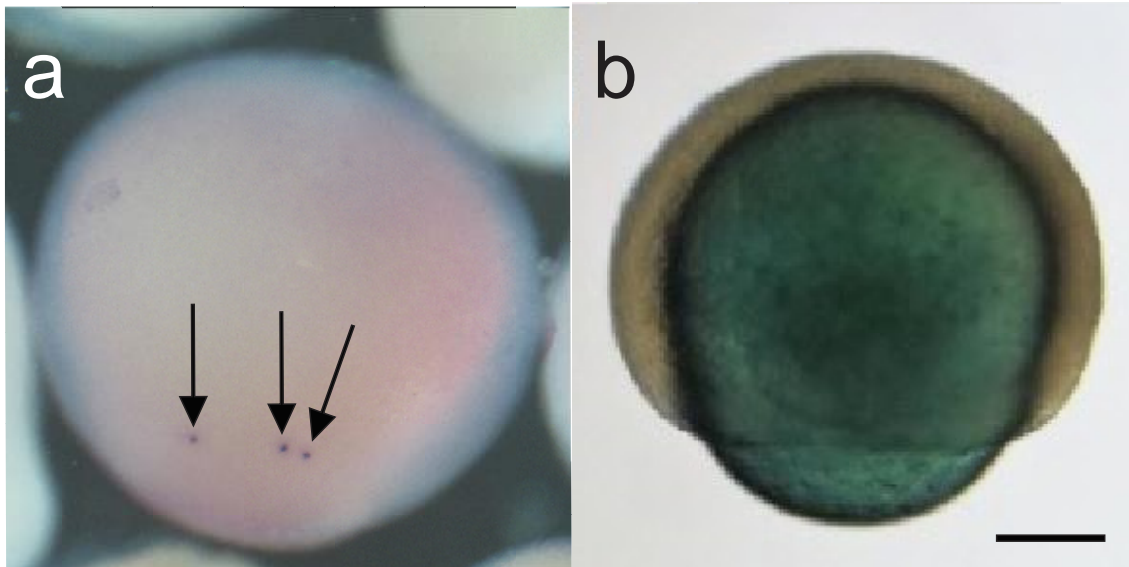


Fig. 5: Gastrula stage embryos of the marbled catfish *P. mangurus*. **a**, expression of *nanos-3* in *P. mangurus* embryo at 90% epiboly stage; **b**, *P. mangurus* embryo at 90% epiboly stage. Arrowheads indicate *nanos3* signals. Scale bar indicates 250 μm

At the early segmentation period, *nanos-3*-positive cells were observed beginning to gather around both sides of the embryonic body. From neurula stage, *nanos-3*-positive cells formed clusters on the region near of the firsts somites (Fig. 6a, c). At the 8- to 28-somite stage, many *nanos-3*-positive cells were clustered on both sides of the gonadal ridge of the embryonic body (Fig. 6b, d).

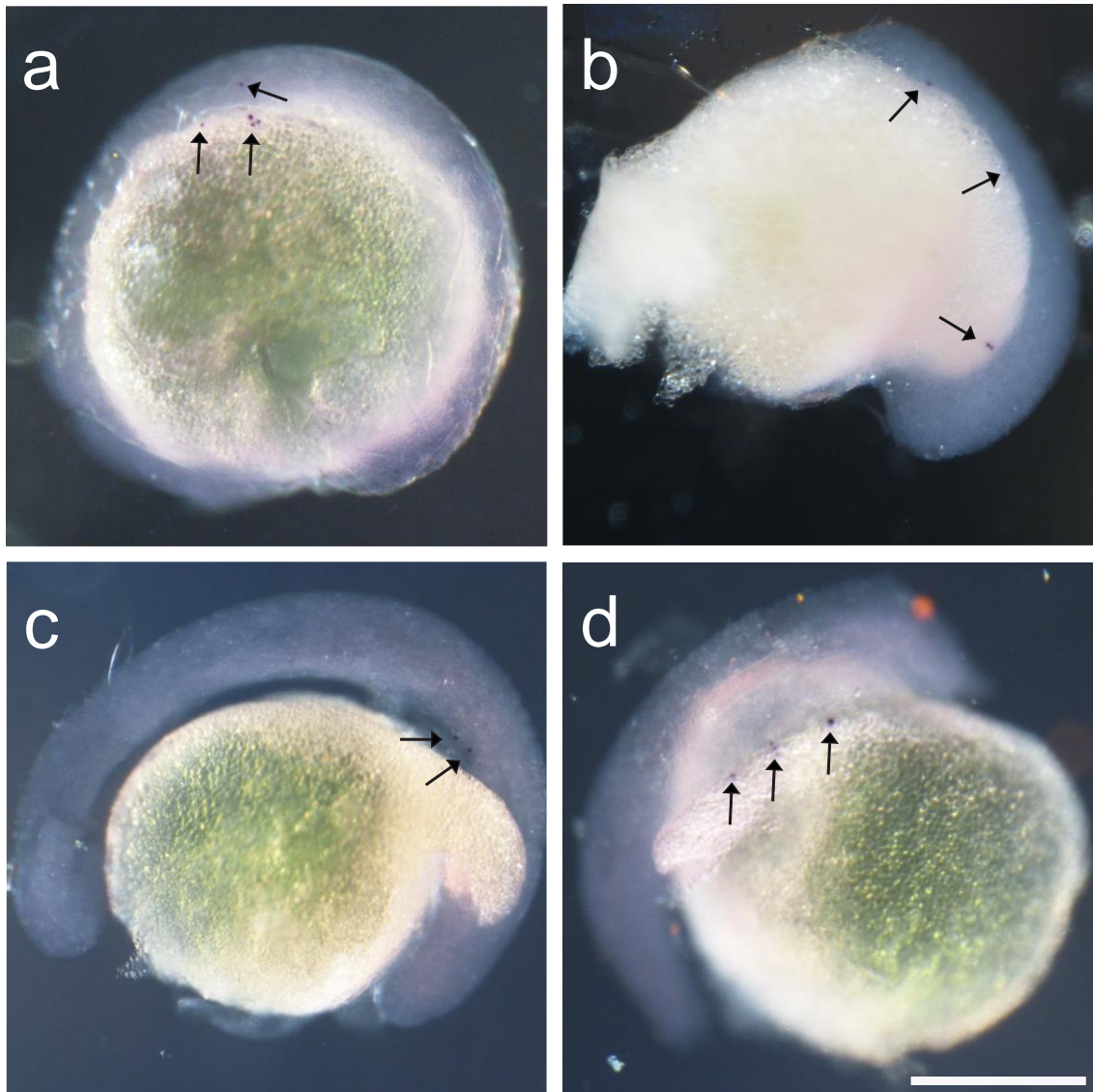


Fig. 6: Expression of *vasa* during segmentation embryonic stages of the marbled catfish *P. mangurus*. Embryos were hybridized with a *P. mangurus vasa* antisense probe. **a**, neurula ~1-somite stage; **b**, 8 somite stage; **c**, 20 somite stage; **d**, 28 somite stage. Arrowheads indicate *vasa* signals. Scale bar indicates 250 μm .

The distribution of the *nanos-3* transcripts during the initial steps of development of *P. mangurus*, from early cleavage to 28 somite stages, can be observed in the Fig. 7.

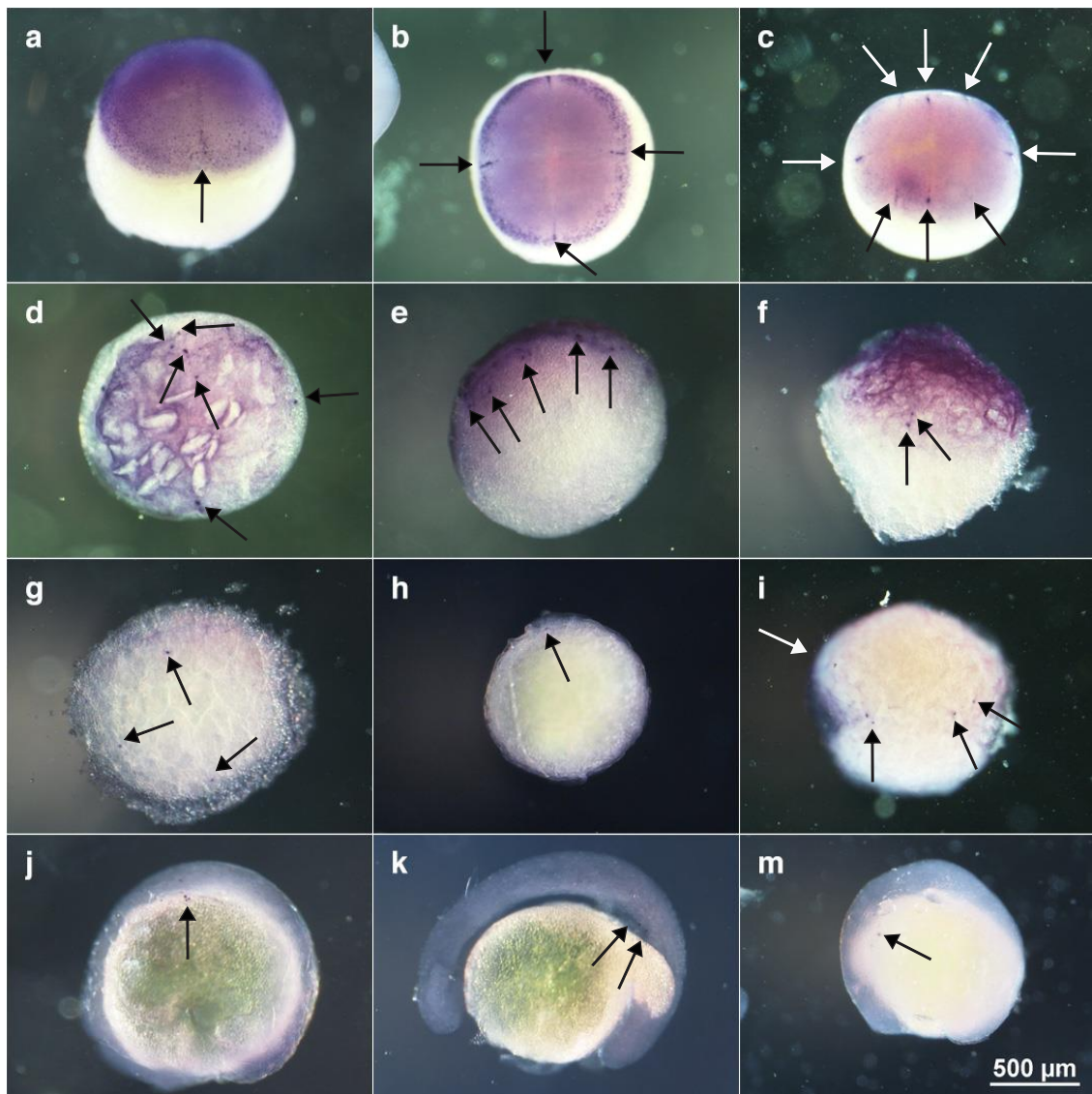


Fig. 7: Expression of *nanos-3* during early embryonic development of the marbled catfish *Pseudopimelodus mangurus*. Embryos were hybridized with a *P. mangurus nanos-3* antisense probe. **a**, 2-cell stage; **b**, 4-cell stage; **c**, 8-cell stage; **d**, 32-cell stage; **e**, 128-cell stage; **f**, 512-cell stage; **g**, 1k-cell stage; **h**, ring~50% epiboly stage; **i**, 100% epiboly stage; **j**, 100% epiboly stage; **k**, 1-somite stage; **l**, 20-somite stage; **m**, 28-somite stage. Arrowheads indicate *nanos-3* signals. Scale bar indicates 500 μm .

DISCUSSION

In this study, were tracked, using whole-mount *in situ* hybridization method with a specific *P. mangurus nanos-3* RNA anitsense probe, the germ cell lineage in the early embryonic development of marbled catfish *Pseudopimelodus mangurus*. The *nanos-3* sequence was confirmed using Nucleotide BLAST program (<https://blast.ncbi.nlm.nih.gov>) with 85.88% identity of *nanos-3* *Pangasianodon*

hypophthalmus. Using protein BLAST, it was observed 75.35% identity with *nanos-3 Pangasianodon hypophthalmus*, 62.44% identity with *nanos-3 Ictalurus punctatus* and 41.84% identity with *nanos-3 Danio rerio*. This result appoints to greater similarity between *nanos-3* sequences from Siluriform species, being the zf-*nanos* superfamily domain is the region more conserved. Nanos is a protein that binding in specific 3' untranslated region of a messenger RNA and repress its translation (HASHIMOTO et al., 2010).

The *nanos-3* RNA probe, synthesized from genetic material extracted from the own marbled catfish eggs, was effective to provide transcript signals of *nanos 3* mRNA expression in *P. mangurus* preserved embryos. Those signals were observed in *P. mangurus* embryos after fertilization from the early cleavage, aggregated at the ends of cleavage furrows on first cell boundaries (Fig. 1); at the blastula period, grouped in individualized blastomeres distributed on blastoderm marginal part, some of them in pairs (Fig. 4); at gastrulation, *nanos 3*-positive cells clusters began to rearrange and rally on the embryonic shield (Fig. 5a), to 28 somites segmentation stage, PGCs *nanos 3*-positive cells clustered on both sides of the gonadal ridge (Fig. 6d).

These results in the *P. mangurus* embryos suggest that the *nanos-3*-positive cells correspond to the PGCs, with the *P. mangurus nanos-3* probes signals matching topographically with the PGC migratory patterns reported in other fish species (FUJIMOTO et al., 2006), with distinctions not yet fully clarified. A possible particular feature of *P. mangurus* PGC migration route, the phenomena of in pars composition of the *nanos 3*-positive cells, observed at blastula period (Fig. 3 b), may imply a particularity of this species early embryonic development.

The positive results of *P. mangurus nanos-3* RNA antisense probe, observed here, corroborate the premise of high conservation of those germ cell markers in several teleost species observed here, corroborate the premise of high conservation of those germ cell marker in several teleost species, such as zebrafish, goldfish, loach, herring, medaka, ice goby, and carp (KUROKAWA et al., 2006; SAITO et al., 2011; YOON; KAWAKAMI; HOPKINS, 1997), including the neotropical siluriform, and the possibility of using those genes in conservation efforts.

In conclusion, the primordial germ cells in the early embryonic development of marbled catfish *Pseudopimelodus mangurus* were tracked by the whole mount in situ hybridization method, with a specific *P. mangurus nanos-3* RNA antisense probe. The *nanos 3* can be considered a specific PGC-gene marker, with the function preserved in

several teleost species. Therefore, descriptions of the *P. mangurus* germ cell lineage in more detail are needed to elucidate the general principles of how they originate and migrate. Our results provide information useful for future investigation of mechanisms of development and subsidies for conservation efforts of neotropical siluriform species, with the use of biotechnology tools.

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