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Lumbar glands in the frog genera *Pleurodema* and *Somuncuria* (Anura: Leiuperidae): histological and histochemical perspectives

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Abstract

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The skin in the lumbar region of nine species of *Pleurodema* and in the monotypic genus *Somuncuria* (Anura: Leiuperidae) bears macroglands [lumbar gland (LG)]. Lumbar glands of *Pleurodema bibroni*, *P. borellii*, *P. brachyops*, *P. bufoninum*, *P. cinereum*, *P. cordobae*, *P. kriegi*, and *P. thaul*, as well as *Somuncuria somuncurensis*, were examined using histological and histochemical methods. The epidermis and the dermis of LGs are described. Also, skin of LGs presents characteristic features as the interruption of the Eberth–Katschenko layer and the presence of a differentiated type of gland only observed in macrogland and not previously described for *Pleurodema* or *Somuncuria*; this is termed lumbar serous gland. These glands are filled with a granular product, which occasionally is immersed in a matrix. Differences in the secretory products of mucous and serous glands are described, as well as inter- and intraspecific variability of gland structure. The mode of toxin expulsion from macroglands and the homology between lumbar and inguinal glands among anuran families are discussed.

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Introduction

Amphibian skin is a complex organ formed by an epidermis and an underlying dermis, which possesses a *stratum spongiosum* and a *stratum compactum* (Elias and Shapiro 1957; Fox 1994). The Eberth–Katschenko (EK) layer lies between the dermal strata; it is formed by an amorphous substance in which calcium salts are deposited (Elkan 1968; reviewed by Toledo and Jared 1993). Two types of dermal glands developed in all living adult amphibians are considered synapomorphies of the group (Parsons and Williams 1963) – the mucous glands, usually associated with respiration and water balance, and the serous (or granular or poison) glands, related to defense mechanisms (Toledo and Jared 1995). Also, mixed (or seromucous) glands have been found exclusively in urodeles (Delfino *et al.* 1986 *in* Brizzi *et al.* 2002), whereas lipid glands occur in some anurans (Blaylock *et al.* 1976). In several anuran species, an aggregation of numerous secretory units in certain regions of the body constitutes the so-called macroglands. The most common are parotoid, paracnemid, lumbar, and hedonic glands (Quay 1972; Toledo and Jared 1995).

Leiuperidae includes seven neotropical anuran genera (Grant *et al.* 2006), five of which bear a pair of dorsolateral sacral macroglands, called lumbar glands (LGs) (*Edalorhina*, *Pleurodema*, *Somuncuria*) or inguinal glands (*Eupemphix* and *Physalaemus*). We differentiate LG and inguinal glands based on their form and position; the LG is prominent, oval, elevated, and visible when animal rests, whereas the inguinal gland is flat, subcircular, and partial obliterated by hind limb when animal rests. Eight of the fourteen members of the widely distributed South American genus *Pleurodema* have LGs (*P. bibroni*, *P. borellii*, *P. brachyops*, *P. bufoninum*, *P. cinereum*, *P. cordobae*, *P. kriegi*, and *P. thaul*; taxonomy follows Frost 2011 and Kolenc *et al.* in press). The monotypic *Somuncuria* endemic to the Meseta de Somuncurá (Río Negro Province, Argentina) possesses slightly developed LGs. *Somuncuria* bears osteological and karyological similarities with

Pleurodema (Lynch 1978); therefore, Grant *et al.* (2006) included *Somuncuria* in Leiuperidae. Species possessing inguinal or LGs sometimes display a deimatic behavior, in which the animal, when feels frightened, turns against the predator, lowers the head, and elevates the sacral region of the body, simulating a large animal with a pair of big eyes (reviewed by Toledo *et al.* 2011). This behavior was observed in *Pleurodema bibroni*, *P. borellii*, *P. brachyops*, *P. bufoninum*, *P. kriegi*, and *P. thaul* (Cei 1958, 1962; Donoso-Barros 1969; Vaz-Ferreira 1984; Martins 1989; Kolenc *et al.* 2009) and other leiuperid genera (Sazima and Caramaschi 1986; Lenzi-Mattos *et al.* 2005; Borteiro and Kolenc 2007).

Morphological information about *Pleurodema* LGs is scarce. Previous studies (Birabén 1929; Adam 1954; Rada de Martínez and Finol 1986; Toledo and Jared 1989; Mangione and Lavilla 2004) provided structural or ultrastructural descriptions of the glands of three species (*P. borellii*, *P. brachyops*, and *P. thaul*). The LGs of *S. somuncurensis* have not been studied.

The goals of the present study include the following: (1) a description of the morphology of LGs of *Pleurodema* and *Somuncuria*; (2) an analysis of morphological and histochemical variations of LGs in *Pleurodema borellii* and comparison with LGs of other species of the genus, as well as of *Somuncuria somuncurensis*; and (3) a description of the inter- and intraspecific variabilities.

Materials and Methods

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). LGs of *Somuncuria somuncurensis* and eight species of *Pleurodema* were examined (see list in Appendix). The samples were obtained from specimens housed at herpetological collections or recently collected in field trips and sacrificed by immersion in a 10% aqueous solution of tricaine methanesulfonate (MS-222). All specimens were fixed in 10% formalin and stored in 70% ethanol.

LGs were removed under stereomicroscope, as well as dorsal skin for comparative purpose. The dehydration process varied depending on the preservation of the specimen. Dehydration began in 70% ethanol for samples derived from ethanol-preserved specimens. The skin of recently collected specimens was washed with fresh phosphate buffer (pH 7.2) and dehydrated in ascending ethanol series (50–96%). Samples were cleared in butyl alcohol, paraffin-embedded, sectioned in transverse and sagittal plane (4–5 μm thick), and mounted onto microscope slides. Sections were stained with hematoxylin and eosin (HE; Martoja and Martoja-Pierson 1970), Masson trichrome stain (Bradbury and Gordon 1990), and Masson-Goldner's trichrome stain (M&G; Martoja and Martoja-Pierson 1970) for general cytology and histology. In addition, selected sections were tested with the following histochemical stains to characterize the secretory products of dermal glands: periodic acid-Schiff-hematoxylin (PAS-H; Cook 1990) for neutral carbohydrates, Alcian blue (AB) 8GX at pH 2.5 (Cook 1990) for primarily carboxylated acidic glycosaminoglycans, and Ninhydrin/Schiff (NS; Stevens 1990) for proteins. Stained sections were examined using a Nikon Eclypse 200 microscope, and the images were captured using a Sony DS-U2 digital camera. Measurements of dermal glands were taken with an ocular micrometer using a minimum of 10 randomly selected sections (in a transverse plane).

Results

Macroscopically, the LGs of *Pleurodema* are elongated and markedly protuberant, whereas those of *S. somuncurensis* are not well developed and are more easily observed with magnification (Fig. 1). The measurements of dermal glands are presented in Table 1. LGs of *Pleurodema borellii* will be described in detail, and only the differences between these and the macroglands of the other examined species will be presented in text. Table 2 summarized essential features of LGs, i.e., epidermis layers, specialized cells within epidermis, presence of EK layer in LG, chromatophores, and features of dermal glands.

LG of Pleurodema borellii

The dorsal skin (Fig. 2A) of the trunk of *Pleurodema borellii* differs from that in the lumbar region (Fig. 2B). The epidermis of the LG of *P. borellii* consists of five to seven layers that vary in form from cuboidal cells in the deepest layer to progressively more flattened and completely keratinized cells in the superficial layer. In the epidermis, cytoplasmatic processes of melanophores (Fig. 2C) and elongated flask cells (Fig. 2C insert) occur sporadically.

The superficial *stratum spongiosum* of the dermis of LGs consists of loose connective tissue with multicellular glands and blood vessels, whereas the deeper *stratum compactum* consists of dense connective tissue formed by collagen fibers. The EK layer, which occurs between both *strata* of the dermis of dorsal skin, is interrupted where LGs develop (Fig. 2B). The most superficial portion of the *stratum spongiosum* contains pigment cells (Fig. 2D). Iridophores are located in the uppermost region. They are oval and bear basal nuclei and birefringent granules in their cytoplasm; the granules stain turquoise-green with MG. Xanthophores are scarce and occur either below or between the iridophores. The deepest layer contains melanophores, uniformly distributed in some regions (Fig. 2E).

Three multicellular dermal glands can be distinguished in the *stratum spongiosum*, mucous glands (Fig. 2E), and two types of serous glands (Fig. 2B, F–I). These glands consist of an intra-epidermal duct, a neck, a secretory unit, and a contractile sheath (myoepithelium).

Duct-lining cells of mucous glands are keratinocytes that cross the epidermal layers to form a horny lining (Fig. 2E).

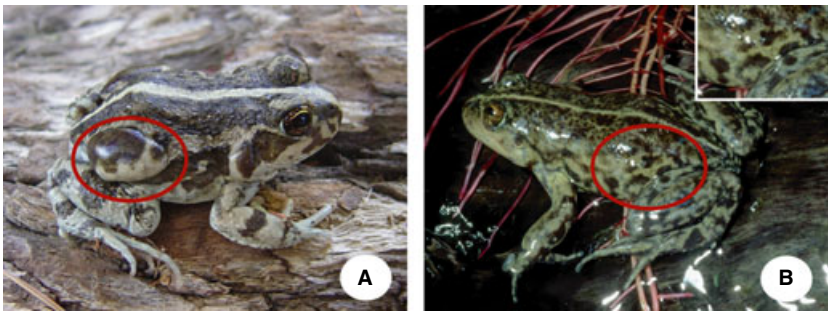

Fig. 1—Differences in lumbar gland development (red circle). — **A**. *Pleurodema bufoninum*. — **B**. *Somuncuria somuncurensis* (Photograph: B. Blotto). Insert: magnification of lumbar gland.

Table 1 Measurements (μm) of dermal glands developed in lumbar glands of *Pleurodema* and *Somuncuria* species. Data are expressed as mean values ± standard error

	Mucous glands		**Ordinary serous glands**		**Lumbar serous glands**	
	Width of acini	Length of acini	Width of acini	Length of acini	Width of acini	Length of acini
P. bibroni	77.50 ± 10.90	52.50 ± 2.89	–	–	392.00 ± 61.84	760.00 ± 103.54
P. borellii	55.75 ± 2.27	33.75 ± 2.20	145.83 ± 12.02	105.83 ± 20.28	172.50 ± 16.06	994.00 ± 127.62
P. brachyops	42.25 ± 2.37	24.00 ± 2.18	–	–	184.00 ± 18.51	325.00 ± 21.56
P. bufoninum	60.96 ± 3.43	30.87 ± 1.40	157.78 ± 13.82	166.67 ± 15.00	224.00 ± 11.93	1495.00 ± 74.39
P. cinereum	41.63 ± 2.31	39.19 ± 1.55	123.75 ± 6.05	633.50 ± 44.93	1851.43 ± 214.65	2446.67 ± 441.50
P. cordobae	69.75 ± 7.15	76.75 ± 4.28	–	–	360 ± 41.46	800.0 ± 30.55
P. kriegi	73.00 ± 3.51	46.75 ± 4.08	–	–	335.00 ± 98.11	1070 ± 126.10
P. thaul	38.75 ± 4.62	44.38 ± 4.83	218.00 ± 23.56	583.00 ± 62.65	165.00 ± 15.00	255.00 ± 55.0
S. somuncurensis	46.43 ± 3.53	30 ± 2.62	–	–	14.00 ± 0.76	80.75 ± 9.36

The neck of the gland is lined by simple, cubic epithelium. The acini of mucous glands consist of simple, tall, cylindrical secretory cells (mucocytes) orderly arranged around a wide lumen. Mucocytes differ in their staining reactions and in the morphology of secretory products (Table 1, Fig. 2E, insert). First, in both the transitional region between acinus and neck and in the basal region of the secretory unit, mucocytes with abundant strongly acidophilic granules and dark purplish-red granules stained positively with PAS and AB are observed; these granules are dispersed throughout much of the cytoplasm. Second, mucocytes located in the lateral region of the acinus have a pale cytoplasm composed of a PAS- and AB-negative foamy secretion and condensed nuclei, which are flattened against the basement membrane. Third, mucocytes with abundant, weakly acidophilic, PAS-positive granules, and AB-negative granules are basally located.

The ducts and necks of serous glands have the same features as the mucous glands. Furthermore, below the epithelium lining the neck, a layer of undifferentiated cells with acidophilic cytoplasm and ovoid nuclei is observed (Fig. 2G). Serous glands have an internal secretory layer that is syncytial and surrounded by myoepithelial cells (Fig. 2H). The syncytium contains ovoid nuclei with conspicuous nucleoli. The secretion is stored within the whole syncytium, and glands lack a proper lumen (Fig. 2F–H). Two types of serous glands can be identified based on the secretory unit size and morphology and the dye affinity of the secretions. In sagittal section, a gland type termed 'ordinary serous gland' (OSG) was observed in the most anterior and posterior regions of the macrogland; these glands are present in dorsal and lateral skin, as well as in lumbar skin (Fig. 2A, F, H, I). OSGs are more or less round (Table 1) and filled with a translucent, finely granular secretory product that is strongly acidophilic (green) when stained by MG. This secretion stains negatively with PAS, AB, and NS (Fig. 2F, I).

The second gland type, the 'lumbar serous gland' (LSG), is larger than the ordinary one (Table 1), oval, and occupies most of the macrogland (Fig. 2B, F, H, I). The contiguous, lateral surfaces of LSGs are separated by thin sheaths of loose connective tissue. The secretory units are ellipsoidal, with the long axes oriented perpendicular to the epidermis. Secretory units contain both strongly and weakly acidophilic granules when stained with MG; both kinds of granules are PAS- and AB-negative and NS-weakly positive. Usually, these granules are located in the upper part of the secretory unit, whereas the rest of the secretory unit is filled with a nonstructured homogeneous content (Fig. 2B, I). Weakly acidophilic granules vary in size and appearance; they may be ellipsoidal or round and are characterized by a homogeneous content or areas with material of different densities,

Table 2 Features of epidermis and dermis and histochemistry of dermal glands and secretory products of lumbar glands of *Pleurodema* and *Somuncuria* species

	Epidermis	Dermis				
					Serous glands	
	Epidermis layers/specialized cells	Eberth–Katschenko layer in LG	Chromatophores observed	Mucous glands	Ordinary serous glands	Lumbar serous glands
P. bibroni	3	Absent	Melanophores, iridiophores	Mucocytes PAS+, AB+	Not observed	WA granules PAS–, AB–, NS–, nonhomogeneous matrix
P. borelli	5–7/flask cells, cytoplasmic processes of melanophores	Absent	Melanophores, iridiophores, xantophores	Mucocytes diversity: SA granules PAS+, AB+; Foamy secretion PAS–, AB–; WA granules PAS–, AB–	SA fine-spotted content PAS–, AB–, NS–	SA and WA granules PAS–, AB–, NS+ nonstructured homogeneous secretion
P. brachyops	4/Flask cells, pillar cells	Absent	Melanophores, iridiophores	Mucocytes PAS+, AB–	Not observed	Intraspecific variation (see text)
P. bufoninum	3–4/flask cells, pillar cells, cytoplasmic processes of melanophores	Absent	Melanophores, iridiophores xantophores,	Mucocytes diversity: SA granules PAS+, AB+; Foamy secretion PAS–, AB–; WA granules PAS–, AB–	SA fine-spotted content PAS–, AB–, NS–	SA and scarce WA granules PAS–, AB–, NS+
P. cinereum	3–4/flask cells, cytoplasmic processes of melanophores	Absent	Melanophores, iridiophores, xantophores	Mucocytes diversity: WA granules PAS–, AB+; SA granules PAS+ and AB–	SA fine-spotted content PAS–, AB–, NS–	WA and SA granules PAS–, AB–, NS++ nonstructured homogeneous secretion
P. cordobae	4/pillar cells, cytoplasmic processes of melanophores	Absent	Melanophores, iridiophores	Mucocytes PAS+, AB+	Not observed	WA granules PAS–, AB–, NS+ homogeneous matrix
P. kriegi	4/flask cells, cytoplasmic processes of melanophores,	Absent	Melanophores, iridiophores	Mucocytes PAS+, AB+	Not observed	WA granules PAS–, AB–, NS+ homogeneous matrix
P. thaul	8/flask cells, pillar cells, cytoplasmic processes of melanophores	Absent	Melanophores, iridiophores, xantophores	Mucocytes PAS+, AB+	SA fine-spotted content PAS–, AB–, NS–	WA and SA granules PAS–, AB–,NS+++ homogeneous matrix
S. somuncurensis	3/pillar cells	Absent	Melanophores, xantophores	Present, no histochemical data	Not observed	WA granules NS+ homogeneous matrix

LG, lumbar gland; WA, weakly acidophilic (MG stain); SA, strongly acidophilic (MG stain); PAS, periodic acid Schiff; AB, Alcian Blue; NS, Ninhydrin/Schiff; +, positive reaction; –, negative reaction.

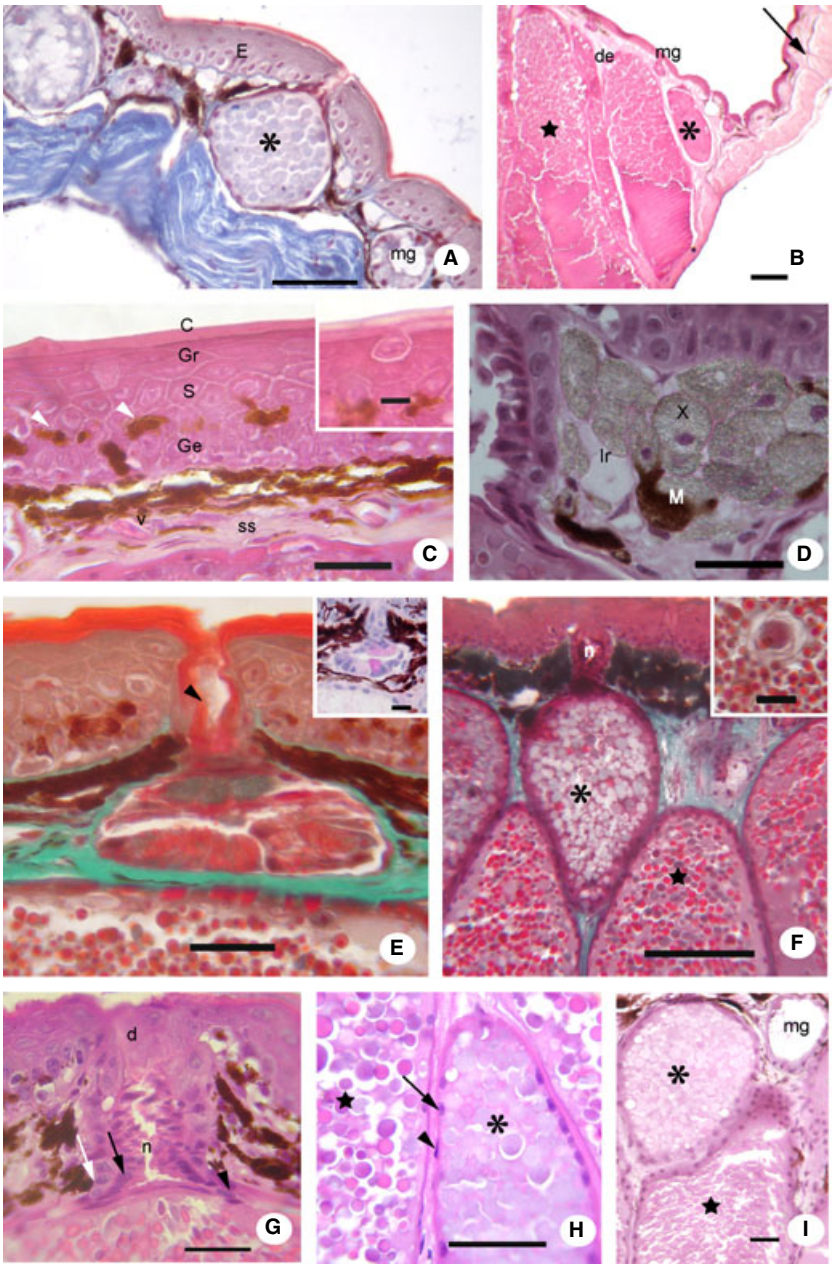

Fig. 2—Skin of *Pleurodema borellii*. — **A**. Dorsal skin of *Pleurodema borellii* with an ordinary serous gland (OSG; asterisk) and a mucous gland (mg). Epidermis (E). Masson trichrome stain. Scale bar 50 μm. — **B**. General view of macrogland, in which three types of dermal glands are developed—mucous gland, OSG, and lumbar serous gland (LSG; star). The EK layer (arrow) is interrupted by macrogland. Note the accumulation of secretory granules in the upper region of the secretory unit of the LSG, whereas the rest of the unit is filled with a homogeneous secretory material. Dermis (de). H&E. Scale bar 50 μm. — **C**. Detail of the epidermal layers: *stratum corneum* (C), *stratum granulosum* (Gr), *stratum spinosum* (S), and *stratum germinativum* (Ge). Note cytoplasmatic processes of melanophores within epidermis (arrowheads) and a blood vessel (v) in the *stratum spongiosum* (ss) of the dermis. H&E. Scale bar 10 μm. Insert: Flask cells frequently are found in the epidermis. H&E. Scale bar 5 μm. — **D**. Iridophores (Ir), melanophores (M), and xanthophores (X) beneath epidermis. H&E. Scale bar 20 μm. — **E**. Mucous gland showing heterogeneous features of the mucocytes in the same gland. Note the epithelium (arrowhead) lining the duct. M&G. Scale bar 10 μm. Insert: Mucous gland with PAS-positive cells. PAS-H. Scale bar 5 μm. — **F**. An OSG with its neck (n) and a LSG. M&G. Scale bar 50 μm. Insert: Detail of granules inside a LSG. M&G. Scale bar 5 μm. — **G**. Transitional region between the duct (d) and the neck of a LSG. Note the simple cubic epithelium lining the neck (white arrow), the undifferentiated cells (black arrow), and the nuclei (arrowhead) of the myoepithelial cells. H&E. Scale bar 10 μm. — **H**. Limit between OSG and LSG; note the nucleus of secretory syncytium (arrow) and the myoepithelial cells (arrowhead). H&E. Scale bar 25 μm. — **I**. Mucous gland, OSG, and LSG. NS. Scale bar 20 μm.

while other granules begin to disrupt into concentric layers (Fig. 2F insert).

LGs of other Pleurodema *species and of the* Somuncuria somuncurensis

Pleurodema bufoninum (Fig. 3) shows mucous glands lined by cylindrical or cuboidal cells that have pale cytoplasm. Some specimens have a few round OSGs. The secretory epithelium of LSGs bears ovoid nuclei with nucleoli, whereas the lumen is filled with a granular content. Strongly acidophilic granules are of varying morphologies. Some granules contain a dense, homogeneous material, whereas others have a white halo with a highly homogeneous acidophilic material, and a third type contains foamy material.

Pleurodema cinereum (Fig. 4) shows the EK layer in dorsal skin, but it is not present in the LG. Three types of dermal glands are developed. Mucous glands possess two different mucocytes – (1) globoid cells with a spherical, basal nucleus and weakly acidophilic granulations and (2) cells located near the neck of the gland with strongly acidophilic granulations.

Pleurodema thaul (Fig. 5) shows three types of dermal glands; LSGs have a nonstructured, homogeneous secretion with weakly and strongly acidophilic granules. Strongly acidophilic granules are scarce, vary in size, and have a secretion that may be evenly distributed or disposed as fine dots. Occasionally, granules are present inside the necks of LSGs.

Pleurodema kriegi, *P. cordobae*, and *P. bibroni* (Figs 6–8) possess their mucous glands with strikingly wide lumena, while OSGs are absent. LSGs contain few and large secretory units; only three to five secretory units in transverse section are observed.

Pleurodema brachyops shows scarce mucous glands, while OSGs are absent. LSGs are intraspecifically variable. One specimen (CENAI 8784) is strongly acidophilic and contains

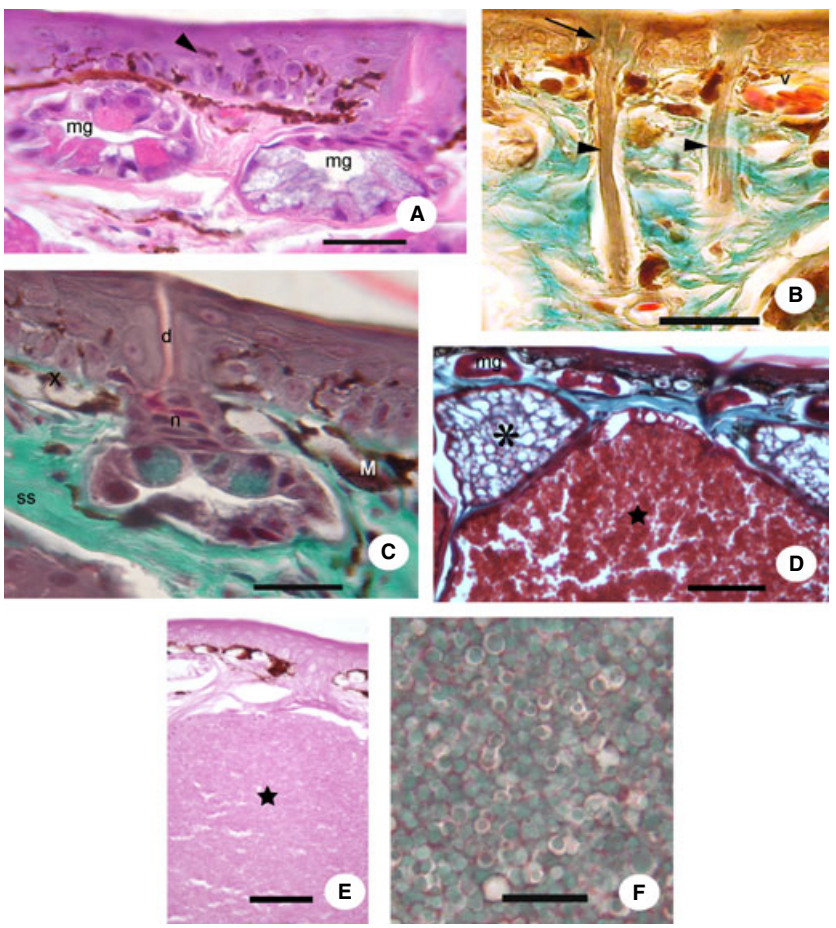

Fig. 3—Lumbar gland of *Pleurodema bufoninum*. — **A**. Detail of mucous glands (mg) observed in the *stratum spongiosum* of the dermis and cytoplasmatic processes of melanophores within epidermis (arrowheads). H&E. Scale bar 10 μm. — **B**. Smooth muscle cells (arrowheads) extending to pillar cells (arrow). Note also a blood vessel (v). Scale bar 100 μm. M&G. — **C**. Mucous gland with its duct (d), neck (n), and both weakly and strongly acidophilic granules in the *stratum spongiosum* (ss) of the dermis, along with melanophores (M) and xantophores (X). M&G. Scale bar 10 μm. — **D**. Mucous gland, ordinary serous gland (OSG, asterisk), and lumbar serous gland (LSG, star). M&G. Scale bar 50 μm. — **E**. LSG with weakly NS-positive granules. NS. Scale bar 50 μm. — **F**. Different-sized granules inside a LSG. M&G. Scale bar 10 μm.

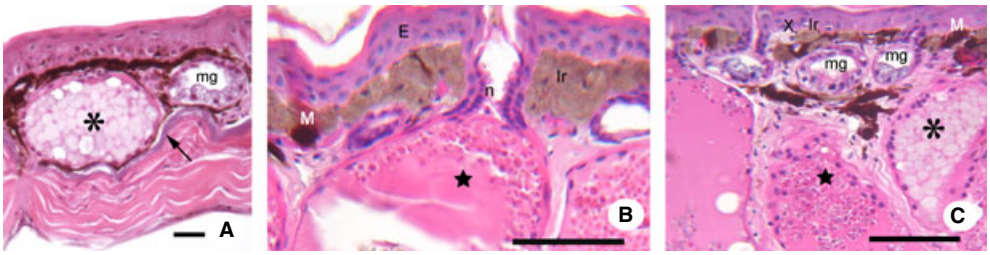

Fig. 4—Lumbar gland of *Pleurodema cinereum*. — **A**. Dorsal skin with a mucous gland (mg) and an ordinary serous gland (OSG, asterisk). Note the Eberth–Katschenko layer (arrow) between the *strata spongiosum* and *compactum* of the dermis. H&E. — **B**. Iridophores (Ir) and melanophores (M) under epidermis (E). Note also a lumbar serous gland (LSG, star) with its neck (n). H&E. — **C**. Iridophores, melanophores, xanthophores (X), mucous glands, OSG, and LSG observed in the dermis. H&E. Scale bar 20 μm.

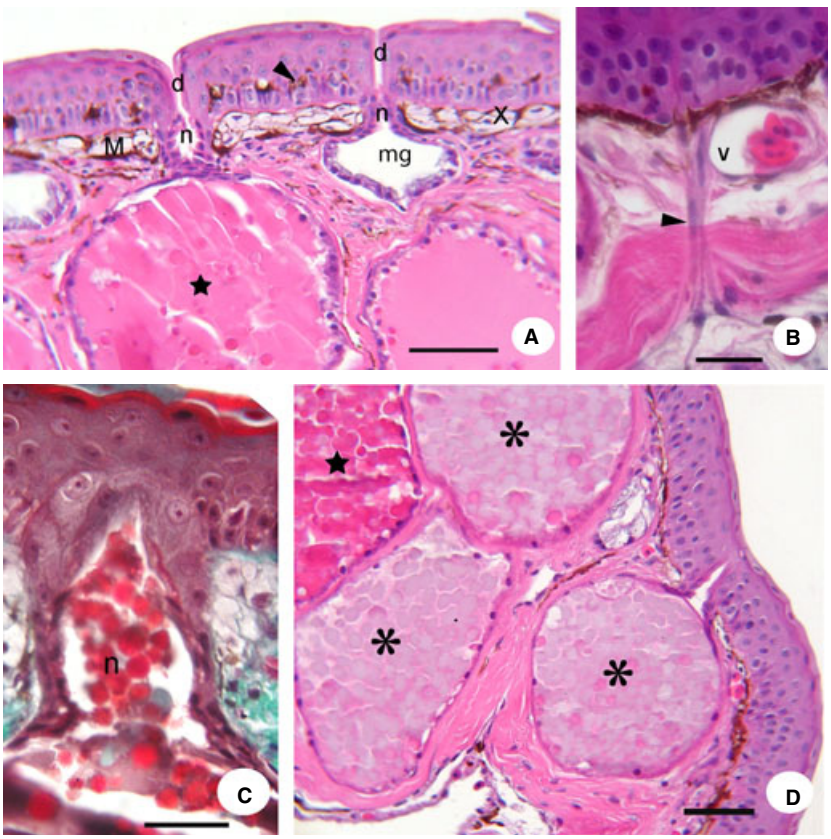

Fig. 5—Lumbar gland of *Pleurodema thaul*. — **A**. Mucous gland (mg) and lumbar serous gland (LSG, star) with their ducts (d) and necks (n). Note cytoplasmatic processes of melanophores within epidermis (arrowheads) and xanthophores (X) and melanophores (M) in the dermis. H&E. Scale bar 10 μm. — **B**. Smooth muscle cells (arrowheads) originating in the dermis extend to pillar cells. Note also a blood vessel (v). H&E. Scale bar 10 μm. — **C**. Granules of a LSG inside its neck. M&G. Scale bar 10 μm. — **D**. Transitional zone between macrogland and lateral skin (placed at right side of the image); note ordinary serous glands (asterisks) and a LSG. H&E. Scale bar 50 μm.

PAS- and NS-positive and AB-negative granules (Fig. 9A), whereas another specimen (UIS-A 98) possesses secretory units filled with a weakly acidophilic matrix, with homogeneous and nonhomogeneous phases and scarce PAS-, AB-, and NS-negative granules (Fig. 9B).

Somuncuria somuncurensis (Fig. 10) shows mucous glands with cubic and plane mucocytes, oval secretory units, and wide lumena free of secretion. OSGs are absent. Although LSGs are larger than mucous glands, they are smaller than LSGs observed in *Pleurodema*.

Discussion

Lumbar glands in *Pleurodema* and *Somuncuria* are specialized regions of the skin, where LSGs are the main glandular component. Macroscopically, LGs in both males and females

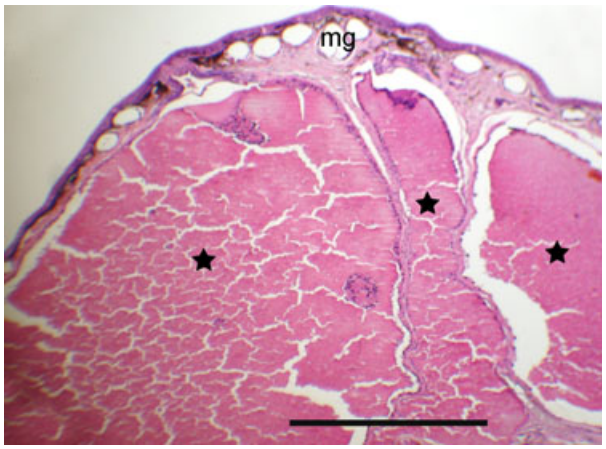

Fig. 6—Lumbar gland of *Pleurodema kriegi*. Note a mucous gland (mg) and three enormous lumbar serous glands (stars). H&E. Scale bar 50 μm.

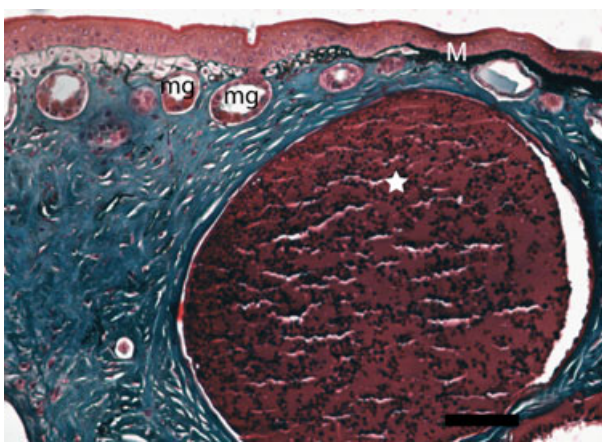

Fig. 7—Lumbar gland of *Pleurodema cordobae*. **—**. Melanophores (M), mucous glands, and a lumbar serous gland (star). M&G. Scale bar 20 μm.

develop similarly; there is no evidence of sexual dimorphism. The histological structure of *Pleurodema* and *Somuncuria* LGs resembles that of macroglands studied in other anurans (Toledo *et al.* 1996; Lenzi-Mattos *et al.* 2005).

Epidermal cells

In addition to the typical cells of the epidermal layers, three specialized cell types occur between these layers – viz., cytoplasmatic processes of melanophores, flask cells, and pillar cells. Toledo *et al.* (1996) described melanophores in epidermis of *Pleurodema thaul*, but probably they correspond to cytoplasmatic processes of dermal melanophores. Flask cells differentiate from epidermal cells during metamorphic climax and are retained throughout postmetamorphic life (Ecker

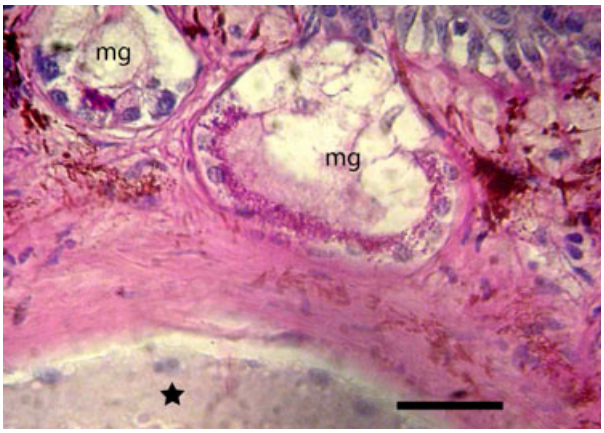

Fig. 8—Lumbar gland of *Pleurodema bibroni*. Mucous glands (mg) and a lumbar serous gland (star). PAS-H. Scale bar 20 μm.

1889; Whitear 1975). It has been proposed that flask cells are involved either in chloride transport (Rozman *et al.* 2000; Wolfram *et al.* 2000) or in absorbing water (Katz *et al.* 2000).

The walls of another epidermal cell type interdigitate with the walls of adjacent dermal smooth muscle cells by means of indentations. These cells were defined as basal layer cells in *Rana temporaria* (Whitear 1974). More recently, Linsenmair *et al.* (1999) termed this cell type as pillar cells for *Hyperolius viridiflavus* (terminology adopted in the present work). On the basis of their ultrastructural characteristics, Linsenmair *et al.* (1999) suggested that pillar cells hold the epidermis during molting.

Flask cells and pillar cells have not been previously observed in *Pleurodema* LGs. However, both cell types are probably more common than reported, because studies on macroglands usually are focused on dermal glands while epidermis remains undescribed.

Chromatophores

Kobelt and Linsenmair (1986) stated that the presence of two or more layers of iridophores in *Hyperolius nitidilus*, as we observed in *P. borellii*, *P. cinereum*, and *P. thaul*, might influence hydric equilibrium. On the other hand, the arrangement of both xantophores and iridophores just beneath the dermal–epidermal junction and underlain by melanophores was termed a dermal chromatophore unit (DCU) by Bagnara *et al.* (1968). The DCU was described for anurans capable of rapid color change (Bagnara *et al.* 1968) and was reported for *P. thaul* (Toledo and Jared 1989). We did not observe all three chromatophores in all species, while in those species having three cell types, their arrangement did not correspond exactly with this model. Although melanophores usually underlie other chromatophores, their processes do not always extend upward around an iridophore. Furthermore, iridophores and xanthophores are not arranged exactly as

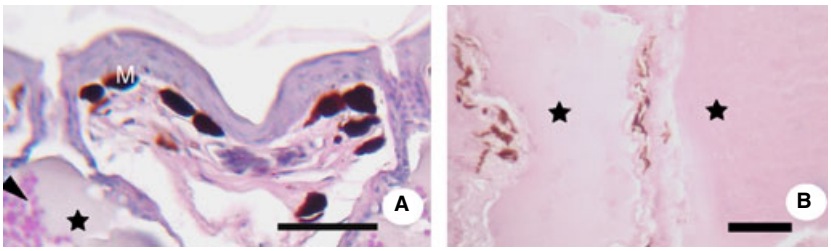

Fig. 9—Lumbar gland of *Pleurodema brachyops*. — **A**. Note melanophores (M) under epidermis. Lumbar serous gland (LSG, star) containing PAS-positive granules (arrowhead). Specimen CENAI 8784. PAS-H. — **B**. Detail of two contiguous LSGs with PAS-negative content. Specimen UIS-A 98. PAS-H. Scale bars 20 μm.

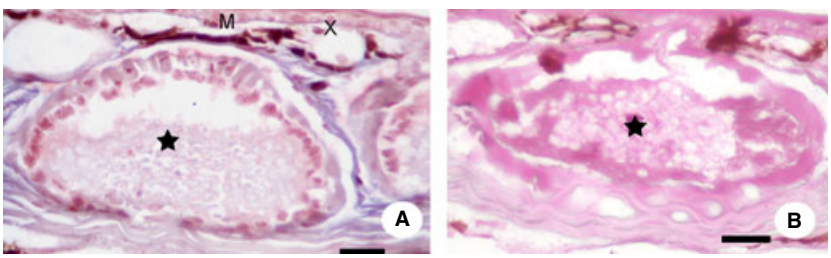

Fig. 10—Lumbar gland of *Somuncuria somuncurensis*. — **A**. Note thin epidermis and melanophores (M) and xantophores (X), along with a lumbar serous gland (LSG, star). Masson trichrome stain. — **B**. Weakly NS-positive granules in a LSG. NS. Scale bars 20 μm.

described for the DCU. However, DCU is a model proposed for explaining the distribution of pigment cells and not a rigid structure. As far as we know, *Somuncuria somuncurensis* and Argentinean species of *Pleurodema* do not undergo dramatic color changes; we have only observed individuals becoming more pale (Daiana Paola Ferraro, pers. obs.). All species of *Pleurodema* and *S. somuncurensis* usually have a uniform distribution of melanophores under the epidermis; possibly, this organization produces the dark blotches on lumbar skin. This coloration may deter potential predators or contribute to reproductive success (Toledo and Haddad 2009).

Dermal glands

LGs of both *Pleurodema* and *Somuncuria* are formed by clusters of mucous and serous glands, in which a region of undifferentiated cells (or intercalary cells) was observed [for details, see Dawson (1920) and Delfino (1980) *in* Delfino *et al.* (1998)].

In *Pleurodema,* the secretory product of mucous glands varies in appearance (i.e., granular or spongy) and histochemistry. The secretory granules within the mucocytes contain both neutral glycoconjugates and acidic glycosaminoglycans. These results are consistent with the previous reports for *P. thaul* (Toledo and Jared 1989; Toledo *et al.* 1996), *P. borellii* (Mangione and Lavilla 2004), and other anurans (e.g., Brizzi *et al.* 2002; Felsemburgh *et al.* 2007; Jared *et al.* 2009). According to the scheme proposed by Harrison *et al.* (1987), variability in staining within a given mucocyte may reflect a temporal sequence of biosynthesis of the mucous secretory granule. Collectively, histochemical results may indicate that mucous gland secretion is a heterogeneous substance to which different mucocytes contribute. Consequently, the final product is a mixture of secretions delivered into the same lumen. This mucus regulates water loss or gain through the skin; in addition, it may be a defensive barrier against bacterial or fungal infection and act as a lubricant to reduce friction in water and minimize abrasive and mechanical damage to the skin out of water (Clarke 1997). On the basis of the presence of abundant rough endoplasmatic reticulum, Toledo *et al.* (1996) suggested that the mucous glands of *P. thaul* contain proteins.

The names of two types of serous glands reported here – OSGs and LSGs – follow the terminology of Delfino *et al.* (1999) and are equivalent to G_1 and G_2 glands, respectively, of Lenzi-Mattos *et al.* (2005). In some *Pleurodema* species, a few OSGs were observed; these glands have a morphological organization similar to that previously described in other leiuperids (Delfino *et al.* 1999; Lenzi-Mattos *et al.* 2005) and in the dorsal skin of *Pleurodema*. Histochemical results in *Pleurodema* suggest that secretory product of these glands contains neither neutral nor acidic mucosubstances. Two types of serous glands have not been previously reported in *Pleurodema* (Birabén 1929; Adam 1954; Rada de Martínez and Finol 1986; Toledo and Jared 1989; Mangione and Lavilla 2004); therefore, we cannot compare our observations.

Lumbar serous glands, present in all examined species, are an additional glandular type observed only in the macrogland. The secretory units of LSGs in three species of *Pleurodema*

species were few in number and exceptionally wide (compare Fig. 2B with Fig. 6). The three species that share this structure are *P. bibroni*, *P. cordobae*, and *P. kriegi*, a complex of phylogenetically related, cryptic, and polyploid species (Barrio 1977; Kolenc *et al.* 2009; Valetti *et al.* 2009). Conversely, our data suggest that *S. somuncurensis*, a species with slightly developed LGs, possesses the smaller secretory units. However, these observations should be confirmed with statistical analyses of gland size and density.

Granule morphology of LSGs varies greatly, as has previously been reported for serous glands of other anuran species, e.g., *Physalaemus biligonigerus* (Delfino *et al.* 1999, 2001), *P. albonotatus* and *Leptodactylus chaquensis* (Alvarez *et al.* 2005), and *Rhinella icterica* (Almeida *et al.* 2007). We observed intraspecific variability (i.e., polymorphic nature of secretions) in secretory product of LSGs of *P. bufoninum*, *P. cinereum*, and *P. thaul*. This variability is primarily a function of post-Golgian maturation, because all anurans share the same biosynthetic apparatus in these glands, although maturation follows specific pathways in different anuran families (see Alvarez *et al.* 2005). Toledo and Jared (1989) reported serous glands in *P. thaul* to possess an acidic mucopolysaccharide homogeneous secretion (weak AB-positive) and neutral mucopolysaccharide granules (weak PAS-positive). These histochemical results differ from our observations. However, as we previously stressed, Toledo and Jared (1989) did not recognize two types of serous glands, although their Fig. 1 seems to illustrate what we call LSG. Moreover, we used specimens from Argentina, whereas Toledo and Jared's (1989) came from Chile. *Pleurodema thaul* is a species with a broad distribution in Chile but a restricted range in Argentina (Ortiz and Díaz-Páez 2006; Ferraro and Casagranda 2009), and its variability should be examined more closely.

Protein content was confirmed by NS-positive reaction in LSGs of all species studied (except *P. bibroni*), as was reported in G_2 gland of *Eupemphix nattereri* (Lenzi-Mattos *et al.* 2005). We found PAS-positive secretory granules in LSGs from a specimen of *P. brachyops* from Venezuela, indicating the presence of neutral glycoconjugates, whereas specimen from Colombia had PAS-negative granules. *Pleurodema brachyops* inhabits a broad region of dry llanos and savannas in northern South America (Dunn 1944; Barrio-Amorós 1998), and its variability should be explored throughout the taxon's whole range for a better understanding of our observations.

Mangione and Lavilla (2004) described histological features of the lumbar skin of those *Pleurodema* species lacking externally evident LGs: *P. diplolister*, *P. guayapae*, *P. marmoratum*, *P. nebulosum*, and *P. tucumanum*; the recently described *Pleurodema alium* was not included (Bastos Maciel and Nunes 2010). Also, macroglands of *P. borellii* were described for comparison. These authors observed a similar proportion of mucous and serous glands in lumbar skin of species without LGs, stressing that this proportion differs from that found in dorsal skin; however, they did not provide meristic data. The secretion of the mucous glands is AB-positive in *P. diplolister* and *P. marmoratum*, whereas secretions are both AB- and PAS-positive in other species examined, as we observed in this work. Mangione and Lavilla (2004) described one type of serous gland with two phases (amorphous and granulose) in all species, except in the cryptic pair *P. guayapae* and *P. nebulosum*, in which the amorphous phase was absent. Furthermore, these authors observed different histochemical affinities of both phases. Mangione and Lavilla (2004) also provided a brief description of the LG of *P. borellii* for the purpose of comparing this species with those lacking LGs. Although they did not identify two types of serous glands, their Fig. 10 (*P. borellii*) seems to depict our LSG. These authors reported PAS-positive granules in serous glands of *P. borellii*. However, based on our observations, both OSGs and LSGs stain negatively for AB and PAS, indicating the absence of acidic glycosaminoglycans and neutral glycoconjugates (except one specimen of *P. brachyops*).

Eberth–Katschenko layer and expulsion of toxins

The EK layer has not been observed in LGs of any examined species, because macroglands may preclude its presence, but it was observed elsewhere in the dorsal skin, accordingly with description of the leiuperid *E. nattereri* (Lenzi-Mattos *et al.* 2005). On the contrary, Mangione and Lavilla (2004) observed a subepithelial EK layer in *Pleurodema borellii*. In toads, EK layer can be present or absent (Toledo and Jared 1993; Almeida *et al.* 2007; and references therein). In line with the defensive function of macroglands, the absence of EK layer removes rigidity to this region, facilitating glandular compression to express toxins when a predator attacks (Lenzi-Mattos *et al.* 2005). The expulsion of toxins from macroglands in both leiuperid and bufonid anurans seems to require external pressure. In toads, defensive behavior includes inflating the lungs (exerting pressure on granular glands), lifting the body, and then jetting the venom if the predator should attack and/or bite (Jared *et al.* 2009). Possibly, pressure exerted on leiuperid glands occurs in a similar fashion as that of *Rhinella*. However, leiuperid toxins are extruded slowly, not as jets of venom. This mechanism may be correlated with morphology of the gland neck. In *Rhinella jimi*, the epithelial duct is lined by a group of cells, leaving only a narrow slit (Jared *et al.* 2009), whereas in *Pleurodema* and *Somuncuria* the gland neck is covered by simple cubic epithelium. These morphologies may influence the expulsion of toxins, either as venom jets (when epithelial plugs of parotoid macroglands are broken) or as little drops (in inguinal and LGs; Sazima and Caramaschi 1986; Toledo *et al.* 1992; Lenzi-Mattos *et al.* 2005; Jared *et al.* 2009). Also, myoepithelium encircling the syncytial secretory unit of both mucous and serous glands plays an important role in gland discharge (Delfino *et al.* 1982). Neural control is adrenergic, and gland discharge is caused by contraction of the peripheral myocytes (Holmes *et al.* 1977; Holmes and Balls 1978; Delfino *et al.* 2006).

Homology of macroglands developed in sacral region of the body of other anurans

Primary homologies are established based on observations of the organisms and should be tested in a phylogenetic analysis (De Pinna 1991). Differentiation of inguinal and LGs in leiuperid genera is based only on a topographical criterion (Introduction). As previously described and discussed, these glands are not sexually dimorphic, are associated with defensive behavior, show a similar and conservative structure, and share the most important histological features. On the basis of this evidence, we consider inguinal and LGs of leiuperids to be homologous.

In Hylidae, Eleutherodactylidae, and Myobatrachidae, macroglands were described. In Hylidae, inguinal glands have been detected in 14 species of the *Scinax perpusillus* and *Scinax catharinae* groups (Hylinae; Faivovich *et al.* 2010 and references therein). In *Scinax,* intraspecific variability was observed with regard to both macroscopic development and degree of development between males and females (Faivovich 2002). In Hylidae and Eleutherodactylidae, inguinal glands vary from an irregular patch of glandular tissue to a well-developed gland (Dixon 1957; Lynch 1968; Faivovich 2002). Inguinal glands of *Uperoleia* (Myobatrachidae) are compact, prominently elevated, and in several species, are associated with 'flash' coloration during defensive postures (Brodie *et al.* 1998; Doughty and Roberts 2008).

Topographically, the location of inguinal glands developed in Hylidae, Eleutherodactylidae, Myobatrachidae is similar to that observed in leiuperids, and they are developed in both sexes (although more evident in males than females of *Scinax*). However, the external morphology and the degree of development of the glands differ. To our knowledge, these glands have not been histologically studied. Future studies on inguinal glands of hylids, eleutherodactylids, and myobatrachids are necessary for a comparison of these structures with those of Leiuperidae; until then, no proper assessment of their primary homology can be made.

Males of *Cycloramphus dubius* and *C. fuliginosus* (Cycloramphidae) possess iliac glands located posteriorly on the abdomen. They are sexually dimorphic, and their proteinaceous secretion probably functions as a pheromone (de-Lucas *et al.* 1996; Figueira Gonçalves and Brito-Gitirana 2008). On the basis of this evidence, we do not consider these glands to be homologous with leiuperid macroglands.

Duellman and Veloso (1977) regarded LGs of *Pleurodema* to be derived based on a precladistic analysis. Given that species lacking externally evident LGs possess abundant serous glands in their lumbar skin (Mangione and Lavilla 2004), the evolutionary polarity of this character should be evaluated in a phylogenetic context. A similar situation was observed in mantellid frogs; although members of the subfamily Mantellinae possess femoral glands, *Boophis ophistodon* (subfamily Boophinae), a species without externally recognizable femoral glands, possesses patches of glandular tissue in the femoral skin (Vences *et al.* 2007). In addition, Vences *et al.* (2007) suggested that the presence of femoral glands in mantellids and in the genera *Indirana* and *Petropedetes* (currently included in families Ranixalidae and Petropedetidae, respectively) is explained by convergence, based on their molecular phylogeny. Finally, these authors suggested that macroglands of diverse functions may have evolved numerous times from generalized granular glands in different anuran clades.

Future investigations should include descriptions of the ultrastructure of macroglands, as well as the toxin composition of secretions released by serous glands. Also, venom-release mechanisms among anuran families should be examined. Such knowledge will help us to understand the origin and evolution of both chemical and behavioral defense in anurans, in addition to other animals.

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Appendix

Examined specimens are housed in the following institutions: Museo de La Plata (MLP A., La Plata, Argentina), Museo Argentino de Ciencias Naturales 'Bernardino Rivadavia' (MACN, Buenos Aires, Argentina), Centro Nacional de Investigaciones Iológicas (CENAI, housed at MACN), Fundación Miguel Lillo (FML, Tucumán, Argentina), and Escuela de Biología, Universidad Industrial de Santander (UIS-A, Bucaramanga, Colombia). Recently collected specimens from Argentina are marked with an asterix (*).

Pleurodema bibroni. CENAI 5330 ♂ (Fig. 8) URUGUAY: MONTEVIDEO: Barra de Santa Lucía. – *Pleurodema*

borellii. MLP A. 2014 ♂ (Fig. 2B–C) ARGENTINA: Catamarca: Mutquín; MLP A. 4649★ ♂ (Fig. 2A,D–I) ARGENTINA: Jujuy: El Carmen Department: between km 1664 and km 1665 on Ruta Nacional 9, 2 km from El Carmen. – *Pleurodema brachyops*. CENAI 8784 ♂ (Fig. 9A) VENEZUELA: Guarico: Estación Biológica de Calabozo; UIS-A 98 ♀ (Fig. 9B) COLOMBIA: Cesar: Municipio de Valledupar: Finca La Dorada. – *Pleurodema bufoninum*. MLP A. 1464 ♀ (Fig. 3B, F) ARGENTINA: Santa Cruz: Puerto Deseado, MACN 40037★ ♂; stream on North shore of Lago Burmeister, MACN 40041★ ♂; near Cabo Blanco, MLP A. 3985★ ♂ (Fig. 3E) Río Negro: Pilcaniyeu Department: Intersection of Ruta Nacional 23 and Río Pichi Leufú, MLP A. 4048★ juvenile (Fig. 3D) Pilcaniyeu Department: pond near intersection of Ruta Nacional 40 and El Porteño stream; MLP A. 4020★ ♂ (Fig. 3A, C) Chubut: stream on Ruta Nacional 40 between Kms 1450 and 1451; MACN 39794★ ♂ near Puesto Salazar. – *Pleurodema cinereum*. FML 14975 ♂ PERU: Puno: near Puno. MLP A. 4678★, 4684–85★ ♂♂ ARGENTINA: Jujuy: Tilcara Department: Juella. MLP A. 4694★ ♀. Cochinoca Department: Ruta Nacional 11, on road to Casabindo. MLP A. 4702★ ♂ (Fig. 4) Cochinoca Department: Casabindo. – *Pleurodema cordobae*. MACN 39911★ ♀ (Fig. 7) ARGENTINA: Córdoba: Calamuchita Department, Estancia Los Tabaquillos. – *Pleurodema kriegi*. MLP A. 3527★ ♂ (Fig. 6) ARGENTINA: Córdoba: Pampa de Achala; MLP A. 4728★ ♀ ARGENTINA: Córdoba: Parque Nacional Quebrada del Condorito, footpath between points 4 and 5. – *Pleurodema thaul*. MLP A. 1275 ♂ ARGENTINA: Río Negro: Tronador: Nahuel Huapi. MLP A. 4068★ ♂ (Fig. 5A–D), 4070★ ♂ ARGENTINA: Chubut: Cushamén Department: Parque Nacional Lago Puelo, near Pitranto Grande. – *Somuncuria somuncurensis*. MACN 33929 ♀ (Fig. 10C–D) ARGENTINA: Río Negro: Somuncurá, Valcheta stream, Estancia El Rincón.