

Distribution of major toxins in *Rhinella marina* parotoid macroglands using Desorption-Electrospray-Ionization mass spectrometry imaging (DESI-MSI)

Pedro L. Mailho-Fontana^a, Andreia M. Porcari^b, Marcos N. Eberlin^c, Carlos Jared^a,
Marta Maria Antoniazzi^a, Daniel C. Pimenta^d, Juliana M. Sciani^{b,*}

^a Laboratório de Biologia Estrutural, Instituto Butantan, Av. Vital Brasil 1500, 05503-900, São Paulo, Brazil

^b Universidade São Francisco, Av. São Francisco de Assis 218, 12916-900, Bragança Paulista, Brazil

^c Escola de Engenharia, Universidade Presbiteriana Mackenzie, Rua da Consolação 930, 01302-907, São Paulo, Brazil

^d Laboratório de Biofísica e Bioquímica, Instituto Butantan, Av. Vital Brasil 1500, 05503-900, São Paulo, Brazil

ARTICLE INFO

Keywords:

Rhinella marina
DESI-MSI
Steroids
Alkaloids
Imaging
Parotoid
Skin glands

ABSTRACT

Amphibian cutaneous glands secrete toxins used in different vital functions including passive defense. Through Desorption Electrospray Ionization-Imaging we analyzed the distribution of the major toxins of the toad *Rhinella marina* parotoid macroglands. Alkaloids and steroids showed characteristic distribution and intensity within the glands and were also present at lower levels on the skin surface. A comprehensive overview of toxins distribution in toads' skin might help to understand their full biological role within the amphibians.

1. Main text

Amphibians are known to possess cutaneous glands distributed throughout the body. Basically, two different types of cutaneous glands can be found: (i) mucous glands, generally associated to maintenance of humidity and cutaneous respiration, and (ii) granular (or poison) glands, generally associated with chemical defense against predators and/or microbial infection (Toledo and Jared, 1995)(Jared et al., 2009).

In some species, the cutaneous glands are grouped in large numbers, forming conspicuous protrusions, known as macroglands, among which the parotoids are the most common (Mailho-Fontana et al., 2017). Histologically, the parotoids in toads of genus *Rhinella* are defined as large accumulations of hypertrophied poison glands arranged side by side and inserted into the dermal connective tissue (Mailho-Fontana et al., 2018).

It is widely accepted that the mucous glands secrete mucins involved in physiological functions (Daly, 1995), while the granular glands would have the functions of producing and storing content rich in a huge diversity of key substances for the defense system against microorganisms and predators (Raaymakers et al., 2017). However, no definitive demonstration of the exclusivity of the granular glands in the production of defensive toxins has yet been performed, in spite of the efforts of

several groups in the thorough characterization of the skin secretion composition (Liu et al., 2010; Li et al., 2016; Zhang et al., 2019).

Recently, our group has started to investigate such issues by an integrative approach using biochemical and immunohistochemical methods, together with classic histochemical stains in order to analyze the content and distribution of toxins in the amphibian skin. First we developed a method for retrieving soluble proteins from viscous amphibian skin secretion (Mariano et al., 2018) aiming at posterior proteomic identification (Mariano et al., 2018). In another study we purified and biochemically identified an enzyme in the skin secretion of the leaf frog *Phyllomedusa distincta*, which was unequivocally localized by immunohistochemistry in all types of skin glands, including the mucous glands (Sciani et al., 2019). In a third study, by the use of immunohistochemistry and mass spectrometry we were able to identify and localize tetrodotoxin in the skin of the newt *Taricha granulosa*, not only inside the granular glands but also within the mucous glands (Mailho-Fontana et al., 2019). In addition, we have also performed a molecular phenotypic approach in the characterization of different South-American toads, by the analysis of their low molecular mass components in the skin secretion (Sciani et al., 2013). Despite the very informative data obtained, molecules localization within the tissues still remains poorly known (Mailho-Fontana et al., 2018).

* Corresponding author. Multidisciplinary Research Laboratory - São Francisco University, SP, Brazil.

E-mail addresses: juliana.sciani@usf.edu.br, jmsciani@gmail.com (J.M. Sciani).

<https://doi.org/10.1016/j.toxcx.2020.100033>

Received 14 February 2020; Received in revised form 30 March 2020; Accepted 31 March 2020

Available online 28 April 2020

2590-1710/© 2020 The Author(s).

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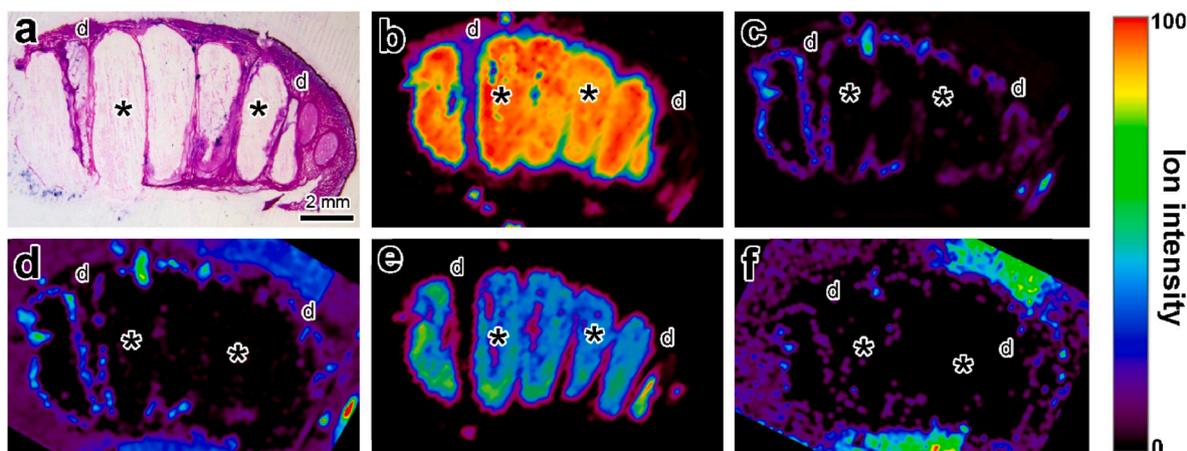


Fig. 1. Transverse histological section of *Rhinella marina* parotoid macrogland. (a) Hematoxylin-eosin staining. (b) Dehydrobufotenin m/z 203.098. (c) Marinobufagin m/z 401.202. (d) Telocinobufagin m/z 403.217. (e) Argininesuberoyol-marinobufagenin m/z 713.383. (f) Hellebrigenin m/z 417.216, as negative control. Poison gland (*). Dermis (d).

In the present work, by performing Desorption-Electrospray-Ionization (DESI) mass spectrometry imaging (MSI), for the first time, we were able to precisely localize toad toxins in the parotoid macroglands.

In order to achieve our goal, we analyzed *Rhinella marina* parotoids (male) (Suppl. Fig. 1) from Belterra (state of Pará, Brazil) under collection permit provided by the Brazilian Federal Government (SISBIO #48080-2). After euthanasia, the parotoid macroglands were removed, immediately frozen in -80°C and sectioned (16 μm thick) in transversal and longitudinal axis (Suppl. Fig. 1), using a Leica CM1860 cryostat. All methods were approved by the Ethics Committee on Animal Use of Instituto Butantan (CEUAIB, protocol #1046/13).

The glass slides containing the histological sections were imaged using a 2-D Omni Spray DESI imaging platform (Prosolia Inc, USA) coupled to a Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, USA), in a spatial resolution set to 200 μm . Analysis was performed under positive ion mode by spraying methanol (MeOH), dimethylformamide (DMF)/acetonitrile (ACN) (1:1) or MeOH/H₂O (1:1) (v/v) and ion images were assembled using BioMap (<http://www.maldi-msi.org>). After DESI-MSI, the sections were stained with hematoxylin-eosin (HE).

Fig. 1 contains the main findings of our analyses: HE-stained tissue section and ion images in a color-coded representation of the whole gland, where red represents abundance and black represents absence.

Fig. 1A shows a transverse section of a parotoid macrogland stained with HE. Fig. 1B-F shows serial transverse sections obtained by DESI-MSI of four most abundant toxins previously identified in this species (Scianni et al., 2013), namely: dehydrobufotenin (Fig. 1B, m/z 203.098), marinobufagin (Fig. 1C, m/z 401.202), telocinobufagin (Fig. 1D, m/z 403.217), argininesuberoyol-marinobufagenin (Fig. 1E, m/z 713.383), and hellebrigenin (Fig. 1F, m/z 417.216), a molecule absent in *R. marina* skin secretion (Scianni et al., 2013), used as a negative control. All these structures were confirmed by MS/MS, as shown in the Suppl. Fig. 2, considering their fragmentation profile, in comparison to the already described ones (Scianni et al., 2013). Methanol was the best solvent to solubilize those molecules and get an appropriated spectrum. The mixture MeOH/H₂O did not improve the signal, neither solubilized different compounds, whereas DMF/ACN yielded low quality spectra.

Two interesting conclusions can be drawn from our analysis: 1) the low molecular mass toxins (alkaloids and steroids) are unequivocally stored within the glands that compose the parotoid macroglands, and 2) there is a large variation in concentration among these toxins, which is in accordance to the relative concentrations previously assessed by RP-HPLC (Mailho-Fontana et al., 2018) (Scianni et al., 2013). Moreover, the location varies for each of the molecules. While alkaloids (Fig. 1B and E) are distributed throughout the glands, steroids are concentrated in the gland's periphery (Fig. 1C and D). Such differences in location may be related to the compatibility of each molecule stored in the gland to

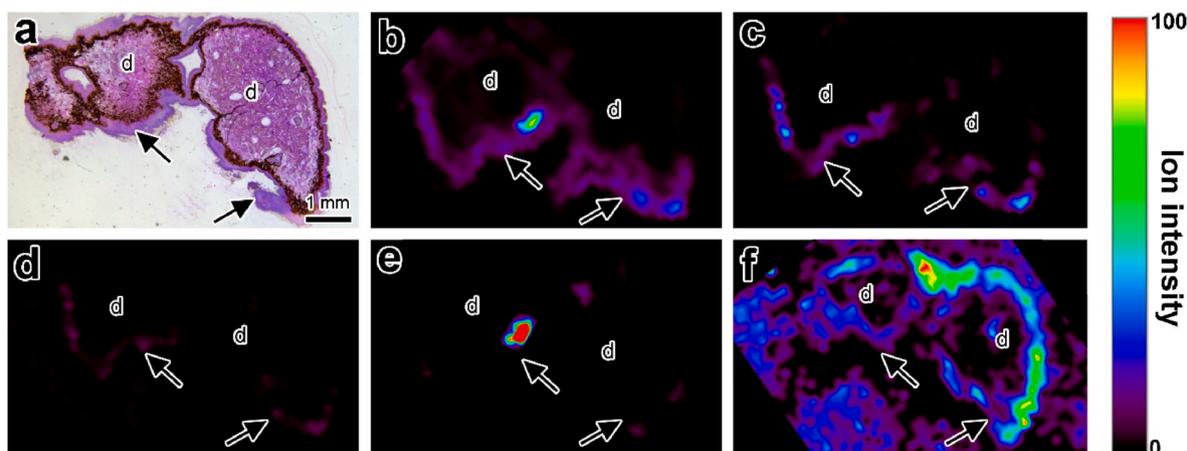


Fig. 2. Longitudinal histological section of *Rhinella marina* parotoid macrogland in a superficial plan. (a) Hematoxylin-eosin staining. (b) Dehydrobufotenin m/z 203.098. (c) Marinobufagin m/z 401.202. (d) Telocinobufagin m/z 403.217. (e) Argininesuberoyol-marinobufagenin m/z 713.383. (f) Hellebrigenin m/z 417.216, as negative control. Dermis (d). Epidermis (arrows).

specific cytoplasm locations or to timing and constancy of toxin production.

The analysis of longitudinal sections by DESI-MSI revealed that the dermis is not consistent with any ion-image (Fig. 2), corroborating the results showed in Fig. 1. On the other hand, more superficial sections revealed that the epidermis clearly exhibits the presence of the analyzed molecules (Fig. 2), although in lower intensity when compared to the interior of the glands. Therefore, such molecules seem to be constitutively secreted by the cutaneous glands onto the skin surface, and may act in the chemical defense against microorganisms, as already described for *Rhinella marina* poison (de Medeiros et al., 2019).

In conclusion, the present work demonstrates for the first time the actual tissue distribution of different toxins in the skin of a toad. The use of DESI-MSI, mainly if integrated to other morphological approaches, can provide relevant information about amphibian poison sources, contributing to the better understanding of its multifunctional role.

Funding

This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior [grant #001 to PLMF], Financiadora de Estudos e Projetos [grants #01.09.0278.04 and #01.12.0450.03], Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) [406385/2018-1 to DCP] and Fundação de Amparo à Pesquisa do Estado de São Paulo [grant #2014/15334-4 and #2017/10488-1 to PLMF, #2018/03265-9 to CJ and #2019/04314-6 to AMP]. DCP, CJ and MMA are CNPq fellow researchers [grants #303792/2016-7, #305266/2019-5 and #309589/2017-7].

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Pedro L. Mailho-Fontana: Conceptualization, Methodology, Investigation, Writing - original draft. **Andreia M. Porcari:** Methodology, Investigation, Writing - review & editing. **Marcos N. Eberlin:** Methodology, Resources. **Carlos Jared:** Conceptualization, Funding acquisition, Writing - review & editing. **Marta Maria Antoniazzi:** Conceptualization, Validation, Writing - review & editing. **Daniel C. Pimenta:** Conceptualization, Funding acquisition, Formal analysis, Writing - original draft. **Juliana M. Sciani:** Conceptualization, Investigation, Resources, Validation, Writing - original draft, Writing - review & editing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.toxcx.2020.100033>.

[org/10.1016/j.toxcx.2020.100033](https://doi.org/10.1016/j.toxcx.2020.100033).

References

- Daly, J.W., 1995. The chemistry of poisons in amphibian skin. Proc. Natl. Acad. Sci. U.S.A. 92, 9–13. <https://doi.org/10.1073/pnas.92.1.9>.
- de Medeiros, D.S.S., Rego, T.B., dos Santos, A.P.d.A., Pontes, A.S., Moreira-Dill, L.S., Matos, N.B., Zuliani, J.P., Stábeli, R.G., Teles, C.B.G., Soares, A.M., Sperotto, A.R.d.M., Moura, D.J., Saffi, J., da Silva Caldeira, C.A., Pimenta, D.C., Calderon, L.A., 2019. Biochemical and biological profile of parotoid secretion of the Amazonian *Rhinella marina* (Anura: bufonidae). BioMed Res. Int. 2019 <https://doi.org/10.1155/2019/2492315>.
- Jared, C., Antoniazzi, M.M., Jordão, A.E.C., Silva, J.R.M.C., Greven, H., Rodrigues, M.T., 2009. Parotoid macroglands in toad (*Rhinella jimi*): their structure and functioning in passive defence. Toxicon 54, 197–207. <https://doi.org/10.1016/j.toxicon.2009.03.029>.
- Li, J.F., Fang, H., Yan, X., Chang, F.R., Wu, Z., Wu, Y.L., Qiu, Y.K., 2016. On-line comprehensive two-dimensional normal-phase liquid chromatography × reversed-phase liquid chromatography for preparative isolation of toad venom. J. Chromatogr. A 1456, 169–175. <https://doi.org/10.1016/j.chroma.2016.06.008>.
- Liu, Y., Xiao, Y., Xue, X., Zhang, X., Liang, X., 2010. Systematic screening and characterization of novel bufadienolides from toad skin using ultra-performance liquid chromatography/electrospray ionization quadrupole time-of-flight mass spectrometry. Rapid Commun. Mass Spectrom. 24, 667–678. <https://doi.org/10.1002/rcm.4436>.
- Mailho-Fontana, P.L., Antoniazzi, M.M., Rodrigues, I., Sciani, J.M., Pimenta, D.C., Brodie, E.D., Rodrigues, M.T., Jared, C., 2017. Parotoid, radial, and tibial macroglands of the frog *Odontophrynus cultripes*: differences and similarities with toads. Toxicon 129, 123–133. <https://doi.org/10.1016/j.toxicon.2017.02.022>.
- Mailho-Fontana, P.L., Antoniazzi, M.M., Sciani, J.M., Pimenta, D.C., Barbaro, K.C., Jared, C., 2018. Morphological and biochemical characterization of the cutaneous poison glands in toads (*Rhinella marina* group) from different environments. Front. Zool. 15, 46. <https://doi.org/10.1186/s12983-018-0294-5>.
- Mailho-Fontana, P.L., Jared, C., Antoniazzi, M.M., Sciani, J.M., Pimenta, D.C., Stokes, A. N., Grant, T., Brodie, E.D., Brodie, E.D., 2019. Variations in tetrodotoxin levels in populations of *Taricha granulosa* are expressed in the morphology of their cutaneous glands. Sci. Rep. 9 <https://doi.org/10.1038/s41598-019-54765-z>.
- Mariano, D.O.C., Di Giacomo Messias, M., Prezotto-Neto, J.P., Spencer, P.J., Pimenta, D. C., 2018. Biochemical analyses of proteins from *duttaphrynus melanostictus* (*Bufo melanostictus*) skin secretion: soluble protein retrieval from a viscous matrix by ion-exchange batch sample preparation. Protein J. 37, 380–389. <https://doi.org/10.1007/s10930-018-9780-z>.
- Raaymakers, C., Verbrugge, E., Hernot, S., Hellebuyck, T., Betti, C., Peleman, C., Claeys, M., Bert, W., Cavelliers, V., Ballet, S., Martel, A., Pasmans, F., Roelants, K., 2017. Antimicrobial peptides in frog poisons constitute a molecular toxin delivery system against predators. Nat. Commun. 8, 1–8. <https://doi.org/10.1038/s41467-017-01710-1>.
- Sciani, J.M., Angeli, C.B., Antoniazzi, M.M., Jared, C., Pimenta, D.C., 2013. Differences and similarities among parotoid macrogland secretions in south american toads: a preliminary biochemical delineation. Sci. World J. 2013 <https://doi.org/10.1155/2013/937407>.
- Sciani, J.M., Neves, A., Vassão, R.C., Spencer, P., Antoniazzi, M.M., Jared, C., Pimenta, D. C., 2019. The Amphibian diacylglycerol O-acyltransferase 2 (DGAT2): a “paleo-protein” with conserved function but unique folding. Protein J. 38, 83–94. <https://doi.org/10.1007/s10930-019-09814-x>.
- Toledo, R.C., Jared, C., 1995. Cutaneous granular glands and amphibian venoms. Comp. Biochem. Physiol. - Part A Physiol. [https://doi.org/10.1016/0300-9629\(95\)98515-1](https://doi.org/10.1016/0300-9629(95)98515-1).
- Zhang, Y., Yuan, B., Takagi, N., Wang, H., Zhou, Y., Si, N., Yang, J., Wei, X., Zhao, H., Bian, B., 2019. Comparative analysis of hydrophilic ingredients in toad skin and toad venom using the UHPLC-HR-MS/MS and UPLC-QqQ-MS/MS methods together with the anti-inflammatory evaluation of indolealkylamines. Molecules 24. <https://doi.org/10.3390/molecules24010086>.