



Intraovarian embryonic development in *Girardinus microdactylus* (Cyprinodontiformes: Poeciliidae) in the Pedernales River, Artemisa, Cuba

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Abstract: In viviparous teleosts the ovary evolved as the only reproductive structure available in females. In Poeciliidae, after the intrafollicular fertilization process, gestation occurs, given by an intrafollicular embryonic development. Eight post-fertilization stages have been determined for this family; however, they have not been studied in the Cuban endemic genus *Girardinus*. In this work we describe, for the first time, the embryonic stages of *Girardinus microdactylus*, a poeciliid exclusive of the western region of Cuba. The fish for this study were collected in the Pedernales River during the two seasons of the year. The gonads of twelve fertilized females were subjected to the classical histological technique and analyzed by bright-field optical microscopy. Embryonic stages were identified from the end of the second post-fertilization stage. The notochord is among the first structures to appear, as well as the muscular, digestive and sensory systems. The eyes are among the first organs to complete their development, so they are born with a complete and differentiated visual system. Viviparity confers protection to the egg and the fry are born with the ability to swim, flee from predators and search for food, which guarantees the success of this species in unstable environments.

Key words: intrafollicular, ovary, post-fertilization, freshwater fish.

Desarrollo embrionario intraovárico en *Girardinus microdactylus* (Cyprinodontiformes: Poeciliidae) en el Río Pedernales, Artemisa, Cuba. Resumen: En los teleósteos vivíparos el ovario evolucionó como la única estructura reproductora disponible. En Poeciliidae, luego del proceso de fertilización intrafollicular ocurre la gestación, dada por un desarrollo embrionario intrafollicular. Para esta familia se han determinado ocho etapas post-fertilización; sin embargo no se han estudiado en el género endémico de Cuba *Girardinus*. En este trabajo se describen, por primera vez, los estados embrionarios de *Girardinus microdactylus*, poecílido exclusivo de la región occidental de Cuba. Los peces para este estudio se colectaron en el río Pedernales durante las dos estaciones del año. Las gónadas de doce ejemplares, se sometieron a la técnica histológica clásica, y se analizaron al microscopio óptico de campo claro. Se identificaron los estadios embrionarios a partir del final de la segunda etapa post-fertilización. El notocordio es de las primeras estructuras en aparecer, al igual que los sistemas muscular, digestivo y sensorial. Los ojos son de los primeros órganos en culminar su desarrollo, por lo que nacen con un sistema visual completo y diferenciado. La viviparidad confiere protección al huevo y los alevines nacen con la capacidad de nadar, huir de los depredadores y buscar alimento, lo que garantiza el éxito de esta especie en ambientes poco estables.

Palabras clave: intrafollicular, ovario, post-fertilización, pez dulceacuícola.

Introduction

Viviparity in teleosts presents characteristics different from other viviparous vertebrates (Blackburn, 2005). This is a complex process in which the ovary evolved as the only reproductive structure available in females (Uribe *et al.*, 2010). During the embryonic development of viviparous teleosts, the left and right ovaries fuse into a single organ, which has a central lumen that is in contact with the germinal epithelium (Grier *et al.*, 2016). On the other hand, teleosts are the only group of vertebrates in which Müllerian ducts do not develop during embryogenesis, which is why they lack an oviduct. Because of this, in viviparous teleosts, gestation also occurs in the ovary. The communication of the germinal part of the ovary with the outside is established by the posterior area of the ovary lacking germ cells called the gonoduct (Turner, 1947; Campuzano-Caballero *et al.*, 2014). In this area, sperm are received during insemination and it is where the offspring emerge at birth (Uribe *et al.*, 2019). Therefore, in viviparous teleosts, all reproductive processes occur in the ovary: oogenesis, sperm is received (Campuzano-Caballero *et al.*, 2014) and stored until fertilization (Potter, 2000), fertilization of the oocytes occurs (Greven, 2010) and then pregnancy (Uribe & Grier, 2018).

Among the freshwater teleost fishes we find the representatives of the family Poeciliidae, which present a marked sexual dimorphism. Adult males have the anal fin modified into an elongated and slender copulatory organ called gonopodium that allows internal fertilization (Fink, 1971). After the intrafollicular fertilization process in poeciliids, gestation takes place, given by intrafollicular embryonic development, the ovulation does not occur and the follicle is maintained (Uribe *et al.*, 2010).

Based on the morphological changes, eight post-fertilization stages have been determined for the Poeciliidae (Haynes, 1995): (1) Blastodisc, (2) primitive embryo, (3) embryo with optic vesicle, (4) embryo early eyes, bud of limbs, (5) embryos middle eyes, (6) embryos developed eyes, (7) embryos with highly developed eyes, (8) mature embryo. Arcanjo *et al.* (2014), for his part, described histological sections of the stages proposed by Haynes (1995).

Viviparity is one of the characteristics that has allowed poeciliids to dominate the fresh and brackish waters of the Cuban archipelago (Rosen and Bailey, 1963), among the most representative of which, we find the endemic genus *Girardinus* (Lucinda, 2003).

Ponce de León *et al.* (2011) analyzed the relationships between egg size and hatchling size and between the newborn and the female, in six species of the genus. Of these same species, Ponce de León (2012) described the changes in the morphology of the nutrient and gas transport structures, also showing the discoblastula and embryos in intermediate and advanced gestation. In Ponce de León and Uribe (2021), the morphology of embryonic and maternal structures related to embryonic nutrition was compared in four species of Cuban poeciliids, including *Girardinus creolus*, with the extreme matrotrophic species *Heterandia formosa*. A recent work described the ovary and ovogenesis of *Girardinus microdactylus* (Torres Martínez *et al.*, 2023). On the other hand, Cabrera (2007) macroscopically characterized the ontogenic states of *Gambusia puncticulata*. However, there are no investigations that detail all embryonic stages at a histological level in *Girardinus* species. This work aims to describe the phases of embryonic development of *Girardinus microdactylus* from Pedernales River, in Western Cuba using optical microscopy.

Materials and Methods

The studied fishes were collected in the Pedernales river Artemisa province, in Western Cuba (22°84'00.79"N, 82°89'40.63"W). It is a permanent water body of running water (lotic), with mountain origin. The locality was visited four times between 2020 and 2022, and a total of 48 adult females of *Girardinus microdactylus* were collected. The fishes were identified, according to their morphological characteristics, using the field guide for the Poeciliidae family from Cuba (Ponce de León & Rodríguez, 2010). To classify as adults, we used as distinctive characters the combination of total length and the presence or absence of the gravid point. The adult specimens used in the study were taken alive to the laboratory, transported in 4-liter tanks filled with two liters of water and the rest of air. For the histological technique, a total of 12 samples were used, 6 females from each season of the year (rainy and less rainy). The specimens used were euthanized with Tricaine Methanesulfonate (MS 222) and dissection was performed on each one by applying a lateral incision in the abdominal cavity, from the operculum to the gonopore, to extract the gonads. These were weighed (PG) and quickly fixed in 10% formalin until the moment of histological processing (Barbeito & Diessler, 2022). Each sample was placed in an automatic tissue processor. They were

first dehydrated by increasing alcohol concentrations (70, 80, 96, and 100%) for 30 minutes. They were then clarified with xylol and finally kept in paraffin until they were included in the form of blocks which, once cold, were cut with a rotating microtome. Sections 4 µm thick were obtained, stretched in a histological bath and then placed on slides previously smeared with albumin. The sections were deparaffinized with xylol and rehydrated with decreasing concentrations of ethanol (100, 96, 80 and 70%). They were then rinsed in running water and stained with hematoxylin-eosin (H-E), toluidine blue, and Masson's trichrome stains. Histological preparations were analyzed on a Novel brightfield optical microscope (4X, 5X, 10X, 40X and 100X objectives and 10X eyepiece) coupled to a digital camera.

The embryonic stages were determined by combining the most used classification for the Poeciliidae family given by Haynes (1995), and the histological evidence provided by Arcanjo *et al.* (2014). It should be noted that in this study the stages began to be counted after fertilization and not from oogenesis, so the numbering of the stages does not coincide with that of these authors. (Fig.1).

In the histological sections analyzed in this study, it was impossible to observe the stages of zygote, cleavage, blastula, or gastrula, proposed by Kimmel *et al.* (1995), so the description of the embryonic stages of *Girardinus microdactylus* began with the segmentation period according to Kimmel *et al.* (1995), between the second and post-fertilization stages according to Haynes (1995) and Arcanjo *et al.* (2014).

Pregnant females were found with embryos in different stages of development. The first stage observed in this study corresponds to the primitive embryo or second post-fertilization stage (2), where the first outlines of organs are observed. (Fig. 2A). Cartilage and muscle can be seen in the caudal region, which will be the vertebrae and notochord (Fig. 2A). In the optic vesicle embryo stage (3), the eyes can be seen, differentiating the layers of the retina, where, although poorly developed, the pigmented epithelium is already present, but neither the lens nor the cornea is defined (Fig. 2B).

In the early eyed-embryo/limb bud stage, the lens is forming and has no layer that limits it (Fig. 2C). In this figure we can also see the brain, the cartilage of what will be the gill arches and the primordia of the pectoral fins. In the middle eyed-embryo stage, the embryo is more robust, the brain is more developed, as are the gill arches and

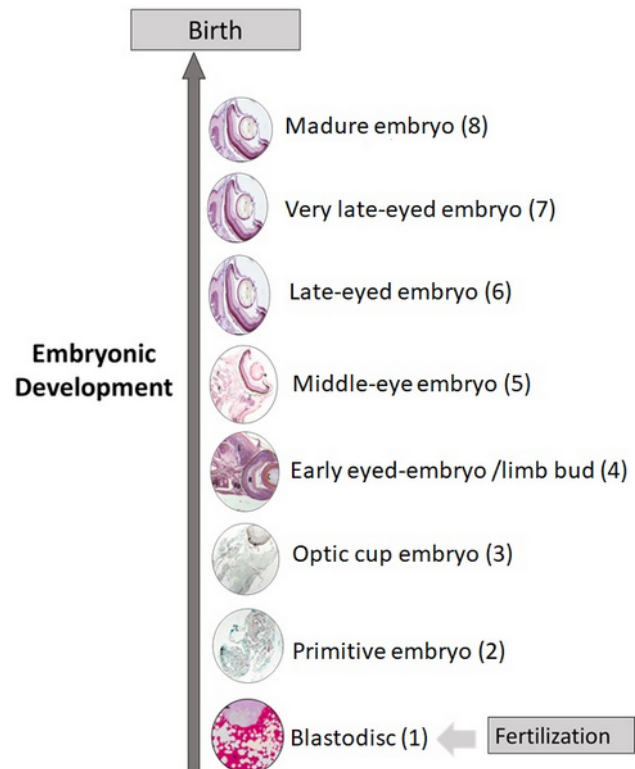


Figure 1. Schematic representation with histological sections of the post-fertilization stages proposed by Haynes (1995) for the family Poeciliidae. The image corresponding to the blastodisc stage is taken from Ponce de León (2012) corresponding to the species *Gambusia punctata*, the remaining images correspond to the species *Girardinus microdactylus* obtained in this study. From stage 6 (late eye embryo) the eye of *G. microdactylus* is fully formed and therefore remains without obvious variations until hatching.

filaments (Fig. 3A). The notochord and well-developed muscle fibers are clearly visible (Fig. 3B). The body wall epithelium is simple and undifferentiated (Fig. 3B). A defined dorsal fin with its rays (Fig. 3C) and caudal fin primordia (Fig. 3B) can be seen. The esophagus, intestine and liver are also observed (Fig. 3D). It still has abundant yolk, the mouth and anus are not fully developed, indicating that the embryo is not yet ready for exogenous feeding (Fig. 3B). The spinal cord is observed between the tail myomeres (Fig. 3B).

In the late-eyed embryo stage the yolk is reduced and connected to the embryo by the middle portion (Fig. 4). The oral cavity is distinguished (Fig. 4A), the gills and pectoral fins are completely formed (Fig. 4B). The pharynx and esophagus are observed (Fig. 4C). The intestinal lumen is visible, with folds inside, showing the columnar epithelial cells of the mucosa and goblet cells (Fig. 4D). The liver is visible and large. The heart is in a ventral

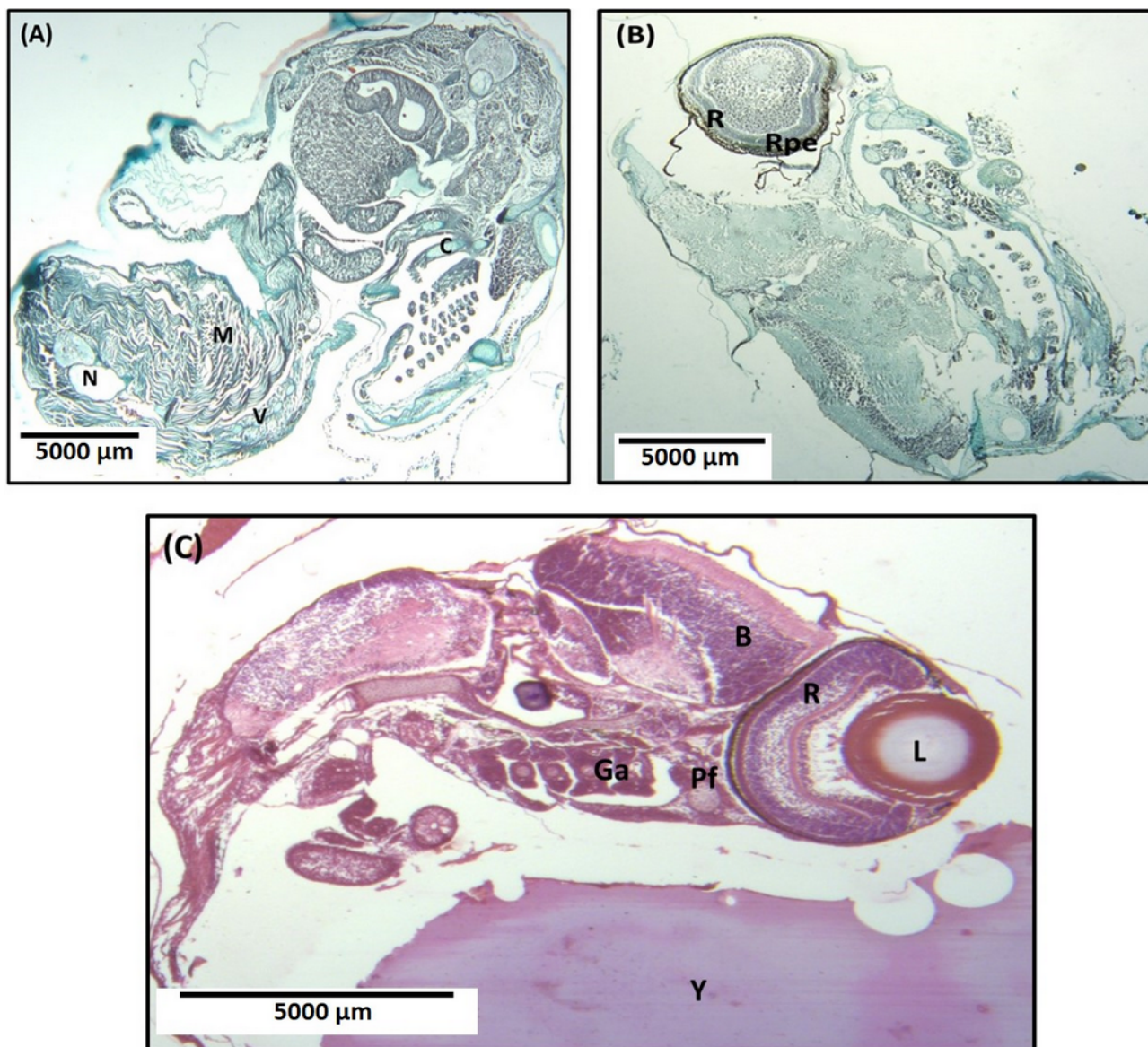


Figure 2. Photomicrographs of histological sections of *Girardinus microdactylus* embryos in different post-fertilization stages. (A) Embryo in the primitive embryo stage, the first sketches of organs can be seen (N: Notochord, M: muscle, V: vertebrae, C: cartilage) (B) Embryo in the optic vesicle embryo stage (R: Retina, Rpe: Retinal pigmented epithelium). (C) Embryo in the early eyed-embryo/limb bud (L: Lens, R: Retina, Pf: Pectoral fin, Ga: Gill arches, B: brain, Y: yolk). A y B: Masson's trichrome, 40X; C: H-E, 40X.

position, attached to the yolk. In the caudal region the vertebrae can be seen and the tail is flexed over the head. The epithelium of the body wall can be seen with a scaly appearance (Fig. 4C). The brain and eyes occupy a large part of the head (Fig. 4A). The cornea of the eye (Fig. 5) is best distinguished at this stage. It is mainly made up of a superficial layer of flat, elongated and scaly cells; a corneal stroma with keratocytes and a simple layer of endothelium, isolates the cornea from the aqueous humor (Fig. 5). Regarding the iris, only the pigmented epithelium is observed, which is derived from the pigmented epithelium of the retina (Fig. 5). At this stage, the

well-defined lens is also observed (Fig. 5), which is a spherical structure (Fig. 5), made up of collagen fibers on the inside that make up the lens, followed by a basement membrane and then a simple cuboidal epithelium, externally surrounded by the lens capsule, which is an acellular layer (Fig. 5B). The retina is made up of eight layers, the external limiting membrane can be seen in the analyzed sections, but not the internal one (Fig. 5B). The choroid (Fig. 5A) is located between the retinal pigment epithelium and the sclera. It is made up of blood vessels that occupy part of the posterior region of the eye. The sclera (Fig. 5A) is composed of

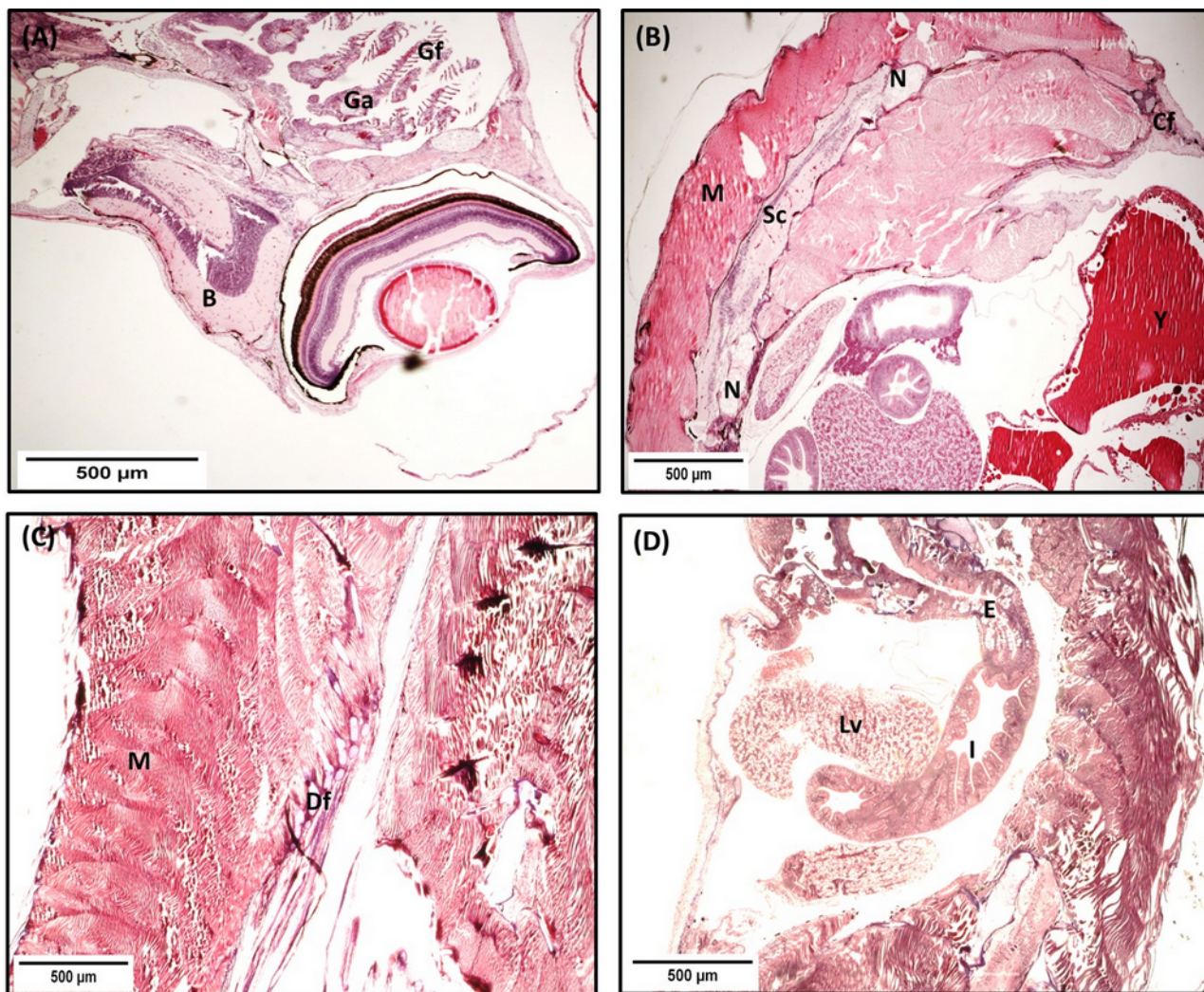


Figure 3. Photomicrograph of *Girardinus microdactylus* embryo at the middle-eyed embryo stage, (B: encephalon, Ga: gill arch, Gf: Gill filament, M: muscle, N: notochord, Sc: spinal cord, Y: yolk, Df: dorsal fin, E: esophagus, Lv: liver, I: intestine). H-E.

dense connective tissue, cartilage and a very thin lamina fusca that covers the cartilage in the anterior part of the eye, while in the posterior region it is surrounded by regular dense tissue.

Regarding the sixth stage: late-eyed embryo, the embryo of *Girardinus microdactylus* is completely formed, however, it still has yolk to be reabsorbed; this must occur in the seventh and eighth stages proposed by Haynes (1995), which were not observed in this study.

Discussion

In poecilids, the progressive processes of differentiation and growth of embryos occur within the ovarian follicle. In general, the post-fertilization stages in *Girardinus microdactylus* coincide in several aspects with other teleost species. This study follows the standardized classification proposed by

Haynes (1995) for the post-fertilization stages, which is still in force (Cabrera, 2007; Arcanjo *et al.*, 2014) with its own adaptations for each species.

The first post-fertilization stage proposed by Haynes (1995) consists of the blastodisc, which could not be observed in the analyzed sections. The embryonic development of *G. microdactylus* could be observed in this study from the second post-fertilization stage which coincides with the segmentation phase defined by Kimmel *et al.*, (1995). Organogenesis begins with the notochord, which is common in all vertebrates, at least in one period of life. This support structure is responsible for inducing the formation of the neural tube and the establishment of the anteroposterior axis. Most of it degenerates and persists only between vertebrae, forming intervertebral discs (Gilbert, 2000). In the first organ outlines, the digestive tube,

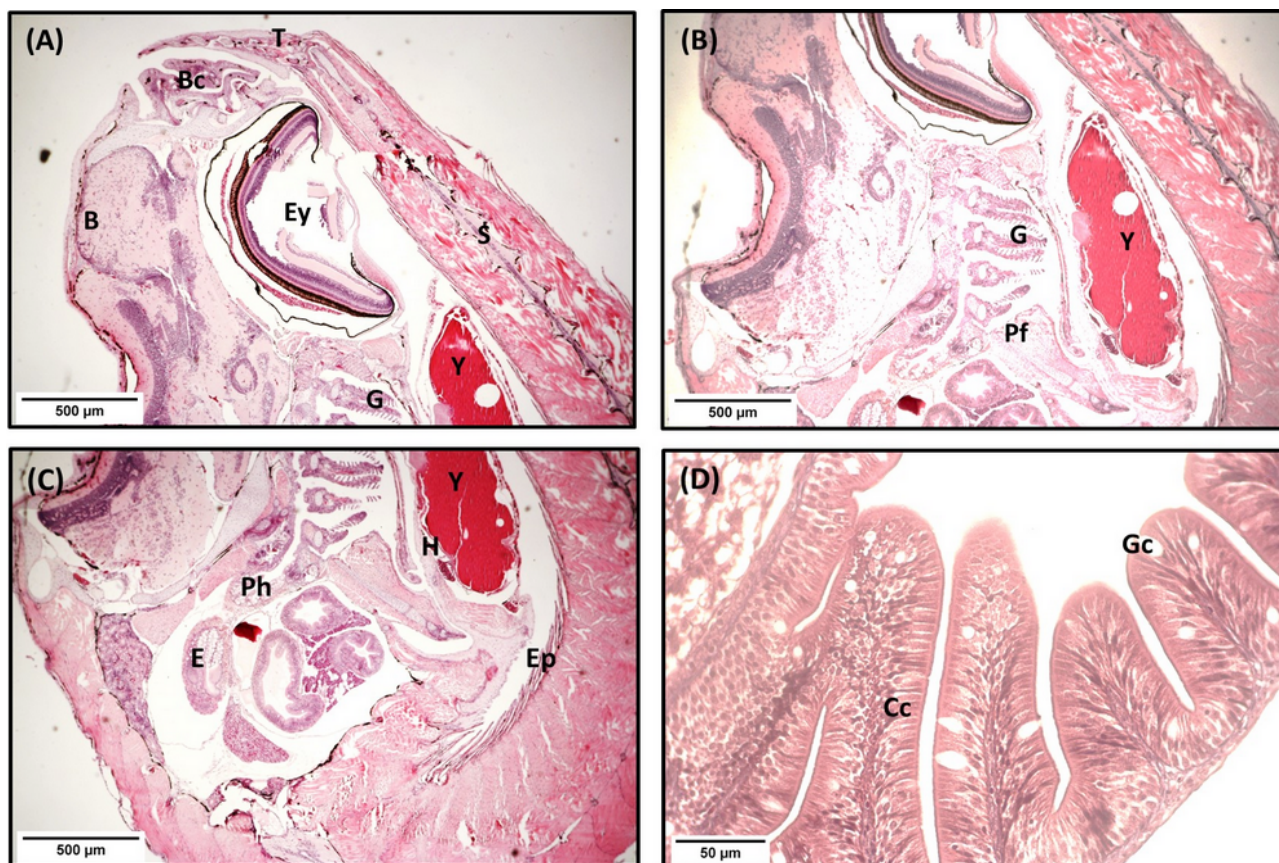


Figure 4. Photomicrograph of *Girardinus microdactylus* embryo at the late-eyed embryo stage. A anterior region, B and C intermediate region, D Enlargement of intestinal folds. (B: Encephalon, Bc: Buccal cavity, Ey: eye, T: tail, S: spine , G: gills, Pf: pectoral fin, Y: yolk, Ph: pharynx, E: esophagus , H: heart , Ep: Epithelium of body wall , Cc: columnar cells of intestinal mucosa, Gc: goblet cells) H-E.

cardiovascular system, muscles, integument and then the sensory system are formed with the formation of eyes, as in other poeciliids *Poecilia vivipara* (Arcanjo *et al.*, 2014). The liver also begins its development early, being involved in the reabsorption of yolk reserves (Cortés-Ortiz *et al.*, 2020).

The *G. microdactylus* embryo in the fourth stage has the head larger than the trunk. The gill arches are observed, which indicates that the respiratory system has already begun its development. Its eyes are enlarged and more developed than in the previous stage, which coincides with *Poecilia vivipara* (Arcanjo *et al.*, 2014), although *G. microdactylus* already has the retinal pigment epithelium.

In the fifth stage, *G. microdactylus* embryos have abundant yolk, as does *Gambusia puncticulata* (Cabrera, 2007). The vertebral column and fins are distinguishable as in *Poecilia reticulata* (Martyn *et al.*, 2006) and in *Poecilia vivipara* (Arcanjo *et al.*, 2014). There is a proportionality between the head and the rest of the body also noted in *Poeciliopsis*

infans (Silva-Santos, 2016). The eyes and brain show greater development. The oral cavity is not yet distinguished, probably because it is submerged in the yolk as in *Poecilia vivipara* (Arcanjo *et al.*, 2014).

By the end of gestation, the embryo is well developed with the dorsal and caudal fins, the latter flexed over the head. It has reduced yolk and well-developed internal structures are observed. This coincides with what was observed by Ponce de León (2012) in several species of Cuban poeciliids and in *Gambusia puncticulata* (Cabrera, 2007), *Poecilia reticulata*, (Martyn *et al.*, 2006), *Poeciliopsis infans* (Silva-Santos, 2016), *Poecilia vivipara* (Arcanjo *et al.*, 2014). The position of the heart coincides with what has been observed in other fish such as *Carassius auratus* (Tsai *et al.*, 2013) and *Fundulus heteroclitus* (Chernick *et al.*, 2021).

The digestive system of *Girardinus microdactylus* is duct-shaped with no external morphological differentiation, as found by Rodríguez-Rodríguez *et al.* (2004) for the sister species *G. metallicus*, which according to these

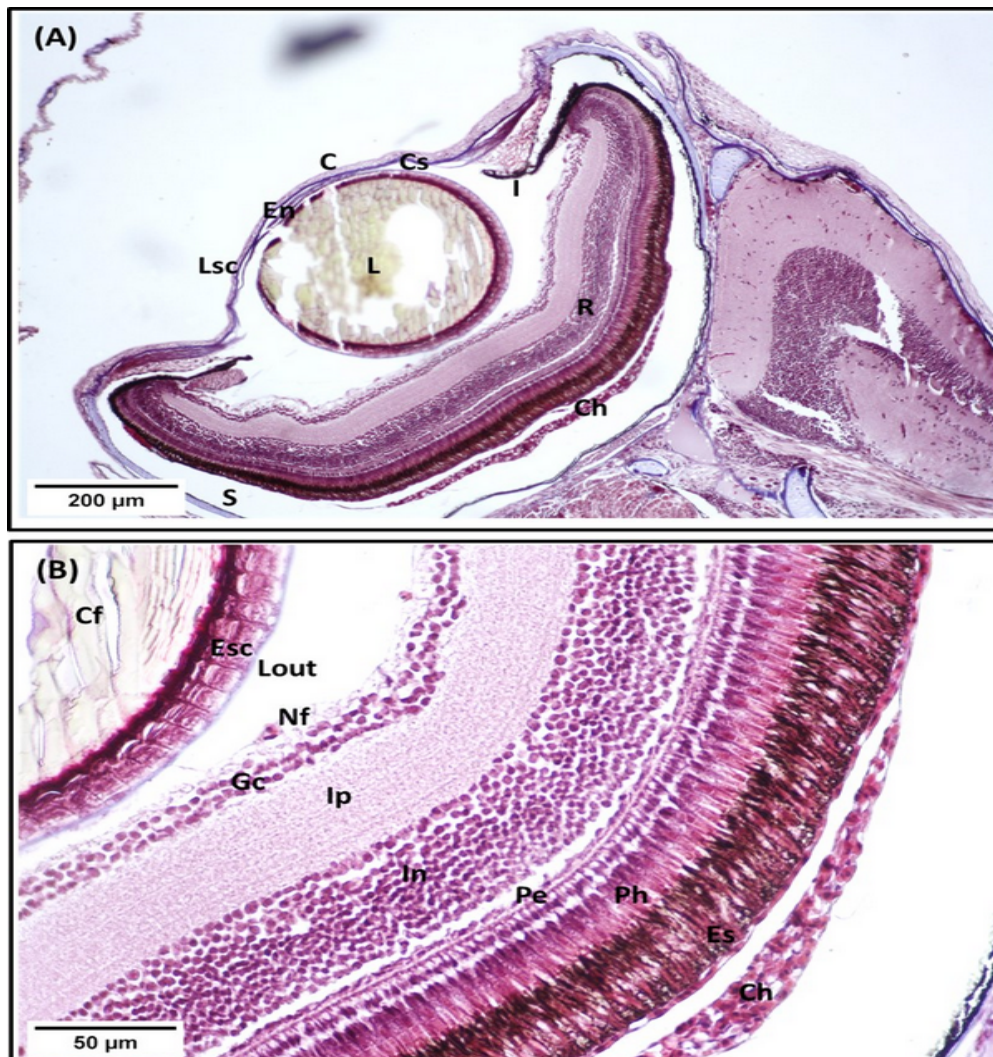


Figure 5. Photomicrograph of the eye of a *Girardinus microdactylus* embryo. A: Shown all the structures of the eye B: magnification of the retinal region. Retina (R), choroid (Ch), sclera (S), lens (L), iris (I), cornea (C), superficial layer of the cornea (Lsc), endothelium (En), corneal stroma (Cs), collagen fibers (Cf), simple cuboidal epithelium (Esc), outer layer of the lens (Lout), nerve fibers (Nf), ganglion cells (Gc), inner plexiform layer (Ip), inner nuclear layer (In), plexiform layer external (Pe), photoreceptor cells (Ph), external segments (Es). H-E.

authors lacks a stomach. In the first portion of the digestive tract of *G. microdactylus* embryos at an advanced stage of development, a stratified epithelium and abundant goblet cells are observed in the pharynx and esophagus, as in *Algansea lacustris* (Cortés-Ortiz *et al.*, 2020). These cells, besides protecting the mucosa, could be related to an agastric digestion. The intestine is elongated and thin and coiled in a spiral shape, as corresponds to omnivorous fish (Poey, 1858).

Regarding the formation of the eye, some authors agree that the retinal pigmentation is delayed in development (Martyn *et al.*, 2006; Silva-Santos, 2016; Arcanjo, *et al.*, 2014) but in species such as

Gambusia puncticulata (Cabrera, 2007) and *G. microdactylus* retinal pigmentation appears in the early stages. As evidenced in this study, the eyes are not one of the first organs that begin to form, but they are one of the first to complete their development. The retina is the innermost layer of the eye, responsible for registering and transducing light stimuli, converting them into nervous impulses that are interpreted as images in the brain. In turn, it is composed of eight layers and two membranes (Ali & Anctil, 1976). It can be subdivided into pigment epithelium, which is the outermost region or layer that adheres to the choroid, and the nervous retina, where the photoreceptors and neurons responsible

for carrying information to the optic nerve are located. The retinal pigment layer according to Arcanjo *et al.* (2014) appears in the sixth stage, however, in *G. microdactylus* embryos it is observed early in development. In the last stage of development of *G. microdactylus*, almost all layers of the retina can be easily observed as in *Paracheirodon axelrodi* (Tovar *et al.* 2009), however, in juveniles of *Ariopsis seemanni* only six layers are observed (Tovar *et al.*, 2013). The layer of nerve fibers coming from the ganglion cells that give rise to the optic nerve in *G. microdactylus* is not as thin as in *Ariopsis seemanni* (Tovar *et al.*, 2013). The retina in *G. microdactylus* is relatively thick compared to the size of the eye, having a wide layer of photoreceptor cells and long outer segments, the resolution power is favoured (Frohlich *et al.* 1995).

To compensate for the lack of refraction caused by the cornea in fish, the lens is generally circular (Gratzek & Matthews 1992), which coincides with what was observed in *G. microdactylus* in this study and in other species such as *P. axelrodi* (Tovar *et al.*, 2009), *Eremophilus mutisii* and *Oncorhynchus mykiss* (Tovar *et al.*, 2008) and *A. seemanni* (Tovar *et al.*, 2013). The lens is the only structure that fulfills optical functions in teleosts; it focuses light through accommodation mechanisms, which occur due to the movement of the lens through the pupil plane (Gratzek & Matthews, 1992). A greater ratio between the size of the lens and the size of the eye favors visual resolution, since more light is focused on the retina (Tovar *et al.*, 2009). Given the characteristics of the lens and retina of *G. microdactylus*, it can be inferred that it has a high level of visual resolution.

The choroid is the layer of blood vessels and connective tissue that supplies nutrients to the internal parts of the eye (Tovar *et al.*, 2009). Photoreceptors consume a large amount of oxygen and the greater the density and length of these cells, the greater the nutrient requirement in the retina (Tovar *et al.*, 2013). The vascularization of the choroid shows the needs for oxygen and nutrients in the retina and could be related to the variable thickness throughout this structure.

On the other hand, the sclera is a firm layer that surrounds the choroid, provides rigidity and gives the shape of the eye, and is a structure that is related to swimming speed in fish (Munz, 1971). The presence of cartilage in the sclera may help maintain the shape of the eye to balance the distortion produced during swimming (Nakamura & Yamaguchi, 1991)

Most teleost fish larvae have an undifferentiated visual system when they hatch and shortly afterwards it is composed of the lens and undifferentiated retina (Blaxter, 1975). However, in the case of *G. microdactylus* fry, at the time of birth they already have a complete and functional eye. What could be an adaptive advantage of viviparity over oviparity, since the fry at birth are capable of receiving visual stimuli that will allow them to search for food and flee from predators.

It is necessary to deepen the study of reproduction in Cuban species of poeciliids, and include in these investigations the male sex, which undoubtedly plays an essential role in this process. Knowledge of the reproductive characteristics will allow us to draw up accurate and well-founded strategies for managing the populations of these fish.

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Ethical statement

The authors declare that the specimens used in this study were handled and euthanized ethically. They were collected under the auspices of the Taxonomy project of some zoological groups of Cuba and the Caribbean, with specialized training, divulgation and environmental education activities. (Code: P-211-LH-005-038).

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