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Structural analysis of embryogenesis of *Leiarius marmoratus* (Siluriformes: Pimelodidae)

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Summary

Embryological studies in fish species are useful to the understanding of their biology and systematics. The available biological data in *Leiarius marmoratus* are scarce and additional information about its reproductive biology is needed, mainly because this species has been commercially exploited and used in production of hybrid lineages. In order to evaluate the temporal–morphological embryonic modifications in *L. marmoratus*, samples of nearly 200 embryos were collected at random at different stages of development, starting from fecundation (time zero). Embryos were fixed in modified Karnovsk's solution and 2.5% glutaraldehyde, processed and analysed under optic and electron microscopy. The incubation period of *L. marmoratus* was equal to 14.42 h at a mean temperature of 28.3 ± 0.07°C. The following stages of embryonic development were established: zygote, cleavage, gastrula, organogenesis and hatching. These stages were divided into phases, as follows: cleavage – phases of 2, 4, 8, 16, 32 and 64 cells and morula; gastrula – phases of 25, 50, 75 and 90% of epiboly and blastopore closure; and organogenesis – neurula, segmentation and pre-larval phases. The embryogenesis of *L. marmoratus* was typical of neotropical teleosteans, with peculiarities in species development.

Keywords: Embryology, Morphology, Neotropical fish, Ontogenesis, Siluriform, Ultrastructure

Introduction

Embryological analyses of fish species are important to achieve a detailed knowledge about their biology and systematics, particularly related to ontogenetic variation in morphology, growth, feeding, behaviour and mortality. Besides serving to identify new stocks and for fisheries management, such studies might be useful for the identification of aquatic habitats in which species are recruited (Nakatani *et al.*, 2001).

An understanding of the ontogeny of species is also favourable to the development of biotechnological processes for environmental monitoring by evaluating the effects of toxic substances over the ontogenetic pattern in aquatic fauna. Moreover, embryonic studies can assist fish culture practices and provide insights on evolution, heredity and the development of structural traits in organisms (Lagler, 1959; Flores *et al.*, 2002; Botero *et al.*, 2004; Ninhaus-Silveira *et al.*, 2006; Marques *et al.*, 2008).

Compared with the high number of native fish in Brazil, little information is known about their reproductive biology and most studies have focussed on commercially important species for fisheries and aquaculture. Reports on embryogenesis in Characiformes species can cite *Brycon cephalus* (Alexandre *et al.*, 2010), *B. insignis* (Andrade-Talmelli *et al.*, 2001),

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B. orbignyanus (Ganeco *et al.*, 2008), *Leporinus piau* (Borçato *et al.*, 2004), *Prochilodus lineatus* (Ninhaus-Silveira *et al.*, 2006) and *B. amazonicus* (Nakaghi *et al.*, 2013). Amongst Siluriformes, embryonic traits are known for *Rhamdia hilarii* (Godinho *et al.*, 1978), *R. sapo* (Cussac *et al.*, 1985), *Pseudoplatystoma corruscans* (Cardoso *et al.*, 1995), *Parauchenipterus galeatus* (Sanches *et al.*, 1999), *Pimelodus maculatus* (Luz *et al.*, 2001; Buzzolo *et al.*, 2011), *Rhinelepis aspera* (Perini *et al.*, 2009), and *R. quelen* (Amorim *et al.*, 2009; Rodrigues-Galdino *et al.*, 2009).

The species *Leiarius marmoratus* is widespread in South American basins (Amazonas, Essequibo & Orinoco). This catfish may reach about 100 cm in length, and is characterized by 9–10 rays in its dorsal fins and dark patches over a yellowish-brown background along the body and fins (http://www.fishbase.org; Reis *et al.*, 2003). According to Ramírez-Gil & Ajiaco-Martinez (1997), this fish reaches a mean weight of 12 kg, being found in deep freshwater environments with a pH range of 5.8–7.2 and a mean temperature of 24–26°C. In its natural habitat, this fish presents a piscivorous diet (Layman *et al.*, 2005).

Since 1986, this catfish has been bred artificially via hormonal induction (Kossowski, 1996). According to Cruz-Casallas *et al.* (2008), *L. marmoratus* is easily adapted to captivity and artificial feeding using dry food, yielding high growth rates during early life stages in preliminary studies. Usually, this species has been selected to produce hybrids in crosses with *P. reticulatum* and *Pimelodus blochii* (Kossowski & Madrid, 1985, 1991; Kossowski, 1991, 1992, 1996a,b).

Even though this species has been routinely raised in captivity, information about its reproductive biology in specialized literature is scarce. Therefore, the goal of the present study was to characterize the morphological events that take place during embryogenesis of *L. marmoratus* using light and electron microscopy.

Materials and methods

The work was carried out during the spawning season of *L. marmoratus* (Fig. 1*A*) from January to February 2010. To obtain the embryos, fish specimens were selected from the broodstock available in National Centre for Research and Conservation of Continental Fish, Chico Mendes Institute for Biodiversity Conservation–CEPTA/ICMBio, Pirassununga, São Paulo, Brazil and Muriti Fishculture Farm, Nova Mutum, Mato Grosso, Brazil.

Analysis of embryonic development

To analyze the temporal–morphological changes in embryos of *L. marmoratus*, random samples of nearly 200 eggs were collected at different times of embryonary development, considering the moment of fecundation as time zero. The eggs were incubated at 28.3 ± 0.07°C, and the collection of samples occurred at 5 min during the first h of development and each 10 min up to the first 2 h of embryogenesis. Subsequent samplings were performed in 1-h intervals up to larval hatching. The collected embryos were divided into two fractions: one was fixed in a solution of 2% glutaraldehyde and 4% paraformaldehyde in sodium phosphate buffer 0.1 M, pH 7.3 for 24 h. Afterwards, the material was transferred into 70% ethanol (light microscopy). The second parcel was fixed and stored in 2.5% glutaraldehyde solution diluted in sodium phosphate buffer 0.1 M, pH 7.3 (scanning electron microscopy).

The prefixed material was then transported to the Neotropical Ichthyology Laboratory (LINEO) – Departament of the Biology and Zootecny, UNESP/FE, Ilha Solteira, São Paulo. Brazil.

Analysis using light microscopy

Fifty embryos of each sample were selected for *in toto* analyses. The chorion was removed using watchmaker's forceps and a needle, stained with Harris haematoxylin–eosin (HE), analyzed and photographed in a stereomicroscope Motic SMZ 168, equipped with a digital camera Moticam 2500/5.0 Mega Pixels USB 2.0.

For histological studies, the selected embryos were embedded in glycol methacrylate (Technovit 7199/historesin) and microtomed into serial transversal and sagittal cuts of 3 and 2 μm. The cuts were placed onto histological slides and then stained with Harris haematoxylin–eosin. Then the histological slides were analyzed using a light microscope (Olympus–CX41) and microphotographed (Moticam 2500–5.0 MPixel USB 2.0).

Analysis by scanning electron microscopy (SEM)

For SEM, the prefixed embryos in 2.5% glutaraldehyde were post-fixed in osmium tetroxide, dehydrated in critical-point Balzers, metalized in a Balzers Metalizer (MED-010 Balzers Union), analysed and electromicrographed using a scanning electron microscope (SEM Quanta 200 - FEI).

Ethics statement

The methodological procedures used for the development of this work is in accordance with the Ethical Principles of Animal Experimentation adopted by the Brazilian College of Animal Experimentation (COBEA) and was approved by the Ethics Committee on Animal Use (CEUA), Faculty of Engineering,

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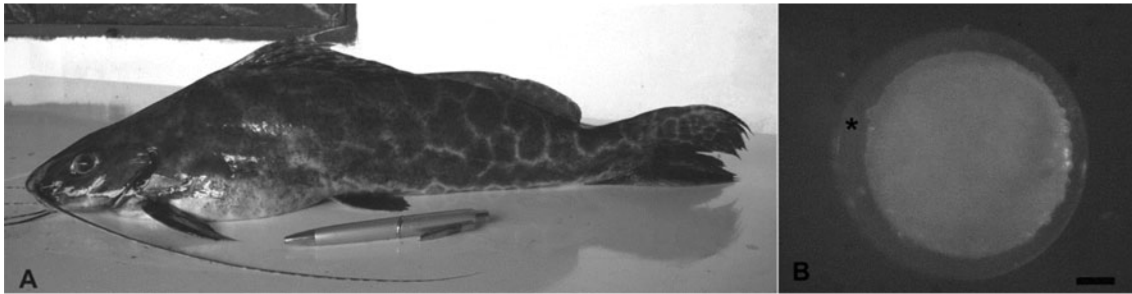

Figure 1 (*A*) *Leiarius marmoratus*; (*B*) hydrated egg. Asterisk – perivitelline space. Bar – 100 μm.

UNESP/Ilha Solteira, São Paulo, Brazil (Protocol no. 007/2011/CEUA).

Results

The eggs of *L. marmoratus* are spherical, yellowish and demersal with a narrow perivitelline space and a well defined transparent chorion after hydration. Fat droplets in the yolk sac were absent during the entire embryonic development. No gelatinous layer was detected surrounding the eggs of *L. marmoratus* after fertilization (Fig. 1*B*). After hydration the perivitelline space and the diameter of the eggs showed about 74.51 ± 20.40 μm and 930.88 ± 27.01 μm, respectively.

Embryogenesis

The incubation period of *L. marmoratus* comprised 14 h at a mean temperature of 28.3°C. The embryonic stages observed were: (i) zygote; (ii) cleavage: 2-cell, 4-cell, 8-cell, 16-cell, 32-cell, 64-cell and morula; (iii) gastrula: epiboly (morphogenetic movement in which the blastoderm covers the yolk vesicle) at 25, 50, 75 or 90% and blastopore closure; (iv) organogenesis: neurula, segmentation and pre-larval phases; and (v) hatching (Figs. 2 and 3).

Some degree of heterogeneity was observed in embryo development, i.e., at the same moment, embryos in different stages or phases of embryogenesis were detected.

Zygote stage

After fertilization and egg hydration, the perivitelline space increased and animal and vegetal poles were defined. The animal pole is composed of active cytoplasm and a nucleus, being identified *in vivo* as a more transparent area, while the vegetal pole is denser *in vivo* and encompasses the yolk vesicles. As animal and vegetal poles were clearly distinguished and a large amount of yolk was present, the eggs were classified as telolecithal. For the first 30 min of incubation, all embryos observed were in the zygote stage (Fig. 2*A*).

Cleavage stage

After 40 min incubation the first cell divisions were observed. The cleavage type was meroblastic or incomplete, according to the following pattern: the first cleavage was vertical, giving rise to two blastomeres of similar size; the second one was vertical and perpendicular to the first division, forming four blastomeres, the third cleavage was vertical and parallel to the first one, giving rise to eight blastomeres in a 4 × 2 arrangement; the forth cleavage was vertical and parallel to the second one, producing 16 blastomeres in a 4 × 4 formation; the fifth cleavage was vertical and parallel to the first one, giving rise to 32 blastomeres in a 4 × 8 formation; the sixth cleavage was horizontal, producing two cell layers with 64 blastomeres in total. During the morula phase (>100 cells), the cells were arranged in several layers, composing a ‘half-berry’-shaped cell mass (Fig. 2*B*–*H*). Although most embryos followed the above mentioned cleavage pattern, odd cleavage patterns giving rise to 6, 13 or 15 blastomeres were sometimes observed. Up to fifth cleavage the blastomeres exhibited incomplete division, once the cleavage groove was unable to cope with the large amount and high density of yolk.

At this stage, the cells (blastomeres) increased in number but their size decreased, displaying a more homogeneous morphology up to the fifth cleavage (Fig. 2*A*–*E*). From the sixth cleavage onwards, the division was horizontal, giving rise to blastomeres of different sizes and complete cleavage (Fig. 2*G*). In this period, embryos with detached blastomeres and deformities in the yolk vesicle were observed as well as a high number of deformed and unviable eggs.

Up to the 64 cell phase, histological analyses showed no distinguishable layer between blastoderm and yolk as the yolk globules penetrated into blastomeres in a scattered way. In addition, individualized nuclei were absent in blastomeres. The first nuclei and nuclear

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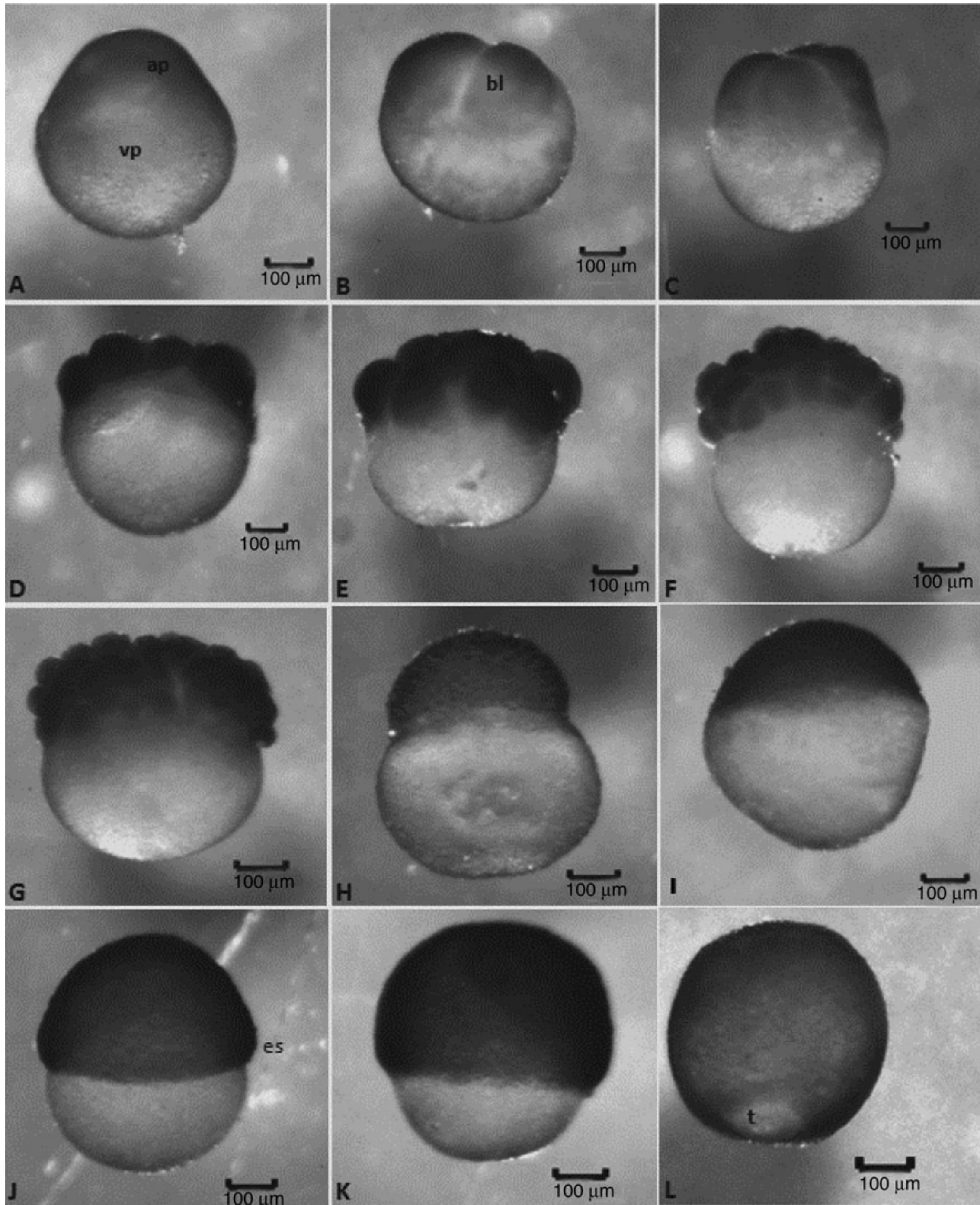

Figure 2 Embryonic development stages of *Leiarius marmoratus*. Zygote stage: (*A*) 1-cell embryo (post-fertilization without chorion). Cleavage stage: (*B*) 2-cell embryos; (*C*) 4-cell embryos; (*D*) 8-cell embryos; (*E*) 16-cell embryos; (*F*) 32-cell embryos; (*G*) 64-cell embryos; (*H*) morula. Gastrula stage: (*I*) 25% of epiboly; (*J*) 50% of epiboly; (*K*) 75% of epiboly; (*L*) 90% of epiboly. Staining: haematoxylin–eosin (HE). ap, animal pole; bl, blastomere; t, blastopore closure; vp, vegetal pole.

divisions could be visualized from the morula phase onwards, as well as the beginning of the formation of the yolk syncytial layer or periblast (Fig. 4*A*). There was no formation of a blastocele but of a more compact blastoderm that, at the end of cleavage stage, assumed a flattened half-berry shape that covered one pole of the yolk sac (Figs. 2*H* and 4*A*). This stage ended after 2–3 h of incubation.

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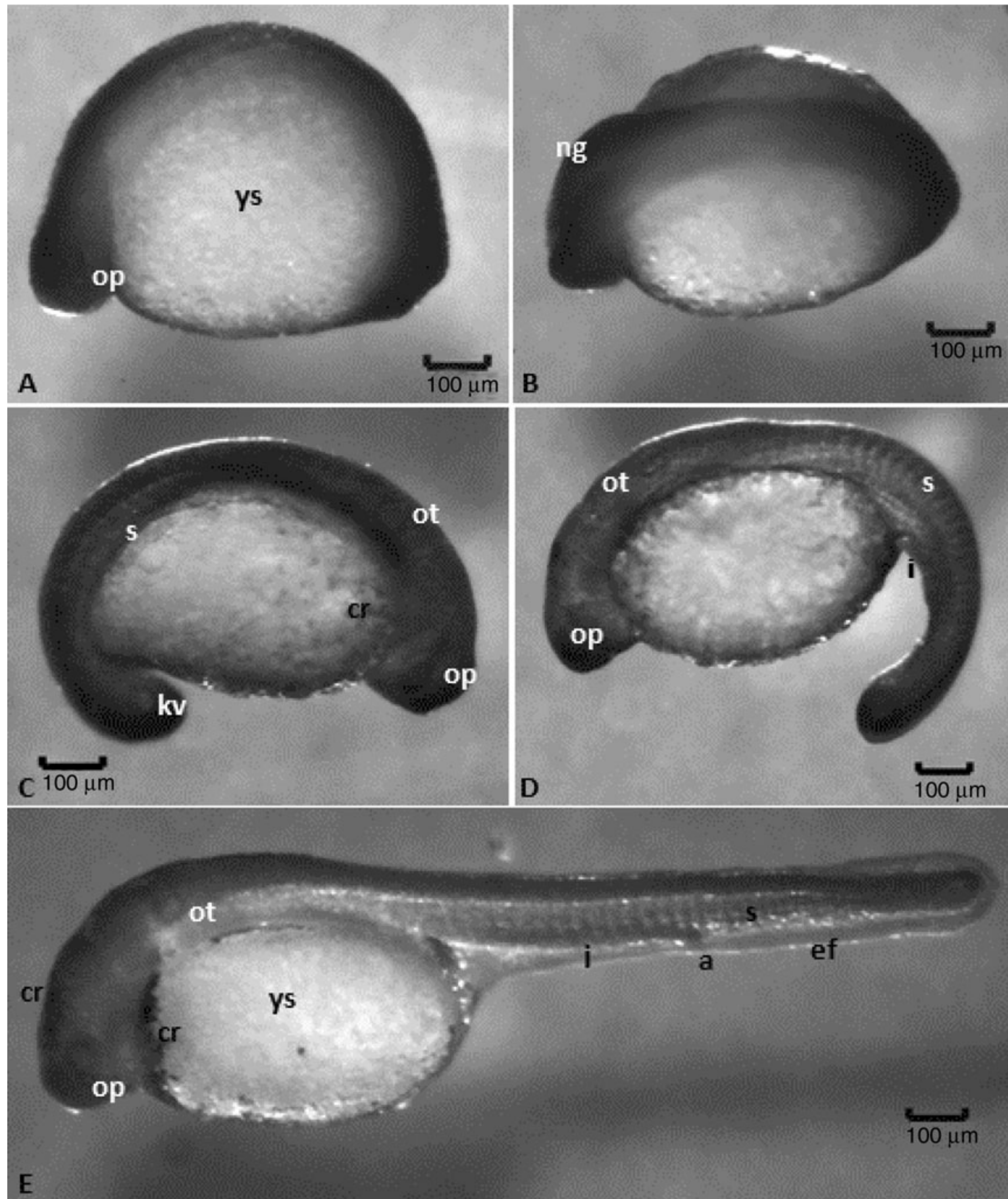

Figure 3 Embryonic development stages of *Leiarius marmoratus*. Organogenesis stage: (*A*, *B*) neurula; (*C*) embryo with about 15 somites, optic vesicle, attached tail and pigmentation of body; (*D*) 24 somites, free tail, rudiments of the posterior intestine. Hatching stage: (*E*) 30 somites, posterior intestine well developed, anal pore delimited. Staining: haematoxylin–eosin (HE). a, anal pore; cr, chromatophore; ef, embryonary fin; I, intestine; kv, Kupffer's vesicle; ng, neural groove; op, optic vesicle; ot, otic vesicle; s, somites; ys, yolk sac.

Gastrula stage

This stage was characterized by the beginning of morphogenetic movements (epiboly) in which the blastoderm cells undergo rapid mitotic divisions. As a result, the surface cells became flattened while the intern cells intercalated with external cells (intercalation) thereby extending blastoderm up to the full covering of the yolk vesicle (Fig. 2I–L).

Morphogenetic movements were observed from the third hour of embryogenesis; in the fourth hour of incubation the *L. marmoratus* embryos reached the 50% epiboly stage with half of the yolk sac covered (Fig. 2*J*).

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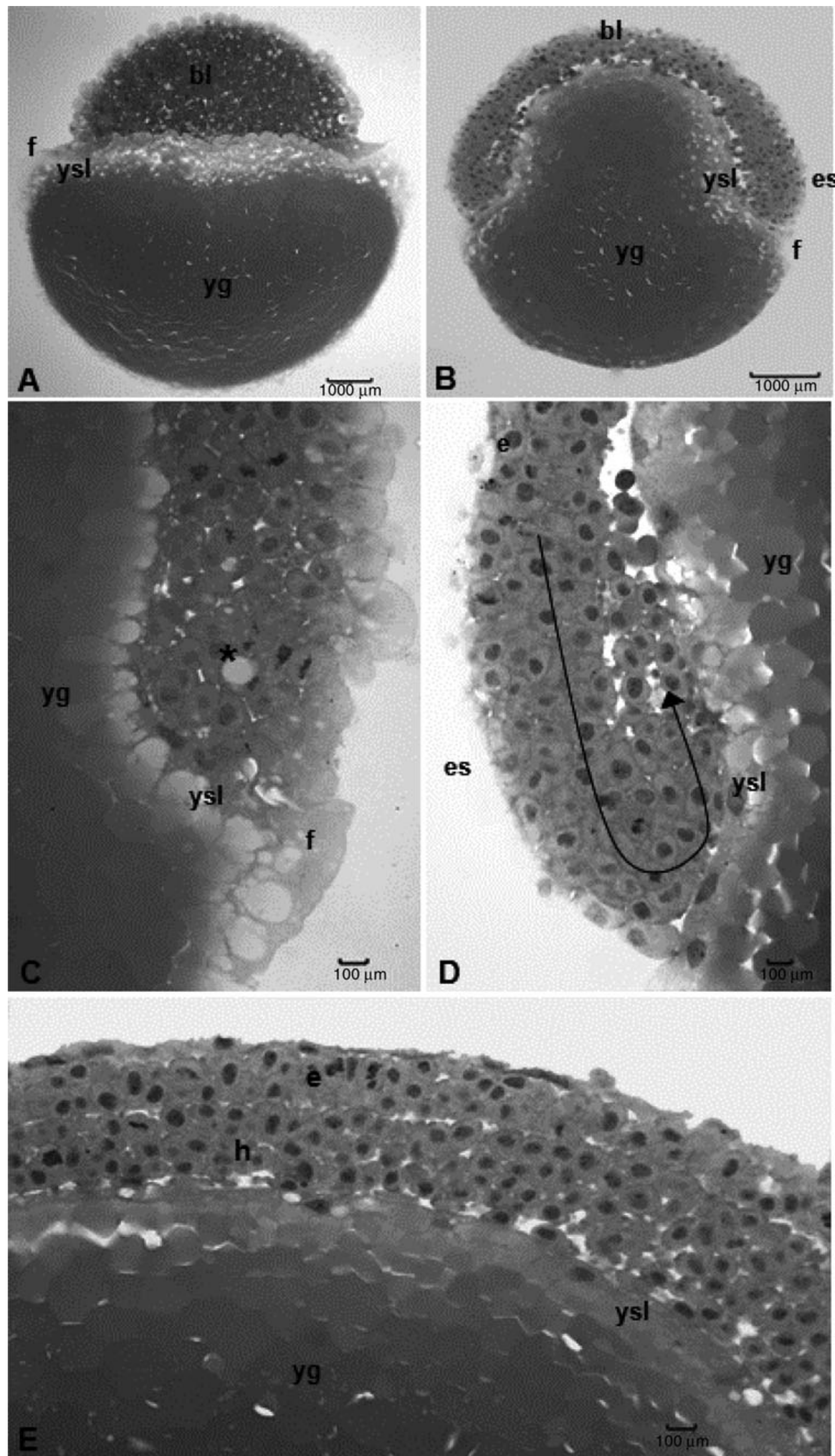

Figure 4 Histological sections of *Leiarius marmoratus* embryos. (*A*) Cleavage stage, morula phase. (*B–E*) Gastrula stage, 50% epiboly phase. Staining: haematoxylin–eosin (HE). bl: blastomeres; e, epiblast; es, embryonic shield; f, fringe; h, hypoblast; yg: yolk globules; ysl, yolk syncytial layer. Arrow – morphogenetic movement of involution. Asterisk: penetration of yolk globules in the embryo.

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Simultaneously, cell involution started inasmuch as the expanding layer folded to form a second layer, which extended towards an opposite direction in relation to the first one, determining the thickening of blastoderm border, called germ ring. Cells accumulated perpendicularly to the germ ring, forming the embryonic shield. These morphogenetic movements then determined the formation of epiblast and hypoblast as well as convergence, directing the cell layers in the embryonic shield to establish the head–tail and dorsal–ventral axes (Fig. 4*B*–*E*).

At 5 h, all embryos presented 75% of yolk covered by blastoderm (Fig. 2*K*). At the 6 h, epiboly in embryos reached 90%, with just a small yolk portion still exposed: the yolk plug or blastopore (Fig. 2*L*). Light microscope analyses revealed the periblast had formed a fringe along the blastoderm border since its formation up to the blastopore closure (Fig. 4*C*). Periblast was characterized by a cytoplasmatic layer with several nuclei with patched yolk globules in cytoplasm and no membrane among them (Fig. 4*C*–*E*).

Even though most embryo cells presented a single nucleolus, several cells with two nucleoli were observed. The gastrula stage ended between the 6–7 h of embryonic development, when the whole yolk vesicle became covered by embryonic cells and the blastopore was closed by the periblast.

Organogenesis stage

This stage started after 7–8 h of incubation and was typified by the origin of rudimentary organs and systems from embryonic layers. Somites, notochord, neural tube, optic and otic vesicles, encephalon and early delimitation of intestines were observed as the embryos grew and elongated, mainly through the head–tail axis (Figs. 5 and 6). After 7 h of embryogenesis, all embryos reached the neurula phase with differentiation between cephalic and caudal regions and neural keel (Fig. 3*B*). At this point, the thickening of dorsal epiblast determined the formation of neural keel that later fused to give rise to the neural plate. The notochord was composed of elongated and aligned cells (Fig. 5*D*, *E*), placed underneath the neural plate. The mesendoderm that will produce the somites (segmentation phase) after segmentation was visualized to be lateral to the notochord (Figs. 3*A*–*E* and 6*B*–*D*).

The differential growth of neural tube portions gave rise to the encephalic region, divided into three regions: prosencephalon, mesencephalon and rhombencephalon. After 8 h of development, the embryos present the optic vesicle and segmentation of the first somites took place. At 9 h, it was possible to visualize the Kupffer's vesicle, an egg-shaped structure at the tail region, composed of a ciliated cubic cell layer and a central lumen (Figs. 3*C* and 5*A*, *B*). The otic vesicle could also be defined at this period, being located between the optic vesicle and the first somite and characterized by a layer of elongated cells organized in parallel with nuclei in the basal region. Two otolithes were observed within the otic vesicle (Figs. 3*C*–*E* and 5*D*). Kupffer's vesicle could be no longer visualized after 10 h of embryogenesis as the first pigmented cells (chromatophores) appeared throughout the yolk sac membrane, near the cardiac region (Figs. 3*C*, *E* and 5*C*).

The pre-larval phase was detected at 11 h of incubation once embryos presented a shape similar to recently hatched larvae, which include: evident tail detached from the yolk sac, absence of Kupffer's vesicle, notochord visible from head to tail (Figs. 5*D* and 6*B*) and a well defined rudimentary intestine. Somites in this phase undergo myogenesis, leading to differentiation of myoblasts with spherical and large nuclei and myomeres with flattened nuclei (Figs. 5*E* and 6*A*).

Based on SEM analysis of *L. marmoratus* embryos this structure was located under the otic vesicle and comprised two pairs of solid elevations with a depression observed between them. This structure is probably the beginning of the formation of the operculum (Fig. 6*C*).

Spasmodic movements were another feature of this stage, which increased as long as embryos developed. At the end of this stage, the embryos presented vigorous swimming movements that play a major role in chorion rupture.

Hatching stage

This stage started after 13–14 h of incubation and was characterized by chorion softening, followed by its rupture and presence of free-swimming larvae. The larval heads were attached to the anterior region of the yolk sac. They lacked individualized fins, but had an embryonic fin along the ventral and dorsal portions of caudal region (Figs. 3*E* and 7*B*). Light microscopy allowed the observation of a rudimentary heart, located in front of the yolk sac (Fig. 5*F*), and the development of an optic calyx, formed by cells with elongated nuclei and involving a cell mass from which the crystalline arises (Fig. 5*C*, *F*). At this stage, the chromatophores were concentrated in the membrane that surrounds the yolk sac, mostly located at anterior and posterior regions, but also in the head of *L. marmoratus* (Fig. 5*C*).

Differentiation of notochord cells were also observed, changing from elongated and aligned cells into egg-shaped cells with several clear areas among them. Analysis by SEM revealed the development of olfactory organ in the form of a small circular

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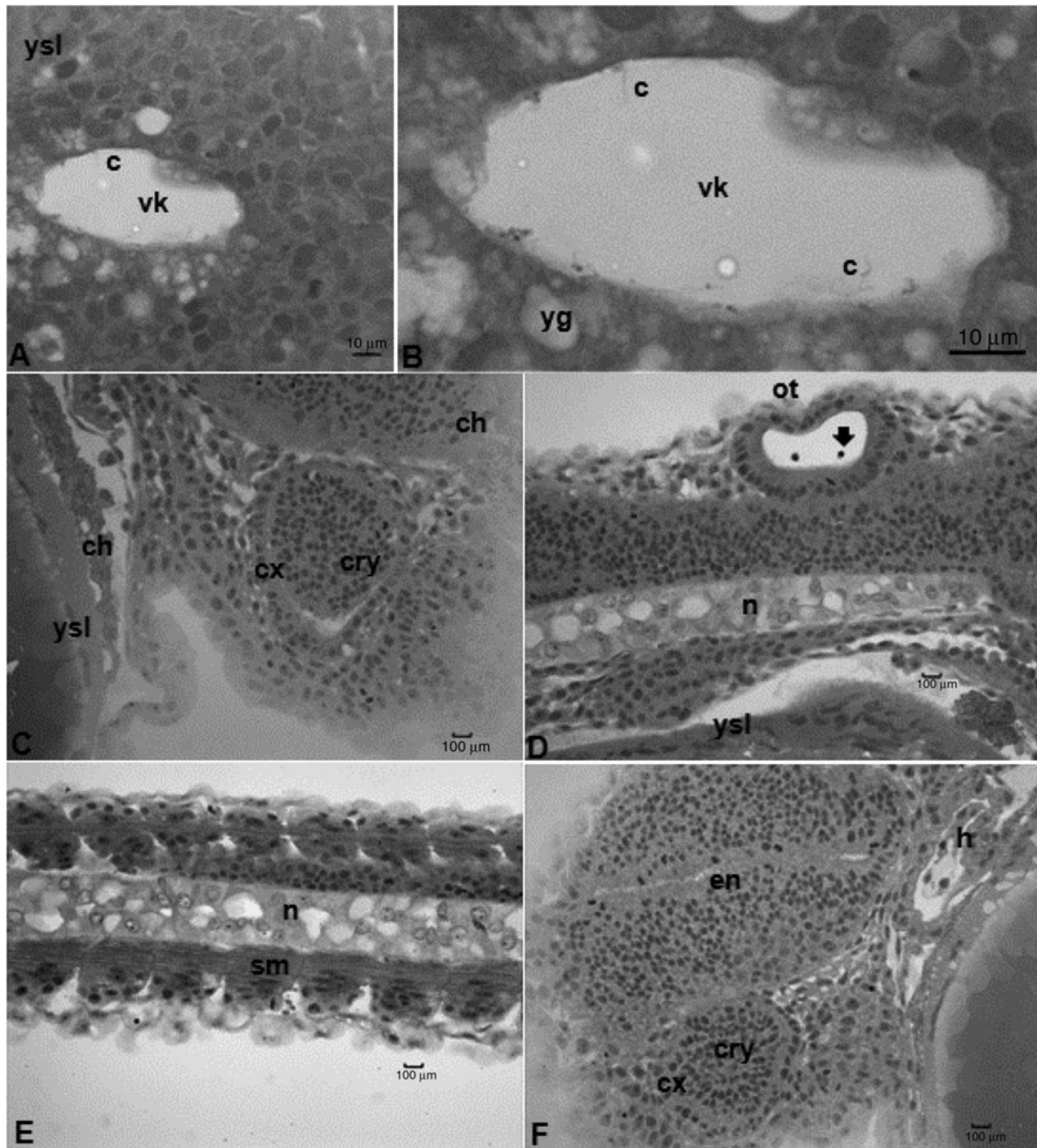

Figure 5 Histological sections of *Leiarius marmoratus* embryos in organogenesis stage. (*A*) Neurula phase. (*B*, *C*) Segmentation phase. (*D*, *E*) Hatching stage. (*F*) Staining: haematoxylin–eosin (HE). c, cilium; ch, chromatophore; cry, crystalline; cx, optical calyx; en, cephalic vesicle; h, heart; n, notochord; op, optic vesicle; ot, otic vesicle; sm, somites in myogenesis; vk, Kupffer's vesicles; yg, yolk globules; ys, yolk sac; ysl, yolk syncytial layer. Arrow – otolithes.

depression with cilium-like structures in the inner portion (Fig. 7*E*, *F*).

Based on SEM analysis of *L. marmoratus* embryos, yolk veins were observed at the dorsal region of the yolk sac (Fig. 7*A*, *B*). Furthermore, the structures identified as possibly forming the operculum were more prominent, between both the cellular clumps as a depression (Fig. 7*A*, *D*). Hatching of *L. marmoratus* larvae occurred after 14 h of development, thus completing the embryonic cycle.

Discussion

Fish eggs are classified as macrolecithal due to their large amount of yolk, and telolecithal as the yolk is located in the vegetative pole, and the cytoplasm and organelles are at the animal pole (Ganeco *et al.*, 2008; Marques *et al.*, 2008). These characteristics have been reported for all species of neotropical fishes studied, including *Prochilodus lineatus* (Ninhaus-Silveira *et al.*, 2006), *P. corruscans* × *P. reticulatum* hybrids (Faustino

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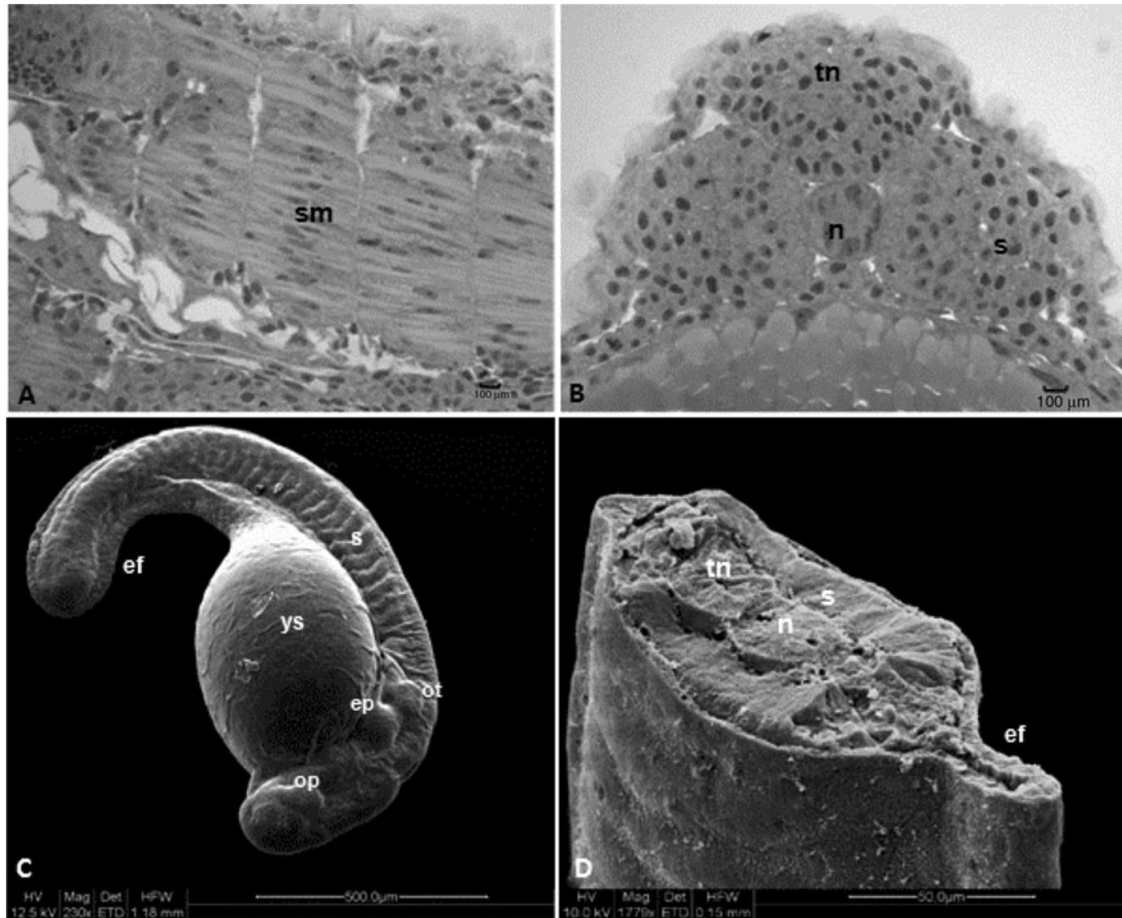

Figure 6 Structural and ultrastructural analysis of *Leiarius marmoratus* embryos in organogenesis stage, segmentation phase. (*A*, *B*) Histological sections staining with Harris haematoxylin–eosin. (*C*, *D*) Scanning electron microscopy. ef, embryonary fin; n, notochord; op, optic vesicle; ot, otic vesicle; s, somites; sm, somites in myogenesis; tn, neural tube; ys, yolk sac; ysl, yolk syncytial layer.

et al., 2007), *P. corruscans* (Marques *et al.*, 2008), *Zungaro jahu* (Nogueira *et al.*, 2012), *Rhinelepis aspera* (Perini *et al.*, 2009), *B. cephalus* (Alexandre *et al.*, 2010) and the present study with *L. marmoratus*.

Rheophilic species such as *Prochilodus lineatus* (Ninhaus-Silveira *et al.*, 2006), *B. orbignyanus* (Ganeco *et al.*, 2008), *Piaractus mesopotamicus* and *Colossoma macropomum* (Ribeiro *et al.*, 1995) produce eggs with a large perivitelline space after hydration that protects embryos against injury during embryogenesis, favouring their survival in fast-flowing waters. Conversely, the perivitelline space in eggs of *R. hilarii* is reduced (Godinho *et al.*, 1978), in a similar manner to the pattern observed in hydrated eggs of *L. marmoratus.* Such differences in the perivitelline space are possibly related to the reproductive strategies of these species and the habitat in which the eggs develop, such as the bottom of rivers in the case of *L. marmoratus.*

The presence of a gelatinous hyaline layer surrounding the eggs has been observed in *Steindachneridion parahybae* (Honji *et al.*, 2012), hybrids between *P. corruscans* and *P. reticulatum* (Faustino *et al.*, 2007), *Rhinelepis aspera* (Perini *et al.*, 2009) and *Pimelodus maculatus* (Buzzolo *et al.*, 2011). According to Rizzo *et al.* (2002), this gelatinous layer is typical of Siluriformes, being composed of coiled delicate fibrils. Nonetheless, this structure was absent on the chorion of the presently studied species.

In bony fishes, fat droplets may be present in the yolk mass in distinct number and size according to each fish family, as reported in the freshwater *B. cephalus* (Lopes *et al.*, 1995) and marine *Abudefduf sexfasciatus* (Shadrim & Emel'yanova, 2007). However, no fat droplets were detectable in the yolk of *L. marmoratus*, following the pattern observed in *Prochilodus lineatus* (Ninhaus-Silveira *et al.*, 2006), *B. orbignyanus* (Ganeco *et al.*, 2008) and *B. insignis* (Andrade-Talmelli *et al.*, 2001).

Brummett & Dumont (1981) observed that, right after the rupture of cortical alveoli, a protoplasmatic cover accumulates at the animal pole, thus forming the blastodisc, in which the zygote is cleaved into

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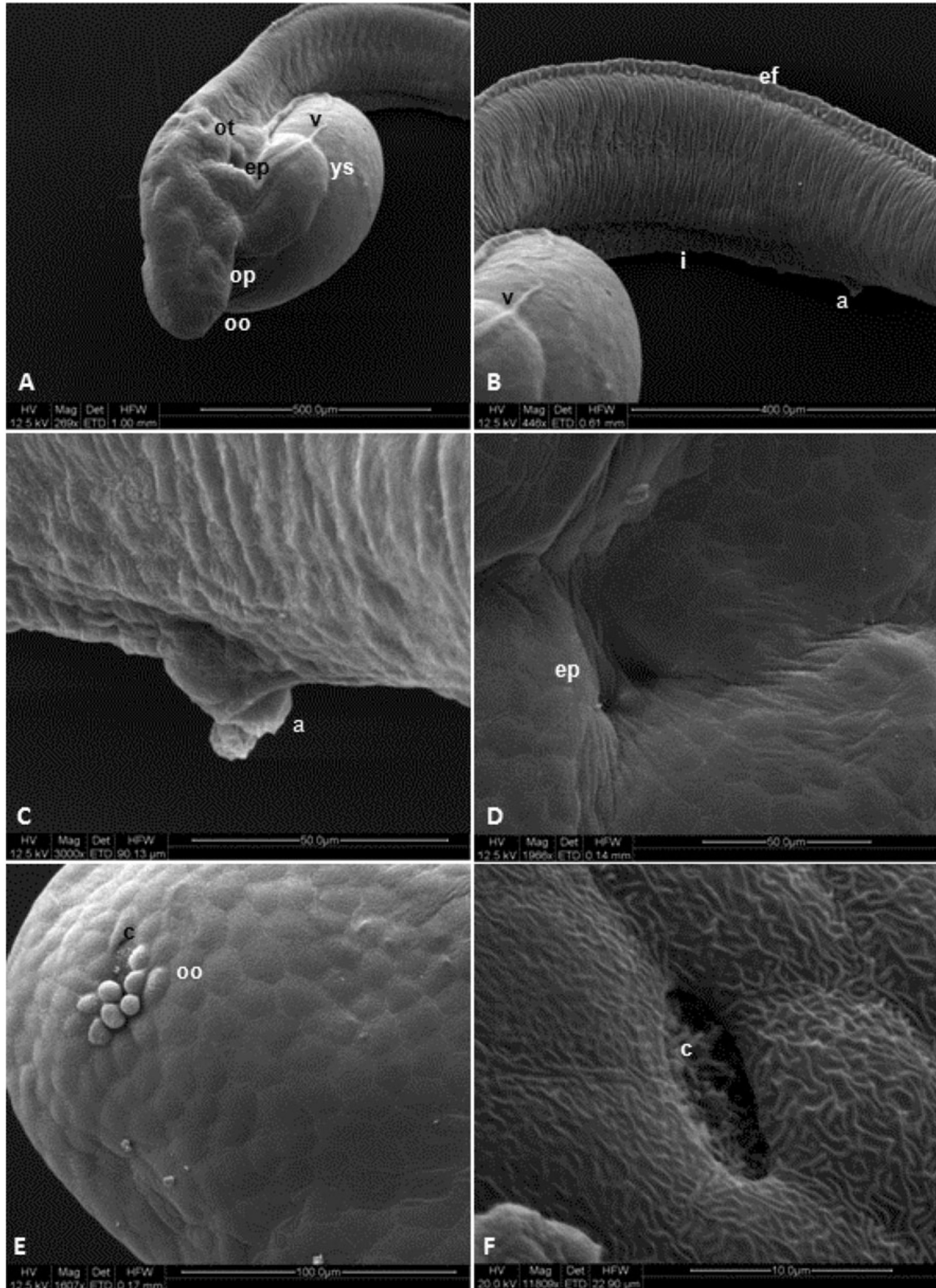

Figure 7 Ultrastructural (SEM) analysis of *Leiarius marmoratus* embryos in hatching stage. a, anal pore; c, olfactory cilium; ep, non-identified structure; ef, embryonary fin; i, intestine; oo, olfactory organ; op, optic vesicle; ot, otic vesicle; v, yolk veins; ys, yolk sac.

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blastomeres. According to Faustino *et al.* (2010a), a cytoplasm movement has taken place after the cortical reaction in eggs of hybrids between *P. corruscans* and *P. reticulatum*, determining the differentiation of both animal and vegetative poles and the beginning of cleavage – changes that are also observed in *L. marmoratus* embryos.

The stages observed through the embryonic development of the species analysed in this study (zygote, cleavage, gastrula, organogenesis and hatching) are similar to those reported in *P. corruscans* × *P. reticulatum* hybrids (Faustino *et al.*, 2007; Shadrim & Emel'yanova, 2007), *P. corruscans* (Marques *et al.*, 2008) and *Zungaro jahu* (Nogueira *et al.*, 2012). The only exception is the blastula stage, described by these authors but not found in the present study, because blastocele formation was not observed in *L. marmoratus* embryogenesis.

According to Zhang *et al.* (2009), holoblastic cleavage results in a ball-shaped cell layer, while meroblastic cleavage forms the blastodisc. However, the features that typify the blastula stage are the formation of peripheral cells in morula and the formation of a cavity filled with liquid named the blastocele. In species with oligolecithal eggs and holoblastic cleavage, the blastocele gives rise to the blastocyst (Moore & Persaud, 2003), whilst in individuals with telolecithal eggs and meroblastic cleavage it originates a cavity below the blastoderm or in irregular spaces among blastoderm cells as often reported (Kimmel & Law, 1985; Trinkaus, 1992; Kimmel *et al.*, 1995; Ninhaus-Silveira *et al.*, 2006; Marques *et al.*, 2008; Ganeco *et al.*, 2008). In *Brachidanio rerio*, irregular spaces interspersed with blastoderm cells were observed that suggested that this stage should be defined as stereoblastula as it lacked a typical blastocele (Kimmel *et al.*, 1995). In other species such trout (Lagler *et al.*, 1977) a typical blastocele has been reported between the blastoderm and the periblast. However, in fish such as *Oreochromis niloticus* (Morrison *et al.*, 2001), the hybrids between *P. corruscans* and *P. reticulatum* (Faustino *et al.*, 2010a) and the species in the present study, neither a characteristic cavity nor irregular spaces, both indicators of stereoblastula, could be identified.

The type of cleavage, meroblastic or partial, observed in *L. marmoratus* has been reported in most teleosteans (Lagler *et al.*, 1977; Ninhaus-Silveira *et al.*, 2006; Ganeco *et al.*, 2008; Marques *et al.*, 2008; Amorim *et al.*, 2009; Perini *et al.*, 2009; Alexandre *et al.*, 2010; Faustino *et al.*, 2010b; Buzzolo *et al.*, 2011).

The first cleavage divided the blastodisc in two cells of similar size, as described in *Xiphister atropurpureus* (Wourms & Evans, 1974), *Cynolebias* (Carter & Wourms, 1991), *Oryzias layipes* (Iwamatsu, 1994) and *Brachidanio rerio* (Kimmel *et al.*, 1995). Cleavage usually begin from the centre to the edges of the blastodisc (Matkovik *et al.*, 1995; Shardo, 1995), as corroborated by the results in *L. marmoratus.*

In *O. latipes* (Iwamatsu, 1994), blastomeres were arranged in a single layers composed of four central and 13 peripheral blastomeres when eggs reached the 16-cell stage; at the 32-cell stage, the blastomeres formed four series of eight cells each. The same result has been reported for *Alosa sapidíssima* (Shardo, 1995) and *B. orbignyanus* (Ganeco *et al.*, 2008). Yet, most teleosteans bear four series with four cells each in this phase, forming a single cell layer (Trinkaus, 1992; Shardo, 1995). This 4 × 4 arrangement has been also observed in *Catostomus commersoni* (Long & Ballard, 1976), *Danio rerio* (Kimmel *et al.*, 1995), *Oreochromis niloticus* (Morrison *et al.*, 2001) and in the present species.

Morrison *et al.* (2001) suggested that variation in both embryogenesis and embryo development (asynchrony and malformations) is related to temperature at incubation and age of broodstock. In *Prochilodus lineatus*, Ninhaus-Silveira *et al.* (2006) reported that, although using young breeders (1–2 years old) and a constant water temperature, a higher asynchrony in embryonic development and an accentuated variation in blastomere division occurred during incubation at 24°C rather than at 28ºC. Kimmel *et al.* (1995) and Morrison *et al.* (2001) also observed that asynchrony in embryonic development might be present even in a single spawn incubated under optimal conditions. Hisaoka & Firlit (1960) verified that mitotic divisions in zebrafish are synchronic up to the 64-cell phase, but they become asynchronic when the embryos reach 64 cells. The results in the experiment with *L. marmoratus* supported these findings, indicating that genetic and environmental traits are essential to the embryonic development of bony fishes.

In *Z. jahu*, Nogueira *et al.* (2012) reported that the yolk globules penetrated the blastomeres after fragmentation, in a similar manner to the observations in the presently studied species. According to Ninhaus-Silveira *et al.* (2006), such fragmentation might facilitate the absorption of yolk globules by cells.

The periblast or yolk syncytial layer is an important structure to the embryonic development of teleosteans (Ninhaus-Silveira *et al.*, 2007). Kimmel *et al.* (1995) stated that this layer is an organ found only in teleosteans as an extra-embryonic structure that contributes in the formation of an embryo body (Balinsky, 1970). This cell layer, also called the periblast, can be differentiated from other blastoderm cells as it is more basophilic than the latter (Hisaoka & Firlit, 1960). It also plays a key role in yolk breakage, making it available to the embryos, which allows their development (Balinsky, 1970).

The formation of the periblast during the morula phase has been reported for *Catostomus commersoni*

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(Long & Ballard, 1976), *P. lineatus* (Ninhaus-Silveira *et al.*, 2006), *Z. jahu* (Nogueira *et al.*, 2012), and in the species studied here. Conversely, the periblast is firstly observed at the start of the blastula stage in other fish species (Wourms & Evans, 1974; Iwamatsu, 1994; Kimmel *et al.*, 1995; Ganeco *et al.*, 2008; Faustino *et al.*, 2010a; Faustino *et al.*, 2010b).

As the yolk syncytial layer increases, it involves the yolk, independently of the blastoderm, serving as a primary cause of epiboly movement (Devillers, 1961; Betchaku & Trinkaus, 1986, 1993; Ninhaus-Silveira *et al.*, 2007). According to these authors, the peripheral yolk syncytial layer contracts to facilitate the migration of nuclei to the inner portion, thereby completing its formation as the syncytial layer grows towards the vegetative pole along with the blastoderm, which is firmly attached, thus triggering epiboly. The present results corroborate this suggestion.

The gastrula stage initiates when the first epiboly movements begins and ends when the blastopore is closed by the blastoderm and the tail bud is formed. These findings agree with the reports for *B. orbignyanus* (Ganeco *et al.*, 2008), *P. lineatus* (Ninhaus-Silveira *et al.*, 2006), *R. aspera* (Perini *et al.*, 2009), *B. gouldingi* (Faustino *et al.*, 2010b), *Pimelodus maculatus* (Buzzolo *et al.*, 2011) and in the species studied here.

According to Trinkaus (1992), the cells presenting epiboly around the yolk also undergo involution on the edges of the germ ring while the embryonic shield is converted, directing these cells to anterior and dorsal positions. These movements form the chordomesoderm that precedes the formation of the notochord. The cells adjacent to the paraxial mesoderm cells form the mesoderm somites (Kimmel *et al.*, 1995). This affirmation is supported by results in *L. marmoratus* and other species such *Prochilodus lineatus* (Ninhaus-Silveira *et al.*, 2006), *B. orbignyanus* (Ganeco *et al.*, 2008), *Zungaro jahu* (Nogueira *et al.*, 2012), *R. quelen* (Amorim *et al.*, 2009) and hybrids between *P. corruscans* and *P. reticulatum* (Faustino *et al.*, 2007). Conversely, *Oreochromis niloticus* embryos cannot extend over the entire vegetative pole because of the size of the yolk sac and then present a rudimentary organogenesis (segmentation of somites) prior the cessation of epiboly movements (Morrison *et al.*, 2001).

By the end of the gastrula stage, the first mesoderm tissues were observed along both sides of notochord, arranged in segments called somites, as described in *Brachidanio rerio* (Hisaoka & Firlit, 1960; Kimmel *et al.*, 1995), *Oreochromis niloticus* (Galman & Avtalion, 1989), *Oryzias latipes* (Iwamatsu, 1994) and *B. orbignyanus* (Ganeco *et al.*, 2008).

Kimmel *et al.* (1995) regarded the neurula as a phase within the organogenesis stage. Brummett & Dumont (1978) and Morrison *et al.* (2001) observed Kupffer's vesicle in the early steps of the organogenesis stage. In *L. marmoratus*, this vesicle appeared at the neurula phase. According to Hisaoka & Firlit (1960), Kupffer's vesicle represents a remnant structure from the archenteron located above the periblast and below the notochord. In *Oncorhynchus keta* (Mahon & Hoar, 1956 cited by Hisaoka & Firlit, 1960), Kupffer's vesicle is described as an oblique and elongated cavity with columnar epithelial walls, which is separated from the periblast by a layer of endoderm cells, similar to the pattern observed by Ninhaus-Silveira *et al.* (2006) in *P. lineatus* and in *L. marmoratus* from this study.

Brummett & Dumont (1978) hypothesized that this vesicle might have a digestive activity bring helpful to yolk absorption, once cilium-like cells were present in Kupffer's vesicle and the intestine of *Fundulus heterocliltus*. Essner *et al.* (2005) showed that, in *Danio rerio*, Kupffer's vesicle contained fluids and cilia and suggested that this transitory embryonic structure could be responsible for the organ asymmetry during development. In the species analysed in this study, cilia were detected on the epithelium of this vesicle. In contrast, these features were not observed in neotropical species, such as *B. cephalus* (Alexandre *et al.*, 2010) and *Pimelodus maculatus* (Buzzolo *et al.*, 2011). By non-visualization these cilia could be linked to the monociliar feature to the cells lining Kupffer's vesicle, as demonstrated in zebrafish (Okabe *et al.*, 2008), and the histological technique used by the authors cited.

According to Gilbert (2010), the neural tube arises from a solid cell cord at neural plates, forming a string-like structure that migrates into the embryo to form the tube. The uppermost portion of neural tube undergoes drastic changes by expanding into three primary vesicles: anterior brain (prosencephalon), middle brain (mesencephalon) and posterior brain (rhombencephalon). In *L. marmoratus*, the anterior region of neural tube expanded to form these three regions, as reported for *Prochilodus lineatus* (Ninhaus-Silveira *et al.*, 2006), *B. orbignyanus* (Ganeco *et al.*, 2008), *Brachidanio rerio* (Kimmel *et al.*, 1995), *R. sapo* (Cussac *et al.*, 1985) and *P. corruscans* (Marques *et al.*, 2008).

In *R. sapo* (Cussac *et al.*, 1985), the notochord cells form vacuoles that move the nuclei towards the peripheral region. Falk-Petersen (2005) reported that the notochord is composed of vacuolated cells separated by thin cell membranes. The histological analyses of this structure in the species analyzed in this work corroborate these previous reports.

The development of circulatory system begins with the formation of the heart and it continues through the larval development (Langeland & Kimmel, 1997; Yelon & Stainier, 1999; Hu *et al.*, 2000). According to Hu *et al.* (2000), the heart is the first definitive organ to develop and it is functional during embryogenesis.

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Other authors reports that the heart initially appears as a single tube with a multilayer wall (Morrison *et al.*, 2001; Hall *et al.*, 2004). Rodrigues-Galdino *et al.* (2009) also describes yolk veins with circulating blood in *R. quelen.* The present data in *L. marmoratus* support the above-mentioned reports.

The V-like embryonic muscles formed by myotomes are established in early somitogenesis (Patterson *et al.*, 2008). Muscular contractions occur initially in individual myotomes and then spread to coordinated series of myotomes. These contractions in late embryos arise in bursts as the circuits of motor and sensory reflexes develop functionally (Zhang *et al.*, 2009). According to Rodrigues-Galdino *et al.* (2009), when the somites are arranged in a V-shape, the embryos display lateral body flexion. These movements were firstly detected in pre-larval embryos of *L. marmoratus.*

The otic vesicle was established in early organogenesis and otolithes were detected at the end of this stage, following the pattern described by Zhang *et al.* (2009). Rodrigues-Galdino *et al.* (2009) observed a pair of otolithes similar in form to small granules attached to the inner surface of each otocyst.

The olfactory plates are formed when the embryo are near the hatching stage (Blaxter, 1988) as observed in the studied species. In *Z. jahu* (Nogueira *et al.*, 2012) and *B. gouldingi* (Faustino *et al.*, 2010b), the olfactory organ was observed at the hatching stage with a small amount of rudimentary cilia. The olfactory plates with cilia are present in the borders before the eyes (Zhang *et al.*, 2009).

The chorion rupture in bony fishes is favoured by hatching glands. Prior to hatching, these glands secrete proteolytic enzymes, chorionases, which degrade the inner layers of the chorion, facilitating the exit of embryos (Zhang *et al.*, 2009). According to these authors, both tail and body movements are also helpful to the hatching process. Chorion deterioration and embryo movements were also observed in *L. marmoratus* in the pre-larva phase near to the hatching stage.

The analysis of chromatophores and pigments on eyes and body are important traits in taxonomy and species identification (Meijide & Guerrero, 2000). The cells from the reticular epithelium and skin melanophores are the first to show pigments during embryogenesis (Kimmel *et al.*, 1995), as observed in *L. marmoratus*, as well as other species such as *Z. jahu* (Nogueira *et al.*, 2012) and *R. aspera* (Perini *et al.*, 2009).

Cussac *et al.* (1985) also detected three pairs of a similar structure to that observed in *L. marmoratus*, so-called anterior mesoderm bundles, located at the dorsal region in embryos of *R. sapo.* These authors showed that the first anterior mesoderm bundle remains undifferentiated at hatching, while the second one differentiates into the anterior and outer wall of opercular cavity. In the third bundle, the cells were arranged in parallel to form the branchial fissures. A similar structure was observed using SEM by Faustino *et al.* (2010a) in *P. corruscans* and *P. corruscans* × *P. reticulatum* hybrids, but identified as primordial barbels.

The embryogenesis period in *L. marmoratus* was similar to that reported by Faustino *et al.* (2010a) in hybrids of *P. corruscans* and *P. reticulatum.* Variation in embryonic periods is related to sensitivity of teleostean embryogenesis to environmental changes, mainly in temperature.

A fast embryonic development is typical of teleosts species with seasonal reproductive strategies, high fecundity and no parental care (Vandevalle *et al.*, 2005) such as *L. marmoratus.* Blaxter (1988) reports that most recently hatched fish larvae lack mouth, anus, gills, swimming bladder, fins, pigmentation and visual accuracy. Overall, these features were also observed in *L. marmoratus* larvae, differing in the presence of pigmentation in the region of the yolk sac and head, an anal pore and a single embryonic fin.

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