Parotoid macroglands in toad (Rhinella jimi): Their structure and functioning in passive defence

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A B S T R A C T

When toads (Rhinella) are threatened they inflate their lungs and tilt the body towards the predator, exposing their parotoid macroglands. Venom discharge, however, needs a mechanical pressure onto the parotoids exerted by the bite of the predator. The structure of Rhinella jimi parotoids was described before and after manual compression onto the macroglands mimicking a predator attack. Parotoids are formed by honeycomb-like collagenous alveoli. Each alveolus contains a syncytial gland enveloped by a myoepithelium and is provided with a duct surrounded by differentiated glands. The epithelium lining the duct is very thick and practically obstructs the ductal lumen, leaving only a narrow slit in the centre. After mechanical compression the venom is expelled as a thin jet and the venom glands are entirely emptied. The force applied by a bite of a potential predator may increase alveolar pressure, forcing the venom to be expelled as a thin jet through the narrow ductal slit. We suggest that the mechanism for venom discharge within all bufonids is possibly similar to that described herein for Rhinella jimi and that parotoids should be considered as cutaneous organs separate from the rest of the skin specially evolved for an efficient passive defence.

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1. Introduction

Amphibian skin glands are a synapomorphy of the group and are usually present in great numbers on the whole body of all species. Among amphibian skin glands the most conspicuous are undoubtedly the parotoids. Parotoids (for etymology and the recommendation to use parotoid instead of “paratoid” or “parotid” see Cannon and Palkuti, 1976; Tyler et al., 2001) are multiglandular structures, which can be found as paired protuberances, postorbital in position, in a variety of Anura (Wilber and Carroll, 1940; Lutz, 1971; Toledo et al., 1992) and Urodela (Phisalix, 1922; Luther, 1971; Brodie and Smatresk, 1990; Toledo and Jared, 1995). Because parotoids consist of an aggregation of numerous secretory units, they have been named macroglands to differentiate them from the common mucous and granular glands of the remaining skin (see Toledo and Jared, 1995).

There seems to be no doubt that parotoids as well as the granular glands distributed over the body of a toad, from which the parotoids are derived (Toledo et al., 1993; Toledo and Jared, 1995), are involved in chemical defence including defence against microorganisms (Tempone et al., 2008). Behavioural studies in both Anura and Urodela have demonstrated that many species show characteristic defence behaviour when threatened. Urodela, for example,
position their body, tilting it toward the source of contact (for review see Brodie, 1983) and passively release unpalatable or toxic cutaneous glandular contents onto the body surface, rather than actively spraying them at predators (e.g., Brodie, 1983; Brodie and Smatresk, 1990). Anura, especially toads, inflate their lungs and also assume special postures to present the parotoids to the source of danger, but seem to spray their venom only in response to physical pressure (e.g., Hinsche, 1928; Toledo and Jared, 1995).

Secretions of the granular cutaneous glands (and parotoids) may be very toxic to animals (Lutz, 1966; Toledo and Jared, 1989a, 1995; Barthalmus, 1989; Clark, 1997). In bufonids they contain steroids such as bufogenines and bufotoxins that, when in contact with the buccal mucosa of many vertebrates, especially of snakes, have cardioacceloratory properties increasing the strength of the heart beat and decreasing heart rate (Habermehl, 1981). The venom can also exert a marked effect as a local anaesthetic (Habermehl, 1981). In the case of a bite, after venom ingestion, the potential predator can show intense salivation and excitation, paralysis, trembling and convulsions, often leading to death (Vital Brazil and Vellard, 1925, 1926; Garrett and Boyer, 1993; Pineau and Romanoff, 1995; Sakate and Lucas de Oliveira, 2000; Sonne et al., 2008).

Anuran granular or venom glands are syncytial in nature (Fox, 1986; Delfino, 1991; Terreni et al., 2003). They are irregularly distributed over the whole body surface, but are found also in aggregated forms in certain parts of the body such as the parotoid and the paracnemic macroglands of bufonids (Hostetler and Cannon, 1974; Toledo and Villa, 1987; Toledo et al., 1992) or the lumbar macroglands of some leptodactylids (Toledo and Jared, 1989b, 1995; Toledo et al., 1996; Lenzi-Mattos et al., 2005). Studies on the structure and function of such aggregations are limited. Specifically in toads, there are some articles that describe their secretory syncytia, the secretion granules and the myoepithelial layer (Hostetler and Cannon, 1974; Cannon and Hostetler, 1976; Toledo et al., 1992; Barthalmus, 1994; Almeida et al., 2007). In addition, a special vascularization net irrigating the toad parotoids has been described in detail (Hutchinson and Savitzky, 2004). On the other hand, the biochemistry and biological effects of toad skin, including the parotoids, are relatively well studied (Pas- net irrigating the toad parotoids has been described in detail (Hutchinson and Savitzky, 2004). On the other hand, the biochemistry and biological effects of toad skin, including the parotoids, are relatively well studied (Pas-
citrate (Reynolds, 1963) and examined in a LEO 906E transmission electron microscope.

3. Results

3.1. Anatomy of the parotoid glands and venom discharge induced by external pressure

The parotoids are conspicuous glandular structures, which are easily dissected and removed from the body when the dorsal skin around them is cut. Externally the parotoid skin seems similar to the rest of the dorsal skin, except for the presence of a large number of well defined pores (Fig. 1). When the macroglands are horizontally cut into two pieces and the pasty secretion is removed, a honeycomb-like structure is revealed (Fig. 1(2,3)), composed of many alveoli. Transverse histological section through the parotoid shows that each alveolus contains a very large bottle-shaped gland, full of secretion and closed on the top by a thick ductal epithelium apparently forming a plug (Fig. 1(4)). Serial sections, however, reveal that there is a very narrow ductal lumen forming a slit (Figs. 1(4) and 3(14)) through which the secretion can pass when the gland is squeezed. Since the parotoid forms a convex structure, the bottle-shaped glands are larger and more elongated in the central region and gradually decrease in size toward its borders. The pores on the surface of the parotoids measure from 0.5 to 1 mm, depending on the individual, and appear as circular depressions, almost entirely obstructed internally by solid tissue, except for the small slit in the centre. Each pore is externally surrounded by smaller pores forming a special pore pattern on the parotoid surface (Figs. 1(6) and 3(16)).

A constant manual pressure applied to the parotoids forces the venom to be discharged only from the pores corresponding to the glands which were effectively squeezed between the fingers. The venom is expelled in the form of thin jets (Fig. 1(3)). It is sticky and has a colour varying from white to yellowish.

3.2. Histology and histochemistry

The dorsal skin has an irregular surface and is composed of the epidermis containing around six cellular layers, and the dermis with the stratum spongiosum, where mucous and granular glands are observed, and the stratum compactum, mainly constituted by thick collagen fibres (Fig. 2(7)). The mucous glands are arranged just below the epidermis and have a typical acinar form, with large lumina and thin ducts opening in the skin surface. The secretory epithelium reacts differentially to PAS and Alcian Blue, these differentiated glands are much larger, with diameters around 370 μm, whereas the common mucous glands have diameters around 100 μm (Fig. 2(9,10)). Also, the acini of the differentiated glands are formed by high prismatic cells, full of a homogeneous secretion very reactive to Bromophenol Blue (Fig. 2(10)) and to PAS (Fig. 2(11,12)), and sparse cells, very positive to Alcian Blue and negative to Bromophenol Blue, differently from the granular glands of the dorsal skin (compare Fig. 2(10,11)). The syncytial nuclei are arranged in a single peripheral row. Each gland is enveloped by a monolayer of thin myoepithelial cells, with flat and elongated nuclei (Fig. 4(23)).

The large ducts of the bottle-shaped glands are surrounded by a number of glands clearly different from mucous and granular glands both in size and histchemistry (compare Fig. 2(9,10), and Fig. 2(12,13)). In histological transverse sections, these glands appear circularly arranged around the main duct (Fig. 3(15)), forming a rosette-like arrangement. In whole mount preparations the small pores of these glands can be seen surrounding the main pore of the large syncytial granular gland (Fig. 3(16)). Although similar to the common mucous glands in terms of their typical acinar structure and their positive reaction to PAS and Alcian Blue, these differentiated glands are much larger, with diameters around 370 μm, whereas the common mucous glands have diameters around 100 μm (Fig. 2(9,10)). Also, the acini of the differentiated glands are formed by high prismatic cells, full of a homogeneous secretion very reactive to Bromophenol Blue (Fig. 2(10)) and to PAS (Fig. 2(11,12)), and sparse cells, very positive to Alcian Blue, pH 2.5, located mainly in the apical secretory epithelium (Fig. 2(12)). Occasionally the lumen contains secretion.

Longitudinal serial sections reveal that bundles of collagen fibres surround each duct, forming a distinct arrangement from the rest of the stratum compactum (Fig. 3(14)). At about 500 μm from the skin surface, the duct lining epithelium is thickened and practically obstructs the ductal lumen, leaving only a narrow slit (measuring about
Fig. 1. (1) Female *Rhinella jimi* exhibiting the right parotoid macrogland localized at the postorbital region. The arrows point to the glandular pores. (2) A parotoid sectioned according to a frontal plane, from which the venom was withdrawn. Notice the alveolar, honeycomb-like internal structure. (3) Higher magnification of the alveoli showing the walls (arrows) and floors (asterisks). (4) Longitudinal section of two parotoid bottle-shaped glands (G). The arrows point to the pores obstructed by a thick epithelium. D, dermis. Paraffin, HE. (5) Venom jets squirting from the pores, after parotoid manual compression. (6) View of the pores on the parotoid surface. Note the small slit (large arrows) in the duct centre. Each pore is surrounded by smaller pores (small arrows).

Fig. 2. (7) *Rhinella jimi* dorsal skin. The dermis shows many mucous (*) and granular (g) glands. The calcified dermal layer (ca) is present dividing the stratum spongiosum (ss) from the stratum compactum (sc). E, epidermis; v, blood vessels. Paraffin, HE. (8) *Rhinella jimi* ventral skin. When compared to the dorsal skin, it is much thinner and with a small number of glands, which are mainly mucous (*). The dermis is well vascularized, with many blood vessels (v) under the epidermis (E). sc, stratum compactum; ss, stratum spongiosum. Paraffin, HE. (9) *Rhinella jimi* parotoid macrogland. Apical portion of the macrogland where four glandular types are distinguishable: common mucous gland (*), common granular gland (g), differentiated gland (dg) and parotoid granular gland (G). Note that the differentiated cells are much larger than the mucous cells. A thick calcified layer (ca) is seen in the upper dermis (D) underlining the epidermis (E). A small part of the ductal epithelium (d) is observed in the parotoid granular gland. Paraffin, HE. (10) *Rhinella jimi* parotoid macrogland. Equivalent section to Fig. 2(9) stained with Bromophenol Blue. Mucous glands (*) and parotoid granular gland (G) are negative, the common granular gland (g) is positive and the differentiated glands (dg) are highly positive to the method. E, epidermis; D, dermis, d, ductal epithelium. Paraffin. (11–13) *Rhinella jimi* parotoid macrogland. Comparison of the four gland types present in the macrogland by PAS-AB (pH 2.5). The parotoid granular gland (G) is positive to Alcian Blue, while the common granular glands (g) are negative. The cells of the mucous glands (*) are mainly positive to Alcian Blue (arrows, Fig. 2(12)) while in the differentiated glands (dg) most cells are positive to PAS, with a few cells positive to Alcian Blue (arrows, Fig. 2(11)). E, epidermis; D, dermis; d, duct; s, secretion. Paraffin.
40 \mu m) in the centre. Longitudinal sections through the epithelium reveal that the cells arranged towards the ductal slit are flatter than the basal cells (Fig. 3(17)). In addition, the whole ductal epithelium is quite reactive to PAS (Fig. 3(18)). The transmission electron microscopy of these cells shows several cytoplasm inclusions containing material of medium electron density (Fig. 3(19,20)). The connections among the central flatter cells consist of very loose interdigitations (Fig. 3(20)).

The connective tissue surrounding the large syncytial glands is divided into two strata, a loose and richly vascularized stratum, closely adjacent to the gland myoepithelia, and a dense stratum (Fig. 4(21,22)). The dense stratum is continuous with the superficial stratum compactum, and envelopes each gland, structuring the honeycomb-like framework of the parotoid (see Fig. 1(2,3)). The parotoid base is flat and constituted of the same dense connective tissue.

After parotoid compression, conspicuous structural changes are observed in the alveoli affected by squeezing: they are empty and almost completely free of secretion. The periphery, where the syncytial nuclei are located, collapses...
Fig. 4. (21) *Rhinella jimi* parotoid macrogland. Two granular parotoid glands, one non-compressed filled with secretory product (G) and another compressed, with the syncytium (sy) completely collapsed in the centre, appearing in orange. Note the difference in volume between the loose connective tissue (lt) in both glands. The dense connective tissue (dt) is unchanged. Blood vessels (arrows). Paraffin, Mallory trichrome staining. (22) *Rhinella jimi* parotoid macrogland. Connective tissue between two parotoid granular glands (G). The loose (lt) and dense (dt) connective tissues are clearly distinguish. Blood vessels (arrows) are abundant in the loose connective tissue. Paraffin, HE. (23) *Rhinella jimi* parotoid macrogland. Part of the syncytium (sy) of a non-compressed alveolus, sheathed by the myoepithelial layer (my) and full of granular secretory product (G). The arrow points to a blood vessel. Paraffin, HE. (24) *Rhinella jimi* parotoid macrogland. A compressed parotoid gland, equivalent to the one observed in Fig. 4(21), with the syncytium (sy) completely collapsed in the center. The arrows point to blood vessels. lt, loose connective tissue, dt, dense connective tissue. Paraffin, HE. (25) Higher magnification of Fig. 4(24), where rests of the secretory product are seen inside the syncytium, which is enveloped by the myoepithelial layer (my). lt, loose connective tissue, dt, dense connective tissue. Paraffin, HE.
together with the surrounding myoepithelium: both (syncytium and myoepithelium) are now seen as a wrinkled structure in the centre of the alveolus (compare full and empty alveoli in Fig. 4(21)). The loose vascularized connective tissue, adjacent to the myoepithelium, appears expanded occupying a larger space between the collapsed gland and the dense connective tissue, which is unchanged (Fig. 4(21,24,25)). The comparison of a gland full of secretion with a squeezed gland in Picrosirius stained sections viewed under polarized microscopy shows the difference between non-expanded and expanded loose connective tissue surrounding each of the wrinkled syncytia (compare Fig. 5(26,27) with Fig. 5(28,29)).

Serial histological sections of the compressed glands did not show any significant modification of the ducts after secretion release except for a discrete widening of the ductal slit and the presence of secretion in the ductal lumen.

4. Discussion

*Rhinella jimi* has a wide distribution in Brazilian Catinga. *Rhinella schneideri*, which is a close relative of *R. jimi* (probably consisting of a complex of species), lives in the central Brazilian Cerrados (dry open woodlands). Both species, despite the presence of the paracnemic macroglands (characteristic of the *Rhinella schneideri* group) in their hind limbs, comprise large individuals, up to 300 mm in adult females. One of the diagnostic features which enables the recognition of *Rhinella jimi* as a distinctive species in the *Rhinella schneideri* group is the presence of macroglands also in the forelimbs (*Stevaux, 2002*). This characteristic has already been described by (*Toledo and Jared, 1995*, Fig. 2(13)) who referred to such glandular accumulations as radioulnar macroglands, in order to distinguish them from the paracnemic macroglands, previously studied by *Toledo and Villa (1987)*. Different from other anurans inhabiting open and dry regions, *Rhinella jimi* and *Rhinella schneideri* remain completely exposed to the environment for long periods, without running the apparent risk of desiccation, a fact that is probably associated with the presence of a calcified dermal layer (*Elkan, 1976; Toledo and Jared, 1993b*), covering the entire body, including the parotoids. In both species parotoid macroglands are very prominent, a fact commented on by *Lutz (1925)* in the original description of *Rhinella*.
the soil (Sazima and Caramaschi, 1986). We have many
the second after vigorously rolling and rubbing it against
that this mammal rejected the first frog but could ingest
after biting a specimen several times on the tail (Nowak
don et al., 1979) and a hedgehog avoided
efts of the salamander Notophthalmus viridescens
predator. In urodelans, chickens learn avoidance of the
head and most of the skin were discarded by the
times observed in the field half-eaten dead toads where
avoid poison. Experiments in captivity in which frogs
as many animals are known to have found ways to
detail in toads and depends on the predator's capabilities,
which is followed by spraying the venom, if the predator
should attack and/or bite his victim. Even if the toad is
the defensive strategy may be efficient at the level of
species defence, since the predator may be able to
associate the stereotyped defence behaviour with the
distasteful or harmful venom when grasping another toad.
However, this system seems not to have been explored in
detail in toads and depends on the predator's capabilities,
as many animals are known to have found ways to
avoid poison. Experiments in captivity in which frogs (Eupemphix nattereri, formerly Physalaemus nattereri)
were offered to the procyonid Nasua nasua (coati) showed
that this mammal rejected the first frog but could ingest
the second after vigorously rolling and rubbing it against the
soil (Sazima and Caramaschi, 1986). We have many
times observed in the field half-eaten dead toads where
the head and most of the skin were discarded by the
 predator. In urodelans, chickens learn avoidance of the
efts of the salamander Notophthalmus viridescens (Brandon et al., 1979) and a hedgehog avoided Pleurodeles waltl
after biting a specimen several times on the tail (Nowak and Brodie, 1978).

Although the external pressure is probably the most
important factor for parotoid venom release, lung inflation
must be significant in the defence of Rhinella jimi since,
besides making the animal appear larger, the lung pressure
against the body is possibly carried over to the parotoid
floor and transferred to the parotoid bottle-shaped glands.
The state of turgidity thus produced and the probable aid of
the individual glandular myoepithelial layer make it such
that, by external pressure, the secretion is expelled through
the duct slit causing venom jets to squirt out.

The dense connective tissue surrounding each bottle-
shaped gland forms a resistant framework responsible for
the shape maintenance of the parotoid, which appears
macroscopically unaltered even after venom release. On
the other hand, the loose connective tissue seems to form
part of the secretion release system. Also, the loose tissue
must have a significant role in syncytium and myoepithelial
maintenance, since it is highly vascularized and must be
involved in gland nutrition and transport of precursor
molecules for venom synthesis (Hostetler and Cannon,
1974; Cannon and Hostetler, 1976; Ersparser, 1994;
Hutchinson and Savitzky, 2004). Immediately after the
explosive emptying, the syncytium is totally collapsed and
the space between the dense connective tissue and the
myoepithelium is filled by the obviously expanded loose
connective tissue. Simultaneously with the syncytium
secretion refilling, the loose connective tissue is gradually
pushed towards the dense connective tissue walls.

The ultrastructure of compressed and non-compressed
parotoids in Rhinella ictericus (formerly Bufo ictericus),
suggests that the alveoli, when full of venom, is under
a constant internal pressure, since organelles are crowded
in a small cytoplasm volume. After discharge, however,
the organelles are easily recognized and distributed in a larger
volume of cytoplasm (Toledo et al., 1992). Our histological
results strongly indicate that similar features must occur in
the parotoid alveoli of Rhinella jimi. When the alveoli are
full, it is most probable that the normal pressure of the
venom on the loose connective tissue maintains a condition
of relative internal turgidity. The moment Rhinella jimi
feels threatened its defence mechanism of inflating its
lungs may contribute to the increase of the internal
pressure of each alveolus. Since the collagen surrounding the
alveolus and the duct appears very resistant, when an
external force is applied to the parotoid, e.g., by a bite, the
individual alveolar pressure is increased to a threshold
level, forcing the venom to eject through the narrow slit by
compressing the ductal epithelial lining cells. The ductal
thick epithelium could be compared in this context with
a small nozzle. Only the alveoli directly compressed cause
the venom discharge, liberating simultaneous multi-
directed “shots” inside the predator’s mouth. This diffuse
mechanism of venom elimination in a spray form can
reinforce the envenomation through breathing mucosa.
The parotoid alveoli which were not directly compressed
remain practically intact and ready to be triggered in the
event of a new attack.

This putative mechanism does not contradict the fact
that granular glands of the body skin and also the parotoid
glands to some extent may discharge their secretion by
contraction of the myoepithelium. Both mechanisms may
complement each other at the moment the animal is
threated. Contraction of the myoepithelium is triggered by
an adrenergic mechanism evoked by orthosympathetic
stimulation (Holmes and Balls, 1978; Delfino et al, 1982;
Nosi et al., 2002). In addition, the myoepithelial cells in
macroglands may have the additional function of homo-
genizing the voluminous gland secretion by constant gentle
contractions.

The thick epithelium present in the parotoid ducts,
although previously shown elsewhere (Lobo, 2005; Lobo...
et al., 2005; Jared et al., 2007) was herein described in detail, with suppositions of its role in venom release. In the same way, although the large differentiated glands in the parotoids and their special arrangement around each pore of the bottle-shaped glands have already been shown (Lobo, 2005; Lobo et al., 2005; Jared et al., 2007), they were herein described in more detail. The function of these differentiated glands is entirely unknown as yet, but based on their specific location, one should expect their secretions to have a role in venom release or in making part of the venom itself. In fact, Almeida et al. (2007) reported that in Rhinella ictericra, the parotoid venom is composed of a mixture of products released by different gland types. Almeida et al. (2007), however, did not make a distinction between the common mucous cells and the herein named differentiated glands, which they called mixed glands due to the protein and mucous nature of their secretory product. We have shown the same type of histochemical results in R. jimi. Also, Almeida et al. (2007) did not notice the clear association of these mixed glands with the large parotoid ducts which have already been described by Lobo (2005), Lobo et al. (2005) and Jared et al. (2007).

Another morphological aspect of R. jimi skin deserving attention is the difference between the calcified dermal layer of the dorsal skin and of the parotoid macrogland skin. In the parotoid this layer is much thicker and is located more superficially, just below the epidermis and above the bottle-shaped glands. Considering anuran integument in general, this observation seems quite unexpected since the calcified layer is usually localized below the glands, even when they are large (personal observations). This unusual more external position of the calcified dermal layer in the parotoid probably confers to the macrogland a more intense superficial mechanical resistance, and may help, at the moment of a bite, to canalize the internal venom pressure towards the ducts, forcing the venom to be released through the ducal slits. Association of the calcified dermal layer distribution pattern with the mechanism of gland discharge has already been proposed for Physalaemus nattereri (presently Eupemphix nattereri) (Lenzi-Mattos et al., 2005), whose posterior dorsal skin is provided with a pair of black and circular macroglands resembling two black eyes. These macroglands appeared to be the only region of the frog’s integument where the calcified dermal layer is absent, probably facilitating the venom to be expelled when the attack of a predator is directed to the macroglandular region (Lenzi-Mattos et al., 2005).

Besides R. jimi, we have examined the parotoid of many different species of Rhinella (R. schneideri, R. ictericra, R. margaritifera, R. crucifer and R. granulosa). In all the studied species the morphological and histochemical patterns of the parotoid macroglands were very similar to what we have described for R. jimi, including the duct arrangement, the differentiated glands around the ducts and the location of the calcified dermal layer. This similarity is a strong indication that the structure of the parotoid macrogland herein described may constitute a basal character within the genus.

Based on the fact that parotoid macroglands are common to the whole genus Rhinella, and that the general morphology of these structures is quite similar in many toad species, we suggest that the mechanism for venom release within the genus is possibly similar to that we have described for Rhinella jimi. In addition, supported by the structural complexity herein shown, the parotoid macroglands can no longer simply be considered as mere gland aggregations but should be regarded as highly differentiated structures, characteristic of bufonids, forming cutaneous organs separate from the rest of the skin, which were specially evolved for an efficient passive defence.

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Conflict of interest

The authors have no conflicts of interest.

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