Morphological Changes in Skin Glands During Development in *Rhinella Arenarum* (Anura: Bufonidae)

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ABSTRACT

Avoiding predation is critical to survival of animals; chemical defenses represent a common strategy among amphibians. In this study, we examined histologically the morphology of skin glands and types of secretions related to chemical skin defense during ontogeny of *Rhinella arenarum*. Prior to metamorphic climax the epidermis contains typical bufonid giant cells producing a mucous substance supposedly involved in triggering a flight reaction of the tadpole school. An apical layer of alcianophilic mucus covers the epidermis, which could produce the unpleasant taste of bufonid tadpoles. Giant cells disappear by onset of metamorphic climax, when multicellular glands start developing, but the apical mucous layer remains. By the end of climax, neither the granular glands of the dorsum nor the parotoid regions are completely developed. Conversely, by the end of metamorphosis the mucous glands are partially developed and secrete mucus. Adults have at least three types of granular glands, which we designate type A (acidophilic), type B (basophilic) and ventral (mucous). Polymorphic granular glands distribute differently in the body: dorsal granular glands between warts and in the periphery of parotoids contain protein; granular glands of big warts and in the central region of parotoids contain catecholamines, lipids, and glycoconjugates, whereas ventral granular glands produce acidic glycoconjugates. Mucous glands produce both mucus and proteins. Results suggest that in early juveniles the chemical skin defense mechanisms are not functional. Topographical differences in adult skin secretions suggest that granular glands from the big warts in the skin produce similar toxins to the parotoid glands. Anat Rec, 299:141–156, 2016.

Key words: skin glands; parotoid gland; ontogeny; histology; bufonid

INTRODUCTION

Avoiding predation is critical to the survival of animals and has led to the development of a wide variety of defensive strategies (Edmunds, 1974; Toledo, Sazima and Haddad, 2011). Among them, chemical defenses represent a common strategy to various animals, and, particularly in amphibians, skin glands are responsible for the synthesis, storage and release of unpalatable or toxic substances that function as a defense mechanism (Daly, 1995; Toledo and Jared, 1995; Clarke, 1997). The skin of extant adult amphibians consists of an epidermis and an

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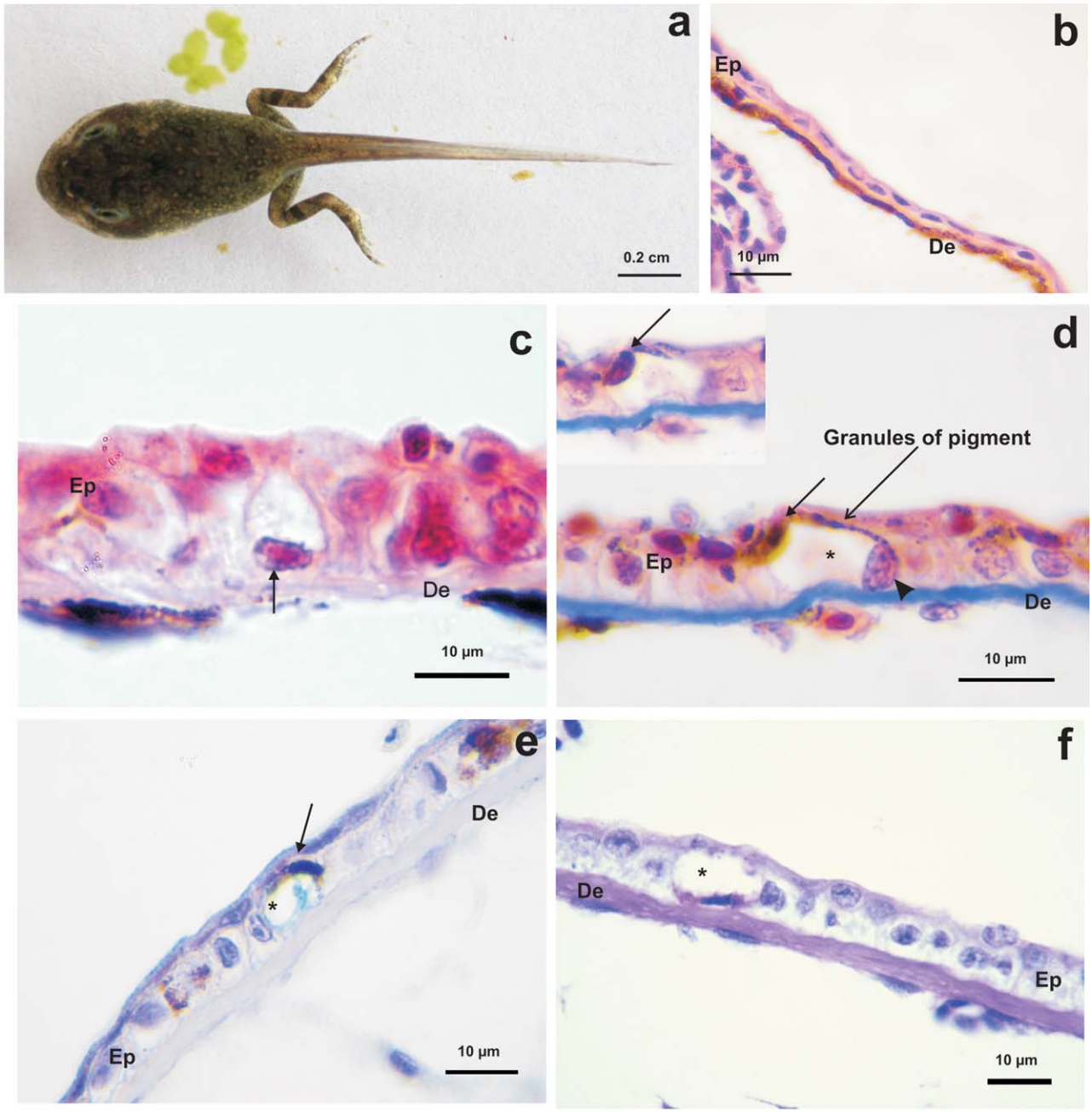


Fig. 1. *Rhinella arenarum* skin during the larval period. (**a**) Tadpole in stage G39. (**b**) Dorsal skin of tadpole in stage G27. H–E staining. Notice the single-layer epidermis without glandular cells. (**c**) Dorsal skin of tadpole in stage G30. MMT staining. Arrow points to mucous cell. (**d**) Dorsal skin of tadpole in stage G30. MMT staining. Notice the nucleus of the giant cell (arrowhead) opposite the nucleus of the melanophore (arrow). Inset focuses on the nucleus of the melanophore (arrow). (**e**) Dorsal skin of tadpole in stage G35. AB staining. Notice the Alcian blue positive content inside the giant cell. Arrow points to the nucleus of the melanophore. (**f**) Dorsal skin of tadpole in stage G35. PAS-H staining. Notice the PAS positive content inside the giant cell. Giant cell (*); Epidermis (Ep); Dermis (De).

underlying dermis with two types of glands, considered synapomorphies of Lissamphibia: mucous glands, usually associated with respiration and water balance, and granular (also known as serous or venom) glands, related to defense mechanisms (Duellman and Trueb, 1994). Granular glands synthesize a wide variety of chemical compounds, e.g., proteins, lipids, catecholamines, alkaloids, or glycoconjugates, depending on the group of amphibians (Toledo and Jared, 1995; Jared et al., 2009; Antoniazzi et al., 2013; Ferraro, Topa and Hermida, 2013). Among anurans, true toads belonging to the family Bufonidae produce highly toxic skin secretions, which are composed of cardiotonic steroids, called bufadienolides, and biogenic amines like catecholamines,

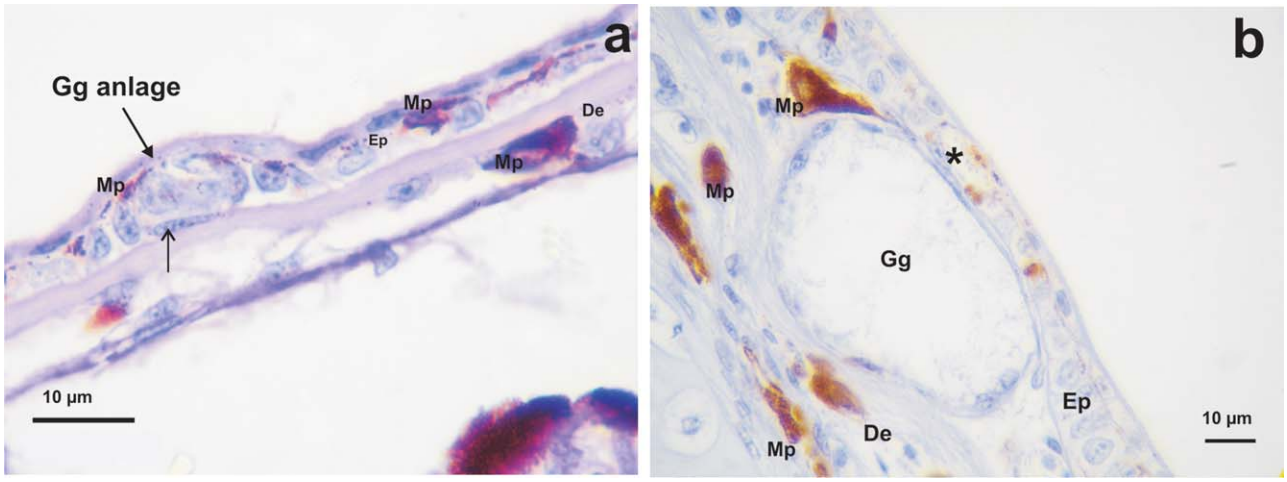


Fig. 2. *Rhinella arenarum* skin during prometamorphosis. (**a**) Dorsal skin of tadpole in stage G36. H–E staining. Notice an early anlage of a multicellular gland. The thin arrow points to a future myoepithelial cell. Observe melanophore prolongations (Mp) in the epidermis. (**b**) Dorsal skin of tadpole in stage G40. Haematoxylin staining. By late prometamorphosis, both multicellular glands and giant cells occur in the skin. Giant cell (*); Granular gland (Gg); Melanophore prolongation (Mp); Epidermis (Ep); Dermis (De).

with a powerful vasopressor action in vertebrates (Toledo and Jared, 1995; Maciel et al., 2003; Cunha-Filho et al., 2005; Sciani et al., 2013).

Toads with larval development experience the transition from an aquatic to a terrestrial habitat during their life cycle and defense mechanisms against predators may vary as a function of habitat type. Tadpoles of bufonids are particularly unpalatable or toxic to some predators (reviewed by Gunzburger and Travis, 2005; Hayes et al., 2009; Jara and Perotti, 2009) and toxicity of individuals is associated with the content and variety of bufadienolides (Hayes et al., 2009.). As an aditional defense mechanism, tadpoles of bufonids possess giant cells (also known as Riesenzellen), which only release their secretion when the skin is injured, triggering a "flight reaction" in the tadpole school (Pfeiffer, 1966; Fox, 1988; Delfino, Brizzi and Feri, 1995).

In adult anurans, granular glands are scattered throughout the skin or arranged in multiglandular structures called macroglands (Toledo and Jared, 1995). In *Rhinella* toads, postorbital/supratympanic macroglands by the name of parotoids are related to antipredator defense (Jared et al., 2009; van Bocxlaer et al., 2010). Parotoids are not merely accumulations of granular glands but specialized regions of the integument which contain different gland types, with a histological organization that confers functional features beyond a typical passive defense mechanism (Almeida et al., 2007; Jared et al., 2009). According to some studies, parotoids do not appear to be fully developed by the end of the metamorphic climax, only becoming functional later during the juvenile stage (Freeland and Kerin, 1991; Phillips and Shine, 2006). Based on these observations, chemical defense mechanisms vary during the ontogeny of anurans, and morphological changes during development are crucial for defining the patterns of activity and habitat use to avoid intense predator pressure (Freeland and Kerin, 1991).

In the present work, we studied the morphology of structures associated with chemical skin defense throughout ontogeny of *Rhinella arenarum*, a bufonid toad native to Argentina, Uruguay, Bolivia and Southern Brazil, by using conventional histological and histochemical techniques to describe the ontogenetic temporal pattern of change in secretions during development.

MATERIALS AND METHODS

Animals

Specimens of *Rhinella arenarum* were collected in the Parque Nacional de la Memoria, Buenos Aires City and in La Reja, Buenos Aires Province, Argentina, during the spring breeding seasons of 2012 and 2013. A total of 50 tadpoles, 1 juvenile with a snout-vent length (SVL) of 3.31 cm, and 8 adults (5 males and 3 females) with SVLs between 10.81–12.23 cm were employed for this study. Tadpoles were maintained in dechlorinated tap water under a natural photoperiod and temperature and fed *ad libitum* with boiled chard. All tadpoles were staged according to Gosner (1960). After metamorphosis, 10 newly metamorphosed toadlets were reared under natural outdoor conditions for 10 days. Tadpoles and adult toads were euthanized by immersion in 0.1% or 1% aqueous solution of MS222 (tricaine methanesulfonate; Sigma-Aldrich, St. Louis, MI), respectively. Because the MS222 typically takes several minutes to anesthetize tadpoles and this could cause skin irritation that affects the histological appearance of the glands, the effect of another anesthetic, benzocaine, was also tested. It was observed that the skin of specimens euthanized with benzocaine was similar to specimens euthanized with MS222.

This study was carried out according to the regulations specified by the Institutional Animal Care and Use Committee of the Facultad de Ciencias Exactas y Naturales, UBA (Res C/D 140/00). The Conservation category of *R. arenarum* is "Least concerned" according to the IUCN Red List criteria (IUCN, 2012).

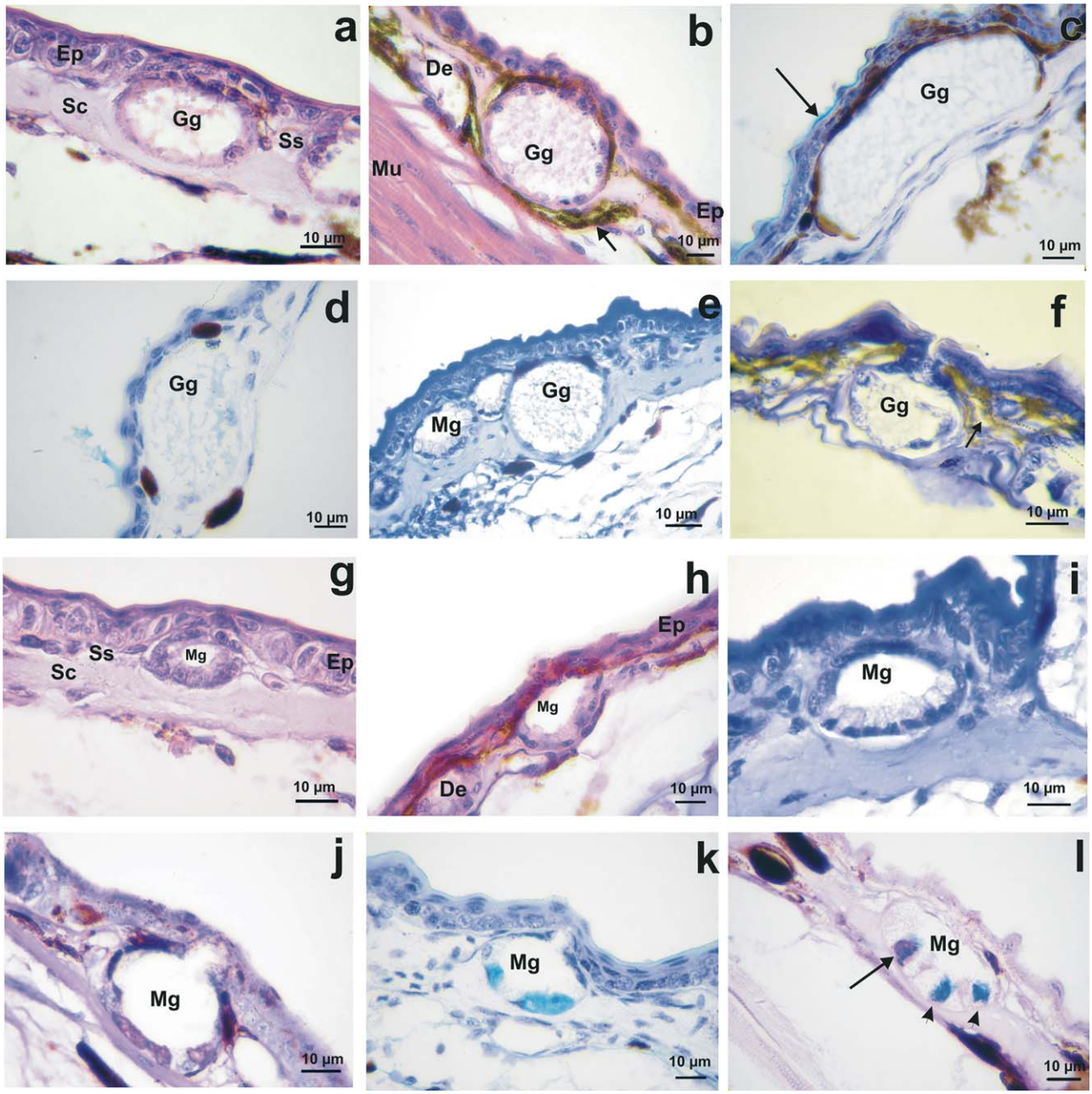


Fig. 3. *Rhinella arenarum* skin in the trunk during the metamorphic climax. (**a**)–(**f**) Development of granular glands. (**g**)–(**l**) Development of mucous glands. (a) Dorsal skin of tadpole in stage G42. H–E staining. Notice that the secretory cells form a syncytium from the beginning of gland development. (b) Dorsal skin of tadpole in stage G46. H–E staining. Arrow points to a melanophore. (c) Dorsal skin of tadpole in stage G46. AB staining. Notice the apical layer of alcianophilic mucus (arrow). (d) Ventral skin of tadpole in stage G46. AB staining. Note the absence of alcianophilic mucus inside granular glands by the end of metamorphosis. (e) Dorsolateral skin of tadpole in stage G46. Coom staining. Note the absence of protein content inside granular glands by the end of metamorphosis. (f) Dorsal skin of tadpole in stage G46. Chromaffin reaction is negative inside granular glands. Arrow points to brown melanophores below the epidermis. (g) Ventral skin of tadpole in stage G42. H–E staining. (h) Dorsal skin of tadpole in stage G46. Notice melanophore prolongations in the dermis as brownish-red deposits. H–E staining. (i) Dorsal skin of tadpole in stage G46. Coom staining is negative in mucous glands by the end of metamorphosis. (j) Dorsal skin of tadpole in stage G46. PAS-H staining. Notice that only some cells are positive for neutral glycoconjugates (notice the presence of cells with pink cytoplasm). (k) Dorsolateral skin of tadpole in stage G46. AB staining. Notice that only some cells are positive for acidic glycoconjugates (notice the presence of cells with blue cytoplasm). (l) Dorsal skin of tadpole in stage G46. PAS + AB staining. Note that only one cell is positive for both PAS and AB (arrow). Arrowheads point to cells that are only positive to AB. Dermis (De); Epidermis (Ep); Granular gland (Gg); Mucous gland (Mg); Muscle (Mu); *Stratum compactum* (Sc); *Stratum spongiosum* (Ss).

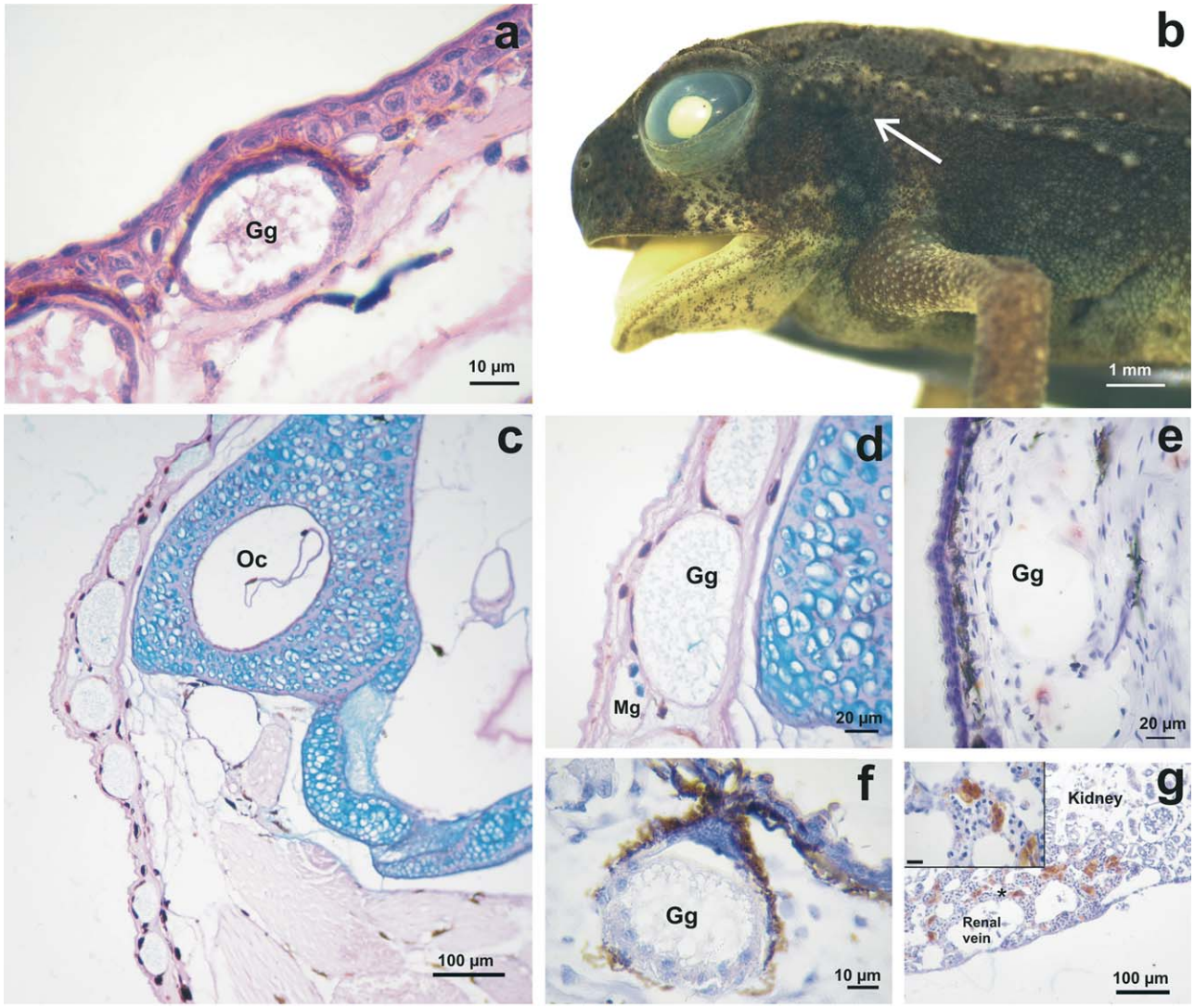


Fig. 4. *Rhinella arenarum* skin in the postorbital region during the metamorphic climax. (**a**) Tadpole in stage G42. H–E staining. Notice that by the beginning of the metamorphic climax granular glands in the parotoid region have a similar morphology to granular glands in the rest of the body but are larger and closer together. (**b**) Lateral view of tadpole in stage G46 fixed with Bouin's fluid. White arrow points to developing parotoid gland. (**c**) Transverse section of tadpole in stage G46. PAS + AB method. Note the protrusion of the skin corresponding to the parotoid gland. (**d**) Detail of the skin near the otic capsule (Oc) in (c). (**e**) Tadpole in stage G46. Sudan Red III method show negative results by the end of metamorphosis. (**f**) Tadpole in stage G46. Chromaffin reaction is negative in granular glands by the end of metamorphosis. Brown structures surrounding the gland are melanophores. (**g**) Positive control of the chromaffin reaction. Transverse section of interrenal gland of adult *R. arenarum* (*). Inset in (g) shows detail of brown chromaffin cells. Scale bar: 20 µm. Granular gland (Gg); Mucous gland (Mg).

Histological and Histochemical Procedures

After euthanasia, samples of approximately 50 mm^2 of dorsal and ventral skin, as well as parotoid glands from adult specimens were rapidly excised and washed in phosphate buffer (pH 7.2). For histological studies, except for lipid detection and chromaffin reaction, skin samples of adults and whole tadpoles ranging from stages G27 to G46 were fixed in Bouin's solution for 24 h, embedded in paraffin, and then sectioned in a transverse plane at 6 µm (Kiernan, 1999). Sections were stained with haematoxylin and eosin (H-E) or modified Masson's trichrome (MMT) stain for general cytology and histology. MMT is different from the original Masson's trichrome in that the acid fuchsin solution also contains orange G. In addition, the following histochemical stains were performed on selected sections to characterize the secretory products of the different gland types: periodic acid-Schiff-haematoxylin (PAS-H; Kiernan, 1999) for neutral glycoconjugates, Alcian blue 8GX at pH 2.5 plus haematoxylin or safranin (AB; Kiernan, 1999) for primarily carboxylated acidic glycosaminoglycans, and Coomassie blue R250 (Coom; Kiernan, 1999) for proteins. The combination of PAS and AB was employed to detect cosecretion of both neutral and acidic glycoconjugates according to Kiernan (1999). We analyzed at least 3 individuals of each Gosner stage and at least 3 different skin samples of adults. Mucous glands of adult skin and cartilage of

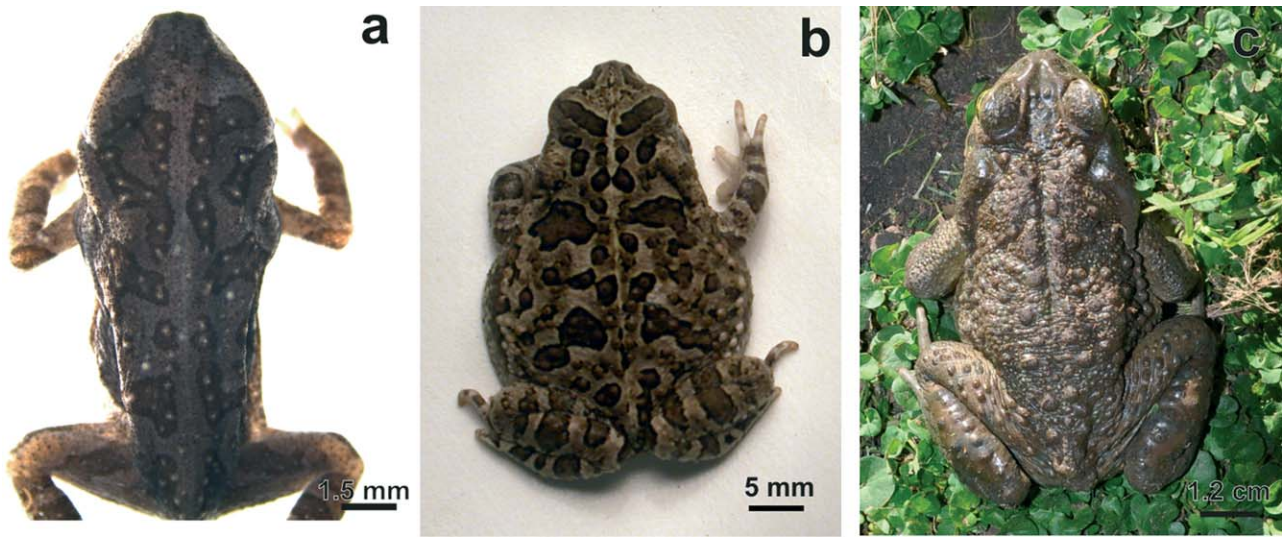


Fig. 5. *Rhinella arenarum*. (**a**) Dorsal view of 10-day-old juvenile showing the cryptic coloration pattern of the skin. Formalin-fixed specimen. (**b**) Dorsal view of older juvenile collected in the field showing the cryptic coloration pattern of the skin. (**c**) Dorsal view of an adult specimen without the cryptic coloration pattern.

tadpoles were used as positive controls for PAS and AB; a keratinized portion of the dorsal epidermis of adults was used as a positive control for Coom.

For detection of catecholamines in gland secretions, skin samples were subjected to the chromaffin reaction according to Kiernan (1999). Afterwards they were sectioned in a transverse plane at 6 μm, mounted onto microscope slides, deparaffinized in xylene and mounted with synthetic mounting medium. Some sections were also stained with haematoxylin to better visualize the topographic context. The interrenal gland of a male adult *R. arenarum* was used as a positive control of the chromaffin reaction due to the presence of catecholamine producing chromaffin cells (Regueira et al., 2013; Fig. 4g). Positive chromaffin cells with this technique exhibit the same characteristics as those detected by immunohistochemistry using an antibody against tyrosine hydroxylase, the key enzyme in the production of catecholamines (Regueira et al., 2013). This control validates the chromaffin reaction as a useful technique to detect catecholamines.

Lipid derived secretions, i.e. bufadienolides, other cardiotonic steroids and lipids in general, were detected in frozen sections using Sudan Red III and haematoxylin staining (Kiernan, 1999). Tadpoles and skin fragments were fixed in a 10% formalin-calcium solution for 2 days and embedded in a polyacrylamide solution to improve the quality of frozen sections (Hausen and Dreyer, 1981). Samples were frozen at −70°C and sectioned in a transverse plane at 20 μm with a cryostat. Intestine of tadpoles and mouse fat cells served as positive controls of the Sudan III technique (data not shown).

Fragments of skin and tadpoles were analyzed with a stereoscopic microscope Leica EZ4D and macroscopic images were captured with an incorporated digital camera. Low magnification pictures of the specimens were captured with a Nikon coolpix 995 3.34 Mp digital camera. Stained sections were examined using a Zeiss Primo Star microscope, and images were captured using a Canon PowerShot A640 digital camera.

RESULTS

The Skin in the Larval Period

Tadpoles of *R. arenarum* had a dark brown dorsal skin with longitudinally arranged light brown spots (Fig. 1a). Up to stage G30, the skin consisted of a single layer of epidermis, with highly heterochromatic cell nuclei, and without any glandular cells. Melanophores only occurred in the thin dermis, which consisted of a thin layer of dense connective tissue (Fig. 1b). At stage G30, the skin developed into a two-layer epidermis with two types of scattered glandular cells: mucous cells (Fig. 1c) and big secretory cells, with the characteristics of the giant cells (Riesenzellen) described by Pfeiffer (1966; Fig. 1d). The former were cylindrical cells with basal nuclei and a clear cytoplasm, which contacted the basal membrane and the surface of the epidermis, typical characteristics of mucus-secreting cells (Fig. 1c). The latter were scattered throughout the epidermis, both in dorsal and ventral skin, located always adjacent to an epidermal melanophore. The eccentric nucleus of the giant cell always lay opposite the nucleus of the melanophore, and numerous fine pigment granules covered the apical side of the cell (Fig. 1d). Giant cells were round or oval with a main axis between 20 and 28 μm long, and did not appear to reach the skin surface (Fig. 1d–f). By stage G35, giant cells tested positive for acidic and neutral glycoconjugates (Fig. 1e,f), and negative for proteins and lipids (data not shown). The larval epidermis also contained an external layer of a mucus-like substance, which stained positive for acidic glycoconjugates (AB; Fig. 1e). The first multicellular gland anlage was observed in the epidermis of tadpoles in stage G36 in the dorsal middle region of the body, a stage by which external myoepithelial cells and central secretory cells

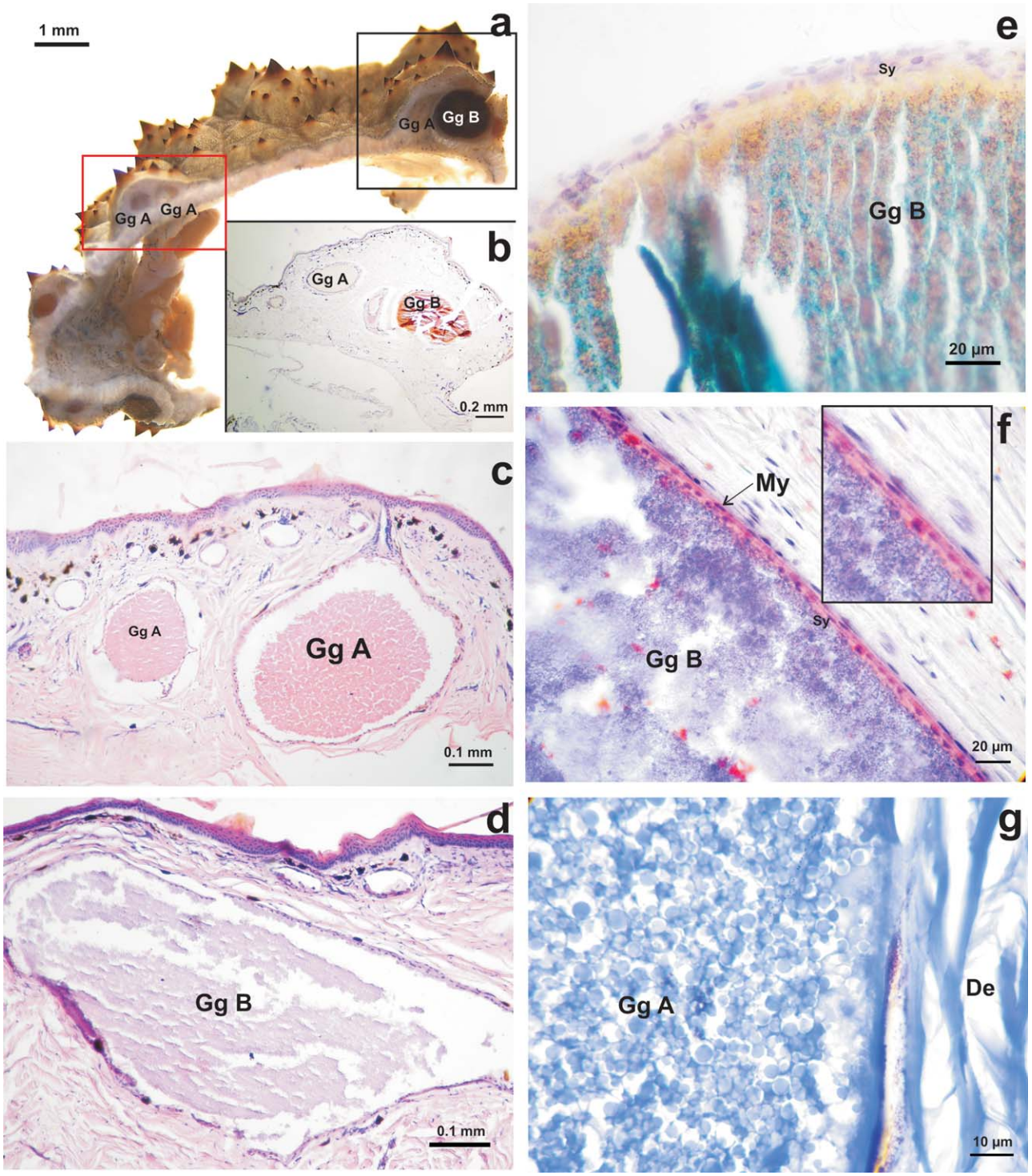


Fig. 6. Granular glands in dorsal skin in the trunk of adults of *Rhinella arenarum*. (**a**) Macroscopic view of a section of skin fixed for analysis of the chromaffin reaction. Black rectangle highlights a big wart with two types of glands; red rectangle shows a small wart with only one type of gland. (**b**) Histological section of the big wart shown in (a). Chromaffin reaction and haematoxylin. Notice the presence of granular glands type A and B, the latter being positive for the detection of catecholamines. (**c**) Small wart with granular glands type A. H–E staining. (**d**) Big wart with granular glands type B. H–E staining. (**e**) Granular gland type B. Chromaffin reaction, haematoxylin and AB staining. Note that the content of granular glands type B has acidic glycoconjugates and catecholamines. (**f**) Granular gland type B. Sudan Red III and haematoxylin staining. Notice that the cytoplasm of the syncytium around the basal nuclei and myoepithelial cells stain positive for lipid. Arrow points to myoepithelial cell. Inset shows higher magnification of syncytium and myoepithelial cells. (**g**) Granular gland type A. Coom staining shows the presence of proteins inside granular glands type A. Dermis (De); Granular gland type A (Gg A); Granular gland type B (Gg B); Myoepithelial cells (My); Syncytium (Sy).

TABLE 1. Skin glands in adults of *R. arenarum*.

Region of the body	Dermal gland type	Histological characteristics	Distinctive features
Dorsal skin	Mucous gland	AB (+), PAS (+), Coom (+), Sudan III (−), Chromaffin (−)	Proteins and glycoconjugates are produced by the same cells in the secretory epithelium
	Granular gland A	Acidophilic granules, AB (−), PAS (−), Coom (+), Sudan III (−), Chromaffin (−)	Granular content. Produces proteins
	Granular gland B	Basophilic content, AB (+),PAS (−), Coom (−), Sudan III (+), Chromaffin (+)	Produces catecholamines, lipid−derived products and acidic glycoconjugates
Ventral skin	Mucous gland	= Mucous gland in dorsal skin	= dorsal skin
	Ventral granular gland	AB (+), PAS (−), Coom (−), Chromaffin (−)	Gland content with spongy appearance. Produces acidic glycoconjugates
Parotoid skin	Peripheral granular gland	= Granular gland A in dorsal skin	Large Granular gland A
	Central granular gland	= Granular gland B in dorsal skin	Large Granular gland B
	Duct gland	Acidophilic cytoplasm, AB (−), PAS (+), Coom (+), Sudan III (−), Chromaffin (−)	Differentiated mucous glands around the duct of Central granular gland
	Granular gland A	= dorsal skin	= dorsal skin
	Mucous gland	= dorsal skin	= dorsal skin

Histological techniques and abbreviations: AB: Alcian blue 8GX at pH 2.5 (acidic glycoconjugates); PAS: Periodic acid-Schiff (neutral glycoconjugates); Coom: Coomassie blue R250 (proteins); Sudan Red III (lipids); Chromaffin reaction (catecholamines).

were already differentiable (Fig. 2a). By the end of prometamorphosis, granular glands contained syncytial acini, while giant cells were still present in the epidermis (Fig. 2b).

The Skin During the Metamorphic Climax

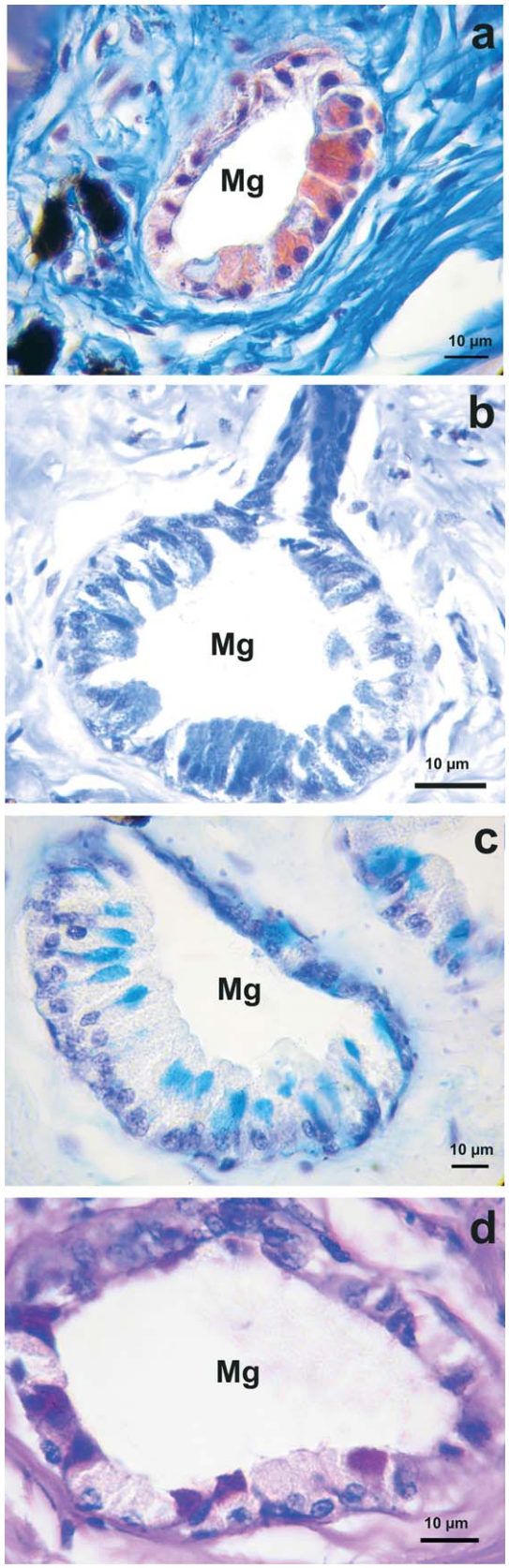
By stage G42, the epidermis of *R. arenarum* consisted of 2 or 3 layers of epidermal cells (Fig. 3a,g). The dermis became thicker and a thin *stratum spongiosum* became visible between the epidermis and the dense connective tissue of the *stratum compactum* (Fig. 3a,g). At the end of the metamorphic climax, granular glands developing in the trunk of the body contained granular content and acidophilic cytoplasm surrounding the basal nuclei, and were surrounded by dermal melanophores (Fig. 3b). Histochemical analysis showed that the glands tested negative for acidic glycoconjugates, both in the dorsal and ventral regions of the body, and for proteins and catecholamines (Fig. 3c–f). In addition, as occurred in larval skin, the epidermis of metamorphosing tadpoles had an external layer of a mucus-like substance which stained positive for acidic glycoconjugates (Fig. 3c). At this time, the acini of mucous glands in the trunk of the body consisted of simple, cylindrical secretory cells with basal nuclei, orderly arranged around a narrow lumen (Fig. 3h). Histochemical analysis showed that the secretory cells of mucous glands in late metamorphosing tadpoles were negative for proteins (Fig. 3i) but positive for acidic and neutral glycoconjugates (Fig. 3j,k). In addition, while some cells tested positive for both AB and PAS, other cells only tested positive for one type of glycoconjugate (Fig. 3l). Granular and mucous glands at the end of metamorphosis already displayed a neck and a short duct (Fig. 3f,j,k).

Histological studies of the skin in the postorbital region of tadpoles showed that by stage G42 granular glands in the parotoid region had similar morphology to the granular glands in the rest of the body, but were larger and located closer together (Fig. 4a). Tadpoles at the end of the metamorphic climax and early juveniles up to 10 days postmetamorphosis exhibited small protrusions in the parotoid region followed by a lateral skin fold (Fig. 4b). Transverse histological sections of the postorbital region showed a protuberance with enlarged dermal glands in the skin attached to the otic capsule (Fig. 4c). The staining with AB-PAS showed a slight positive reaction to acidic glycoconjugates in the accumulated secretion of the granular glands and in some cells of the nearest mucous gland (Fig. 4d). On the other hand, the study of lipid-derived products or catecholamines in the granular glands of parotoids in newly metamorphosed toadlets showed negative results (Fig. 4e,f).

In addition to the developing dermal glands, juveniles possessed cryptic coloration patterns not present in adults (Fig. 5a–c). The back of juveniles had symmetrically arranged brown patches, delimited by dark brown lines (Fig. 5a,b).

The Skin of Adults: The Trunk Region

Dorsal skin of the adult trunk exhibited different types of epidermal projections, as occurs in other anurans (Fig. 6a). As we did not observe differences between males and females for the studied skin regions, results for each sex are not shown separately. Table 1 summarizes skin gland types observed in adults. The biggest warts contained granular glands of two types: **Type A** (Acidophilic) small granular glands, which stained negative for catecholamines and had an acidophilic granular



content (Fig. 6a–c), and **Type B** (Basophilic) large granular glands, positive for detection of catecholamines, with basophilic content (Fig. 6b,d). The accumulated secretion of granular glands type B also tested positive for acidic glycoconjugates (Fig. 6e), whereas the cytoplasm of the syncytium around the basal nuclei tested positive for lipids (Fig. 6f). As observed in Fig. 6f, myoepithelial cells surrounding the syncytium were also positive for lipids. On the other hand, the syncytium of granular glands type A tested negative for lipids (data not shown) and their granular content tested positive for proteins (Fig. 6g). The smaller warts only contained granular glands type A (Fig. 6a). Mucous glands were uniformly distributed in large and small warts and were found in the apical portion of the *stratum spongiosum* (Fig. 7). The secretory cells of mucous glands consisted of different types of cylindrical tall cells with basal oval nuclei (Fig. 7a) that tested positive for proteins (Fig. 7b). Some of these cells were also positive for acidic and neutral glycoconjugates (Fig. 7c,d).

A different type of granular gland was present in ventral skin. It had a spongy appearance and tested positive for acidic glycoconjugates (Fig. 8a) and negative for proteins (data not shown). Ventral skin mucous glands had the same morphology and histochemical characteristics of dorsal mucous glands (Fig. 8b–d).

The Skin of Adults: The Parotoid Gland

Fully developed parotoid glands could be easily dissected and removed from the body when the dorsal skin was cut around them. Macroscopic analysis of transverse sections of this macrogland showed tightly packed large glands in the dermis, with central glands being larger than peripheral glands (Fig. 9a). In transverse histological sections, macroglands were characterized by the accumulation of large, elongated, syncytial granular glands arranged side by side deep in the dermis, below the layer formed by the much smaller regular skin glands (Fig. 9b,c). Central glands had basophilic content (Fig. 9b), and were negative for proteins (Fig. 9c) and positive for acidic glycoconjugates and catecholamines (Fig. 9d,e). The cytoplasm of the syncytium around the basal nuclei and myoepithelial cells tested positive for lipids (Fig. 9f). These results show that central glands in the parotoids are similar to granular glands type B from dorsal skin. On the other hand, peripheral glands had an acidophilic granular content (Fig. 9b), were positive for proteins (Fig. 9c) and negative for acidic glycoconjugates and catecholamines (Fig. 9d,e), and the syncytium was also negative for lipids (data not shown). All these characteristics suggest that peripheral glands are enlarged dorsal granular glands type A.

Immediately below the epidermis, the dermis contained regular granular glands type A and mucous

Fig. 7. Mucous glands in dorsal skin in the trunk of adults of *Rhinella arenarum*. (**a**) Notice the presence of different types of secretory cells in the epithelium. MMT staining. (**b**) Coom staining shows that the secretory cells of mucous glands are positive for protein content. (**c**) AB staining shows acidic glycoconjugates inside some cells of the epithelium. (**d**) PAS-H staining shows neutral glycoconjugates in some cells of the epithelium. Mucous gland (Mg).

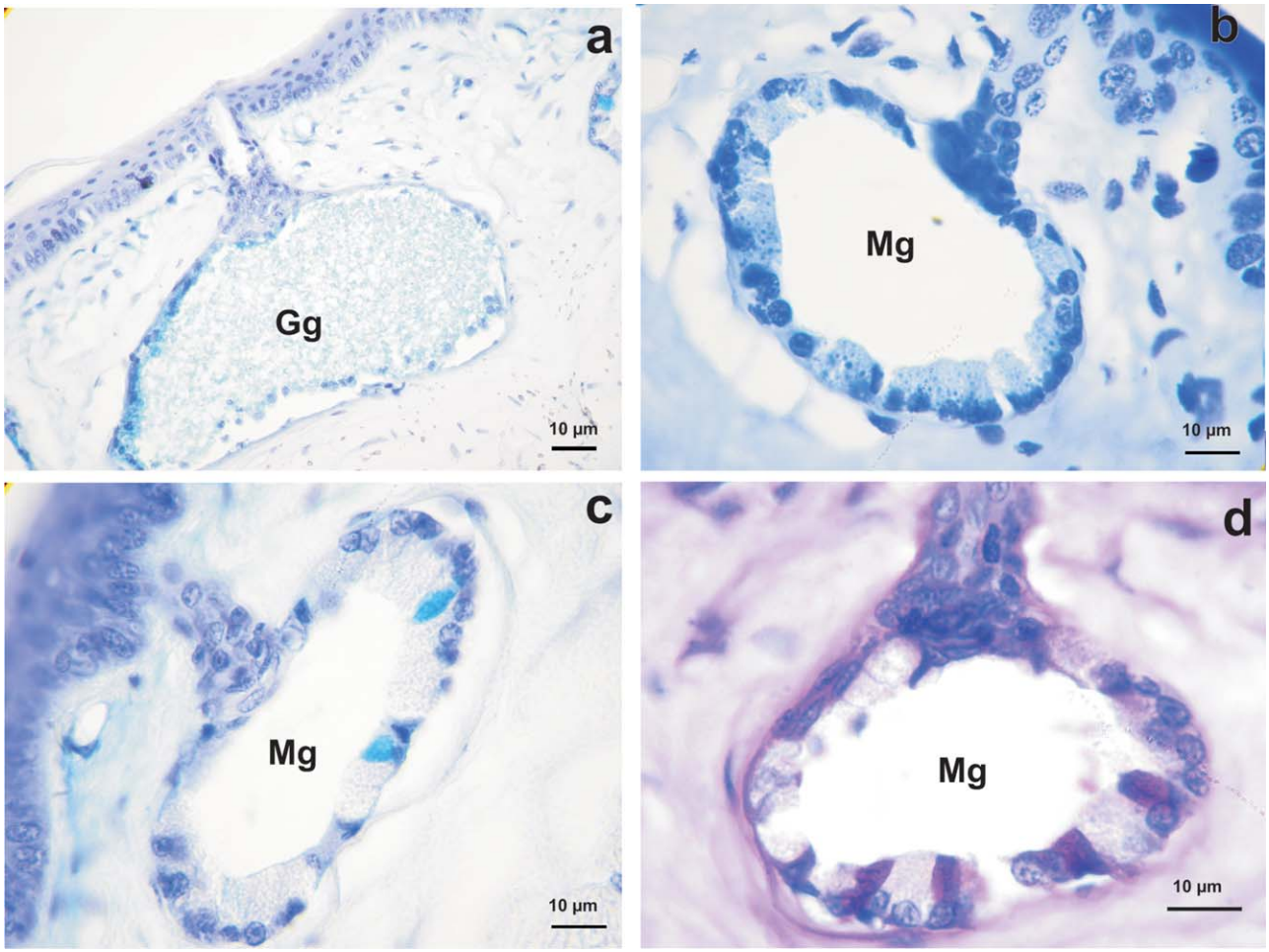


Fig. 8. Ventral skin in the trunk of adults of *Rhinella arenarum*. (**a**) Granular gland. (**b**)–(**d**) Mucous gland. (a) AB staining. Notice the presence of content with spongy appearance inside ventral granular glands, positive for acidic glycoconjugates. (b) Coom staining. (c) AB staining. (d) PAS-H staining. Note that ventral mucous glands have the same morphology and histochemical characteristics of dorsal mucous glands. Granular gland (Gg); Mucous gland (Mg).

glands with the same histochemical characteristics as those from the dorsal skin (Fig. 9b,c). In addition, a different type of mucous gland was observed around the duct of central glands, with their ducts in turn opening into the main duct of the big central glands (Fig. 10a). The secretory cells of these differentiated mucous glands consisted of tall cells with central nuclei, full of homogeneous acidophilic secretion (Fig. 10b), strongly reactive to the detection of proteins and neutral glycoconjugates (Fig. 10c,d).

Ontogenetic changes and timing of appearance of different secretory structures of the skin are summarized in Fig. 11.

DISCUSSION

Defensive strategies against predators vary with habitat type; larval anurans with indirect development must therefore change their defensive strategies throughout the life cycle when switching from an aquatic larva to a terrestrial adult (Pfeiffer, 1966; Toledo and Jared, 1995; Gunzburger and Travis, 2005; Hayes et al., 2009; Toledo et al., 2011). Particularly in bufonids, it is well documented that the structures of the skin related with chemical defenses change along the ontogenesis (Pfeiffer, 1966; Delfino et al., 1995, 2001; Toledo and Jared, 1995). During larval stages, the epidermis of *Rhinella arenarum* presents glandular giant cells (Riesenzellen), which in other bufonids secrete pheromones that trigger a flight reaction of the tadpole school (Pfeiffer, 1966; Fox, 1988). Riesenzellen appear to be a unique feature of Bufonidae since they have been described in tadpoles of *Bufo bufo, Epidalea calamita, Amietophrynus regularis, Rhinella granulosa*, and results from our work with *Rhinella arenarum* (Pfeiffer, 1966; Fox, 1988; Delfino et al., 1995; Chammas et al., 2014), but were not described in other anurans. Presence of giant cells appears to be associated with social behavior of tadpoles since in all the species where they have been described, tadpoles present gregarious behavior, and contrary to nonsocial tadpoles, gregarious individuals could easily respond to alarm substances (Caldwell, 1989; Kehr,

1994; Delfino et al., 1995; Griffiths and Foster, 1998). Because social tadpoles occur in species with large egg clutch sizes (van Bocxlaer et al., 2010), histological studies that might account for the presence of this type of cell in Bufonidae species with small egg clutch sizes, such as the basal bufonids *Melanophryniscus* spp. and *Dendrophryniscus* spp., would be particularly interesting. Furthermore, it would be an appealing idea to evaluate if the presence of giant cells in tadpoles is a trait related with a fast range-expansion phenotype in extant species of Bufonidae, whose adults are not dependent on the constant availability of water bodies, possess parotoid glands, inguinal fat bodies, large sizes, and large egg clutch sizes, among other traits (van Bocxlaer et al., 2010). In addition to our proposal, it would be interesting to evaluate if species of non bufonid anurans with social behavior have secretory cells with similar characteristics to giant cells. Gregarious tadpoles occur in diverse species of anurans, including the genus *Scaphiopus*, *Xenopus, Rhinophrynus, Lithobates, Pseudacris*, *Hypsiboas, Leptodactylus*, among others (Wassersug, 1973; AmphibiaWeb, 2005).

Despite some initial misunderstanding in the past regarding the distinctive characteristics of giant cells, Fox (1988) clarified that these cells are secretory, tightly attached to an epidermal melanophore. In *R. arenarum*, giant cells produce acidic and neutral glycoconjugates and do not seem to contact the exterior. Regarding the study of the chemical nature of alarm substances, two types of approaches have been taken: ultrastructural and histochemical studies. As in *R. arenarum*, Le Quang Trong (1973) also described acidic glycoconjugates inside giant cells of *Amietophrynus regularis*. Delfino et al. (1995) reported that giant cells of *B. bufo* have a well developed smooth endoplasmic reticulum and mitochondria with tubular cristae, which, according to the authors, could be related to steroid biosynthesis. In *R. arenarum*, the analysis of lipid content in giant cells showed negative results and these differences between species could be due to a different chemical nature of the alarm substance produced by each species. Another disparity between studies regarding giant cells in different species is whether they contact the exterior or not. Differences could be related to the methodologies employed in each study or, as mentioned by Fox (1988), to the fact that giant cells only open to the exterior temporarily. Except for the study by Delfino et al. (1995) in *B. bufo*, ultrastructural studies in that same species and in *Rhinella granulosa* have demonstrated that giant cells do make contact with the exterior of the skin (Fox, 1988; Chammas et al., 2014). According to Fox (1988), the alarm substance would be secreted from giant cells in response to rapid movements of body muscles during escape from danger. On the contrary, behavioral experiments by Pfeiffer (1966) showed that the alarm substance was only secreted when the skin was damaged. Evidently, the mechanism of secretion of the alarm substance from giant cells is still not clear.

By the beginning of the metamorphic climax, the skin of *R. arenarum* no longer contains giant cells, and multicellular glands are not fully developed. Anlagen of multicellular glands appear during prometamorphosis as in other anuran species (Delfino et al., 1995, 2001; Terreni et al., 2003). The ontogenetic shift from unicellular glands to complex glands suggests that tadpoles of *R. arenarum* are not dependent on a chemical skin defense system during metamorphic climax. Nevertheless, both prometamorphic and metamorphosing tadpoles of *R. arenarum*, as those of other anuran species, possess an apical layer of acidic glycoconjugates covering the epidermis, which may produce an unpleasant taste of bufonid tadpoles for predators (Gunzburger and Travis, 2005; Chammas et al., 2014). Multicellular glands develop from epidermal cells as in all other amphibian species (Delfino et al., 1998; Terreni et al., 2003), and differentiate into one of two lineages of glands: acinar glands with lumen (mucous glands), and syncytial glands (granular glands). Some cells of the acinar glands begin to produce glycoconjugates by the end of the metamorphic climax but they still do not produce the protein secretion characteristic of adults. Syncytial glands from the trunk of the body or in the parotoid region of tadpoles and early toadlets do not yet produce the proteinaceous or basophilic secretion of granular glands types A or B of adults. Our results suggest that early toadlets would count on mucus producing glands to help in avoiding dehydration in their new terrestrial habitat but are not yet protected by a chemical skin defense mechanism. Even though we found immature granular glands at the end of metamorphosis, a chemical study of toxin profiles in the related species *Rhinella marina* (*R. arenarum* belonging to the *R. marina* group; Maciel et al., 2010) indicates that the increased content of bufadienolides and their toxicity is coincident with the formation of granular glands towards the end of prometamorphosis (Brodie, Formanowicz and Brodie, 1978; Hayes et al., 2009). Differences between our histological results and the chemical study of toxin profiles in these two species could be due to interspecific differences in the ontogeny of the chemical defense, to the lower sensitivity/specificity of the histochemical analysis, or to the fact that the chemical study of toxin profiles was performed in whole tadpoles instead of skin fragments (Hayes et al., 2009). Unlike most anurans, where the adult skin develops during metamorphic stages (Fabrezi et al., 2010; Saporito et al., 2010), or even during prometamorphic stages in some ceratophryd frogs (Quinzio and Fabrezi, 2012), *R. arenarum* seems to have a delayed pattern regarding development of the adult skin. Although other works have also studied the morphological development of the skin in other bufonid species (Hayes, 1995; Hayes and Gill, 1995; Chammas et al., 2014), they do not specifically mention the existence of a delayed pattern in the ontogenesis of skin glands. Therefore, whether this characteristic is typical of bufonids in general or an exclusive trait of *R. arenarum* remains unresolved.

The fully developed skin of adults of *R. arenarum* contains different gland types with a characteristic topographic distribution. Interestingly, both granular and mucous glands in ventral skin produce mucus, although mucous glands also synthesize proteins which may contribute to the antibiotic and antifungal function of the skin mucus (Toledo and Jared, 1995). Dorsal skin of *R. arenarum* has polymorphic granular glands, as previously described in other bufonid species (Delfino et al., 1999); however, their distinct topographic distribution has not been previously described. In contrast to ventral skin, granular glands located between the warts of dorsal skin produce a proteinaceous secretion (granular glands type A), and secretion of mucous glands has high

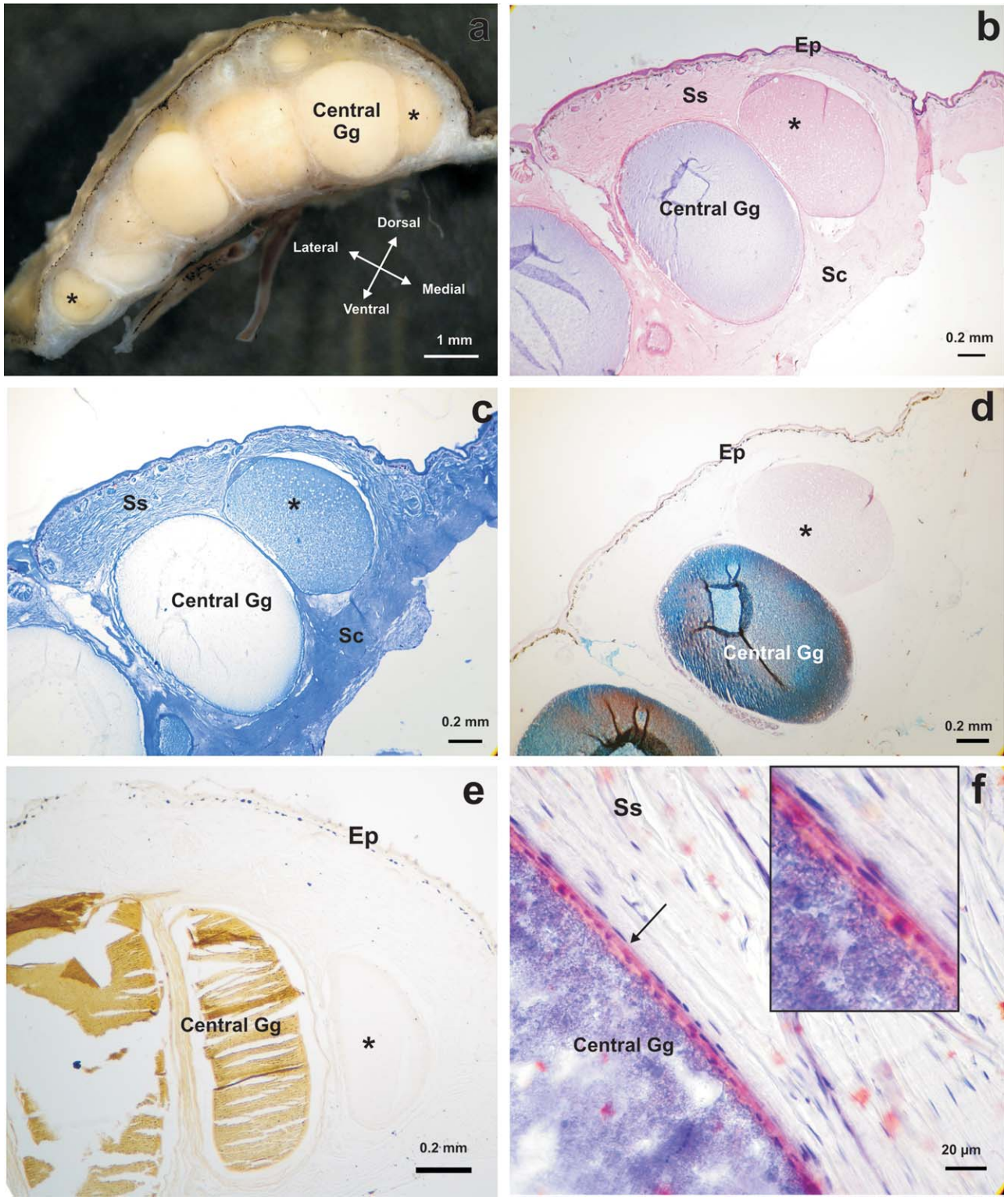


Fig. 9. Parotoid gland of adults of *Rhinella arenarum*. (**a**) Macroscopic view of transverse section of the parotoid gland. (**b**)–(**l**) Histological transverse sections of a parotoid gland. (b) Dorsal portion showing peripheral and central glands. H–E staining. (c) Coom staining. Notice the difference in staining between central and peripheral glands. (d) AB and safranin staining. Observe the presence of acidic glycoconjugates inside central glands. (e) Chromaffin reaction and haematoxylin. Note the presence of catecholamines inside central glands. (f) Detail of the central gland. Sudan Red III and haematoxylin staining. Notice that the cytoplasm of the syncytium around the basal nuclei and myoepithelial cells stain positive for lipid. Inset shows higher magnification of syncytium and myoepithelial cells. Epidermis (Ep); Granular gland (Gg); Peripheral gland (*); *Stratum compactum* (Sc); *Stratum spongiosum* (Ss).

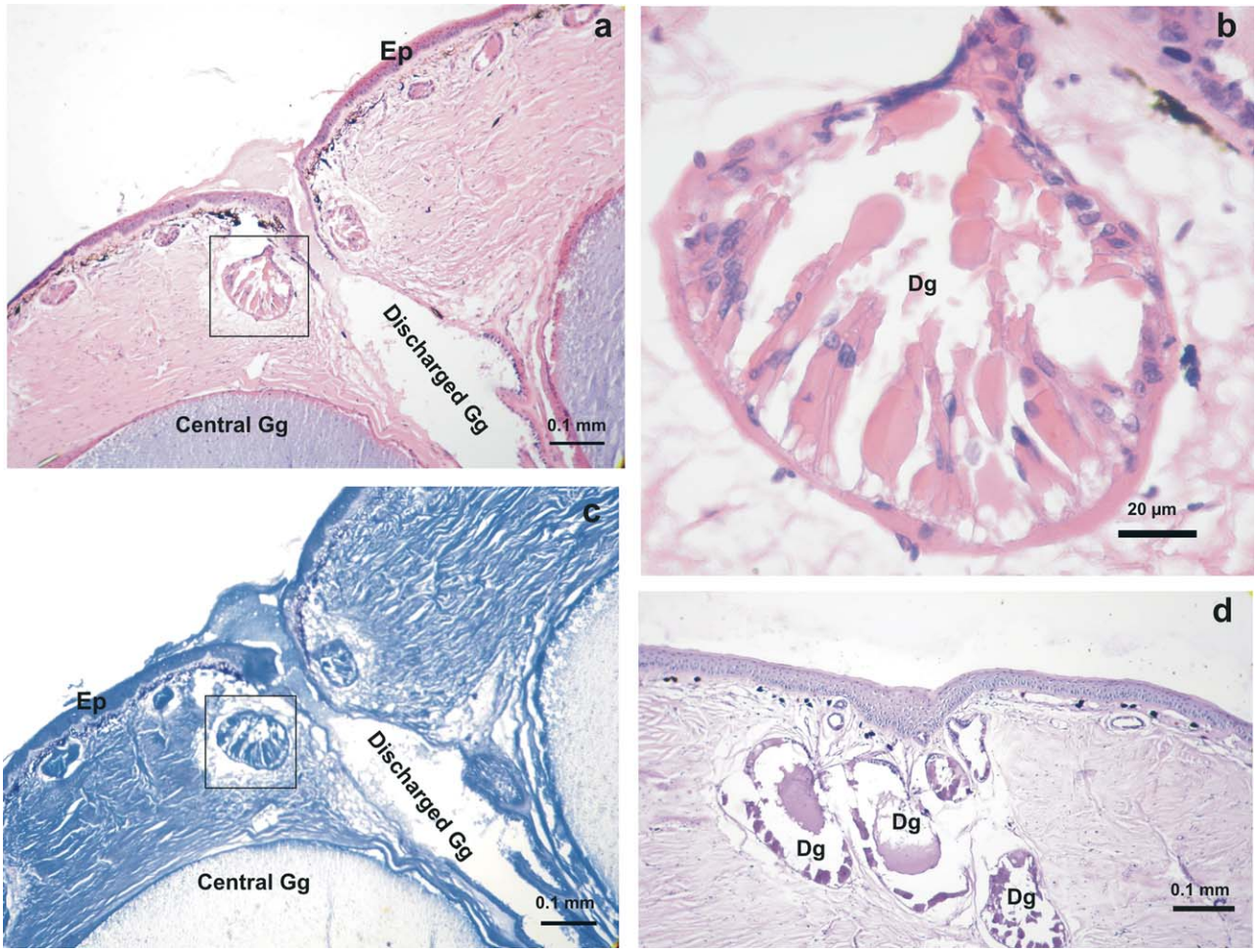


Fig. 10. Specialized mucous glands (duct glands) around the duct of big granular glands in parotoid of *Rhinella arenarum*. (**a**) H–E staining. Note that the duct of specialized mucous glands opens into the duct of the big central glands. (**b**) Detail of the rectangle drawn in (a) shows that secretory cells are tall cells with central nuclei. (**c**) Coom staining. Rectangle shows specialized mucous gland. (**d**) PAS-H staining. Notice the presence of proteins and neutral glycoconjugates inside cells of the specialized mucous glands. Duct gland (Dg); Epidermis (Ep); Granular gland (Gg).

protein content. Interestingly, granular glands in large dorsal warts produce catecholamines, a lipid-derived secretion, and acidic glycoconjugates (granular glands type B), similar to secretions typical of glands of bufonids found in the central portion of parotoid glands (Toledo and Jared, 1995). Our results show that the skin of *R. arenarum* presents at least three types of granular glands, which we designate A, after their acidophilic content, B, with basophilic content, and ventral, with mucus secretion. As was the case for granular glands type B of *R. arenarum*, granular glands in the head of the hylid frog *Litoria caerulea* present a Sudan positive peripheral cytoplasm and a Sudan negative central secretion (Warburg et al., 2000). As in both *R. arenarum* and *L. caerulea* the central content of the gland is negative to lipids, we hypothesize that the maturation processes during biosynthesis of poisonous secretion alters the lipid-derived product to a nonreactive molecule. In addition, both myoepithelial cells and the syncytium of granular glands were found to be positive to lipids in *R. arenarum*. It had previously been suggested by Hostetler and Cannon (1974) and Cannon and Hostetler (1976), that myoepithelial cells are involved in the synthesis of steroid-derived secretions in granular glands of toads though the functional role of myoepithelial cells in venom synthesis is still unknown.

Parotoid glands of *R. arenarum* exhibit the same organization as in other bufonid species (Hostetler and Cannon, 1974; Cannon and Hostetler, 1976; Toledo, Jared and Brunner, 1992; Felsemburgh et al., 2009; Jared et al., 2009; Chammas et al., 2014), which suggests that this is a highly conserved characteristic in this group. As the basal bufonid *Rhaebo guttatus* also presents a complex parotoid macrogland composed of highly packed glands (Jared et al., 2011; Mailho-Fontana et al., 2014), parotoids in bufonids seem to be a specialized macrogland different from parotoids of other groups. For instance, parotoids of hylids do not share the same complex arrangement as those of bufonids, and their secretion products are of a different chemical nature (Antoniazzi et al., 2013). Another example of a macrogland consisting of a complex arrangement of glands is

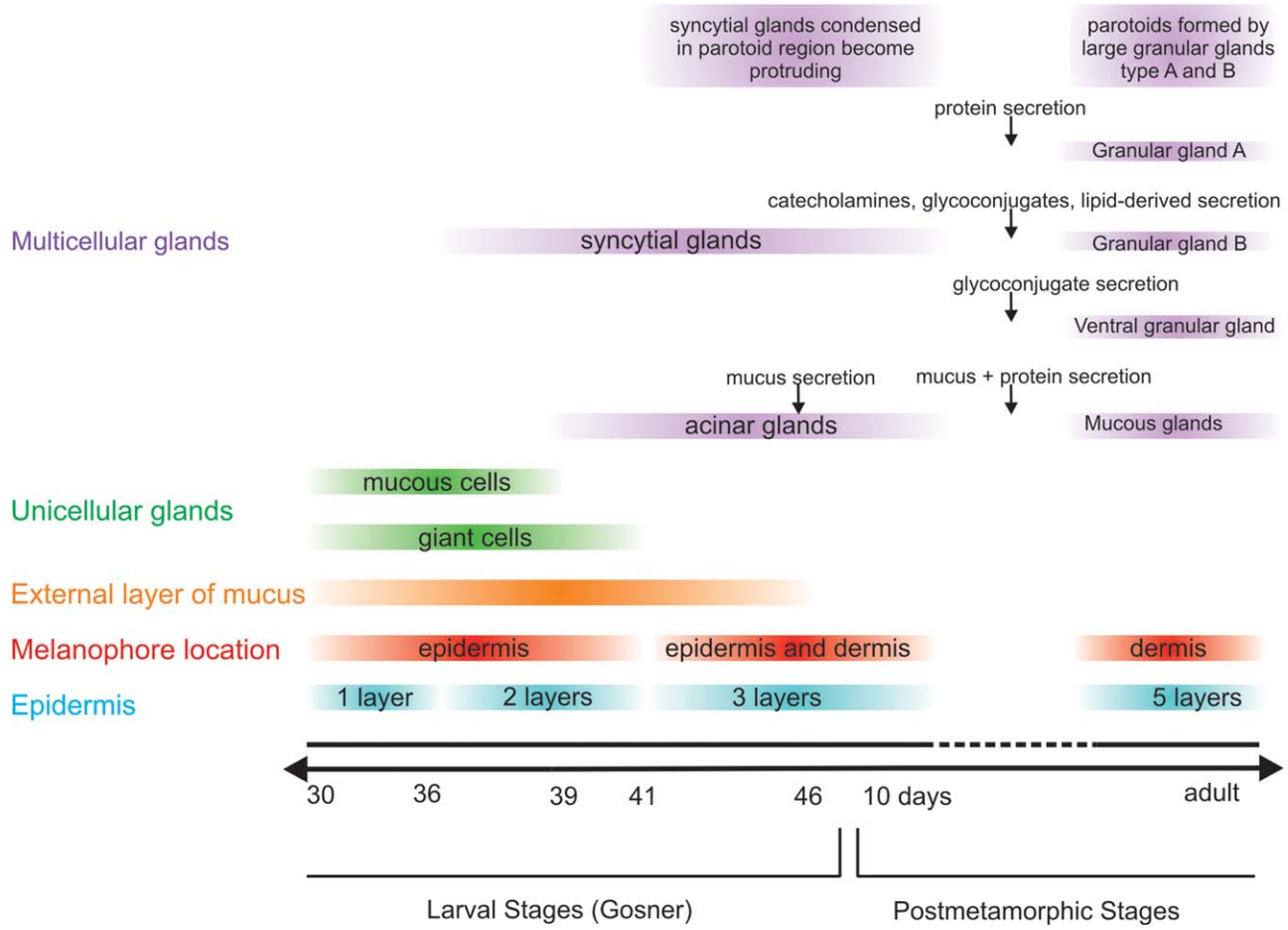


Fig. 11. Summary of the relative timing of developmental changes in the skin during the ontogeny of *Rhinella arenarum*. The graph is based on data from this study.

the femoral gland of some mantelline frogs, which consists of circularly arranged granular glands that secrete their products into a central external depression (Vences et al., 2007). These examples highlight the fact that, in some groups of anurans, skin macroglands are more than a mere accumulation of dermal glands. In contrast to what was described in other bufonids, in *R. arenarum* we observed that large peripheral and central glands constitute two different types of granular glands. Central glands produce catecholamines, a lipid-derived product, and acidic glycoconjugates, whereas peripheral glands produce a proteinaceous secretion. Secretions from parotoids in bufonids are composed mainly of biogenic amines and bufadienolides, proteins being present in extremely low abundance (Rash et al., 2011), signaling that the contribution of the peripheral glands to the total secretion of parotoids would be minimal. Our study of the ontogeny of the postorbital/supratympanic region showed that these diverse types of glands differentiate from similar syncytial glands, suggesting that the differentiation of the glands occurs during postmetamorphic development. A particular characteristic of the parotoid gland of bufonids is the presence of differentiated mucous glands around the duct of the large glands (Jared et al., 2009; Antoniazzi et al., 2013); in *R. arenarum*, these mucous glands were only observed around the duct of the central glands. Glands surrounding the ducts differ from the other mucous glands of the body, both in their function and their morphological characteristics. Regarding their function, Jared et al. (2009) described that enlarged specialized mucous glands discharge their content into the upper portion of the duct of central glands contributing to the secretion of these glands or contributing to the formation of a plug in the duct.

In summary, our results are consistent with previous studies (Warburg et al., 2000; Jared et al., 2009; Antoniazzi et al., 2013) in concluding that it would not be accurate to differentiate syncytial and acinar glands of toads based only on the appearance and/or chemical nature of the secretion that they produce (i.e. granular/serous and mucous glands), since it is far too variable. Additionally, we observed topographical differences in skin secretions of *R. arenarum*. Interestingly, granular glands from the big warts in the skin of *R. arenarum* produce toxins with similar characteristics to that of parotoid glands, i.e. catecholamines and lipid-derived secretions, but do not display the same organization as

the macroglands. We showed that early juveniles have nonfunctional parotoids, but also a cryptic coloration pattern that is not present in adults. A study concerned with the covariation in the development of parotoid and granular glands in the rest of the body in relation to animal body size during postmetamorphic growth would be necessary to complete our understanding about the ontogeny of chemical defenses.

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