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Structural and ultrastructural description of larval development in *Zungaro jahu*

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Summary

The *Zungaro jahu* is an important large catfish of the order Siluriformes that is in danger of extinction due to habitat destruction. Studies on its biology are scarce and the majority relates only to nutrition or parasitology. In order to provide greater information on its morphology and aid husbandry and larviculture studies, the aim of this study was to characterize larval development in *Z. jahu* from hatching to total yolk absorption. Samples were collected at pre-established times, processed, stained, and analyzed under stereomicroscopy, light microscopy, and scanning electron microscopy. Total yolk absorption was observed by 60 hours post-hatching (hph) at 28.75 ± 0.59°C. The newly hatched larvae showed slightly pigmented body, the outline of the digestive tract, evident eyes, and the first swimming movements. Mouth opening took place at 12 hph and the connection between the oral cavity and the rudimentary intestine was observed at 24 hph. Were analyzed the main larval organs and systems: digestive organs, heart, gill arches, sensory system, thyroid, kidney, and swim bladder. As the larvae grew, these organs became more mature and functional. The development of the sensory and feeding structures was observed at the start of larval development, and thus before depletion of endogenous energy reserves, the strategy for this species is to increase its chances of survival in the environment.

Keywords: Fish, Histology, Morphology, Ontogeny, Teleostei

Introduction

Initial fish development is a highly dynamic process, with ontogenetic changes resulting in morphofunctional changes of the main body systems, a consequence of the fast morphological and physiological evolution of fish embryos and larvae during their development (Osse *et al.*, 1997).

In fish, the larval stage can last from days to months. During this period, the larva doubles in length and increases in weight up to 100 times. Progressive differentiation of adult characteristics takes place through a process of morphogenesis up to the juvenile stage, when the larvae looks like the adults (Nakatani *et al.*, 2001). Thus, several teleostei fish undergo great changes in body shape, which directly affect the growth and survival of young specimens (Gisbert *et al.*, 2000).

Fish larvae are morphologically different from the adults and have different ecological demands in regards to habitat, feeding, and behavior (Leis & Trnski, 1989 *apud* Sanches *et al.*, 2001). Thus, studies on fish ecology cannot be considered adequate without previous knowledge of the initial development of the species (Sanches *et al.*, 2001). Furthermore, the great similarity between the larvae of different species and

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the lack of comparative literature make identification studies difficult.

The catfish *Zungaro jahu*, commonly known as 'jaú', is a vulnerable species in danger of extinction due to loss of habitat. It can be found in the Paraná-Paraguay and Uruguay River basins in South America and is one of the largest neotropical fish, reaching up to 150 kg and 144 cm in length (Agostinho *et al.*, 2004). It reaches first maturation with approximately 70 cm total length, migrates during the reproductive period, has free-floating eggs, and spawns between the months of December and February. Information on its biology and ecology are scarce and the majority of data available is related to nutrition and parasitology (Agostinho *et al.*, 2004).

Larval ontogenesis studies under controlled conditions enable the monitoring of the morphological changes taking place, providing information on environmental demands and feeding needs during the initial phases of development. Therefore, studies aimed at understanding the development of larval body systems are amongst the most useful for larvae and juvenile husbandry techniques, which in turn increases the chances of survival and, consequently, success in incubation, larviculture, and juvenile production.

Thus, the aim of this study was to observe and describe the ontogeny of the digestive and sensory systems and of other major organs in *Z. jahu* larvae from hatching to total yolk absorption through stereomicroscopy, light microscopy (LM), and scanning electron microscopy analysis (SEM).

Materials and methods

All procedures were performed according to the ethical principles established by the Brazilian College of Animal Research (Colégio Brasileiro de Experimentação Animal – COBEA) and the study approved by the Ethics Committee in the Use of Animals (Comissão de Ética no Uso de Animais – CEUA, protocol no. 10348/16) of the UNESP – Universidade Estadual Paulista, Jaboticabal-SP, Brazil.

Broodstock of the species *Z. jahu* Ihering, 1898 (= *Paulicea luetkeni* Steindachner, 1875) from the fishery Usina Hidrelétrica Engenheiro Souza Dias (Companhia Energética do Estado de São Paulo, Castilho-SP, Brazil) were subjected to induced reproduction and dry fertilization, according to the techniques by Woynarovich & Horváth (1983).

The females ($n = 5$) received two injections of common carp (*Cyprinus carpio*) pituitary extract (0.8 and 5.0 mg/kg body weight) in the coelomic cavity at 12-h intervals. The males ($n = 8$) received a single dose (2.5 mg/kg body weight) concomitantly to the females' second dose.

Approximately 12 h after the effect of the hormone, the gametes were obtained by manual craniocaudal massage of the abdomen and subsequently mixed and hydrated.

Embryos were placed in 60-litre incubators with continuous water flow, where they remained for the whole study period. *Artemia nauplii* and plankton were provided from the second day after hatching, and 'lambari' (*Astyanax altiparanae*) and 'curimbatá' (*Prochilodus lineatus*) larvae from the third day post-hatching.

The following physical-chemical characteristics of the hatcheries water were recorded: temperature and dissolved oxygen (mg/l), by YSI 550 A equipment, pH, conductivity (μS/cm), by YSI 63 equipment, and alkalinity (mg/l) ammonia (μg/l) by Goltermann *et al.* (1978) method.

Samples were collected at the time of hatching (time zero) and then every 4 h until total yolk absorption. At each sampling time, 10 larvae were fixed in Karnovsky solution (2.5% glutaraldehyde + 2.5% paraformaldehyde) for 24 h, washed in 0.1 M sodium cacodylate buffer (pH 7.4), and stored in 70% alcohol for further analysis according to the following methodologies.

Stereomicroscopy and morphometry

Larvae were observed and photomicrographed using the LEICA DFC 280a photomicrography equipment attached to a LEICA MZ8 stereomicroscope. Larval total length was obtained using the program IM 50-LEICA.

Light microscopy (LM)

Samples were processed for embedding in historesin (kit Leica Historesin®) according to the manufacturer's recommendations. Samples were cut into 4-μm sections and every fifth section mounted onto slides (semi-serially) and stained with hematoxylin–phloxin (HP) or periodic acid Shiff (PAS) (Tolosa *et al.*, 2003). Slide analysis and photodocumentation were carried out using a LEICA DM 2500 photomicroscope.

Scanning electron microscopy (SEM)

After being washed in sodium cacodylate buffer, the larvae were post-fixed in 1% osmium tetroxide solution for 2 h, washed again in the same buffer, dehydrated in increasing graded ethanol, dried to critical point with liquid CO_2 (BAL-TEC dryer), mounted onto copper stands, coated with palladium-gold, and observed and electron micrographed using a scanning electron microscope (JEOL-JSM 5410).

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Results

The mean values for the physical–chemical water variables were: temperature = 28.75 ± 0.59°C; pH = 8.18 ± 0.035; dissolved oxygen = 7.33 ± 0.21 mg/l; alkalinity = 19.00 ± 1.73 mg/l; conductivity = 32 ± 2 μS/cm and ammonia = 12.47 ± 1.79 μg/l.

Z. jahu larvae hatched 13 h after fertilization with mean total length of 3.79 ± 0.11 mm. Total yolk absorption was observed at 60 hours post-hatching (hph) and mean total length was 7.05 ± 0.33 mm.

At hatching, the larvae were transparent with pigmentation on the yolk sac and on the cephalic and ventral regions (Fig. 1*a*), which increased with larval development. A highly developed embryonic fin covering the dorsal and ventral regions of the body was also observed (Fig. 1*a*).

Digestive system

The digestive system was divided into cephalic gut (oral cavity and pharynx), anterior gut (esophagus and stomach, when present), midgut (intestine *per se*), and hindgut (rectum and anus).

At hatching, a straight and simple tube was observed in the ventral region of the larvae, corresponding to the outline of the primitive gut (Fig. 1*a*). This tube was lined with a single layer of prismatic cells throughout its extension and had no connection to the oral cavity. At 8 hph, the gut began to show small invaginations and, by 36 hph, the first digestive tract fold could be seen past the proximal region of the midgut (Fig. 1*b*, *c*). From 24 to 60 hph, there was an increase in the number of invaginations, leading to multiple folds in the midgut. After complete yolk absorption, the midgut had a simple prismatic lining epithelium with striated borders and mucus-producing cells (Fig. 1*d*).

By 12 hph, the oral cavity was opened (Fig. 1*e*) and, from 16 hph, the oral cleft began to elongate. From 20 hph, the lining epithelium of the oral cleft was elevated due to the emergence of teeth and a high number of taste buds was observed on the labial borders and inside the mouth (Figs 1*f* and 2*a*). By 24 hph, small teeth at the early stages of development could be seen in the maxilla and mandible (Figure 2*a*, *b*) and a large number of mucus-producing cells were present on the labial borders (Fig. 2*b*).

At 24 hph, the cephalic gut communicated with the anterior gut through the esophagus opening (Fig. 2*c*). At 28 hph, the differentiation of the proximal region of the midgut began with an increase of its lumen and the number of mucus-producing cells increased as differentiation progressed (Fig. 2*d*). The esophagus, lined by a single layer of goblet cells, showed some mucus-producing cells. The pharynx, also composed of goblet cells, did not show significant alterations in its structure. At 28 hph, the first pharyngeal teeth could be seen (Fig. 2*e*). Anal opening occurred at 16 hph.

The liver appeared at 16 hph and became more evident at 20 hph, occupying all the area anterior to the yolk sac and behind the pericardial cavity (Fig. 2*f*). The liver stained positive to PAS at 37 hph, indicating glycogen storage. There was an increase in the number of hepatocytes and in the quantity of hepatic glycogen with time (Fig. 2*g*).

The pancreas, located between the liver and the yolk sac, was also visualized at 16 hph. It initially displayed few zymogenic granules (Fig. 2*f*) that increased significantly in number until total yolk absorption. The first pancreatic islet was observed at 24 hph (Fig. 2*h*).

Reabsorption of the yolk sac took place through the yolk syncytial layer; however, the presence of food inside the digestive tube of the larvae before complete yolk absorption indicates an overlapping of endogenous and exogenous feeding.

Gill arches

The outline of the gill arches in *Z. jahu* was present soon after hatching. The start of chondrogenesis and blood supply occurred at 20 hph, and by 24 hph the gills were branching out (Fig. 3*a*). At 8 hph, the branchiostegal membrane began developing (Fig. 3*b*) and was totally formed and covering the gill arches by 28 hph (Fig. 3*c*). The gill arches were well developed after complete yolk absorption (Fig. 3*d*).

Heart

A rudimentary heart was present at hatching and located in the pericardial cavity, anterior to the yolk sac and abdominal cavity. At 24 hph, there were two heart chambers (atrium and ventricle). The atrium had a thin wall while the ventricular myocardium was composed of several cell layers and, from 28 hph, the trabeculae started to develop (Fig. 3*e*). By 32 hph, blood flowed through the heart chambers, coming in by the sinoatrial orifice in the atrium, flowing through the atrioventricular orifice and into the ventricle, and then reaching the bulbus arteriosus by the ventricle-bulbus orifice (Fig. 3*f*) before leaving the heart by the ventral aorta to be oxygenated at the gill arches.

Pronephros/mesonephros

The pronephros is temporary structure and was present soon after hatching. With the development, the pronephros transforms in a mesonephros. It was shaped as two straight lateral tubules composed of

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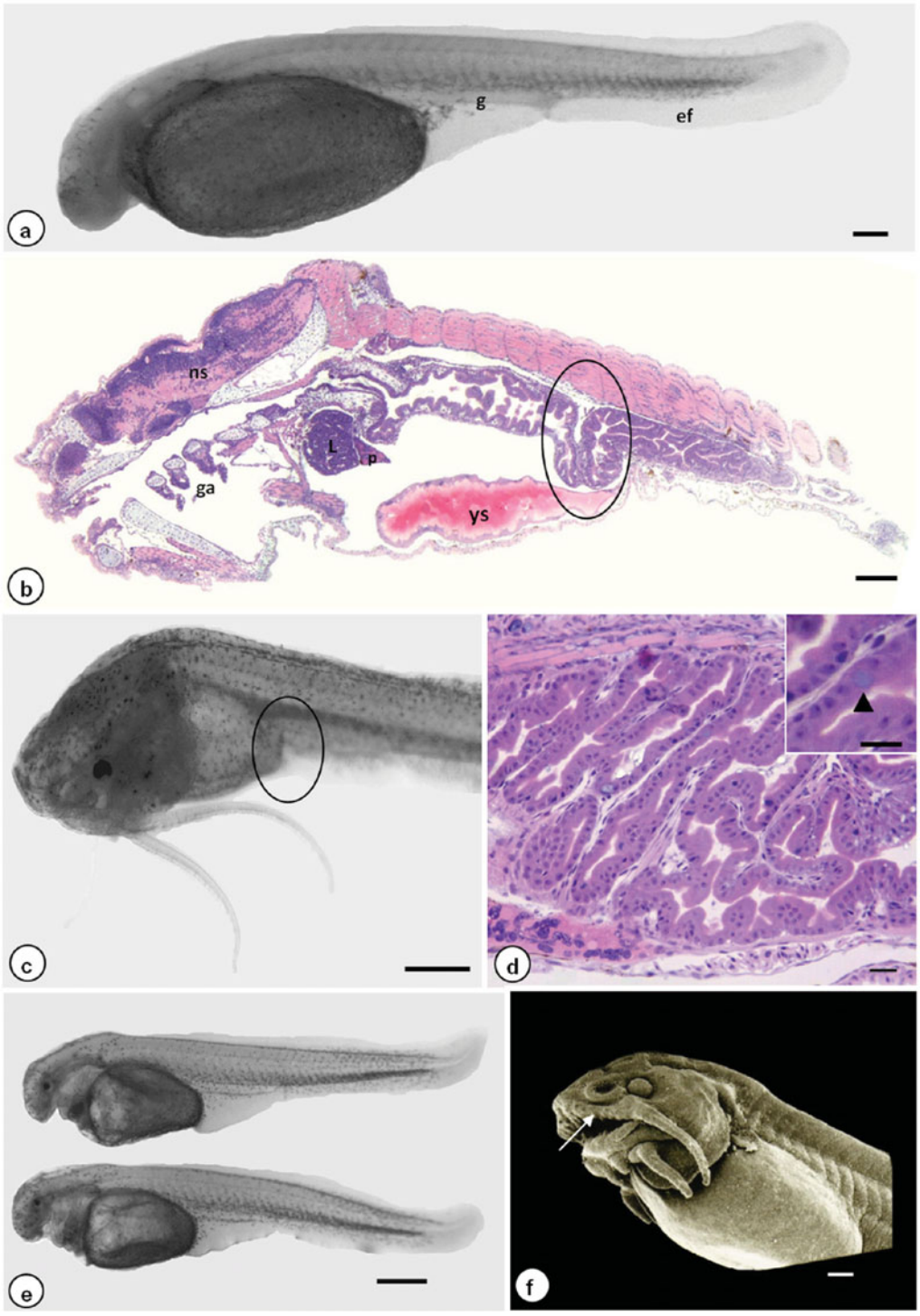

Figure 1 Photomicrography (*a*–*e*) and electron micrography (*f*) of Z. jahu larvae. (*a*) Hatching – primitive gut; (*b*, *c*) 36 hph – first digestive tract fold (circles); (*d*) 60 hph – detail of the gut wall, mucus-producing cell (arrowhead); (*e*) 12 hph – opening of the oral cavity; (*f*) 20 hph – emergence of teeth, taste buds on lip borders and inside the mouth (arrow). Abbreviations: ga = gill arches, l = liver, g = gut, ef = embryonic fin, p = pancreas, ns = nervous system, ys = yolk sac. Bars: (*a*, *e*) = 500 μm; (*b*, *f*) = 100 μm; (*c*) = 500 μm; (*d*) detail = 30 μm. Staining: (*b*) = hematoxylin–phloxin (HP); (*d*) = periodic acid Shiff (PAS).

simple cuboidal epithelium. It was located above the gut and followed its entire extension. The cranial region became convoluted at 16 hph, while the caudal region remained straight (Fig. 3*g*). As larval development progressed, the mesonephros became more convoluted and its lumen more evident (Fig. 3*h*). The mesonephros tubules were joined and opened into the urogenital papilla, together with the intestine.

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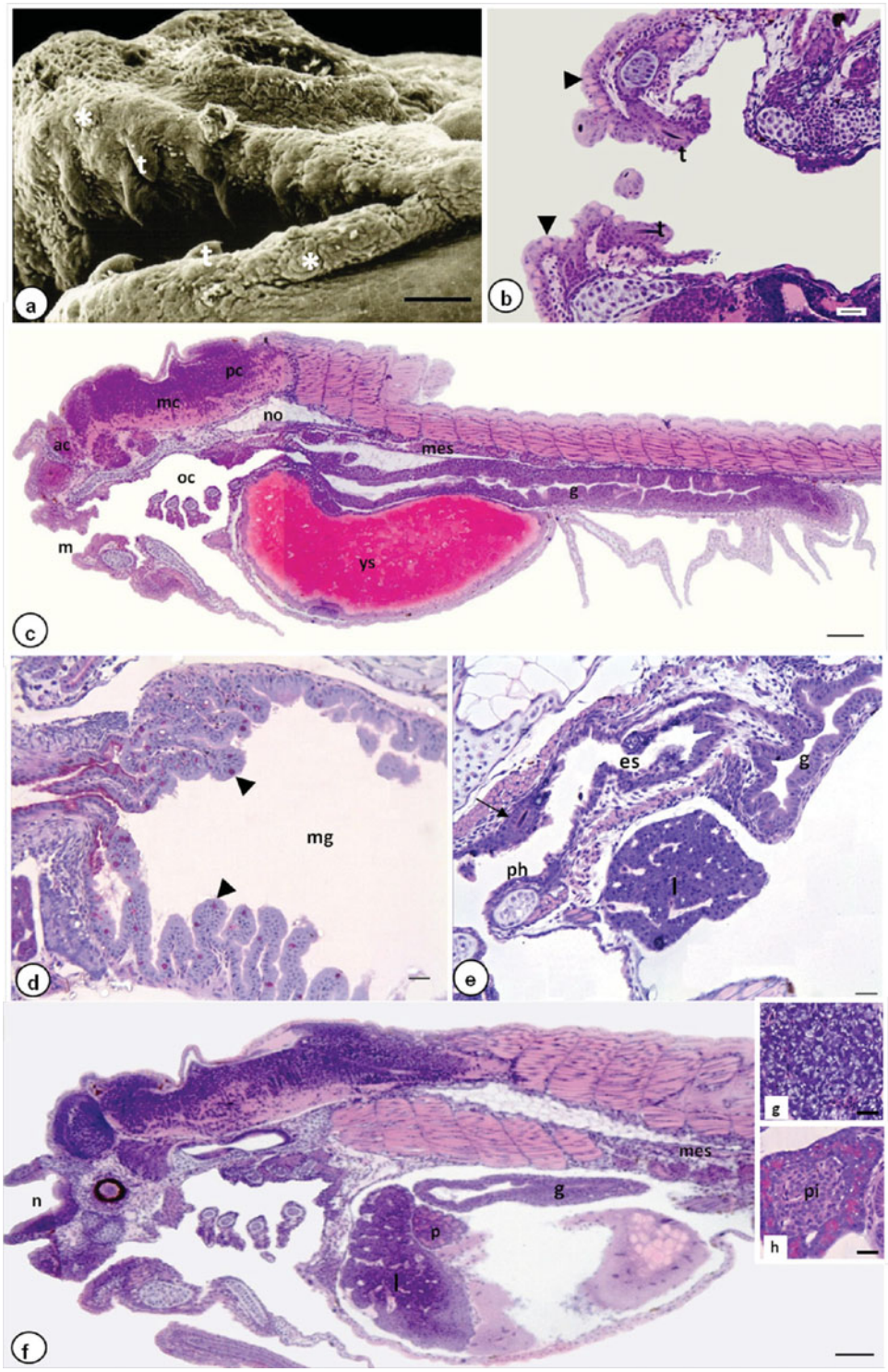

Figure 2 Electron micrography (*a*) and photomicrography (*b–h*) of *Z. jahu* larvae. (*a*, *b*) 24 hph – teeth in the maxilla and mandible, taste buds (∗), mucus-producing cells (arrowhead); (*c*) 24 hph – opening of the esophagus; (*d*) 28 hph – mucous-producing cells (arrowheads, stained pink); (*e*) 28 hph – pharyngeal teeth (arrow); (*f*) 20 hph – liver and pancreas; (*g*) 60 hph – detail of the liver; (*h*) 24 hph – pancreatic islet. Abbreviations: m = mouth, ac = anterior cerebrum, mc = mid cerebrum, oc = oral cavity, pc = posterior cerebrum, t = teeth, es = esophagus, l = liver, ph = pharynx, g = gut, mg = midgut, pi = pancreatic islet, n = nares, no = notochord, p = pancreas, mes = mesonephros, ys = yolk sac. Bars: (*a*) = 50 μm; (*b*, *c*, *e*) = 100 μm; (*d*) = 30 μm; (*g*, *h*) = 20 μm. Staining: (*b*, *c*, *e–h*) = hematoxylin–phloxin (HP); (*d*) = periodic acid Shiff (PAS).

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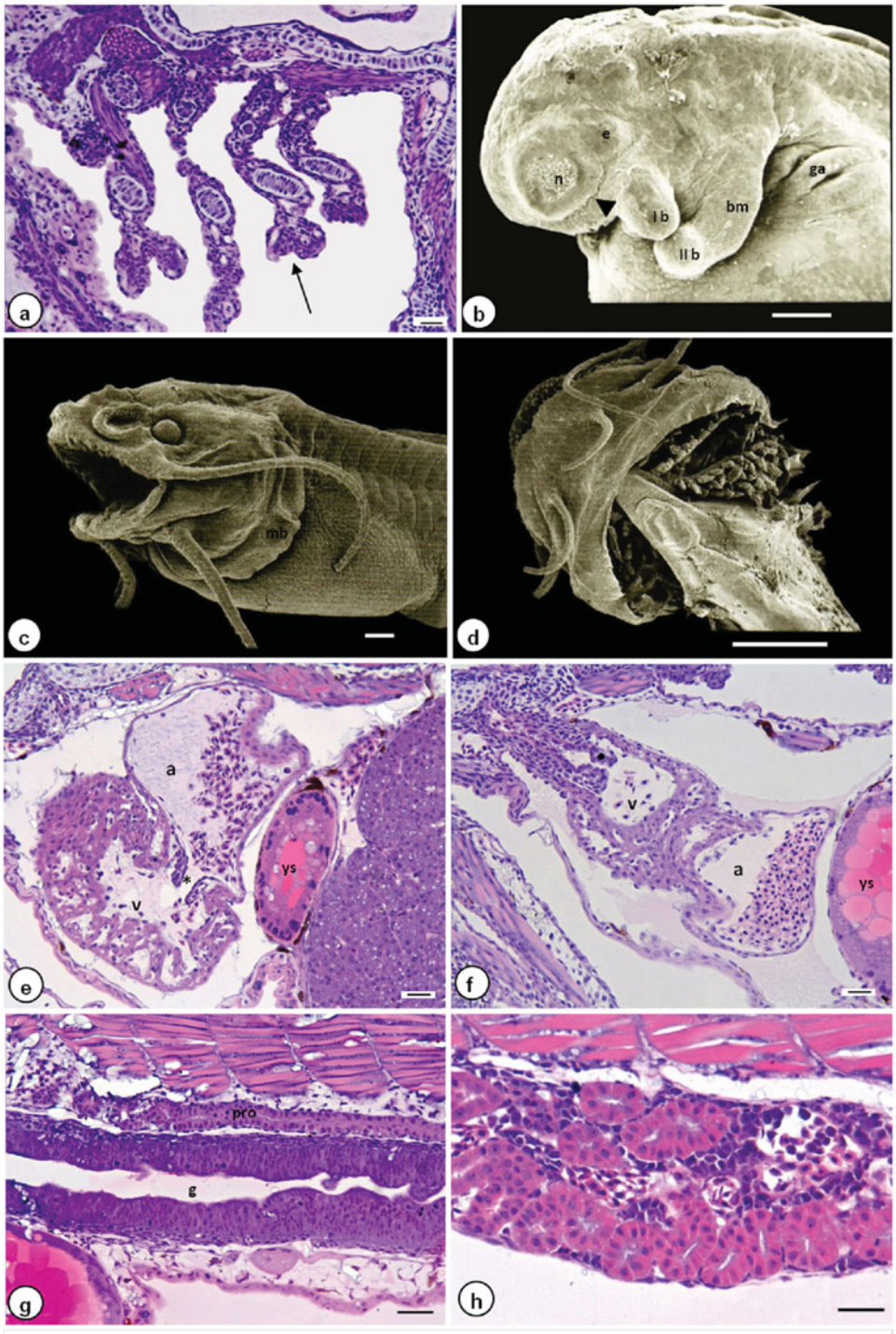

Figure 3 Electron micrography (*b*, *c*, *d*) and photomicrography (*a*, *e–h*) of *Z. jahu* larvae. (*a*) 24 hph – gills branching out (arrow); (*b*) 8 hph – branchiostegal membrane, nasal fold (arrowhead); (*c*) 28 hph – branchiostegal membrane covering the gill arches, presence of the first two pairs of barbels; (*d*) 60 hph – gill arches; (*e*) 28 hph – heart, detail of the atrium and ventricle with trabeculae and atrioventricular orifice (∗); (*f*) 32 hph – heart, detail of the ventricle-bulbus orifice (•); (*g*) 16 hph – mesonephros; (*h*) 60 hph – mesonephros with evident lumen. Abbreviations: a = atrium, ga = gill arches, I b = first pair of barbels, II b = second pair of barbels, g = gut, bm = branchiostegal membrane, n = nares, e = eye, mes = mesonephros, v = ventricle, ys = yolk sac. Bars: (*a*, *e*, *f*, *h*) = 30 μm; (*b*, *c*) = 100 μm; (*d*) = 500 μm; (*g*) = 50 μm. Staining: (*a*, *e–h*) = hematoxylin–phloxin (HP).

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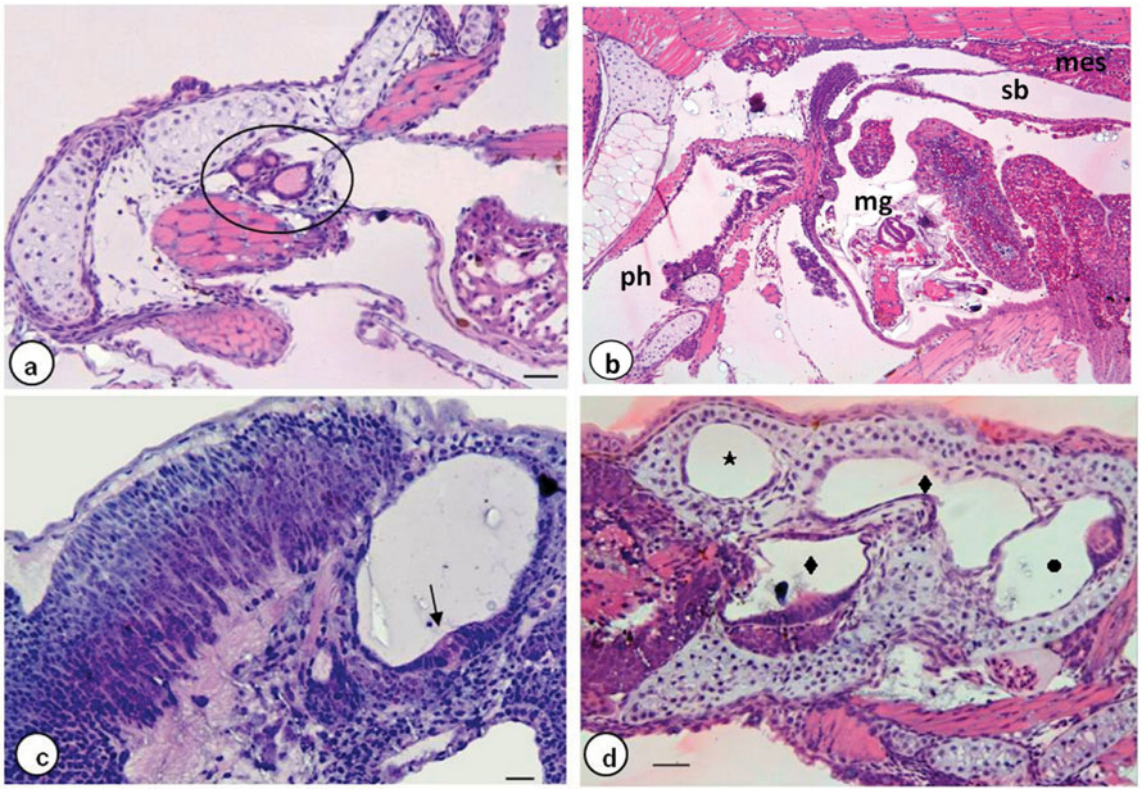

Figure 4 Photomicrography of *Z. jahu* larvae. (*a*) 56 hph–thyroid follicles (circle); (*b*) 24 hph – swim bladder; (*c*) 8 hph – inner ear, detail of neuromast (arrow); (*d*) 60 hph – inner ear with anterior (⋆), lateral (♦), and posterior canals (●). Abbreviations: mg = midgut, ph = pharynx, mes = mesonephros, sb = swim bladder. Bars: (*a*, *c*) = 30 μm; (*b*) = 50 μm; (*d*) = 20 μm. Staining: hematoxylin–phloxin (HP).

Thyroid

The first thyroid follicles were observed from 56 hph. They were located above the ventral aorta and consisted of a single layer of cuboidal cells, with colloid in their interior (Fig. 4*a*).

Swim bladder

With the opening of the esophagus at 24 hph, the swim bladder developed between the stomach and pronephros (Fig. 4*b*). It was composed of a thin layer of squamous epithelium, with a pneumatic duct connecting it to the esophagus. It did not show significant alterations throughout larval development.

Encephalon and sensory system

Encephalon

The differentiation of the neural tube regions into prosencephalon, mesencephalon, and rhombencephalon occurred concomitantly to somitogenesis. At 24 hph, there was the differentiation of the telencephalon and diencephalon regions of the anterior portion of the cerebrum, the mid ventral and dorsal portions of the cerebrum, and the development of a prominent transverse cerebellum that separated the mid and posterior cerebrum (Fig. 2*c*).

Inner ear

The inner ear developed from the otic vesicle. At 8 hph, structures similar to free neuromasts began to form inside the ear (Fig. 4*c*) and were well developed by 24 hph, when the anterior and posterior canals were observed. At total yolk absorption, the inner ear already showed well-defined anterior and posterior canals (Fig. 4*d*).

Eyes

During somitogenesis, the optic vesicle was already being developed. At 8 hph, retina pigmentation was observed. At the end of yolk absorption, this pigmentation was intense, the eye was well developed with nuclear layers (internal and external) connected through the thick plexiform layer (Fig. 5*a*), while the nucleus of the cones and rod receptors began to differentiate into two layers.

Nares

At hatching, it was observed the initial development of the nares, with olfactory cells containing small

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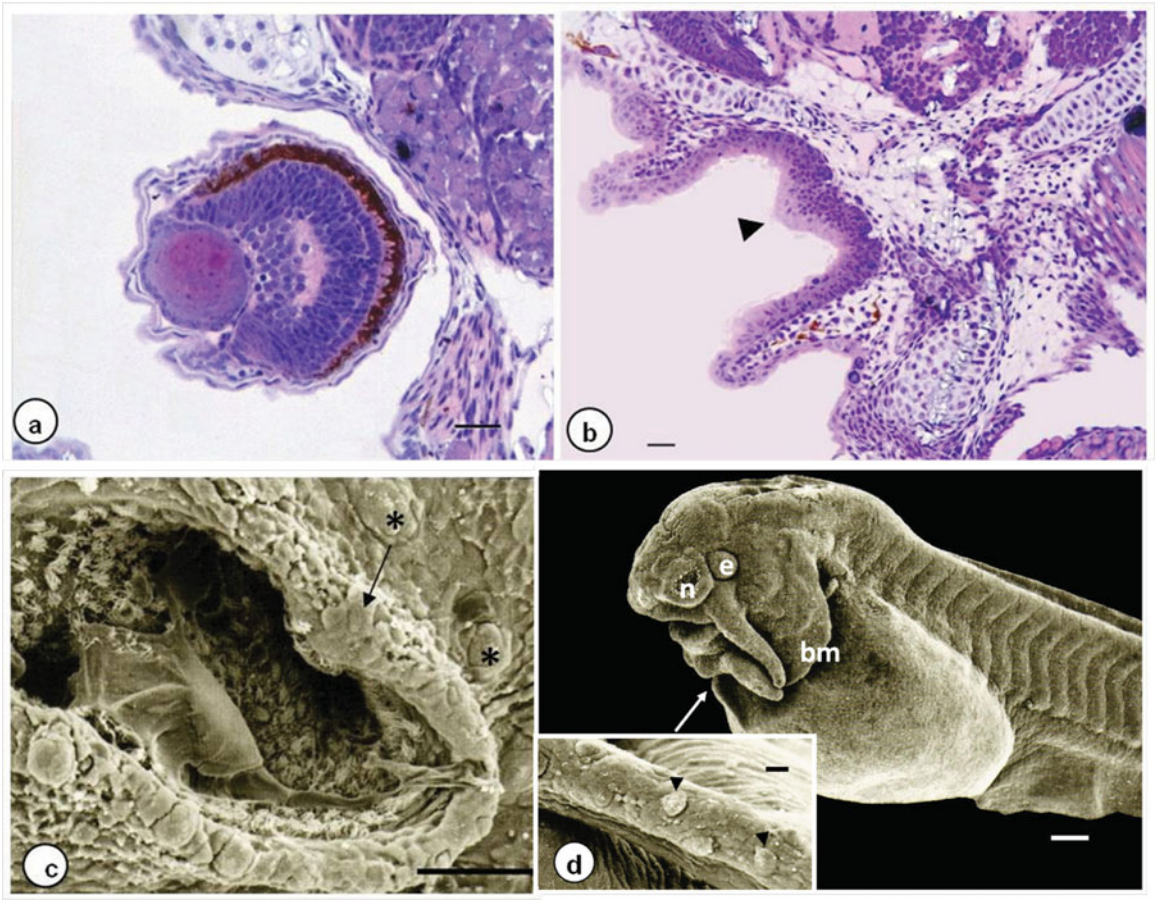

Figure 5 Photomicrography (*a*, *b*) and electron micrography (*c*, *d*) of *Z. jahu* larvae. (*a*) 60 hph – eye; (*b*) 0 hph – nares with cilia (arrowhead); (*c*) 48 hph – division of the nares (arrow), sensory buds (∗); (*d*) 16 hph – primordium of the third pair of barbels (arrow); (*d*, inset) 24 hph – detail of the barbel highlighting the sacciform papillae on the ventral surface (arrowhead). Abbreviations: bm = branchiostegal membrane, n = nares, e = eye. Bars: (*a*) = 30 μm; (*b*, *c*) detail = 50 μm; (*d*) = 100 μm. Staining: (*a*, *b*) = hematoxylin–phloxin (HP).

cilia (Fig. 5*b*). At 8 hph, the nares were more evident and the nasal fold began to form (Fig. 3*b*). After 16 hph, the olfactory organs were well developed, with strands of cilia on the sensory plaque. At 28 hph, there was a protuberance in the interior of the nares and the start of lamellae formation that began to divide and had several sensory buds around it by 48 hph (Fig. 5*c*). At 60 hph, the nares were lined by a ciliated pseudostratified prismatic epithelium.

Neuromasts

Neuromasts were observed mainly in the cephalic region at 44 hph. These structures were characterized by a group of sensitive cells with a protruding gelatinous cupula and a layer of supporting cells over the basal lamina.

Barbels

The primordium of the first pair of barbels (maxillary barbels) was observed soon after hatching (Fig. 4*f*). At 4 hph, the primordium of the second pair of barbels appeared (first mentonian pair) and began to elongate. At 16 hph, the primordium of the third pair of barbels (second mentonian pair) could be seen (Fig. 5*c*). At 28 hph, the first two pairs of barbels were elongated and sharp, whereas the maxillary barbels were longer than the first mentonian pair (Fig. 3*c*) and the third pair of barbels began to sharpen by the last analyzed time. By 24 hph, several papillae could be seen on the ventral surface of the barbels, where tasting buds developed (Fig. 5*c*, detail). Under LM, the barbels showed a layer of epithelial cells and three cell layers, two longitudinal and external and one transversal.

Discussion

The degree of differentiation of newly hatched larvae varies among species according to egg size (Blaxter, 1969). Species with small eggs and high fecundity

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rates have faster embryonic development and poorly developed larvae at hatching, as observed in *Z. jahu* and in the majority of migratory species. Conversely, species with large eggs and low fecundity rates have slower embryonic development and well developed larvae at hatching (Sato *et al.*, 2003).

In fish, the development of the body structures and the start of exogenous feeding are important events for larval survival (Balon, 1986). The opening of the mouth in *Z. jahu* was observed at 12 hph, close to the time reported by Maciel *et al.* (2010) in *Brycon orbignyanus* (9 hph). Total yolk absorption occurred at 60 hph, as also observed by Landines *et al.* (2003) in larvae of spotted sorubim (*P. coruscans*). Yolk absorption occurred through the yolk syncytial layer, in agreement with reports by Gomes *et al.* (2007) in the larvae of three species of trahiras (*Erythrinidae* sp.).

The transition from endogenous to exogenous feeding is a critical stage in early development, often characterized by high mortality rates (Gordon and Hecht, 2002). In the majority of fish species, larvae hatch with a simple and undifferentiated digestive tract and later develop digestive organs (Govoni *et al.*, 1986). This was observed in the *Z. jahu* larvae, which had a rudimentary digestive tube at hatching that became more developed by the time of total yolk absorption and could then be divided into cephalic gut, anterior gut, midgut, and hindgut.

Stomach and gastric glands development could not be seen during the period analyzed. According to Watanabe and Kiron (1994), to compensate for the lack of gastric enzymes, the fish depend on their ability to select food, mechanical digestion, and pancreatic and gut enzymes that can act in an alkaline environment. According to Gonzáles *et al.* (2002), the efficiency of food digestion and absorption in the gut is due to the presence of folds and microvilli. Nevertheless, the mature liver and pancreas aid food digestion by producing digestive secretions that digest proteins, fats, and sugars.

A few mucus-producing cells were observed in the esophagus, just past its connection to the midgut. The early presence of these cells in the larvae of *Z. jahu* indicates that the larvae are morphologically prepared to protect themselves against food abrasiveness (Galvão *et al.*, 1997). The pharyngeal teeth in carnivore fish aid in the apprehension of food by preventing the scape of prey (Rodrigues *et al.*, 2006). In *Z. jahu*, these teeth were present at 28 hph, soon after the opening of the esophagus, when the larvae began exogenous feeding.

Some authors have reported that hepatocytes store glycogen during the larval stage (Hamlin *et al.*, 2000; Micale *et al.*, 2006) and, according to Hamlin *et al.* (2000), glycogen storage in the liver can be considered as the start of hepatocyte function, which remains throughout the larval and juvenile stages. In *Z. jahu* larvae, there was an increase in hepatic glycogen as development progressed, suggesting an improvement in hepatic function.

Similarly to reports by Sampaio *et al.* (2015) in larvae of *B. orthoataenia*, *S. brasiliensis*, *L. abtusidens*, and *P. argenteus*, the pancreas in *Z. jahu* had an endocrine and exocrine portion before total yolk absorption. The endocrine portion is composed of Langerhans islets (Gonzáles *et al.*, 2002) and their presence suggests that the larvae are able to efficiently assimilate sugars. Well-differentiated liver and pancreas can be seen in larvae that begin exogenous feeding soon after hatching and become functional before total yolk absorption, as in *Paralabrax maculofasciatus* (Peña *et al.*, 2003), *Amphirion percula* (Gordon and Hecht, 2002), *A. lupus* (Falk-Petersen and Hansen, 2001), and *Melanogrammus aeglefinus* (Hamlin *et al.*, 2000).

The gills are vital structures for fish health, as they are the main site for gas exchange as well as being involved in osmoregulation, acid-base balance, and excretion of nitrogen compounds (Maciel *et al.*, 2010). The outline of the gill arches in *Z. jahu* was observed soon after hatching and was well developed by the time of total yolk absorption. The development of heart in *Z. jahu* was similar to that observed in zebrafish (*Danio rerio*), appearing during somitogenesis and showing thickening only of the ventricular myocardium during larval ontogenesis (Hu *et al.*, 2000). According to Hu *et al.* (2000), the heart is the first definite organ to develop and become functional during embryogenesis.

In *Z. jahu* larvae, the sensory system developed concomitantly to the digestive system. The eyes, inner ear, and the nares were the first sensory structures to appear, while the taste buds were the last and could only be identified after the opening of the mouth and elongation of the barbels. Taste buds can be found on the epithelium of the lips, oropharyngeal cavity, barbels, head, and occasionally throughout the whole body (Hansen *et al.*, 2002).

The development of the sensory organs is important for larval survival and is closely related to the onset of exogenous feeding, scape from predators, and vertical migration in the water column (Matsuoka, 2001). Kawamura *et al.* (2003) suggest that the development of sensory organs accompanies behavioral changes with important implications to ecology and fish farming, as high mortality rates in larvae under farming conditions could be related to the stress caused by high agitation of the water, aeration, and exaggerated flow, amongst other environmental disturbances that affect chemical and mechanical sensitivity at the different stages of the life cycle.

Total yolk absorption in *Z. jahu* was fast (60 hph), a characteristic of predatory species such as *B.*

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amazonicus (Neumann, 2008), *P. fasciatum* (Pérez *et al.*, 2001), and *B. gouldingi* (Faustino et al., 2015). The development pattern of *Z. jahu* prioritized the formation of sensory and feeding structures that enable piscivorous expression at the start of development, before the depletion of endogenous energy reserve, thus ensuring fast growth and increasing the chances of larval survival.

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