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The inguinal macroglands of the frog *Physalaemus nattereri* (Leptodactylidae): structure, toxic secretion and relationship with deimatic behaviour

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Abstract

Amphibian skin is characterized by the presence of mucous glands, related to cutaneous breathing, reproduction and water balance, and granular glands, related to the production of toxins used in defence. In some species the granular glands can form accumulations in certain regions of the body. This is the case for inguinal macroglands of the leptodactylid frog *Physalaemus nattereri*, where these structures form a pair of black discs associated with deimatic behaviour. The morphology of the inguinal macroglands and their secretion were studied in this species and correlated to deimatic behaviour. The inguinal macroglands are formed from elongated granular glands that, in contrast with the granular glands of the rest of the skin, have small spherical granules with a proteinic content. In the dermis of the whole body, except for the inguinal macroglands and the inguinal region, a well-developed calcified dermal layer is observed. During deimatic behaviour these macroglands discourage a potential predator from attacking, but if visual cues are insufficient and the predator persists in the attack, a toxic secretion is eliminated in its mouth. This elimination is favoured by the absence of a calcified dermal layer in the macroglands, which makes the dermal region softer than the rest of the dorsal skin.

Key words: Amphibia, integument, inguinal macroglands, skin secretion, *Physalaemus nattereri*, defence

INTRODUCTION

Amphibian integument plays an important role in the defence against predators and microorganisms (Zug, 1993), which is directly associated to the presence in the dermis of mucous and granular glands responsible for the secretion of mucus and toxins, respectively (Lutz, 1966; Stebbins & Cohen, 1995; Toledo & Jared, 1995). In anurans, the mucous glands are basically formed by simple acini, and the granular glands are formed by secretory syncytia without lumen. Both types of glands are involved by a myoepithelial monolayer (Delfino, Nosi, Brizzi *et al*., 2001; Delfino, Nosi & Giaci, 2001) generally associated with the expulsion of the secretion. A great variety of chemical compounds is present in the secretion of the granular glands. For this reason, amphibian skin has been considered as a valuable source for the prospecting of new bioactive compounds (Toledo & Jared, 1995; Clarke, 1997).

In many species the granular glands form clusters, or macroglands (Toledo & Jared, 1995), which are strategically located on the body surface. In toads (bufonids) macroglands are common, forming the parotoids in the post-orbital region (Lutz, 1966; Hostetler & Cannon, 1974; Duellman & Trueb, 1986; Pough *et al*., 2001). In certain frogs, as in the leptodactylids, lumbar and inguinal macroglands are present in the dorsal skin (Toledo & Jared, 1989*a*, 1995; Toledo, Jared & Brunner, 1996). This is the case for *Physalaemus nattereri*, a frog occurring in mid-west and south-east Brazilian savanna (Cei, 1980). One of the most conspicuous characteristics of this species is the presence, both in males and females, of a pair of black and round inguinal glands, emarginated by a white contour (Vizotto, 1964; Lynch, 1970; Sazima & Caramaschi, 1986), giving the impression of two large black eyes.

In this paper, the morphology and histochemistry of the inguinal macroglands of *Physalaemus nattereri* are studied, and a preliminary analysis made of some biochemical and toxinological parameters of the bulk secretion extracted from these macroglands and their correlation with defensive behaviour. The morphological data revealed a specialized glandular structure, with

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secretion granules totally different from those observed in the rest of the skin. The analysis of the secretion demonstrated a high toxic potential.

Our results lead to the conclusion that the defence of *P. nattereri* may occur in two successive phases. First, the frog may try to intimidate a possible predator by exhibiting the large and false 'black eyes' when harassed. If this display is not effective and the predator persists in trying to bite the anuran, it may then be affected by the toxic secretion expulsed from the macroglands inside its mouth.

MATERIAL AND METHODS

Animals

Ten adult specimens of *Physalaemus nattereri* Steindachner, 1863 (snout–vent length 3.5–4.0 cm), were collected in the district of Rio Claro, State of São Paulo (Brazil). The animals were maintained for 4 months in the vivarium of the Laboratory of Cellular Biology of the Instituto Butantan in terraria containing humid earth at the bottom and dry branches. Food was supplied every other day and was composed of beetles *Tenebrio molitor*, crickets *Gryllus* sp. and cockroaches *Pycnocellus surinamensis*. During this period, behavioural observations were made and the secretion of the inguinal glands of 5 individuals was extracted monthly. The other non-extracted animals were then killed with an intraperitoneal overdose of Thionembutal, and samples of the skin and inguinal glands were removed for morphological studies. Voucher specimens were deposited in the collection of the Museum of Zoology, University of São Paulo (MZUSP).

Behavioural observations

The observations were made when the frogs were manipulated during the maintenance of the terraria (every 3 days). To stimulate defensive behaviour, the frogs were teased with the observer's forefinger. The defensive display was photographically registered and notes were taken.

Histology

Fragments of the dorsal, ventral and inguinal skin and both inguinal macroglands were removed from the frogs. For histological preparation the skin was cut in strips of *c*. 3 × 5 mm using a razor blade. Skin samples and the entire glands were fixed in 4% paraformaldehyde in PBS 0.1 M, pH 7.2 for 24 h. The material was embedded either in Leica historesin or paraffin.

For general study of the tissues, 2 μm historesin sections were stained with toluidine-fuchsin (Junqueira, 1995) and 5–7 μm paraffin sections were stained with haematoxylin-eosin. Collagen fibres were analysed using picro-Sirius staining followed by polarized microscopy (Junqueira, Bignolas & Brentani, 1979).

Histochemistry

Historesin and paraffin sections were submitted to the following histochemical methods: periodic-acid Schiff (PAS) and alcian blue pH 2.5 for identification of neutral and acidic mucosubstances, respectively; von Kossa for identification of calcium phosphate; bromophenol blue for identification of proteins (Bancroft & Stevens, 1996).

Photomicrography

Photographs were obtained in a Leica DMLB microscope equipped with a Leica MPS60 photographic system. Sections stained with picro-Sirius were photographed under polarized light in an Olympus BX-60 microscope equipped with a PM-C35DX photographic system.

Transmission electron microscopy

Skin and inguinal macrogland fragments (*c*. 1 mm^3) were fixed in Karnovsky solution, pH 7.2 (Karnovsky, 1965) for 24 h, post-fixed in 1% osmium tetroxide, contrasted in 2% uranyl acetate, dehydrated in ethanol and embedded in epoxy resin. Ultrathin sections were examined in a LEO 906E, operating at 80 kV.

Collection of secretion from the inguinal macroglands

The secretion of the inguinal glands of 5 frogs was collected by covering the macroglands with 2-cm^2 filter paper and squeezing them laterally between the thumb and the forefinger. The paper and resulting secretion was then soaked in 1 ml of PBS and crushed with a micropipette tip. After centrifugation, the resulting milky solution was stored at − 20 °C and then lyophilized.

Lethality and LD_{50} calculation of the macrogland secretion

The tests were made in BALB/c mice aged 2 months and weighing 18–20 g, obtained from Biotério de Criação de Animais Isogênicos do Instituto Butantan, SP, Brazil. The mice were treated and maintained under strict ethical conditions according to animal welfare international recommendation (Committee Members, International Society on Toxinology, 1991). The lethal toxicity of *P. nattereri* macrogland secretion was assessed in BALB/c mice by intraperitoneal injection of different amounts of the secretion (5, 10, 15, 25, 40 and 80 μg per animal) diluted in 200 μl of PBS. Five mice were used for each toxin dose. The LD_{50} was calculated by probity analysis of death occurring within 72 h of sample injection.

Electrophoresis

Samples of *P. nattereri* macrogland secretion were solubilized in non-reducing sample buffer, run on 12% SDS-PAGE (Laemmli, 1970) and stained with silver (Morrissey, 1980).

Zymography

Gelatinase activity of *P. nattereri* secretion was analysed by zymography (Kleiner & Stetler-Stewensow, 1994). Samples (3 μg) were run under non-reducing conditions on a 10% polyacrylamide gel containing 1% gelatine. The gels were washed twice for 30 min at room temperature in 2.5% Triton X-100, and incubated overnight at 37 °C in zymography buffer (50 mm Tris-HCl, 200 mm NaCl, 10 mm $CaCl_2$, 0.05% Brij-35; pH 8.3). Gels were stained in Coomassie brilliant blue solution (40% methanol, 10% acetic acid and 0.1% Coomassie brilliant blue).

RESULTS

Defensive behaviour of *Physalaemus nattereri* in captivity

When threatened, *P. nattereri* (Fig. 1a), usually presents a stereotyped set of postures and behaviours, consisting of puffing up the body laterally by inflation of the lungs, turning the back to the threatening agent and elevating the hind parts (Fig. 1b, c). In this posture the black inguinal glands are exhibited together with the prominent coccyx, giving the observer the impression of a large face with a pair of black eyes (Fig. 1b). The more the frog is mechanically stimulated, the more conspicuous this deimatic display is observed. When intensively harassed during the deimatic displays, the black skin covering the macroglands may turn whitish as a consequence of a milky secretion.

General morphological and histochemical characteristics of the skin

The skin is characterized by a large number of mucous and granular glands in the more external region of the dermis, the stratum spongiosum, just below the epidermis. The dorsal skin, when compared to the ventral skin, has a larger number of pigment cells (Fig. 2a), mainly chromatophores, with their characteristic black granules (Fig. 2c). The epidermis is composed of at least four layers of cells. The more superficial stratum corneum is formed by a single layer of flat nucleated cells (Fig. 2a).

The dermis is composed of the stratum spongiosum, filled by loose connective tissue, and the stratum compactum, characterized by the dense connective tissue consisting mainly of collagen fibres. Chromatophores and cutaneous glands are located within the stratum spongiosum (Fig. 2a).

The mucous glands are acinar, with a simple duct and larger diameter measuring *c*. 45 μm (Fig. 2a). The acini are formed by cells whose secretion is positive to PAS and alcian blue pH 2.5 methods.

The granular glands (G_1) are syncytial, without a lumen (Fig. 2a), and have a sole excretory duct. They are larger than the mucous glands, with a maximum diameter of *c*. 80 μm. The secretory syncytium is characterized by peripheral nuclei and by many large compacted granules. When stained with toluidine-fuchsin (Fig. 4a) or haematoxylin-eosin, these granules seem to be non-homogeneous both in size and stain affinity. Histochemistry reveals that these granules are negative

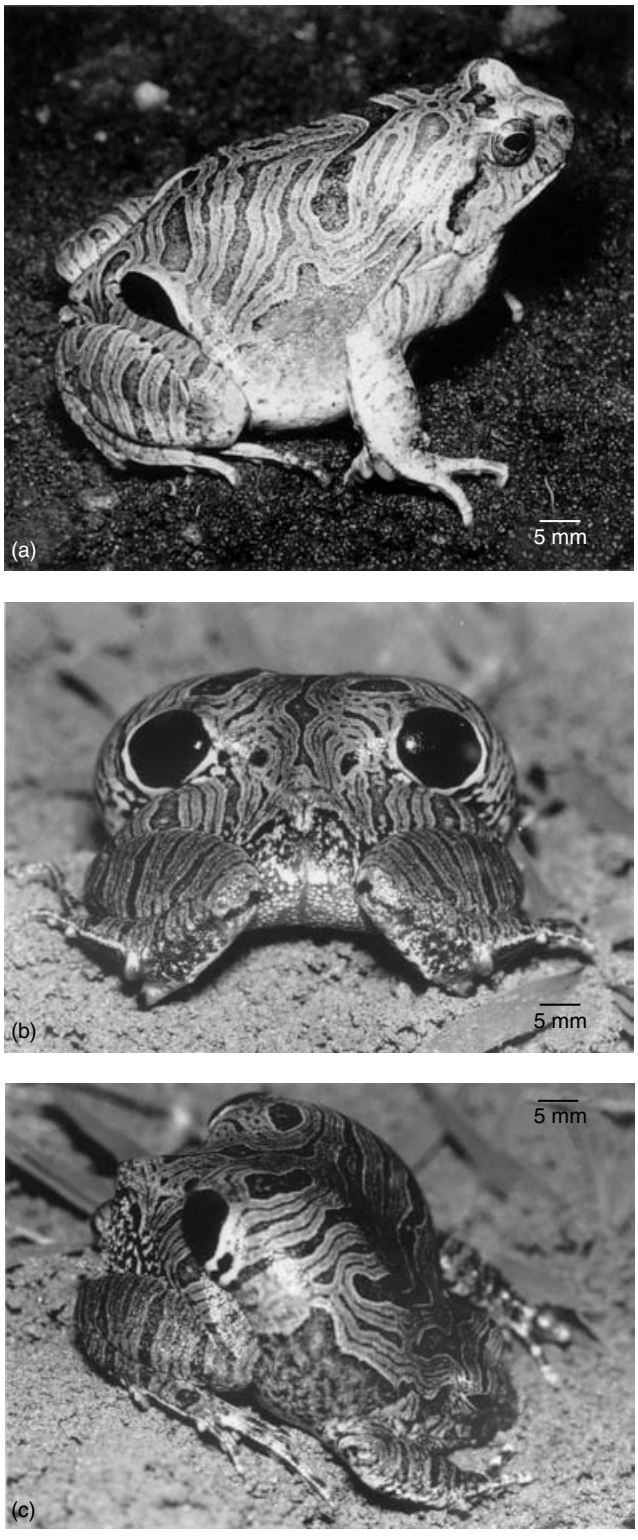

Fig. 1. *Physalaemus nattereri*. (a) Normal lateral view: (b, c) during defensive (deimatic) behaviour, exhibiting the inguinal macroglands: (b) back view; (c) lateral view.

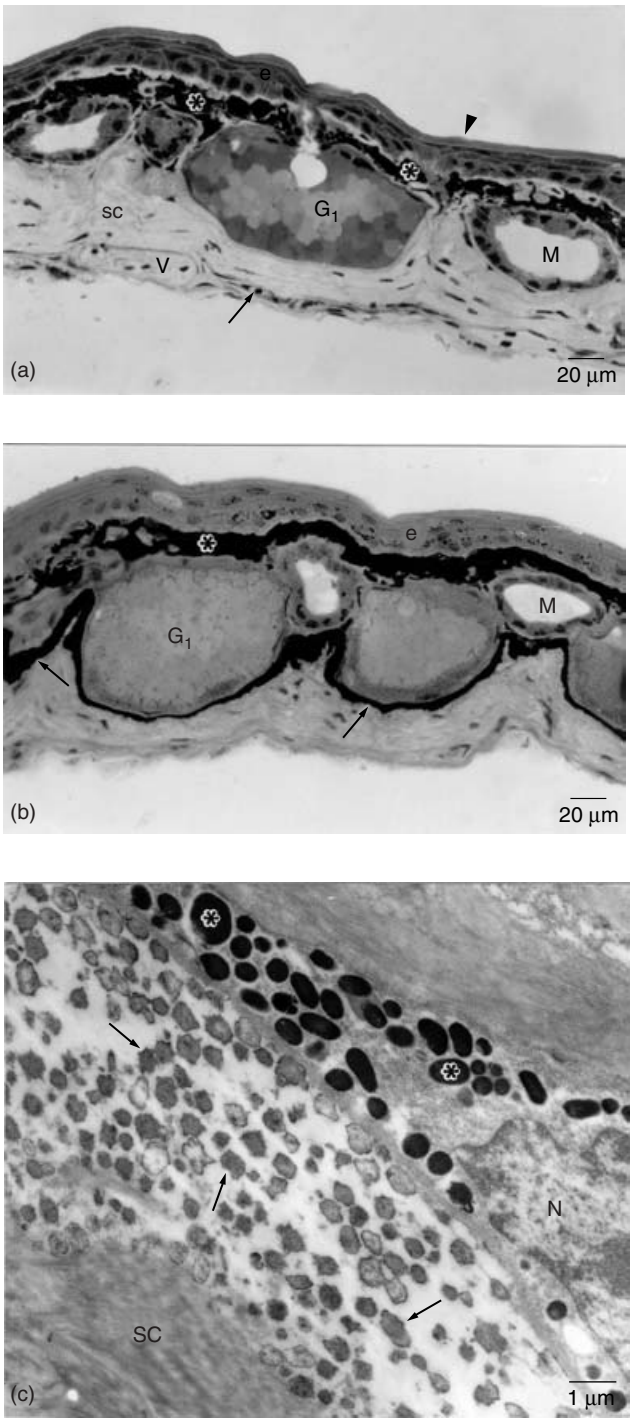

Fig. 2. *Physalaemus nattereri*. Histological section of dorsal skin: (a) where the two types of glands are observed; M, mucous gland; G_1, granular gland; arrowhead, stratum corneum; e, epidermis; *, chromatophores; sc, stratum compactum; arrow, subcutaneous tissue; v, blood vessel (historesin/toluidine blue-fuchsin staining); (b) where the calcified dermal layer is evidenced as a black line (arrows) basally contouring the cutaneous glands (historesin/von Kossa reaction), abbreviations as (a). (c) Transmission electron micrograph of the calcified dermal layer, consisting of globular structures (arrows) immersed in an electron-transparent matrix; upper right region shows a melanophore with melanosomes (*). N, Melanophore nucleus; sc, stratum compactum.

to PAS and alcian blue pH 2.5 and moderately positive to bromophenol blue (Fig. 4b). When observed at a transmission electron microscope (Fig. 4c), the granules are shown to be enclosed by a membrane and are different sizes and electron densities, despite all being made up of many circular subunits.

Both mucous and granular glands are enclosed by a monolayer of myoepithelial cells. These glands present the same morphological and histochemical characteristics in both dorsal and ventral skin.

Between the stratum spongiosum and the stratum compactum, a well-defined, calcified dermal layer (Eberth–Kastschenko layer) is present, contouring the mucous and granular glands. This layer is evidenced by the method of von Kossa (Fig. 2b), which indicates the presence of calcium phosphate. The calcified layer is well developed and continuous in the dorsal skin (Fig. 2b) but less developed and discontinuous in the ventral skin. Transmission electron microscopy shows that it is composed of irregular globules, with an homogeneous content of medium electron density, immersed in an electron transparent matrix (Fig. 2c).

The skin is internally delimited by a thin layer of subcutaneous tissue rich in blood vessels (Fig. 2a).

Histology and histochemistry of the inguinal macroglands

The inguinal macroglands comprise a pair of well-defined cutaneous structures. They are characterized by a cluster of granular glands (Fig. 3a, b), that are here referred to as G_2 to differentiate them from those present in the rest of dorsal and ventral skin (G_1). G_2 are elongated and much larger than G_1, measuring *c*. 340 µm high and 140 µm wide. Similarly to G_1, they are connected to the exterior by a sole duct (Fig. 3b, insert), do not have lumen and are enclosed by a myoepithelial monolayer (Fig. 4d). They are located side by side in the dermis in a honeycomb-like arrangement, each alveolus corresponding to one syncytium (Fig. 3a, b). The alveoli are immersed in dense connective tissue, forming a framework evidenced by the picro-Sirius method followed by polarized light microscopy (Fig. 3a). Under these conditions, it is possible to distinguish at least two types of collagen fibres in this framework, appearing in green and orange. At the transmission electron microscope, the epidermis lining the macroglands shows a microrugosity at the surface (Fig. 3c, insert). Among the keratinocytes, it bears melanophores and many sparse melanin granules, which are found even within the cornified layer (Fig. 3c). In the same way, the dermis in this region is highly pigmented, exhibiting an exceptional number of melanophores (Fig. 3c). This heavy pigmentation is responsible for the black colour observed in the inguinal macroglands.

Granules present in G_2 are much smaller than the granules in G_1 (compare Fig. 4a & 4d). They are spherical and very eosinophilic when submitted to haematoxylin-eosin staining, and have an intense affinity to toluidine blue (Figs 3b & 4d). They are strongly positive to bromophenol

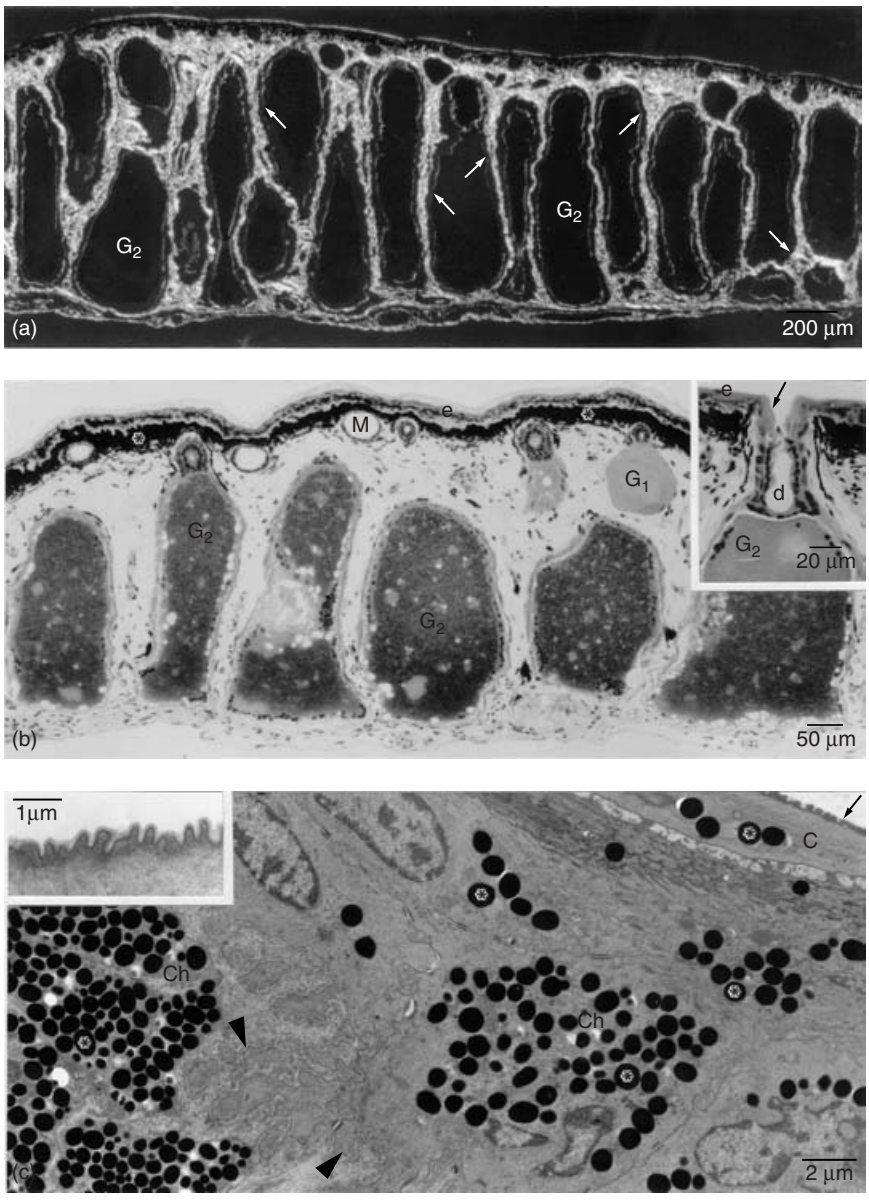

Fig. 3. Transversal section of the inguinal macrogland of *Physalaemus nattereri*: (a) collagen fibres (arrows) form an alveolar honeycomb-like structure, each alveolus corresponds to a granular gland (G_2) (low magnification under polarized light; paraffin/picro-Sirius method); (b) granular glands G_2 are morphologically distinct from those of the rest of the skin (G_1); insert: d, duct of a G_2 gland; arrow, glandular pore; e, epidermis; *, chromatophores; M, mucous gland (historesin and toluidine blue-fuchsin). (c) Transmission electron micrograph of the pigmented epidermis lining the inguinal macrogland. Note the presence of: Ch, chromatophores; *, isolated melanosomes within the keratinocytes, including c, cells of the stratum corneum; arrowheads, basal layer of the epidermis; arrow, microrugosity of the skin surface which is shown in higher magnification in the insert.

blue, indicating proteinic material in their content (Fig. 4e). Similarly to G_1, the spherical granules of G_2 are negative to PAS and alcian blue pH 2.5. When observed at the transmission electron microscope, the granules are heterogeneous in size, electron density and morphology of their content (Fig. 4f).

Just below the epidermis (and above the granular alveoli) mucous glands (Fig. 3b) are observed, histologically and histochemically similar to those of the rest of the skin.

In contrast to dorsal and ventral skin from the rest of the body, there is no trace of a calcified dermal layer in the dermis of the macroglands. This observation was confirmed by the negative result to the von Kossa method.

Biochemical characterization of *Physalaemus nattereri* macrogland secretion

Analysis of the secretion on SDS-PAGE followed by silver staining reveals many bands from 24 to 120 kDa (Fig. 5a). To verify the presence of gelatinases in *P. nattereri* macrogland secretion, samples were analysed for expression of these components by zymography. The

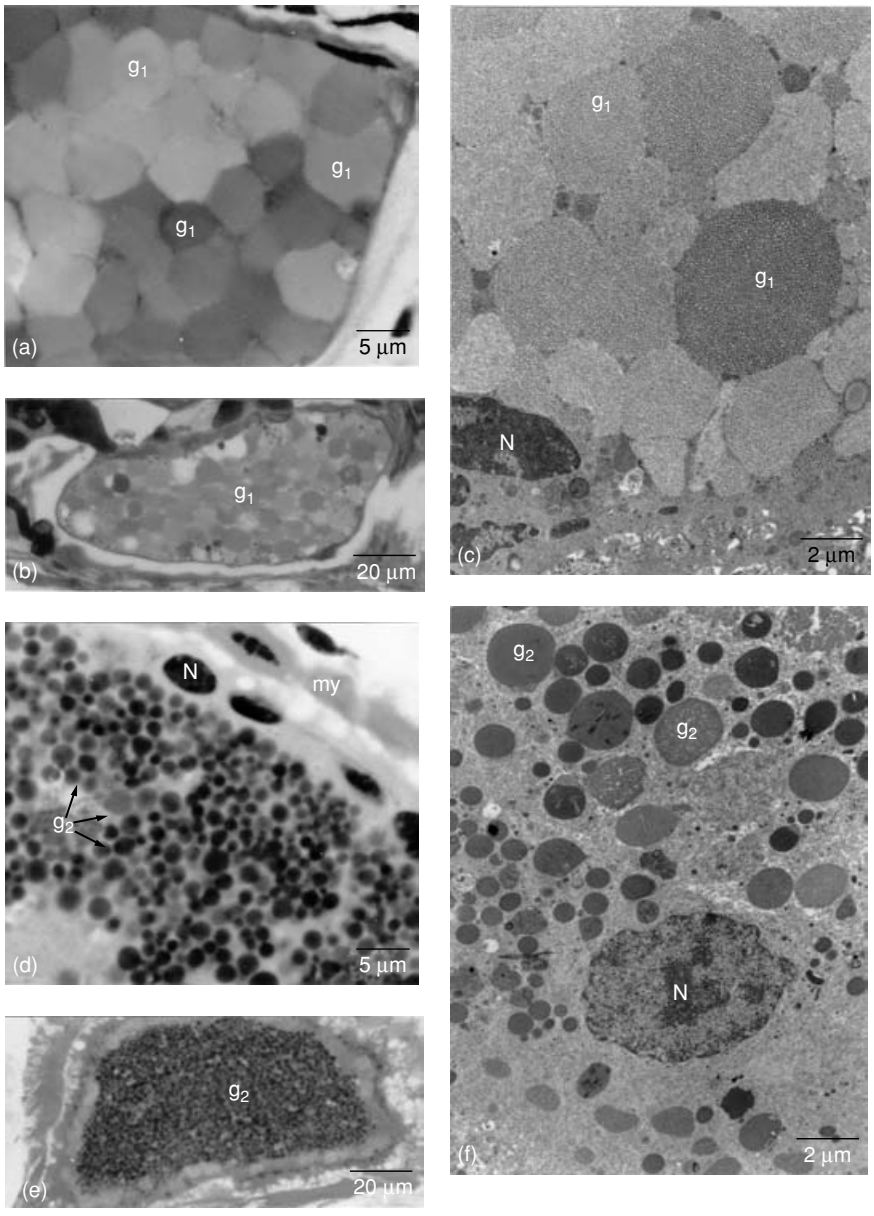

Fig. 4. Dorsal skin of *Physalaemus nattereri*: (a) histological high magnification; g_1, G_1 granules (historesin/toluidine blue-fuchsin); (b) histological section submitted to bromophenol blue reaction; g_1, granules of the granular gland G_1 are weakly positive to this histochemical method (historesin/bromophenol blue); (c) transmission electron microscopy of the granular gland G_1; g_1, granules are compacted and present a substructure that varies in electron density from one granule to another; N, nucleus of the secretory syncitium; (d) histological high magnification of the granular gland G_2 of the inguinal macrogland; g_2, granules appear spherical, not homogeneous in size but smaller than g_1, and with distinct tinctorial properties; secretory syncytium with peripheral nuclei (N); my, myoepithelial layer (historesin/toluidine blue-fuchsin). Granular gland G_2: (e) histological section submitted to bromophenol blue reaction; g_2, granules are strongly positive to this histochemical method (historesin/bromophenol blue; (f) transmission electron micrograph of part of a granular gland G_2; syncytial cytoplasm bears heterogeneous granules (g_2) with different sizes and electron densities. An evident morphological difference in their contents is also noted; N, nucleus of syncytium.

secretion contains three gelatinases with relative masses of *c*. 50, 61 and 70 kDa (Fig. 5b).

Toxicity of *P. nattereri* macrogland secretion in BALB/c mice

Groups of five BALB/c mice were injected intraperitoneally with different doses of secretion and the death survival ratio was determined after 72 h. The LD_{50} value, calculated by probit analysis at 95% confidence, is 27 µg (1.34 mg/kg).

DISCUSSION

To avoid ambiguity in gland classification, the term macroglands is used here instead of glands to designate

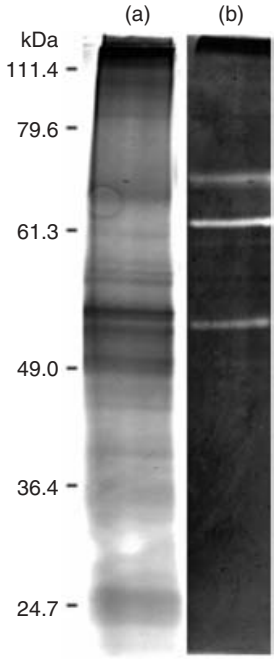

Fig. 5. (a) Electrophoretic analysis of *Physalaemus nattereri* secretion: samples (5 μg) were separated by SDS-PAGE (12% gel) under non-reducing conditions, and silver stained. A large number of bands from 24 to 120 kDa are revealed. (b) Zimography analysis: samples (3 μg) were run under non-reducing conditions on a 10% polyacrylamide gel containing 1% gelatine, and the reaction developed as described in material and methods. Three bands of gelatinases (relative mass *c*. 50, 61 and 70 kDa) were revealed. Gels were stained in Coomassie brilliant blue solution.

the secretory inguinal clusters, which are well-defined, multiglandular structures. The term glands is reserved only for the mucous and granular glands of the body skin that are single secretory units (Toledo & Jared, 1995).

Physalaemus nattereri, as well as other leptodactylids such as *Pleurodema brachyops* (Martins, 1989), *Pleurodema thaul* (Toledo & Jared, 1989*a*; Toledo *et al*., 1996) and *Physalaemus deimaticus* (Sazima & Caramaschi, 1986), have inguinal macroglands simulating large black eyes, which are an essential part of the deimatic behaviour (from the Greek word *deimos* = fear), common to all these species. This term, proposed by Edmunds (1974), refers to a set of intimidating stereotyped postures and actions adopted by species of many different taxa, such as insects, molluscs, fishes, reptiles, birds and mammals, when confronting possible predators.

Information about the structure of inguinal macroglands is still scarce. In *P. nattereri* these glands were only preliminarily studied by Vizotto (1964). Furthermore, information on the biochemical and pharmacological effects of their secretion in leptodactylids is almost non-existent (Toledo & Jared, 1989*b*; Toledo & Jared, 1995).

Physalaemin from *Physalaemus fuscomaculatus* is one of the few compounds already isolated from the skin of leptodactylids (Toledo & Jared, 1995). It is a bioactive peptide that when subcutaneously or intravenously injected in mice causes a considerable stimulating effect over the gastro-intestinal motility. This substance is also active on blood pressure, serving as a potent hypotensive agent (Bertaccini, Cei & Erspamer, 1965*a*,*b*).

The alveolar architecture of *P. nattereri* inguinal glands is similar to that observed in the parotoids of *Bufo jimi* and *Phyllomedusa distincta* (Jared *et al*., 2003). In all these species, the macroglands are composed of syncytial granular alveoli, disposed side by side in a honeycomb-like arrangement, which are morphologically similar to the granular glands of the rest of the skin, except for their larger dimensions. Therefore, the appearance of such structures in bufonids, hylids and leptodactylids seems to indicate the probable occurrence of adaptive convergence as a response to predator attacks. Although the general structure of the macroglands seems to be constant, their position varies in each group and distinctive morphological characteristics have been observed (Jared *et al*., 2003). In *P. nattereri*, these characteristics are: (1) the granular glands G_1, present in the skin, are morphologically distinct from those glands composing the inguinal macroglands (G_2); (2) the ducts of G_2 glands, unlike those of *Bufo* parotoids and similar to those of *Phyllomedusa* parotoids (Jared *et al*., 2003), are not obstructed by epithelial plugs; (3) unlike the rest of the dorsal skin, the inguinal macroglands do not show a calcified dermal layer (Eberth-Kastschenko layer) (Elkan, 1976; Toledo & Jared, 1993*a*,*b*).

Following the pattern already observed in the parotoids of bufonids and hylids (Phyllomedusinae), the alveoli of the inguinal macroglands of *P. nattereri* are supported by a framework of collagen fibres which, under polarized light, brighten in yellowish orange and green. According to Montes & Junqueira (1991), these colours may indicate the presence of collagen fibres type I and III, respectively.

In *P. nattereri*, the absence of epithelial plugs obstructing the ducts in the macroglands may indicate that there is no internal pressure inside the alveoli of G_2, contrary to observations in *Bufo ictericus* and *Bufo jimi*, where the alveolar secretion seemed to be under constant pressure (Toledo *et al*., 1992; Jared *et al*., 2003). Because of these differences, the inguinal macrogland of *P. nattereri* eliminates its content in the form of small drops, which accumulate on the external surface of the epidermis. In contrast, in *Bufo ictericus* and *Bufo jimi*, the manual compression of the parotoid provokes the rupture of the epithelial plugs and the consequent expulsion of the toxic secretion in the form of strong jets (Jared *et al*., 2003).

The elimination of the secretion as small drops has already been observed by Sazima & Caramaschi (1986) in *Physalaemus deimaticus*, in which a simple mechanical stimulus by touch is sufficient for the secretion to be eliminated. During the defensive display, *P. nattereri* may show a milky secretion over the gland skin. One of six frogs mechanically stimulated during deimatic behaviour showed the black skin of the macrogland covered by a layer of milky secretion, turning the 'eyes' cloudy. Furthermore, in nature, it has already been observed that, at the moment of the defensive display, *P. nattereri* may show little drops of secretion over the dark macrogland skin (D. Pavan, pers. comm.). The conspicuous microrugosity observed on the

surface of the macrogland epidermis may facilitate the retention of glandular secretion over the skin surface. On the other hand, the elimination of glandular secretion in many species of anurans is controlled by the myoepithelial layer, which involves the granular glands (Delfino, Nosi, Brizzi *et al*., 2001; Delfino, Nosi & Giachi, 2001). In the pipid *Xenopus laevis*, it is known that the elimination of the cutaneous secretion is controlled by an adrenergic action over the myoepithelium and follows a holocrine mechanism of secretion (Barthalmus, 1994). The same mechanism may occur in *P. nattereri* when harassed, as Vizotto (1964) has already suggested.

In *P. nattereri*, the black colour of the inguinal glands is certainly related to the great quantity of melanin granules observed in the dermal and epidermal strata. The 'black eyes' that are formed in this way, together with the white contour which stands them out even more, create an efficient visual warning which, in many cases, must be sufficient to discourage the attack of potential predators, visually oriented. In the case the predator insists in the attack, trying to bite the frog, its oral mucosa will be in contact with the gland secretion, causing its poisoning.

On the other hand, in *Physalaemus fuscomaculatus*, the inguinal glands are not black and consequently do not stand out from the cryptic pattern of the dorsal skin. Even though these glands are exhibited in postures similar to those observed in *P. nattereri*, they probably do not have the same visual impact (Sazima & Caramaschi, 1986). In *P. fuscomaculatus*, therefore, the defensive display may be less efficient. As the natural history of both *P. nattereri* and *P. fuscomaculatus* is still poorly known, differences regarding pigmentation of macroglands may be related to predators or predation strategies of these species.

One of the most important results of our analysis of *P. nattereri* skin is the histological and ultrastructural differences observed between the granular glands G_1 and G_2. This finding seems unreported for anurans and suggest a high degree of integument specialization, in which different glandular secretions were developed in specific regions of the body, possibly associated with deimatic behaviour.

The inguinal macroglands release secretion with high toxicity, capable of envenoming and killing a potential predator. An LD_{50} of *c*. 27 μg is very high when compared with, for example, the viperid snake *Bothrops jararaca* ($LD_{50} = 45$ μg; Sanchez, Freitas *et al*., 1992), responsible for the largest number of snakebites in Brazil (Ministério da Saúde, Fundação Nacional da Saúde, 1998). As an average of 8 mg of protein is obtained from each individual, the toxic compounds in the macroglands of one frog would have the potential to kill *c*. 150 mice. Furthermore, the gelatinases in the inguinal secretion detected by zimography may contribute to its toxicity, as proteases capable of digesting gelatine are abundant in the venom of snakes and arachnids and play an important role in the pathology of envenomation (Sanchez, Cordeiro *et al*., 1995; Almeida *et al*., 2002).

Toxins from the inguinal macroglands seems to be efficient in the chemical defence of *P. nattereri*. Sazima & Caramaschi (1986) reported observations, both in nature and in captivity, of predation (or predation attempts) on *P. nattereri* by potential vertebrate predators. These authors report that in nature, under the perch of an owl *Tyto alba*, four dead but intact specimens of *P. nattereri* were found together with regurgitated pellets, indicating an immediate toxic effect of the skin secretions on the bird. Additionally an experiment in captivity where a specimen of *P. nattereri* was offered to the procyonid *Nasua nasua* (coati) showed that the frog was mouthed and immediately rejected, fleeing alive. Just after, the coati repeatedly rubbed its snout with the forelimbs, demonstrating that it was disturbed. A second specimen of the frog offered to the same animal was vigorously rolled and rubbed against the soil before ingestion. Another possible natural predator of *P. nattereri* is the colubrid snake *Waglerophis merremii* (formerly *Xenodon merremii*), known for its specialization in preying on toads (Brazil & Vellard, 1926; Fonseca, 1949). This assertion was supported by Sazima (1973) who observed this snake preying on *P. nattereri* in captivity.

The toxic granules of the inguinal macroglands G_2 showed a strong positivity to the bromophenol blue method, indicating a high proteinic content in the secretion, which coincides with our electrophoretic results. The same histochemical method, however, when applied to G_1, indicates a weak positivity of its granules. These results reinforce the evident morphological differences observed between the G_1 and G_2 granular glands. These histochemical findings lead to the conclusion that these differences are probably not only morphological but also chemical. On the other hand, in the same way the compound physalaemin was identified in the skin of *Physalaemus fuscomaculatus* (Bertaccini *et al*., 1965*a*,*b*), it is possible that analogue peptides may also occur in the skin of *P. nattereri*.

Another important fact in this context is the absence of the calcified dermal layer in the inguinal macrogland, contrasting with the rest of the body skin. Without a calcified layer, the integument of this region must be more malleable, facilitating glandular compression at the moment of the predator's bite and enabling a more efficient elimination of the toxic secretion inside its mouth.

In contrast to the prominent post-orbital parotoids of bufonids, hylids and urodelans, the position of the inguinal glands of *P. nattereri* suggests that they were not selected to avoid the same predator strategy expulsing the secretion at the moment of the predator's bite. Their position, their similarity with eyes and the fact they are not able to expulse the glandular content in the form of jets, indicate that their evolution, probably was initially more due to the selection of visual displays capable of intimidating the predator than to the efficiency of secretion expulsion. The backwards posture and the spontaneous elimination of secretion in the form of small drops deposited on the skin surface, however, may favour the contact of toxins with the predator's oral mucosa.

Sazima & Charamaschi (1986) emphasize a few reports of people walking in the field that confused *Physalaemus deimaticus* (a species that has the same association posture/eyes as those observed in *P. nattereri*) with a snake

head. Similar situations with *P. nattereri* have also been experienced during field expeditions. In fact, the 'face' exhibited by *P. nattereri* during deimatic display is very convincing. It is composed of the pair of 'eyes', which are enhanced by the white contours, a 'nose' formed by the coccyx, and a 'mouth' formed by the hindlimbs juxtaposed to the body. Furthermore, the appearance of the face is enhanced by the lateral inflation of the lungs and the elevation of the hind parts out of the substrate.

This paper was about a leptodactylid presenting deimatic behaviour and having eye-like inguinal macroglands. To contribute to the knowledge of these structures, this line of investigation could be continued with a comparative study of other leptodactylids with inguinal macroglands, such as *P. fuscomaculatus* (deimatic behaviour but no black pigmentation) and species of *Ciclorhamphus* (neither deimatic display nor pigmentation).

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