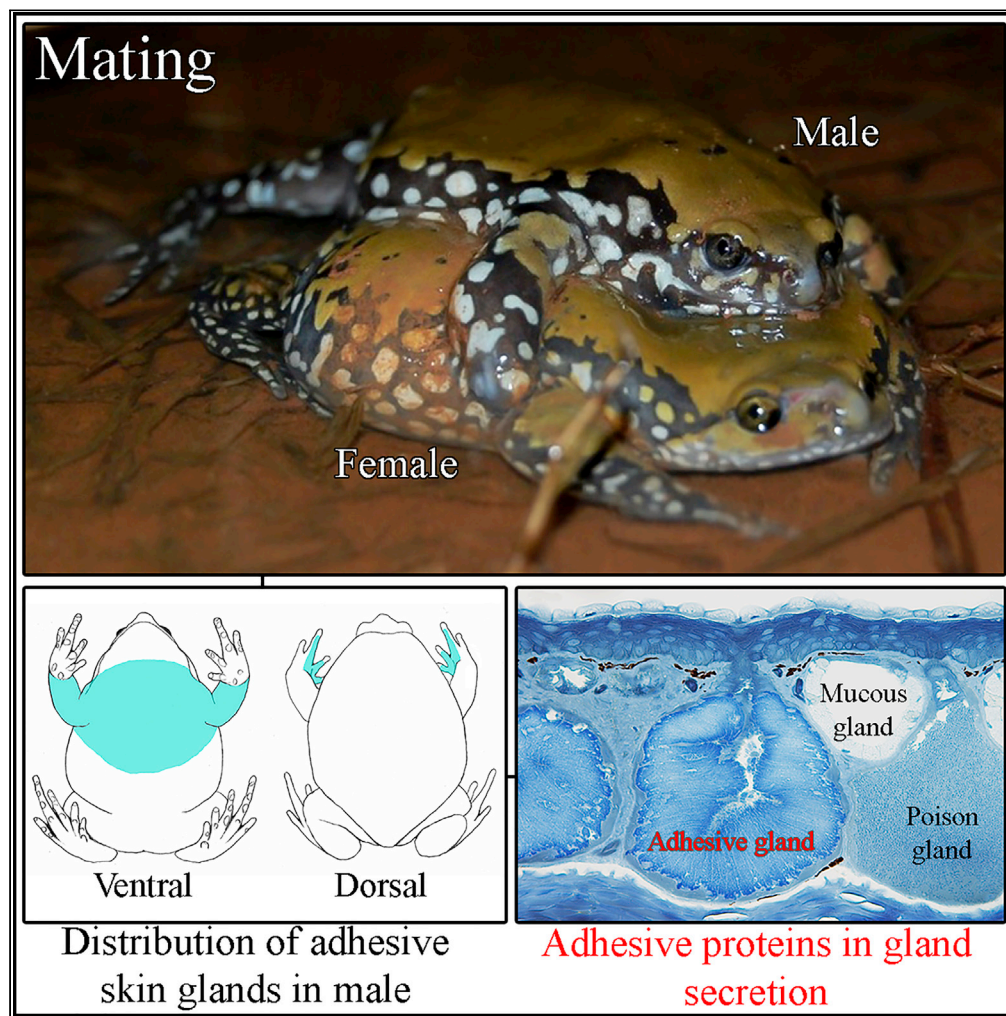


Article

Reproductive behaviour, cutaneous morphology, and skin secretion analysis in the anuran *Dermatonotus muelleri*



Marta Maria Antoniazzi, Pedro Luiz Mailho-Fontana, Fausto Nomura, ..., Fernando Rogério Carvalho, Denise Cerqueira Rossa-Feres, Carlos Jared

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Highlights

*Dermatonotus muelleri* mating involves peculiar male adherence to the female's back

Adhesion phenomenon is possibly related to arm shortening and round-shaped body

Differentiated adhesive glands are distributed in the male's anterior ventral skin

Male skin secretion contains compounds related to the adhesive properties

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## Article

Reproductive behaviour, cutaneous morphology, and skin secretion analysis in the anuran *Dermatonotus muelleri*

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## SUMMARY

Despite the common poison and mucous glands, some amphibian groups have differentiated glands associated with reproduction and usually present on the male ventral surface. Known as breeding glands or sexually dimorphic skin glands (SDSGs), they are related to intraspecific chemical communication during mating. Until recently, reproduction associated with skin glands was recognized only in salamanders and caecilians and remained unexplored among anurans. The Brazilian microhylid *Dermatonotus muelleri* (Muller's termite frog) is known for its very toxic skin secretion. Despite the slippery body, the male adheres to the female back during reproduction, as they have differentiated ventral glands. In this paper, we have gathered data proposing an integrative approach correlated with the species' biology and biochemical properties of their skin secretions. Furthermore, we suggest that the adhesion phenomenon is related to arm shortening and rounded body that make amplexus inefficient, although constituting important adaptive factors to life underground.

## INTRODUCTION

One of the most striking features shared throughout the Amphibia is the existence of numerous glands scattered across the surface of the skin or forming accumulations in certain regions of the body. The glandular types common to all amphibians are the mucous glands, associated with lubrication and moistening of the body surface, and the poison (or granular) glands, with defensive function, which in many taxonomic groups are also found in the form of glandular accumulations (Jared et al., 2009; Mailho-Fontana et al., 2014, 2018). In addition, in some groups there are differentiated glandular types such as lipid-secreting glands of Phyllomedusinae tree frogs, with waterproofing function (Nosi et al., 2002; Antoniazzi et al., 2013), and glands associated with reproduction that are mostly associated with males. In anurans, this last type has been studied in the last two decades and includes the glandular accumulations present in frogs of the genus *Cycloramphus* (Gonçalves and Brito-Gitirana, 2008), in certain mantellid frogs (Vences et al., 2007; Poth et al., 2012), as well as the glands generally associated with nuptial pads, usually present on the hands, but also occurring in other parts of the body such as arms, lips, mental, pectoral, and lateral regions, and even on the toes (Luna et al., 2018). Such glands known as breeding glands or sexually dimorphic skin glands (SDSGs) (Thomas et al., 1993; Brizzi et al., 2002; Brunetti et al., 2012, 2015; Luna et al., 2018) are in general related to intraspecific chemical communication during reproduction.

While the reproductive function associated with amphibian skin glands has long been known among Caudata (Noble, 1931; Lanza, 1959; Sever, 1976, 1989; Houck and Verrel, 1993), until recently, within the vast anuran universe, the little information available referred exclusively to morphological aspects (Thomas et al., 1993; Romero de Perez and Ruiz Carranza, 1996; Brizzi et al., 2002, 2003; Vences et al., 2007; Gonçalves and Brito-Gitirana, 2008; Siegel et al., 2008; Poth et al., 2012). One possible reason for the scarcity of information is that, historically, communication among anurans has been associated almost exclusively with emission and reception of sounds (Brunetti et al., 2015), leaving aside the role of the skin and cutaneous secretions in reproduction. However, evidence that other forms of communication,

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**Figure 1.** A mating couple of *Dermatotonotus muelleri*

including chemical and tactile stimuli, are also widely used among Anura species is gradually increasing (Haddad and Giarretta, 1999; Haddad and Sawaya, 2000; Haddad et al., 2005; Hödl and Amézquita, 2001; Stephenson and Verrell, 2003; Giasson and Haddad, 2006; Toledo et al., 2007; Taylor et al., 2011; Grafe et al., 2012; Preininger et al., 2013; Mariano et al., 2015). Recently, some research groups have been dedicated to the biochemical analysis of the secretions of SDSGs, trying to understand the mechanisms of action among individuals of different skin chemical compounds produced (Brunetti et al., 2012, 2015).

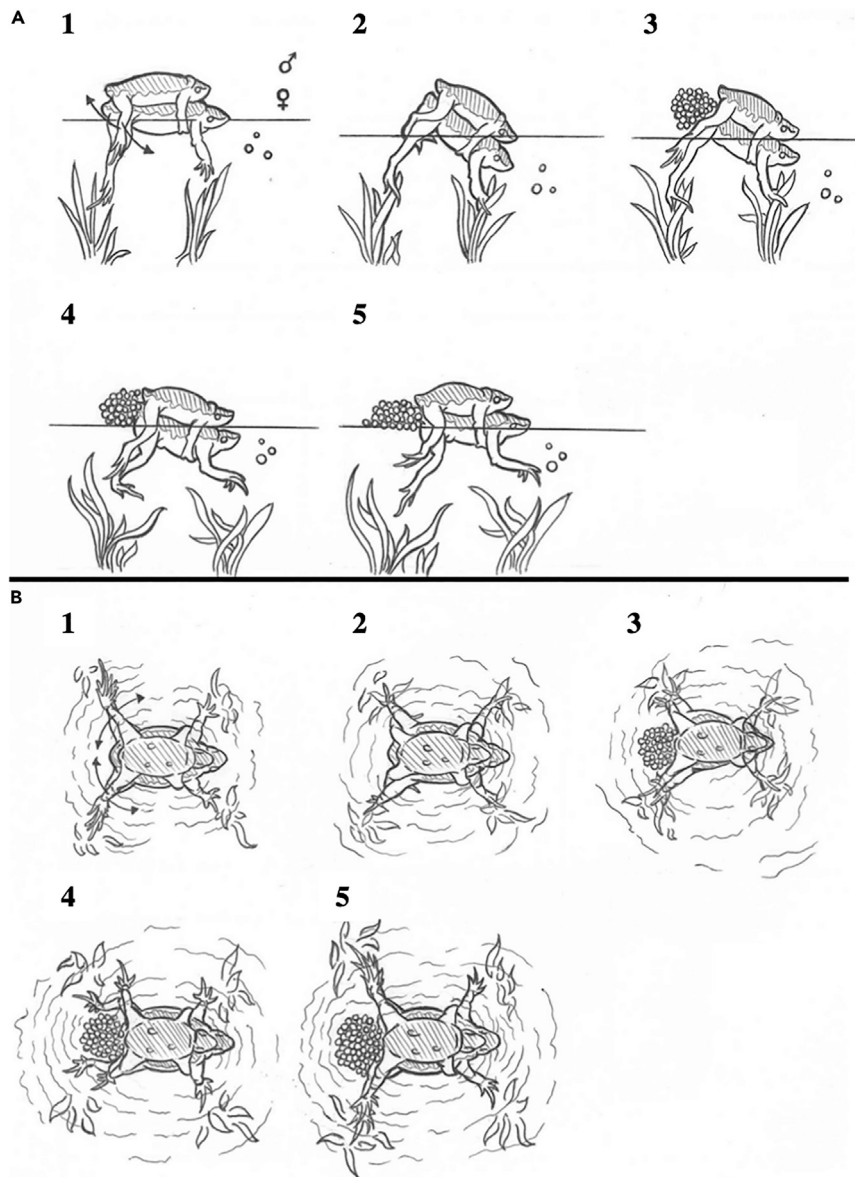
In addition to compounds acting in chemical communication, the skin of some anurans produce substances that favour the attachment of male and female, in order to ensure mating. That is the case of the family Microhylidae, in which has long been known that during mating some species produce skin adhesive, which help the amplexus between males and females. The theme was explored mainly in the North American species *Gastrophryne carolinensis* (Conaway and Metter, 1967; Metter and Conaway, 1969; Holloway and Dapson, 1971; Siegel et al., 2008), but also in *Gastrophryne olivacea* (Fitch, 1956), *Kaloula picta* (Inger, 1954), and *Dermatotonotus muelleri* (Nomura, 2003). In addition, microhylids generally have a very secretory and slippery body and, at least for the Neotropical species *Dermatotonotus muelleri*, popularly known as Muller's termite frog, there are numerous anecdotal reports about the high toxicity of the species. In fact, during fieldwork, we have witnessed the death of other amphibian species when placed in direct contact with *D. muelleri* during specimen collection. Also, in one of our expeditions into the caatinga (interior of the Brazilian State of Rio Grande do Norte), one of our workers experienced great irritation of eye mucosa accompanied by intense pain by accidental contact of the skin secretion in the eyes. The biochemistry and the biological actions of the skin secretions of *D. muelleri* were recently analyzed (Cavalcante et al., 2017). Here, we aim the identification of different components of the skin secretion in this species, particularly those related to the adhesive properties of SDSGs in the males.

In this paper, we describe the general morphology of the skin and of the cutaneous glandular types present in males and females of the microhylid *Dermatotonotus muelleri*, focusing on the morphological characterization and distribution on the body of the cutaneous glands in general and of the adhesive glands. We also compared the chemical profiles of the cutaneous secretion of both sexes, trying to identify compounds responsible for the adhesive properties in this species.

## RESULTS

### Reproductive behaviour

Couples of *Dermatotonotus muelleri* (Figure 1) laying eggs were observed in the water, generally about 1 meter from the margin of the waterbody. The ethogram with the complete sequence of egg-laying behaviour is represented in Figure 2 and Table 1. During the elevation phase, the male used the feet to stretch the body backwards, placing a small portion of the ventral skin close to the female's cloacal opening. Subsequently, the male positioned the legs around the female body with the feet below her cloacal opening, forming a small channel through which the semen may flow. The male's legs and feet



**Figure 2. Oviposition behaviour of *Dermatotonotus muelleri***

(A) Lateral view.

(B) Dorsal view. The numbers indicate the behavioural sequence and correspond to the description in the ethogram in Table 1. Same numbers in figures A and B indicate the same phase.

placed in such position restricted the space for egg liberation by the female during the oviposition phase. After about 12 h of embracing, with periodical sequences of oviposition with simultaneous egg fertilization, males and females forced body separation. The male supported the forelimbs on the female's back and raised the shoulder girdle while the female tried to swim and dive through branches of vegetation that eventually would interpose between them, helping the couple to separate. In general, the male rotated the body about 160° in relation to the female's perpendicular axis, both swimming in opposite directions, until their final separation.

### General aspects of the skin

The skin of *Dermatotonotus muelleri* is very secretory, which makes the surface of these anurans constantly moist and slippery. The skin secretion, besides the obvious mucous appearance, contains toxins. This

**Table 1. Ethogram of the oviposition behavior of *Dermatonotus muelleri***

BEHAVIOR	DESCRIPTION
1. Amplexus	FEMALE: gets in the water. MALE: Approaches the female laterally or from behind; jump on the female and attempt to hold her with the anterior members. AMPLEXUS: Axillary, adhered by sticky mucus
2. Elevation	FEMALE: Lifts the body over the water while immersing its head and shoulder girdle; may hold on to submerged vegetation or move the hind limbs in swimming movements to keep the body stabilized. MALE: Flexes the hind limbs and brings the heels close to its cloacal opening; distends its hind limbs and stretches its ventral skin and making a channel to the female cloaca; positions its heels below the female's cloacal opening.
3. Oviposition	FEMALE: lays the eggs in the space between the males' hind limbs. MALE: releases the sperm into the channel created by its stretched skin.
4. Sub elevation	FEMALE: flexes its legs, returning to the initial position; the eggs get in contact with the water surface, start to float, and their jelly capsule grows in volume; MALE: slides its heels into the joints of the female's hind limbs as she flexes the hind limbs.
5. Waiting	FEMALE: selects a new site or restarts the oviposition sequence. MALE: remains in latency until the female begins to move when he aids on fluctuation and displacement by moving the hind limbs or start the oviposition sequence.

Numbers correspond to the behavioral stages in [Figure 1](#)

reinforce that during fieldwork or even in routine dealing in captivity, it is necessary to keep *D. muelleri* specimens separate from the other amphibians to avoid deaths by poisoning.

### Morphological analysis of the skin

The analysis of *Dermatonotus muelleri* skin shows that, in both males and females, especially in the dorsal region, very large glands occupy most of the volume of the stratum spongiosum; much smaller glands are distributed among them just below the epidermis, which is composed of 4–5 cell layers ([Figures 3A](#) and [3B](#)). The large size of the glands allows their visualization in skin cross sections even with naked eyes. Skirting the bottom of the glands, a thin and continuous dermal calcified layer is present, delimiting the stratum spongiosum and the collagen fibres of the stratum compactum ([Figure 3A](#) insert).

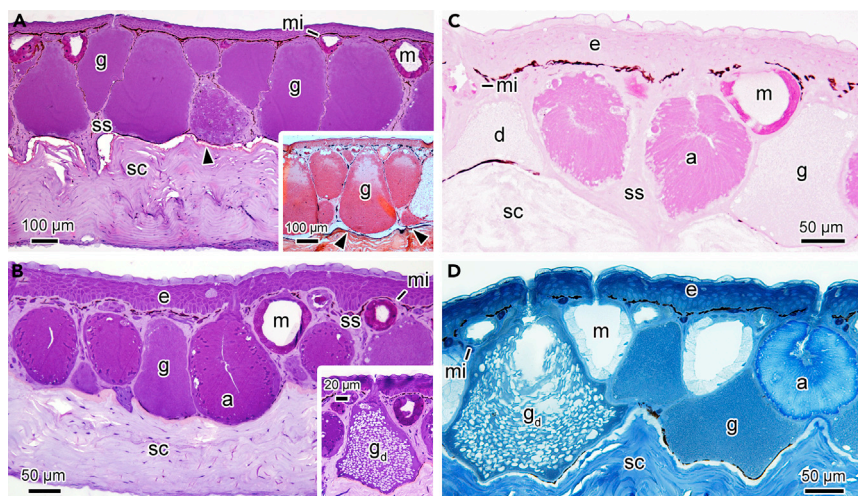
A detailed histological analysis allows the classification of the glands in three different types: the poison (or granular) glands ([Figure 3A](#)), the mucous glands ([Figures 3A](#) and [3B](#)), and the mixed glands ([Figure 3A](#)). Exclusively in the ventral surface of males, a fourth glandular type was also identified, which, following the denomination already in use in literature, was named adhesion gland ([Figure 2B](#)). All types of skin glands are surrounded by a monolayer of myoepithelial cells and communicate with the skin surface through ducts lined by epithelia.

### Poison (granular) glands

The poison glands, compared to the mucous glands, are much larger, mainly in the dorsal region. Here, they form a side-by-side palisade arrangement, occupying most of the volume of the stratum spongiosum of the dermis ([Figures 3A](#) and [3B](#)). These glands are negative to periodic acid-Schiff (PAS) ([Figure 3C](#)) and predominantly positive to bromophenol blue ([Figure 3D](#)), but some, although similar in size and shape, show positive cytoplasm but negative granules to bromophenol blue (named as differentiated poison glands) ([Figures 3B–3D](#)). Poison glands are formed by a secretory syncytium, characterized by peripheral nuclei ([Figure 4A](#)), absence of lumen ([Figures 3](#) and [4A](#)), and an electron-dense cytoplasm ([Figures 4B–4D](#)). This mainly contains secretion granules that are formed in the periphery as small spherical vesicles that gradually grow by coalescence ([Figure 4B](#)), as they accumulate within the gland, acquiring varied shapes, densities, and textures ([Figures 4C](#) and [4D](#)). Other organelles such as mitochondria and endoplasmic reticulum are hardly visible among the granules.

### Mucous glands

The mucous glands are spherical and acinar but clearly distinct from the mixed glands by the larger size and volume of the lumen ([Figures 3](#) and [5A](#)). They are composed of two cell types with visible basal nuclei ([Figure 5A](#)). The predominant type has the cytoplasm full of coalescent granules with flocculent content, is



**Figure 3. Histology of the skin in *Dermatodonotus muelleri***

(A) Dorsal skin of a male showing a large number of skin glands, mainly poison glands (g), but also mucous (m) and mixed (mi) glands, occupying most of the volume of the stratum spongiosum (ss). The arrowheads point to the calcified dermal layer, which is highlighted in black using von Kossa histochemistry (insert).

(B) Ventral skin of a male in the pectoral region. Besides the poison glands and the mucous (m) and mixed (mi) glands, a fourth type is present, the adhesive gland (a). The insert shows a differentiated poison gland ( $g_d$ ) found less frequently in the skin of both sexes.

(C) PAS of the same region shown in (B), demonstrating the presence of mucopolysaccharides in the adhesive glands (a), and in the mucous (m) and mixed (mi) glands.

(D) Bromophenol blue histochemistry of the dorsal skin, where the two types of poison glands, both positive for proteins, can be distinguished, type 1 (g), with positive granules, and type 2 ( $g_d$ ), with negative granules and positive cytoplasmic matrix. The mucous glands (m) are negative, while the mixed glands (mi) are positive to the reaction, as well as the adhesive glands. e, epidermis; sc, stratum compactum.

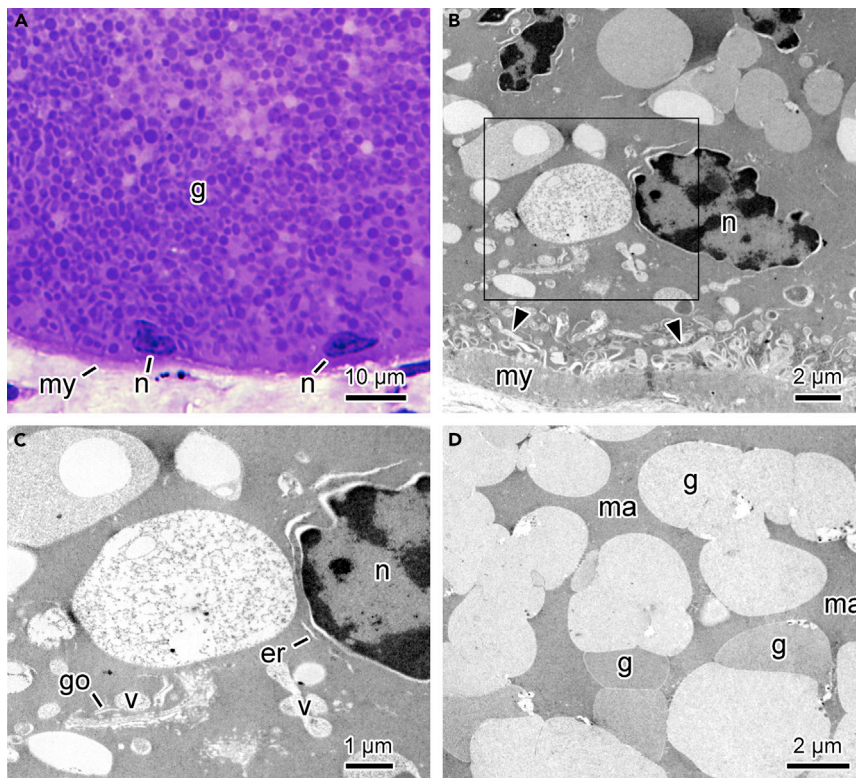
very reactive to PAS (Figure 3C) and is electron lucent at transmission electron microscopy (TEM) (Figures 5B–5D). At the cell basis, around the nuclear area, the cytoplasm exhibits other organelles such as the Golgi apparatus (Figures 5B and 5C). The other cell type is much less numerous and appears usually compressed among the cells of predominant type (Figures 5B and 5D). The cytoplasm shows abundant endoplasmic reticulum (Figure 5B) and contains granules that are not reactive to the performed histochemical methods (Figures 3C and 3D), and at TEM appear homogeneous and with medium electron density (Figures 5B–5D).

### Mixed glands

The mixed glands constitute the smallest among all glandular types (Figure 3). Histologically, they show acinar shape with a central lumen and an epithelium comprising at least two cell types (Figure 6A) clearly distinguishable through histochemistry (Figures 3C, 3D and 6A insert). The inner epithelial surface delimiting the lumen forms numerous microvilli, indicating secretion through exocytosis (Figure 6B). The most numerous cells show basal nucleus and cytoplasm full of roundish granules. Depending on the cell, they show different levels of affinity to toluidine blue (Figure 6A) and different levels of electron density when observed by TEM (Figure 6B), although they are always bromophenol blue-negative (Figure 6A insert). The cytoplasm exhibits exuberant rough endoplasmic reticulum (Figures 6C and 6D). The other cell type, much less abundant, is not always visible, and the apical region sneaks through the other cells, assuming a typical hourglass shape. The cytoplasm presents granules at the apical region that are much smaller in comparison with the predominant type that are positive to PAS (Figure 3C) and to bromophenol blue (Figure 5A, insert). At TEM, these granules are of medium electron density and often show electrodense internal areas (Figure 6B). At the cell base, a profusion of cell organelles is seen, such as rough endoplasmic reticulum lamellae, Golgi apparatus, and mitochondria (Figure 6D).

### Adhesive glands

The adhesive glands are observed exclusively in males and were found in only six of the 38 regions analyzed. They are in an extensive ventral area covering the pectoral region and the arms and extending



**Figure 4. Skin poison gland in *Dermatonotus muelleri***

(A) Histology of part of a poison gland (g) syncytium, showing the external myoepithelial layer (my), the nuclei (n) in the periphery, and the internal cytoplasmic mass, full of secretion granules.

(B) TEM of the syncytium periphery, where the granules are secreted by the cellular machinery. Note the myoepithelial layer (my) and the interdigitations (arrowheads) connecting it with the syncytium.

(C) Higher magnification of the area delimited in (B), evidencing the endoplasmic reticulum (er), the Golgi apparatus (go), and many secretory vesicles (v).

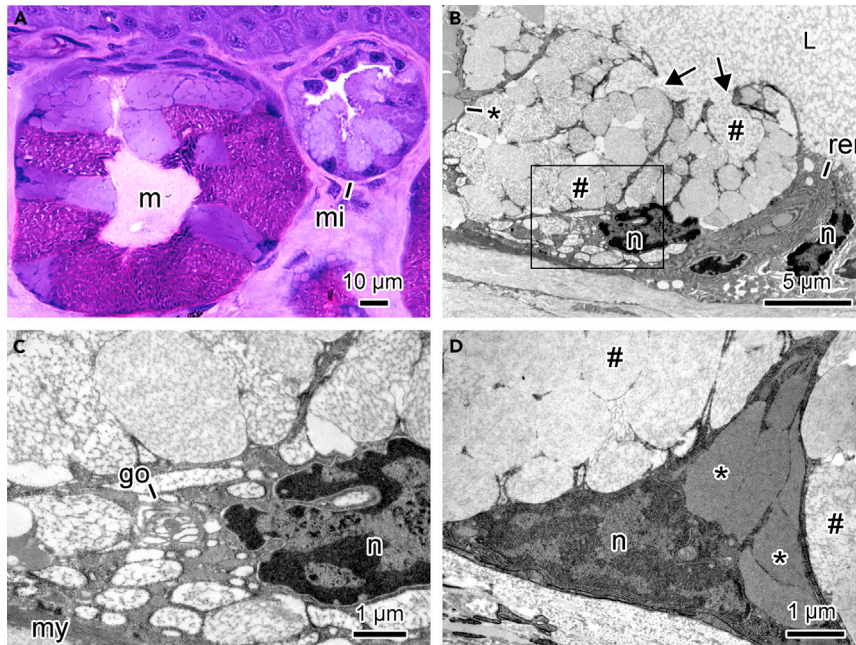
(D) TEM of the internal region of the syncytium where granules (g) of different sizes and electron densities are fusing, contrasting with the homogeneous electron-dense matrix (ma).

to the dorsum of the forelimb (Figure 7). The adhesive glands show typical acinar organization but are more elongated than the mucous and mixed glands (Figures 3, 7, and 8). Compared to the poison glands, they are much smaller in size although larger than the mucous and mixed glands (Figure 3). Histologically, they are formed by a simple columnar epithelium organized around a narrow lumen (Figures 8A and 8B). The cells are all similar, with the cytoplasm filled with spherical granules (Figure 8B) highly positive to PAS (Figure 3C) and bromophenol blue (Figure 3D), respectively, indicating the presence of neutral mucous substances and proteins. At TEM, the spherical or polygonal granules are homogeneously electron-dense (Figures 8C and 8D) and are secreted through an apocrine process, among the scarce and short apical microvilli facing the glandular lumen (Figure 8C). The columnar cells exhibit an exuberant rough endoplasmic reticulum, mostly prominent in the basal region, close to the nucleus, and in a peripheral position throughout the cell (Figure 8E).

#### Biochemical analysis of the cutaneous secretion

The SDS-PAGE revealed protein bands distributed through a broad molecular mass range (Figure 9). Besides the proteins common to both males and females, we identified exclusive proteins between them: females have at least one protein (number 1) absent in males, and males have three proteins (numbers 2, 3, and 4) not found in females. Moreover, proteins 5 and 6, shared by both sexes, seem to be much more expressed in males.

By HPLC analysis (C18 column,  $\lambda = 214$  nm), several peaks along the gradient were observed (Figure 10), indicating a secretion rich in compounds of low molecular mass (<600 Da) as previously evidenced by



**Figure 5. Skin mucous gland in *Dermatotonus muelleri***

(A) Histological section containing a mucous gland (m) and a mixed gland (mi), emphasizing their difference in size and in the secretory cell morphology.

(B) TEM showing the predominant secretory cells, with flocculant granules that are released (arrows) in the lumen (L) and the less frequent cells, with granules more homogeneous, with medium electron density (\*). Note the well-developed endoplasmic endoplasmic reticulum (rer).

(C) High magnification of the region delimited in (B). The granules are produced at the cell basis, close to the nucleus. Note the area of the Golgi apparatus (go).

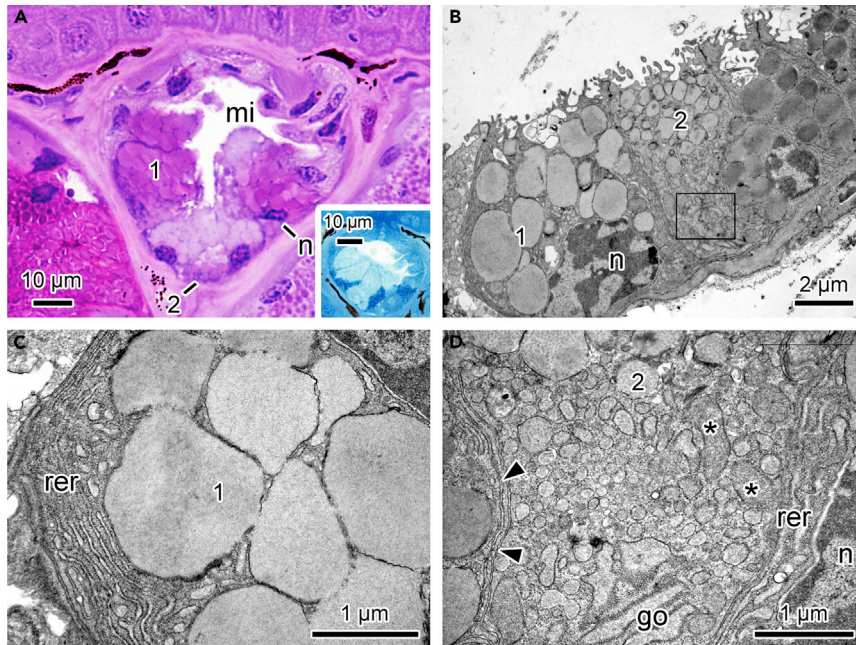
(D) TEM focusing the frequent cell type, exhibiting more electron dense granules (\*), which are also seen in (B). my, myoepithelial layer; n, nucleus; #, flocculant granules.

mass spectrometry analysis (Cavalcante et al., 2017). However, the secretion of males and females did not show characteristic peaks indicating a similar molecular composition, with variations only in intensity of some compounds (Figure 10).

These six main protein bands (numbers 1, 2, 3, 4, 5, and 6) obtained by SDS-PAGE were excised and processed for proteomics analyses and it was possible to identify the proteins listed in Table 2. The best fragmentation patterns for each digested band were sequenced once more and the resulting peptides were analyzed by BLAST algorithm for protein identification. It is noteworthy to mention the presence of several proteins associated with binding processes, such as mucin, SET and MYND, cadherin, glue protein, neurexin, and desmocollin. Moreover, two apparently sex-regulated proteins were also identified: prostaglandin receptor and the corticosteroid 11-beta-dehydrogenase. It was also possible to identify one matrix-related enzyme, one mitochondrial-related protein, one blood pressure-related controlling enzyme, one growth factor-like protein, one Glu-receptor, one cytoskeleton regulator protein, one phospholipase, besides two uncharacterized proteins.

## DISCUSSION

Most microhylids, including the Neotropical species, are fossorial, with drop-shaped bodies, small heads, narrow mouths, and very short limbs in relation to the body, feeding on ants and termites, and having an explosive pattern of reproduction (Duellman and Trueb, 1986). Among the microhylids of the Brazilian fauna already examined by our group (such as *Stereocyclops incrassatus*, *Elachistocleis bicolor*, and *Chiasmocleis leucosticta*, in addition to *Dermatotonus muelleri*), we note that the dorsal skin is very rich in poison (or granular) glands, in addition to a large number of mucous glands, which indicates that they are highly poisonous species with a high cutaneous secretory ability (Toledo and Jared, 1995). Especially in *D. muelleri*, the poison glands on the dorsum show a very large size and elongate shapes,



**Figure 6. Skin mixed gland in *Dermatotonotus muelleri***

(A) Histological section focusing a mixed gland (mi), where two types of cells are recognized by the size and shape of their granules. The most abundant type (1), with basal nuclei (n) and roundish granules, and a less common type (2), with smaller granules in apical disposition. The insert shows a mixed gland stained with bromophenol blue.

(B) Part of the glandular epithelium in TEM, showing the difference in shape of the two cell types, as well as the general aspect of their granules. Among them, a cell type 2, with apical granules of smaller size.

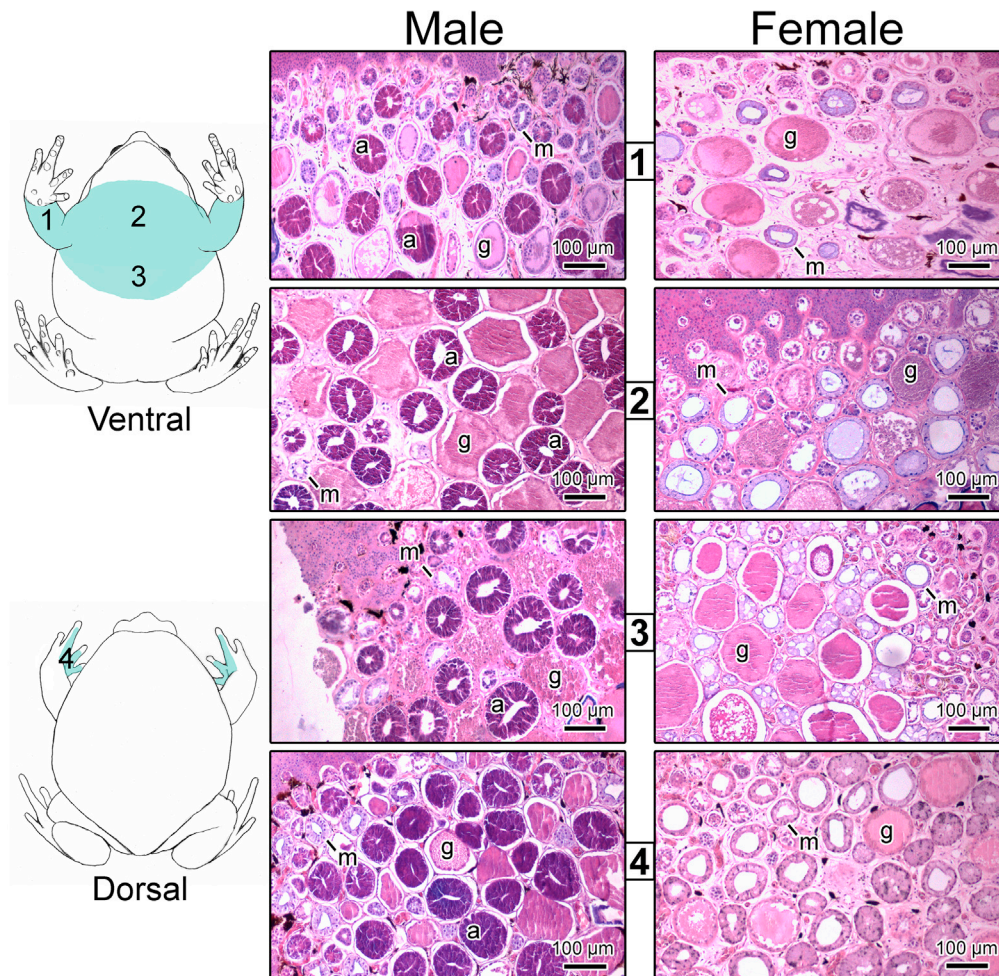
(C) Detail of the cytoplasm of type 1 cell in TEM. The granules are roundish and with homogeneous content and occupy most of the cell volume. At the periphery, the well-developed rough endoplasmic reticulum is observed.

(D) Higher magnification of an equivalent region to the area delimited in (B). The basal cytoplasm of cell type 2 shows well developed rough endoplasmic reticulum (rer), Golgi apparatus (Go), and mitochondria (\*). The arrow points to the cell limits.

one juxtaposed to the other, occupying a considerable part of the dermal volume, a condition that, from our knowledge, is not common in other anurans.

In our histological studies in different body regions of *Dermatotonotus muelleri*, the skin of males and females was very similar in terms of the general structure and distribution of glands. However, exclusively in male ventral skin, many differentiated glands were identified spread over the pectoral region and along the ventral extension of the arms. Owing to the specific body location, such glands were promptly related to adhesion that occurs during the amplexus, similarly to what was observed in other microhylids (Inger, 1954; Fitch, 1956; Conaway and Metter 1967; Metter and Conaway 1969; Holloway and Dapson 1971; Nomura, 2003; Siegel et al., 2008). In addition, the males of the other microhylid species examined presented the same type of glands, suggesting that this character is plesiomorphic among Microhylidae. Our observations indicate that each species shows variations regarding the disposition of such glands, which seems to reflect the variation in body shape and the differences in the form the males position their bodies over the females during the amplexus. The adhesion glands we observed, at least in general appearance, are very much alike the SDG glands observed in other anurans such as those of the Cophomantini hyelines (Brunetti et al., 2015) and the specialized mucus glands observed in some species of *Rana* (Brizzi et al., 2002). All these glands are typically acinar, formed by columnar cells filled with small, homogeneous spherical granules. In the case of the adhesive glands, it seems clear that the lumen tends to be narrow and that the secretory process is apocrine (Siegel et al., 2008).

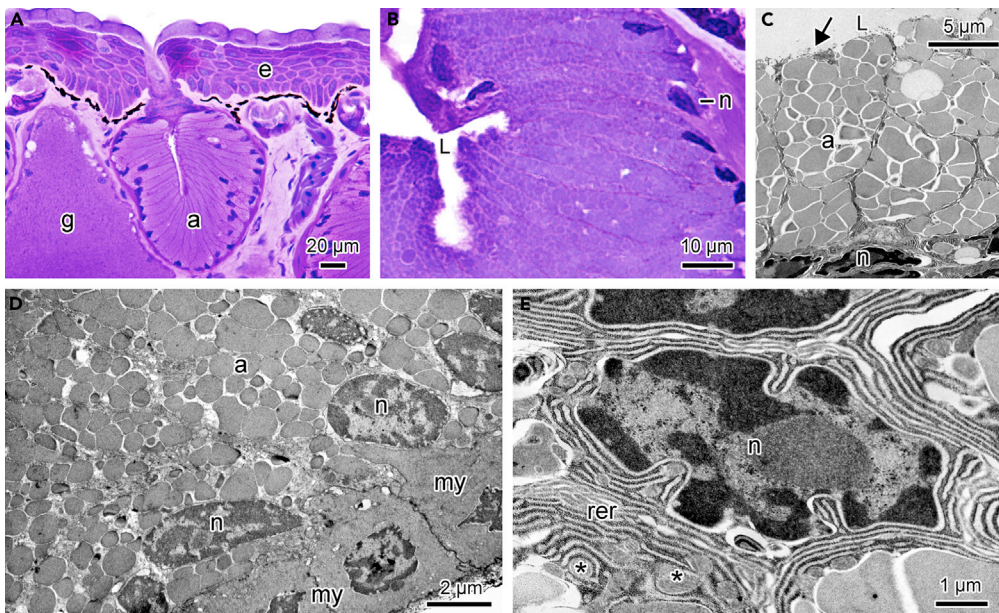
The round-shaped body and the short forelimbs of *D. muelleri*, together with the large size compared to most microhylids (which gave rise to its popular Brazilian name “ball frog”), are possibly related to fossoriality (Ceï, 1980), a lifestyle that, in general, tends to reduce the surface area in relation to the body



**Figure 7. Distribution of the adhesive glands in *Dermatonotus muelleri* male skin**

From the extensive sampling conducted in the skin of males and females, four significant regions were chosen to represent the area (in green) where adhesive glands (a) are present, contrasting with the females that do not show this type of gland. (1) ventral forearm, (2) pectoral region, (3) anterior ventral region, (4) dorsum of the forepaw. Note that the adhesive glands stain intensely with the hematoxylin. g, poison (or granular) glands; m, mucous glands.

volume. This species spends the entire dry season buried, emerging only in the short rainy season, when it explosively reproduces, in episodes concentrated in a few days, with the aggregation of a high number of individuals in the same water body (Ceï, 1980; Lavilla et al., 1995; Vizotto, 1967; Nomura et al., 2009). To compensate for the short limbs and the highly secretory and slippery skin, which renders amplexus difficult, *D. muelleri*, similarly to other microhylids, uses an adhesive secretion that holds the couple together during the entire amplexus (Halliday, 1983; Duellman and Trueb, 1986; Nomura, 2003). Our results about the location and distribution of the adhesive glands in the anterior ventral portion of the male's body match with the occurrence of the amplexus through an axillary embrace. The male tightly clasps the female with the dorsum of his hands, which is the only dorsal portion of the body where we observed adhesive glands in the skin. While the adherent skin appears to overcome problems related to amplexus, the globular body and short limbs of *D. muelleri* still cause difficulties for pairing the two cloacal openings (Duellman and Trueb, 1986) necessary to fertilization success (Halliday, 1983). However, the behavioural adaptations for aquatic oviposition here described seem to compensate for the mechanical difficulties. Thus, the behaviour of males stretching their ventral skin to near the cloacal opening to facilitate sperm transport and the use of the legs to generate a proper area to receive the female eggs may compensate for the unfavourable adaptations of the body for fossorial habits. In this context, freedom of the male legs and posterior ventral skin, not sticking to the female's body, seems essential. Once more, the negative result for adhesive gland prospection in the male posterior skin area matches our morphological and behavioural findings.



**Figure 8. Skin adhesive gland in males of *Dermatonotus muelleri***

(A) General view of the adhesive gland (a). Note the elongated and homogeneous secretory epithelium around a narrow lumen.

(B) Higher magnification of the cells composing the secretory epithelium (a), full of small granules and with basal nuclei (n).

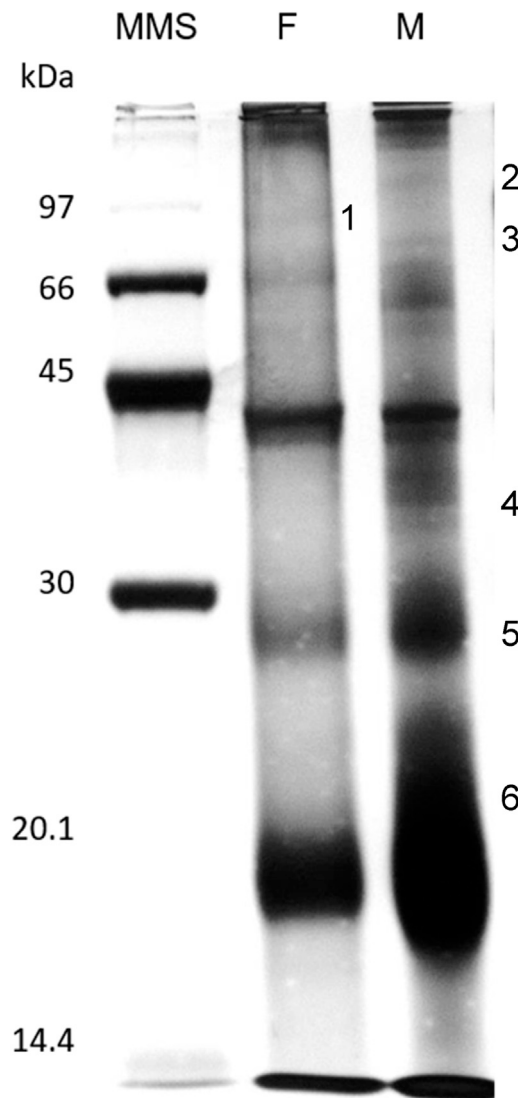
(C) TEM of the apical secretory granules with homogeneous medium electron density. They are quite compact, occupying most of the cytoplasm. The arrow points to the microvilli facing the lumen (L).

(D) TEM of the basal region of the secretory cells (a). Note the nuclei (n) and the myoepithelium (my) enveloping the gland.

(E) TEM of a transversal section at the basal region of the glandular cells, evidencing the exuberant rough endoplasmic reticulum (rer) and mitochondria (\*) circundating the nuclei. g, poison gland; L, lumen; n, nucleus.

Studies carried out by other authors, mainly in *Gastrophryne carolinensis* (Conaway and Metter, 1967; Metter and Conaway, 1969; Holloway and Dapson, 1971; Siegel et al., 2008), showed the presence of differentiated glands in specific areas of the male's ventral skin, which they called breeding glands. In this species, an experiment was carried out involving castration and injection of hormones in males, demonstrating the influence of sex hormones in the development of the adhesive glands (Metter and Conaway, 1969). In *Dermatonotus muelleri* and the other species we have observed, the areas with such glands correspond mainly to the anterior ventral face, especially the pectoral region and the arms, which corresponds with that observed by for *G. carolinensis* (Siegel et al., 2008). Among the microhylids, all the studies carried out until now point to the male's exclusive participation in the couple's adhesion process due to the ventral adhesive glands, indicating that this is most likely a common phenomenon in the whole family. However, different processes of adherence can occur, as observed in members of family Brevicipitidae, such as in *Breviceps adspersus* (Poynton, 1964) and *B. gibbosus* (Visser et al., 1982), where the adhesive secretions from the males seem to mix with skin secretions produced on the female's back. That probably occurs by forming a sticky mixture that fixes the male on her top since the male's size is about three times smaller, making amplexus inviable (Visser et al., 1982).

Some other anurans also have adhesive compounds in their skin related to reproduction. In the African genus *Hemiscus*, for example, the hands and forearms of males seem to secrete a sticky secretion (Rödel, 2000) that may facilitate the amplexus. Other cases such as the North and Central American species *Rhinophryne dorsalis* (Hughes and Wylie, 2021) and the Australian *Myobatrachus gouldii* (Roberts, 1981; Vertucci et al., 2017) should be investigated for the possible presence of glands in their skin with adhesive function. It calls attention that all anurans mentioned above, including *Dermatonotus muelleri*, are fossorial (or semi-fossorial) and live in dry environments. Such a lifestyle is probably related to their globose body, usually with short legs. This body shape may difficult the amplexus, justifying the need for adhesion of the amplexant couple in some cases. However, despite sharing the globose body shape, other dry-dwelling fossorial anurans such as the Brazilian semi-arid *Proceratophrys cristiceps* and *Pleurodema diplolister* (Jared et al., 2019) do not show adhesive glands (personal



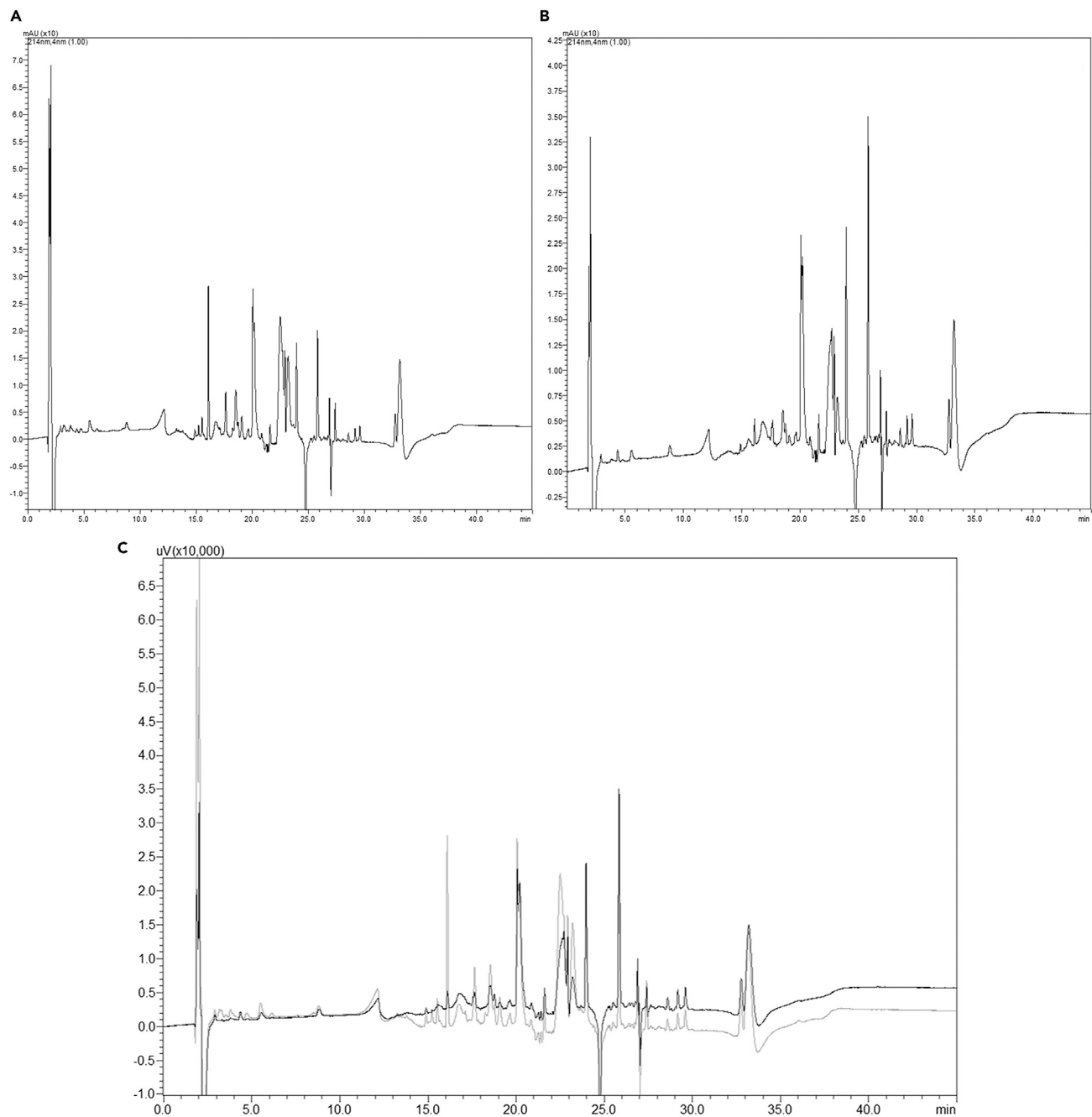
**Figure 9. SDS-PAGE 12% under reducing conditions of *Dermatonotus muelleri* secretion**

MMS = molecular mass standard; M = male; F = female. The numbers correspond to the bands that were analyzed *de novo*.

observations). On the other hand, the amphibian phylogeny points to a close relationship among the groups with adhesive glands used in reproduction. In the past, the African genus *Breviceps* was even considered part of the family Microhylidae and presently is part of the family Brevicipitidae, a sister group of Hemisotidae to which the genus *Hemisus* belongs (Hime et al., 2021).

Adhesive secretions, primarily directed to defence, are spread over all amphibian orders. Among anurans, the better known cases are the Australian burrowing genus *Notaden* (Williams et al., 2000; Tyler, 2010), *Corythomantis greeningi* (Jared et al., 1999, 2015), *Trachycephalus venulosus* (Toledo et al., 2011), and *Eupsophus vertebralis* (Suárez-Villota et al., 2021). In salamanders, they are present in the genus *Plethodon*, which adhesive secretions associated with the use of the tail for defence, and in the caecilian *Dermophis mexicanus* (Evans and Brodie, 1994; von Byern et al., 2017).

The adhesive glands used in defence usually produce copious milky secretion in extensive areas of the skin and, in general, large amounts of sample can be extracted, serving for studies on their chemical,



**Figure 10. HPLC profile of *Dermatonotus muelleri* secretion, in a C18 column**

(A) Female; (B) Male; (C) Comparison between female (grey) and male (black).

biochemical, and physical properties (Tyler, 2010; von Byern et al., 2017; Suárez-Villota et al., 2021). The same does not occur with the secretions from the adhesive glands related to reproduction, at least in *Dermatonotus muelleri* and the other microhylids we have examined. These glands are microscopic and located in very limited areas of the skin and secrete tiny volumes, and they may undergo differentiation (or activation) only during the reproductive period. Furthermore, the adhesion mechanism may occur more subtly, probably by intimate contact between the male and female epidermis. In this paper, we focused on the adhesion phenomenon through the topography of the glands in the skin and their matching with the areas of contact between males and females. As a biochemical complement, we were able to

**Table 2. Protein matching of the *de novo* sequenced tryptic peptides derived from the differentially secreted proteins in males and females of *Dermatonotus muelleri***

Gender	Band	<i>de novo</i> sequencing (blast score) <sup>a</sup>	Protein/function (annotated and/or predicted) <sup>b</sup>
♀	1	<u>APLLSDSSHMR</u> <sup>c</sup> (22.7)	glutamate receptor
♀	1	<u>RLCSDGNRALGPGPRER</u> (26.5)	multiple epidermal growth factor-like domains protein 8 isoform X1
♀	1	<u>NSVHTFVGG</u> <sup>d</sup> (24.8)	calcium-independent phospholipase A2
♂	2	<u>RTSGPSGC</u> <sup>d</sup> (20.6)	mucin-5B-like (gel-forming mucin that contribute to the lubricating and viscoelastic properties of secretion) <sup>e</sup>
♂	2	<u>QERSVTARAGLQSNR</u> (21.8)	uncharacterized protein LOC100492277 isoform
♂	2	<u>HLVAEAEASSVASGSWK</u> (24.0)	SET and MYND domain-containing protein 4 (DNA and/or protein binding protein)
♂	3	<u>APLLSDSSQRR</u> (23.5)	phosphatase and actin regulator 4-A (down regulates cell migration)
♂	3	<u>RLDAMGGSVTR</u> (22.3)	prostaglandin F2 receptor negative regulator-like
♂	3	<u>VLPERTAPAAAGVFKFK</u> (25.2)	cadherin-6 isoform X1 (calcium-dependent cell adhesion proteins)
♂	3	<u>MSDYKFQAK</u> (21.0)	endothelin-converting enzyme 2 isoform X1
♂	3	<u>WRVSPLLLNRGR</u> (22.7)	salivary glue protein Sgs-3-like
♂	4	<u>FGSVGAHK</u> (21.0)	corticosteroid 11-beta-dehydrogenase isozyme 2 (enzyme expressed in epithelial tissues such, but also in the placenta and testis)
♂	4	<u>WEAWPAPPR</u> (21.4)	NXPE family member 3-like isoform X2 (forms very tight complex with alpha neuexins, involved in adhesion between dendrites and axons)
♂	4	<u>TLLDLTEKK</u> (22.7)	desmocollin 3 precursor (involved in cell-cell adhesion by differential adhesiveness between cells)
♀ ♂	5	<u>LLTEDYRFKI</u> <sup>d</sup> (33.3)	mitofusin 2 (involved in mitochondrial fusion)
♀ ♂	5	<u>EAHVPSYN</u> <sup>d</sup> (21.8)	carbohydrate (chondroitin 4) sulfotransferase 13 precursor (chondroitin 4 is predominant in cartilage, and cell surfaces and extracellular matrices)
♀ ♂	6	<u>LLAENVQFLEK</u> (23.5)	uncharacterized protein LOC100135405

<sup>a</sup>Score calculated according to [Altschul et al. \(1997\)](#).

<sup>b</sup>All belonging to *Silurana tropicalis*, except for phosphatase and actin regulator 4-A which are derived from *Xenopus leavis*.

<sup>c</sup>The underlined part of the sequence represents the region of the peptide that was select by the BLAST algorithm for the alignment, yielding the score presented below the *de novo* sequenced peptide.

<sup>d</sup>Non-Arg or Lys C-terminal peptides could be derived from non-specific trypsin cleavage, C-terminal protein/protein-fragment, or misinterpretation of the automated *de novo* sequencing (Peaks) or ion search (Mascot).

<sup>e</sup>This brief function description is derived from the corresponding UniProt entry for the matched protein.

approach the protein content of the adhesive secretion using an indirect via, verifying by exclusion which compounds were exclusively present in males.

Regarding the morphology of the adhesive glands in *Dermatonotus muelleri*, it is similar to that described in *Gastrophryne carolinensis* ([Holloway and Dapson, 1971](#); [Siegel et al., 2008](#)), in leptodactylids ([Brizzi et al., 2002](#)) and also to the SDSGs present in numerous anuran families ([Thomas et al., 1993](#); [Brizzi et al., 2003](#); [Brunetti et al., 2012, 2015](#); [Luna et al., 2018](#)). All these glands have a typical acinar arrangement in which

a columnar epithelium releases spherical granules of homogeneous size in a narrow central lumen. At the ultrastructural level, the columnar cells have a similar morphology to that of *G. carolinensis*, including the apocrine type of secretion (Siegel et al., 2008).

*Dermatonotus muelleri* adhesive glands were highly positive for PAS and bromophenol blue, indicating that there is an expressive presence of proteins in the secretion in addition to polysaccharides. These results differ from those found in *Gastrophryne carolinensis* (Siegel et al., 2008; Holloway and Dapson, 1971), where the secretion was positive only for PAS and was interpreted as being exclusively mucous. Such differences in results may be related to the season when the individuals were collected since the composition of the glandular secretions may vary according to the reproductive cycle.

In addition to the poison glands, histological, histochemical, and ultrastructural examination of the mucous and mixed glands of *Dermatonotus muelleri* showed similarities to glands present in the skin of other anuran species (Brizzi et al., 2002; Duellman and Trueb, 1986; Stebbins and Cohen, 1995; Almeida et al., 2007).

In most amphibian species, the poison glands are related to the production of toxins for defence and are usually rich in protein content (Toledo and Jared, 1995). In this work, the presence of protein content in *Dermatonotus muelleri* secretion of the poison gland type 1 was accessed by histochemistry. Using a proteomic approach, Cavalcante et al. (2017) identified peptidases among the proteins, such as metalloproteinases and serinoproteinases, that may have a relevant role in secretion toxicity. It is worth emphasizing the sparse presence of poison gland type 2 in the *D. muelleri* body, which is poor in protein content and has an unknown function, possibly representing a different developmental stage of the abundant poison gland type 1.

It is also worth mentioning that we were not able to identify any peptide in the venom study of *Dermatonotus muelleri* secretion (Cavalcante et al., 2017). This molecule class is quite common among low molecular weight compounds in many anuran amphibians (Toledo and Jared, 1995). On the other hand, we found many sugar molecules, probably related to the production of mucous and mixed glands (Cavalcante et al., 2017).

Cavalcante et al. (2017) carried out the proteomics in the cutaneous secretion of males and females of *D. muelleri* identified 22% of binding proteins. Conversely, for the biochemical analyses presented here that focused mainly on the study of adhesive secretions, we used skin secretion extracted separately from males and females collected in the field during reproduction, thus characterizing specimens physiologically prepared for mating. The secretion was extracted as soon as the animals were brought to the laboratory. This proceeding ensured a reliable comparative analysis between males and females, detecting the exclusive or shared compounds in each sex. Except for the presence of adhesion glands exclusive to males, we did not observe significant variations in the morphological pattern of the skin between the sexes. Then, we inferred that at least most compounds detected only in males must have come from the adhesion glands.

The general profile obtained by HPLC indicated low molecular mass compounds. Still, it did not show significant qualitative differences between male and female secretion, which seemed to be related only to peak intensity. In contrast, differences in protein content between males and females were observed by comparing SDS-PAGE profiles and subsequent protein identification in both sexes. Males showed exclusive band 4 (a protein linked to sex-specific tissues) and bands 5 and 6 (proteins that might be related to energy production and matrix organization), but also some more subtle differences in bands 1 (females), 2, and 3 (males). Such bands contained most of the binding-related proteins, including the salivary glue protein from which we obtained sufficient similarity for protein identification.

The salivary gland secretion (Sgs) genes encode proteins that make up a glue produced by *Drosophila* larvae used to attach them to surfaces when they undergo metamorphosis (Da Lage et al., 2019). Moreover, a salivary glue protein is possibly present in *Ophiophagus hannah* (King cobra), as derived from the automated annotation of whole-genome shotgun for this snake (Vonk et al., 2013). Therefore, the presence of this glue protein in *Dermatonotus muelleri* is a herpetological relevant finding, bearing a significant correlation with the observed biological phenomenon.

In addition to the identification of an adhesive (glue) protein, we also showed the presence of other proteins in the skin secretion of *Dermatonotus muelleri* whose functions are directly linked to binding processes (cadherin, NXPE, and desmocollin), lubrication (mucin), and proteolysis (the membrane-anchored metallopeptidase endothelin-converting enzyme 2). It is possible to speculate that while the adhesive proteins may play a relevant role at the beginning of mating, ensuring success in egg fertilization, the peptidase may be necessary at the end of this event, allowing male and female separation through proteolytic cleavage of the adhesive proteins. Nevertheless, this hypothesis needs further studies to be confirmed. Finally, more profound biochemical studies about this theme may reveal novel compounds with adhesive function with the potential to generate biological adhesives for medical or industrial purposes (Tyler, 2010).

Our data show that the adhesive glands are exclusively present in the ventral skin of male *Dermatonotus muelleri* and secrete binding proteins that are probably responsible for the adhesive action during amplexus, guaranteeing reproductive success. We intend to enlarge the data obtained by studying other microhylids (such as *Stereocyclops incrassatus*, *Elachistocleis bicolor*, and *Chiasmocleis leucosticta*) to expand our knowledge about Microhylidae skin, its toxicity, and its relationship with reproduction. In addition, through an integrative approach, this study defines parameters that may be used as characters for future phylogenetic applications.

### Limitations of the study

This work describes the reproductive behaviour of adhesion of the male *Dermatonotus muelleri* (Muller's termite frog) to the female's back. This species of anuran amphibian of the Microhylidae family is widely distributed in South America, though generally challenging to access in the field due to its burrowing habits. This limitation restricts the scope of studying the biology of this species in its environment, which is only possible in the few days of explosive reproduction. With strategically organized fieldwork, we observed the reproductive behaviour and collected enough specimens to describe the cutaneous morphology and demonstrate the presence of adhesive glands in regions of the male's ventral skin, compatible with the nuptial embrace. It was also possible to show that compounds from the cutaneous secretion, exclusive to males, are related to adhesion. Although there is a strong indication that these compounds come solely from adhesive glands, it would be necessary to evidence their presence composing the secretion of these glands. Also, concerning these compounds, this work was restricted to proteins only. Its continuation remains open, exploring other classes of low molecular weight compounds. Moreover, it would be necessary to expand this study in all microhylids, confirming the possibility of adhesive glands being a synapomorphy of the family.

### STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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## AUTHOR CONTRIBUTIONS

C.J. and M.M.A. conceived the research. All authors collected the data and contributed with ideas. C.J. and M.M.A. conducted the analysis and writing with input from all authors.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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## STAR★METHODS

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Historesin Embedding kit	LEICA Microsystems	Cat # 702218500
Experimental models: Organisms/strains		
<i>Dermatonotus muelleri</i>	Collection Laboratory of Instituto Butantan, São Paulo, Brazil	IBSPCR-3828
<i>Dermatonotus muelleri</i>	Collection Laboratory of Instituto Butantan, São Paulo, Brazil	IBSPCR-3829
<i>Dermatonotus muelleri</i>	Collection Laboratory of Instituto Butantan, São Paulo, Brazil	IBSPCR-3830
<i>Dermatonotus muelleri</i>	Collection Laboratory of Instituto Butantan, São Paulo, Brazil	IBSPCR-3831
<i>Dermatonotus muelleri</i>	Collection Laboratory of Instituto Butantan, São Paulo, Brazil	IBSPCR-3832
<i>Dermatonotus muelleri</i>	Collection Laboratory of Instituto Butantan, São Paulo, Brazil	IBSPCR-3833
<i>Dermatonotus muelleri</i>	Collection Laboratory of Instituto Butantan, São Paulo, Brazil	IBSPCR-3834
Software and algorithms		
Standart Protein BLAST	U.S. National Library of Medicine, National Center for Biotechnology Information	<a href="https://blast.ncbi.nlm.nih.gov/Blast.cgi">https://blast.ncbi.nlm.nih.gov/Blast.cgi</a>
Amphibian database	UniProt	<a href="https://www.uniprot.org/">https://www.uniprot.org/</a>
Peaks Studio 7	Bioinformatics Solutions Inc., Canada	<a href="https://www.bioinfor.com/">https://www.bioinfor.com/</a>

### RESOURCE AVAILABILITY

#### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Carlos Jared ([carlos.jared@butantan.gov.br](mailto:carlos.jared@butantan.gov.br)).

#### Materials availability

This study did not generate new unique reagents.

#### Data and code availability

All data reported in this paper will be shared by the lead contact upon request.

This paper does not report original code.

Any additional information required to reanalyse the data reported in this paper is available from the lead contact upon request.

### EXPERIMENTAL MODEL AND SUBJECT DETAILS

*Dermatonotus muelleri* (Boettger, 1885), (Figure 1) is an endemic species in the Cerrado-Caatinga-Chaco domain in Brazil, Bolivia, Paraguay, and Argentina. The distribution range in Brazil covers the states of the Midwest and Northeast, and Minas Gerais, São Paulo, and Espírito Santo (Ceil, 1980; Lavilla et al., 1995; Vizotto, 1967; Nomura et al., 2009; Almeida et al., 2011). This domain is characterized by a prolonged dry season and savanna vegetation (Duellman, 1999). For this work, adult males and females of *Dermatonotus muelleri* from São José do Rio Preto, SP, Brazil, were studied in December, period when a few annual explosive reproduction events occur. They were first observed in the field for the description of the reproductive behaviour. Following, nine males and five females were collected from the observed population and brought to Instituto Butantan, SP. Gender of the individuals, was confirmed by the dark colour of the mentonian region of males and the flagrant larger size of the females. The animals were maintained in plastic boxes containing humid soil as substrate for the animals to dig and find shelter similarly to their behaviour in nature. They were fed weekly with cockroaches, young crickets and tenebraria.

Animals were obtained under collecting permit provided by Instituto Chico Mendes de Conservação da Biodiversidade (Sistema de Autorização e Informação em Biodiversidade #18163-3). All aspects of the study were carried out under protocols approved by the Ethics Committee on Animal Use of Instituto Butantan (process # 1273240920). Voucher specimens are deposited in the Collection Laboratory of Instituto Butantan (IBSPCR-3828, IBSPCR-3829, IBSPCR-3830, IBSPCR-3831, IBSPCR-3832, IBSPCR-3833, IBSPCR-3834).

The all-occurrence method, in which all instances of a given behaviour performed during a given time period are recorded, was applied to observe the oviposition behaviour and to construct an ethogram (Lehner, 1996). Egg-laying behaviour was observed between April/2001 and June/2003, only during the day ( $n = 26$  pairs), from 8:00 h to 14:00 h, in an artificial temporary pond (60 m length x 31 m width x 2 m deep) located in a matrix of pasture and corn crops in the municipality of Vitória Brasil, São Paulo State, Brazil (20°12'S, 50°29'W). The pond margins were covered by grass and sparse bush, except for one side deprived of vegetation measuring about 8 m in length. Around two-thirds of the margin edges had a slope of less than 12° inside the water, forming extensive shallow water areas. The maximum depth of the pond varied during the rainy season, from a few centimetres (e.g., 5 cm) in the first rains, generally in October, to almost 2 m (in the central area of the pond) in May, at the end of the rainy season. The pond bottom was mainly composed of clay, which gave the water a red coloration.

## METHOD DETAILS

### Skin sample collection and tissue fixation

The animals were euthanized with a lethal intraperitoneal dose (50 mg/kg) of Thiopental. Thirty-eight skin fragments of approximately 1 cm<sup>2</sup> were removed from each animal, representative of the whole skin covering all parts of the body. For histological preparation, the samples were fixed for 24 h in 4% paraformaldehyde in 0.1 M PBS buffer, pH 7.2, and embedded respectively in glycol methacrylate or paraffin. For electron microscopy study, samples were also fixed in Karnovsky solution (5% glutaraldehyde and 4% paraformaldehyde, in 0.1 M cacodylate buffer, pH 7.2).

Following sample collection, the animals were fixed in formalin and preserved in 70% ethanol and sent to the Butantan Institute herpetological collection.

### Histology and histochemistry

After fixation, the samples were dehydrated in crescent ethanol series (70%–100%), embedded in paraffin (for cross-section and longitudinal sectioning) and sectioned 5µm-thick using a microtome Microm HM 340E, with disposable steel blades. The sections were then stained with haematoxylin-eosin and used for morphological and morphometrical analysis. Some of the samples were also embedded in historesin (Leica) and sectioned 2µm-thick using the same microtome with the aid of glass knives. The sections were then stained with toluidine blue-fuchsin for general histological observation.

For histochemistry, the sections were submitted to bromophenol-blue, periodic acid-Schiff (PAS) for basic chemical characterization of the glandular content (for proteins, neutral polysaccharides, respectively).

### Photomicrographs

Photomicrographs were obtained using an Olympus BX51 microscope and an Olympus SZ stereomicroscope, equipped with an Olympus CCD camera (Q Color 5) assembled to the software Image-Pro Express for image capture and morphometry.

### Transmission electron microscope (TEM)

For transmission electron microscopy (TEM), fragments of the skin of two males were extracted from the areas where the adhesive glands were and fixed according to Karnovsky (5% glutaraldehyde and 4% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.2), post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer and contrasted in 0.5% aqueous uranyl with 13.3% sucrose. After dehydration, the samples were embedded in epoxy resin (Polybed, Electron Microscopy Science). Ultrathin sections were contrasted with 2% uranyl and lead citrate and examined under the LEO 906E transmission electron microscope, operating at 80 kV.

### Cutaneous secretion extraction

Just after the microhylids arrived in the vivarium, the skin secretion was extracted separately from males and females by submerging each animal in a beaker containing distillate water. A second extraction was performed after 15 days from the animals arriving for comparison with the fresh, just arrived, secretion. The obtained solutions were lyophilized, kept at  $-20^{\circ}\text{C}$ , and resuspended in appropriate buffers, according to the biochemical essays.

### QUANTIFICATION AND STATISTICAL ANALYSES

Samples were submitted to one dimensional polyacrylamide electrophoresis gel (SDS-PAGE) under reducing conditions (Laemmli, 1970). The gel was stained with silver, and the bands considered to be different between male and female were excised and digested for protein identification (Shevchenko et al., 1996).

The resulting tryptic peptides were separated by RP-HPLC (20A Proeminence, Shimadzu Co., Japan) and analysed by mass spectrometry (ESI-IT-ToF, Shimadzu Co., Japan). The chromatographic separation was performed using a C18 column (Supelco, 100 Å, 5  $\mu\text{m}$ , 50  $\times$  2.1 mm) in a linear gradient of B over A in 20 min (solvents A:  $\text{H}_2\text{O}$  containing 0.1% formic acid and B: 90% acetonitrile in  $\text{H}_2\text{O}$ , containing 0.1% formic acid), under constant flow of 0.2  $\text{mL}\cdot\text{min}^{-1}$ .

Mass spectrometric analyses were performed in positive ionization (ESI+), at  $200^{\circ}\text{C}$  interface temperature, 4.5 kV interface voltage, 1.7 kV detector voltage and in a 50 to 2000  $m/z$  range. For MS/MS spectra, ions were automatically selected and collided with Argon (50% energy), in a 100–1,500  $m/z$  range.

For protein identification, MS/MS experimental data were compared to an Amphibian database (downloaded from the UniProt) using in house Mascot Server. Alternatively, these spectra were also *de novo* sequenced by Peaks Studio 7 (Bioinformatics Solutions Inc., Canada). The obtained sequences were manually checked and submitted to Standard Protein BLAST (Basis Local Alignment Search Tool), in non-redundant mode, using Amphibia Taxonomy filter (Altschul et al., 1997).