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Structural analysis of the *Pimelodus maculatus* (Lacépède, 1803) embryogenesis (Siluriformes: Pimelodidae)

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The fish embryonic development comprises the events between the egg fertilization up to larvae hatching, being useful for the identification of viable eggs in productivity and survival studies as well as in raising experiments of several species. The goal of the present study was to characterize the embryonic development of *Pimelodus maculatus* (Siluriformes; Pimelodidae). The embryogenesis was typical of teleosteans, but with differences in relation to other species such as duration of development, type of blastocoel, moment of somite segmentation among others. Six stages of embryonic development were defined: zygote, cleavage, blastula, gastrula, organogenesis (divided in phases: early segmentation and late segmentation) and hatching with a period of incubation equal to 13 hours at 29 ºC and 17 hours at 25 ºC. The extruded oocytes presented a mean diameter of 812 μm before and 1066 μm after hydration. When fertilized, they presented a yellowish coloration and a gelatinous layer surrounding the chorion. The cleavage pattern is described as: 2; 4; 8 (4x2); 16 (4x4); 32 (4x8) and 64 (2x4x8) blastomeres up to morula phase (+64 cells). It was also possible to observe at this phase, the beginning of the formation of the yolk syncyctial layer (YSL). Afterwards, the blastula and gastrula stages followed. The end of gastrula was characterized by the formation of the yolk plug. Subsequently, the differentiation between cephalic and caudal regions began, along with the embryo elongation, structuring of optic, Kupffer's and otic vesicles besides a previously unidentified structure in the yolk syncyctial layer. The end of this stage is typified by the tail detachment. The late segmentation phase was distinguished by a free tail, presence of more than 30 somites, optic and otic vesicles, development of posterior intestine, pigmentation of cephalic and caudal regions of yolk sac and embryo growth. The recently-hatched larvae presented a primordial digestive tract, quite evident and pigmented eyes, closed mouth, encephalic vesicles and a mean length of 3410 μm.

O desenvolvimento embrionário de peixes compreende eventos que ocorrem desde o ovo fertilizado à eclosão das larvas, podendo auxiliar na identificação dos ovos viáveis em estudos de produtividade e sobrevivência, como também nas pesquisas de cultivo desses animais. O objetivo do presente estudo foi caracterizar o desenvolvimento embrionário do *Pimelodus maculatus* (Siluriformes; Pimelodidae). A embriogênese foi característica de teleósteos, apresentando variações que difere de outras espécies como, tempo de desenvolvimento, tipo da blastocele, momento de segmentação dos somitos, entre outros. Seis estágios de desenvolvimento embrionário foram definidos: zigoto, clivagem, blástula, gástrula, organogênese (dividido em fases: segmentação inicial e segmentação final) e eclosão com período de incubação de 13 horas à 29ºC e de 17 horas à 25ºC. Os ovócitos extrusados apresentaram diâmetro médio de 812 μm antes da hidratação e após 1066 μm. Após a fertilização, apresentaram coloração amarelada e uma camada gelatinosa envolvendo o córion. O padrão de clivagens foi descrito como segue: 2; 4; 8 (4x2); 16 (4x4); 32 (4x8) e 64 (2x4x8) blastômeros até a fase de mórula (+64 células). Também foi possível observar nesta fase, o início da formação da camada sincicial do vitelo (CSV). Em seguida foram observados os estágios de blástula e gástrula. O final da gástrula caracterizou-se pela formação do tampão vitelino. A seguir, iniciou-se a diferenciação das regiões cefálica e caudal, o alongamento

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do embrião, estruturação das vesículas ópticas, de Kufper, óticas e de uma estrutura não identificada na literatura localizada na camada sincicial do vitelo. O final deste estágio é demarcado pelo desprendimento da cauda. A fase de segmentação final caracterizou-se pela cauda livre, presença de mais de 30 somitos, vesícula óptica e ótica, desenvolvimento do intestino posterior, pigmentação nas regiões cefálica e caudal do saco vitelino e crescimento do embrião. As larvas recém-eclodidas apresentaram esboço do trato digestório, olhos bem evidentes e pigmentados, boca fechada, presença das vesículas encefálicas e com comprimento médio de 3410 μm.

Key words: Embryonic development, Induced spawning, Morphology, Yolk syncytial layer.

Introduction

Brazil presents a remarkable hydrographic and climatic potential as well as the richest fish fauna of the world, therefore being one of the most promising countries for aquaculture expansion. Although aquaculture technologies for neotropical fish species in Brazil are in development, it is still necessary to increase the information about biological features of species with potential to fishculture (Ninhaus-Silveira *et al*., 2006; Castagnolli, 1992).

The species *Pimelodus maculatus* belongs to the family Pimelodidae, order Siluriformes, It is an omnivorous and widespread catfish popularly known as "mandi, mandi-amarelo or mandi-pintado". This species is widely distributed in Brazil (Nomura, 1978), being found in Paraná, Plata and São Francisco river basins (Godoy, 1987; Reis *et al.*, 2003), Colombia, Maranhão, Jequitinhonha, Doce e Paraíba rivers (Fowler, 1951 apud Nakatani *et al.,* 2001). According to Castagnolli (1979) and Weingartner & Zaniboni Filho (2004), the *Pimelodus maculatus* is a species with potential for aquaculture and has interesting features, such as good quality meat and the absence of intramuscular bones, as well as adapting easily to artificial feeding, which represents a positive aspect to consider the feasibility of intensive breeding. Accordingly, studies suggest that improvements in technologies for building are important.

The early development and the embryologic description are important features to be studied in fish species since the knowledge of their biological features is a fundamental tool for both fishculture and fishery biology (Matkovic *et al.*, 1985). In addition, the embryological approach can contribute to studies regarding evolutionary relationships, inheritance pattern, mechanisms of development and the role of environmental influences over structural features of the organisms (Lagler, 1959). Morrison *et al.* (2001) suggest that the variations in the rate of embryogenesis and embryo development (asynchrony and malformations) are related to the breeders' age and the temperature of incubation.

According to Nakatani *et al.* (2001), in spite of several reports about embryonic development and larvae culture, there are few studies addressing the complete early development of native fish species (e.g., Ribeiro *et al.*, 1995; Andrade-Talmelli *et al.*, 2001; Ganeco, 2003; Pereira *et al*., 2006; Ninhaus-Silveira *et al.*, 2006 and Faustino *et al.*, 2007). Data about the embryonic development of *Pimelodus maculatus* are restricted to the studies by Luz *et al.* (2001) and Sato (1999).

Therefore, the goal of the present study was to characterize, under light microscopy, the several morphological events during the embryonic development stage of the *Pimelodus maculatus.*

Material and Methods

The experiment was carried out with adult specimens of *Pimelodus maculatus* from CEPTA – Centro de Pesquisa e Gestão de Recursos Pesqueiros Continentais – ICMBIO (Instituto Chico Mendes de Conservação da Biodiversidade), Pirassununga, São Paulo, Brazil. To obtain the embryos, two collections were performed in November 2007, during the reproductive period of this species that ranges from October to March (Vazzoler, 1996).

The spawning induction of breeders followed the procedure described by Woynarovich and Hórvath (1983), using a crude common carp (*Cyprinus carpio*) pituitary extract; three females with approximately 0.5 kg in weight each received two applications with a dosage of 0.5 mg and 5 mg/kg, respectively, in a 10-hour interval. Two adult male specimens weighing 0.5 kg, were induced to spawn with an application of 1mg/kg simultaneously with the second application of females. After about six hours from the last inductive dosage, the eggs were extruded and the semen was collected. The "dry" method was used for fertilization, where the eggs are mixed to semen without contact with water. Afterwards, water was added to activate the spermatozoa and hydrate the eggs. After washing to remove the excess of semen, the eggs were divided into two sets for incubation at different temperatures (25 ºC and 29 ºC). The incubators were connected to a closed heated water system coupled with a thermostat. To evaluate possible morphotemporal alterations in embryos of *Pimelodus maculatus*, samples of 200 embryos were collected at random at different development stages, regarding the moment of fecundation as time zero. In the eggs incubated at 29 ºC, the collections were performed each 15 minutes up to the first two hours of embryogenesis, and then each hour up to hatching. The samples of eggs incubated at 25ºC were obtained each five minutes up to the first hour, each 10 minutes up to the second development hour and then each hour up to larvae hatching. The embryo samples were fixed in modified Karnovsky's fixative solution (4% paraformaldehyde and 2% glutaraldehyde, in 0.1 M phosphate buffer, pH 7.2). After 24 hours, the samples were washed in

0.1M sodium phosphate buffer, pH 7.2 and kept in refrigerated 70% alcohol (10 ºC).

Stereoscopic analysis

The pre-fixed material was soon after processed, analyzed and photomicrographed in the Laboratório de Ictiologia Neotropical – L.I.NEO, Departamento de Biologia e Zootecnia - FEIS/UNESP, Ilha Solteira, São Paulo, Brazil.

For analysis, 50 embryos of each sample were selected and the chorion was removed using a watchmaker tweezer and a needle. The embryos were stained with Harris hematoxylin and eosin (HE), analyzed and photographed under a trinocular stereo microscope Coleman equipped with a Motic 2000 digital camera (Motic Instruments Inc.).

Light microscopy analysis

Twenty representative pre-fixed specimens from each development stage were embedded individually and oriented in glycol metacrylate plastic resin for microtomy. Serial transversal and sagittal histological cuts of 3 to 5µm were obtained and stained with Harris hematoxylin and eosin (HE) and toluidine blue, analyzed and photographed in a microscope Carl Zeiss Jena equipped with a Moticam 2000 digital camera (Motic Instruments Inc.).

Results

Embryogenesis

The embryonic development of *Pimelodus maculatus* from fertilization up to larvae hatching comprised a period of 13 hours at 29 ºC and 17 hours at 25 ºC. The following stages were established: zygote, cleavage, blastula, gastrula, organogenesis (divided in phases: early segmentation e late segmentation) and hatching (Tables 1 and 2; Figs.1-3). Heterogeneity was observed during the embryo development, *i.e*., embryos at different embryonic stages were observed at the same moment. In *Pimelodus maculatus,* the temperature at 25 ºC yielded both a higher asynchrony in the embryonic development and a wider variation in the blastomere division and the embryo formation was more homogeneous than incubation at 29 ºC.

Zygote stage. 0-0.23h (29 ºC); 0-0.42h (25 ºC)

Right after spawning, the oocytes were spherical, transparent and yellowish, being classified as telolecithal, once they present a large amount of yolk restricted to the vegetative pole. After fertilization, the egg became hydrated with an increased perivitelline space (31.3%), pronuclei fusion and cytoplasm reorganization with definition of both animal and vegetal poles (Figs. 4a-c). The animal pole is composed of active cytoplasm and a nucleus, being identifiable *in vivo* and in light microscope analyses because it is more transparent (Fig. 4c). On the other hand, the vegetal pole was denser *in vivo* with a faint coloration in the total preparations. The eggs presented a gelatinous surrounding layer (Figs. 4a-b) from spawning, when the oocytes were still dehydrated, up to the end of gastrula. During the entire embryonic development, fat drops in the yolk vesicle were not observed.

The eggs of *Pimelodus maculatus* were measured *in vivo* and presented nearly 812 µm and 1066 µm in diameter prior and after hydration, respectively.

Table 1. Embryonic development of *Pimelodus maculatus*, at 29 °C.

Time (hours)	Stage	Observations
0-0.23	Zygote	Animal pole without cleavage
0.40	Cleavage	100% with 2 cells
0.65	Cleavage	7% with 4 cells
		93% with 8 cells
1.00	Cleavage	73% with 16 cells
		27% with 32 cells
1.25	Cleavage	6% with 16 cells
		94% with 32 cells
1.50	Cleavage	100% with 64 cells
1.75	Cleavage	100% morula
	Blastula	
2.00	Cleavage	100% morula
	Blastula	
2.25	Gastrula	100% with 25% of epiboly
3.00	Gastrula	100% with 50% of epiboly
4.00	Gastrula	100% with 75% of epiboly
5.00	Gastrula	100% with 90% of epiboly
6.00	Organogenesis	Early segmentation phase - 100% neurula
7.00		Early segmentation phase- 11 somites, optic vesicle, otic vesicle, Kupffer's vesicle, pigmentation of embryo body and attached tail.
8.00		Early segmentation phase - 14 somites, optic and otic vesicles, Kupffer's vesicle in regression, pigmentation of embryo body and attached tail.
9.00	Organogenesis	Late segmentation phase - 19 somites, optic vesicle, otic vesicles, pigmentation of embryo body and free tail.
10.00		Late segmentation phase - 24 somites, free tail, optic and otic vesicles and pigmentation of embryo body.
11.00		Late segmentation phase - More than 30 somites, growing larva, pre-hatching, pigmentation of the yolk
12.00		membrane close to the head and tail.
13.00	Hatching	100% hatched larvae.

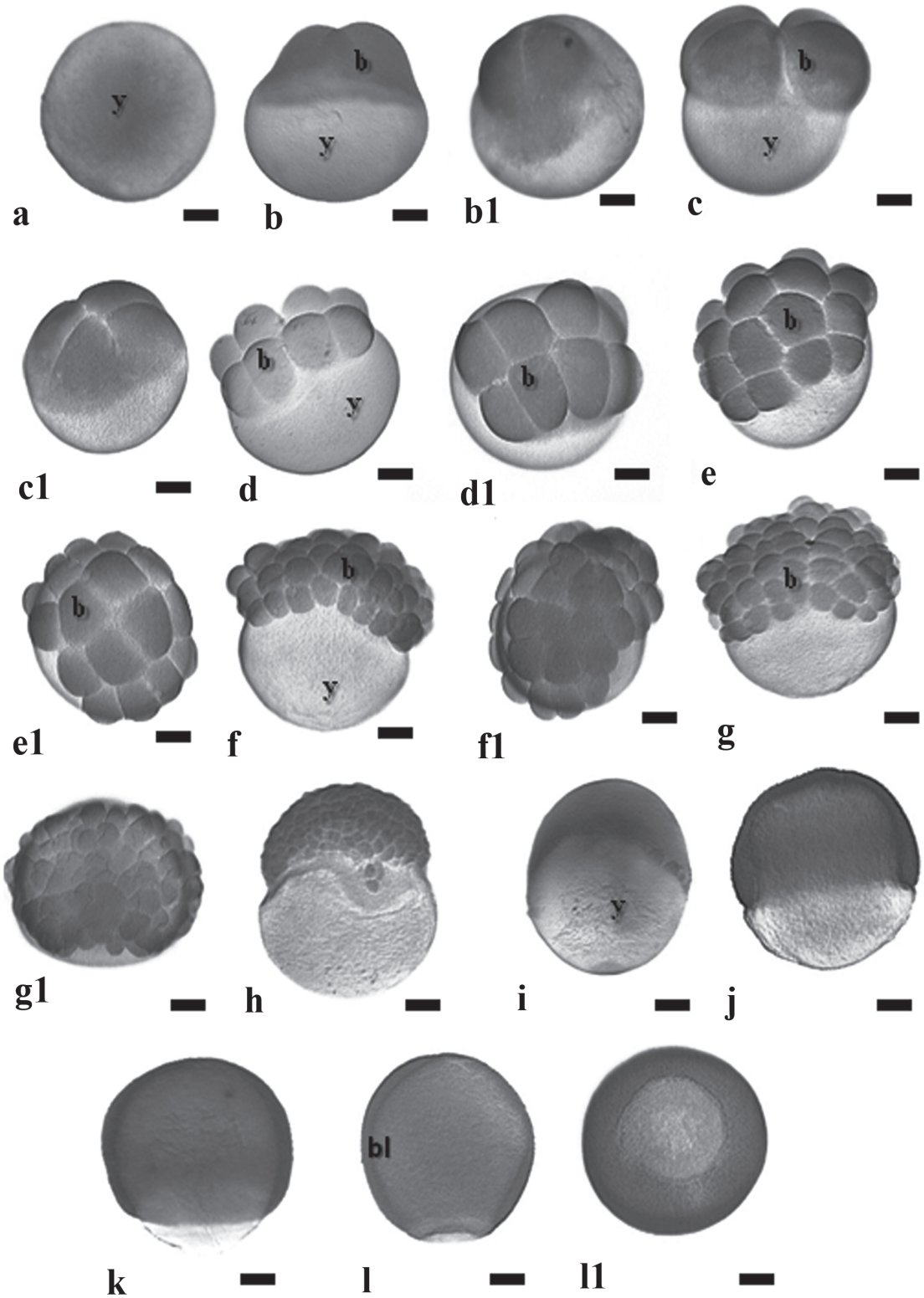

Fig. 1. Embryonic development stages zygote, cleavage and gastrula in the *Pimelodus maculatus*. **a** - post-fertilization without chorion; **b** and **b1** - 2-cell embryos; **c** and **c1** - 4-cell embryos; **d** and **d1**- 8-cell embryos; **e** and **e1** - 16-cell embryos; **f** and **f1** - 32-cell embryos; **g** and **g1** - 64-cell embryos; **h** - morula; **i** - gastrula (25% of epiboly); **j** - gastrula (50% of epiboly); **k** - gastrula (75% of epiboly); **l** - gastrula (90% of epiboly); **l1** - detail of the blastopore closure. Staining: HE. y: yolk, b: blastomeres, bl: blastoderm. Scale bar: 81 μm.

Table 2. Embryonic development of *Pimelodus maculatus*, at 25 ºC.

Time (hours)	Stage	Observations
0-0.42	Zygote	Definition of animal and vegetal poles
0.50	Cleavage	100% with 2 cells
0.67	Cleavage	44% with 2 cells and 56% with 4 cells
0.75	Cleavage	100% with 4 cells
0.83	Cleavage	67 % with 4 cells and 33 % with 8 cells
0.91	Cleavage	80% with 8 cells and 20% with 4 cells
1.00	Cleavage	30% with 4 cells, 33% with 8 cells and 37% with 16 cells
1.17	Cleavage	3% with 4 cells, 46% with 8 cells, 8% with 16 cells and 43% with 32 cells
1.34	Cleavage	19% with 16 cells, 50% with 32 cells and 31% with 64 cells
1.51	Cleavage	16% with 32 cells and 84% with 64 cells
1.68	Cleavage Blastula Gastrula	41% with 64 cells, 43% at morula, 16% in epiboly of 25%
1.85	Cleavage Blastula Gastrula	25% with 64 cells, 56% at morula and 19% in epiboly of 25%
2.00	Blastula Gastrula	86% at morula and 14% in epiboly of 25%
3.00	Blastula Gastrula	80% at morula and 20% in epiboly of 25%
4.00	Gastrula	56% in epiboly of 50%, 26% in epiboly of 75% and 18% in epiboly of 25%
5.00	Gastrula	32% in epiboly of 50% and 68% in epiboly of 75%
6.00	Gastrula	100% in epiboly of 90%
7.00	Gastrula Organogenesis	6% in epiboly of 90% and 94% early segmentation phase
8.00		Early segmentation phase - 6% at neurula and 94% with attached tail, optic vesicle, 5 somites and Kupffer's vesicle
9.00		Early segmentation phase - 12 somites, beginning of body pigmentation, Kupffer's, optic vesicle, attached tail
10.00		Early segmentation phase - 20 somites, otic, optic and Kupffer's vesicles, attached tail, pigmentation of embryo body
11.00		Late segmentation phase - Free tail, absence of Kupffer's vesicle, 24 somites, otic and optic vesicles, pigmentation of embryo body
12.00	Organogenesis	Late segmentation phase - Free tail, more than 30 somites, otic and optic vesicles, pigmentation
13.00		Late segmentation phase - Free tail, more than 35 somites, pigmentation, loose chorion
14.00		Late segmentation phase - More than 40 somites, increased pigmentation at head and tail, free tail, otic and optic vesicles
15.00		90% at pre-hatching and 10% hatched.
16.00	Hatching	62% pre-hatching and 38% hatched
17.00		100% hatched larvae

Cleavage stage. 0.40- 2.00h (29 ºC); 0.50 – 1.85h (25 ºC)

Twenty-four minutes after fertilization at 29 ºC and 30 minutes at 25 ºC, the first cleavages could be noticed initiating from the center towards the blastodisc margins (Fig. 1b1). In *Pimelodus maculatus*, the type of cleavages are discoid meroblastic (Figs. 5a-b), *i.e*., they occur in the animal pole according to the following pattern: first cleavage was vertical, originating two blastomeres (Figs. 1b-b1), the second cleavage was vertical and perpendicular to the first one, giving rise to four blastomeres (Figs.1c-c1); the third cleavage was vertical and parallel to the first one, originating 8 blastomeres (Figs.1d-d1), under a 4x2 arrangement; the forth cleavage was vertical and parallel to the second one, producing 16 blastomeres in a 4x4 formation (Figs.1e-e1); the fifth cleavage was vertical and parallel to the first one, giving rise to 32 blastomeres in a 4x8 formation (Figs.1f-f1); the sixth cleavage was horizontal, forming two cell layers with a total of 64 blastomeres in a 2x4x8 formation (Figs.1g-g1), *i.e*., the embryo presents two cell layers where the superior layer has undergone complete cleavage. For as long as the cleavage continues, the number of blastomeres increases and their size diminishes. Until the third cleavage the cells were homogeneous, but from the fourth cleavage plane onwards blastomeres of distinct sizes could be observed. At this embryonic stage, individualized nuclei were undetectable (Figs.5b-c), but several yolk globules could be visualized in the cytoplasm (Fig.5a).

The morula phase was characterized by a blastoderm with more than 64 cells, forming a "half-berry' like cell mass (Fig. 1h). It was also possible to observe at this stage, the beginning of the formation of the yolk syncyctial layer, named periblast (Fig. 5d).

The yolk syncyctial layer (YSL) of *Pimelodus maculatus* embryos is characterized by a cytoplasm layer with several nuclei, containing yolk globules within the cytoplasm. The nuclei in this layer are initially from the nearby blastomeres that, when in contact with the cytoplasm layers encompassing the yolk, release their contents into it.

Image analyses indicate the yolk globules penetrate the YSL as fragmented portions, and then they are particularized and transferred to the blastomeres where the yolk globules are remounted (Figs. 6a-b).

Blastula stage. 1.68 – 3.00h (25 ºC); 1.75 – 2.00h (29 ºC)

At the beginning of this stage, the blastoderm presented

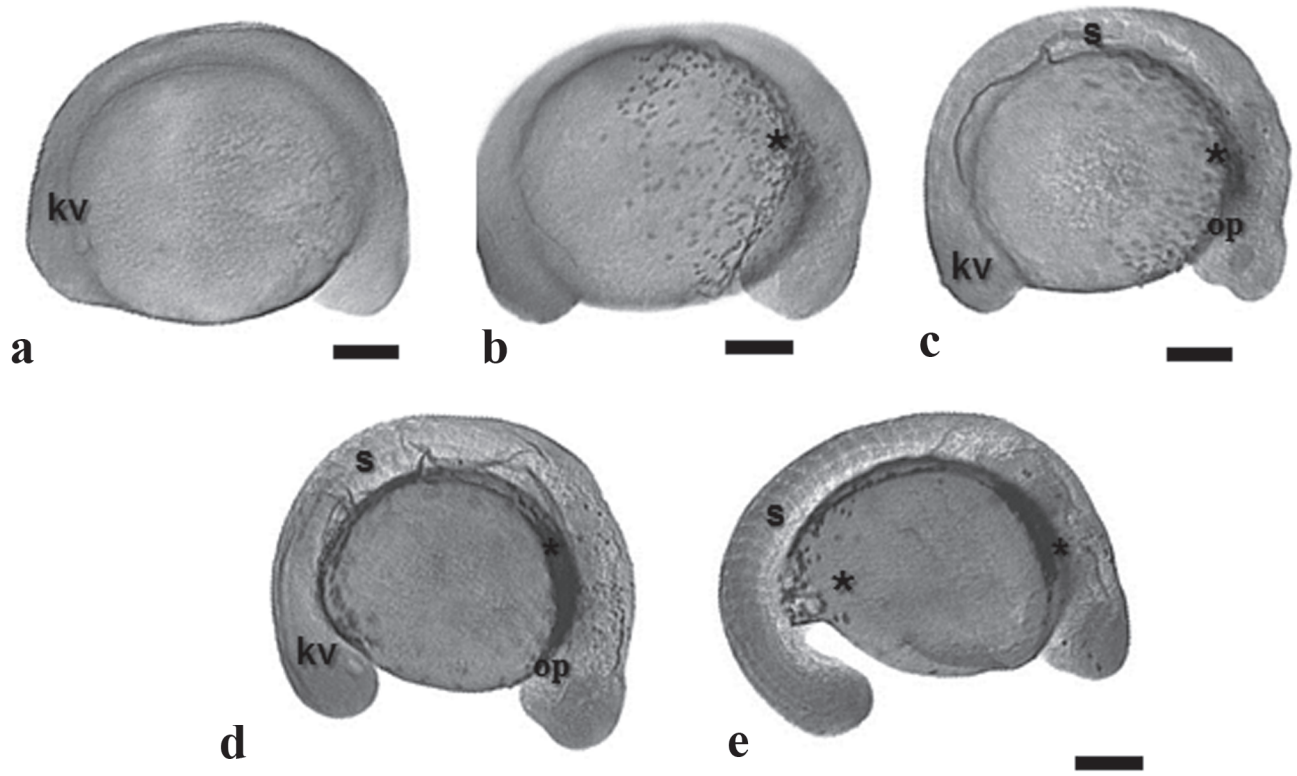

Fig. 2. *Pimelodus maculatus* embryos in segmentation stage: **a** - neurula; **b** - embryo with about 11 somites, optic vesicle, attached tail and pigmentation of embryo body; **c** - embryo with about 14 somites, optic and Kupffer's vesicles, pigmentation of embryo body and attached tail; **d** - embryo with about 19 somites, Kupffer's, optic and otic vesicles, attached tail e pigmentation of embryo body; **e** - 24 somites, presence of otic and optic vesicles, Kupffer's vesicle is absent and free tail. Staining: HE. asterisks: evolution of body pigmentation in embryo; op: optic vesicle; kv: Kupffer's vesicle; s: somites. Scale bars: **a**: 82 μm; **b**: 81.4 μm; **c**: 80.4 μm; **d**: 81.3 μm; **e**: 81.1 μm.

a cup-like shape. The cells were still dividing but the cleavage planes were undetermined. The main characteristic of this stage is the appearance of irregular spaces among the blastomeres - the pseudoblastocoel. The end of this stage was characterized by the first epiboly movements (Figs. 5d and 6a-d).

Gastrula stage. 2.25h – 5.00h (29 ºC); 1.68h – 7.00h (25 ºC)

The gastrula stage was characterized by the occurrence of morphogenetic movements – epiboly, convergence and cell involution (Figs. 1i-j-k-l), that ultimately will form the first germinative follicles and determine the embryonic axis.

During epiboly, the yolk syncyctial layer expands along with the embryo until recover all the yolk mass, forming the yolk plug. The periblast gives rise to a fringe in front of the blastoderm border, since its formation up to blastopore closure (Figs. 6c-e-f and 7a-b). The morphogenetic movements of convergence and cell involution (or migration) started from the blastoderm border at about 50% of epiboly, forming the germination ring or the embryonic shield, and finished with the formation of two embryonic layers, the epiblast and the hypoblast (Fig. 7c).

This stages ends when the yolk plug is completely closed.

Organogenesis stage. 6.00-12.00h (29 ºC); 7.00-14.00h (25 ºC)

Early segmentation phase. 6.00-8.00h (29 ºC); 8.00-10.00h (25 ºC)

This phase lasted from the sixth up to the eighth hour at 29 ºC and from seventh to tenth hour at 25 ºC. At this stage, the optic vesicle was present (Figs. 2c-e and 7e), as well as the Kupffer's vesicle (Figs. 2a-c-d and 7f), the notochord (Figs. 7d and 8b), and the somites that have a mesoderm origin and will give rise to the muscles (Figs. 8a-b). The tail is short and attached to the yolk sac, which is pigmented at the cephalic and caudal extremities due to the presence of punctiforme chromatophores (Figs. 2c-d).

During segmentation, the yolk syncyctial layer becomes a thin layer encompassing the yolk completely (Fig. 7d), the neural tube begins to be formed (Fig. 8b) and, as long as its sections undergo differential growth, the prosencephalon, mesencephalon and rhombencephalon regions can be identified.

The Kupffer's vesicle, whose function remains unknown, disappeared after nine hours at 29 ºC and 11 hours at 25 ºC,

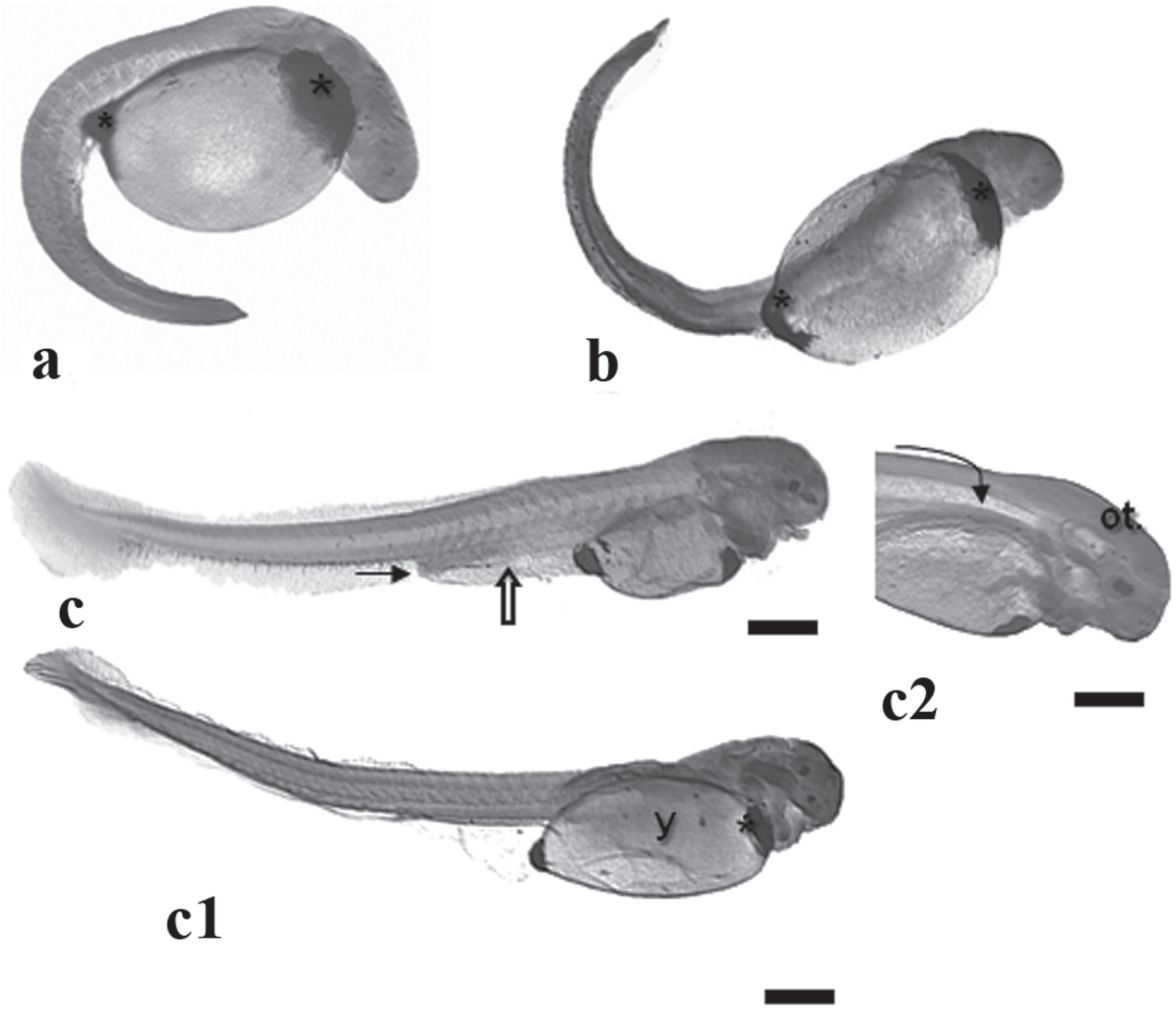

Fig. 3. *Pimelodus maculatus* embryos in organogenesis (late segmentation phase) and hatching stage. **a** – more than 30 somites, growing larva; **b** - pre-hatching embryo; **c, c1** and **c2** –hatched larvae. **C2** – detail of the otic vesicle and notochord. Staining: HE. asterisks: pigmentation close to head and tail; black arrow: anus; large arrow: posterior intestine; ot : otic vesicle; black curved arrow: notochord; y: yolk. Scale bars: **a:** 81 μm; **b:** 81 μm: **c:** 110 μm; **c1:** 81 μm; **c2:** 110.4 μm.

during the neurula phase. Some embryos from this stage on presented an unidentified structure within the yolk syncyctial layer, below the embryo body, that resembles a tube but lacks any delimited membrane (Fig. 7f).

This stage ends with the tail detachment and growth (Fig. 2e).

Late segmentation phase. 9.00-12.00h (29 ºC); 11.00-14.00h (25 ºC)

Started at the 12th hour and lasted up to 14th hour at both 29 ºC and 25 ºC, being characterized by the presence of a free tail, more than 30 somites and embryo growth (Fig.3).

At this phase, the embryos presented a well developed otic vesicle (Fig. 9a), the notochord extends from the cephalic up to caudal region (Fig. 8c), the posterior intestine developed from the endoderm, reaching the urogenital pore and the intestinal lumen can be observed (Figs. 8d and 9e). The somites are undergoing myogenesis to eventually form the muscles (Figs. 8e-f).

The chromatophores that were located at the edges of the yolk sac started migrating and now can be observed in several parts of the embryo body. They are also changing from a punctiforme a dendritic morphology (Figs. 9c-f).

Hatching stage. 13.00h (29 ºC); 17.00h (25 ºC)

At this stage, the embryos presented a mean total length of 3410 μm (Fig. 3c) and vigourus movements that are important to break the chorion, more flexible now.

At hatching, the somite myogenesis was quite advanced (Fig. 9b), and heart beats could be clearly noticed *in vivo*. Under histological cuts, the hearts is evident (Fig. 9d), the mouth is closed once the digestive system is under

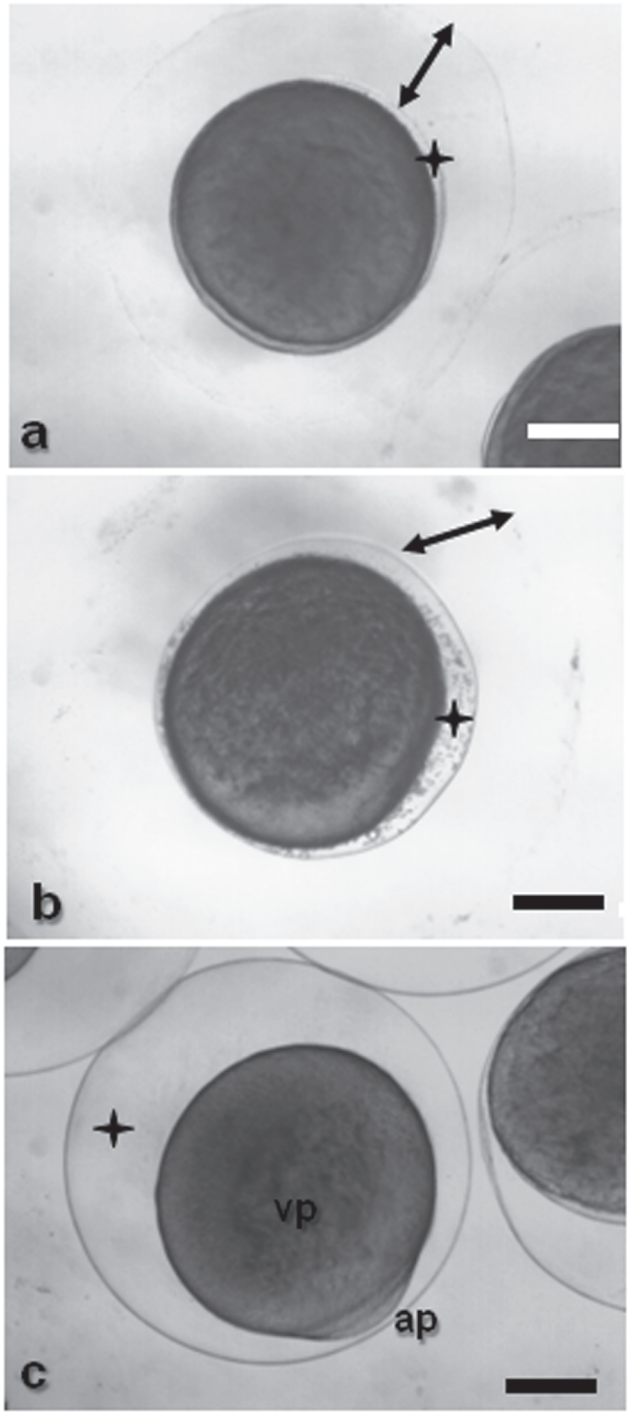

Fig. 4. *Pimelodus maculatus* egg. **a** - fertilized and non-hydrated egg; **b** - hydrating egg and **c** - hydrated egg. star: perivitelline space; double arrow: gelatinous layers; vp: vegetal pole; ap: animal pole. Scale bars: **a:** 75 μm; **b:** 868 μm; **c:** 1100 μm.

development, the eyes are intensively pigmented, the chromatophores are spread over several body parts, and the cephalic structures are well developed and defined (Figs. 8d and 9a-e).

The tube-like structure formerly seen within the YSL remains up to late segmentation phase, composing a single structure at the cephalic and caudal ends and double at the dorsal region (Figs. 8b-f-h).

Discussion

The morphological events identified during the embryogenesis of *Pimelodus maculatus*, as well as the short duration of embryonic development, were similar to those reported in other teleosts: *Brycon orbignyanus* (Ganeco, 2003), *Prochilodus lineatus* (Ninhaus-Silveira *et al.,* 2006), *Pseudoplatystoma coruscans* (Marques *et al.,* 2008, Landines *et al.,* 2003).

In this present study, after fertilization, egg hidratation was observed, which lead to a increase of its volume. The morphometric values of *Pimelodus maculatus* eggs were similar to those obtained by Luz *et al.* (2001), 900 ± 20 μm for non-hydrated eggs e 1090 ± 10 μm for hydrated ones at temperature the 23.1 ± 0.5°C, but lower to those reported by Sato (1999); 1050 to 1200 μm (non-hydrated) and 1730 to 1950 μm (hydrated eggs) at temperature between 24 and 25°C. Species such as *Prochilodus lineatus* (Ninhaus-Silveira *et al*., 2006), have eggs with a large perivitelline space that protects the embryo against injuries during embryogenesis, contributing to improved survival in running water. In *Rhamdia hilarii* eggs, the perivitelline space has been reduced (Godinho *et al*., 1978), as was also observed in *Pimelodus maculatus* eggs, after hydration of the eggs. Taking into account the functions assigned to the chorion and the perivitelline space, such as mechanical protection, osmotic regulation, fluctuating, nutrition and prevention of polyspermy (Shardo, 1995), this difference in size of the perivitelline space occurs due to different reproductive strategies of species above mentioned and the environment in which eggs develop. The yellowish colour is characteristic of the oocytes of Siluriformes (Sato *et al*., 2003; Marques *et al*., 2008) and is associated with the presence of carotenoid pigments obtained from food. These pigments constitute an endogenous oxygen supply when the respiratory system is inefficient at obtaining exogenous oxygen (Balon, 1977; Amorim *et al*., 2009 apud Perini *et al.*, 2010).

The gelatinous layer surrounding the oocytes and the eggs of the *Pimelodus maculatus* provided some adherence to them, as observed in *Rhamdia hilari* (Godinho *et al.*, 1978), *Pseudoplatystoma coruscans* (Landines *et al.*, 2003), *Rhinelepis aspera* (Perini *et al.*, 2010) and in surubins hybrids (*Pseudoplatystoma corruscans* x *Pseudoplatystoma fasciatum*) (Faustino *et al.*, 2007). According to Rizzo *et al.* (2002), this gelatinous layer found in Siluriformes is composed of a net of several delicate fibrils, being observed in either adhesive or non-adhesive eggs, such as those from *Pimelodus maculatus,* as well as in eggs from other teleosteans like Perciformes, Cypriniformes and Cyprinodontiformes (Riehl & Patzner, 1998). More studies concerning the constitution and function of the jelly coat and therelationship between egg surface and adhesiveness are necessary.

The egg cleavages in *Pimelodus maculatus* occurred only

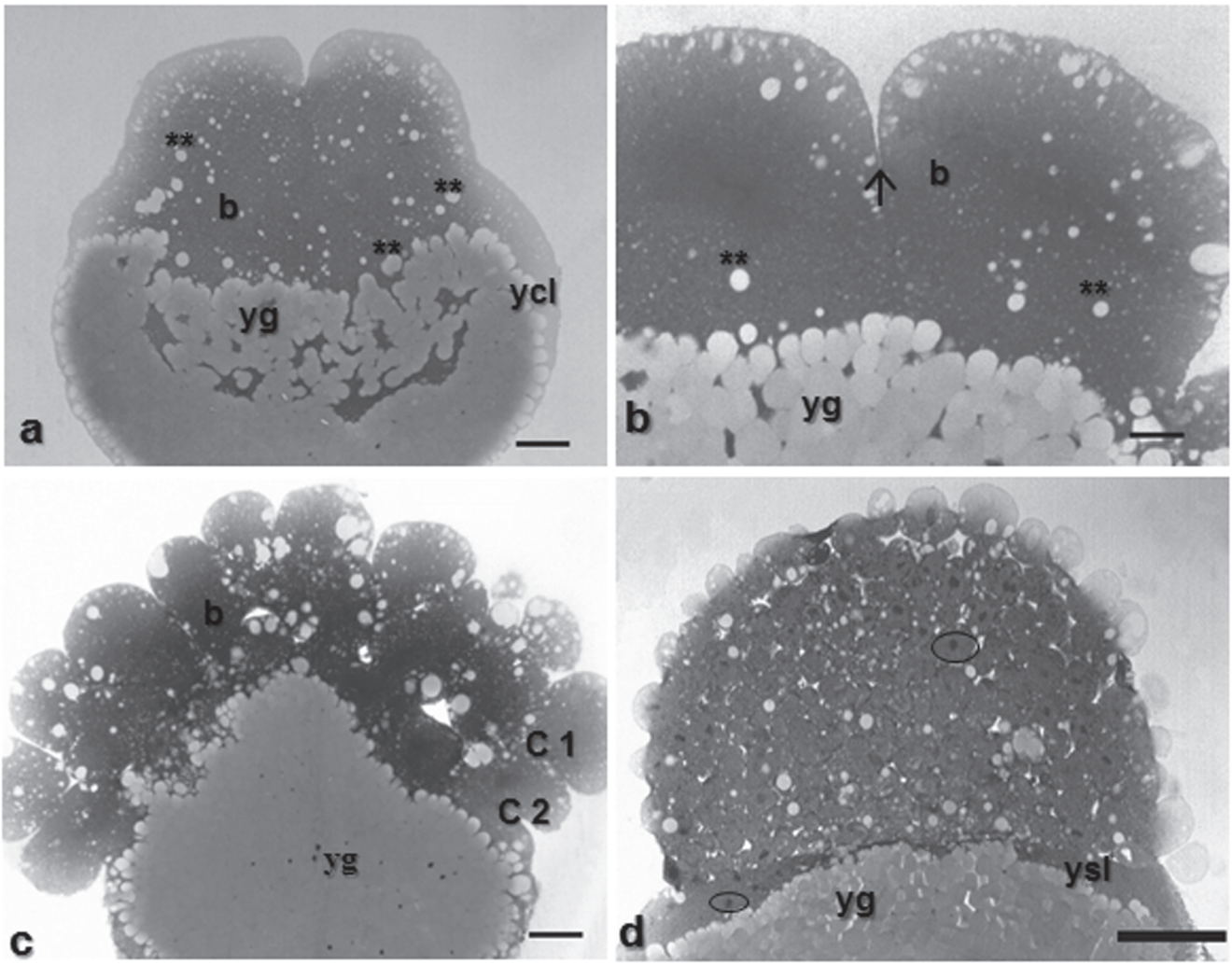

Fig. 5. Histologicals sections of *Pimelodus maculatus* embryos in cleavage stage. **a** - with 2 cells, staining: HE, showing details of the yolk globules penetration into embryo; **b** - with 16 cells, staining: toluidine blue, showing detail of the meroblastic division; **c** - visualization of the penetration of yolk globules in blastomeres, first complete and partial second cleavages, staining: toluidine blue; **d** - morula phase, staining: HE, characterized by the formation of the yolk syncyctial layer and individualized nuclei. b: blastomeres; ycl: yolk cytoplasm layer; yg: yolk globules; two asterisks: penetration of yolk globules in the embryo; arrow: partial or meroblastic division; C1: total cleavage; C2: partial cleavage; ysl: yolk syncyctial layer; circle: individualized nucleus. Scale bars: a: 210 μm; b: 83.8 μm; c: 83.8 μm; d: 526.4 μm; e: 42.6 μm.

in the animal pole, while the vegetative pole was composed of yolk. Such division pattern is typical of fish eggs and it is known as meroblastic or partial, once it involves exclusively the animal pole (Balinsky, 1970; Leme dos Santos & Azoubel, 1996). The mitotic divisions during the cleavage take place in order to promote a new balance in the relationship between the nucleus and the cytoplasm, *i.e.*, the high volume of the zygote cytoplasm is divided into increasingly smaller cells, while the cytoplasm volume does not increase (Gilbert, 2003). This feature was visualized in *Pimelodus maculatus*: as the number of blastomeres increased, their size decreased corroborating the reports by Ganeco (2003), Nakagui *et al.* (2006) and Castellani *et al.* (1994), being possible to observe several events of mitotic divisions. Ninhaus-Silveira *et al.* (2006) reported that the yolk globules were fragmented during the cleavage stage, what is likely to facilitate their absorption by the cells, thus corroborating the observations in *Pimelodus maculatus*.

Kimmel *et al.* (1995) and Morrison *et al.* (2001) reported that even within the same spawn and incubation at optimal conditions, the period of embryonic development shows asynchrony. Analyzing the abovementioned results, it can be shown that the morphological changes during embryogenesis, besides being species-specific, are closely related to temperature variation. At higher temperatures, the incubation time is shortened, while, at lower temperatures, this period is increased. In the present study with *Pimelodus maculatus*,

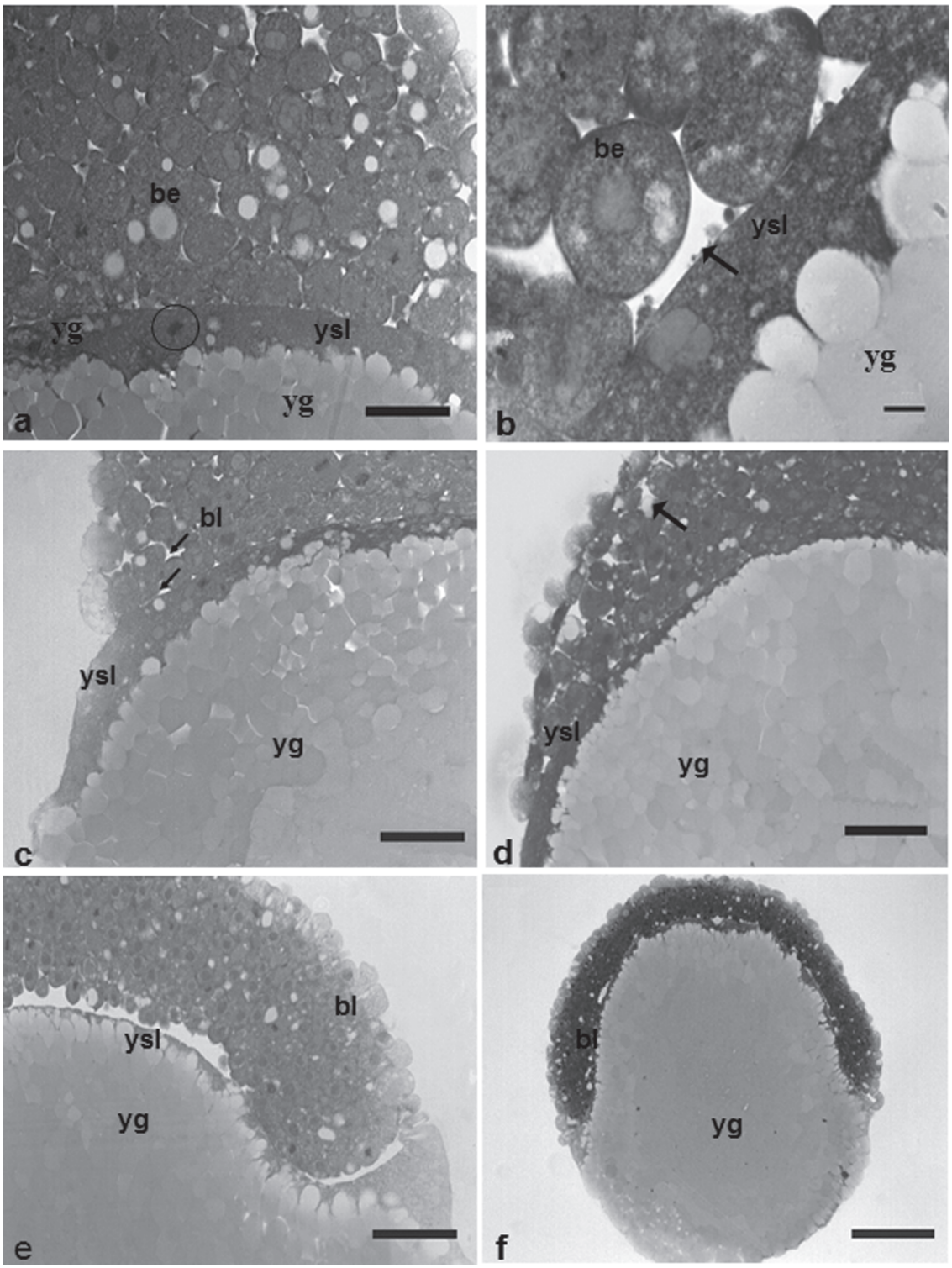

Fig. 6. Histologicals sections of *Pimelodus maculatus* embryos. **a-** detail of the nucleus of the syncyctial layer, at morula phase, staining: HE; **b** - detail of the penetration of yolk globules as fragments into the yolk syncyctial layer, staining: HE; **c**- embryo section at blastula stage, initiating epiboly, staining: toluidine blue; **d**- embryo section at gastrula stage (epiboly of 25%), expansion of yolk syncyctial layer along with the embryo to form the yolk plug, staining: toluidine blue; **e** - embryo section at gastrula stage (epiboly of 25%), staining: HE. **f** - embryo section at gastrula stage (epiboly of 50%), staining: toluidine blue; ysl: yolk syncyctial layer; be: blastomeres with euchromatic nucleus; circle: presence of nucleus in the yolk syncyctial layer; yg: yolk globules; bl: blastoderm; black arrows: irregular spaces; bl: blastoderm. Scale bars: **a:** 68.2 μm; **b:** 65.6 μm; **c:** 52.5 μm; **d:** 52.5 μm; **e:** 83.8 μm **f:** 210 μm.

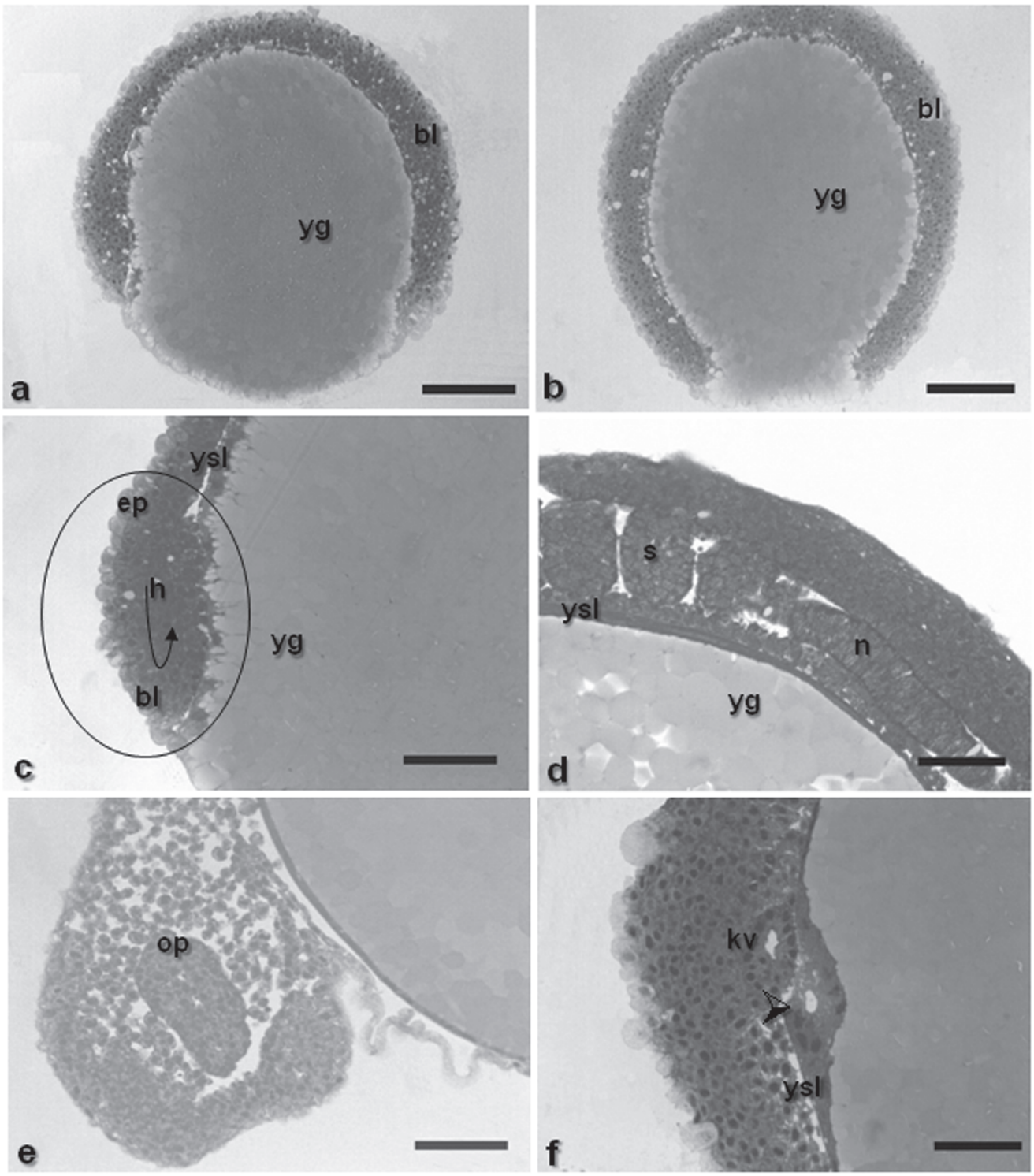

Fig. 7. a - embryo section at gastrula stage (epiboly of 75%), staining: HE; **b** - embryo section at gastrula stage (epiboly of 90%), blastopore closure. Staining: HE; **c** - embryo section at gastrula stage (epiboly of 50%), staining: toluidine blue. **d** - neurula phase, with details of somites and notochord, staining: toluidine blue; **e** - neurula phase, with details of the optic vesicle, staining: HE. **f** - neurula phase, with details of the Kupffer's vesicle and unidentified structure. Staining: HE. yg: yolk globules in the embryo; bl: blastoderm; ysl: yolk syncyctial layer; s: somites; n: notochord; circle and black arrow: embryonic shield; ep: epiblast; h: hypoblast; op: optic vesicle; kv: Kupffer's vesicle; ysl: yolk syncyctial layer; arrowhead: unidentified structure. Scale bars: **a:** 210 μm; **b:** 210 μm; **c:**106.6 μm; **d:** 52.5 μm; **e:** 83.8 μm; **f:** 52.5 μm.

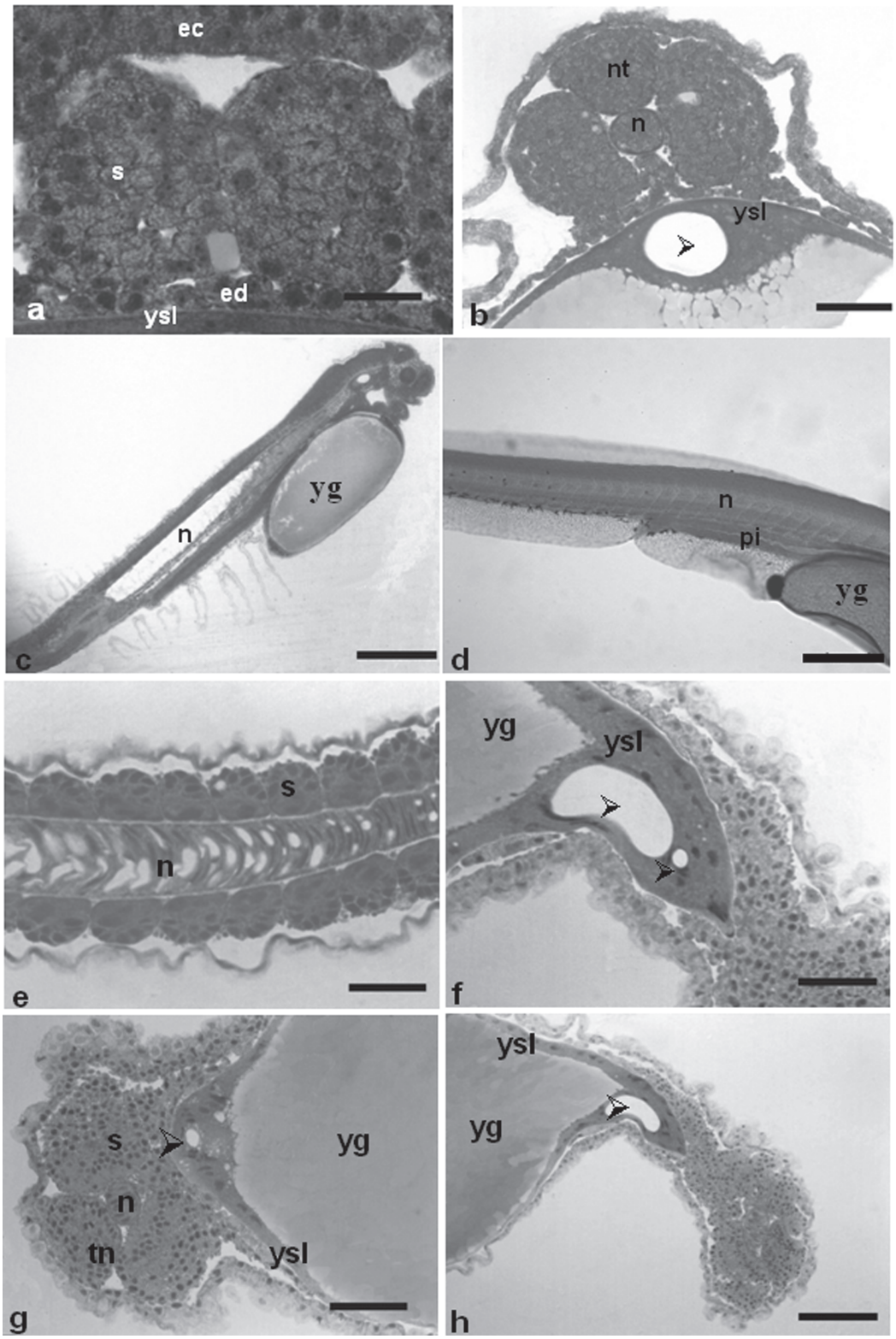

Fig. 8. a - segmentation stage, showing the somites that will form the muscles; **b** - segmentation stage with formation of the neural tube; **c** - overall detail of the embryo, highlighting the notochord; **d-** posterior intestine under development; **e** - development of notochord up to caudal region, presence of somites; **f** - unidentified double structure across the yolk syncyctial layer; **g** - unidentified structure in the yolk syncyctial layer; **h** - unidentified single structure across the yolk syncyctial layer. Staining: HE. ec: ectoderm; ed: endoderm; ysl: yolk syncyctial layer; s: somites; nt: neural tube; n: notochord; arrowhead: unidentified structure; pi: posterior intestine; yg: yolk globules in the embryo. Scale bars: **a:** 20.8 μm; **b:** 65.6 μm; **c:** 139.3 μm; **d:** 100 μm; **e:** 42.6 μm; **f:** 68.2 μm; **g:** 83.8 μm; **h:** 173.2 μm.

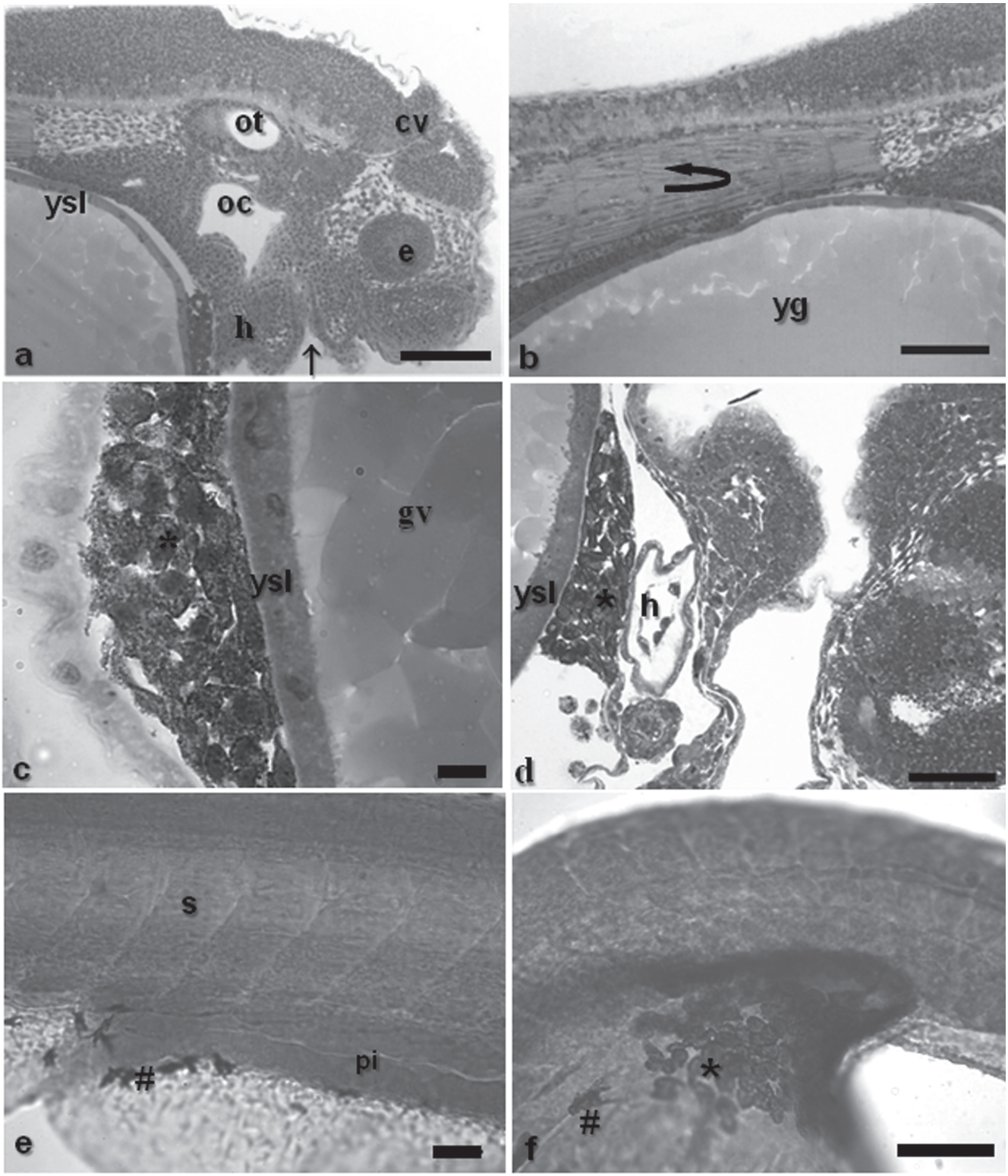

Fig. 9. a - head detail, showing the otic vesicle, oral cavity, cephalic vesicle, closed mouth and eye. **b** - myogenesis process; **c** and **d** - detail of punctiformes chromatophores caudal and cephalic extremities of the yolk sac, respectively, and heart; **e** - development of posterior intestine; **f** - dendritic chromatophores at the end of the yolk sac. Staining: HE. ot: otic vescile; oc: oral cavity; cv: cephalic vesicle; arrow : closed mouth; ysl: yolk syncyctial layer; e: eye; h: heart; yg: yolk globules in the embryo; curved arrow: myogenesis process; number sign: dendritic chromatophores; asterisks: punctiformes chromatophores; pi.: posterior intestine. Scale bars: **a:** 139.3 μm; **b:** 139.3 μm; **c:**10 μm; **d:**10 μm; **e:**141,9 μm; **f:**35,6 μm.

despite the utilization of young breeders and a constant water temperature, incubation at 25 ºC led to a higher asynchrony of embryonic development and a variation in blastomere division. Some authors such as Arezon *et al.* (2002) and Ninhaus-Silveira *et al.* (2006) have also shown that, coupled with a natural variation, the water temperature during incubation also interferes with the homogeneity of embryo development.

According to the results by Long & Ballard (1976) in *Catostomus commersoni* and Matkovic *et al.* (1985) in *Rhamdia sapo*, the formation of the yolk syncyctial layer (YSL) begins during morula phase, as reinforced by the data from the present experiment. However, Cardoso *et al.* (1995), analyzing the embryogenesis of *Pseudoplatystoma coruscans* and González-Doncel *et al.* (2005) in *Oryzias latipes* reported that this layer begins to be formed during the blastula stage.

This layer can be regarded as an extra-embryonic organ being found only in teleosteans, and thus not contributing for the embryo formation, but playing a role in the yolk breakage, turning it viable for the embryo cells (Kimmel *et al.*, 1995). In *Pimelodus maculatus*, yolk globules could be observed in the yolk syncyctial layer close to the blastoderm, differently from the report by Ninhaus-Silveira *et al.* (2007) in *Prochilodus lineatus*. The YSL membrane projections that extended between the yolk globules, as well as the presence of small yolk portions in YSL, indicate that the vitellinic material is partitioned and then transferred into the blastoderm. It can be inferred that the nutritive content passes through the plasmatic membrane under the form of small molecules and then it is reconstituted in the cytoplasm of blastoderm cells.

The blastula stage is characterized by the formation of blastocoele (formation of a large space between the yolk and the blastomeres). In this species the formation of a blastocoele has also been observed, represented by irregular spaces among some blastoderm cells, as described by several authors for other teleostean species (Marques 2008, Trinkaus, 1984; Ganeco, 2003). Regarding the irregular spaces that arise between blastoderm cells, Kimmel *et al.* (1995) have suggested that this stage could be better defined as the stereoblastula, since the formation of the blastocoel cavity has not yet formed. However, other authors have not differentiated between these stages in teleosteans and merely report the presence of the blastocoel (Lagler *et al.* 1977 apud Faustino *et al.,* 2010).

The gastrula stage begins with the first epiboly movements (Leme dos Santos & Azoubel, 1996) and is completed by closure of the blastopore by the blastoderm and the formation of a tail button (Kimmel *et al*., 1995). These observations are in accordance with what was detected in *Pimelodus maculatus*. The gastrula stage began after two hours and fifteen minutes of development at 29 ºC and after one hour and forty minutes at 25 ºC. In *Pimelodus maculatus*, the formation of the embryonic shield becomes visible when the blastoderm encompasses 50% of the yolk sphere, being in accordance with previous reports in other species (Kimmel *et al*., 1995; Ganeco, 2003; Ninhaus-Silveira *et al.*, 2006). On the other hand, in *Oreochromis niloticus*, Morrison *et al.* (2001) reported that, due to the size of yolk, the embryo is not able to extend over the entire vegetal pole and, thus, rudimentary organogenesis (somite segmentation) starts before the epiboly movement is finished. The blastopore closure was observed five hours after fertilization at 29 ºC and six hours after fertilization at 25 ºC. Previous studies with the same species carried out by Luz *et al.* (2001) showed the beginning of gastrulation after two hours of embryonic development at 23.1 ± 0.5 ºC and the blastopore closure occurred five hours and fifty minutes after fertilization.

Following the gastrulation, comes the organogenesis stage. The formation of somites is necessary in the organization of the segmental pattern of vertebrate embryos. These structures are transitory muscular precursors that develop into cell blocks (Gilbert, 2003). The beginning of the somitogenesis in *Pimelodus maculatus* took place after blastopore closure and the end of epiboly, as also reported for *Rhamdia quelen* (Amorim *et al.*, 2009); *Leporinus piau* (Borçato *et al.,* 2004); *Prochilodus lineatus* (Ninhaus-Silveira *et al.*, 2006) and three trahira species (Gomes *et al.,* 2007). In *Pimelodus maculatus*, the embryo was differentiated after seven hours at 29 ºC and nine hours at 25 ºC. In the same species, Luz *et al*. (2001) observed that the embryo differentiation occurred after 10 hours and 50 minutes of incubation at 23.1 ± 0.5 ºC.

As mentioned previously, the morphological changes during embryogenesis, being beside species-specific, are closely related to temperature variation. However Kimmel *et al.* (1995) point out that comparisons of embryos raised at different temperatures should be made with caution, because there is not assurance that all features of the embryo change coordinately their rates of development when the temperature is changed. The difference in hatching time might be due to environmental conditions water flow, alkalinity, pH (Cussac *et al*., 1985), may be related to biotic factors such as age, size of the breeding or nutritional factors. In addition, other environmental factors not detected in the water could have affected developmental period of the embryos.

No information was available to help us identify the structure located within the YSL and further studies are required to elucidate its function, if any.

The anterior region of the neural tube in the studied species expanded to form the prosencephalon, mesencephalon and rhombencephalon regions, similar to the patterns described in *Pseudoplatystoma coruscans* (Marques *et al*., 2008) and *Prochilodus lineatus* (Ninhaus-Silveira *et al.,* 2006).

Brummett & Dumont (1978) and Morrison *et al.* (2001) found the Kupffer's vesicle stage in the early stages of organogenesis, as in our study this vesicle is observed in early neurula and at the end of this phase disappears. According Hisaoka & Firlit (1960) Kupffer's vesicle represents a reminiscent structure of arquenteron and is located above of the periblasto and below of the notochord. In *Oncorhynchus keta* (Mahon & Hoar, 1956 apud Hisaoka & Firlit, 1960) Kupffer's vesicle cavity is described as an oblique and elongated, with walls of columnar epithelium, which is separated from periblasto by a layer of endodermic cells, similar

to that described by Ninhaus-Silveira *et al.* (2006) in *Prochilodus lineatus*. Brummet & Dumont (1978) raised a hypothesis that this could have a digestive function, favorable to yolk resorption by the embryo, since several ciliated cells were observed inside Kupffer's vesicle and the intestines of *Fundulus heteroclitus*. *Pimelodus maculatus* Kupffer'svesicles showed a histological constitution as described above, but was not detected ciliated cells.

Studies about chromatophores and body or eye pigmentation are helpful as taxonomic traits for species identification (Meijide & Guerreiro 2000). Nakatani *et al*. (2001) described two types of chromatophores: punctiforme, when present as small spots, and dendritic, when they assume an irregular shape with projections into distinct directions. In this work, the pigmentations has intensified and irregularly distributed, the chromatophores started migrating throughout the body by using pseudopodia for moving and then changed from punctiforme into a dendritic shape. Some fish species present only dendritic chromatophores, such as *Rhinelepis aspera* (Perini *et al.*, 2010), others have just dot-like chromatophores, like *Rhamdia quelen* (Amorim *et al.,* 2009) and *Hoplias lacerdae* and *Hoplerythrinus unitaeniatus* (Gomes *et. al.,* 2007), whereas *Hoplias malabaricus* (Gomes *et al.*, 2007) presents both types, just like observed in the present work.

Right after hatching, the larvae of *Pimelodus maculatus* were pigmented, differently from what has been observed by Faustino *et al.* (2007) in surubins hybrids and by Marques *et al.* (2008) in *Pseudoplatystoma coruscans.* In the present experiment, the larvae present a mean total length higher than that reported by Luz *et al.* (2001) at temperature 23,1 ± 0,5°C and Sato (1999) at temperature between 24 and 25°C.

The present data, therefore, showed that the study of embryonic development is essential to a better knowledge of some biological features of a species, helpful to elucidate issues related to fish rearing at this stage, being also useful to further taxonomic, ecological and conservational studies in the *Pimelodus maculatus.*

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