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EMBRYOGENESIS OF *MYLOSSOMA DURIVENTRE* (PACU-PEVA)

EMBRIOGÊNESE DE MYLOSSOMA DURIVENTRE (PACU-PEVA)

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SUMMARY

This paper tested a semi-natural reproduction and embryo development of Mylossoma duriventre embryo until hatching. Six females with bulging and softness coelomic cavity and eighteen males that release sperm under light belly compression were selected. Two doses of luteinizing hormone (0.0125 mg/kg and 0.125 mg/kg) were apllied for females performing a semi-natural reproduction.After fertilization the embryos were transferred to an incubator and were evaluated every 15-min intervals during the first hours of development and at one-hour intervals until hatching. The embryos are megalecites with meroblastic division, being visualized six different stages of development: fertilization, cleavage, blastula, gastrula, segmentation and pharyngula. In gastrulation (120-min post-fertilization), there is formation of "tail bud" and appearance of somites during segmentation (510-min post-fertilization), after the blastopore closure. The hatching occurred with 20 hours of development, at a temperature of 24°C. It was possible to reproduce the pacu peva in captivity and the embryonic development showed some specificity when compared to other species. The results of the reproduction and embryogenesis are needed because these are the critical phases that limit the survival of the larvae in fish farming.

KEY-WORDS: Embryos. Embryo development. Fish. Hatching rate. Morphogenesis.

RESUMO

Este estudo descreve a reprodução seminatural e o desenvolvimento morfológico de embriões de *Mylossoma duriventre* até a eclosão. Foram selecionadas seis fêmeas com protuberância e maciez de cavidade celomática e dezoito machos que espermiaram por compressão da cavidade celomática. Foram aplicadas duas doses de hormônio luteinizante (0.0125 mg/kg and 0.125 mg/kg) para fêmeas caracterizando a reprodução semi-natural. Depois da fertilização os embriões foram transferidos para a incubadora e foram avaliados em intervalos de 15 min durante a primeira hora de desenvolvimento e intervalos de uma hora até a eclosão. Os embriões são megalécitos com divisão meroblástica, sendo visualizado seis diferentes estágios de desenvolvimento: fertilização, mórula, blástula, gástrula, segmentação e faringula. Na gastrulação (120min pós fertilização), ocorre a formação do "botão da cauda" e aparecimento dos somitos durante a segmentação (510 min pós fertilização), depois o fechamento de blastóporo. A eclosão ocorreu com 20 horas de desenvolvimento à temperatura de 24ºC. É possível reproduzir pacu-peva em cativeiro e seu desenvolvimento embrionário apresenta especificidades quando comparado com outras espécies. Os resultados em relação à reprodução e a embriogênese são necessários pois, é a fase crítica que limita a sobrevivência das larvas em sistemas produtivos e para o entendimento da biologia da espécie.

PALAVRAS-CHAVE: Desenvolvimento embrionário. Embrião. Morfogênese. Peixe. Taxa de incubação.

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INTRODUCTION

Mylossoma species are economically important in several states of Brazil. In Mato Grosso, for example, specialized fishing is performed all over the Cuiabá River using different fishing strategies which increases the catching capacity of this species (Resende et al 1998). *Mylossoma duriventre* (pacu-peva) is known in the basins of the Paraguay, Lower Paraná and Lower Uruguay rivers. Considered a herbivorous fish, highly dependent on floodplains and of great economic importance for commercial and sport fishing (Mateussi et al 2018). A recent study by Lourenço et al 2020 highlighted the bioecology and production of artisanal fisheries in the Middle Madeira in the vicinity of the municipality of Humaitá and found that two species of pacu (Mylossoma spp) added up to a production of 20 tons (10.5 ton for *M. aureum* and 9.5 ton for *M. duriventre*).

Embryonic and larval development depends of multifactorial parameters, such as thermal stability and a high oxygen supply (Ayala and Arias Clavijo 2003). Natural systems facilitate the distribution of embryos along the riverbeds and in areas recently flooded. Those places are rich in food and shelter, but wich cam also accommodate potential predators (Mago-Leccia 1970; Machado-Allison 1990; Fregadolli 2003).

With the destruction of breeding fish habitat, contamination of river waters and overfishing, it is necessary to study about its cultivation in captivity. However, the production of fry is still a difficulty since pacu-peva does not replicate naturally in ponds or tanks. For this reason, it becomes necessary to induce, with hormones, their reproduction and incubate the eggs, in order to access larvae development in laboratory (Bock and Padovani 2000).

To induce spawning of fish it is necessary the application of conventional techniques of hormonal induction. This phase is complete when oocytes are at vitellogenesis stadium and central germinative vesicle, requiring hormonal induction to ensure the final maturation and spawning (Sato 1999). According to Mylonas et al. 2010, for many species it is necessary to employ artificial gamete collection and fertilization, since, sponyaneous spawning is not going to happen, losing the opportunity of fryes.

The embryonic development comprises staging of the morphological structure that comprises eggs, embryos and larvae until the total absorption of the yolk sac. The knowledge of morphological development of the embryo is an important tool for artificial breeding, because the embryos exhibit asynchronous growth (Kimmel et al. 1995) and multiparameters factors are known to impaire theis development. Their understanding provides for aquaculture, the identification of eggs and larvae in nature and taxonomic studies (Nakatani et al. 2001) and important parameters in the analysis of evolutionary relationships among species (Futuyma 2002; Silveira 2004).

The knowledge of the reproductive biology of Mylossoma duriventre, its behavior towards induction techniques and the details of its initial development are essential to establish reproductive techniques in cultivation or conservation, so that there is a comparison with the species in the natural environment and to contribute with fish development. Thus the objective of this study was to evaluate the morphological development of the *Mylossoma duriventre* embryo until hatching deriving of semi-natural reproduction.

MATERIAL AND METHODS

Spawning was conducted in January/2011, in the Department of Fish Culture of Medicine Veterinary School from Federal University of Goiás. Six mature females were visually identified by external characteristics such as coelomic cavity bulging and softness and hyperemic urogenital papilla and 18 males with sperm release under mild abdominal pressure were used.

Females were induced with two doses of LH (Lutropin®) with a priming dose of 0.0125 mg/kg and six hours later with a resolving dose of 0.125 mg/kg. Hormone application was intramuscularly and close to the pectoral fin of the animals. In males no hormonal induction was made, characterizing a semi-natural reproduction. The fish were placed in polyethylene water tanks of 500L, with constant water flow and average temperature of 26°C. The ratio between female: male was of 1:3.

Evaluation of embryonic development was performed according to Kimmel et al. (1995), in a stereomicroscope (4x), and the time was recorded after fertilization. Photomicrographs were taken from 10 embryos in each sampling period and analyzed by computer with Image J software (Schneider et al. 2012).

Embryo development was observed every 15 minutes during the first two hours. After this period, observations were made every hour until hatching. The hatching rate was calculated by the ratio of total number of larvae (100) that hatched by the total assessed in percentage at the end of experiment.

Morphometric measurements were taken according to the time of embryo development (Figure 1). Between stages of fertilization and blastocyst evaluations were made:

A. Horizontal perivitelline space
B. Vertical perivitelline space vertical
C. Diameter of the yolk sac
D. Height of the yolk sac

At segmentation phase measurements were taken: area, diameter and height of the somites.

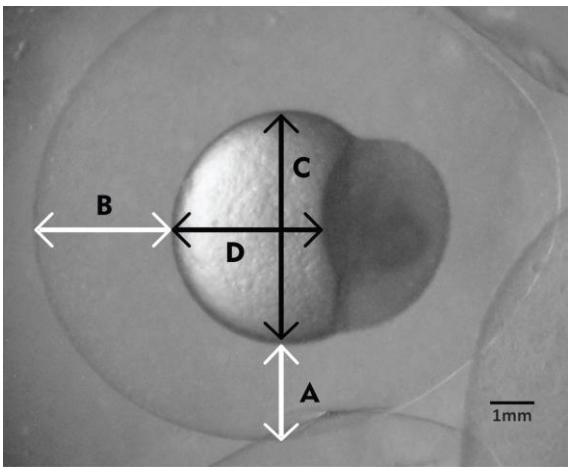

Figure 1 - Morphometric measurements embryos of *Mylossoma duriventre* from semi-natural reproduction. A) Horizontal perivitelline space; B) Vertical perivitelline space; C) Diameter of yolk sac and D) Height of the yolk sac.

RESULTS AND DISCUSSION

General Description

Pacu-peva is a total spawning fish with external fertilization, their embryos development is meroblastic, with clear separation between animal and vegetal pole. This is a pioneering work of induction of pacu-peva in captivity, showing that this fish can be produced on commercial scale.

Embryo development is asynchrony and has been reported by Kimmel et al. (1995) with *Danio rerio* (zebrafish). A standardized method for assessment of the phases of embryo development has also been mentioned by this author and is used to describe morphologically different stages of the embryos. In this experiment we identified seven stages of development: Zygote, Cleavage, Blastula, Gastrula, Segmentation and Pharyngula. Tsai et al. (2013) divided goldfish (*Carassius auratus*) embryonic development into seven periods Zygote, Cleavage, Blastula, Gastrula, Segmentation, Pharyngula, and Hatching based on Kimmel et al. (1995) for the zebrafish.

Embryos were kept at a temperature of 24°C and hatched approximately 20 hours after fertilization with a hatching rate of 60%. Different temperatures will alter the rates of embryonic growth, increasing or decreasing the speed of their development (Kimmel et al. 1995; Fornari et al. 2012). Studies show that embryos of ectodermic animals develop faster at higher temperatures, due to the temperature-induced changes in enzyme activity during organogenesis (Ojanguren and Braña 2003).

Fertilization

The eggs had an enormous amount of yolk and the embryos were translucent, with a large perivitelline space, of the megalecite type. This feature determines that cytoplasm and nucleus are reduced to a minimum portion of the total area, called germinal disc, which will give rise to the embryo (Figure 1). At 0 hours post-fertilization (hpf) the yolk had an average of 2.2 mm for both the diameter and for height and the perivitelline space averaged was of 4.6 mm (Table 1).

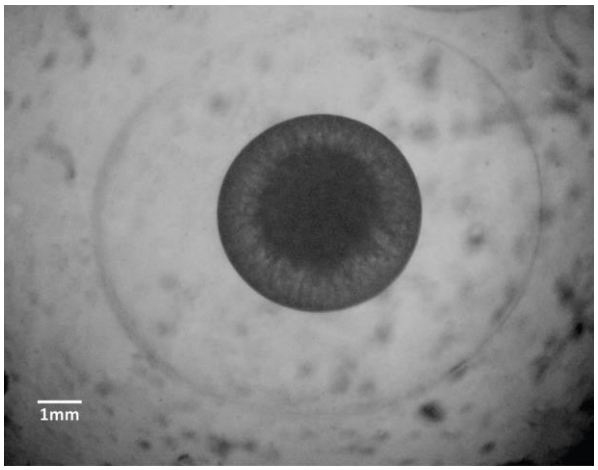

Figure 2 - Image of the zygote 0 hours post-fertilization embryos of *Mylossoma duriventre* from semi-natural reproduction.

After 0,5 hpf was possible to see an increase in the yolk sac of approximately 0.3 mm, and the perivitelline space increased to approximately 5.2 mm, at this stage the embryo is identified as zygote (Table 1). It was also possible to observe complete segregation between animal and vegetal poles and the beginning of cytoplasmic movements (Figure 3), the same results were observed by Langeland and Kimmel (1997).

Initially the embryo has formed only by one cell that started a mitosis and consequently proliferation. The cleavages occurred only in the region occupied by animal pole, leaving only the mass of undivided yolk (vegetal pole). Once the cleavage had started, the size of blastomeres decreased and the number increased. As the embryo development was observed, the measures of perivitelline space and yolk sac have also changed, as shown in Table 1.

Table 1 - Mean of the yolk sac and chorionic sac (mm) related to hours post-fertilization and embryonic development of *Mylossoma duriventre* (N = 10 embryos; mean ± SD).

Stage	Morphologic features	Hours post-fertilization	Yolk sac		Perivitellinic space	
			Diameter	High	Horizontal	Vertical
Zygote	Fertilization	0	2.21±0.17	2.18±0.19	4.65±0.38	4.63±0.36
Cleavage	One cell	0.5	2.50±0.52	2,57±0.45	5.25±1.07	5.42±1.14
	1K-cell	1.5	2.36±0.62	2.52±1.16	5.82 ± 1.87	5.78±1.96
Blastula	High	2.0	2.30±0.40	2.24±0.48	4.92±0.82	4.93±0.86
	30-40% epiboly	2.3	2.18±0.27	2.16±0.28	4.45±0.74	4.38±0.70
Gastrula	50-60% epiboly	3.3	2.30±0.45	2.38±0.49	4.73±0.88	4.86±0.86
	70-80% epiboly	4.3	2.19± 0.25	2.17± 0.27	4.87± 0.69	4.98± 0.72
	90% epiboly	5.3	2.32± 0.25	2.30± 0.18	4.92± 0.33	4.99± 0.38
	Bud	7.5	2.79± 0.30	2.86± 0.38	5.64± 0.66	5.73± 0.70

Cleavage Period

The period of the cleavage occurs between 0,5 and 1,5 hpf after fertilization, with a duration of 1 hpf. This same period for zebrafish was found at 2,15 hpf (Kimmel et al. 1995). These data are important because they demonstrate the specificity of development and so the importance of the morphological study of embryonic development in each species. The difference in incubation period is related to the size of the egg and the reproductive strategies of species (Sato 1999; Nakatani et al. 2001).

The cleavage was discoidal meroblastic in which cell division occurs only in the animal pole. During cleavage, was also visible a decrease in size of cells from the animal pole (Figures 3A; 3B and 3C). Although the cells proliferate cytoplasmic bridges connect them and to the yolk sac (Kimmel and Law 1985). These bridges are nothing more than catenins and cadherins that form gap junctions between the yolk sac and animal pole (Pelegri 2003). As reported by Kimmel et al. (1995) the end of cleavage occurs with at the phase of 64 embryo cells, when an overlapping of cells occurs, in which a cellular surface layer forms an envelope around the blastoderm.

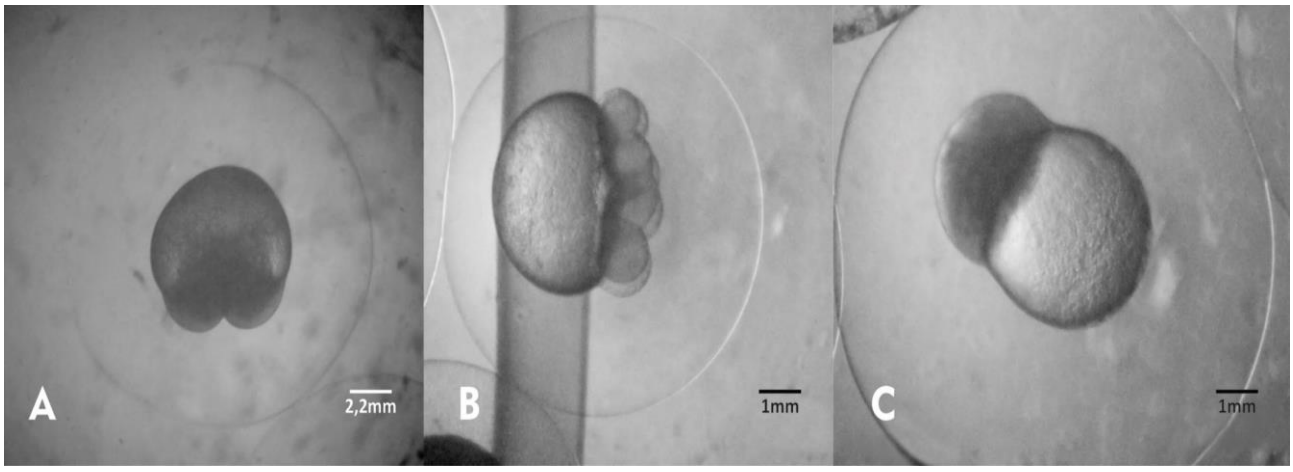

Figure 3 - Two-cell stage (A), sixteen cell stage (B) and 1K cell stage (C) embryos of *Mylossoma duriventre* from semi-natural reproduction.

Blastula Period

The period of blastocyst started at 2,0 hpf and its duration was approximately of 50 minutes. During this period the blastoderm had a round shape, the yolk sac was formed and the movements of epiboly started. The round shape acquired by the blastoderm (Figure 4A) is due to mitoses performed by cells in the animal pole, which overlaps one each other. The vitellin syncytial layer is formed by marginal cells lining the yolk sac. These structures determine the multi-compartimentalization of fish embryos. This period is also cited as the moment of the start of gene transcription of the zygote (Botta et al. 2010).

In figure 4B the cells began movement toward the yolk sac, called epiboly movements. From this moment the names given to the embryos are related to the percentage of cells lining the yolk sac. During these movements the blastoderm initiates the formation of layers, decreasing the thickness of it (Figure 4C). The epiboly movements also determined a constriction of the yolk sac, forming a dome shaped structure, indicated by the arrow in Figure 4C.

To coordinate the movements occurring during epiboly, the embryonic cytoskeleton provides two distinct arrangements of microtubules. One of the arrangements forms a dense network in the yolk syncytial layer (YSL), which originated from the marginal blastomers, the other one stretches from the YSL toward the vegetative pole (Krezel and Driever 1994). In *Fundulus* sp., desmossomes are responsible for the link between the yolk sac and the blastoderm, and this attracts one another as it occurs epiboly movements (Betchaku and Trinkaus 1978).

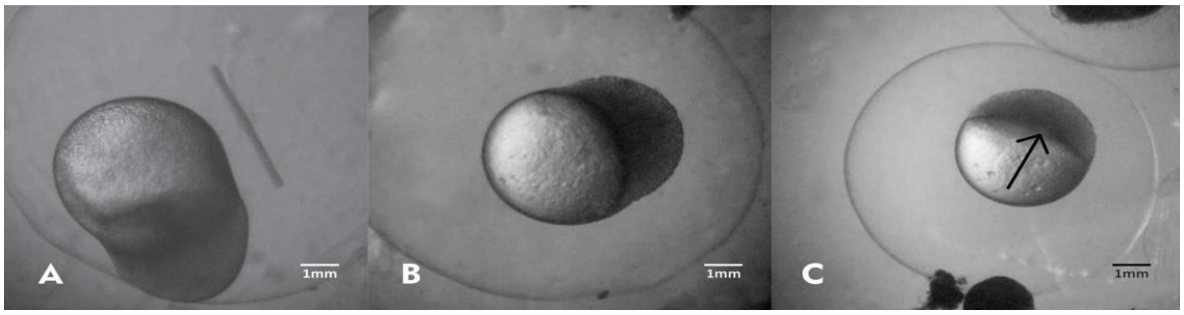

Figure 4 - *Mylossoma duriventre* embryos at different stages of the blastula. Embryo at high (H), oblong embryo stage (B) and the embryo stage dome indicated by the arrow (C).

Gastrula Period

Gastrula period started with 50% epiboly at 3,3 hpf (Figure 5A), lasting 4,16 hours. At this moment the displacement of blastoderm cells occurrs, leading to separation and differentiation of the epiblast and hypoblast and the germ layers formed the notochord (Mullins 1999; Stickney et al. 2000). The epiboly continued determining a change in conformation of blastoderm, from oval shape to a multicellular layer paving, occurred between 4,3 and 6,6 hpf (Figures 4 and 5). At the end of this phase blastoderm covered the entire yolk sac, causing closure of the blastopore (Figure 5B). During this movement, at 50% of epiboly, there is the formation of the germ ring. This morphological structure appeared as a cell thickness on the edges of the animal pole (Figure 5A).

The lowest mean values of both the yolk sac and the chorion during this developmental stage can be caused by an involution of the cells during migration over the yolk sac (Botta et al 2010). This involution is associated with convergence, involution and expansion of the blastoderm during the formation of germ ring. The movement of epiboly continued from 70-80% (Figure 5C), and at 5,3 hpf the embryo was with 90% of epiboly (Figure 6A). The gastrula stage ended at 7,5 hpf, when the beginning of segmentation was observed. The final stage of gastrulation ended with the bud stage (Figure 6C), in which there was an accumulation of cells, just before closure of blastopore, forming a structure called tail bud, which contributes to the formation of the tail (Kimmel et al. 1995).

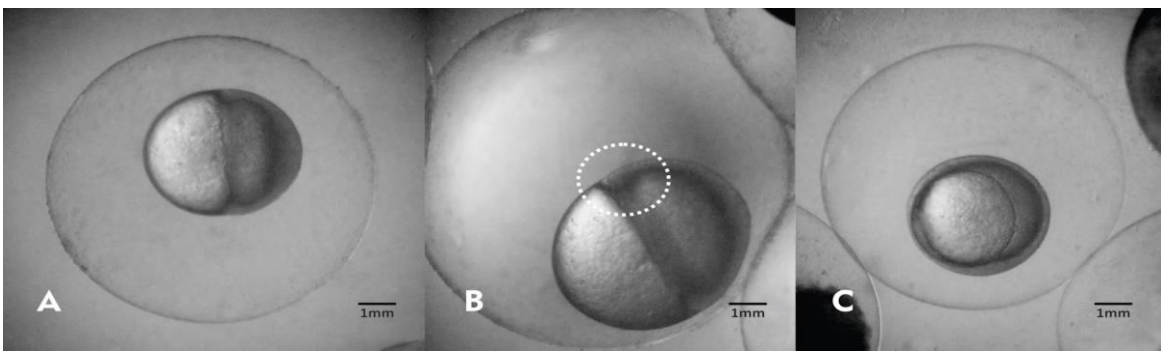

Figure 5 - *Mylossoma duriventre* embryo with 50% epiboly stage and formation of germ ring (A); shield embryo (accumulation of cells indicated by the dotted line) in (B); 70% of epiboly stage (C).

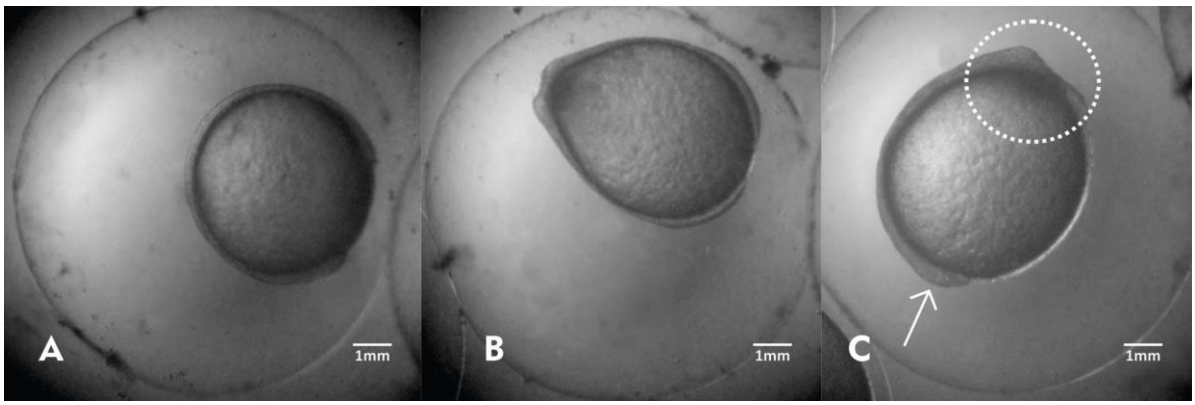

Figure 6 - *Mylossoma duriventre* embryo with 90% of epiboly (A); in closure of blastopore (B); formation of the tail bud (dotted line) and head (white arrow) (C).

Segmentation Period

The segmentation phase started from the formation of the first pair of somites with 8,5 hpf ending with 10,5 hpf. For evaluation of the somites, measures of area were taked such as width and diameter. When the embryos were with 16 somites, the area was of 2.65 ± 0.53 mm, width of 0.38 ± 0.09 mm and diameter of 0.17 ± 0.05 mm. At 24 somites stage the area was 3.27 ± 0.72 mm, width of 0.53 ± 0.09 mm and diameter of 0.15 ± 0.02 mm. After hatching, the somites showed an area of 4.53 ± 0.75 mm, width of 0.60 ± 0.09 mm and diameter of 0.17 ± 0.03 (Figure 7).

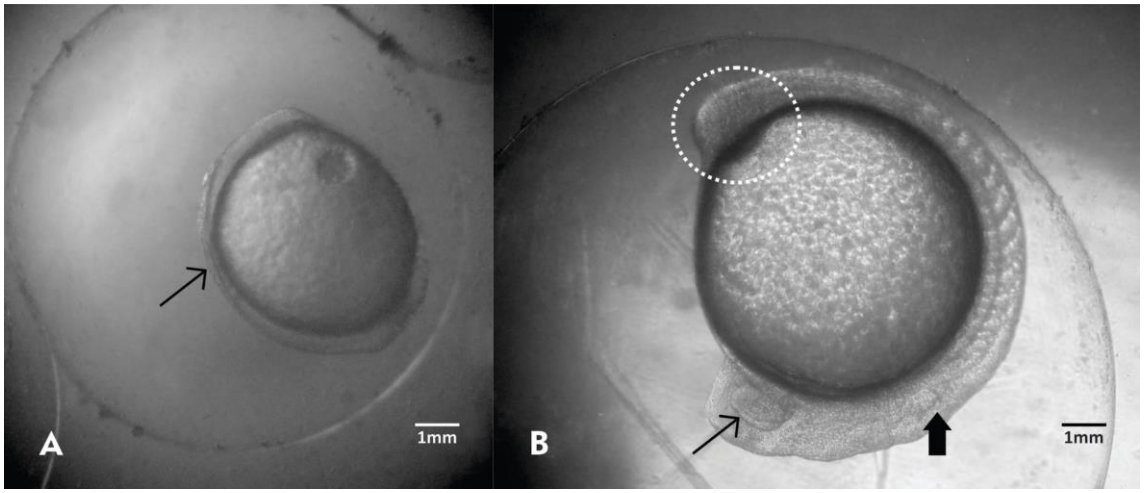

Figure 7 - *Mylossoma duriventre* embryo with three somites (arrow) (A). Embryo at approximately 18 somites (B), with the presence of optic vesicle (black arrow), optical vesicle (filled black arrow) and tail (dotted line).

The appearance of the first somites occurred at 8,5 hpf in pacu-peva while in zebrafish occurred at 10,7 hpf (Kimmel et al. 1995). The somites at the front part of the embryos appeared first and then the finals ones. At this time there was also an increase in the length of the embryo. The area and width of somites increased as the number of somites increased as well. However the diameter remained similar. So we can infer that the embryo growth occurred primarily by increasing the width and area of the somites. The somites originally will give rise to muscle after differentiation (Kimmel et al. 1995).

After 9 hpf the optic vesicle was present so as the otic vesicle (Figure 7B). The Kupffer's vesicle has now disappeared (Figure 7B), and appears to be an allantoic rudiment. If we observe the fate map, epithelial cells lining the vesicle will later form tail and mesodermal derivatives, including notochord and muscle in zebrafish (Melby et al. 2003). During somitogenesis these structures were observed also in *Pseudoplatystoma coruscans* (Cardoso et al. 1995), *Leporinus taeniatus* (Padilha 2003); *Orthotaenia brycon*, *Leporinus obtusienses*, *Prochilodus argenteus* and *Salminus argentius* (Sampaio 2006).

In figure 7B brain differentiation started between 10,5 and 14 hpf. The cranial portion begins do appear prominent transversal structures that determines the formation of diencephalon and mesencephalon (Kimmel et al. 1995). From this moment the start of histogenesis and organogenesis of the embryo started, which occurs initially by the formation of the epiblast and hypoblast (Kimmel et al. 1995). These factors are similar to those observed for *Prochilodus lineatus* studied by Botta et al. (2010), but there are some differences. The 3 somites embryo appears at 8,5 hpf before closure of blastopore, while for pacu-peva this event was observed after the closure of blastopore.

Pharyngula Period

At figure 7, when compared to figure 6B, there is an increase in the number of protrusions existing in the cranial region of the embryo. These projections are responsible for the formation of the telencephalon, diencephalon, midbrain and cerebellum. The central nervous system is formed from the transformation of the anterior multilayered neural plate to the pseudostratified epithelium of the neural tube. This requires cell interdigitation and intercalation across the layers, being progressively and different from what occurs in mammals (Clarke 2009).

Neural system appears in the zebrafish during "prim stage", in pharyngula phase. In pacu peva, morphogenesis occurs at this moment too, at 17 hpf. It is also possible to observe a reduction of the head in addition to the constriction of the yolk sac to release the tail. Kimmel et al. (1995) determined that to zebrafish the tail separation occurs together within yolk sac separation but the embryo connection with the yolk continues.

Also were detected small reflexive movements of the larvae in this phase, determined by uncoordinated contraction of the myotomes. These movements continue to improving, until they are coordinated and the larvae can move its body through the water (Kimmel et al. 1995).

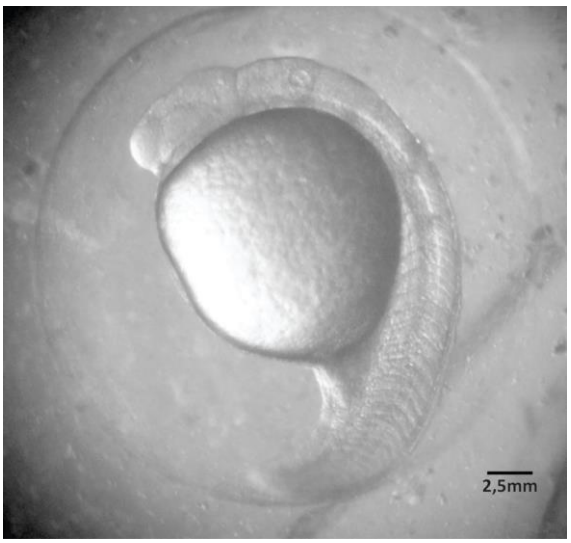

Figure 8 - *Mylossoma duriventre* embryo from semi-natural reproduction with 17 hpf.

CONCLUSION

This was the first report of induced spawning of pacu-peva and its early embryonic development. Under the conditions of this study we can conclude that the early embryonic development of this species occurs in approximately 20 hours in temperature of 24°C. This paper may be useful as a reference for future studies and reproductive biotechnologies such as cryopreservation of semen and embryos. In addition, the results obtained regarding embryonic development are essential for a better understanding of biology this species.

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