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Skin Glands, Poison and Mimicry in Dendrobatid and Leptodactylid Amphibians

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ABSTRACT In amphibians, secretions of toxins from specialized skin poison glands play a central role in defense against predators. The production of toxic secretions is often associated with conspicuous color patterns that warn potential predators, as it is the case of many dendrobatid frogs, including *Ameerega picta*. This species resembles the presumably nontoxic *Leptodactylus lineatus*. This study tests for mimicry by studying the morphology and distribution of skin glands, components of skin secretion, and defensive behavior. Dorsal skin was studied histologically and histochemically, and skin secretions were submitted to sodium dodecyl sulfate polyacrylamide gel electrophoresis, reversed phase high performance liquid chromatography and assays for proteolytic activity. We found that poison glands in *A. picta* are filled with nonprotein granules that are rich in carbohydrates, while *L. lineatus* glands present protein granules. Accordingly, great amounts of proteins, at least some of them enzymes, were found in the poison of *L. lineatus* but not in that of *A. picta*. Both species differ greatly on profiles of gland distribution: In *L. lineatus*, poison glands are organized in clusters whose position coincides with colored elements of the dorsum. These regions are evidenced through a set of displays, suggesting that poison location is announced to predators through skin colors. In contrast, *A. picta* presents lower densities of glands, distributed homogeneously. This simpler profile suggests a rather qualitative than quantitative investment in chemical defense, in agreement with the high toxicity attributed to dendrobatids in general. Our data suggest that both species are toxic or unpalatable and transmit common warning signals to predators, which represents a case of Müllerian mimicry. J. Morphol. 273:279–290, 2012.

KEY WORDS: amphibia; defensive behavior; skin morphology; poison glands; mimicry

INTRODUCTION

In amphibians, cutaneous secretion of toxins is one of the main defense mechanisms against predators (Toledo and Jared, 1995; Jared et al., 2009; Toledo et al., 2010, 2011). The characteristic chemical defense of these animals depends on skin glands, especially granular or poison glands, which are mainly related to toxins against microorganisms and predators, while mucous glands are usually related to other vital functions, such as respiration and water balance (Fox, 1986, 1994; Clarke, 1997; Rollins-Smith et al., 2002, 2005). Poison glands secrete a wide diversity of peptides, biogenic amines, steroids and alkaloids, which present a broad spectrum of biological activity (Daly et al., 1987; Schwartz et al., 2007). The morphology and the patterns of gland distribution along the body vary among species, sometimes as a function of its ecology and natural history (Toledo and Jared, 1995). For example, some amphibians exhibit clusters of poison glands in certain body regions, strategically positioned to be activated through the predator's bite (Lenzi-Mattos et al., 2005; Jared et al., 2009).

Some organisms present patterns of bright and contrasting skin colors, associated to the presence of toxins or unpalatable substances. Such aposematic patterns indicate danger to potential aggressors and may confer protection against predation (Mallet and Joron, 1999; Servedio, 2000; Ruxton et al., 2004; Toledo and Haddad, 2009). Aposematic coloration may favor the evolution of mimicry (Sherrat, 2002, 2008), because mimicry depends on immediate recognition of an organism by its predators (Wüster et al., 2004). In Batesian mimicry (Bates, 1861), possibly the most widespread form, potential predators avoid an undefended species due to its resemblance to an aposematic one (Pasteur, 1982). Among vertebrates, the classic

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example is that of coral snakes (Elapidae), whose venom presents a high lethality. Several species of nonvenomous dipsadids and colubrids resemble coral snakes, suggesting the possibility of numerous Batesian relationships (Greene and McDiarmid, 1981; Brodie, 1993). Mullerian mimicry (Müller, 1878) occurs when a single color pattern developed in several nonrelated, poisonous species. In this case, similarity benefits the entire group of species, referred to as comimics (Sherrat, 2008). Wickler (1968), taking into account the toxicity present in all comimics, cast doubt on the term mimicry, as in Mullerian mimicry the predator is not truly deceived, differently of Batesian mimicry.

Among amphibians, the highest diversity of aposematic color patterns is found in poisonous frogs of the Dendrobatidae family, whose skin secretions are composed of a variety of dietary alkaloids, some of which are highly toxic to vertebrates (Daly et al., 2005). Dendrobatids were repeatedly suggested as anuran model system to study evolution of mimicry (Lynch, 1985; Symula et al., 2001; Toledo and Haddad, 2009; Vitt and Caldwell, 2009). For example, the Amazonian poison-dart frog *Ameerega picta,* presumably is imitated by the sympatric leptodactylid *Leptodactylus lineatus*. Although *L. lineatus* was repeatedly assumed as a nontoxic anuran (Nelson and Miller, 1971; Duellman, 1978; Lamar and Wild, 1995), no data are available about the biochemical properties of the skin secretion of this species. Toledo and Haddad (2009) discussed a similar case (with Allobates femoralis instead of *A. picta*), suggesting a Batesian (although not concluding which species is the model and which is the mimic) or a Müllerian mimicry ring.

In this article, we present a comparative study on poison gland morphology, glands distribution, basic toxinology, and defensive behavior in the poison-dart frog *A. picta* and in its possible mimic, the frog *L. lineatus*.

MATERIAL AND METHODS

Animal Collection and Behavioral Observations

A. picta (Fig. 1a) and *L. lineatus* (Fig. 1b) were observed and collected in Jacareacanga (Pará State) and Paranaíta (Mato Grosso State) at the Brazilian Amazon during June and November 2008 and February 2009. Additionally, 15 individuals of each species were maintained in the Laboratory of Cell Biology, Instituto Butantan, for additional observations of defensive behavior and extraction of skin secretions. Frogs were kept in terraria (40 × 50 × 60 cm) containing leaf litter, bark and pots as shelters. Water was offered ad libitum and animals were fed two times a week with young crickets. Temperature was kept between 22–28°C and high air humidity was maintained by manual spray of water three times a week.

The frogs were handled according to the procedures indicated in the Guidelines for Animal Experimentation established by the Brazilian College for Animal Experimentation.

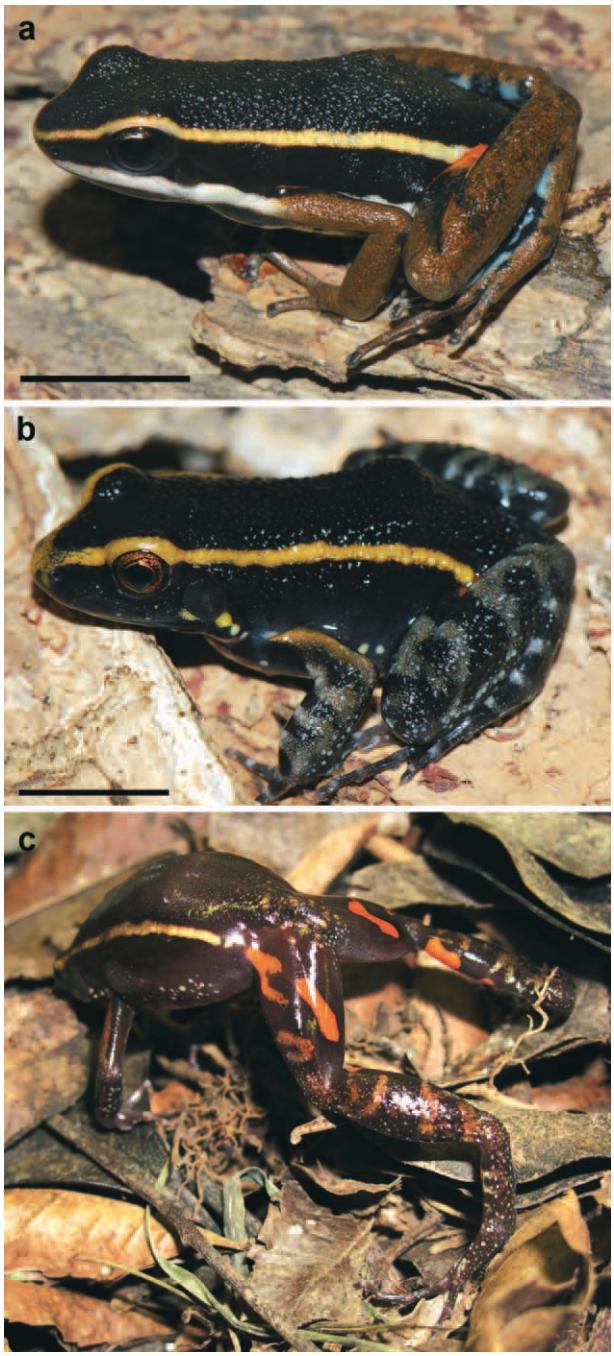

Fig. 1. Studied animals. (**a**) *A. picta*. (**b**) *L. lineatus*. (**c**) *L. lineatus*, defensive behavior: full body-rising with legs vertically stretched. Exposing the posterior portion of the dorsum, this posture exposes and emphasizes colored stripes and spots. Bars: 1 cm.

Histological Study of the Skin and Glandular Distribution

Adult specimens of both species (N = 8) and young *L. lineatus* (*N* = 2; <3.4 cm; Bernarde et al., 2009) were sacrificed with an overdose of thionembutal (30 mg/kg) and fixed by immersion and injection with paraformaldehyde (4%) in phosphate buffer saline (PBS) buffer (0.1 mol l^{-1}, pH 7.2) for 8–10 h. Transversal

stripes of skin were removed with blade at five points along the dorsum (including head) and three points of the dorsal surface of the right thigh (Fig. 2), sampling regions of black (dorsum and thigh), yellow (dorsum) and orange (thigh) color. Whole heads from both species were decalcified in a solution of 4% ethylenediaminetetraacetic acid (EDTA), pH 7.2, in constant agitation for 2–3 months. The skin fragments and the decalcified heads were dehydrated in ethanol series (70–100%) and embedded in paraffin. Skin fragments were also embedded in historesin (Leica Microsystems Nussloch GmbH, Nussloch/Heidelberg, Germany). Material embedded in paraffin was sectioned at 5 μm thickness and used for histochemical reactions and histological study. Material embedded in historesin was sectioned at 2 μm, stained with toluidine blue-fuchsin and studied histologically. Photomicrographs were obtained in an Olympus BX52 microscope coupled to an Olympus charge-coupled device (CCD) camera, using Image Pro Express software (Media Cybernetics, Bethesda) for image capture.

Histochemical Study

Sections were submitted to the following reactions: bromophenol blue for protein identification, periodic acid-Schiff (PAS) for identification of carbohydrates in general and alcian blue pH 2.5 for identification of acid carbohydrates (Bancroft and Stevens, 1996; Kiernan, 1999).

Quantitative Analysis of Glandular Distribution

Paraffin sections of both species were subjected to a quantitative analysis in order to evaluate differences in the number of poison glands at different body regions. We studied the dorsum skin at the level of forelimb insertion and the skin of the right thigh at the midpoint between knee joint and pelvis (Fig. 2). At the dorsum, we sampled the skin at the trunk centre and that of the right yellow dorsal stripe. At the thigh, we sampled skin from the centre of the orange femoral spot and the black skin anterior to the spot. We used skin fragments of 3 × 3 mm of five individuals of each species. For each individual, we sampled six sections of 5 μm in each of the four regions, spaced 25 sections (125 μm) apart from each other in order to avoid sampling of a given gland in different sections. We photographed each section and positioned a bar of 1 mm on its central point using Image Pro Express software. Then, we counted the number of poison glands contained in the interval delimited by the bar.

Transmission Electron Microscopy (TEM)

Fragments of dorsal and thigh skin of 2–3 specimens of each species were removed and fixed in Karnowsky fixative (5% glutaraldehyde and 4% paraformaldehyde in cacodylate buffer, 0.1 mol l^{-1}, pH 7.2; Karnowsky, 1965). The material was post fixed in osmium tetroxide (1%) in cacodylate buffer (0.1 mol l^{-1}), contrasted with aqueous uranyl acetate (1%), dehydrated in ethanol series (70–100%) and embedded in Epon resin using propylene oxide as an intermediary (Bozzola and Russell, 1999). For prior examination by light microscopy, semi thin sections were obtained in a Sorvall MT6000 ultramicrotome, (Du Pont Company, Wilmington) and stained with toluidine blue (1%) solution in borax (1%). The ultrathin sections were obtained in a Sorvall MT6000 ultramicrotome using a diamond knife. After contrast in uranyl acetate and lead citrate (1%), sections were examined in a TEM LEO 906E (LEO Electron Microscopy, Cambridge, England).

Extraction of Skin Secretions

Crude skin secretions were collected through glandular stimulation by manual compression of *A. picta* and *L. lineatus* within a beaker containing distilled water. The resulting solution was lyophilized and the dry matter was diluted to 1 mg ml^{-1} for biochemical analyses.

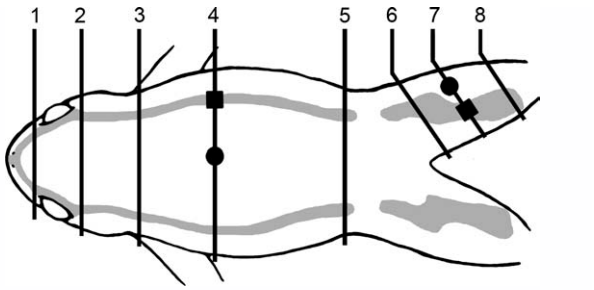

Fig. 2. Skin regions studied. 1–8 indicate points along the body at which transversal stripes of the skin of *L. lineatus* and *A. picta* were removed for histological study. Circles and squares indicate regions used in the quantitative analysis of poison glands' density. Circles: black skin (dorsum and thigh), squares: yellow stripe (dorsum) and orange spot (thigh).

Electrophoresis (SDS-PAGE)

The presence of proteins in skin secretions was evaluated by electrophoresis in 12% polyacrylamide gel (PAGE) containing sodium dodecyl sulfate (SDS) under reducing conditions. The gel was then stained with coomasie brilliant blue and/or silver stained, following Laemmli (1970).

Chromatography (RP-HPLC)

Aqueous extracts of skin secretions were analyzed by reversed phase high performance liquid chromatography (RP-HPLC) with a binary system (20A Prominence, Shimadzu Co., Japan). Samples were injected in a C18 column (ACE C18, 5 mm, 100 Å, 50 × 4.6 mm), using solvents (A) trifluoroacetic acid (TFA)/H_2O (1:1,000) and (B) TFA/acetonitrile/H_2O (1:900:100) at a constant flow of 1.2 ml min^{-1}, with a gradient of 0–100% of solvent B in 20 min, after isocratic elution at 0% for 5 min. Eluted content was monitored by a detector Shimadzu SPD-M20A (Photo Diode Array detection). The wavelength of 245 nm was selected for presentation due to the larger number of components detected when compared to the other typical wavelengths (214/220/222, 280 and 339 nm).

Enzymology

For detection of proteolytic activity, particularly of the serinopeptidase type, we determined the Vmax of hydrolysis of the fluorogenic peptidic substrate Z-Arg-MCA. Titration 96-well plates containing 100 μl of Z-Arg-MCA solution (100 mmol l^{-1}) in ammonium acetate buffer (100 mmol l^{-1}, pH 7.5) were placed for 5 min in a thermally controlled chamber (37°C). We then added a solution (1 μg μl^{-1}) of *A. picta* or *L. lineatus* skin secretions. Changes in fluorescence were recorded over 60 min through a spectrofluorimeter Spectra Max Gemini XPS (Molecular Devices, Sunnyvale). For detection, we used λ_{ex} = 320 nm and λ_{em} = 420 nm.

Data Analysis

Data on the distribution of poison glands on dorsal surfaces were analyzed using analysis of variance, modeling species and skin region as fixed factors and individual and section (nested within individual) as random factors. In all analysis, normality of data was determined graphically through distribution of dependent variables and residuals in histograms and q-q plots, as well as through values of skewness and kurtosis, between +2

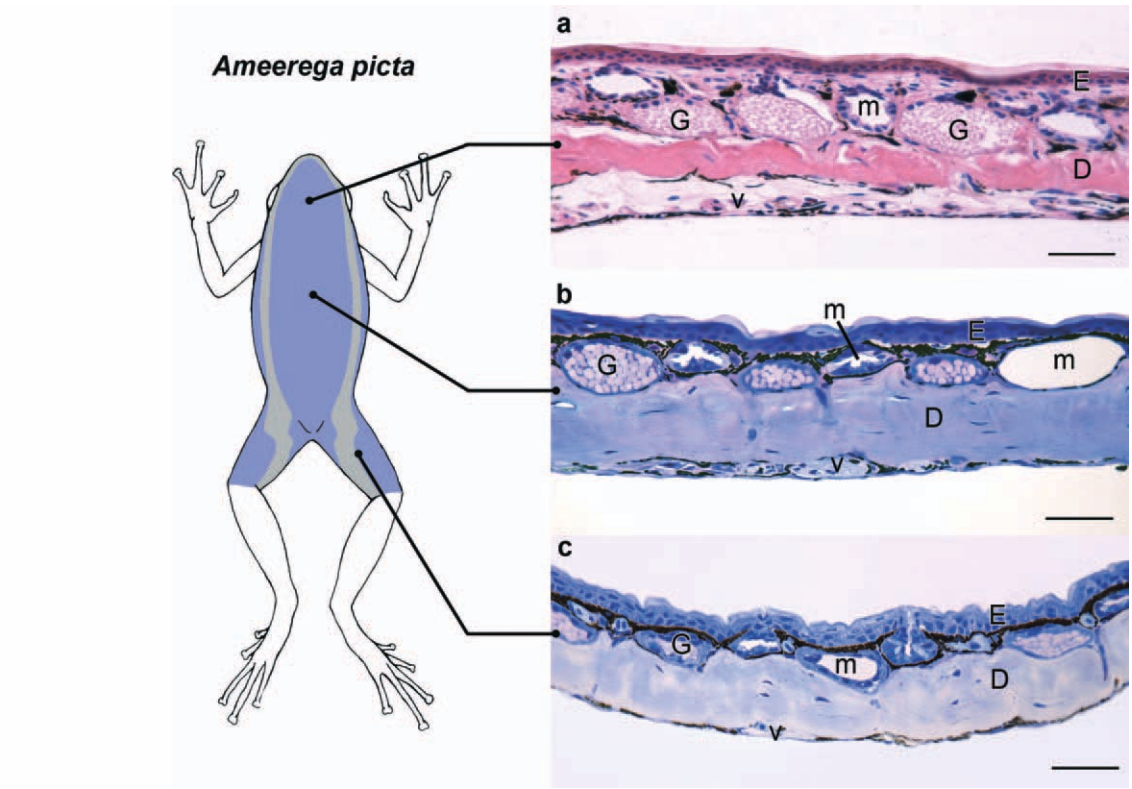

Fig. 3. *A. picta*. Distribution of poison glands at the dorsal surface. (**a**) Head skin. Glands present homogeneous distribution in similar densities to that of the dorsum. Hematoxylin-eosin. (**b**) Dorsum skin. Glands have uniform distribution, with no evidence of glandular accumulations. Toluidine blue-fuchsin. (**c**) Thigh skin. Glandular distribution follows the pattern observed at the rest of the dorsal surface. Toluidine blue-fuchsin. D, dermis; E, epidermis; G, poison (granular) glands; m, mucous glands; v, blood vessels. Bars: 50 μm.

and −2. Homogeneity of variances among groups was evaluated using Levene's test. Analyses were performed in statistical package for the social sciences, IBM (SPSS) 17.

RESULTS

Natural History Observations

A. picta (Fig. 1a) is a diurnal species that occurs mainly at the banks of streams, where they are found in high numbers and often vocalizing. Their conspicuous colors contrast with occupied substrates, mainly bare rocks presenting small herbaceous vegetation and rocks covered by a thin layer of leaf litter. Individuals are territorial and occupy a fixed area close to one or more shelters, mainly crevices between rocks.

L. lineatus (Fig. 1b) was captured by pitfall traps only, especially at rainy nights. Observations on its behavior come strictly from captivity, where it proves to be a nocturnal species that remains under leaf litter or inside cracks and narrow holes during the day. Individuals are excellent jumpers and present characteristic displays of defense (sensu Toledo et al., 2011) when threatened: fleeing, or full body-rising with legs vertically stretched (Fig. 1c), puffing up the body, and body-tilting toward the attacker's direction. When captured, individuals can produce short and shrill distress calls. When manipulated, *L. lineatus* produces an abundant foamy, viscous skin secretion with an intense bitter smell. Even after 18 months in captivity, this secretion continues to present such characteristics.

Distribution of Poison Glands

In *A. picta* poison glands are distributed uniformly across the dorsal surface (Fig. 3a,b). There is no significant difference in the density of glands between the midpoint of the dorsum (black skin) and the right yellow stripe ($F_{1,\ 29} = 3.6$, $P = 0.068$. See Table 1). By contrast, the distribution of poison glands in the dorsum of *L. lineatus* is very heterogeneous. Glands are concentrated in two dorsolateral bands whose position corresponds to that of the yellow stripes, also encompassing the bordering black skin (Fig. 4b). These bands are extremely rich in poison glands, extending from the postorbital region to the base of the hindquarters. Conversely, such glands are very rare in the central region of the dorsum (Fig. 4c), with a significantly lower density than that of the stripes region ($F_{1,\ 29} = 890.24$, $P < 0.001$. Table 1). Histologically, these bands reveal a juxtaposed array of

TABLE 1. Mean values ± standard deviation on the number of glands in different regions of the dorsal skin of L. lineatus and A. picta

	Dorsum	Stripe	Spot	Ant spot
A. picta	3.23 ± 1.5	3.97 ± 2.02	1.97 ± 1.35	2.7 ± 1.51
L. lineatus	0.3 ± 0.53	11.33 ± 1.82	0.13 ± 0.34	8.5 ± 1.79

Dorsum: black skin at the centre of the dorsum; Stripe: yellow skin of the right dorsal stripe; Spot: orange skin from the right femoral spot; Ant Spot: black skin of anterior position to the femoral orange spot.

elongated poison glands, measuring about 150 μm in height and 110 μm in width (Fig. 4b). Likewise, there is a high density of glands in the head skin (Fig. 4a). Although the only two juvenile individuals of *L. lineatus* that we found were not included in this quantitative analysis, the histological study reveals an identical distribution of skin glands when compared to adults.

Histological analysis of the skin at the tights of *A. picta* shows a uniform distribution of glands, like the rest of the dorsum (Fig. 3c). In contrast, the skin at the tights of *L. lineatus* exhibit glandular clusters distributed according to skin colors, in a fashion similar to the dorsum region. Each side of the orange femoral spot is bordered with elongated glandular aggregates that extend from the cloacae to the knee joint, while the spot itself presents significantly lesser and almost no poison glands ($F_{1,\ 29} = 655.08$, $P < 0.001$. Table 1). The histology of these aggregates is the same as the glandular bands associated with the dorsal yellow stripes (Fig. 4d). Also in *A. picta*, the incidence of glands is higher in the black skin adjacent to the

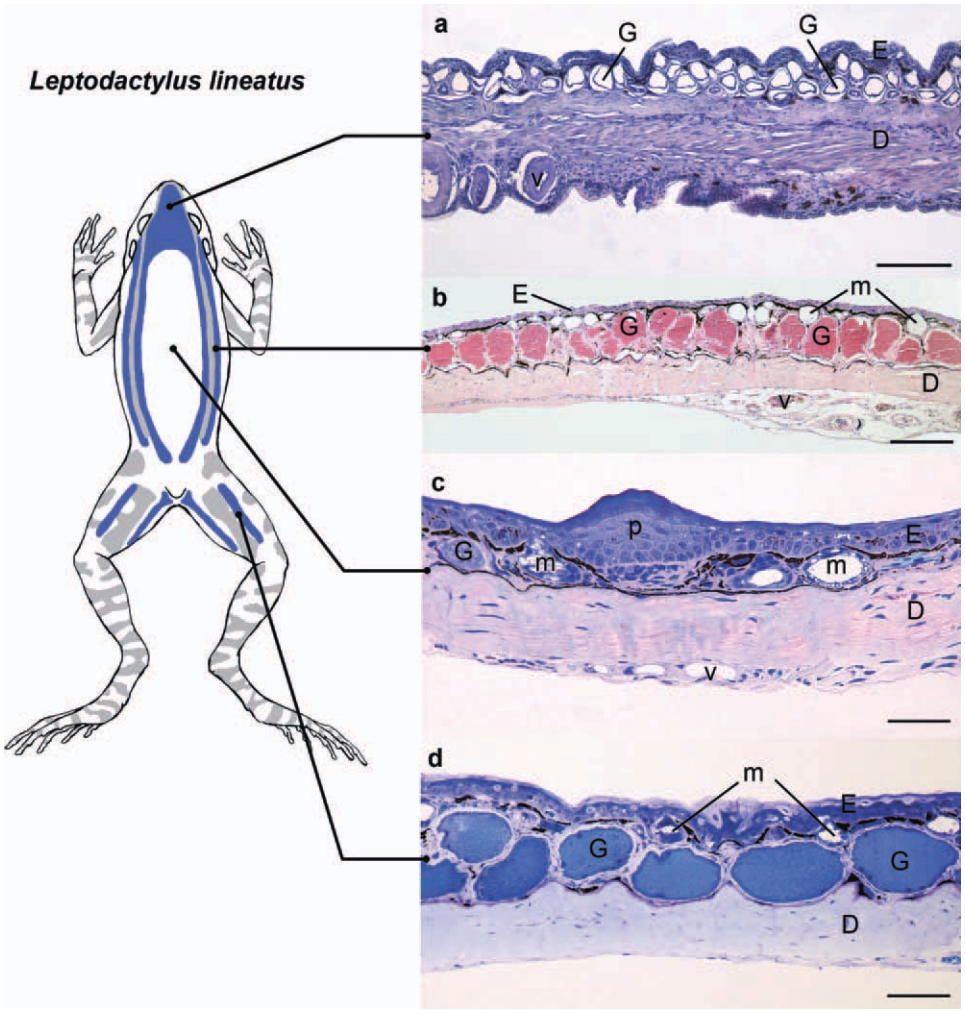

Fig. 4. *L. lineatus*. Distribution of poison glands at the dorsal surface. (**a**) Head skin. Note a large incidence of poison glands. Hematoxylin-eosin. Bar: 200 μm. (**b**) Dorsal skin at the yellow stripes. Note a large concentration of poison glands, slightly elongated dorsoventrally, juxtaposed side by side. Hematoxylin-eosin. Bar: 200 μm. (**c**) Skin at the center of the dorsum. Poison glands when present are very scarce and isolated. There are epidermal protuberances (p) in which the outermost epidermal layer, the *stratum corneum*, is thicker than in the rest of the skin. Toluidine blue-fuchsin. Bar: 50 μm. (**d**) Black thigh skin anterior to the orange spot. A large concentration of poison glands is arranged in a similar fashion to that of dorsum clusters in (b). Toluidine blue-fuchsin. Bar: 100 μm. D, dermis; E, epidermis; G, poison (granular) glands; m, mucous glands; v, blood vessels.

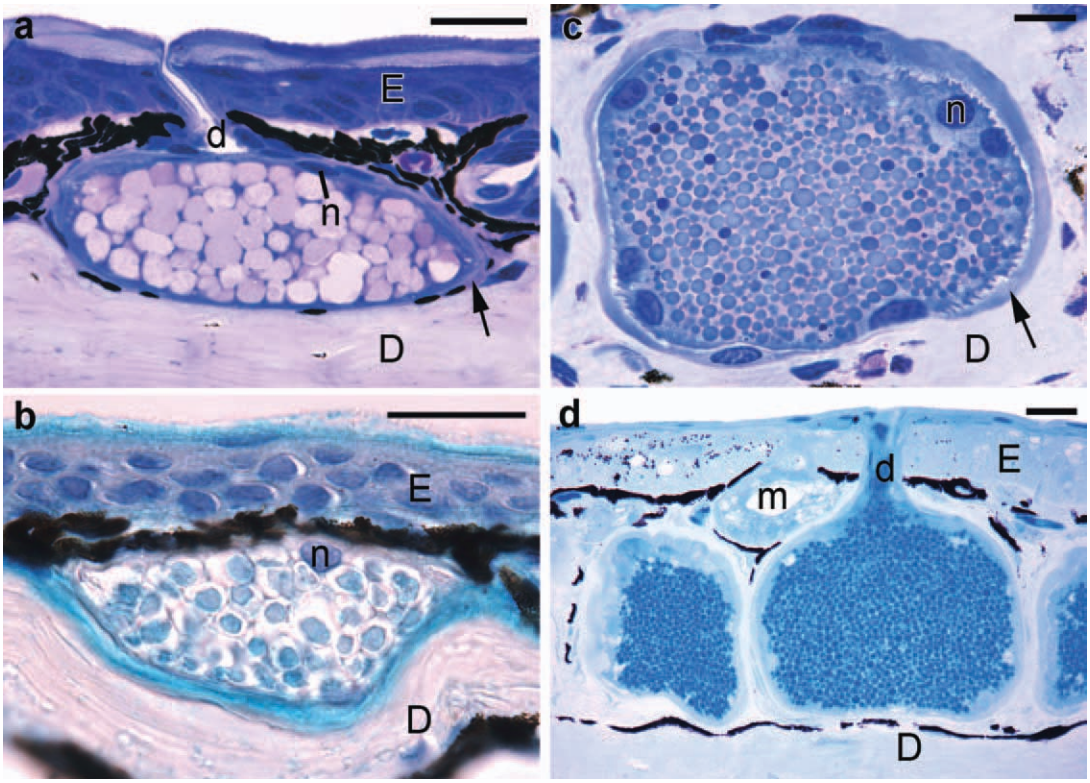

Fig. 5. Morphology of poison glands. (**a**) *A. picta*. The glandular alveolus is filled with large granules of heterogeneous size and shape. Nuclei (n) occupy the periphery of the secretory syncytium. There is a duct (d) for extrusion of glandular content. A myoepithelial layer (arrow) surrounds the alveolus. Toluidine blue-fuchsin. Bar: 20 μm. (**b**) *A. picta*. Secretory granules react to alcian blue pH 2.5, indicating acid carbohydrates in skin secretions. Bar: 10 μm. (**c**) *L. lineatus*. Densely juxtaposed spherical granules fill the alveolar glands. Nuclei (n) occupy the periphery of the secretory syncytium. A myoepithelial layer surrounds the alveolus (arrow). Toluidine blue-fuchsin. Bar: 20 μm. (**d**) *L. lineatus*. Differently from *A. picta*, glands strongly react to bromophenol blue, indicating the massive presence of proteins. The duct (d) is visible in this section. Bar: 20 μm. D, dermis; E, epidermis; m, mucous glands.

femoral spot rather than in the spot itself ($F_{1, 29} = 4.59$, $P = 0.041$).

Morphology of the Poison Glands

Granular glands of *A. picta* and *L. lineatus* are located in the stratum spongiosum of the dermis, along with a large number of chromatophores and blood vessels. The secretory epithelium is syncytial, with nuclei situated at the alveolus periphery and secretion granules filling the central region (Fig. 5a,c). The alveolus is surrounded by a single layer of myoepithelial cells and has a duct that communicates it with the skin surface. Despite these similarities, the glands of both species differ in the morphology of secretory granules and in the affinity for histochemical treatments. In *A. picta*, poison glands are elliptical, with the largest diameter parallel to the epidermis and maximal diameter of about 80 μm (Fig. 5a). Its granules are negative to bromophenol blue, indicating absence of protein material, but react to PAS and alcian blue pH 2.5 (Fig. 5b), indicating the presence of acidic mucosubstances. In *L. lineatus*, glands generally present spherical shape but may be laterally compressed in the gland clusters of dorsum and thighs. Well defined secretion granules are spherical and homogeneous in appearance, although presenting assorted sizes (Fig. 5c). Unlike *A. picta*, a high protein secretion in *L. lineatus* is indicated by intense affinity of poison glands for bromophenol blue (Fig. 5d), while the lack of affinity for PAS and alcian blue at pH 2.5 indicates absence of both neutral and acidic mucosubstances.

In TEM, the poison glands of *A. picta* present a prominent rough endoplasmic reticulum (RER) and a high density of mitochondria, concentrated at the peripheral portion of the secretory syncytium (Fig. 6a,b). The Golgi apparatus abounds, with dictyosomes enclosing electron-dense structures of spherical shape among an electron-transparent medium (Fig. 6a,c). More internally, secretory granules contain an electron-transparent matrix among which electron-dense particles of circular, elongated or irregular shape are observed (Fig. 6d). These particles are remarkably similar to those observed in vesicles derived from

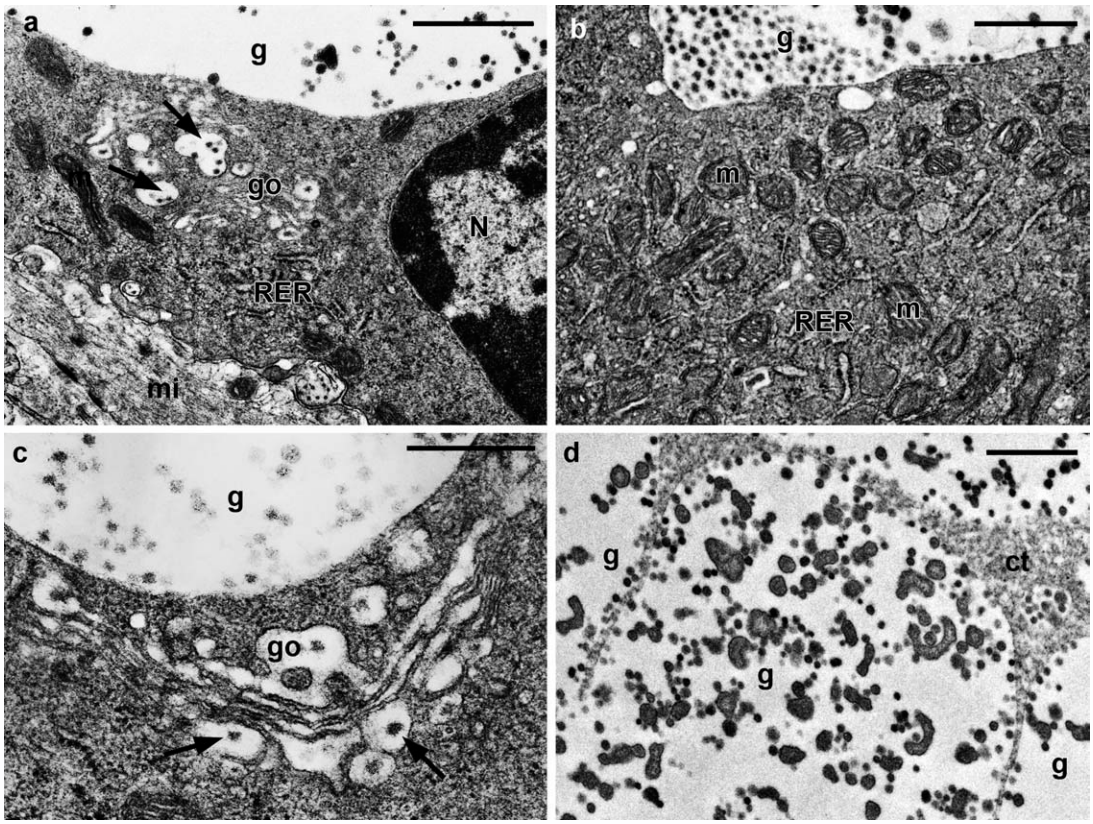

Fig. 6. *A. picta*. Transmission electron microscopy of the secretory syncytium of poison glands. (**a**) Nuclei (N), mitochondria and rough endoplasmic reticulum (RER) occupy the periphery of the glandular alveolus. Golgi dictyosomes (go) exhibit spherical electron-dense particles (arrows). Bar: 1 μm. (**b**) High density of mitochondria (m) with lamellar cristae, close to a secretory granule (g). The RER occurs sparsely. Bar: 0.5 μm. (**c**) Golgi dictyosomes (go), with several budding vesicles (arrows) around it. A large electron-transparent granule encloses circular particles, with aspect similar to those within Golgi budding vesicles. Bar: 0.5 μm. (**d**) Part of large juxtaposed secretory granules full of particles with circular or elongated shape and apparently surrounded by membrane. Bar: 1 μm. ct, cytoplasm; g, secretory granules; mi, myoepithelial layer.

the Golgi apparatus. In contrast, the glands of *L. lineatus* have few mitochondria, concentrated at the periphery of the secretory syncytium. At this same region, a high density of expanded RER lamellae suggests high activity of protein synthesis (Fig. 7b). The Golgi apparatus is moderately developed, with several vesicles budding from its trans face (Fig. 7b). Toward the interior of the syncytium, a high number of immature secretory granules occur. These granules are characterized by an electron-transparent matrix containing portions of electron-dense material at different levels of aggregation (Fig. 7c). The inner portion of the syncytium exhibits a large number of mature secretory granules of spherical shape and various sizes, filled with an electron-dense content (Fig. 7a,b).

Biochemistry of Skin Secretions

Electrophoresis analysis reveals few proteins in the skin secretion of *L. lineatus* (Fig. 8a). Namely, one major band at ~25 kDa and two faint bands at ~50 and ~15 kDa, besides some unresolved material at the low molecular mass region, probably peptides. These results are supported by the presence of an intense nonretained fraction present in the RP-HPLC chromatographic analysis (λ = 214 nm, data not shown). By contrast, the secretion of *A. picta* has virtually no protein content (Fig. 8a,b). Even an overstained silver SDS-PAGE revealed no proteins, at 1 mg ml^{-1} (data not shown). The RP-HPLC profiles of both species were also markedly different. Although not complex, *L. lineatus* skin secretion presented a series of peaks with more hydrophilic behavior, whereas *A. picta* skin secretion was very poor, with few components eluting in the more hydrophobic region of the chromatogram. In spite of the low protein contents, enzymatic activity (serinopeptidase type), could be detected. We found a marked difference among species: 1 mg ml^{-1} solution of *A. picta* skin secretion was able to hydrolyze the fluorogenic substrate at 270 AUF (arbitrary units of fluorescence)/min. On the other hand, a solution of *L. lineatus* skin secretion, at the same concentration, hydrolyzed the same substrate at a velocity of 720 AUF/min.

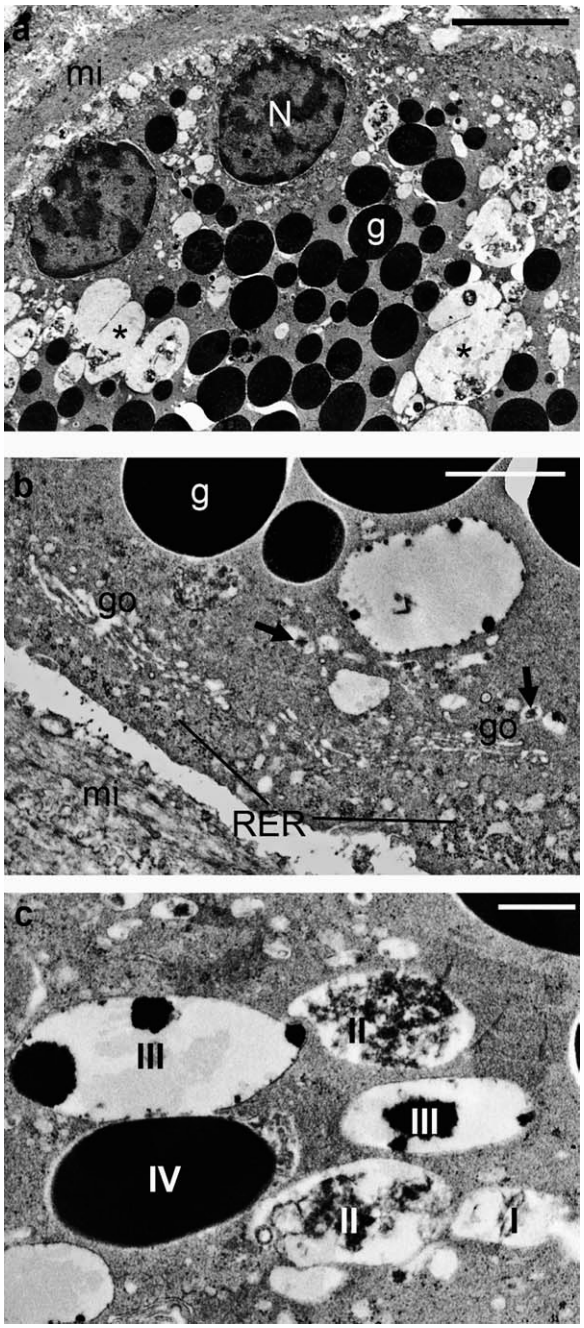

Fig. 7. *L. lineatus*. Transmission electron microscopy of the secretory syncytium of poison glands. (**a**) Mature secretory granules of highly electron-dense content fill the central portion of the syncytium, while nuclei and electron-transparent immature granules (asterisks) occur at peripheral portions. Bar: 5 μm. (**b**) The peripheral RER often presents expanded lamellae, suggesting high activity of protein synthesis. The Golgi apparatus (go) is moderately developed, with many vesicles in its trans face, which often encloses electron-dense particles (arrows). Bar: 1 μm. (**c**) The heterogeneous levels of aggregation of poison content seems to represent stages of maturation of glandular secretions, indicated by the presumed sequence I–IV. Bar: 0.5 μm. g, mature secretory granules; mi, myoepithelial layer; N, nuclei; RER, rough endoplasmic reticulum.

DISCUSSION

Due to the great morphological similarity, a Batesian association involving the poison-dart frog *A. picta* and the allegedly non-toxic frog *L. lineatus* was proposed by several researchers (Nelson and Miller, 1971; Duellman, 1978; Toledo and Haddad, 2009; Vitt and Caldwell, 2009), despite the

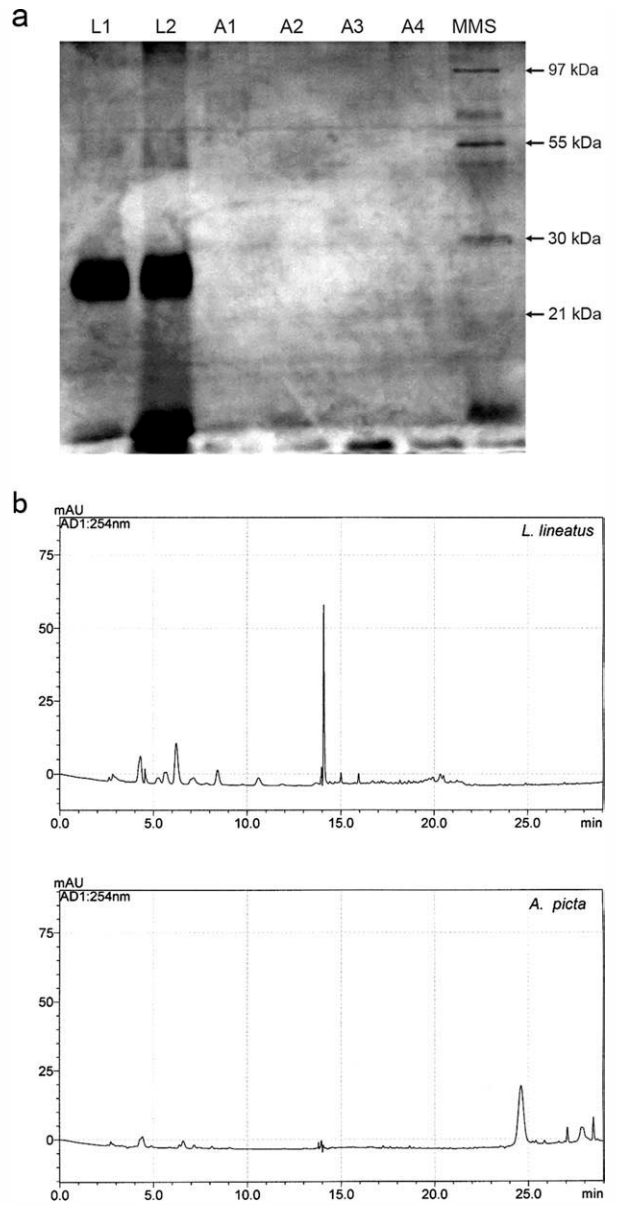

Fig. 8. Biochemistry of the skin secretions. (**a**) SDS-PAGE. L1 and L2 represent secretions of a group of four adult *L. lineatus* each, while A1 to A4 represent secretions of a group of three adult *A. picta* each. MMS is the standard for molecular mass markers. In *L. lineatus*, a major protein band at ~25 kDa can be observed. By contrast, *A. picta* secretions have no observable protein bands. (**b**) C18-RP-HPLC, monitored at 254 nm, *L. lineatus* skin secretion presents peaks with hydrophilic behavior, whereas *A. picta* skin secretion contains few components eluting in the hydrophobic region of the chromatogram.

lack of specific studies on this topic. Some authors also noted that *L. lineatus* is similar to other anuran species, such as the poison-dart frogs *Ameerega hahneli* and *Ameerega trivittata* and the aromobatid *Allobates femoralis* (Lamar and Wild, 1995; Toledo and Haddad, 2009), suggesting that *L. lineatus* could develop a Batesian mimetic relationship with these species as well.

Amphibian skin is a remarkable organ that serves multiple vital functions, such as respiration, water economy and defense against predators and microorganisms (Toled and Jared, 1995; Rollins-Smith et al., 2002, 2005; Lillywhite, 2006; Schadich, 2009). The morphological and toxinological study of this organ, coupled with behavioral observations at the field and in captivity, may contribute to the elucidation of aspects of amphibian biology and natural history that are inaccessible by other methods. Here, we applied this approach as an effort to contribute to the elucidation of a case of mimicry between anuran species.

We observed that the skin of *L. lineatus* and *A. picta* presents the morphological structure found in anurans as a whole (Delfino et al., 1998, 1999; Terreni et al., 2003; Alvarez et al., 2005). However, with regard to poison glands, these two species exhibit marked differences in relation to gland shape, distribution and concentration in specific body parts, as well as size and aspect of secretory granules. Taken together, these features indicate that these two amphibians make use of chemical defenses in quite a different manner.

Based on previous information (Fox, 1986, 1994; Clarke, 1997; Rollins-Smith et al., 2002, 2005) and on our own morphological and histochemical results, we presumed that proteins, alkaloids and other potential toxic compounds here found are mainly secreted by the skin poison glands. However, very little is known about the presence of toxic compounds (together with polysaccharides) in the mucous glands.

Histochemical and biochemical data show that the skin secretion of *L. lineatus* has a high concentration of proteins, although it seems to be not very diverse, as shown by the SDS-PAGE experiment, which presents just one intense band. This was also evidenced ultrastructurally by the intense presence of RER in the secretory syncytium of its poison glands. In fact, proteins are important components of the skin secretions of leptodactylids as a whole (Bevins and Zasloff, 1990; Pukala et al., 2006, Schwartz et al., 2007). By contrast, proteins have virtually no expression in the poison of *A. picta*. In this species, the poison is probably composed of alkaloids, as in other dendrobatids. These substances come from amphibian dietary items such as ants, beetles, millipedes and mites, but their primary source is probably plants or fungi ingested by these invertebrates (Saporito et al., 2003, 2004, 2007; Dumbacher et al., 2004;). Moreover, our histochemical data show that *A. picta* have a skin secretion rich in mucosubstances. This fact is supported ultrastructurally by the presence of a well-developed Golgi apparatus, an organelle that acts in the synthesis of carbohydrate chains and in their incorporation into proteins (Farquhar and Palade, 1998). Since carbohydrates often have allergenic properties (Paschinger et al., 2005), it is possible that they contribute to the toxicity of *A. picta*. The indication of carbohydrates in the poison of a dendrobatid deserves attention, since this group has been studied toxinologically exclusively from the perspective of its alkaloids. Nevertheless, the ultra-violet high performance liquid chromatography (UV HPLC) detection and the technique employed (reversed phase) would not be the best for detecting either alkaloids or sugars. Hydrophilic interaction chromatographic (HILIC) coupled to mass spectrometry (MS) and/or capillary electrophoresis (CE) - MS experiments could yield better results in the investigation of the skin secretion components. Both in *A. picta* and *L. lineatus*, we observed patterns of poison maturation at the ultrastructural level that involve fusion of vesicles, growth of secretory granules, aggregation, and progressive compression of granule contents. Similar patterns have been described for a diversity of anurans (Delfino et al., 1999, 2002; Angel et al., 2003; Terreni et al., 2003).

In general anurans exhibit a homogeneous distribution of skin glands along the dorsal surface (Toledo and Jared, 1995). This is indeed the case of *A. picta*, in contrast with *L. lineatus*, whose poison glands are concentrated in the head and in bands associated with colored elements of the dorsum. Thus, although very similar regarding color patterns, dorsal skin in these two species appears to represent distinct strategies for defense against predation. In *L. lineatus*, the association between skin colors, high concentration of poison glands and a characteristic display of defense strongly indicate that colored stripes and spots signalize regions where the poison is in fact concentrated. Similarly, in the frog *Eupemphix nattereri* two circular gland clusters at the lumbar region are evidenced by an intense black pigmentation surrounded by a white band, composing an arrangement that simulate eyes. This structure, able to secret a poison with high lethality, is exposed at the time of attack through behavioral display (Lenzi-Mattos et al., 2005). Moreover, the high density of glands in the dorsal surface of the head of *L. lineatus* may be related to the fact that birds and snakes usually start ingesting anurans from the head. Similar cephalic aggregates have been reported for other anurans, such as the toad *Melanophryniscus cambaraensis* (Santos and Grant, 2011).

Our data on behavior, skin morphology and toxinology suggest that the defense of *L. lineatus* is based on skin secretions with a pronounced toxicity or unpalatability. Its skin is rich in proteins and peptides and some of them might have toxic effects. Moreover, the peptidase activity identified in the skin secretions, may indicate that the active

peptides can be processed in the skin, or that the enzyme itself can be involved in the toxic or defensive processes. These observations suggest that the mimetic relationship between *L. lineatus* and *A. picta* is Mullerian in nature. The toxinological set presented by *L. lineatus* could lead an attacker to learn to avoid the color pattern shared by these species, benefiting also *A. picta* (or other similar dart-poison frogs). Our findings provide a more complex character for this mimicry pair, since *L. lineatus* is no longer regarded as a passive species that would defend itself only by exposing skin colors to predators, accordingly to that proposed by Toledo and Haddad (2009).

The natural history of these two species is poorly known, and our fieldwork showed important ecological differences between them. Thus, the characteristic glandular distribution in the skin of each species is probably related to their specific ecology (Erspamer, 1994). In fact, *A. picta* has an intense diurnal activity, being very conspicuous in the environment both visually and acoustically. In contrast, *L. lineatus* is very difficult to observe and was captured only in traps. Adults are solitary and nocturnal, spending the day sheltered. These characteristics may impair the efficiency of its color as a warning signal and hence its supposed mimetic defense. In an attempt to explain this incongruence, it was suggested that mimicry might be effective only for young *L. lineatus*, which are not just nocturnal but also diurnal (Regos and Schluter, 1984; Lamar and Wild, 1995). However, reaffirming the hypothesis of Schluter and Regos, (1981), we suggest that adults can also take advantage of mimicry in the case of being surprised by potential aggressors during the day, or during their reproductive period, about which little is known. On such occasions, *L. lineatus* would have the same picnotic characteristics as *A. picta*, its likely diurnal comimic. This hypothesis has support in the fact that, from a morphological standpoint, we did not observe differences between adults and young with regard to the structure of skin, poison glands or poison granules. However, it is important to note that differences in the secretory content of poison glands along ontogeny were described by Schadich et al. (2010) for the Ewing's Tree Frog (*Litoria ewingii*).

Considering the notorious toxicity reported for dendrobatid frogs, a comparison on the dimensions and quantity of poison glands between *A. picta* and *L. lineatus* proves to be surprising. These glands are much smaller in the dart-poison frog, presenting considerably lower concentration. As an effect, it could be expected that the glands of *A. picta* possess a higher efficiency than those presented by the leptodactylid. It is possible that, through the course of the evolutionary history of *A. picta*, there have been a major investment in gland efficiency and quality, which may actually characterize the toxic lineages of Dendrobatidae as a whole. Conversely, due to the high concentration of glands and their pronounced dimensions, the chemical defense of *L. lineatus* may be more dependent on quantitative glandular characteristics. In this species, the presence of a concentrated distribution of glands along the yellow stripes and orange spots is an unprecedented finding. It is possible that this arrangement represents an initial stage in the evolution of cutaneous macroglands. This hypothesis finds support in the defensive behavior of this species, which includes the display of dorsal contrasting colored elements, confirmed to be aposematic in this case as it is related to toxic secretions. Accordingly, several anuran species exhibit similar defensive displays directly associated with the presence of macroglands (e.g., parotoid or inguinal), as it is the case of most bufonids and some leiuperids and hylids (Toledo and Jared, 1995; Lenzi-Mattos et al., 2005; Jared et al., 2009; review in Toledo et al., 2011).

This work aimed to open a range of topics for future studies able to penetrate deeper into the knowledge about the skin morphology and poison biochemistry and pharmacology of the two species here studied. We are convinced that only through a detailed multidisciplinary approach, concomitantly with field studies, the mimetic relationship here exposed can be further elucidated.

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