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Supplemental Information

Morphological Evidence for an Oral Venom System in Caecilian Amphibians

Pedro Luiz Mailho-Fontana, Marta Maria Antoniazzi, Cesar Alexandre, Daniel Carvalho Pimenta, Juliana Mozer Sciani, Edmund D. Brodie Jr., and Carlos Jared

TRANSPARENT METHODS

Animals

Sixteen specimens of *S. annulatus* including males (n=7) (mean snout-vent length [SVL] 37.3 ± 3.1 cm), females (n= 7) (SVL 38.6 ± 4.1 cm) and newly hatched (n= 2) (SVL 3.1± 0.3 cm) were collected in Ilhéus, Bahia state, Brazil. The animals were euthanized with a lethal intraperitoneal injection of Thiopental associated with lidocaine and preserved in Bouin fixative or 4% formaldehyde in PBS pH 7.2 for evaluation of the dental glands. All experimental procedures conform to the relevant regulatory standards and were approved by the Ethics Committee on Animal Use of Instituto Butantan (protocols #3053090916 and #9749270718). The presence of dental glands was also evaluated in *Rhinatrema* sp. and *Typhlonectes* compressicauda, both provided by the Special Laboratory of Zoological Collections of Butantan Institute and *Schistometopum thomense* provided by E.D.B.Jr (N=2/species).

Morphological analysis

The skin of the head of an adult of each sex was partially macerated in sodium hypochlorite and photographed using a stereomicroscope Leica M205A equipped with DMC2900 camera coupled with the software LAS V4.6. Two additional heads of adults of each sex were decalcified in 4% EDTA and embedded in paraffin, sectioned, and stained with hematoxylin-eosin. Tridimensional reconstructions of dental glands were based on serial sections using the software Reconstruct (Fiala, 2005). Fragments of rostral skin and the heads of newborn were embedded in historesin (Leica), sectioned and stained with toluidine blue-fuchsine or treated with bromophenol blue for identification of proteins, periodic acid-Schiff (PAS) for identification of neutral carbohydrates, alcian blue pH 2.5 for identification of acidic carbohydrates, and Sudan black B for identification of lipids (Bancroft and Stevens, 1995). The sections were photographed using an Olympus BX51 microscope equipped with a CCD camera Q Color 5 (Olympus Corporation of the Americas) coupled with the software CellSens. For scanning electron microscopy, one specimen of each sex was dehydrated in a critical point dryer, sputter coated with gold and examined in FEI Quanta 250 microscope.

Oral secretion collection

Twenty minutes prior euthanasia two adult specimens of each sex were injected intraperitoneally with Pilocarpine 10 mg/kg to stimulate the dental glands to secrete. After the euthanasia the upper jaw and lower jaw was gently compressed and the secretion present in the base of the teeth was collected using micropipettes and stored at -20°C.

Biochemical analysis

Micropipette tips were submerged in 25 mL 25 mM Na_2HPO_4, pH 6. Subsequently, the tube was vortexed and centrifuged (1000 x g, 5 min, 4 °C). The tips were then removed from the buffered oral secretion supernatant (termed B) that was submitted to an Ion Exchange solid phase extraction procedure employing Spe-ed COOH cartridges (Applied Separation). After loading the sample, the resin was washed (5 volumes) with 25 mM Na_2HPO_4, pH 6, to displace the unbound proteins (termed UP). Then the resin was washed (5 volumes) with the same buffer containing 1 M NaCl to remove low molecular mass components and remaining bound proteins (termed BP). Protein concentration was estimated by UV absorbance at 280 nm (NanoDrop). According to the UniProt, the majority of the characterize proteins present a pI value around pH 6-7. So, rationale of the performed strong cation exchange was not to bind most of the proteins whereas alkaloids, the major complicators for amphibian protein analyses would bound to the resin. For further details, please see work of Mariano et al. (Mariano et al., 2018).

The buffered secretion and samples resultant from the ion exchange were loaded onto a 12% polyacrylamide gel (PAGE) (20 µg/well) containing sodium dodecyl sulphate (SDS) (Laemmli, 1970), under reducing and non-reducing conditions. The gels were stained with silver. To evaluate zymography activities the dental secretion preparations (40 µg/well) were fractionated by SDS-PAGE 12% in the presence of gelatin (2 mg/mL), casein (2 mg/mL), fibrinogen (0.5 mg/mL) and hyaluronic acid (170 µg/mL) as substrates (Jared et al., 2015). The PLA2 activity of the secretions was determined using the synthetic substrate 4-nitro-3- [octanoyloxy] benzoicacid (Enzo® Life Sciences) in a final concentration of 320 µM, (Moretto Del-Rei et al., 2019 with some modifications). Hydrolysis was determined according to OD at 425 nm in a spectrophotometer SpectraMax® M2 (Molecular Devices) and activity was expressed by increase in absorbance at 425 nm caused by the cleavage of the substrate (OD Abs/min/µg of venom). This assay was performed in triplicates two independent experiments. Results were expressed as mean ± se.

SUPPLEMENTAL REFERENCES

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Supplemental figures and tables

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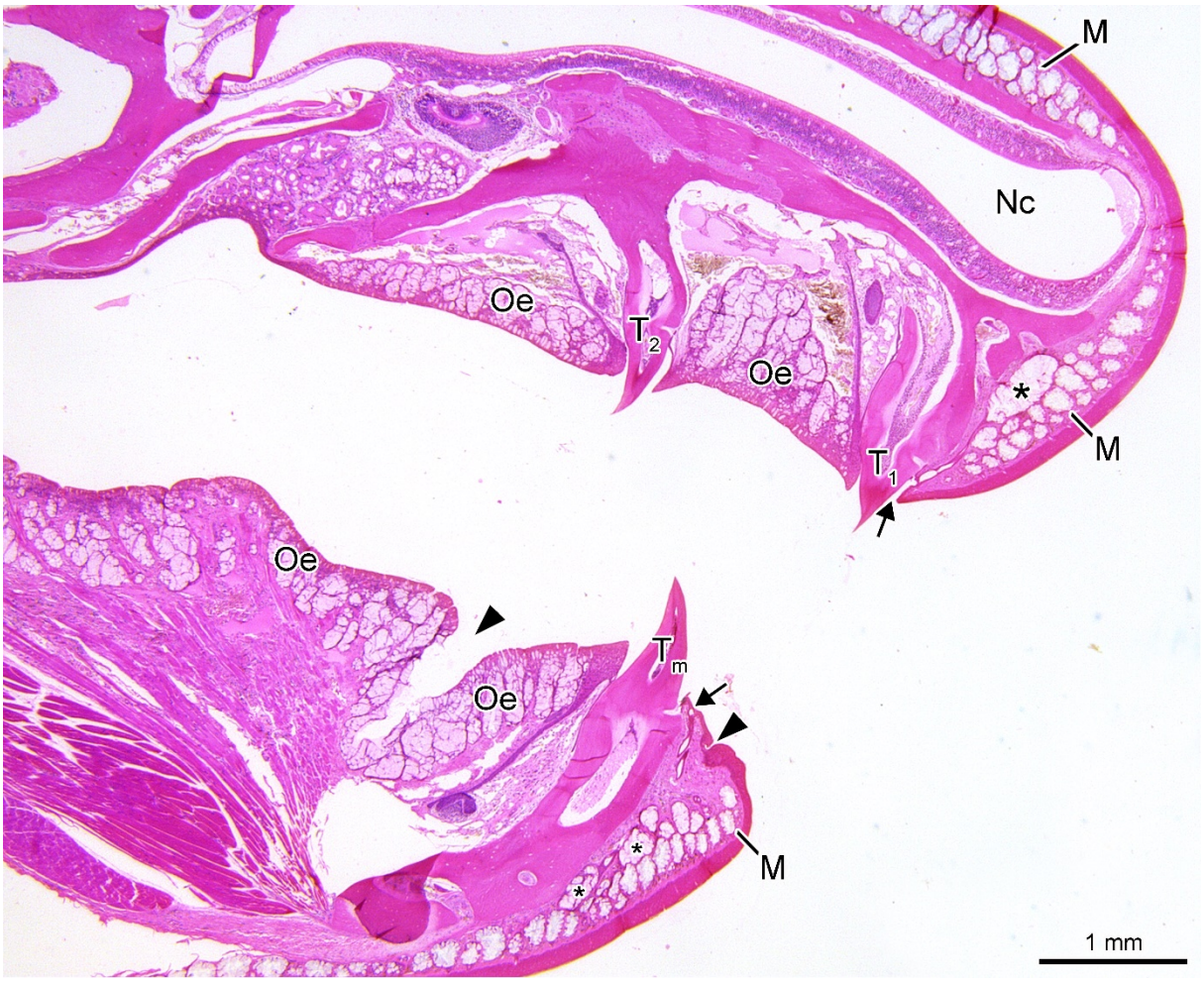

Fig. S1. Distribution of tooth-related glands in *Siphonops annulatus*. Related to Figure 2. The sagittal section of the head clearly shows that in the upper jaw only the outer tooth row (T_1) is associated with the glands (*); there are not dental glands associated with the inner upper tooth row (T_2). Note in the lower jaw the glandular ducts (arrow) leading to the base of the tooth (Tm). Observe the mucous tissue of the oral epithelium (Oe) and the large number of mucous glands (M) located more superficially in the skin. Nc, nasal cavity; Arrowhead, cavity where the tooth is accommodated when the mouth is closed. Histological method: Hematoxylin-eosin, paraffin section.

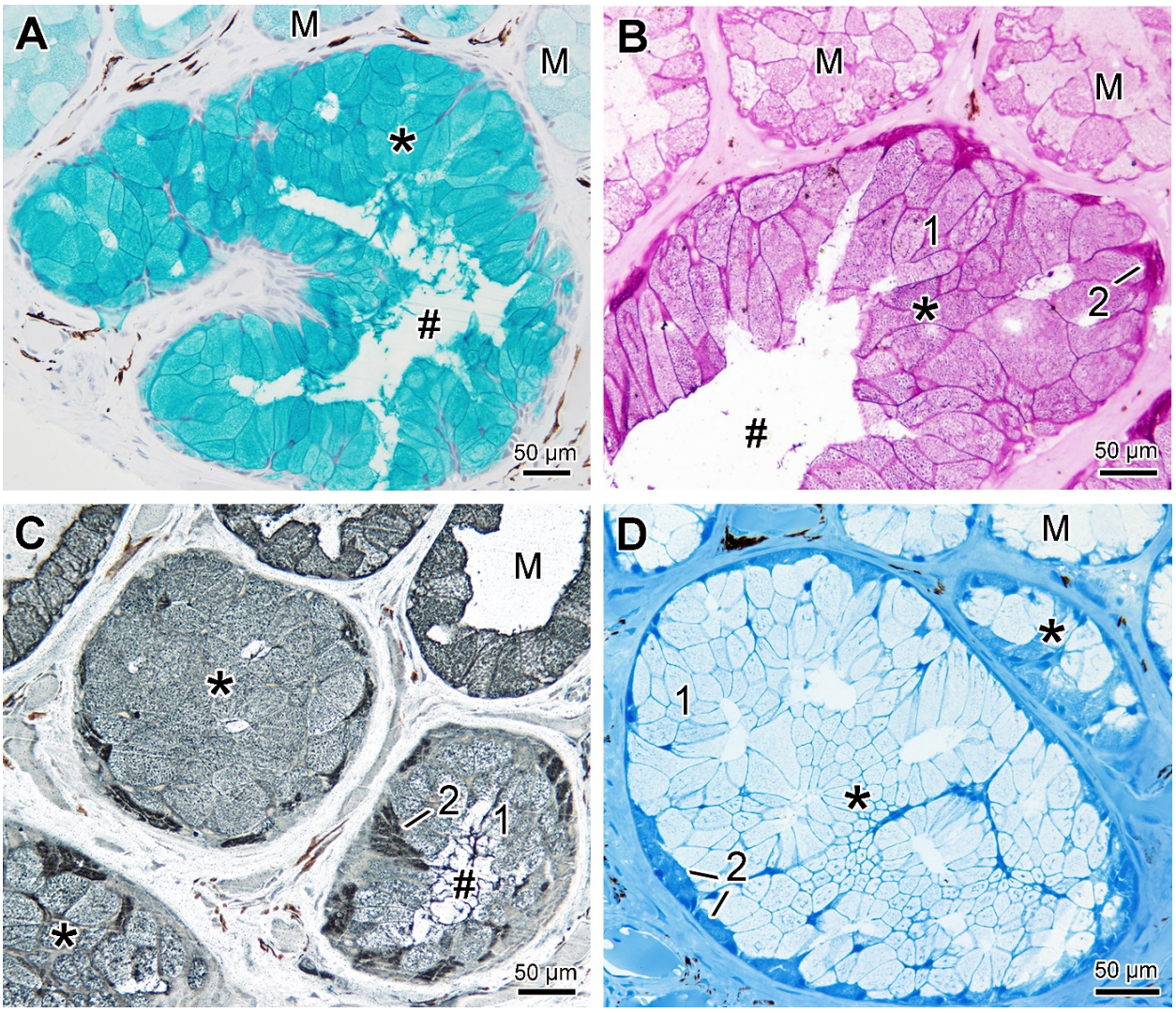

Fig. S2. Histochemical characterization of tooth-related glands in *Siphonops annulatus*. Related to Figure 2. (A) The glands (*) contain a higher amount of acid mucopolysaccharides compared to the skin mucous glands (M). Note the glandular lumen (#). (B) Type 1 (1) cells are richer in neutral mucopolysaccharides than Type 2 cells (2). (C) Both cell types produce lipids, although Type 2 cells are richer in this class of molecules. (D) Type 2 cells have higher protein content, but both cell types have higher protein abundance compared to the skin mucous glands. Histological methods: (A) alcian blue, pH 2.5; (B) periodic acid-Schiff; (C) Sudan black; (D) bromophenol blue, historesin sections.

Fig. S3. *Siphonops annulatus* feeding on a newborn mouse. Related to Figure 3. Note the large amount of secretion (arrow) that accumulates around the caecilian's mouth and on the mouse's skin at the time of the bite.

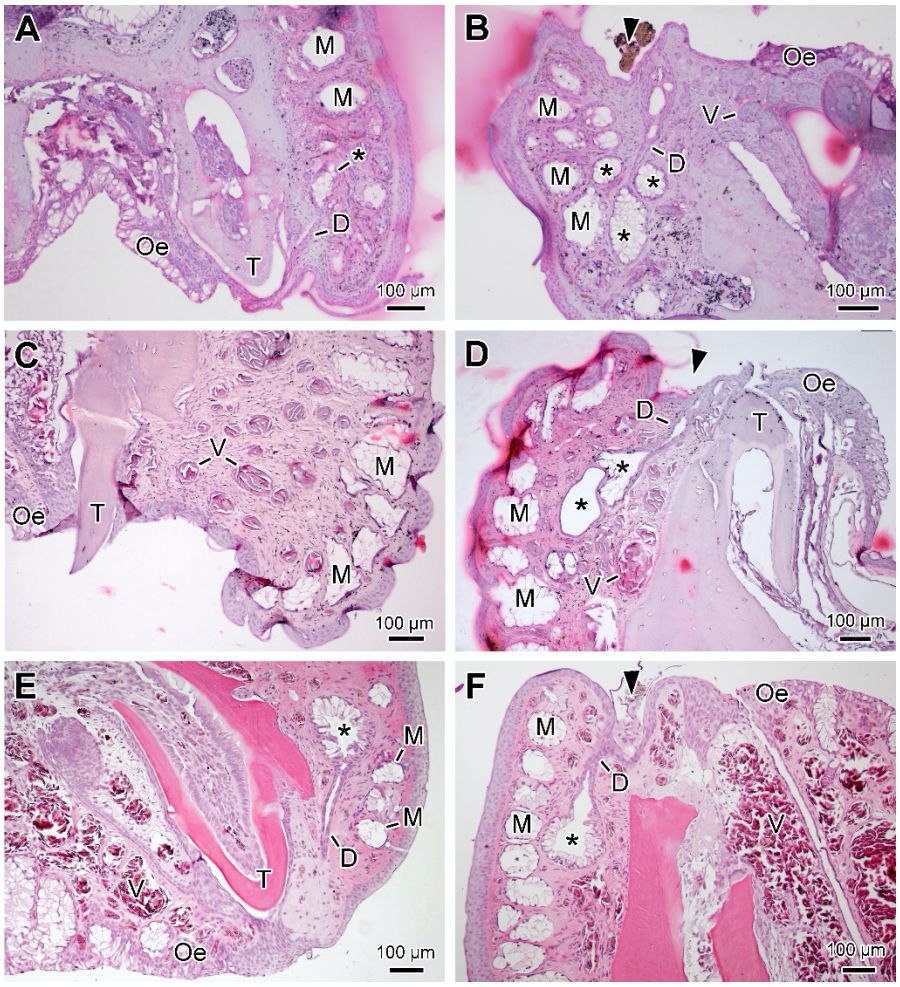

Fig. S4. Dental glands in caecilians of other families. Related to Figure 2. (A) Sagittal section of *Rhinatrema* sp upper jaw showing the dental glands (*) in a family considered as more basal among caecilians. (B) Sagittal section of *Rhinatrema* sp. showing the dental glands associated with the cavities (arrowhead) that accommodate the upper jaw teeth when the mouth is closed. (C) Sagittal section of the upper jaw of the aquatic caecilian *Typhlonectes* compressicauda. Note the absence of the dental glands. (D) Sagittal section of *T. compressicauda*. lower jaw, showing the dental glands. (E) Sagittal section of the lower jaw of *Schistometopum thomense*, showing a dental gland with its duct (D). (F) Sagittal section of a mandibular cavity in *Schistometopum thomense*. M, mucous gland; T, tooth; Oe, oral epithelium; V, blood vessel.

Table S1. Number of dental glands in the different species studied. Related to Figure 2.

Species	Number of glands per maxillary tooth	Number of glands per mandibular cavity
Siphonops annulatus	4 to 7	5 to 9
Rhinatrema sp.	2 to 4	4 to 8
Typhlonectes compressicauda	–	2 to 3
Schistometopum thomense	2 to 4	2 to 4