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# Induced reproduction and early development histology of Oscar *Astronotus ocellatus* (Agassiz, 1831)

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## Summary

Oscar (*Astronotus ocellatus*) is an important fish from the Amazon Basin that has great potential for fish farming, human consumption, sport fishing and fish keeping. This study aimed to evaluate the effect of two hormonal treatments on the induction of artificial reproduction in broodstock and to describe the histological development of embryos and larvae. Broodstocks were selected and induced using two different hormones: (i) extract of carp pituitary (ECP); and (ii) synthetic human chorionic gonadotropin (hCG). Spawnings were transferred to hatcheries, collected at pre-established times, processed and analysed by histology. *Astronotus ocellatus* did not respond well to induced reproduction. From 16 couples of breeding fish, only five out of the eight females released oocytes after the hormonal action time, three with hCG and two with ECP; just one male responded positively to hCG. Oscar eggs were oval, and semi-adhesive, the yolk contained granules, and egg diameter was approximately 1.65 ± 0.057 to 1.98 ± 0.038 mm. Development from the initial collection (IC) point until the total absorption of the yolk lasted 315 h, at an average temperature of 27.45 ± 2.13 ºC. Several events marked embryonic and larval development, including the formation of the optic cup, forebrain, otic vesicle and cephalic divisions. The newly hatched larvae had non-pigmented eyes, and a closed mouth and anus, as well as the presence of adhesive glands on the head. Larval development was characterized by formation of the heart, liver, gaseous bladder, gills, pronephros, brain, fins and also the digestive tract. These results provide important information for the rearing and reproduction of *A. ocellatus*.

Keywords: Cichlids, Embryology, Histology, Ontogeny, Reproduction

## Introduction

*Astronotus ocellatus* is the taxonomic name of the Amazonian cichlid, a fish breed that has great potential for commercial fish farming because their meat is tasty, firm and lacks intramuscular bones (Fontenele & Nepomuceno, 1983). Commonly known as 'Oscar', it is consumed widely in the north and northeast of Brazil and also cultivated worldwide as an ornamental fish (Fontenele, 1982). It has been reported that in the USA, in the southern state of Florida, this species is cultivated in large numbers in aquaria for sport fishing (Fury & Morello, 1994).

Adult specimens reach 33.0 cm in length and weigh up to 1.5 kg (Machado, 1983). Their first gonadal maturation is reached around 10–12 months of age and produces on average about 1500–2000 eggs per clutch (Braga, 1962). It is a partitioned spawning species with parental care behaviour and 3–4 reproductive cycles per year (Silva *et al*., 1993).

Studies that investigate hormonal induction are very important for reproduction research because, for most species, reproductive techniques are not well understood and the focus of major studies in this field has been on exotic species (Andrade & Yasui, 2003).

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Non-migratory species spawn in captivity, but with natural reproduction it is not possible to control the reproductive process, because of the difficulty in implementing a programme to monitor embryo development and broodstock quality (Andrade & Yasui, 2003). In particular, little information is known about the embryonic stages and reproductive potential of Amazonian species (Anjos & Anjos, 2006), especially regarding partitioned spawning fish such as *A. ocellatus*.

Early fish development studies have been of great interest for providing basic knowledge that has helped to improve the understanding of embryo physiology, to preserve fish populations and to improve cultivation techniques (Godinho *et al.*, 2003). Most studies with fish larvae are focused on systematics and phylogeny, without giving particular attention to morphological development in relation to function. Larvae studies are necessary to understand the functional role of morphological characteristics on larval survival, especially those mechanisms related to vital requirements, such as locomotion, feeding and respiration (Urho, 2002). Thus, the aim of the current study was to test two different hormone treatments to induce *A. ocellatus* breeding and to describe the main morphological events during initial development.

## Material and methods

The *A. ocellatus* specimens used for this study were 1–2 years old and were kept in two brick ponds (volume 50 $m^3$). Fish were fed twice a day with commercial extruded feed that contained 40% crude protein.

### Induced reproduction

Sixteen couples of breeding fish (four females and four males for each hormonal treatment) with signs of secondary maturation, such as rounded-off abdomens and prominent, reddish urogenital organs, were selected. These fish were weighed with a digital scale and induced with intramuscular injections of sex hormones without taking them from the water, in order to minimize stress.

*Extract of carp pituitary*
Females received a first dose of 0.5 mg/kg fish live weight and a second dose of 5 mg/kg after 18 h. Males received 1.5 mg/kg as a single dose, simultaneously with the females' second dose.

*Human chorionic gonadotropin hormone (hCG; Vetecor®)*
A dose of 5.0 IU per gram of fish live weight was used and divided into two injections for females, the first corresponding to 10% of the dose and a second dose (90%) applied 18 h after the first. The males received half of the dose given to females as a single dose, at the same time that the females received their second dose.

At 24 h after the last injection, mild abdominal massage was performed in a cranium–tail direction for the extrusion of gametes. The quantity of semen released and the quantity, colour and shape of oocytes were observed.

### Embryonic development

During the reproduction period, plastic cylinders and ceramic bricks were placed alongside the pond to act as a shelter and substrate for spawning, to induce naturally occurring spawnings.

As soon as the spawnings were seen in the pond, they were removed and transferred to incubators that had a continuous water flow. Sample collection was performed according to the following protocol: initial collection (IC; as soon as the spawning was identified in the nursery); every 6 h until 102 h post-hatching (hPH); every 12 h up to 162 hPH; and every 24 h up to 383 hPH.

Samples were placed in modified Karnovsky's solution (paraformaldehyde 2.5% + glutaraldehyde 2.5%) for 24 h and washed in phosphate-buffered saline (pH 7.4). Samples were prepared for HistoResin inclusion by dehydration for 24 h in an 80% ethanol solution and washing twice for 30 min in 90% and 100% ethanol before inclusion using a Leica HistoResin® kit. Samples were also prepared for embedding in Paraplast® (Merck) by dehydration in ethanol at 80, 90, 95 and 100% and clearing them in xylene before infiltration and inclusion in Paraplast. The 3.0 $\mu$m and 5.0 $\mu$m slices were stained with hematoxylin–floxin (HF) or hematoxylin–eosin (HE) (Tolosa *et al.*, 2003). Subsequently, the samples (eggs and larvae) were observed and photographed using a Leica DM 2500 optical microscope.

## Results

### Induced reproduction

Females had an average weight of 499 g and males 437 g. A positive response to hormonal stimulation was considered to be when gametes were observed to be released by extrusion for both females and males. Thus, it was found that five out of the eight females released oocytes after the hormonal treatment action, three with hCG and two with ECP.

The three females treated with hCG spawned 800, 1350 and 1400 oocytes, respectively, that were coloured bright yellow, with an ovoid shape, uniform size and were resistant to the touch (Fig. 1*A*–*C*). Females

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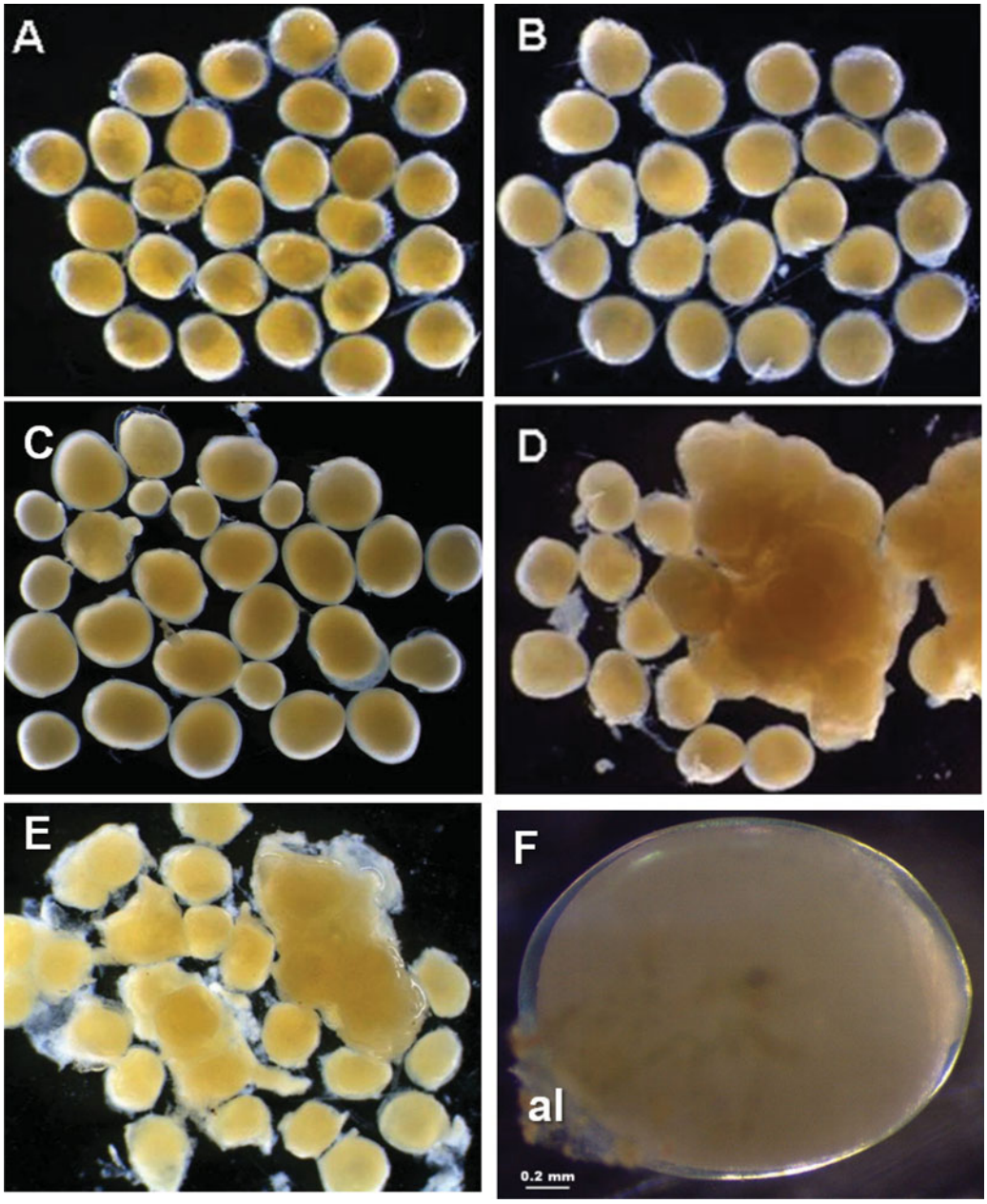


**Figure 1** Photomicrographs of *Astronotus ocellatus* oocytes. (*A–C*) Oocytes from females induced by human chorionic gonadotropin (hCG). (*D*, *E*) Oocytes from females induced by extract of carp pituitary (ECP). (*F*) Oocyte showing the adhesiveness layer (al).

treated with ECP spawned approximately 500 and 1100 oocytes, respectively. The oocytes formed 'lumps', had a colour ranging from pale yellow to yellow-brown and an irregular shape and size (Fig. 1*D*, *E*). The presence of a gelatinous layer surrounding the oocytes was also observed (Fig. 1*E*). Statistically there was no difference between the hormones with regard to gamete release, however the quantity and the quality of oocytes from females induced with hCG was better.

As only one male responded positively to hCG treatment, releasing <1.0 ml of semen, no statistical analysis was made for males.

**Embryo formation and hatching**

The average of physical and chemical water parameters monitored in the incubators were as follows: temperature 27.45 °C; pH 4.83; dissolved oxygen 5.52 mg/l; total alkalinity 52 mg/l; ammonia 117.45 $\mu$g/l and conductivity 35.51 $\mu$S/cm.

*Astronotus ocellatus* eggs are oval, yellow, semi-adhesive, and demersal with a thick chorion; in the present study the eggs were approximately 1.65 ± 0.057 to 1.98 ± 0.038 mm in diameter and the yolk sac showed granules. Eggs from the IC

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were at the gastrula stage, characterized by migration movements forming two layers: (i) the top layer or epiblast; and (ii) the bottom layer or hypoblast (Fig. 2*A*, *B*). Fifteen hours after the IC, the optical cup and forebrain formed, as seen in Fig. 2*C*, as well as the optic vesicle. The cephalic division into rhombomeres and the cerebellar primordium are shown in Fig. 2*D*.

Hatching took place 46–58 h after IC. Newly hatched larvae had non-pigmented eyes, as well as a closed mouth and anus. At this stage the larvae measured approximately 3.25 ± 0.24 mm in length and 1.55 ± 0.03 mm in height. At 7.86 ± 0.11 mm in length the larvae had already absorbed the yolk sac. Before hatching, two pairs of highly developed adhesive glands in the upper and frontal regions of the head, formed by mucus-producing cells, were observed (Fig. 2*E*, *F*).

### Digestive tract

Soon after hatching, the digestive tract could be seen as a straight tube that consisted of a simple epithelium located just above the yolk sac. At 24 hPH, the tube was already open but the mouth was still closed (Fig. 3*A*). After the mouth opened, the digestive tract began to differentiate into the oesophagus, stomach and intestines. From 125 hPH, the presence of numerous goblet cells, which stained strongly purple in HistoResin, was observed in the region of the pharynx and oesophagus. These cells were no longer seen in the region where the stomach primordium started. At 161 hPH, large secretory cells were present in the region of the pharynx and oesophagus, either as isolated cells or in groups. These cells increased in number as the larvae grew and their cytoplasm became voluminous, clear, with a vesicular aspect and very often with a nucleus found in the basal position (Fig. 3*B*). At the same time, the stomach appeared differentiated, with a thin wall and invaginations, lined by pseudo-stratified columnar epithelium whose cells were larger, with quite evident nucleoli. The intestine showed a prominent and thick luminal edge. Enterocytes with large nuclei and evident nucleoli, among them secretory cells, were also observed. As exogenous feeding started (125 hPH), the larvae already presented a digestive tract that was a little more developed, but the presence of digestive glands was not noticed even at the final observation time (383 hPH).

### Oral cavity

After 77 hPH, it was observed that the oral cavity and pharynx were separated by the first branchial arch, which was supported by cartilage. The oral valve and skeletal striated muscle that supports the lower jaw were identified on the back of the maxilla and mandible. The mandibular and oral maxillary valves were observed in larvae 161 h after hatching. Histologically, taste buds and tooth formation were not observed.

### Liver

The liver was very large compared with the larval body size at the time of hatching. Hepatocytes appeared mostly with a single nucleus and vacuolar cytoplasm. Capillaries that contained erythrocytes were also present.

### Skeletal striated muscular tissue

Skeletal striated muscular tissue was observed in the larvae from 6 hPH onwards. The muscular fibres found along the larval body consisted of multi-nucleated myofibrils with peripheral nuclei. Visceral or smooth muscle tissue was also seen associated with the digestive tract in this period.

Bone tissue was still not seen by the last observation time and skeletal support was given only by hyaline cartilage with chondrocytes in lacunae surrounded by the extracellular matrix, which emerged from the 12 hPH onwards.

### Swim bladder

The swim bladder of *A. ocellatus* was located below the notochord and above the digestive tract. At 101 hPH, it was not yet inflated and was composed of stratified squamous tissue, with cells having clearly evident nuclei and nucleoli (Fig. 3*C*) and, at 137 hPH, it was functional, inflated and consisted of a single layer of cuboidal cells with evident nuclei and nucleoli, and basophilic cytoplasm (Fig. 3*D*).

### Eyes

The onset of eye formation was observed prior to hatching and, at 42 hPH, eyes were well developed and pigmented. The retina was surrounded by a pigmented layer composed of cuboidal cells that contained many melanin granules and apical extensions. Subsequently, a photoreceptor cell layer, and outer and inner nuclear layers were also seen. It was observed that the choroid, with coating cells and loose connective tissue, the crystalline lens, cornea and vitreous body were coated with stratified squamous epithelium (Fig. 3*E*, *F*).

### Heart

The heart was completely formed and located in the pericardial cavity of the larvae from 113 hPH onwards. The sinus venosus, sinus atrial valve, atrium, atrium ventricular valve, ventricle with well developed

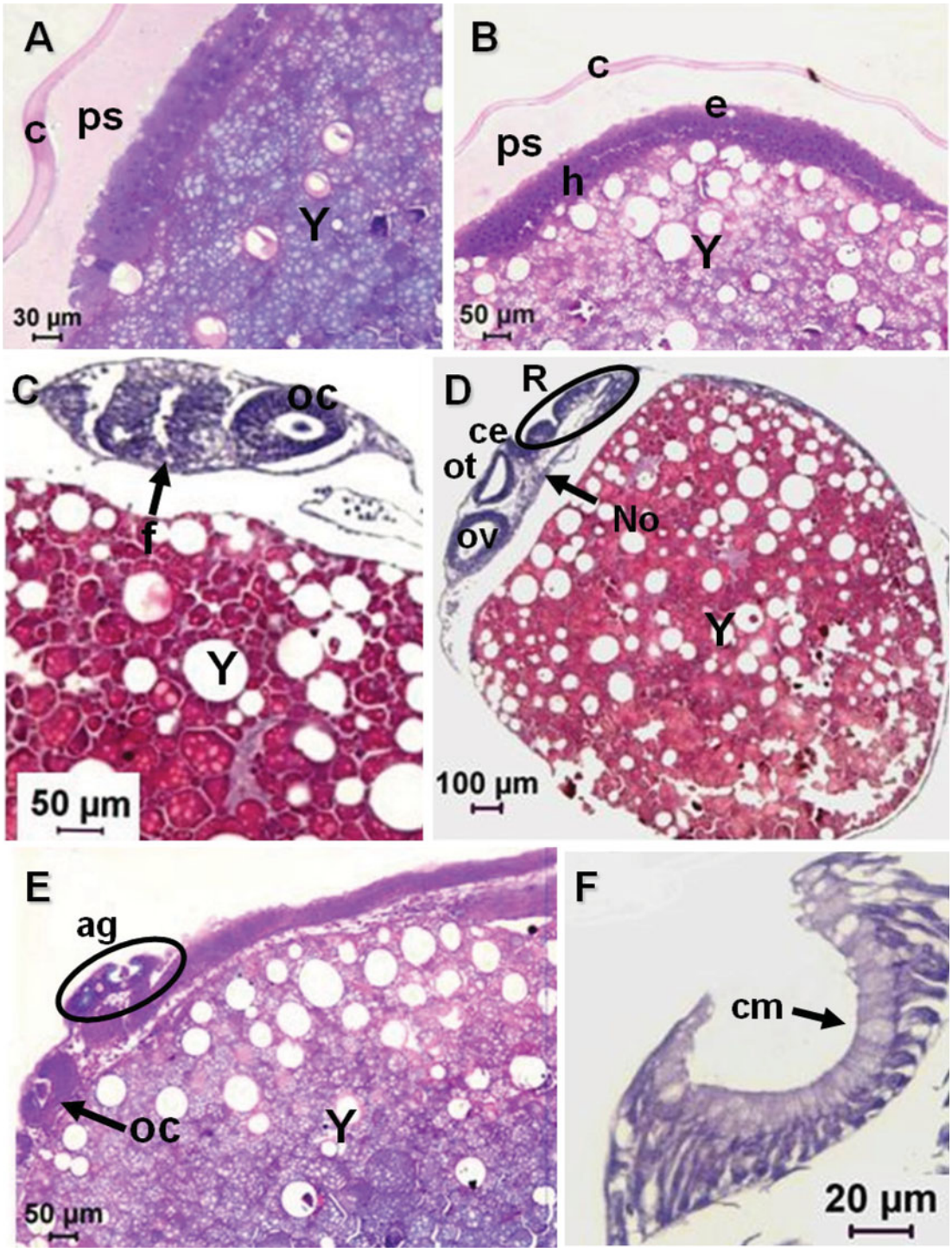


**Figure 2** Photomicrographs of *Astronotus ocellatus* eggs. (*A*, *B*) Eggs showing the chorion and gastrula stage (HistoResin/hematoxylin–floxin (HF)). (*C*) Head formation (Paraplast/hematoxylin–eosin (HE)). (*D*) Optical and otic vesicles, formation of cerebellum, rhombomeres and notochord (Paraplast/HE). (*E*) Pre-larva showing the adhesive glands in the head (HistoResin/HF). (*F*) Details of the adhesive gland (Paraplast/HE). ag: adhesive glands; c: chorion; Ce: cerebellum; e: epiblast; f: forebrain; h: hypoblast; mc: mucus-secreting cells, No: notochord; oc: optical cup; ot: otic vesicle; ov: optical vesicle; ps: perivitelline space; R: rhombomeres; Y: yolk.

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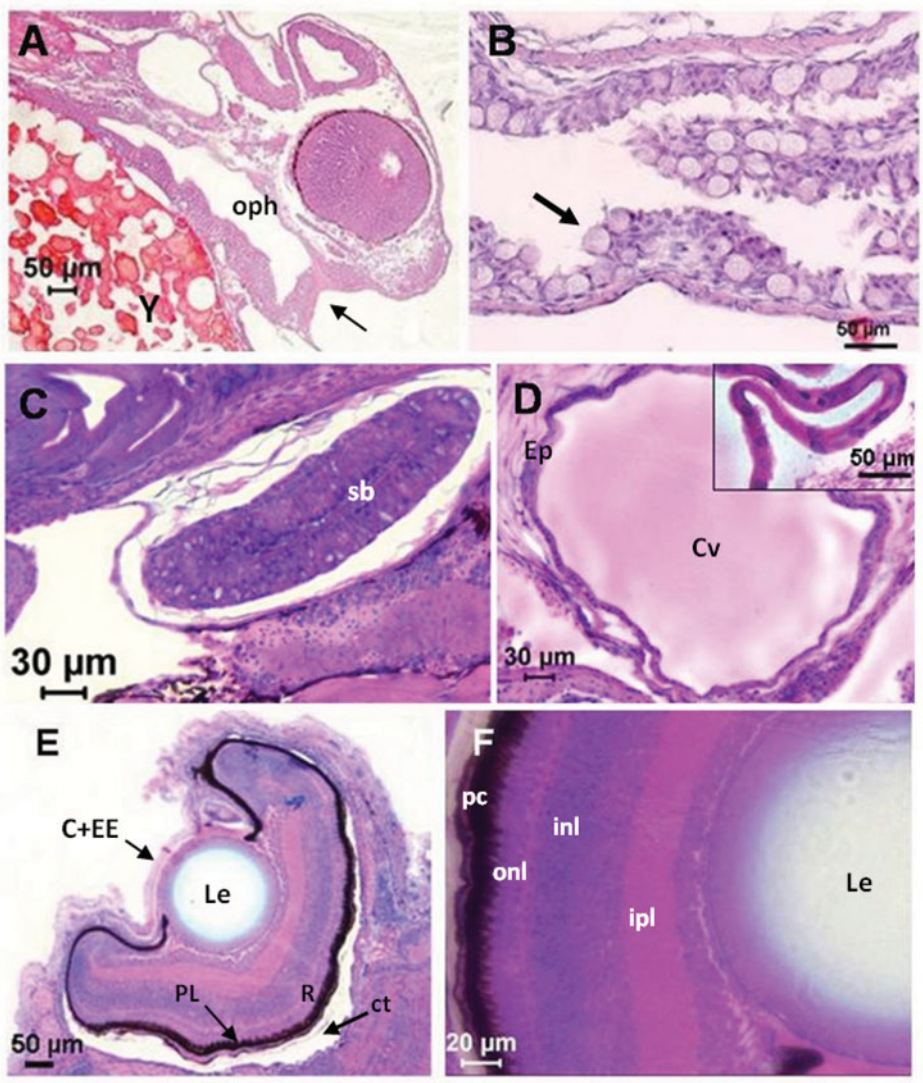


**Figure 3** Photomicrographs of *Astronotus ocellatus* eggs: (*A*) 24 h post-hatching (hPH): oropharyngeal cavity open, mouth still closed (arrow) (Paraplast/hematoxylin–eosin (HE)). (*B*) 137 hPH: transition between oesophagus and stomach (arrow showing oesophagus secreting cells) (HistoResin/hematoxylin–floxin (HF)). Small black arrow: pharynx secretory cell. (*C*) 113 hPH: swim bladder is not inflated (HistoResin/HF). (*D*) 383 hPH: inflated swim bladder (detail shows the tissue) (HistoResin/HF). (*E*, *F*) 137 hPH: detailed layers of the eye (HistoResin/HF). C+EE: cornea and epidermis stratified epithelium; ct: choroidal tapetum; Cv: cavity; Ep: epithelium; inl: inner nuclear layer; ipl: inner plexiform layer; Le: lens; onl: outer nuclear layer; oph: oropharyngeal cavity; pc: photoreceptor cells; sb: swim bladder; PL: pigmented layer; R: retina; Y: yolk.

cardiac striated muscle, the ventricle-bulbar valve and branchial artery were also identified (Fig. 4*A*).

### Gills

Gills were arranged in four branchial arches with primary and secondary lamellae supported by cartilage in larvae from 77 hPH (Fig. 4*B*). The arches and branchial lamellae were found to be coated by the branchial epithelium, composed of cubic cells, a few mucus-secreting cells, chloride cells that had a large nucleus and were distributed at the base of the secondary lamellae and supporting cells. Pseudo-gills were observed on both sides of the roof of the pharynx, inside the ventral operculum, posterior to the eye and anterior to the first gill arch.

### Pronephros

The pronephros was observed in the dorsal part of the body cavity and above the swim bladder of the larvae at 113 hPH. These organs developed coiled structures that united and led into the urogenital papilla. The glomeruli could also be viewed with nearby blood vessels and renal tubules (Fig. 4*C*). These consisted of cells with large nuclei and marked basophilia.

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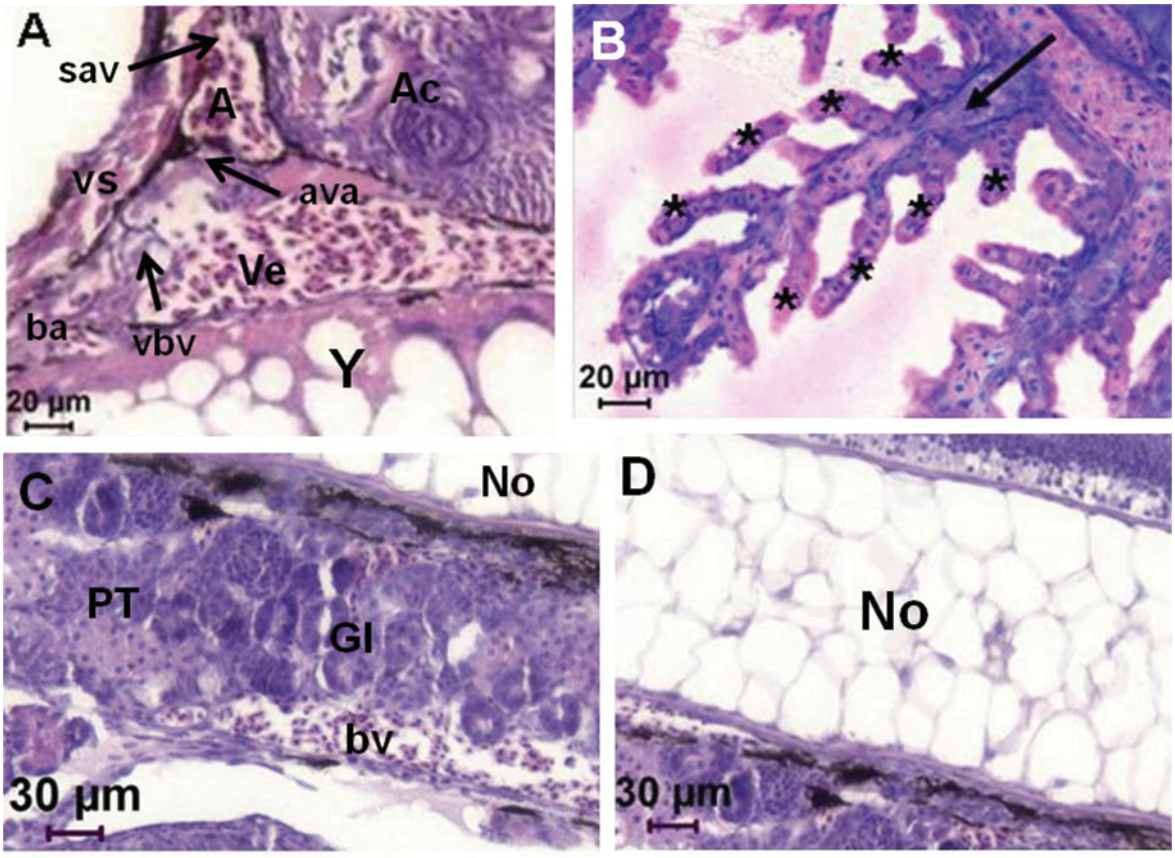


**Figure 4** Photomicrographs of *Astronotus ocellatus* larvae. (*A*) 161 h post-hatching (hPH) heart (Paraplast/hematoxylin–eosin (HE)). (*B*) 101 hPH branchial arch with primary lamellae (arrow) and secondary lamellae (asterisks) (Paraplast/HE). (*C*) 113 hPH pronephros (Paraplast/HE). (*D*) 113 hPH notochord (Paraplast/HE). A: atrium; Ac: abdominal cavity; ava: atrioventricular valve; ba: branchial artery; BV: blood vessel; Gl: glomerulus; No: notochord; PT: proximal tubule; sav: sinoatrial valves; vbv: ventriculo-bulbar valve; Ve: ventricle; vs: Venous sinus; Y: yolk.

### Notochord

The notochord was observed along the whole body length of larvae. It was observed in the embryos even before hatching, but became more noticeable in larvae from 24 hPH and after. The cells were large, with vacuolar cytoplasm and peripheral nuclei (Fig. 4*D*).

### Brain

Although not fully developed in larvae at 89 hPH, it was possible to have a general idea about the location of most of the brain. In the frontal portion, the olfactory lobe was surrounded by pavement cells on the outside and cuboidal cells on the inside. In the dorsal part of the brain, posterior to the lobe, the central pineal organ was observed. Subsequently, the cerebellum could be seen and, above the buccopharyngeal cavity, the hypothalamus.

## Discussion

The methods currently used for hormonal induction, although successful for many species still present obstacles for many other species because there is great difficulty in standardizing the applied dose and there is no established methodology (Andrade & Yasui, 2003), especially for species with partitioned spawning.

In the experiment described here, induction with carp pituitary was used because this technique is the one most favoured by fish farmers (Andrade & Yasui, 2003), and hCG was chosen because it is an inexpensive hormone used successfully in many species (Andrade-Talmelli *et al*., 2002; Carrillo & Romagosa, 2004; Cerqueira *et al*. 2005).

Hormonal induction was more effective in females than in males, but was considered unsatisfactory in both cases. The males in some species did not require hormonal induction because gametes were released easily with abdominal massage. Often hormones are used only to improve the fluidity of the semen produced and not to increase the number of sperm cells (Zaniboni-Filho & Nuñer, 2004). *Astronotus ocellatus* is a species that produces only a small amount of semen, and the number of mature males at any time is significantly smaller than the number

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of mature females over the same period (González *et al*., 1996). We suggest that the males in the current experiment were probably either not yet ready to receive hormone injections or had already spermiated recently, naturally, in the ponds.

Histological analysis of the initial development stages of *A. ocellatus* revealed interesting findings that could improve the understanding of this species' biology, management and morphology. Larval embryology and ontogeny were, in general, very similar to those of other cichlids (*Oreochromis niloticus*, Morrison *et al.* (2001); *Paracheirodon axelrodi*, Anjos & Anjos (2006)), but with some peculiarities.

In cichlids, larvae present a functional stomach before feeding habits change from endogenous to exogenous (Gordon & Hecht, 2002). At this time, it was observed that the stomach of *A. ocellatus* larvae did not have gastric glands. However, from 89 hPH, the bowel already had many folds in the mucosa that, according to Neumann (2008), slowed down the passage of food to allow a longer digestive period, improving feeding use and increasing nutrient absorption capacity. Several round mucus-secreting cells, either isolated or found in groups, were also observed in the pharynx and oesophagus. Although *A. ocellatus* is omnivorous (Nakatani *et al*., 2001), these cells have been also seen in typically carnivorous species such as *Pseudoplatystoma corruscans* (Mangetti, 2006), *Brycon orbignyanus* (Maciel *et al*., 2010) and *Silurus glanis* (Kozarić *et al*., 2008). These authors also noted that cell numbers increased as the larvae got older, concentrating towards the oesophagus and stomach; this phenomenon was also observed in the present study. Kozarić *et al*. (2008) reported that, in *Silurus glanis*, these mucous cells in larvae secreted neutral and acid mucous substances from the fifth day after hatching, suggesting that this event could be a response mechanism of young fish to enable the alimentary canal to tolerate environmental changes and maintain an osmotic balance. The large amount of mucous secreted also works as a lubricant. As fish do not have salivary glands, the cells in the mucosa of the oesophagus may play the same role as the saliva of mammals, protecting the mucosa of the alimentary canal (Kozarić *et al*., 2008; Scocco *et al*., 1998).

Teeth were not formed in Oscar larvae until the last collection time (383 hPH). This factor is presumably correlated with the nature of the diet; omnivorous and herbivorous species, unlike carnivores, do not have well developed teeth (Rodrigues *et al*., 2006).

The maxillary and mandibular oral valves were observed in larvae at 161 hPH. According to Cericato (2005), these valves assisted in mechanical respiration, preventing backflow of water from the oral cavity to the environment during the respiratory effort.

The livers of *A. ocellatus* larvae were relatively large compared with the larval body, and had vacuolar hepatocytes and erythrocytes. The liver, in addition to storing fat and glycogen (Falk-Petersen & Hansen, 2001), is an important organ in the process of yolk absorption (Morrison *et al*., 2001). Prior to the mouth opening, the larvae feed on the yolk reserve, which is absorbed by endocytosis via the yolk syncytial layer (Shahsavarani *et al*., 2002). According to Nakatani *et al*. (2001), species with larger yolk sacs are better prepared to survive longer without relying on exogenous feeding. Tengjaroenkul *et al*. (2002) reported that in order to avoid larval mortality and feed waste, exogenous feeding should begin after the yolk has been completely absorbed.

Circulatory system development started with the heart, during embryo stomatogenesis, and continued throughout larval development (Hu *et al*., 2000). During this period, blood cells are pumped through sinusoids present in the yolk sac and in the fin membranes (Helfman *et al*., 2000). The heart structure develops quickly and is the first functional organ of the larvae. Osman *et al*. (2008) stated that in *Clarias gariepinus*, rapid heart formation indicates an adaptation of the native species to warm waters, as it indicates an increased metabolic rate resulting from the demand for oxygen and nutrients under higher temperatures.

Branchial arches were observed in four pairs and in a pair of pseudobranchiae, on each side of the head. The arches had expansions that extended towards the pharyngeal cavity and varied in shape, size, quantity and distribution, therefore this feature could be a species identification parameter (Eiras-Stofella *et al*., 2001). The lamellae increased the contact surface area with the water and increase gas exchange. Thus, the number of lamellae present in different species is an adaption to suit the environment in which they live (Evans *et al*., 2005).

The eyes of the Oscar developed very quickly, even before the mouth opened, and they were the first sense organ to be fully functional. These characteristics are typical of a species whose effective feeding and escape from predators depend primarily on their vision (Osman *et al*., 2008).

The swim bladder presented squamous stratified tissue when not inflated and consisted of a single layer of cubic cells after inflation. This observation was also described by Morrison *et al*. (2001) for Nile tilapia larvae. In several species, the swim bladder is a hydrostatic organ that assists fish gravity adjustment to different depths. It can also contribute to the respiratory process, work as a sense organ, or even assist in the production of sounds (Machado, 1999).

The description of initial development in *A. ocellatus* is important for the biology, management,

reproduction and characterization of the species. Species development was considered to be slow compared with migratory species. Development of morphological structures that allow food capture even before the depletion of endogenous energy reserves is a mechanism that ensures an increase in larvae survival chances in the environment. The rapid development of structures that allow displacement and predation, such as fins, swim bladder and eyes, characterize the species as a visual predator.

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