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Odorous secretions in anurans: morphological and functional assessment of serous glands as a source of volatile compounds in the skin of the treefrog *Hypsiboas pulchellus* (Amphibia: Anura: Hylidae)

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Abstract

Serous (granular or venom) glands occur in the skin of almost all species of adult amphibians, and are thought to be the source of a great diversity of chemical compounds. Despite recent advances in their chemistry, odorous volatile substances are compounds that have received less attention, and until now no study has attempted to associate histological data with the presence of these molecules in amphibians, or in any other vertebrate. Given the recent identification of 40 different volatile compounds from the skin secretions of *H. pulchellus* (a treefrog species that releases a strong odour when handled), we examined the structure, ultrastructure, histochemistry, and distribution of skin glands of this species. Histological analysis from six body regions reveals the presence of two types of glands that differ in their distribution. Mucous glands are homogeneously distributed, whereas serous glands are more numerous in the scapular region. Ultrastructural results indicate that electron-translucent vesicles observed within granules of serous glands are similar to those found in volatile-producing glands from insects and also with lipid vesicles from different organisms. Association among lipids and volatiles is also evidenced from chemical results, which indicate that at least some of the volatile components in *H. pulchellus* probably originate within the metabolism of fatty acids or the mevalonate pathway. As odorous secretions are often considered to be secreted under stress situations, the release of glandular content was assessed after pharmacological treatments, epinephrine administrated *in vivo* and on skin explants, and through surface electrical stimulation. Serous glands responded to all treatments, generally through an obvious contraction of myoepithelial cells that surround their secretory portion. No response was observed in mucous glands. Considering these morpho-functional results, along with previous identification of volatiles from *H. pulchellus* and *H. riojanus* after electrical stimulation, we suggest that the electron-translucent inclusions found within the granules of serous glands likely are the store sites of volatile compounds and/or their precursors. Histochemical and glandular distribution analyses in five other species of frogs of the hylid tribe Cophomantini, revealed a high lipid content in all the species, whereas a heterogeneous distribution of serous glands is only observed in species of the *H. pulchellus* group. The distribution pattern of serous glands in members of this species group, and the odorous volatile secretions are probably related to defensive functions.

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Introduction

Hundreds of species of amphibians secrete volatile molecules that are perceived due to their particular smells (Myers et al. 1991; Smith et al. 2003, 2004). In most species, these molecules are secreted under stress, and are suggested to have a role in defensive contexts (Myers et al. 1991; Smith et al. 2004; Toledo et al. 2011). Although other biologically active compounds (e.g. amines, peptides, alkaloids) are known to be stored in cutaneous glands, especially serous (or granular) glands (Neuwirth et al. 1979; Erspamer, 1994; Daly, 1995; Brizzi et al. 2003), no study has attempted to assess the source of volatile compounds through morphological and/or histochemical evidences.

The morphology and the patterns of gland distribution along the body vary among species (Toledo & Jared, 1995). In particular, macroglands related to defensive roles against predators have specific locations (Toledo et al. 2011). In other cases, individual glands may be concentrated in specific regions without the macroscopic outgrowth that characterizes the macroglands (Prates et al. 2011). Although the few studies that succeeded in the identification of the volatile molecules analyzed secretions from macroglands (Whitear, 1974; Myers et al. 1991; Smith et al. 2003, 2004; Poth et al. 2012, 2013; Starnberger et al. 2013), a recent study has identified volatile compounds from the skin of species that lack macroglands (Brunetti et al. 2015b).

Amphibians release defensive substances under situations of stress (Gallardo, 1958; Barrio, 1962; Myers et al. 1991; Smith et al. 2000; Faivovich et al. 2013), as the result of α-adrenergic nerve stimulation (Benson & Hadley, 1969; Dockray & Hopkins, 1975). Experimentally, α-adrenergic agonists stimulate serous glands to secrete their content (e.g. Benson & Hadley, 1969; Hoffman & Dent, 1977; Gammill et al. 2012), whereas it was demonstrated that α-adrenergic agonists and stress situations caused after handling have similar physiological effects in *Lithobates catesbianus* (As *Rana catesbeiana*; MbangKollo & deRoos, 1983). More recently, several studies have obtained the glandular secretion after surface electrical stimulation (SES; Tyler et al. 1992). Specifically, SES was used to characterize the volatile secretion in some species of anurans (Smith et al. 2000, 2003; Brunetti et al. 2015b). However, there is no morphological description of the effects of SES on serous gland structure (either related to non-volatile or volatile components), and no study has attempted to investigate the role of α-adrenergic agonists on the secretion of volatile compounds.

Most species of the *H. pulchellus* group release a strong smell when handled (Barrio, 1962, 1965; Faivovich et al. 2013). This odour was likened to 'crushed plants' (Faivovich et al. 2013) or, particularly in *H. pulchellus*, to a skunk-like or fox-like odour (Langone, '1994' [1995]; Gallardo, 1958, 1961). A recent study demonstrated that in this species and in *H. riojanus* (another member of the *H. pulchellus* group), volatiles are a complex mixture of at least 37 different compounds (Brunetti et al. 2015b), which are responsible for the characteristic smell of the secretion of both species. As discussed by these authors, compounds from *H. pulchellus* and *H. riojanus* likely are derived from different biosynthetic origins related to fatty acids metabolism, the mevalonate pathway and degradation of amino acids. These compounds include alcohols, aldehydes, esters, ketones, sulphur derivatives and terpenoids.

In this study, we describe the structure, ultrastructure, histochemical properties, and distribution of serous glands in *H. pulchellus*. We also assess the morphological response of serous glands in this species after application of the neurotransmitter epinephrine *in vivo* and *in vitro*, and after *in vivo* electrical stimuli on the skin surface. Additionally, we present a comparative analysis of gland distribution in the body, as well as the response of serous gland to lipid staining, in five other species of Cophomantini. Our results are examined and discussed in relation to the presence and function of volatile compounds secreted by species of the *H. pulchellus* group, particularly *H. pulchellus*, in a defensive context. We also discuss the co-occurrence of other chemical components in *H. pulchellus*, and the presence of lipid content in serous glands in the hylid subfamily Hylinae.

Materials and methods

Animals

Adult specimens of *H. pulchellus* were collected during different months from three different localities in Argentina: (i) Province of Entre Rios: Departmento Islas del Ibicuy: Bridge Brazo Largo (33°40′S, 58°52′W); (ii) Province of Buenos Aires: Departmento Tigre: General Pacheco (34°40′S, 58°47′W) and (iii) Province of Santa Fe: Department Garay: Helvecia, (31°07′S, 60°07′W). Specimens were euthanized with a lethal dose of lidocaine 20% applied in the pelvic region. Additionally, adult museum specimens from five species of the tribe Cophomantini were examined. These include three other species from the *H. pulchellus* group (*H. curupi*, *H. marianitae* and *H. riojanus*), *H. punctatus* and *Bokermannohyla pseudopseudis*. Voucher specimens are housed in the Herpetological Collection of the Museo Argentino de Ciencias Naturales 'Bernardino Rivadavia'-CONICET (MACN), and in the Departamento de Zoologia, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais (UFMG). See Appendix 1 for complete specimen data. 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).

Light microscopy

Specimens of *H. pulchellus* were fixed by immersion in 10% neutral phosphate buffer formalin (pH 7.2) overnight. Small strips of skin (about 9–16 mm^2) were removed from six different regions: three along the dorsum (cephalic, scapular and middorsal), one from the midlateral, and two from the ventral skin (gular and midventral). Skin samples were washed with the same buffer and dehydrated in ascending ethanol series (70–100%), cleared in xylene, paraffin-embedded and sectioned transversely at 5 μm. Sections were stained with Masson–Goldner's trichrome stain (Martoja & Martoja-Pierson, 1970) for general cytology and histology. Histochemical techniques included Coomassie Blue (Bancroft & Gamble, 2002) to detect proteins/peptides, and combined Alcian Blue-Periodic Acid Schiff Mowry & Winkler (1956) for acid mucopolysacharides and neutral carbohydrates; all samples were counterstained with Carazzi's Haematoxylin. To detect lipids, samples from specimens preserved in ethanol 70%, were washed with tap water, placed in gum sucrose solution at 4 °C for 18 h, and mounted onto a block holder (Anderson & Bancroft, 2002). Transverse sections 10-μm-thick were cut in a cryostat and stained with Sudan black B (SBB) and Sudan III (Pearse, 1985).

Museum specimens of the other five species had been fixed in 10% neutral phosphate buffered formalin, and preserved in 70% ethanol. Skin samples only from scapular and cephalic regions were removed in these specimens. Samples were processed through similar protocols but were only stained for general cytology and with SBB. Photomicrographs were obtained in a Nikon E200 microscope coupled to a Nikon DS-U2 CCD camera. A detailed structural and ultrastructural analysis of skin glands from dorsal, gular, lateral and ventral regions of *H. punctatus*, as well as structural analysis of the gular and lateral regions of *H. curupi*, *H. marianitae*, and *B. pseudopseudis* have been presented previously (Brunetti et al. 2012, 2015a). In the present study, those observations are complemented with the examination of gland distribution and gland size from cephalic and scapular regions of these species. Throughout this paper we will refer to different gland types as ordinary serous glands (OSGs) and ordinary mucous glands (OMGs) according to the terminology proposed by Brizzi et al. (2003), which has also been used in our previous works (Brunetti et al. 2012, 2015a).

Transmission electron microscopy

Small samples of skin from the scapular region from *H. pulchellus* were submitted to ultrastructural analysis using the same methodology described in Brunetti et al. (2012). Semithin sections (1 μm) were stained with 1% Toluidine Blue in 1% Na_2CO_3 and examined under a light microscope. Ultrathin sections were observed with a Zeiss (Oberkochen, Germany) EM 109T transmission electron microscope at 60 keV. The images were recorded with an ES1000W Erlanshen CCD Gatan digital camera.

Analysis of glandular distribution and gland measurements

To investigate differences in the number of mucous and serous glands at different body regions in *H. pulchellus*, as well as differences in serous gland size among regions, paraffin sections of different specimens were subjected to a quantitative analysis. Measures were taken in a single section of 5 μm from two to ten samples of the examined regions in each specimen. Each section was photographed and a bar of 1 mm was positioned on its central point. The total number of glands present within this bar was counted and the value expressed as number of glands/linear mm. Average serous and mucous gland number was calculated from measures taken on each region from different specimens. As only one section was measured from each region in different specimens, they are true replicates. Additionally, the average of the greatest height and width of serous glands was calculated from measurements from three randomly selected glands from the same sections used to count the gland numbers. Similar procedures were used to measure number of glands/linear mm, and gland size in *B. pseudopseudis*, *H. curupi*, *H. marianitae*, *H. punctatus*, and *H. riojanus*, but only from scapular and cephalic regions.

Response of cutaneous glands to epinephrine and electrical stimuli

The response of cutaneous glands to different stimuli was observed *in vivo* and *in vitro*. Two types of *in vivo* stimuli were used: subcutaneous injection of epinephrine and surface electrical stimulation (SES). In the former, 500 μL of epinephrine (FADA) 1 mM and, as a control, physiological solution, were injected subcutaneously into the dorsal lymph sacs. This concentration was chosen because it has been demonstrated to be high enough to produce the discharge of the secretion from OSGs (Hoffman & Dent, 1977; Delfino et al. 2006). The SES treatment followed the protocol described by Tyler et al. (1992). The skin of the frog was moistened with distilled water, and an electrical stimulus (1–2 V, pulse length 2–4 ms) was applied for 8–10 s using two platinum electrodes that were rubbed gently along the dorsum of the animal. This time was enough to observe the secretion release, as well as to perceive the characteristic smell emitted by this species. As a negative control, the electrodes were left on the dorsum of the animal but with the electrical source off.

The responses of cutaneous glands in *H. pulchellus* were observed *in vitro* in incubated integumentary explants. The explants, 9 mm^2 pieces of skin, were removed under anaesthesia (lidocaine 20%). Release of cutaneous secretion during removal of explants and skin samples was minimized by first lightly anesthetizing the frogs, and then placing them on ice until they were completely immobilized. Neither non-volatile secretions nor the characteristic smell were perceived during this procedure. After removal, the skin was washed in physiological solution for 10 min. The explants were incubated in epinephrine at 1 mM in petri dishes at 10 °C for 20 min. Explants used as negative controls were kept in physiological solution under the same experimental conditions. Skin from the scapular region from experimental and control specimens treated with epinephrine were processed for TEM and LM as described in previous sections, whereas the skin from those specimens treated with SES were only processed for LM.

Results

Distribution of skin glands, and size of serous glands in *H. pulchellus*

Two types of glands, ordinary mucous glands (OMGs) and ordinary serous glands (OSGs), have been identified in all body regions. Gland distribution is heterogeneous in the six regions (Table 1; Fig. 1A–F). Ordinary serous

glands have a similar and moderate distribution in the cephalic, middorsal, midlateral, and midventral regions (2–6 glands/linear mm); they are less concentrated in the gular region (1–2 glands/linear mm), and more concentrated in the scapular region (4–9 glands/linear mm). In contrast, OMGs are uniformly distributed in almost all regions examined, except in the scapular region, where they are less concentrated (Table 1). Although there are differences in the mean size (mean maximum width and mean maximum height) of OSGs between the dorsal skin surfaces (cephalic, middorsal, and scapular), midlateral region and ventral surfaces (gular and midventral; Table 1), the size of OSGs in all regions is within the same range of variation.

Morphology and histochemistry of cutaneous glands in *H. pulchellus*

LM observations

Ordinary serous glands and ordinary mucous glands possess a basic structure; an intraepidermal duct, a neck and a secretory portion surrounded by a contractile sheath of myoepithelial cells (MECs). The serous glands possess a syncytial internal secretory layer with peripheral nuclei, a lumen filled with acidophilic granules, and a thick layer of MECs (Fig. 1G). A noticeable feature that characterized OSGs is the strong reaction of their secretory granules to lipid staining (SBB and Sudan III; Fig. 1H,I). Also, the granules react to CooB, indicating the presence of proteins (Fig. 1J). The granules of OSGs are PAS-negative, but react to AB 2.5 at their periphery (Fig. 1K).

Mucous glands are multicellular alveolar glands, formed by a single layer of secretory cells with basal nuclei and an evident lumen (Fig. 1E,F). Mucocytes are flat to cubical cells. Two distinct types of mucocytes are recognized based on their histochemical reaction; the content of some mucocytes react positively to PAS, whereas others are PAS-negative but their apical portion reacts positively to AB pH 2.5 (Fig. 1K). All mucocytes are negative to SBB and to CooB (Fig. 1H,J). The layer of MECs is much thinner than in OSGs.

TEM observations of OSGs

Ordinary serous glands are surrounded by the connective tissue of the dermis (Fig. 2A). A continuous sheath of MECs of variable thickness is found around the secretory portion of OSGs, separated from the collagen by a basement membrane (Fig. 2A,B). Adjacent myoepithelial cells forming this sheath are connected by a great number of desmosomes, which also connect MECs and the glandular syncytium (Fig. 2B). Nuclei of MECs are flat, and the cytoplasm contains a large number of smooth endoplasmatic reticulum (SER; Fig. 2A,B). Nerves are observed in the interstice between MECs and the syncytium (Fig. 2C, D). Syncytial nuclei are located at the periphery of the secretory portion. The cytoplasm encompassing these nuclei contains numerous mitochondria and rough endoplasmatic reticulum (RER), whereas Golgi, SER and free

Table 1 Number of glands per linear mm, and ordinary serous gland (OSG) size from skin samples of six body regions in *Hypsiboas pulchellus*, and from two body regions in five selected species of Cophomantini.[a]

		No glands (glands/linear mm)[b]		OSG size (μm)[c]	
Species	Body region	OSG	OMG	Maximum width (μm)	Maximum height (μm)
Hypsiboas pulchellus	Cephalic $n = 6$	3.6 ± 1.2 (3–6)	5.8 ± 1.5 (4–8)	91 ± 10 (74–112)	65 ± 7 (56–80)
	Scapular $n = 10$	7.3 ± 1.4 (4–9)	3.6 ± 1.2 (2–6)	88 ± 15 (67–135)	68 ± 21 (31–98)
	Middorsal $n = 6$	3.0 ± 1.2 (2–5)	6.8 ± 1.9 (4–10)	91 ± 18 (55–130)	63 ± 15 (40–102)
	Midlateral $n = 8$	3.1 ± 1.3 (1–5)	5.4 ± 0.7 (5–7)	81 ± 21 (45–123)	49 ± 11 (30–76)
	Midventral $n = 3$	4.7 ± 1.1 (4–6)	6.7 ± 2.1 (5–9)	110 ± 13 (74–126)	93 ± 9 (54–133)
	Gular $n = 2$	1.5 ± 0.7 (1–2)	6.0 ± 1.4 (5–7)	100 ± 1 (99–101)	60 ± 17 (48–72)
Hypsiboas curupi	Cephalic $n = 1$	3	6	98 ± 4 (95–101)	63 ± 2 (62–65)
	Scapular $n = 1$	9	7	109 ± 15 (94–125)	81 ± 8 (72–89)
Hypsiboas marianitae	Cephalic $n = 1$	1	6	92	81
	Scapular $n = 1$	5	5	105 ± 25 (77–123)	78 ± 12 (65–89)
Hypsiboas riojanus	Cephalic $n = 2$	3.0 ± 1.4 (2–4)	6 ± 1.4 (5–7)	135 ± 60 (70–213)	72 ± 41 (37–101)
	Scapular $n = 2$	5.0 ± 1.4 (5–6)	4.0 ± 1.4 (4–5)	92 ± 28 (65–112)	62 ± 23 (42–94)
Hypsiboas punctatus	Cephalic $n = 1$	7	2	105 ± 5 (100–109)	64 ± 1 (63–65)
	Scapular $n = 1$	8	4	128 ± 14 (120–144)	93 ± 25 (64–110)
Bokermannohyla pseudopseudis	Cephalic $n = 2$	3.0 ± 0.0	4.0 ± 1.4 (3–5)	110 ± 35 (84–135)	52 ± 24 (34–70)
	Scapular $n = 2$	4.5 ± 2.1 (3–6)	3.5 ± 0.7 (3–4)	99 ± 16 (80–131)	59 ± 21 (32–87)

[a]Values are expressed as mean ± SD, followed by the range in parentheses.
[b]The number of glands/linear mm were counted from a single slide randomly selected from each specimen.
[c]Whenever possible, gland measurements were taken from three randomly selected OSGs from a single slide.

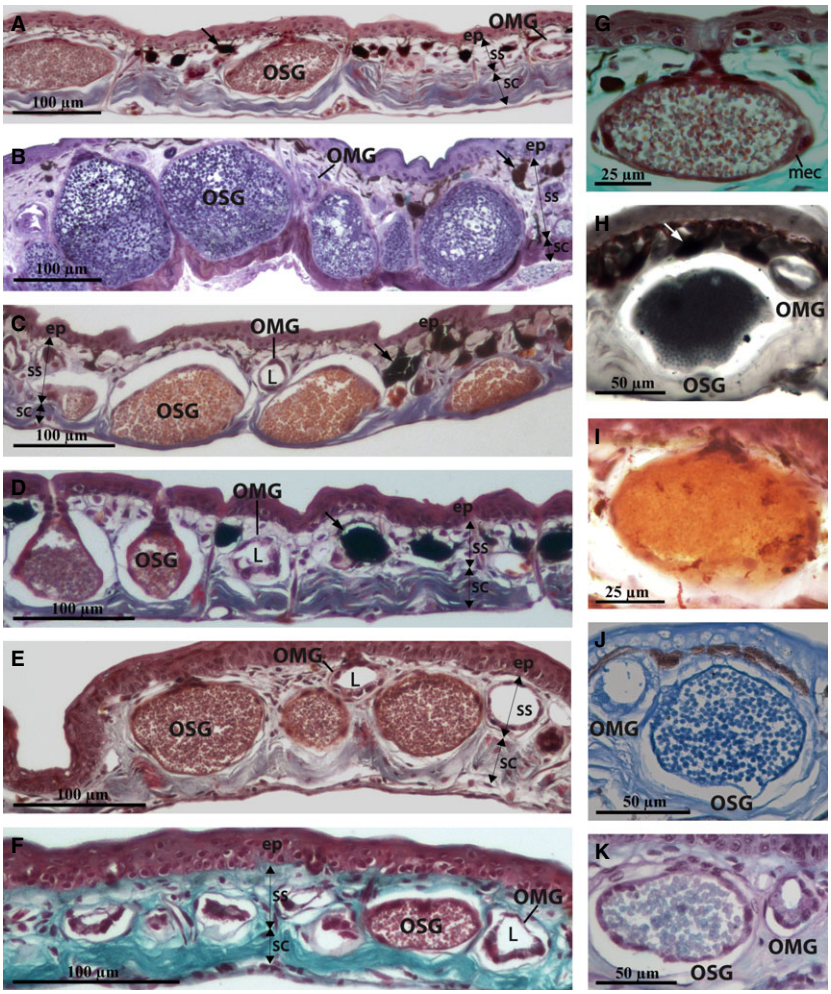

Fig. 1 Light micrographs of different skin regions of *Hypsiboas pulchellus* in cross-section (A–F), and details and histochemical reactions of ordinary serous glands (OSGs). (A) Cephalic region. (B) Scapular region. (C) Middorsal region. (D) Midlateral region. (E) Midventral region. (F) Gular region. Most regions can be recognized by differences in the concentration of OSGs (see also Table 1). (A–K) Ordinary mucous glands (OMGs) and OSGs can be identified by their size, structure, and histochemical properties. Note differences in thickness of the *stratum spongiosum* (ss) in regions with and without OSGs, and the presence of melanophores (arrows). Line arrows indicate the limits of the *stratum compactum* (sc) and ss within the dermis. Staining: (A,C–E) Masson trichrome; (B) Toluidine blue-basic fuchsin; (F,G) Masson–Goldner's trichrome; (H) Sudan Black B; (I) Sudan III; (J) Coomassie Blue R250; (K) Alcian Blue-PAS. Ep, epidermis; L, lumen; mec, myoepithelial cell.

ribosomes are commonly observed in a more central position within the gland (Fig. 2B,C,E,F). Occasionally, these organelles may also occur at the periphery (Fig. 2C).

A distinct feature of OSGs is the presence of a wide lumen full of secretory granules of different size, morphology, and electron density (Fig. 2G). All granules are round to oval, and it was not possible to detect a limiting membrane around them (Fig. 2H). The granules possess a spongy appearance, which is caused by the presence of small and round low electron-dense subunits within a homogeneous matrix of medium to high electron density. Based on their ultrastructural variation, three types of granules are differentiated. The first correspond to the general pattern described above (Fig. 2G,H). The second is similar to the former but is characterized by the presence of a low electron-dense vesicle in an eccentric location having a mesh-like appearance. Occasionally, vesicles from adjacent granules are found merged (Fig. 2H). The third granular type possesses wide low electron-dense regions randomly distributed within the medium to high electron-dense matrix (Fig. 2G).

Response of cutaneous glands of *H. pulchellus* to epinephrine and electrical stimuli

LM observations

Histological examinations revealed that OSGs, but not OMGs, were stimulated by subcutaneous injections of epinephrine (Fig. 3A,B). The discharge pattern of OSGs exhibits a range of variation that may be classified in three categories; without response, partially depleted and fully depleted. An amorphous material that covers most of the space of the gland is the secretory product remaining within partially depleted OSGs. In this case, intact granules are observed exclusively at the periphery of the glands (Fig. 3B). The same concentration of epinephrine administered *in vitro* on isolated skin explants induces on OSGs the release of the whole content, whereas no response was observed in OMGs (Fig. 3C,D). Although stimulation by epinephrine is observed in OSGs with a variable thickness of MECs, it is more evident in those glands with thicker MECs because of the obvious contraction at the surface of the MECs, as well as by the slight separation of neighbour cells (Fig. 3D).

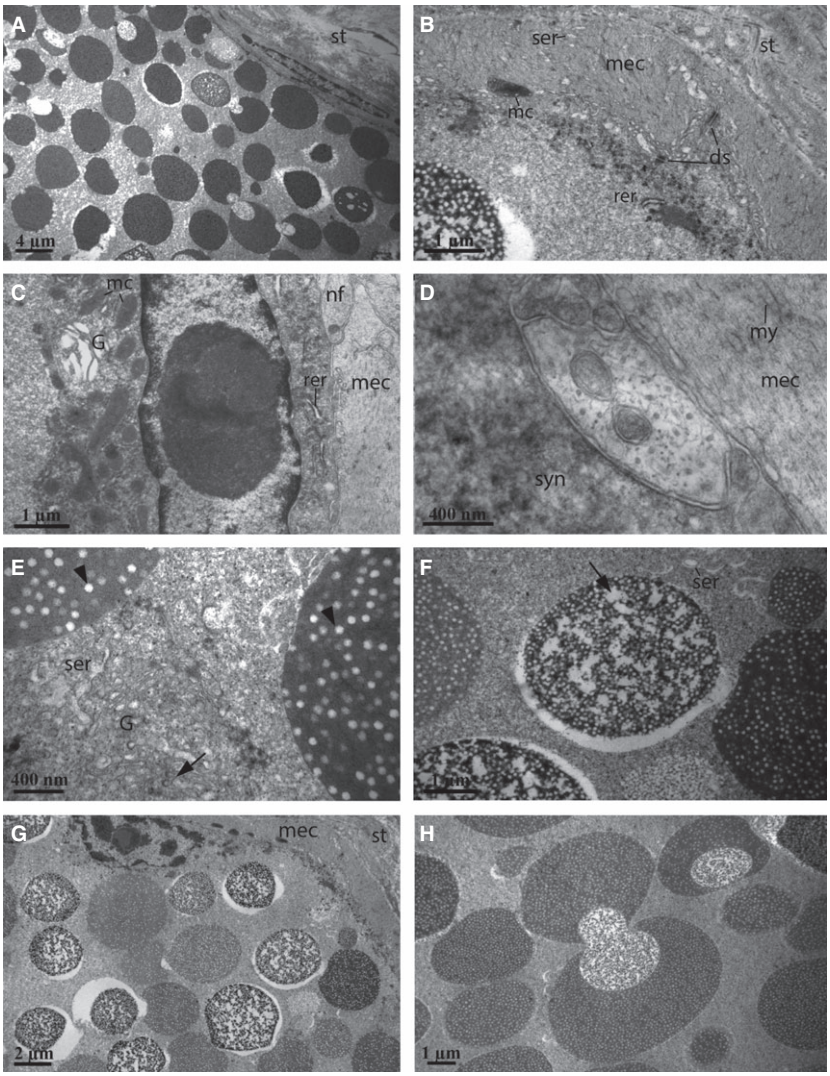

Fig. 2 Transmission electron micrographs of ordinary serous glands (OSGs) of *Hypsiboas pulchellus*. (A) General view of the gland. Note the lumen full of secretory granules of diverse appearance. (B) Detail of the thick sheath of myoepithelial cells (MECs) surrounding the secretory portion. Note the absence of cellular limits in the secretory portion comprising a syncytium (syn) and the presence of desmosomes (ds) among adjacent MECs and between these cells and the syncytium. (C) Detail of the peripheral region of the gland. The perinuclear area contains numerous mitochondria (mc), rough endoplasmatic reticulum (RER) and Golgi (G). Note the presence of a nerve fibre (nf) located at the interstice of the syncytium and MECs. (D) Detail of a nerve fibre. (E,F) The syncytium at a more central region possesses smooth endoplasmatic reticulum (SER) and abundant Golgi. Note in (E) emerging membrane of Golgi (arrows). (E–H) Granular diversity in OSGs. (E) Granules with spongy appearance possess several low electron-dense subunits homogeneously distributed (arrowheads). (F) Granules similar to those described in (E) but with large, low electron-dense areas of irregular shape. (G) Co-occurrence of granules that differ in electron density and appearance. (H) Granules with homogeneous distribution of low electro-dense subunits and a low electron-dense vesicle in an eccentric location having a mesh-like appearance. Note merged vesicles from adjacent granules. my, myofilaments; st, stroma.

As it is observed with epinephrine treatments, both *in vivo* or *in vitro*, SES stimulates OSGs, but not OMGs (Fig. 3E,F). The effect of SES on OSGs is noticed by the partial or full depletion of their content, and by the contraction of MECs which shows a wave-like morphology (Fig. 3F). Glandular content is secreted as an amorphous material, and some nuclei may also be expelled from the gland (Fig. 3E). Control treatments produced no observed effect.

TEM observations

One of the responses observed in OSGs to subcutaneous injection of epinephrine is the disintegration of most granules, thus generating the loss of its shape and amorphous material within the gland (Fig. 4A). However, this material retains some of the substructures found in granules (i.e. rounded low electron-dense subunits within a medium to high electron-dense matrix). Glands that released most of their content possess a flocculent material of medium electron density scattered within the syncytium (Fig. 4B). Neither granules or de-aggregated granular product were observed in these OSGs. The nuclei of the syncytium retain their peripheral location, whereas organelles, mainly mitochondria, are found toward the centre.

As observed in LM, OSGs that were stimulated by epinephrine administered on *in vitro* skin explants discharged the whole granular content (Fig. 4C). In these glands, spherical subunits of medium electron-density and double membrane vesicles of amorphous shape are found in a central position (Fig. 4C), whereas nuclei surrounded by RER and high electron-dense particles are located towards the periphery (Fig. 4D). Also at the periphery, are some small granules that were not secreted. These granules differed from those described in unstimulated glands because they lack spherical low electron-dense vesicles (Fig. 4E). Some high electron-dense particles are found at the borders of the granules, which are similar to those observed in the RER. Myoepithelial cells present a wave-like pattern at the

surface facing the lumen of the gland. The pattern is more evident in thicker MECs (Fig. 4F).

Serous glands in other species of Cophomantini

Skin samples from cephalic and scapular regions of *H. curupi*, *H. marianitae*, *H. punctatus, H. riojanus*, and *B. pseudopseudis* present similar morphological features to those found in *H. pulchellus* (Figs 1A–F and 5A–E). The concentration and distribution of OSGs vary between species, whereas OMGs have a similar concentration and distribution in all species (Table 1). Ordinary serous glands of the three species of the *H. pulchellus* group (i.e. *H. curupi*, *H. marianitae* and *H. riojanus*) are more concentrated in the scapular region than in the cephalic region (5–9 and 1–4 glands/linear mm, respectively), with similar values to those counted in *H. pulchellus*. In *H. punctatus* and *B. pseudopseudis*, OSGs are homogeneously distributed in both regions, but differ between the two species. In the former, the number of glands/mm is similar to those found in the scapular region of the species of the *H. pulchellus* group, whereas in the later the number is similar to those of the cephalic region of species of this group (Table 1). Although the mean size (maximum mean height and width) of OSGs of all the species examined is within the same range of variation, they are slightly larger in *H. curupi*, *H. marianitae*, *H. riojanus*, *H. punctatus* and *B. pseudopseudis* than those of *H. pulchellus.* As in the latter, OSGs from the other five species show a strong reaction to SBB, indicating the presence of large amounts of lipids (Fig. 5F–J).

Discussion

The skin from the six body regions examined in *H. pulchellus*, and from two body regions each in *H. curupi*, *H. marianitae*, *H. riojanus*, *H. punctatus* and *B. pseudopseudis* present two types of glands, OMGs and OSGs, whose morphologies have the general structure found in anurans (Fox, 1986; Toledo & Jared, 1995). In particular, the structure of both glands in these regions is similar to those observed

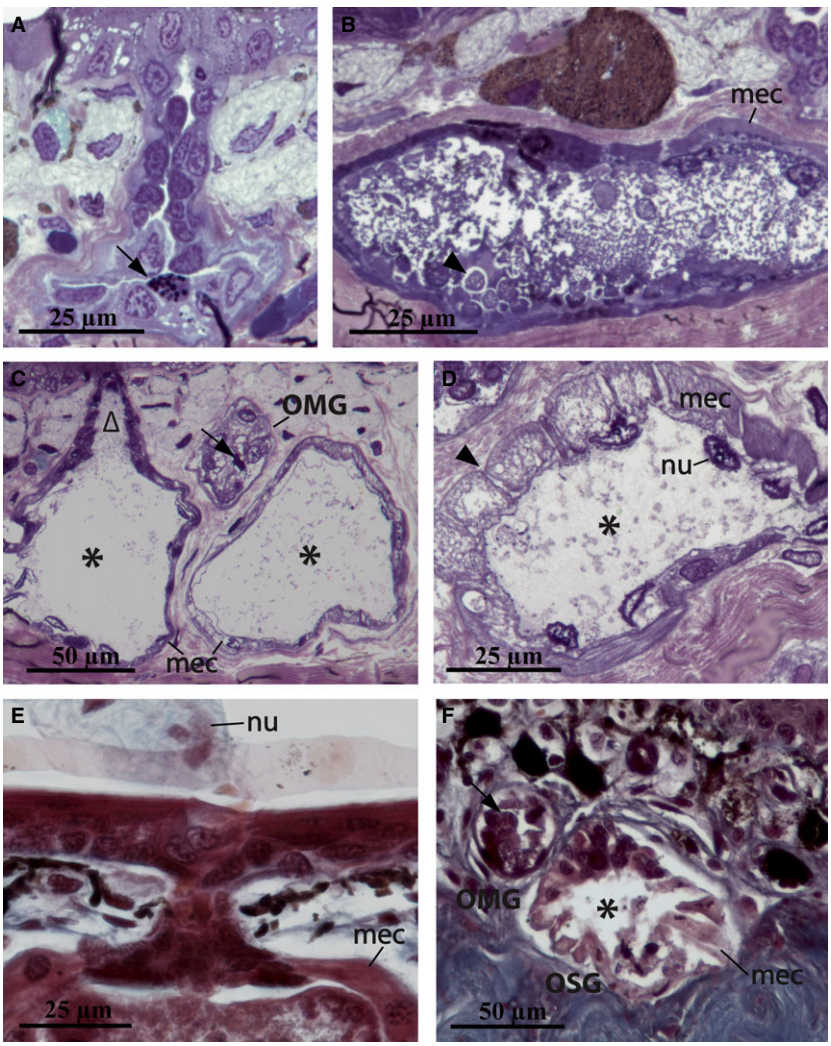

Fig. 3 Light micrographs of the scapular region of *Hypsiboas pulchellus* after different stimuli. (A,B) Subcutaneous injection of epinephrine (*in vivo* treatment). (A) No change is observed in ordinary mucous glands (OMGs). Note the presence of secretory granules within mucocytes (arrow). (B) Part of the content of ordinary serous gland (OSGs) was secreted. Within the gland, an amorphous material is observed covering most of the space, whereas intact granules are located at the periphery (arrowhead). (C, D) Epinephrine administered on isolated skin explants (*in vitro* treatment). (C) No change is observed in OMGs. Note the presence of secretory granules within mucocytes (arrow). Lumen of OSGs is devoid of secretion (asterisk), whereas the neck is dilated (triangle). (D) Myoepithelial cells (MECs) possess a wave-like morphology. An evident interstitial space is observed between adjacent cells (arrowhead). Note the absence of nuclei in OSGs in (C), and peripheral nuclei in (D). (E,F) Surface electrical stimulation (SES; *in vivo* treatment). (E) The secretion of OSGs within the gland, as well as the one that is expelled, possess an heterogeneous appearance. Note some nuclei being secreted with the gland content. (F) No change is observed in OMGs. Note the presence of secretory granules within mucocytes (arrow). Lumen of OSGs is devoid of secretion. Myoepithelial cells exhibit a conspicuous contraction. Staining: (A–D) Toluidine blue-basic fuchsin; (E,F) Masson trichrome. mec, myoepithelial cell, nu, nuclei.

from dorsal, ventral, lateral, and gular regions in *H. punctatus* (Brunetti et al. 2012), and from gular and lateral regions in *H. marianitae*, *H. curupi* and *B. pseudopseudis* (Brunetti et al. 2015a). Despite these similarities, the morpho-physiological evidence presented in this study along with the identification of volatile compounds through SES in *H. pulchellus* (Brunetti et al. 2015b), suggest that these components are stored and secreted within serous glands.

OSGs as source of volatile compounds and their relation with lipid secretions

Most of our knowledge on sources of volatile compounds in vertebrates comes from the study of 'scent glands' in mammals, squamates and crocodylians (e.g. Sokolov et al. 1980; Scully et al. 2000; Weldon et al. 2008). However, until now no association had been explored in vertebrates between ultrastructural data and secretion of volatile compounds. Ultrastructural studies of volatile-producing glands in insects suggest a close relationship among volatile compounds and the presence of electron-translucent inclusions referred in different contributions as vesicles or vacuoles (Sbrenna & Leis, 1983; Sreng, 1985; Liang & Schal, 1993). In particular, Liang & Schal (1993) noticed a positive correlation between the quantity of sexual volatile pheromone and the number of electron-translucent vesicles within the gland in the German Cockroach *Blatella germanica*. Our results showed that OSGs of *H. pulchellus* possess electron-translucent vesicles homogeneously distributed within the gland, and a specific granule type that has a vesicle with mesh-like appearance. As this vesicle is morphologically similar to those identified by Liang & Schal (1993) as the store site of volatile compounds, it seems likely that the volatile substances secreted by *H. pulchellus* might occur within the granules of OSGs in the small electron-translucent subunits and/or in the vesicles that contain the electron-translucent material.

The electron-translucent inclusions found within the granules of *H. pulchellus*, as in other anuran species (e.g. *Litoria caerulea*, *Pseudacris regilla*), resemble lipid vesicles, and/or lipid particles found in different animal tissues (e.g. Higgins & Hutson, 1984; Guyton et al. 1991), which may also explain the presence of SER related with the biosynthesis of lipids and the strong reaction of secretory granules to lipid staining. Morphological and experimental studies in the pheromone-producing cells of Silkmoth (*Bombyx mori*) demonstrated a positive correlation between the number and size of lipid droplets and the pheromone bombykol, a volatile 16-carbon alcohol (Fónagy et al. 2000, 2001). Following their results, the authors proposed that lipid droplets observed in the cells could be carriers of pheromone precursors and/or the pheromone bombykol. Most of the

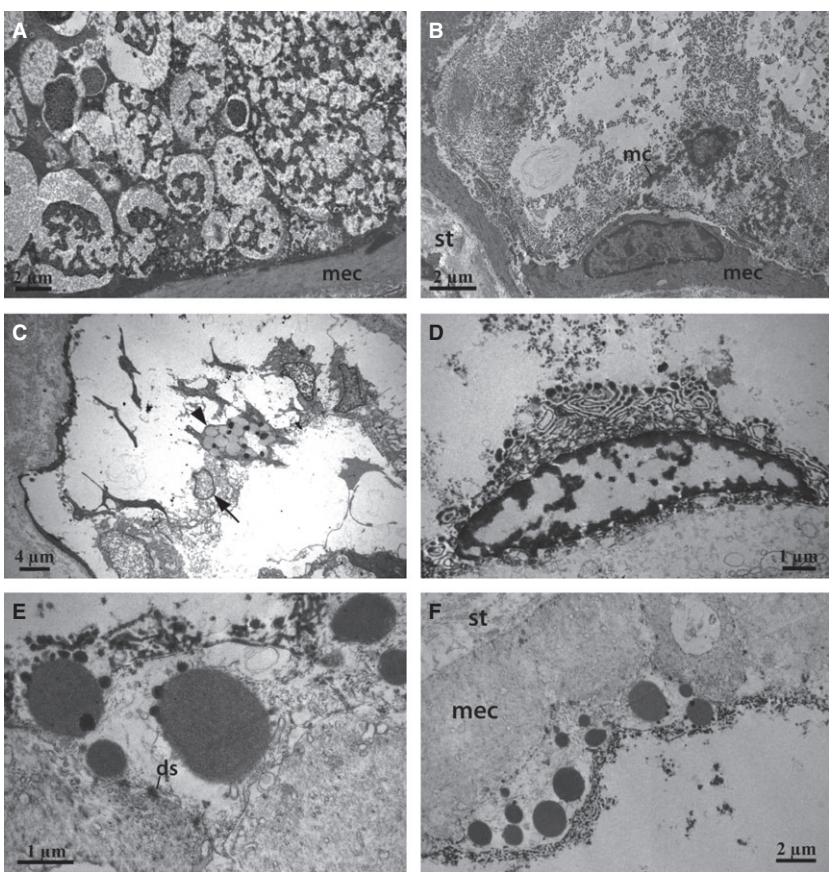

Fig. 4 Transmission electron micrographs of ordinary serous glands (OSGs) from the scapular region of *Hypsiboas pulchellus* after different epinephrine treatments. (A,B) Subcutaneous injection of epinephrine (*in vivo* treatment). (A) Gland with de-aggregated material from granules. Note the electro-dense matrix, and the low electro-dense subunits that were stored in the granules. (B) An OSGs that expelled most of their granular content. Note the flocculent material that possesses similar appearance to that observed among the granules in control specimens (compared to Fig. 3F). (C–F) Epinephrine administered on isolated skin explants (*in vitro* treatment). The gland expelled almost all of its content. (C) Spherical (arrowhead) and double membrane vesicles with mesh-like appearance (arrow) are observed toward the centre of the gland. (D) Nucleus at the periphery of the gland surrounded by large amounts of rough endoplasmatic reticulum (RER) and high electron-dense particles. (E) Small granules of moderate electron-dense content are also observed at the periphery. Note desmosomes (ds) among myoepithelial cells (MECs) and the translucent cytoplasm of the syncytium. (F) Myoepithelial cells with a wave-like pattern. mc, mitochondria; st, stroma.

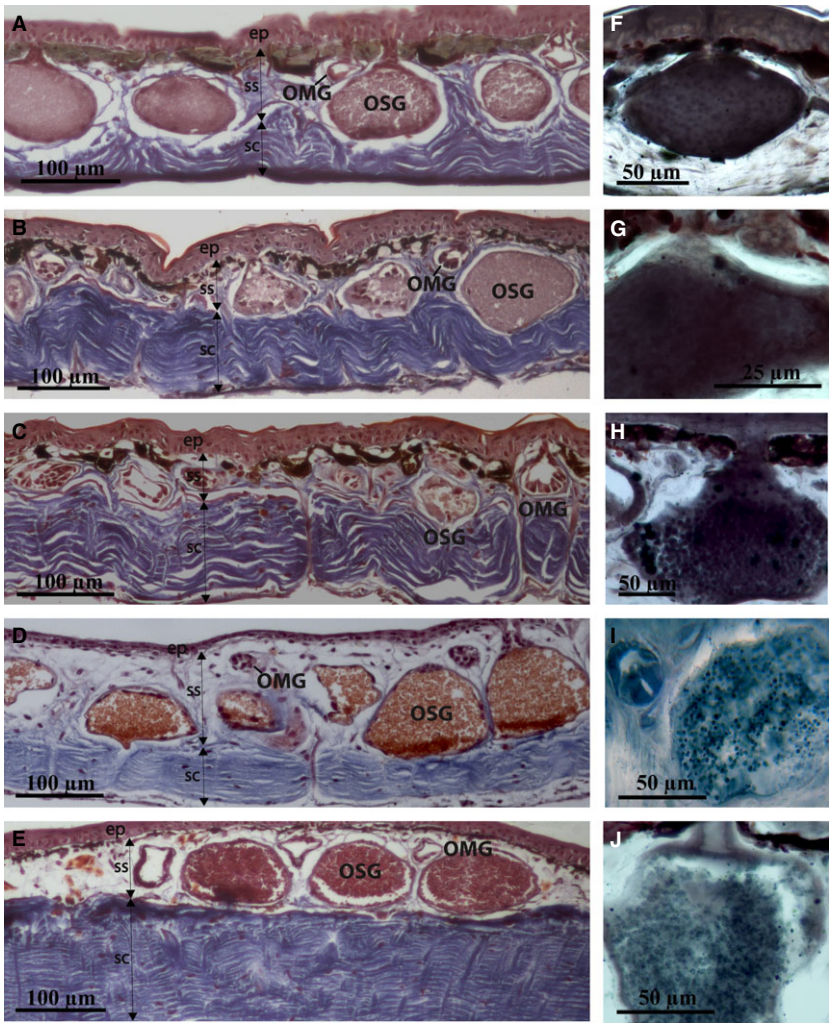

Fig. 5 Light micrographs of scapular regions in cross-section of selected species of *Hypsiboas* and *Bokermannohyla pseudopseudis* (A–E), and histochemical reaction of ordinary serous glands (OSGs) to Sudan Black B (SBB, F–J). (A,F) *Hypsiboas curupi*. (B,G) *Hypsiboas marianitae*. (C,H) *Hypsiboas riojanus*. (D,I) *Hypsiboas punctatus*. (E,J) *Bokermannohyla pseudopseudis*. (A–E) Ordinary mucous glands (OMGs) and OSGs can be identified by their size and structure. Line arrows indicate the limits of the *stratum compactum* (sc) and *stratum spongiosum* (ss) within the dermis. (F–J) In all species the contents of OSGs react positive to lipid staining (SBB). Staining: (A–E) Masson trichrome; (H–J) Sudan Black B. ep, epidermis.

volatiles secreted by *H. pulchellus* and *H. riojanus* are lipophilic compounds (e.g. some alcohols, aldehydes and ketones, esters, and terpenoids) that likely are derived either from the metabolism of fatty acids or the mevalonate pathway (Brunetti et al. 2015b). Although some of the low-molecular weight components could have been lost during preparative procedures for ultrastructural analysis, considering both the results of Fónagy et al. (2000, 2001) and ours, we suggest that the electron-translucent inclusions found within the granules in OSGs are the storage sites of volatile compounds and/or their precursors (e.g. linoleic acid and squalene).

Given that most of the volatiles described so far in species of anurans from different taxonomic groups are lipid soluble compounds, a similar situation is feasible in other species with odorous secretions. It then seems reasonable in these species to expect a granular morphology related with lipid secretions, occurring together with volatile components. For example, this was observed in secretory granules of other species, *Litoria caerulea* (Warburg et al. 2000), from which were identified volatile compounds that are soluble in organic solvents (e.g. 2-pyrrolidone, butyrolactone, and benzaldehyde). However, morphological and chemical studies need to be interpreted cautiously, as the presence of lipids in other anuran species does not imply the occurrence of volatiles. Specifically, electron-translucent inclusions that resemble those of *H. pulchellus* are found in anuran species in which there are no reports of odorous volatile secretions [e.g. *H. punctatus* (Brunetti et al. 2012), *Phyllomedusa sauvagii*, and *P. azurea* (as *P. hypochondrialis*; Delfino et al. 1998)]. Understanding the chemical nature of volatile and lipid compounds, the variability in the fine structure of the skin, and the taxonomic distribution of strong odours in anurans, are necessary to understand better the limits of the inferences discussed here.

OSGs as source of lipid compounds and chemical diversity

The presence of lipid secretion in cutaneous glands has a wide taxonomic distribution in hylids. In Hylinae and

Pelodryadinae, lipids are stored in OSGs (Warburg et al. 2000; Barbeau & Lillywhite, 2005; Brunetti et al. 2012, 2015a; this study), whereas in Phyllomedusinae they are stored in specialized lipid glands (Blaylock et al. 1976). Although the function of lipid secretions is unknown in Cophomantini, in some species, lipids might assist in the reduction of evaporative water loss (EWL) while basking (Centeno et al. 2015). However, the physiological role of lipid secretions to reduce EWL should be carefully interpreted as, according to the results of Withers et al. (1984), the presence *per se* of intradermal lipids is not correlated with a waterproofing barrier, although these lipids might contribute to a structural lipid barrier. Lipids have been detected in OSGs of several other anurans from many families (e.g. Arthroleptidae, Hyperoliidae, Leptodactylidae, Mantellidae, Microhylidae, Pipidae, Ranidae; Dapson et al. 1973; Thomas et al. 1993). An increased knowledge on the taxonomic distribution of lipid accumulation in OSGs would also contribute to an understanding of its physiological role.

In addition to lipids, our histochemical results show that OSGs of *H. pulchellus* possess proteins, as well as acid carbohydrates limited to the periphery of the granules. In addition, biochemical analyses demonstrated the presence of several antimicrobial peptides (Siano et al. 2014). The presence of a protein (and/or peptide) content within the granules of OSGs in this species may also explain the occurrence of some of the volatiles compounds (3-methyl-1-butanol, 2-methyl-1-butanol, and 2-phenylethanol) that would be formed from the degradation of amino acids (Brunetti et al. 2015b). The histochemical and chemical diversity observed from the secretion of OSGs was also evidenced from ultrastructural data because of the great number of mitochondria and both types of endoplasmic reticula (an RER related to the biosynthesis of peptides and proteins, and an SER related to the biosynthesis of lipids). The co-secretion of different biologically active compounds of serous glands is known from several species of amphibians (Erspamer, 1994), suggesting that they may be responsible for different types of functions.

Glandular response to pharmacological and electrical stimuli

Histological examination showed that OSGs from *H. pulchellus* respond to epinephrine treatment either *in vivo* (through subcutaneous injection) or *in vitro* (applied on isolated skin explants), and also to SES. Our data revealed that epinephrine and electrical stimuli have similar effects on OSGs, including the release of the glandular content and a contraction of MECs that surround the secretory portion. Contraction of MECs as a response to catecholamine (i.e. epinephrine or norepinephrine) was observed in several species of amphibians after *in vivo* (Dockray & Hopkins, 1975; Hoffman & Dent, 1977; Gammill et al. 2012) and *in vitro* treatments (Benson & Hadley, 1969; Hoffman & Dent, 1977; Melzer et al. 2011). The morphological features of MECs described in this study correspond to the type II MECs described by Bani & Delfino (1990). This type of MEC is engaged in fast and intense contractions, causing a quick discharge of the secretory product in those skin regions that undergo stimulation.

Ultrastructural observations allowed identification of the common morphological features of adrenergic innervation (i.e. electron-translucent vesicles with electron-opaque inclusions; Whitear, 1974; Dockray & Hopkins, 1975) in the nerve endings of OSGs of *H. pulchellus*. These nerve endings are situated in direct contact with plasma membrane of MECs, which suggests a synchronic activation of these cells favouring a quick discharge of serous product (Sjöberg & Flock, 1976; Bani & Delfino, 1990). Since the release of the volatile secretions of *H. pulchellus* occurs naturally under stress (Gallardo, 1958), and is also possible to reproduce it experimentally after SES (Brunetti et al. 2015b), our findings support the possibility that volatile compounds of this species play a defensive role. Moreover, it is possible to associate the emission of the volatile secretions after handling with the effect of epinephrine on the discharge of OSGs, as handling and the α-adrenergic agonists may exhibit similar physiological effects in some species of anurans (MbangKollo & deRoos, 1983).

Our results have shown some variability in the effects of epinephrine after subcutaneous injection. Because the effect of this stimulus is restricted to the site of injection (Dockray & Hopkins, 1975; Hoffman & Dent, 1977; Gammill et al. 2012), this variation could be the result of slight differences in injection site, and the distance of the processed skin sample from the point of injection. Alternatively, it may be explained by the presence of a modulated product discharge, which is characterized by a mild contraction of MECs, and a fluidified secretory material (Delfino et al. 1996). Our histochemical data agree with these observations, and according to Delfino et al. (1996) are consistent with the use of the secretion of OSGs in homeostasis.

Glandular distribution and biological function

Defence against microorganisms and predators is generally associated with noxious or toxic substances from serous glands (Fox, 1986; Toledo & Jared, 1995). In the family Hylidae the best-known glands associated to a defensive role are the macroscopically evident supratympanic macroglands of some species of the subfamilies Phyllomedusinae and Pelodryadinae (Warburg et al. 2000; Faivovich et al. 2010). These macroglands are characterized by the accumulation of large, elongated, syncytial serous glands that are clearly distinct from OSGs occurring along the body of those same species (Warburg et al. 2000; Antoniazzi et al. 2013). However, in some hylid species, particularly within the subfamily Hylinae, glands known to contain toxins

[e.g. glands in *Trachycephalus typhonius* (as *T. aff. venulosus*), Rigolo et al. 2008] are not macroscopically evident and are neither large nor elongated. According to morphological and histochemical evidence, OSGs in the skin from different regions in *H. pulchellus* are similar to OSGs of *T. typhonius* and to OSGs along the body of some phyllomedusines and pelodryadines. Although from a structural perspective, *H. pulchellus* and the other species of the *H. pulchellus* group do not possess a glandular specialization, the higher concentration of OSGs observed in the scapular region might be related to a distinct mode for defence against predators. For instance, given that anurans are usually ingested from the head (e.g. Albuquerque et al. 2007; Prates et al. 2011; Dorigo et al. 2014), this glandular aggregation may facilitate the release of higher amounts of secretion, including volatiles, during the first contact with a predator.

Gallardo (1958) described that individuals of *H. pulchellus* during predation events emit the characteristic smell and agonistic vocalizations simultaneously. The context in which both signals are produced, suggests that they are implicated in defensive functions. Alternatively, but not excluding an antipredatory role, they may act as alarm signals, alerting conspecifics to a predation risk. Behavioural experiments are needed to analyze the role of odorous volatile secretions as well as its interaction with acoustic signals during predation events.

Conclusions

This study provides the first morphological and functional basis to identify volatile-producing glands in amphibians; further studies in species secreting volatile substances are needed to test the inferences discussed here. Such studies will help to clarify the possible relationship of at least some of these compounds with lipids. They may also provide additional evidence of the great diversity of biologically active substances occurring simultaneously in a single glandular type, which suggests that these glands may carry out defensive and regulatory functions. The functional assessment of the gland discharge against epinephrine and SES supports a defensive role for these compounds. Moreover, the heterogeneous glandular distribution along the body might imply an antipredator mechanism. Behavioural and physiological experiments complemented with systematic observations are required to gain a functional understanding of frog odours, as well as the role of other glandular compounds.

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Conflict of interest

The authors have no conflict of interest.

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Appendix 1

Specimens of *H.* are housed in the herpetological collection of the Museo Argentino de Ciencias Naturales 'Bernardino Rivadavia'-CONICET, Buenos Aires, Argentina (MACN), and specimens of *Bokermannohyla pseudopseudis* are housed in the Departamento de Zoologia, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais. Abbreviations indicate the technique employed on the specimen: light microscopy (LM), and transmission electron microscopy (TEM).

H. pulchellus: MACN 40489 (LM); MACN 40490 (LM); MACN 40491 (Fig. 3F; LM); MACN 40493 (Fig. 3E; LM); MACN 46639 (Fig. 1D; LM); MACN 46641 (Fig. 1E,G,I; LM); MACN 46643 (Fig. 1F,J,K; LM); MACN 47728 (Fig. 1A; LM); MACN 47729 (LM); MACN 47731 (LM); MACN 47732 (LM); MACN 47734 (LM); MACN 47737 (LM); MACN 47740 (LM): Argentina: Entre Ríos: Departamento Islas del Ibicuy: Ruta 12 vieja, próximo a Brazo Largo, MACN 45120 (Fig. 4C–F; TEM, LM); MACN 45121 (Fig. 3C,D; LM); MACN 45124 (Fig. 3A,B; LM); MACN 45125 (Fig. 4A,B; TEM, LM); MACN 45126 (Fig. 1C; 2A,B,E–H; TEM, LM); MACN 45127 (Fig. 1B,H; LM): Argentina: Santa Fe: Departamento Garay: próximo a Helvecia.

H. curupi: MACN 37516 (Fig. 5A; LM); MACN 42591 (Fig. 5F; LM): Argentina: Misiones: Departamento Guaraní: San Vicente: INTA Cuartel Río Victoria.

H. marianitae: MACN 46637 (Fig. 5B,G; LM): Argentina: Salta: Departamento Santa Victoria: Parque Nacional Baritú, Quebrada La Cortadera.

H. punctatus: MACN 47715 (Fig. 5D,I; LM): Argentina: Santa Fe: Departamento La Capital: próximo a Alto Verde, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral.

H. riojanus: MACN 47726 (LM); MACN 47727 (Fig. 5C,H; LM): Argentina: Jujuy: Departamento General Manuel Belgrano: Laguna El Rodeo.

Bokermannohyla pseudopseudis: UFMG 11588 (LM), UFMG 11589 (Fig. 5E,J; LM): Brazil: Goiás: Alto Paraíso de Goiás.