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Evidence for a Trophic Paternal-Larval Relationship in the Frog *Rhinoderma darwinii*

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ABSTRACT.—**Larvae of *Rhinoderma darwinii* have a nonaquatic development inside the vocal sac of the male. Different authors have suggested the existence of a trophic relationship between the male and the larvae. In order to test this possibility, horseradish peroxidase, ^{3}H valine and ^{3}H leucine were injected into the paternal circulation and traced in the larvae at different postinjection intervals. The different tracers were demonstrated in several organs of the larvae thus indicating a possible paternal nourishment. Up to stage 10, the tracers were incorporated into the larvae through their skin and the cephalic and caudal parts of their digestive tract. At later stages, the intestines were the main route for the entry of tracers into the larvae.**

Rhinoderma darwinii is a small anuran living in southern Chile, from Ñuble to Chiloé (Cei, 1962). Gay (1848) observed that some adults of *R. darwinii* carried broods inside an internal sac and wrongly thought that these were viviparous females. De la Espada (1872) established that it was the male and not the female that carried the brood in a *gran saco bucal aereo* (large buccal air sac). This sac was later shown to be the vocal sac which communicates with the buccal cavity through two clefts located on both sides at the bottom of the mouth.

Many authors have considered the possibility that the vocal sac is something more than a simple larval container and have suggested that it might also involve a trophic and a respiratory interchange between host and larvae (Burger, 1904; Krieg, 1924; Wilhelm, 1927).

In 1972 Jorquera et al. described the development of *R. darwinii* and determined that the larval development and metamorphosis occurs inside the male's vocal sac. After external fecundation the first 20 days of embryonic development take place in moist ground. The males who remain in proximity of the cluster pick up the eggs one by one with the mouth and introduce them into the vocal sac. This conduct is stimulated by the beginning of the eggs muscular activity (Cei, 1962). After hatching the larvae remain approximately 52 days and complete metamorphosis within the sac. Some features of the embryonic and larval development were considered as signs of an adaptative mechanism for a paternal dependence in a nonaquatic medium. Larvae taken from the vocal sac and maintained in an aerated Ringer's solution developed more slowly than inside the vocal sac and died before metamorphosis (Jorquera et al., 1972).

Garrido et al. (1975) described the structure of the vocal sac and demonstrated that its internal epithelium displays signs of a secretory activity and indicated that the superficial cells of the larval skin have many pinocytotic vesicles. Jorquera et al. (1982) reported that the larvae of *R. darwinii* are not capable of intestinal absorption prior to the beginning of metamorphosis. These observations led the authors to suggest that the epithelial cells of the sac secrete a substance which then becomes part of the viscous fluid found inside the vocal sac. This material could be incorporated by the larvae through their skin and, when the intestinal epithelium is capable of performing absorptive functions, a buccal route of entry could operate.

The use of tracer molecules that can

be readily detectable both in paternal or larval structures appears the method of choice to obtain direct evidence for a paternal-larval inter-relationship. The use of peroxidase as a tracer molecule has made it possible to study the routes of transport from blood to tissues (Straus, 1957, 1960, 1961 and 1964; Wachstein et al., 1959). The combined use of labeled amino acids and autoradiography is a useful tool for the study of synthesis, transport and location of peptides and proteins (Caro and Palade, 1964; Jamieson et al., 1967; Haddad et al., 1971).

Materials and Methods

Experimental Design Using Peroxidase as a Tracer.—A total of 15 males, carrying larvae in their vocal sac, were injected in the dorsal lymphatic sac with 0.2 mg of peroxidase (Merck = 100 U/mg) per gram of body weight dissolved in 100 µl of 10% Holtfreter solution, pH 7.2.

a) *Detection of peroxidase in the viscous fluid contained in the vocal sac.* Eight hours after the injection of peroxidase, 3 males were anaesthetized by contact with 2% ether in Ringer's solution and a 100 µl sample of the viscous fluid contained in the vocal sac was collected. The peroxidase activity of this material was measured according to the technique of Sánchez-Vizcaino and Cambra (1981). The same procedure was applied to three control males injected with 10% Holtfreter's solution.
b) *Location of peroxidase in paternal and larval tissues.* Eight hours after the peroxidase injection, 12 males were killed by decapitation. The larvae, and the vocal sacs of the males brood carrier were removed and fixed in an aldehyde mixture (TAM) containing 4% paraformaldehyde, 2% glutaraldehyde and 1% acrolein in 0.2 M phosphate buffer to pH 7.4 (Rodríguez, 1968). After 12 h of fixation at 4°C, the samples were washed with several changes of 0.1 M Tris-HCl buffer, pH 7.2, at 4°C, for 8 h.

A total of 123 larvae were classified from stage 3 to 15 according to the table of Jorquera et al. (1972). Larval size is indicated in Table 1. Samples, approximately 1 mm thick, were obtained from different organs of two to three larvae in each of the different developmental stages. The presence of peroxidase in these samples was investigated according to the technique of Graham and Karnovsky (1966).

Two sets of samples were obtained from different tissues (Table 1). One set was embedded in paraplast, and 7 µm thick sections were stained with hematoxylin as a background stain for the peroxidase reaction. The second set of samples was post-fixed in a 1% OsO_4 in buffer phosphate 0.1 M for 1 h at 4°C, and then embedded in a mixture of Epon-Araldite. Ultrathin sections were stained with uranyl-acetate for 10 sec.

Brooding males were injected with 100 µl of 10% Holtfreter's solution and used as controls for detecting endogenous peroxidase. Paternal and larval tissues were removed, fixed and processed as described above.

Experimental Design Using ^3H Leucine and ^3H Valine.—Three males with broods were injected in the dorsal lymphatic sac with ^3H leucine (20 µCi per g of body weight) and one with ^3H valine (10 µCi per g of body weight) diluted in 100 µl of 10% Holtfreter's solution. They were killed by decapitation 9 h after the injection. The larvae and the vocal sacs were fixed in TAM and were washed in 0.1 M, Tris-HCl buffer.

a) *Detection of radioisotopes by scintillation counting.* The whole fixed larvae were extracted individually with 1 ml of 30% KOH, which was neutralized with HCl. After mixing 0.5 ml of each one of the samples with an equal quantity of scintillating liquid, 10

TABLE 1. Presence of peroxidase in different larval tissues.

Stages	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV
Larval total size (mm)	10.2	12	12	13.5	14.6	15.1	16	16.5	16.7	16.3	14.3	10.5	8.5
Number of larvae	3	3	3	3	3	3	3	3	3	3	3	2	2
Dorsal skin	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + -	- - -	- - -	- - -	- - -	- - -
Ventral skin	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + -	- - -	- - -	- - -	- - -	- - -	- - -
Skin of the tail	- - -	- - -	- - -	+ + +	+ + +	+ + +	+ + +	- - -	- - -	- - -	- - -	- - -	- - -
Buccal epithelium	+ + -	- - -	+ + -	+ + +	+ + -	+ + -	- - -	- - -	- - -	- - -	- - -	- - -	- - -
Tongue	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	+ + +	+ + -	- - -	- + -
Pharynx	+ + +	-	+ + +	+ + +	+ + +	+ + +	+ + +	+ + -	+ + -	+ + -	- - -	- - -	+ - -
Esophagus	- - -	- - -	- - -	- - -	+ - -	+ + -	+ + -	- - -	- - -	- - -	- - -	- - -	- - -
Liver	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	+ + +	+ + -	+ + +	+ + +
Fore-gut	- - -	- - -	- - -	- - -	+ + -	+ + +	+ + -	+ + -	+ + +	+ + +	+ + +	+ + +	+ + +
Cloaca	- - -	+ + +	+ + -	+ + +	+ + +	+ + -	+ + +	+ + -	+ + +	+ + +	+ + -	+ + -	+ + +
Kidney	+ + +	+ + -	+ + -	+ + +	+ + -	+ + +	+ + +	- - -	+ + +	+ + +	+ + +	+ + +	+ + +

+ = positive reaction to peroxidase for each larva examined.
- = negative reaction to peroxidase for each larva examined.
! = no information.

minute readings were obtained in the scintillation counter.

b) *Location of radioisotopes by radioautography.* The collected larvae belonged to stages 9, 10, 12 and 14. Pieces of tissue were embedded in paraplast and 7 μm sections were stained with hematoxylin-eosin and immersed in a Kodak NTB2 photographic emulsion. After three months they were developed.

Brooding males injected with 10 μg valine or leucine diluted in 100 μl of 10% Holtfreter's solution and processed like the experimental series were used as controls.

RESULTS

The vocal sac of each male contained a mean of 11 larvae, which were in two or three different developmental stages. These larvae were immersed in a sticky material with air bubbles visible through the wall of the vocal sac (Fig. 1A, B).

Fig. 2 is a sagittal section of a male brood carrier showing: a) topographic position of the vocal sac, b) relationship between the vocal sac and the larvae contained and c) encompased areas that correspond to different micrographs of Figs. 3 and 4.

Locations of Peroxidase.—In the male brood carrier peroxidase was detected in blood vessels, lymphatic spaces and interstitial space of different tissues. Special observations were made of the location of peroxidase in the vocal sac. The different layers of the vocal sac's wall showed peroxidase reaction product (PRP) in the following locations: a) lymphatic endothelium: pinocytotic vesicles (Fig. 3B); b) subepithelial layer: scattered patches associated with collagen fibers (Fig. 3C, C_1); the lumina, pinocytotic vesicles and intercellular spaces of endothelium of capillaries (Fig. 3B); c) vocal sac epithelium: in the basal lamina and pinocytotic vesicles of the basal cells (Fig. $3C_1$). In the intercellular space of the basal and superficial cells and in secretory granules in

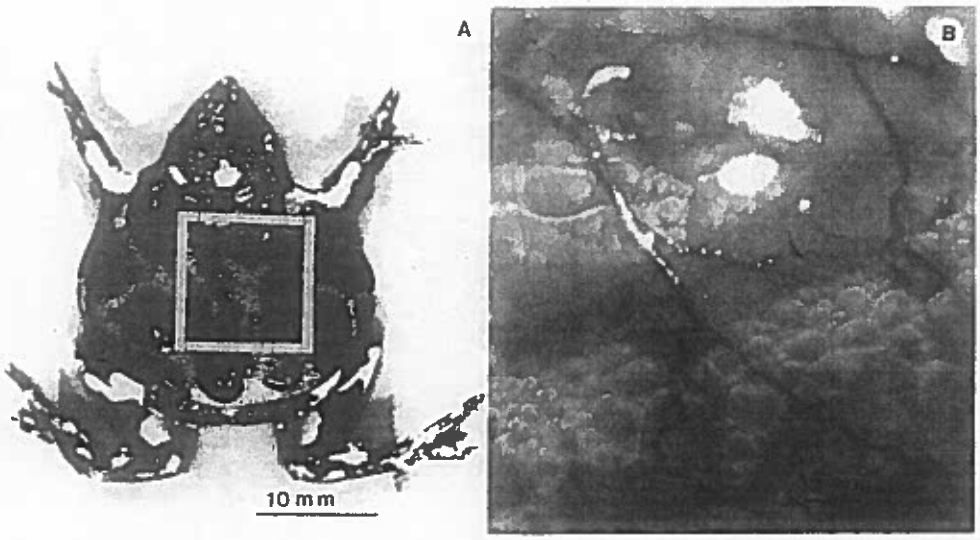

FIG. 1. Vocal sac of the male brood carrier. A. Ventral view of a brooding male. The ventral skin was cut and the vocal sac exposed. Through the wall of the vocal sac a viscous fluid with air bubbles and larvae is visible. B. Magnification of the area encompassed by the rectangle in Fig. 1A, showing air bubbles and blood vessels.

the apical region of superficial cells (Fig. 3C, C_2).

The viscous fluid collected from the vocal sac of the three injected males contained 0.002–0.005 mg/ml peroxidase. Vocal sac fluid from males which had not been injected did not show peroxidase activity.

Under the light microscope the location of PRP between the cells of the vocal sac epithelium suggests a direct passage of peroxidase to the lumen of the vocal sac (Fig. 3A). However the electron micrograph shows tight junctions sealing the space between the superficial cells of the vocal sac epithelium (Fig. 3C).

The location of peroxidase in larval tissues is shown in Table 1. The tracer was consistently present in the skin of larvae from stage 3 to stage 9; it was occasionally found in stages 10 and 11, and absent from the skin of larvae in stage 12. Peroxidase was preferentially located in the cytoplasm of superficial cells. The basal membrane of the epidermis and the underlying connective tissue showed a moderate amount of PRP (Fig. 4A).

In the larval mouth, PRP was found from stage 3 to stage 13. Peroxidase was observed in small vesicles in the superficial cells of the buccal epithelium from stage 3 to stage 9. In the tongue PRP was only detected in stages 12 and 13. In these stages there was an intense peroxidase reaction in the connective tissue along the ventrolateral sides of the posterior third of the tongue, lymphatic endothelium and intercellular spaces of the dorsal epithelium.

The tracer was present in the larval pharynx in all developmental stages. The reaction was restricted to clusters of cells located on the surface of the epithelium. These round cells showed an intense reaction in the whole cytoplasm.

The liver showed PRP from stage 12 on; tracer was located in the intercellular space and in large and round cells distributed throughout the organ.

The midgut and foregut derivatives showed PRP from stage 7 larvae. The PRP was located in the goblet cells, which were scattered or grouped; and in vesicles of different sizes located at different levels of the cytoplasm of the

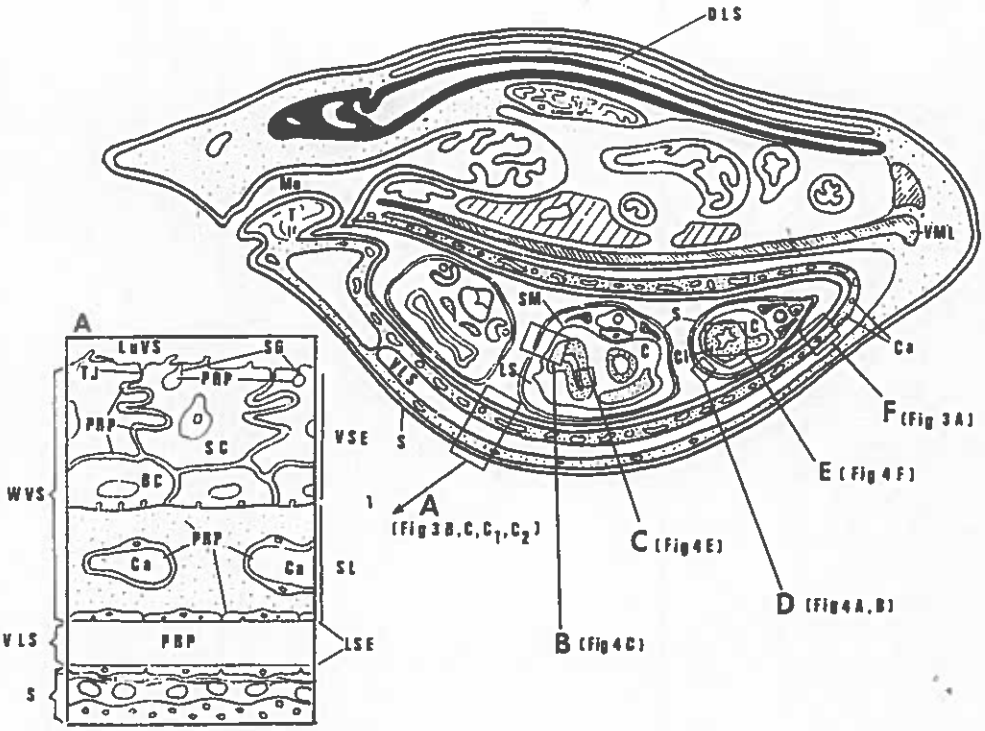

FIG. 2. Diagram of a sagittal view of a brooding male with larvae in the vocal sac. Rectangle A corresponds to the area of the male's ventral skin, ventral lymphatic sac and wall of the vocal sac. High magnification of this area is indicated in the great rectangle (Fig. 3B, C, C_1, C_2). Rectangle B corresponds to the area of skin, lymphatic sac, somatic mesoderm, coelom and intestine in a middle cross larval section (Fig. 4C). Rectangle C corresponds to the area of intestine (Fig. 4E). Rectangle D corresponds to the area of skin in a caudal cross larval section (Fig. 4A and B). Rectangle E corresponds to the area of cloaca (Fig. 4F). Rectangle F corresponds to the wall of the vocal sac (Fig. 3A). BC, basal cell; C, coelom; Ca, capillaries; CL, cloaca; DLS, dorsal lymphatic sac; LS, lymphatic sac; LSE, lymphatic sac endothelium; LuVS, lumen of the vocal sac; Mo, mouth; PRP, peroxidase reaction product; S, skin; SC, superficial cell; SG, secretory granule; SL, subepithelial layer; SM, somatic mesoderm; T, tongue; TJ, tight junction; VLS, ventral lymphatic sac; VML, ventral muscular layer; VSE, vocal sac epithelium; WVS, wall of the vocal sac.

epithelial cells. The cloaca showed PRP from stage 4 to stage 15.

The presence of peroxidase in the mesonephric tissue was detected from stage 3 to stage 15. In stage 3 to 5 the PRP was circumscribed in large cytoplasmic vesicles of macrophages. In more advanced stages, peroxidase was

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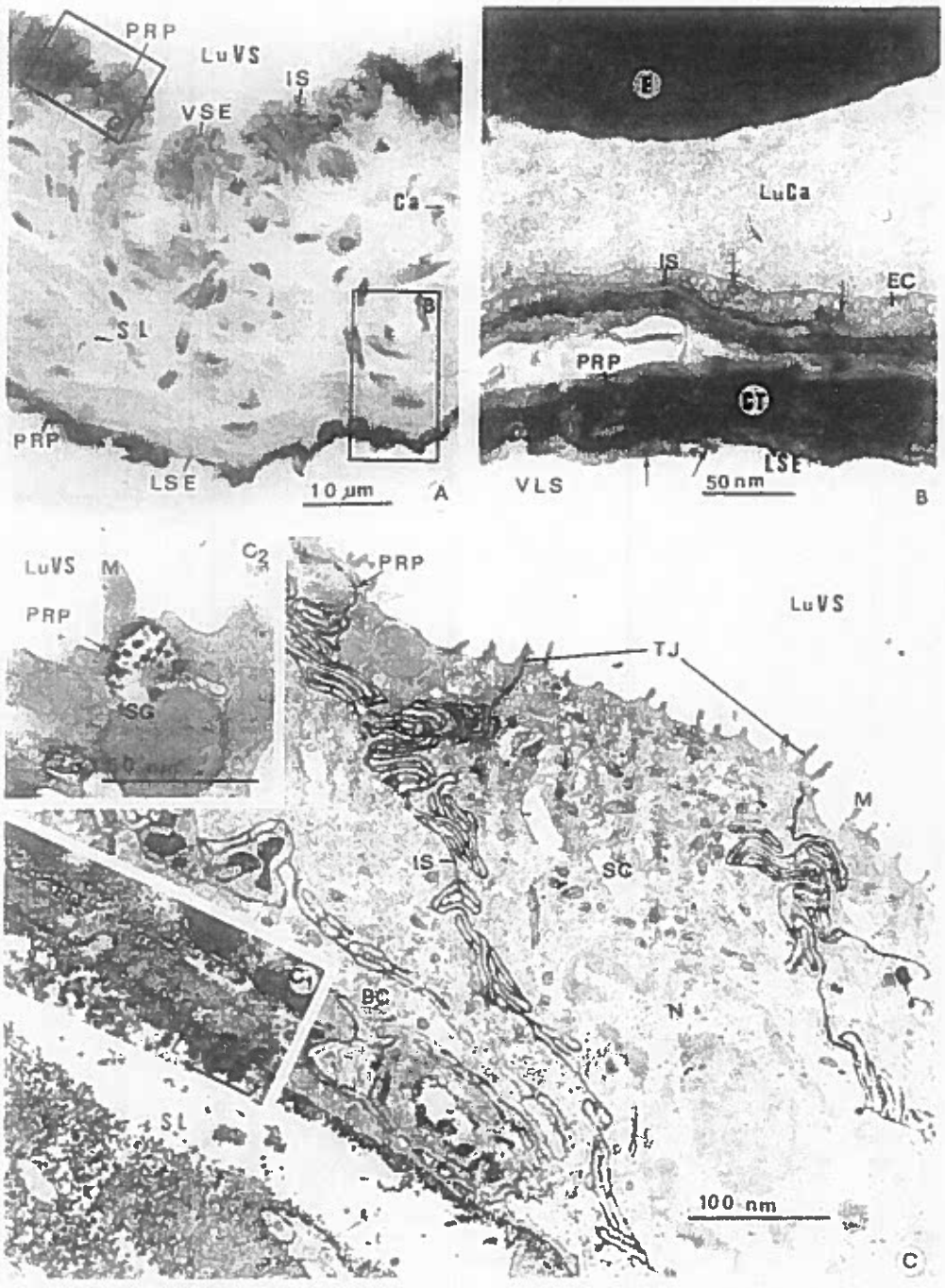
FIG. 3. Location of peroxidase in the vocal sac. A. Cross section of the wall of the vocal sac showing peroxidase reaction product in the lymphatic sac endothelium and in the vocal sac epithelium. B. Magnification of a similar area to those shown in rectangle B in Fig. 3A. Ultrathin section shows peroxidase in the following locations: pinocytotic vesicles in the endothelium of the lymphatic sac; connective tissue of the subepithelial layer; pinocytotic vesicles and intercellular space of endothelium of capillaries; lumen of the capillaries and endogenous peroxidase in erythrocyte. C. Magnification of a similar area to those shown in rectangle C in Fig. 3A. Ultrathin section of the vocal sac epithelium shows peroxidase reaction product in the intercellular space and tight junctions sealing the space between the superficial cells. Inset C_1. Higher magnification of the apposition zone between the epithelial basal cell and the subepithelial layer. Peroxidase is associated with collagen fibers in the subepithelial layer, the basal lamina and pinocytotic vesicles of the basal cells. Inset C_2. Higher magnification of the apical region of a superficial cell. Peroxidase reaction product in secretory granule.



BC, basal cell; BL, basal lamina; Ca, capillary; CT, connective tissue of the subepithelial layer; E, erythrocyte; EC, endothelium of the capillary; IS, intercellular space; LSE, lymphatic sac endothelium; LuCa, lumen of the capillary; LuVS, lumen of the vocal sac; M, microvilli; N, nucleus; PRP, peroxidase reaction product; SG, secretory granule; SC, superficial cell; SL, subepithelial layer; TJ, tight junction; VSE, vocal sac epithelium; VLS, ventral lymphatic sac; short arrows, collagen fibers associated with PRP; long arrows, pinocytotic vesicles with PRP.

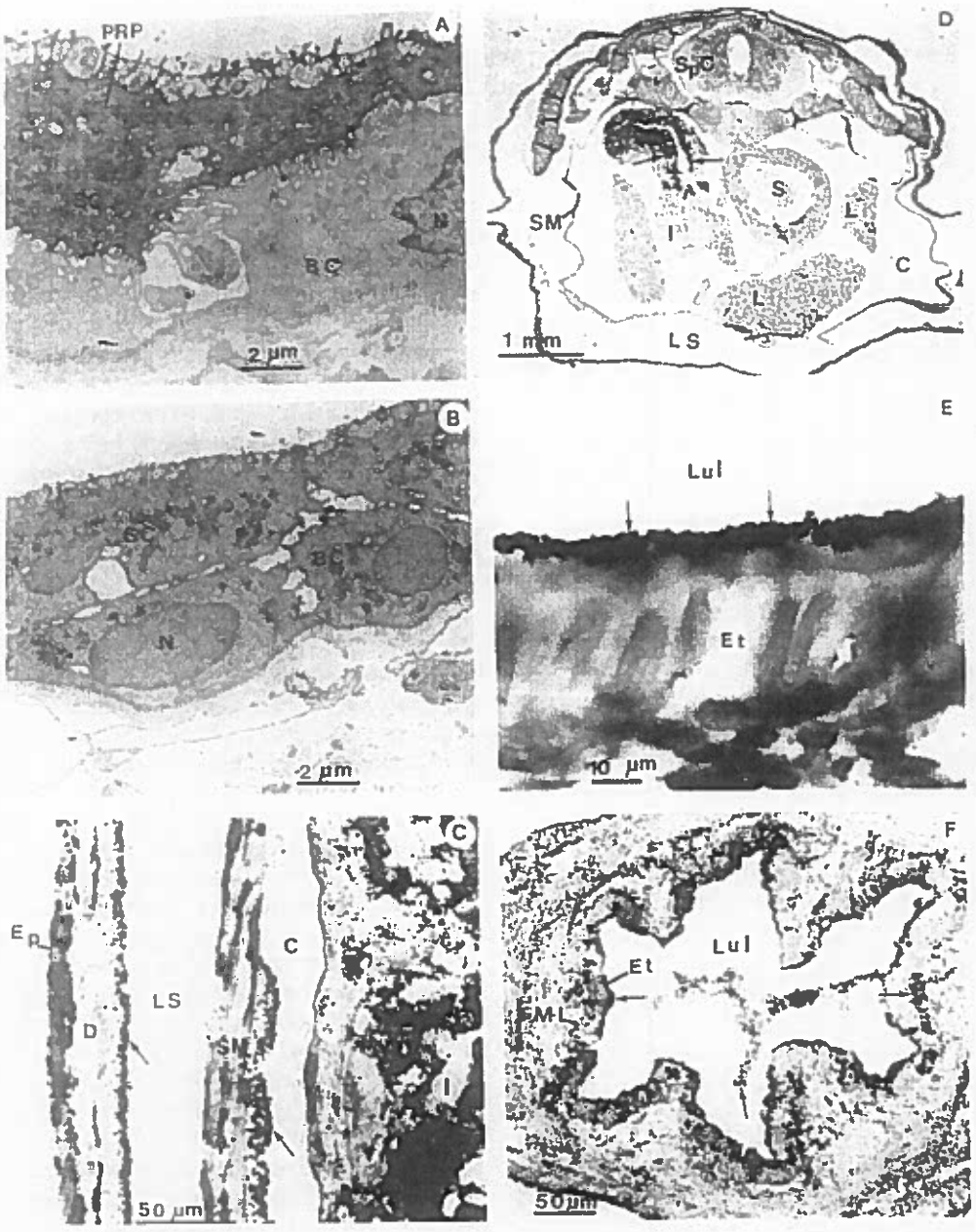


FIG. 4. Location of tracers in larval tissues. A. Ultrathin cross section of larval skin showing peroxidase reaction product in the superficial cells (Stage 8). B. Control of larval skin (Stage 8). Light microscopic radioautography showing ^3H leucine activity in a cross section of larval skin, somatic mesoderm and intestine (Stage 12). D. Light microscopic radioautography of a middle cross section of larva stage 10 showing ^3H leucine activity in the intestine. E. Light microscopic radioautography showing ^3H valine activity in the apical region of the intestinal epithelium (Stage 12). F. Light microscopic radioautography showing ^3H leucine activity in the wall of the larval cloaca (Stage 12). BC, basal cell; C, coelom; D, dermis; Ep, epidermis; Et, epithelium; I, intestine; Li, liver; LS, lymphatic sac; Lul, lumen of the intestine; ML, muscular layer; N, nucleus; PRP, peroxidase reaction product; S, stomach; SC, superficial cell; SpC, spinal cord; SM, somatic mesoderm; arrows, silver grains.

TABLE 2. Scintillation counter readings in larvae after administration of labelled leucine and valine to the host male.

Stages	IX	X	XII	XII	XIII	XIV
Number of larvae	2	1	2	3	2	3
Labeled amino acid	Leu	Leu	Val	Leu	Leu	Leu
cpm/larva	342	692	432	1,972	1,984	1,783
Male body weight (g)	2.4		2.3	2.2		2.0

Leu specific activity = 5.000 μCi/μmol.
Val specific activity = 1.170 μCi/μmol.

confined to small cytoplasmic vesicles of tubular cells.

Controls.—Both light and electron microscopy revealed that in the brooding male and in the larvae the PRP was only observed in the erythrocytes of the examined organs.

Detection of Labeled Amino Acids.—The mean values of readings in the scintillation counter in larvae are shown in Table 2.

Tissue Location of Labeled Amino Acids.—In males injected with leucine or valine, light microscopic radioautography revealed the presence of radioactivity in the inner wall of the vocal sac.

The location of radioactivity in larval tissues at different stages is shown in Table 3. The label was mainly found in the digestive tube. In the mouth the label was detected exclusively in the tongue. Here it was located in the epithelium, basal lamina, muscle and walls of the submaxillary lymphatic sac. In the intestinal epithelium isotopes were observed in cells in an advanced stage of differentiation especially in their apical regions (Fig. 4D, E). In the cloaca, the tracer was located both in the wall and in the lumen (Fig. 4F). Tracers were also detected in the serosa and connective tissues of the intestine.

Isotopic tracers were also found in the epidermis and dermis as well as in the endothelium of the subdermal lymphatic vessels (Fig. 4C). Leucine but not valine was always observed in the spinal cord. This label was observed in the grey matter and meninges in all the stages studied (Fig. 4D). It was also present in the white matter in stage 14.

The incorporation of both isotopes into the skeleton was evident in the more advanced developmental stages. The matrix of the hyaline cartilage of the skull of larvae in stage 12 showed a high concentration of the label.

The radioautography of control larvae samples was negative.

DISCUSSION

The presence in larval tissues of tracers administered into the lymphatic sac of the male carrying them indicates that substances can move from paternal tissues to larval tissues. The presence of peroxidase in the viscous fluid of the host vocal sac suggests that release of substances into this fluid might be an intermediate step in the mechanism of transport between host and larvae. The possibility of paracellular transfer of substance across the vocal sac epithelium to the fluid seems remote by the presence of tight junctions sealing the

TABLE 3. Presence of ^{3}H leucine and ^{3}H valine in different larval tissues.

Stage	11	10	12		14
Isotope	Leu	Leu	Leu	Val	Leu
Number of larvae	2	1	2	2	1
Retina	−−	−	++	−−	+
Spinal cord	++	+	++	−−	+
Nerves	+−	+	++	−−	+
Tongue	++	+	++	++	+
Stomach	−−	+	+−	++	−
Liver	+−	−	++	+−	+
Intestine	++	+	++	++	+
Cloaca	−−	+	++	+−	+
Skin	++	+	+−	++	+
Muscle	+−	+	+−	++	+
Cartilage	−−	−	++	++	−

+ = presence of radioactivity for each larva examined.
− = absence of radioactivity for each larva examined.

apical extremities of intercellular space. The presence of tracer in the pinocytotic vesicles in the basal cells and in secretory granules in the apical region of the superficial cells suggests a transcellular transport. This transport pathway, evidenced by means peroxidase, could be utilized by nutrients substances.

The location of peroxidase in the skin and digestive tract of the larvae points to these structures as the probable route of entry into the larvae of substances provided by the male. The presence of PRP within the epidermal epithelial cells in a diffuse form that occupies most of the cytoplasm should be interpreted with caution. It could represent either a massive absorption of the tracer or diffusion due to a toxic effect of the peroxidase upon the cell membrane of these cells.

Incorporation of peroxidase into the skin only occurs between stages 3 and 10. This is probably due to the differentiation reached by the skin during the late developmental stages. In early stages in which the esophageal plug is still present, the absorption of peroxidase by the digestive tract occurs only in the bucco-pharynx and cloaca. In later stages and parallel to the progressive appearance of the intestinal lumen, peroxidase is also incorportated by other portions of the digestive tube.

The presence of peroxidase in the capillary lumen and mesonephric tissues of the larvae suggests that tracers reach the body fluid of the larvae and then are excreted by the kidney into the cloaca.

The use of radioactive isotopes has confirmed the passage of substances from paternal circulation into the larvae. The increased incorporation of ^{3}H luecine into stages 12 to 14, as compared to that of stages 9 and 10, could be interpreted as a higher demand for nourishment by the larvae. This could also be related to the depletion of stored yolk which, according to Jorquera et al. (1982), occurs in stage 11.

The labeled amino acids were located in most larval tissues but with a higher concentration in differentiating intestinal segments. The presence of radioactive tracers in the skin during the final stages of development and the absence of PRP during the same stages could indicate that either the amino acids had been first incorporated through the digestive tract and then transferred to the skin, or that amino acids, in contrast to large molecules such as peroxidase, are indeed taken up by the skin from the surrounding medium.

Furthermore, the different tissue locations of radioactive amino acids and peroxidase indicate that the latter is processed as a foreign substance, being absorbed and then eliminated by the mesonephros. Labeled leucine and valine are incorporated by many larval tissues such as muscle, cartilage, nerve tissues, meninx, integument, thus suggesting that they might be used for protein synthesis.

In a previous study of morphogenesis and differentiation of the digestive tract of *R. darwinii* Jorquera et al. (1982) postulated that during most larval stages paternally derived nutrients could be transferred to the larvae by use of the skin route of entry. The oral route would only operate from stage 11, when yolk had been depleted and intestinal epithelium had become differentiated. The present findings support this assumption.

This possible feeding of *R. darwinii* larvae by the male seems to be unique among amphibian species. Similar relationships have been described for other amphibians, but the female is usually the one performing the host role. Thus, in *Salamandra atra* once the yolk and the material of embryotrophic eggs are consumed, nourishment is provided by cells derived from the uterine trophic zone (Wiedersheim, 1890; Fachbach, 1969) as well as by substances transferred from maternal capillaries to the uterine epithelium and that become components of the uterine milk (Schwalbe, 1896; Bertín, 1952; Vilter and

Vilter, 1960, 1964; Hafeli, 1971; Salthe and Mecham, 1974). Viviparous caecilians present a similar uterine milk produced by secretory cells of oviducal epithelium (Wake, 1977). This uterine milk could be regarded as an equivalent to the viscous fluid present in the vocal sac of *R. darwinii*. In the viviparous *Nectophrynoides occidentalis*, the embryos are free in the uterine lumen and they incorporate, through their mouths, mucopolysaccharides secreted by the uterine epithelium (Vilter and Lugano, 1959; Xavier, 1973).

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