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Embryonic development and nourishment in the viviparous fish *Poecilia vivipara* (Cyprinodontiformes: Poeciliidae)

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Abstract

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While viviparity confers protection to the embryos during gestation, it increases energetic costs for the mother, which acquires new relations to its offspring. Maternal–fetal transfer of nutrients can occur in different patterns: as lecithotrophy (nourished by yolk) or matrotrophy (nourished by the mother). The development of *Poecilia vivipara* embryos was described macroscopically and microscopically, and the form of nutritional provisioning was identified. Embryonic development was divided into three prefertilization and seven postfertilization stages. The first organ to appear is the notochord, followed by the nervous, digestive and cardiovascular systems, and then by muscles and eyes. Embryonic nutritional provisioning was lecithotrophic, with yolk persisting until the last developmental stages and rich in proteins and polysaccharides. This kind of embryonic nutrition confirms the pattern found in the family Poeciliidae.

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Introduction

Viviparity is not an exclusivity of mammals. While it occurs in all vertebrate taxa, except in birds, its earliest evolutionary appearance was among fishes. In viviparous species, the mother and the embryos develop ontogenetic, morphological, trophic, and physiological relationships (Wourms 1981). Viviparity provides protection to the offspring during their most vulnerable life stage, as well as the possibility to transport them to localities with better conditions for development, such as favorable temperatures or absence of predators (Thibault and Schultz 1978). On the other hand, viviparity increases energetic costs for the female, leading to reduction in brood size (Wourms 1981). Reduction in brood size, however, is compensated by increased size and higher survival rates of descendants (Thibault and Schultz 1978).

While teleosts do not possess specific organs to gestation, they show great resourcefulness in incubating the embryos in the ovary itself (Turner 1940; McMillan 2007). Viviparous family Poeciliidae is one of the four groups of Cyprinodontiformes which developed internal fertilization, associated with the modification of the male anal fin, named gonopodium (Wourms 1981; Vazzoler 1996). This family has follicular gestation, which consists in the maintenance of the developing brood within the ovarian follicle until the young are expelled through the ovarian lumen only in the moment of birth, by effect of contraction of ventral muscles in the mother (McMillan 2007; Rocha *et al.* 2010). This type of gestation has been described in the families Clinidae, Poeciliidae, some species of Labrisomidae, some of Hemiramphidae and for genus *Anableps* (Anablepidae) (Schindler and Hamlett 1993).

Viviparous species present two basic forms of embryonic nutrition, matrotrophy and lecithotrophy, with intermediate forms occurring between them (Blackburn 1992). In the former, the embryo is nourished only by the yolk reserve, accumulated before fertilization, as occurs in oviparous animals. In this case, the ovarian follicle is almost a passive chamber without major modifications, merely hosting the developing embryos (Wourms 1981; McMillan 2007). Matrotrophy takes place when the yolk present in the egg is scarce and the mother transfers nutrients to the embryo after fertilization (Wourms 1981). This type of maternal–fetal nutrient transfer occurs in different, nonexclusive ways, from oophagy, in which nutritional support comes from

surrounding eggs, to placentotrophy, characterized by nutrient transfer via placenta (Blackburn *et al.* 1985).

A placenta is an apposition or fusion of embryonic and maternal organs which allows the exchange of substances between mother and offspring (Blackburn 1992; Pollux *et al.* 2009). Chorioallantoic placenta (the most common type in mammals) is considered the most specialized kind (Blackburn 1992; Pollux *et al.* 2009), but simpler kinds exist, such as the follicular placenta. In this case, the follicular epithelium modifies in such a way that increases its contact surface through formation of villi. In turn, the embryo places its pericardial sac and its yolk sac, both expanded and richly vascularized, next to the follicular epithelium, to exchange nutrients and waste (Wourms 1981; McMillan 2007). In eggs rich in yolk, the follicular epithelium merely becomes fibrous, vascular and intimately related to the enlarged pericardial sac of the embryo, as can be found in some poeciliid fishes (McMillan 2007). Apparently, the only mechanism of transplacental transport is micropinocytosis, and micropinocytic vesicles may be observed in every cell layer that nutrients must cross to reach the embryo (McMillan 2007).

The objectives of this work are: (i) to describe embryonic development, macro and microscopically, in the species *Poecilia vivipara* Bloch and Schneider, 1801 (Cyprinodontiformes: Poeciliidae); (ii) to determine the pattern of embryonic nutrition during gestation.

Materials and Methods

Fish were collected between December 2009 and March 2010, in Acaraú river (04°41′602″S/40°30′104″O and 04°24′072″S/40°33′725″O), in Ceará, northeastern Brazil. Capture was performed with active fishing instruments, like trawls, sieve nets, and hand nets, with meshes of approximately 1 mm.

The fish were fixed with 10% formaldehyde and brought to laboratory. After 24 h, they were washed in running water to remove excess of tissue fixative and then transferred to alcohol 70%. Total weight, gonadal weight, and standard length were measured for each animal, and sex was identified, when possible, by verifying the presence of the gonopodium, a male character. Thirty-three female specimens were dissected to extract gonads and embryos.

Classification into developmental stages

Twenty-one gonads and 160 embryos had their developmental stage classified under a stereomicroscope (Nikon SMZ 1500, images obtained with Nis-Elements F) using morphological characters (e.g., presence of eyes and fins), color, and presence of yolk, in accordance with Haynes (1995). However, the standardized developmental classification of Haynes (1995) was adapted, with the 10th and 11th stages of the original classification lumped into a single stage, due to difficult differentiation between then. The first three stages occur before fertilization, that is, they consist of growing oocytes in the process of maturation. Stages 4–10 were defined postfertilization. The number of embryos in each developmental stage in each female was noted. An attempt was made not to consider malformed or atretic embryos as proper developmental stages.

Histological analysis of gonads

Entire gonads and embryos, comprising all developmental stages found, were processed for histological analyses. The material was dehydrated in a series of increasing alcohol concentrations and embedded into histological resin Leica. Gonads and embryos were cut into 5-μm sections with an automatic microtome (Leica RM 2065, Leica, Nussloch, Germany). Histological sections were stained with Gomori trichrome (Tolosa *et al.* 2003), to obtain a global view of the embryonic components. Additionally, histochemical analyses with Bromophenol blue (Pearse 1985) and Alcian/Periodic acid-Schiff (Alcian/P.A.S.; Junqueira and Junqueira 1983) were performed to reveal proteins and polysaccharides, respectively. Slides were analyzed and photographed with a light microscope Olympus BX41. Histological analyses of embryos and gonads provided information used to determine the pattern of embryonic nutrition in *P. vivipara*.

Results

Standard length of mature specimens varied from 12.28 to 35.98 mm. The smallest male identified had 14.62 mm. The mature individual weights were from 0.06 to 1.13 g, and the lightest male had 0.11 g.

Eggs of the *P. vivipara* exhibited large amounts of stored yolk, present during the whole embryonic development. Oocytes undergoing early oogenesis occurred next to oocytes and embryos belonging to later developmental stages (Figs 1B and 4A), indicating that embryos develop inside the ovary, which constitutes follicular gestation.

Description of developmental stages is as follows. First stage: the oocyte is small, white and opaque (Fig. 1A). Microscopically, it is in intense vitellogenesis, as indicated by the presence of many yolk vesicles. Its membrane forms villi in contact with follicular cell layer, which surround the oocyte. The nucleus possesses nucleolus and a wrinkled nuclear envelope. Polysaccharides are observed mainly in the follicular epithelium and nucleus, and proteins occur all over the oocyte cytoplasm (Fig. 1B).

Second stage: the oocyte is dark yellow with oil droplets that may be observed by transparence (Fig. 1C). The process of vitellogenesis is inferred from the presence of vesicles, mainly in the cell periphery. Oocyte is surrounded by one layer of cuboidal follicular cells, forming the follicular epithelium. Theca of the follicle is located around the outer side of this epithelium. Between the oocyte and the follicular epithelium, there is the vitelline envelope, a translucent acellular layer. Yolk is composed of polysaccharides and pro-

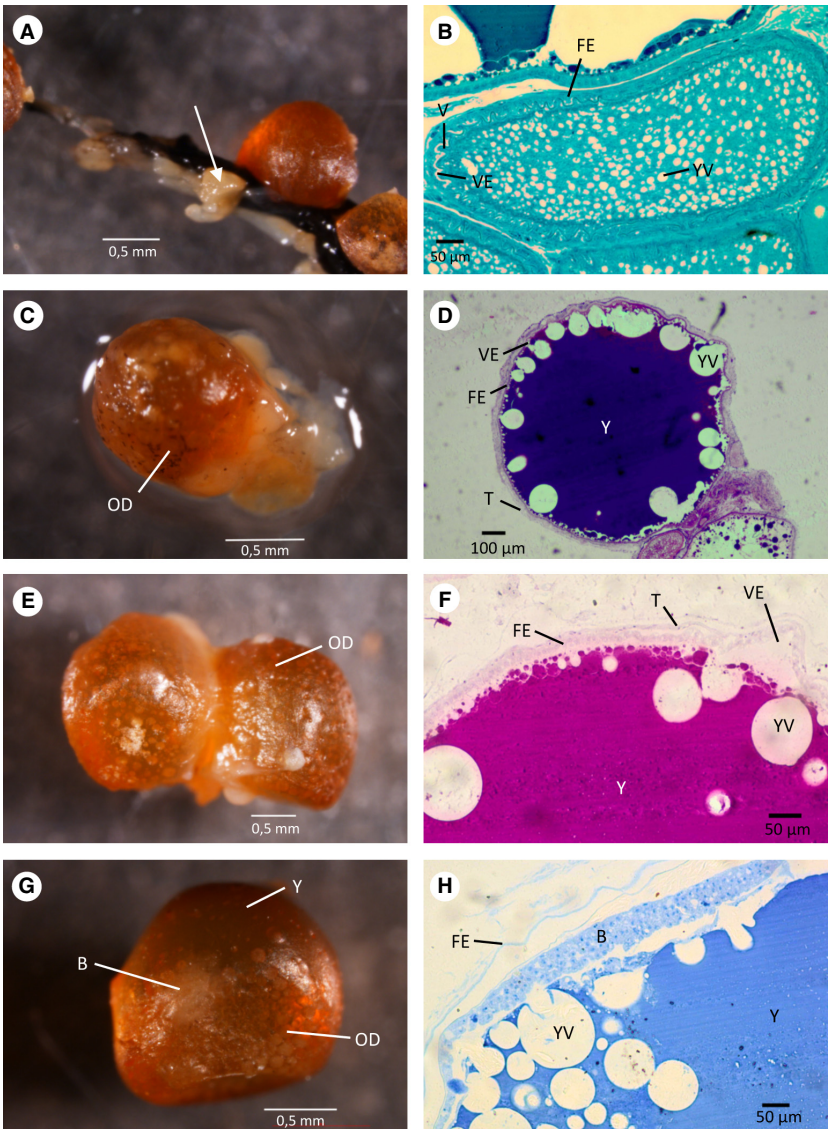

Fig. 1—Stages 1–4 of embryonic development in *Poecilia vivipara*. Oocyte at the 1st stage (arrow), white and immature (**A**) and its histological section showing advanced vitellogenesis (**B**). Macroscopic (**C**) and microscopic (**D**) view of oocyte at the 2nd stage, showing its surrounding layers. Stereoscope view of oocyte at the 3rd stage (**E**) and it observed under the light microscope (**F**). Macroscopic (**G**) and microscopic (**H**) view of embryo at the 4th stage visible as a blastodisc. Stains: B and H: Bromophenol blue; D: Gomori trichrome; F: Alcian/P. A. S. B, blastodisc; FE, follicular epithelium; OD, oil droplets; T, theca; V, villi; VE, vitelline envelope; Y, yolk; YV, yolk vesicle.

teins. Yolk vesicles and the vitelline envelope mainly stained magenta, indicating the presence of abundant polysaccharides and few proteins, which, in turn, suggests the occurrence of glycoproteins (Fig. 1D).

Third stage: the oocyte is in the final of vitellogenesis and is golden yellow, translucent and full of oil droplets equally dispersed. The oocyte becomes mature and ready to fertilization (Fig. 1E). Vesicles storing yolk are no longer restricted to the cell periphery and occur also in the center of the oocyte. This indicates intraoocytic synthesis of yolk. The follicular epithelium becomes columnar, indicating intense production and secretion of nutritional reserves for the oocyte. Besides, this epithelium is more distant from the germinative cell than in previous stages. The villi are retracted, and the vitelline envelope is thicker, characterizing the ovulation process. Theca is barely perceptible. Polysaccharides and proteins are still present in yolk vesicles, but in smaller quantity than in former stages. In turn, the cytoplasm is still full of both substances (Fig. 1F).

Fourth stage: the oocyte has undergone fertilization. The blastodisc is already observed as a white spot on the yolk surface. Oil droplets take place below the embryo, unevenly dispersed (Fig. 1G). The embryo possesses binucleate cells, indicating high mitotic activity and intense clevage. Polysaccharides and proteins from the yolk vesicles are being released into the egg envelope (former vitelline envelope), which is now thicker, and are being consumed by the embryo (Fig. 1H). The follicular epithelium is squamous and more distant from the egg.

Fifth stage: the embryo is visible as a thin white line in the egg periphery. Under stereomicroscope, reduction in quantity of oil droplets is noted (Fig. 2A). In greater magnification, the embryo is elongate and situated in the egg pole. In tail region, mesodermal cells are organized in groups forming somites.

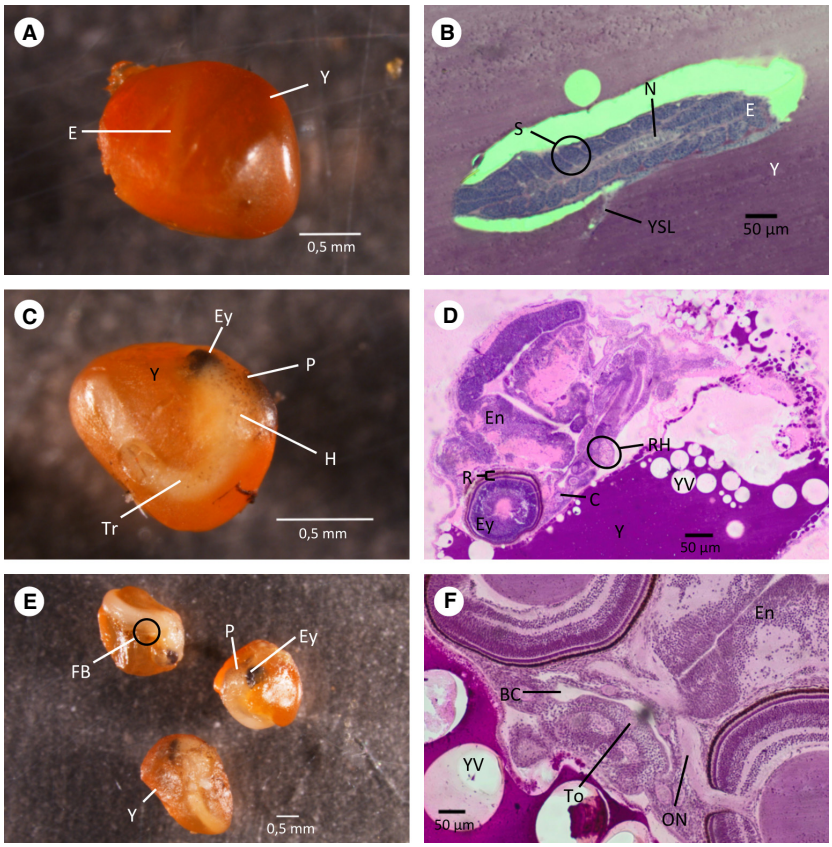

Fig. 2—Stages 5–7 of embryonic development in *Poecilia vivipara*. Embryo at the 5th stage in macroscopic (**A**) and microscopic view (**B**). Macroscopic (**C**) and microscopic (**D**) view of embryo at the 6th stage, evidencing head region with the early development of some organs. Embryos at the 7th stage observed macroscopically (**E**) and microscopically, highlighting yolk vesicles (**F**). Stains: B and D: Gomori trichrome; F: Alcian/P. A. S. BC, buccal cavity; C, cartilage; E, embryo; En, encephalon; Ey, eye; FB, fin bud; H, head; N, notochord; ON, optic nerve; P, pigmentation; R, retina; RH, rudimentary heart; S, somites; To, tongue; Tr, trunk; Y, yolk; YSL, yolk syncytial layer; YV, yolk vesicle.

Embryo is connected to yolk by the yolk syncytial layer, also called periblast. Notochord formation begins in the median portion of the embryo (Fig. 2B). Yolk remains full of polysaccharides and proteins. Follicular epithelium was not observed in sections analyzed, and egg envelope could not be delimitated.

Sixth stage: the embryo has head and trunk surrounding the yolk. Its head is large compared with the trunk and seems to be submerged into yolk, although there is the yolk syncytial layer continuous between the embryonic cells and the yolk. The optic globe is present but not complete. Body pigmentation appears, mostly on top of the head (Fig. 2C). Under the microscope, the embryo has encephalon, cartilage, rudimentary heart, and eyes, without lens and with retina layers in the process of differentiation. The notochord is elongated until tail. Myoblasts, grouped into myomeres (precursors of muscles), are present in the tail portion. Also in the tail portion, early neural tube and early intestine are visible. The whole body is covered by simple, undifferentiated epithelium. Bromophenol blue reveals ossification around the neural tube, indicating the formation of vertebrae. Yolk syncytial layer is visible (Fig. 2D). Histological analyses show intense yolk consumption, evidenced by the formation of vesicles below the embryo. Those yolk vesicles remain rich in polysaccharides and proteins.

Seventh stage: the embryo still has head larger than trunk and has eyes enlarged and better developed, but not complete. Caudal and pectoral fins buds are observed. Pigmentation is pronounced in the head (Fig. 2E). When observed under the microscope, the embryo has eyes with lens and some retina layers, but lacking retinal pigment layer. The optic nerve connects the internal portion of the eyes to the encephalon, forming the optic chiasm. The buccal cavity, tongue, and cartilage of gills can be noted. The notochord and neural tube remain evident, and the covering epithelium is still undifferentiated and simple. The yolk is shapeless, surrounded by yolk syncytial layer and under intense consumption, as indicated by the formation of vesicles containing polysaccharides and proteins (Fig. 2F).

Eighth stage: the embryo circumscribes the yolk mass, which has granular aspect. Body pigmentation reaches tail. The eyes are more developed. The embryo is more robust, with larger trunk and developed fins. Although its snout is still seems to be submerged into yolk, its eyes are already free (Fig. 3A). Microscopically, the embryo has well-developed eyes with retina composed of several layers, but lacking retinal pigment layer. The encephalon, gills, pharynx, intestine, and liver are visible. The tail portion has vertebrae and notochord surrounded by the myomeres (Fig. 3B). The periblast, reduced and with many vesicles, surrounds the yolk. The yolk

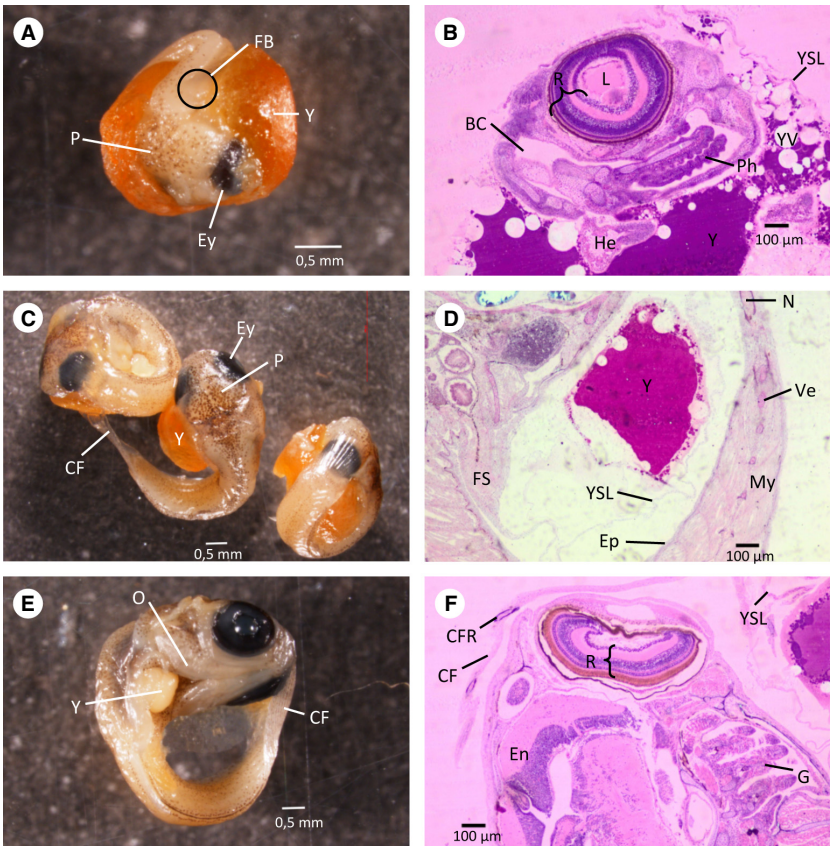

Fig. 3—Stages 8–10 of embryonic development in *Poecilia vivipara*. Stereoscope view of embryo at the 8th stage (**A**) and its histological section showing the head structures (**B**). Embryos at the 9th stage of development observed macroscopically (**C**) and microscopically (**D**). Macroscopic (**E**) and microscopic (**F**) view of an embryo at the 10th stage, showing its main organs. Stains: B and F: Gomori trichrome; D: Alcian/P. A. S. BC, buccal cavity; CF, caudal fin; CFR, caudal-fin rays; En, encephalon; Ep, epithelium; Ey, eye; FB, fin bud; FS, fusiform structure; G, gills; He, heart; L, lens; My, myomere; N, notochord; O, operculum; P, pigmentation; Ph, pharynx; R, retina; Ve, vertebra; Y, yolk; YSL, yolk syncytial layer; YV, yolk vesicle.

is still rich in polysaccharides, and proteins that are being transferred to the embryo through the yolk vesicles.

Nineth stage: the embryo is well developed with whole body pigmented. It has caudal and dorsal fins, and the caudal fin is flexed over the head. The yolk is very reduced and shapeless, connected to the median portion of embryo (Fig. 3C). Microscopically, the encephalon and notochord vestiges are observed. The embryo has already formed gills, heart, pharynx, intestine, liver, pancreas, kidney, and urinary bladder. Fins have ossified rays, and the epithelium covering the whole embryo becomes squamous, but without scales. In the tail portion, the spinal cord and vertebrae are observed among the myomeres. The retinal pigment layer is present (Fig. 3D). Reduced yolk mass is connected to the embryo by periblast, which ends in a fusiform structure composed by embryonic tissue (epithelial and muscle cells) fused to tail region. Yolk continues to secrete its nutritional reserves by vesicles.

Tenth stage: the tail is still flexed over the head. The embryo has all fins, operculum, intense pigmentation, and yolk almost totally absorbed (Fig. 3E). Under the microscope, the encephalon and the eyes are complete and occupy two-thirds of head. Vestiges of the notochord and spinal cord are present in the tail portion, encircled by vertebrae. Intestine, liver, kidney, gill, well-defined myomeres, and fins with rays are present. The covering squamous epithelium now has scales. The yolk mass is small, irregular and surrounded by the yolk syncytial layer. Polysaccharides and proteins are in smaller vesicles, in final absorption (Fig. 3F).

Description of *P. vivipara* embryonic development provided information about its pattern of embryonic nutrition. Yolk persists until the last stages of development connected to the embryo by the yolk syncytial layer. Moreover, no evidence of matrotrophy was found, such as connections between the embryo or the egg envelope and the ovarian wall or other maternal structures (Fig. 4A,B).

Apposition or fusion of embryonic and maternal organs, which characterize a placenta, were not observed. On the contrary, the distance between the embryo and the follicular epithelium increases during development. Vascularization and villi formation in follicular cells were not observed either. The evidence leads us to conclude that *P. vivipara* is lecithotrophic.

Embryos in different stages within the same ovary occurred in only 11 cases of 33, but in those cases, embryos were always in consecutive stages. Two females had embryos in well-distinct stages. In the first case, one oocyte in 3rd stage, two embryos in 6th and one in 7th. In other case, seven oocytes in 2nd stage, six in 3rd, and five in 9th. In both cases, one brood was nonfertilized (2nd and 3rd stages).

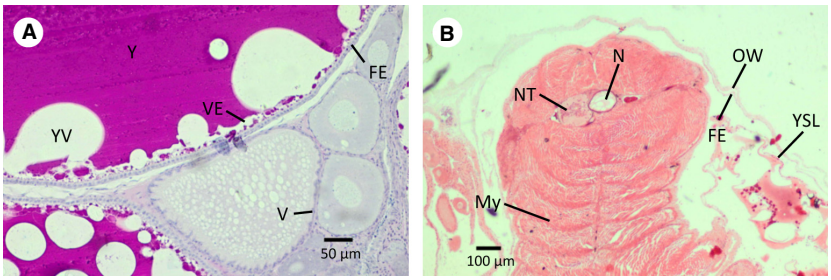

Fig. 4—Evidences of lecithotrophy in *Poecilia vivipara*. Detail of follicular epithelium presenting villi in oocytes in early development and follicular epithelium flat in mature oocytes, indicating that nutrient exchange between embryo and mother diminishes while development progresses (**A**). Detail of yolk syncytial layer in 10th stage with no connection with the mother's ovarian wall (**B**). Stains: A: Alcian/P. A. S.; B: Gomori trichrome. FE, follicular epithelium; My, myomere; N, notochord; NT, neural tube; OW, ovarian wall; V, villi; VE, vitelline envelope; Y, yolk; YSL, yolk syncytial layer; YV, yolk vesicle.

Discussion

The finding of follicular gestation in *P. vivipara*, evidenced by the presence of oocytes near the embryos studied, confirms the pattern observed in the family Poeciliidae (Schindler and Hamlett 1993). Modification of follicular epithelium from cuboidal to columnar, at the 3rd stage, agrees with McMillan's (2007) description of oocyte maturation. Oil droplets were observed macro- and microscopically along the entire embryonic development. Oil droplets are a common type of yolky inclusion in most species of fishes (McMillan 2007; Bone and Moore 2008) whether marine (Unuma *et al.* 2011; Domínguez-Petit *et al.* 2013) or freshwater (Henderson and Tocher 1987; Lahnsteiner 2000). Quantity and size of droplets are distinctive for different fish families (Oliveira-Almeida 2011). The droplets observed in the present study did not have their composition confirmed by histochemical tests, but their lipidic nature can be suggested from the negative reactivity to stains used.

Yolk vesicles grouped below the embryo since the 4th stage indicates the beginning of the consumption of nutrients accumulated during vitellogenesis. Fragmentation of yolk, as was observed in this study, facilitates its uptake by embryonic cells (Ninhaus-Silveira *et al.* 2006). The yolk syncytial layer, or periblast, is formed from the incomplete division of cytoplasm from blastodisc cells, resulting in a multinucleated mass of cytoplasm (Oliveira-Almeida 2011). Eggs of *P. vivipara* are telolecithal and, probably due to its large amount of yolk, cell division is incomplete, as occur in oviparous species studied by Oliveira-Almeida (2011).

The periblast is an extraembryonic structure, placed between embryo and yolk, and during the course of development, it spreads covering the whole blastoderm (Balinsky 1975; Kimmel *et al.* 1995). Periblast is exclusive of teleosts and has great importance in embryonic development, as noted in oviparous fishes (Kimmel *et al.* 1995; Ninhaus-Silveira *et al.* 2007). It is believed that the yolk syncytial layer plays a significant role on degrading and digesting the yolk and making it available for embryo (Lentz and Trinkaus 1967; Balinsky 1975). The periblast has high metabolic activity and presents many ribosomes, mitochondria, vacuoles, and endomembranes and also helps in the epiboly movement (Ninhaus-Silveira *et al.* 2007).

Ninhaus-Silveira *et al.* (2007), studying the embryonic development of *Prochilodus lineatus*, found that the blastoderm covered the whole vegetal pole. That did not occur in *P. vivipara*. Because of its large yolk sac, the embryo starts organogenesis before the epiboly movement is finished. In that way, blastoderm does not cover the whole yolk (Morrison *et al.* 2001). Somites, which give origin to myomeres, originate from mesoderm segmentation and are located in the dorsal portion of the embryo (Nakatani *et al.* 2001; Oliveira-Almeida 2011).

The notochord, observed as the 5th stage, is the first supporting organ, responsible for inducing the formation of the neural tube and establishing the antero–posterior axis (Gilbert 1994). It is present in all chordates at least in one period of life. Its major part degenerates and it persists only between vertebrae, forming intervertebral disks (Gilbert 1994). The early appearance of the notochord shows that the structures to support the body are one of the first to arise in *P. vivipara*, followed by the nervous system. This sequence is common for all vertebrates, including mammals (Gilbert 1994). Formation of the digestive tube (with intestine), cardiovascular system (with heart), muscles, tegument, and sensorial system (with the formation of eyes) then follows in that sequence. According to Hu *et al.* (2000), the heart is the first permanent organ that develops and becomes functional.

At the 7th stage, the respiratory system becomes evident with the appearance of branchial arches and the gills. At the same time, the ossification process becomes apparent. The digestive tube is almost complete by the 8th stage, when the pharynx and the liver develop. Encephalon and eyes are well developed in this stage. Excretory system arises only at the 9th stage, represented by the kidney. In fishes, kidney has its major function in hematopoiesis (Genten *et al.* 2009). Also at the 9th stage, the digestive tube is complete with diffuse pancreas (Genten *et al.* 2009). At the 10th stage, all the sys-

tems of the embryo are complete, and further development consists of consuming the yolk remains or detaching from it.

Evidence of matrotrophy would consist of marked presence of intimate interdigitation of maternal and fetal tissues, whereas no evident exchange of substances would point to lecithotrophy. In this study, the form of embryonic nutrition found is in agreement with the pattern of lecithotrophy or primitive matrotrophy (not specialized) in the family Poeciliidae (Wourms 1981). Description obtained is similar to that of Haynes (1995) for lecithotrophic poeciliids, corroborating our result. According with Thibault and Schultz (1978), lecithotrophy is useful in unstable environments with variation in nutritional supply, because the female can accumulate nutrients in periods of high food availability and store them in oocytes to provide all food necessary for the embryos until parturition. In matrotrophy, because nutrients are supplied gradually to the embryo, declines in food availability to the mother may lead to the abortion of the whole brood. Thus, lecithotrophy in *P. vivipara* may be related to the environmental conditions in which it lives, in a semiarid region, with intermittent rivers and where food supply is variable.

The occurrence of embryos in different developmental stages, inside the same female, is a phenomenon called superfetation. However, the occurrence of embryos in consecutive stages of development in this study was not considered as superfetation. Martyn *et al.* (2006) found embryos in three distinct, but consecutive stages. This difference of stages was attributed to asynchronic fertilization, as the process of fertilization may last for up to 6 days in *Poecilia reticulata* (Thibault and Schultz 1978). In the two cases of embryos in well-distinct stages of development, one brood was nonfertilized, which suggests that these oocytes were ready for fertilization but that it could only occur after parturition of the previous brood. Scrimshaw (1944) says that all species of Poeciliidae have potential for superfetation, but it only occurs in particular conditions, such as constant light and food abundance, being a plastic characteristic of species.

In conclusion, embryonic development in *P. vivipara* is in accordance with the pattern found for others species from the same family, Poeciliidae. The embryo starts its development in the 4th stage, with formation of the blastodisc, followed by the establishment of the antero–posterior axis with the notochord, and then by organogenesis. Embryos only leave the ovary when they are completely formed. Through embryonic development description, with observation of persistent yolk and its attachment to the embryo until the last stage, as well as an apparent lack of important connections between mother and offspring, it is concluded that *P. vivipara* is lecithotrophic.

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