

Comparative Studies of the Digestive Tract Development Between *Rhinoderma darwinii* and *R. rufum*

BORIS JORQUERA, ORLANDO GARRIDO, AND EMILIO PUGIN

Instituto de Embriología, Facultad de Ciencias, Universidad Austral de Chile,
Casilla 567, Valdivia, Chile

ABSTRACT.— A comparative morphological study of the digestive tract during larval development between two species of the genus *Rhinoderma* is presented. The digestive tract of *R. rufum* shows the structure typical of species with free living larvae; a large intestinal mass and early differentiation of the duodenal epithelium. On the other hand, *R. darwinii* with nonaquatic development, lacks the morphology associated with exogenous feeding and the histogenesis of the duodenal epithelium reaches its maximum degree of differentiation only in the last stages of the metamorphosis. For *R. darwinii* a mixed trophic mechanism is suggested involving yolk dependence and a paternal larval relation. This proposition is supported by previous findings which indicate the existence of absorption across the larval skin and inability of the larvae to survive in Ringer's amphibian solution. There is some evidence suggesting that a similar mechanism could operate in *R. rufum*.

The frogs of the genus *Rhinoderma* present an uncommon characteristic of development. After the male has fertilized the oocytes, he remains in proximity to the eggs and takes the embryos up into the vocal sac (Burger, 1904; Wilhelm, 1927, 1932; Cei, 1962). Two species have been identified in the genus: *R. darwinii* Duméril and Bibron (1841) and *R. rufum* (Philippi) Formas et al. (1975).

R. darwinii has direct development without free living larvae. The embryonic period (prehatching) lasts 23 days. Of these, the first 20 occur externally. Once muscular activity begins the embryos are taken into the male's vocal sac where they hatch and remain until the end of metamorphosis. Larval development lasts 52 days. In this species, eggs are large (4 mm), the larvae lack external gills, spiraculum tube, beak and cornified teeth; the caudal fin is poorly developed; and interdigital membrane and digital tubercles are absent (Jorquera et al., 1972).

In *R. rufum* muscular activity occurs after 8 days and the embryos are taken into the vocal sac at this time. The larvae remain in the vocal sac until stage 3 of larval development when horny

jaws are differentiated (Jorquera et al., 1974). At this point the larvae are expelled by the male to complete larval development in an aquatic environment. The duration of this last period is not known. Embryonic and larval characteristics differ from those of *R. darwinii*. The eggs are small (2.4 mm); the larvae have external gills, spiraculum tube, beak and cornified teeth; interdigital membrane and digital tubercles are present on the hind legs (Jorquera et al., 1974).

In summary *R. darwinii* has direct development and paternal care during larval growth, while *R. rufum* presents a form of development, which could be called intermediate, with paternal care during the beginning of the larval stage, and a final free living larval period in an aquatic environment. In the first case, as in most species with direct development, the main source of nutriment during embryonic and larval development is the yolk.

According to our previous investigations the ultrastructure of the vocal sac epithelium of both species of *Rhinoderma* reveals the presence of four cellular types: superficial cells, basal cells,

mucous cells, and ciliated cells (Garrido et al., 1975; Jorquera et al., in press). The superficial cells present different morphological characteristics depending on the presence or absence of larvae in the sac. When larvae are present the superficial cells contain numerous secretory granules which are apparently secreted by a merocrine type of secretion. Near the capillaries, the epithelium has a laminar disposition. On the other hand the ultrastructure of the larval skin reveals a thin epithelium with surface epidermal cells and basal cells. The surface cells contain numerous dense intracytoplasmatic vacuoles some of which communicate with the cellular surface with signs of exo- or endocytosis and some communicate with the endoplasmic reticulum (Garrido et al., 1975). Large vacuoles with dense contents are also present between the surface cells and the basal cells. In spite of the different location of these vacuoles they all have similar ultrastructure features. Extracytoplasmatic vacuoles are present in the larval skin of *R. darwinii* during almost all of the stages of larval development, however in *R. rufum* they are present only until stage 5 (Jorquera et al., in press).

In both species the ultrastructural characteristics of the vocal sac epithelium and the larval skin epithelium, suggest that they may have a functional interrelationship. We postulate that a nutrient substance is synthesized by the sac's superficial cells, released, and then incorporated into the larvae by trans-epithelial absorption. Besides, at least in *R. darwinii*, it is also possible that paternal-larval exchanges may occur by digestive absorption similar to the viviparous anuran *Nectophrynoides occidentalis* in which differentiation of the gut tube begins early in embryonic life (Vilter and Lugano, 1959; Xavier, 1971, 1973, 1977).

The present study reports on the anatomical development of the digestive tract as well as the ultrastructure of the gastric and duodenal mucosa of *R. dar-*

winii and *R. rufum*. The results are discussed taking into consideration the capacity of digestive absorption and yolk dependence during larval development in both species. In addition the role of the vocal sac in the nourishment of the larvae is also considered.

MATERIAL AND METHODS

R. darwinii and *R. rufum* larvae were classified from stages 1-15, according to tables described by Jorquera et al. (1972, 1974).

The gross anatomical characteristics of the digestive tract were studied in 150 dissected specimens. The digestive tracts were illustrated with scale drawings which allowed recognition of the different stages of each species, and a comparative analysis between species at the same stage.

Once the different segments of the digestive tract were identified, samples from the duodenal and cardial regions of the stomach were prepared for electron microscopy. Fragments from the gastric and duodenal walls were fixed in Karnovsky's fluid and post-fixed in an isotonic 1% solution of osmium tetroxide. Samples were then dehydrated in an ethanol-acetone mixture and were embedded in pure araldite (Glauert and Glauert, 1958).

The ultrastructure study was done using 800 to 1000 Å sections mounted on uncovered copper grids. These sections were stained with uranyl-acetate and lead citrate (Glauert, 1965), and observed in a Philips 300 transmission electron microscope.

RESULTS

Rhinoderma darwinii

Anatomical Development of the Digestive Tract.—In stages 1 to 5 of larval development the gastroduodenal loop of the foregut surrounding the pancreas appeared, and the double spiral of the midgut gradually developed (Fig. 1). This latter arrangement underwent only few modifications up to stages 9 and 10

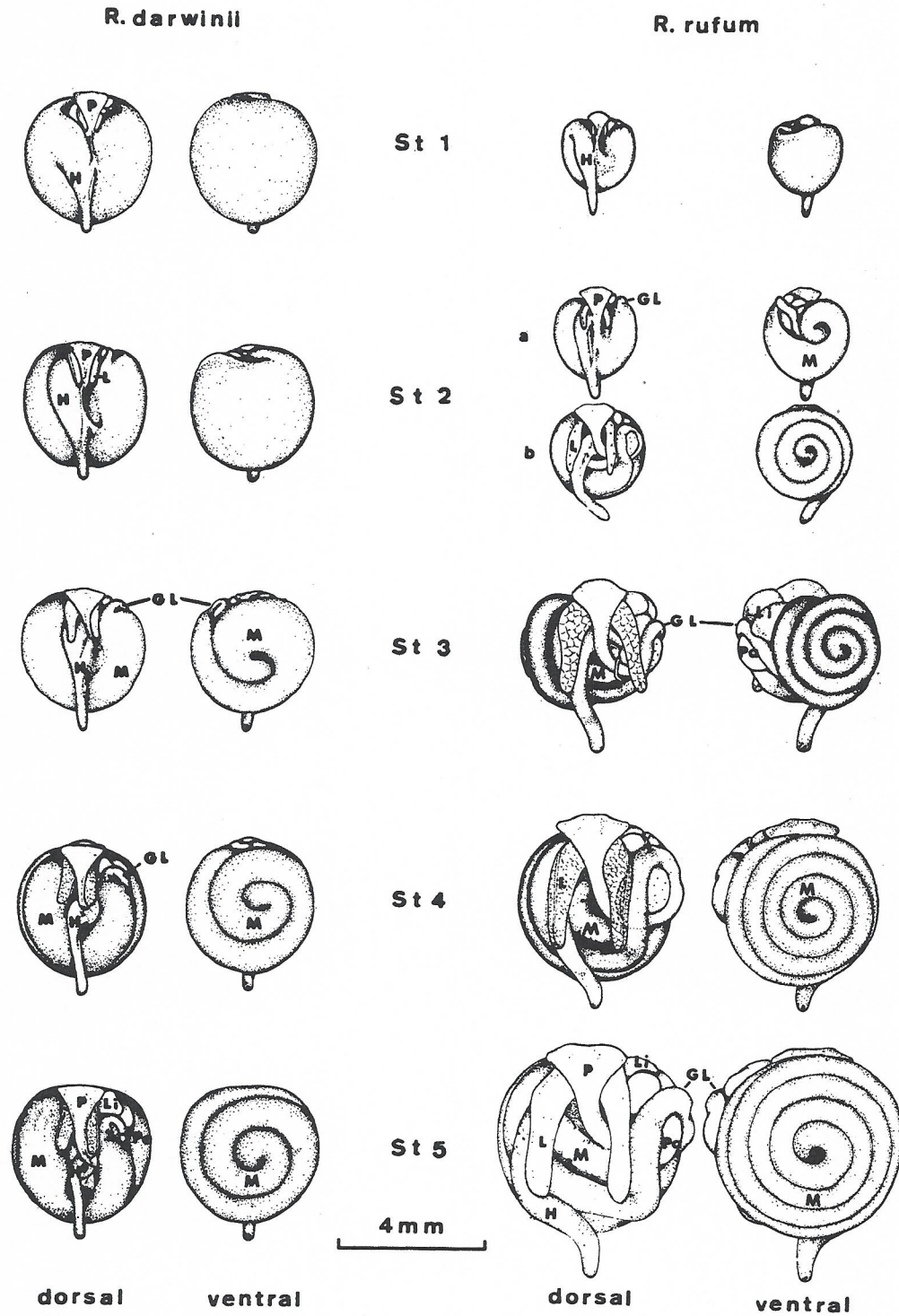


FIG. 1. Anatomy of the digestive tract. Stages 1 to 5 of larval development, in dorsal and ventral view. D, duodenum; GL, gastroduodenal loop; H, hindgut; M, midgut; Li, liver; L, lung; Pa, pancreas and P, pharynx.

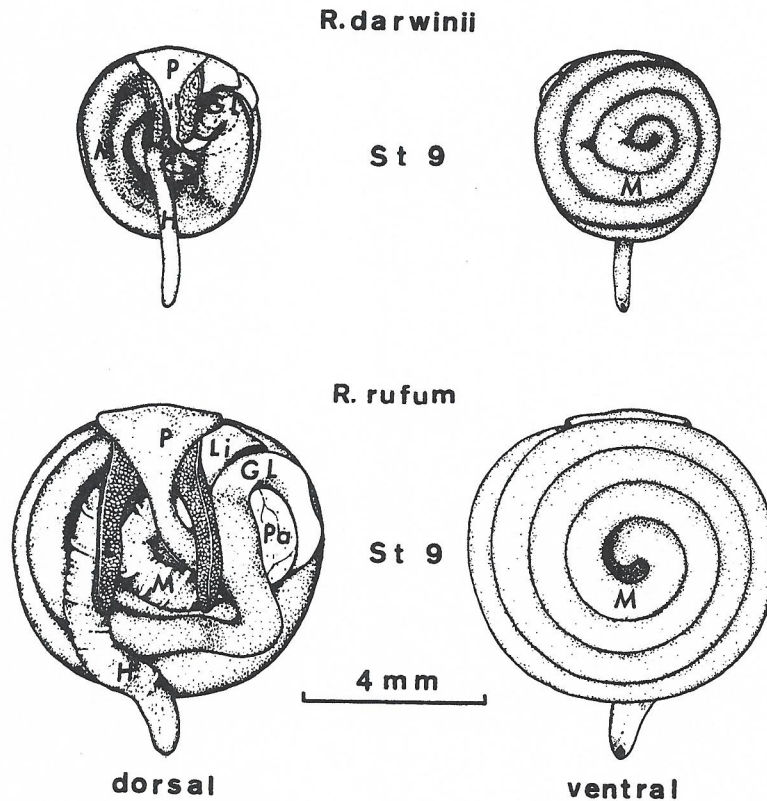


FIG. 2. Anatomy of the digestive tract. Stage 9 of larval development, in dorsal and ventral view.

when the midgut reached its maximum length, completing a double loop of $3\frac{1}{2}$ bends (Fig. 2). Starting from stage 11, the spiral of the midgut began to decrease becoming a simple one with a ventral-left position in stage 12/13. At this point, gastric distention was visible. In stage 13, the reduction of the midgut's spiral ended, becoming a thick transversal loop with an anterior concavity whose left end turned around (in a dorsal-caudal sense) to continue into the rectum. During the terminal stages (14 and 15), the stomach underwent a remarkable growth and prolapsed on the left side. At the same time, the midgut changed in position resulting in a small enclosed loop on the right side (Fig. 3).

During stages 1 to 12 the volume of

the intestinal mass apparently was the same. However, it decreased rapidly during stages 13 to 15.

Histogenesis of the Digestive Tract.—Up to stage 3 of larval development, the duodenal lumen still did not appear and large cells full of cytoplasmic inclusions were found. Among them, the most noticeable were yolk granules of various sizes and large lipidic vacuoles with a homogeneous content. In stage 2, star-shaped nuclei were generally found toward the base of the cell. In stage 3, the nuclei were ovoid and had a basal position (Fig. 4). The duodenal lumen appeared in stage 4. In stage 9 the lumen was well defined and limited by large cells with lipidic and yolk inclusions. Mitochondria and microvilli began to appear. In stages 11–12, the cells

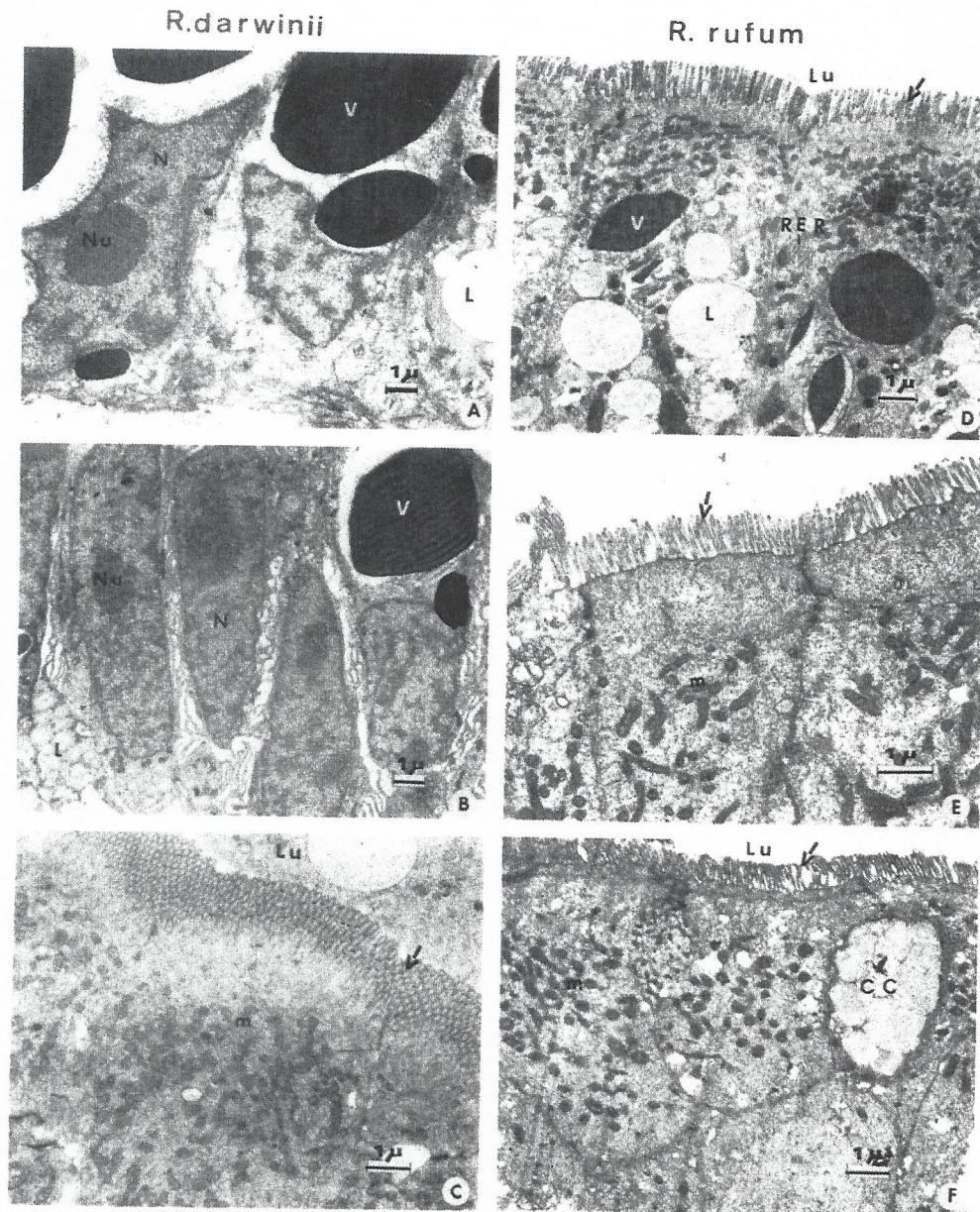


FIG. 4. Histogenesis of the duodenum in different stages of larval development. Illustrations A (stage 2), B (stage 3) and C (stage 15) are *R. darwinii*. Illustrations D (stage 2), E (stage 3) and F (stage 15) are *R. rufum*. CC, caliciform cell; L, lipidic vacuoles; Lu, lumen; m, mitochondria; N, nucleus; Nu, nucleolus; RER, rough endoplasmic reticulum; short arrow, microvilli and V, vitellus.

were cylindrical with ovoid basal nuclei. Microvilli and the organelles were well developed. Yolk inclusions were small and scarce. In stages 13-15 differentiation of the epithelium was com-

pleted, the cells were small, presented no cytoplasmic inclusions and abundant organelles. Mitochondria were numerous. The apical surface of the cells projected numerous microvilli (Fig. 4).

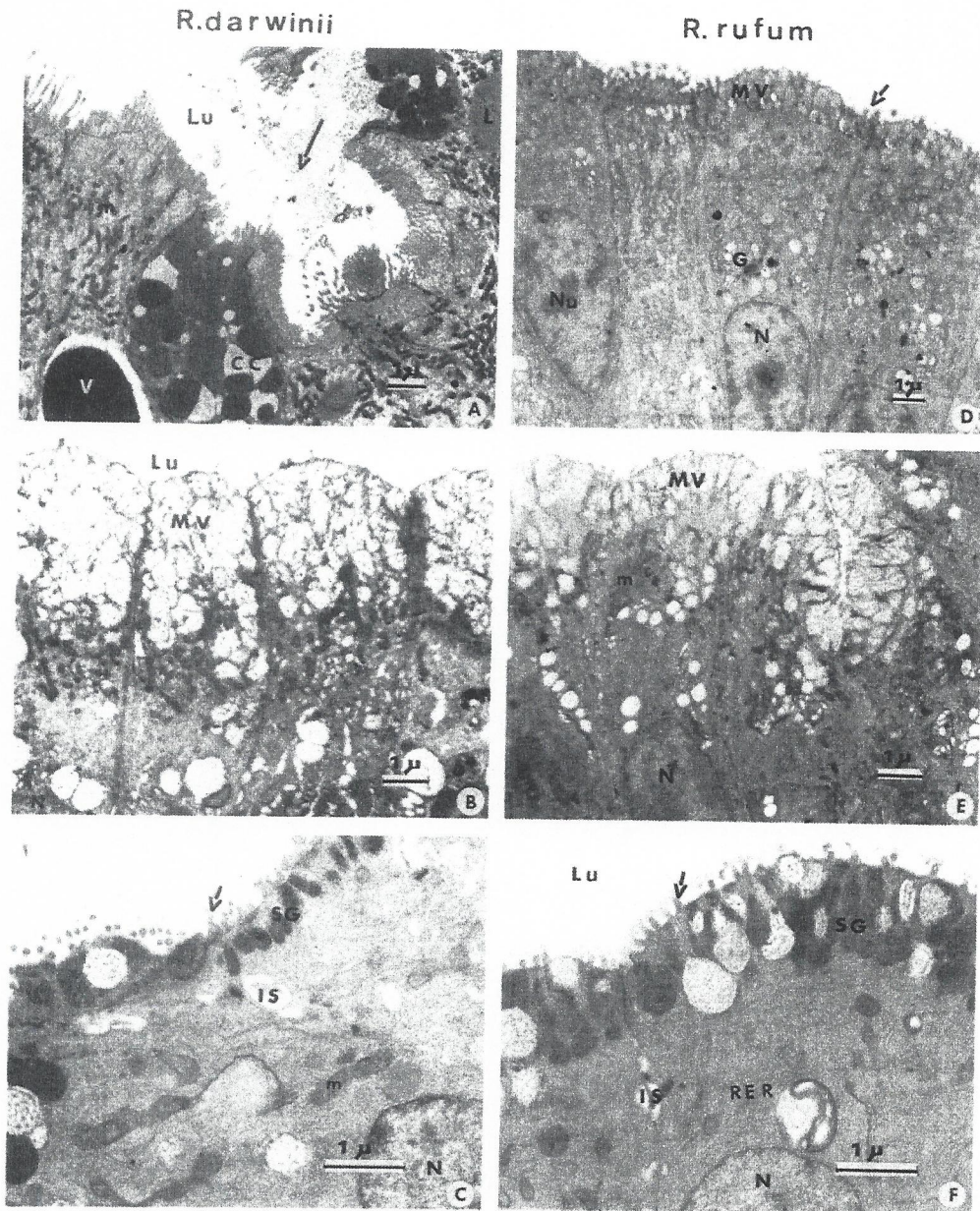


FIG. 5. Histogenesis of the stomach in different stages of larval development. Illustrations A (stage 5), B (stage 7) and C (stage 13) are *R. darwinii*. Illustrations D (stage 5), E (stage 7) and F (stage 13) are *R. rufum*. G, Golgi complex; IS, interstitial space; Long arrow, mucous vacuoles that empty their contents into the gastric lumen; MV, mucous vacuoles and SG, secretory granules.

In stage 5, the gastric lumen appeared. The large cylindrical cells lining the gastric wall displayed numerous lipid and yolk inclusions and a few organelles. Mitochondria and cis-

ternae of the rough endoplasmic reticulum were found, and microvilli appeared at the free surface. Caliciform cells with typical mucous vacuoles opening toward the gastric lumen were

also seen (Fig. 5). Morphological differentiation of the epithelium was completed during stage 7. Cells with an ovoidal basal nucleus and apical cytoplasm containing mucous vacuoles were also observed. These cells presented only few microvilli. Some of these cells presented lipidic inclusions in the apical cytoplasm and vacuoles with an electron dense content in the basal region. There were still some small yolk granules (Fig. 5). In stage 13, crypts radiating around the lumen became evident. The cells lining the crypts had an ovoidal basal nucleus with abundant heterochromatin associated to the nuclear envelope and a prominent nucleolus. The cytoplasm contained some mitochondria and cisternae of the rough endoplasmic reticulum. In their apical pole two types of secretory granules were found: one of low electronic density and a fibrillar (mucous) content, and the other of a high electron density and an irregular profile. The widening of the lateral intercellular spaces was striking (Fig. 5).

Rhinoderma rufum

Anatomical Development of the Digestive Tract.—During stages 1 and 2 of larval development, the gastroduodenal loop and midgut's double spiralization were established (Fig. 1). Spiralization continued until reaching its maximum length with 4 bends in stage 10 (Fig. 2). During stages 11 and 12, the spiral underwent a reduction, turning into a small spiral of 2½ bends and locating itself on the left side during the second half of stage 12. In stage 12/13, the spiral took a ventromedial position to finally be located on the right side in stage 13. As in *R. darwinii*, the gastric dilatation was outlined in stage 12/13, grew rapidly and descended on the left side during stages 13, 14 and 15 (Fig. 3).

During the stages 1 and 2, there was apparently little increase of the intestinal mass; however, it abruptly began to develop in stage 3 reaching its maximum size in stages 9-10 when it

started to decrease progressively until stage 13. Finally, during the stages 12-15 the intestinal mass increased again. However, this was mainly due to the large gastric volume.

Histogenesis of the Digestive Tract.—The lumen of the duodenum was already present in stage 1 of larval development. In stage 2, a premature differentiation of the larvae's duodenal epithelium was observed; in the apical part of the cells, there was a mitochondrial concentration and some cisternae of the rough endoplasmic reticulum. Yolk granules and large lipidic vacuoles still persisted. The apical surface of the cells had abundant microvilli (Fig. 4). In stage 3, the yolk granules disappeared and the morphological cell differentiation was completed. The nuclei were ovoidal and had a basal position. The apical cytoplasm contained a large number of organelles among which mitochondria predominated. A similar appearance was maintained throughout the following stages of metamorphosis (Fig. 4).

In the stomach at stage 5, two cell types were found: cylindrical cells and calciform cells. The former were smaller than those of *R. darwinii* during the same stage, and lacked lipidic and yolk inclusions. The morphological differentiation of the epithelium was completed during stage 6. The gastric crypts and the secretory granules in the apical part of the cell lining these crypts were found during the final stages (13-15) (Fig. 5).

DISCUSSION

R. rufum's digestive tract has the pattern of development typical of an anura with free living larvae. The digestive tract elongates and spirals rapidly, obtaining by stage 3 the absorption surface needed for an herbivorous existence. Once it has reached its maximum length (stages 9-10), the spiral goes through a progressive regression from stage 11 to 15. At the same time, it goes from a left-hand position to a right-

hand one. In the histological aspect, the duodenal epithelium presents in stage 2 sufficient organellar development to allow its cells to be functional. In stage 3, the epithelium reaches the differentiation which it will maintain until the end of metamorphosis. In the stomach's cardiac region, the epithelium undergoes early differentiation and secretory activity apparently begins in stage 5 of metamorphosis.

The development of *R. darwinii*'s digestive tract tends to be direct, deleting the typical modifications of free living larvae. The spiraling of the midgut is slow and progressive, reaching its maximum length in stages 9-10. Even if both species reach maximum length for the double spiral in the same stages of metamorphosis, the shorter length in *R. darwinii* is evident. In both species, reduction of the spiral starts in stage 11. However, this process in *R. darwinii* differs from that of *R. rufum* since the spiral shape disappears in stage 13 becoming a transverse segment with an anterior concavity. In stages 14-15, this segment moves to the right where it becomes a small closed loop similar to that of *R. rufum* at the end of metamorphosis.

The histogenesis of the duodenal epithelium slowly progresses until it reaches, in the last two stages, the same degree of organization that *R. rufum* presents in stage 3. Also, the epithelium of the cardiac region of the stomach presents an earlier differentiation in *R. rufum* compared to *R. darwinii*, even though this differentiation is not as clearly defined as the one observed in the duodenum.

The developmental morphology of the digestive tract indicates that the larvae of *R. darwinii* are incapable of intestinal absorption before stage 11. In *R. rufum* this function is possible starting from stage 2.

R. darwinii larvae taken from the vocal sac and cultivated in aerated Ringer's solution develop slower than inside the vocal sac, and they die prior to meta-

morphosis (Jorquera et al., 1972). These observations suggest an important role of the vocal sac in relation to a respiratory and/or trophic function. The absence of external gills and spiraculum tube and the presence of a thin and richly vascularized integument support the idea that gaseous exchange during larval development is cutaneous (Jorquera et al., 1972). The laminar arrangement of the vocal sac epithelium over the blood capillaries favors this respiratory function similar to one proposed for the marsupial bag in *Gastrotheca riobambae* and *G. marsupiata* (Noble, 1925; Spannhof and Spannhof, 1927; Del Pino et al., 1975). In these species, pouch embryos that have acquired a tadpole shape and developed complete bell gills can be cultured in Ringer's solution (Del Pino et al., 1975). This fact suggests that larvae are capable of uninterrupted gaseous exchanges in culture and that probably no nutrient transfer occurs in the pouch. If we assume that *R. darwinii* is capable of gaseous exchange in Ringer's solution, death would seem most likely to be caused by deficiency of indispensable substances secreted by the epithelium of the vocal sac. The special structure of this epithelium, not described in other larvae-carrying oviparous anurans is different from those of the dorsal pouch of *G. riobambae* where the glands resemble that of ordinary amphibian integument (Del Pino, 1975; Del Pino et al., 1975).

Consequently these facts support the idea of a mixed trophic mechanism fundamentally depending on the abundant reserve of yolk, but also dependent in later stages on the absorption of indispensable nutritive components secreted by the epithelium of the vocal sac.

A similar mechanism has been proposed for the yolk-rich eggs of the viviparous urodele *Salamandra atra* (Greven, 1977). In this species after the embryo has consumed its yolk and the embryotrophic eggs, an intrauterine substance serves as additional nourish-

ment. This possibility has not been analyzed in ovoviviparous anurans of the genus *Nectophrynoides*, but presumably the development of the larvae takes place without nutrients of maternal origin (Lamotte and Lescure, 1977). In the viviparous *N. occidentalis* the embryos are free in the uterine lumen, and they ingest, through the mouth, mucopolysaccharides secreted by the uterine epithelium (Vilter and Lugano, 1959; Xavier, 1973).

In *R. darwinii* the paternal nourishment could be transferred to larvae transepithelially during almost all of the stages of larval development or buccally from stage 10/11 when the yolk has been consumed, and the duodenal epithelium is capable of absorptive functions.

It is suggested that in *R. rufum*, which has a partial dependence on yolk and the vocal sac, a similar trophic mechanism operate during its short stay in the vocal sac (Jorquera et al., in press). Later on, this is replaced by exogenous feeding. The digestive tract and the horny jaws of the mouth are clearly differentiated when the larvae are expelled from the sac.

In summary, the morphological studies reported herein, and our previous investigations on the genus *Rhinoderma*, allow us to make the following conclusions: 1) *R. darwinii* has a direct development, dependent on yolk and vocal sac secretions for nutrients. 2) *R. rufum* during its short permanence in the vocal sac also depends on yolk and vocal sac secretions; however, later the larvae are free. 3) In both species the vocal sac may have a respiratory function.

Ultrastructural and histochemical studies are in progress to determine the nature of the vocal sac secretions and the absorptive capability of the larval skin.

LITERATURE CITED

- ANGEL, F., AND M. LAMOTTE. 1948. Nouvelles observations sur *Nectophrynoides occidentalis* (Angel). Remarques sur le genre *Nectophrynoides*. Ann. Sci. Nat. Zool. 10:115-147.
- BURGER, O. 1904. La neomelia de la *Rhinoderma darwinii* (D&B). Imp. Cervantes. Santiago de Chile. 23 pp.
- CEL, J. M. 1962. Batracios de Chile. Ed. Univ. de Chile. Santiago de Chile. 127 pp.
- DEL PINO, M. E. 1975. Adaptaciones reproductivas para la vida terrestre del sapo marsupial *Gastrotheca riobambae* (Fowler), Anura, Hylidae. Rev. U. Católica Ecuador 8:119-140.
- , M. L. GALARZA, C. M. ALBUJA, AND A. A. HUMPHRIES, JR. 1975. The maternal pouch and development in the marsupial frog *Gastrotheca riobambae* (Fowler). Biol. Bull. 149:480-491.
- DUMERIL, A. M., AND G. BIBRON. 1841. Erpétologie générale. Paris 8:792.
- FORMAS, R., E. PUGIN, AND B. JORQUERA. 1975. La identidad del batracio chileno *Heminectes rufus* (Philippi, 1902). Physis, Secc. C 89:147-157.
- GARRIDO, O., E. PUGIN, AND B. JORQUERA. 1975. Correspondance ultrastructurale entre la bourse gutturale du *Rhinoderma darwinii* et le tegument des larves. Boll. Zool. 42:133-144.
- GLAUERT, A. M., AND R. H. GLAUERT. 1958. Araldite as an embedding for electron microscopy. J. Biophys Biochem. Cytol. 4:191-194.
- . 1965. Section staining, cytology, autoradiography and immunochemistry for biological specimens. In B. K. Blackwell, (ed.) pp. 254-310. Techniques for electron microscopy, Oxford.
- GREVEN, H. 1977. Comparative ultrastructural investigation of the uterine epithelium in the viviparous *Salamandra atra* (Laur) and the ovoviviparous *Salamandra salamandra* (L.) (Amphibia, Urodela). Cell. Tiss. Res. 181:215-237.
- JORQUERA, B., E. PUGIN, AND O. GOICOECHEA. 1972. Tabla de desarrollo normal de *Rhinoderma darwinii*. Arch. Med. Vet. 4:1-15.
- , ———, AND ———. 1974. Tabla de desarrollo normal de *Rhinoderma darwinii* (Concepción). Bol. Soc. Biol. Concepción. 48:127-146.
- , ———, O. GARRIDO, O. GOICOECHEA, AND R. FORMAS. Procedimiento de desarrollo en dos especies del género *Rhinoderma*. Medio Ambiente. In press.
- KRIEG, H. 1924. Biologische reisestudien in Sudamerika. II *Rhinoderma* und *Calyptocephalus*. Zeitschr. Morphol. u. Ökologie der Tiere 3:150-168.
- LAMOTTE, M., P. REY, AND M. VOGELI. 1964. Recherches sur l'ovaire de *Nectophrynoides occidentalis*, Batracien Anoure vivipare. Arch. Anat. Micr. Morphol. Exp. 53:179-224.
- , AND J. LESCURE. 1977. Tendances adaptatives a l'affranchissement du milieu aquatique chez les amphibiens anoures. La Terre et la Vie 31:225-311.
- NOBLE, G. K. 1925. The integumentary, pulmonary and cardiac modifications correlated with increased cutaneous respiration in the amphibia: a solution of the "hairy frog" problem. J. Morphol. 40:341-416.

- SPANNHOF, I., AND L. SPANNHOF. 1927. Beobachtungen zur Brutbiologie und Larvenentwicklung von *Gastrotheca marsupiata*. *Wiss. Z. Univ. Rostock Math. Naturwiss. Reihe* 20:97-104.
- VILTER, V., AND A. LUGANO. 1959. Trophisme intra-uterin et croissance embryonnaire chez *Nectophrynoides occidentalis* Ang., crapaud totalement vivipare du Mont Nimba (Haute Guinée). *C. R. Soc. Biol. Paris* 153:29-32.
- WILHELM, O. 1927. La *Rhinoderma darwini* D&B. *Bol. Soc. Biol. Concepción* 1:11-39.
- . 1932. Nuevas observaciones acerca de la neomelia de la *Rhinoderma darwini*. *Rev. Chil. Hist. Nat.* 36:166-170.
- XAVIER, F. 1971. Recherches sur l'endocrinologie sexuelle de la femelle de *Nectophrynoides occidentalis* (Amphibien Anoure vivipare) These de Doctorat es-sciences C.N.R.S. N°A O 6385. 223 pp.
- . 1973. Le cycle des voies génitales femelles de *Nectophrynoides occidentalis* Angel, Amphibien Anoure vivipare. *Z. Zellforsch.* 140:509-534.
- . 1977. An exceptional reproductive strategy in Anura: *Nectophrynoides occidentalis* Angel (Bufonidae), An example of adaptation to terrestrial life by viviparity. In M. K. Hecht, P. C. Goody, and B. M. Hecht (eds.), pp. 545-552. Major patterns in vertebrate evolution. Plenum Pub. Co., New York.

Accepted: 10 February 1982.