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Bioactive Profiling of Peptide-rich Secretions from male and female *Odontophrynus asper* (Anura: Odontophrynidae)

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Abstract

Amphibian skin secretions represent a chemically diverse arsenal of bioactive peptides that contributes to defense against predators, pathogens, and oxidative stress. This study provides a preliminary characterization of the biochemical composition and in vitro bioactivities of the peptide-rich skin secretions from the common lesser escuerzo (*Odontophrynus asper*), while examining sex-related differences between pooled secretions from male (Oa-M) and female (Oa-F) specimens. Analytical characterization by TLC, HPLC and MALDI-TOF MS confirmed the predominance of peptide components in both secretions, with sex-associated mass profiles: male secretions were enriched in low-mass components (1000–1165 m/z), whereas female secretions featured a greater abundance of medium-to-large peptides (1435–3757 m/z). In vitro assays revealed dual inhibition of acetylcholinesterase and butyrylcholinesterase (up to approximately 40–44%), potent radical scavenging activity (EC_{50}: 48–49 μg/mL) and substantial inhibition of linoleic acid peroxidation (approximately 90–100% after five days). Both secretions also showed strong Fe^{2+} chelating activity (EC_{50}: 107–156 μg/mL). Regarding toxicity assays, female secretions were substantially more toxic against human erythrocytes and *Artemia salina* nauplii than males. Interestingly, bioactive concentrations were much lower than cytotoxic thresholds, suggesting the need for further investigation of the selectivity of individual compounds. These findings altogether

highlight the multifunctional chemical potential of *O. asper*, establishing its secretions as a valuable source of peptides for future chemical and pharmacological research.

Keywords: chemical ecology; amphibian skin secretions; bioactive peptides; cholinesterase inhibition; antioxidant activity; sex related variation.

INTRODUCTION

The highly permeable, naked skin of anurans serves critical physiological purposes such as respiration, osmoregulation, and thermoregulation (Konig, Bininda-Emonds, & Shaw, 2015). However, the lack of physical barriers such as scales, feathers or hair, makes this organ highly vulnerable to an array of environmental stressors and biological threats (Varga, Bui-Marinos, & Katzenback, 2018). To cope with these challenges, frogs and toads possess specialized granular glands that release chemically diverse secretions containing proteins, peptides, alkaloids, amines and steroids. These secretions contribute to protection against predators, microorganisms, and environmental stressors, and their composition is shaped by phylogenetic history, ecological conditions, and species-specific adaptations (Daly et al., 2004; Xu & Lai, 2015). Beyond their reported antimicrobial and antioxidant activities, amphibian-derived molecules have also been associated with antiviral, anti-inflammatory, antitumoral, insulinotropic and enzyme inhibition properties, as well as the modulation of ion channels and receptors (Mo et al., 2014; Patocka, Nepovimova, Klimova, Wu, & Kuca, 2019; Xu & Lai, 2015). This broad range of biological activities has sparked interest in amphibian skin secretions as sources of bioactive compounds, although their ecological functions and biological relevance require species-specific investigation.

The diverse functional profile reported for amphibian skin secretions encompasses several molecular targets and may be relevant to different aspects of amphibian biology. Cholinesterase (ChE)-inhibiting compounds, for example, have been investigated in the context of chemical interactions with predators (e.g. birds, small mammals, insects) and their neurotoxic effects. Inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) can result in the accumulation of acetylcholine and alter cholinergic neurotransmission, potentially leading to muscle spasms and physiological distress (Pope & Brimijoin, 2018; Rodriguez-Ithurralde,

Silveira, Barbeito, & Dajas, 1983). However, the ecological significance of cholinesterase inhibition depends on the identity, concentration, bioavailability, and mode of exposure of the compounds, as well as on the susceptibility of the interacting organism.

Against pathogenic threats, antimicrobial peptides (AMPs) constitute the primary chemical barrier (Rollins-Smith, 2023), by inducing pore formation, membrane destabilization, and disruption of microbial cell envelopes that leads to preventing bacterial, fungal and viral colonization (Siano, Humpola, et al., 2014). Amphibian skin secretions also contain compounds with radical-scavenging and lipid-peroxidation-inhibitory activities, which may be relevant to the maintenance of cutaneous integrity under oxidative conditions (Demori et al., 2019; H. Yang et al., 2009). Skin metal chelating compounds serve as a protective physiological mechanism to prevent metal dyshomeostasis and mitigate oxidative damage (da Rocha Braga, de Menezes Gondim, Pereira, Batista, & Matushima, 2022; Silva Ortiz et al., 2025; Vasconcelos-Teixeira et al., 2022). Together, all these bioactivities constitute a robust chemical strategy worth characterizing, both for its ecological significance and for its bioprospecting potential.

Furthermore, the composition and activity of amphibian skin secretions may vary among species, populations, life stages, ecological contexts, and sexes (de Souza et al., 2020; Ong, Ismail, & Shahrudin, 2022; Sherman et al., 2009). Sex-related variation may result from differences in reproductive investment, habitat use, behavior, predator exposure, or other selective pressures (Antoniazzi et al., 2022; Oliveira et al., 2023; Petroselli, Raices, Jungblut, Pozzi, & Erra-Balsells, 2018). In some amphibians, sex-specific skin peptides have been associated with reproductive communication or interactions among conspecifics, suggesting that the chemical composition of secretions may also be influenced by reproductive biology (King, Rollins-Smith, Nielsen, John, & Conlon, 2005; Wabnitz, Bowie, Tyler, Wallace, & Smith, 2000). Nevertheless, the extent to which sex-related differences affect the overall chemical profile and bioactivity of skin secretions remains insufficiently documented. Comparisons between male and female secretions can therefore contribute to a more comprehensive assessment of intraspecific chemical diversity and help identify compounds or activities that may be differentially represented. Such comparisons are especially relevant when evaluating complex mixtures, in which differences in the relative abundance of individual components may influence the resulting biological activity.

Despite the remarkable chemical diversity of anurans, systematic bioactive characterization has reached only a small fraction of the Neotropical species, with South American frogs particularly underrepresented (Carrillo, Boaretto, Santana, & Silva, 2024; Konig et al., 2015). In Argentina, bioprospecting studies have documented cholinesterase-inhibitory, antioxidant, antimicrobial, metal chelating and cytotoxic activities of several genera, including *Leptodactylus*, *Boana*, and *Rhinella* (Siano, Gatti, et al., 2014; Siano et al., 2018; R. Spinelli, Aimaretti, Lopez, & Siano, 2021; R. Spinelli, Rietmann, Sanchis, & Siano, 2022; R. Spinelli et al., 2019). These studies indicate that Argentine amphibians represent a relevant but still incompletely explored source of chemically diverse secretions. Within this context, information on the chemical composition and biological activities of secretions from the genus *Odontophrynus* remains limited. There is a recent report of a neutral, amphipathic peptide isolated from the skin secretions of *Odontophrynus americanus*, americanin-1 (sequence: NVIHELGNTVDNALRLLTR), exhibiting moderate antimicrobial action against *E. coli* (Cancelarich et al., 2024). Mailho-Fontana et al. also studied the secretions from three types of glands present in the congeneric *Odontophrynus cultripes*, identifying several low-molecular-mass steroids and alkaloids, and eleven new peptides, in what represents the only other report for this genus (Mailho-Fontana et al., 2017).

The common lesser escuerzo, *Odontophrynus asper* (Philippi, 1902), is a native anuran species widely distributed in central and northern Argentina which may also represent an interesting source of peptide-rich secretions (Rosset et al., 2022). Like other members of the genus, *O. asper* is a nocturnal, fossorial frog that spends long periods buried and aggregates at temporary ponds to breed, mainly after heavy rains (Blotto, Pereyra, Faivovich, Dias, & Grant, 2017; Brum, Loebens, Prado, & Cechin, 2020; Miolo, Brum, de Avila, Tozetti, & Cechin, 2025). During these events, calling males and gravid females are likely exposed to conspecifics, predators and microbe-rich aquatic environments. Whether *O. asper* possesses specialized cutaneous macroglands comparable to those described in *O. cultripes* and *O. americanus* has not been documented (Mailho-Fontana et al., 2017).

In the present study, we characterized the biochemical composition, peptide profiles and selected in vitro bioactivities of peptide-rich skin secretions from male and female *O. asper*. Protein quantification, thin-layer chromatography (TLC), reversed-phase HPLC, and MALDI-TOF mass spectrometry were used to obtain a preliminary characterization of the chemical composition and

molecular-mass distribution of the secretions. We further evaluated their cholinesterase-inhibitory activity, DPPH radical-scavenging capacity, inhibition of lipid peroxidation, Fe^{2+}-chelating activity, and toxicity towards *Artemia salina* nauplii and human erythrocytes. Finally, we compared male and female secretions to test three predictions. First, if secretion composition reflects sex-specific reproductive roles, the peptide profiles of males and females should differ, with male secretions potentially enriched in components comparable to the male-associated signaling peptides described in other anurans (King et al., 2005; Wabnitz et al., 2000). Second, because both sexes share the same burrowing lifestyle and face similar oxidative and metal-related challenges to skin integrity, activities associated with basal skin maintenance (radical scavenging, inhibition of lipid peroxidation and Fe^{2+} chelation) should be similar between sexes (Demori et al., 2019). Third, activities more directly related to defense (cholinesterase inhibition and membrane-disrupting toxicity) may differ between sexes if males and females differ in predator exposure or reproductive investment during the breeding period. By combining chemical profiling with multiple in vitro assays, this study provides an initial assessment of the bioactive properties of *O. asper* skin secretions and establishes a basis for future investigations involving the isolation, structural characterization, and functional evaluation of individual peptides.

METHODS AND MATERIALS

Amphibian Collection and Skin Secretion Sampling

Adult specimens of *O. asper* were collected in San José del Rincón, Argentina (31°36'12" S 60°36'42" W), identified and sexed. All the frogs used in this work were manipulated following the recommendations of the American Society of Ichthyologists & Herpetologists (ASIH) (Herpetologists, 2004) and with the supervision and authorization of the bioethical committee of the Facultad de Bioquímica y Ciencias Biológicas – Universidad Nacional del Litoral (Argentina).

To obtain the skin secretions, a non-lethal methodology was employed (Tyler, 1992). Previously moistened with Milli-Q water, specimens were gently stimulated laterally and dorsally from neck to thigh with a 21G platinum bipolar electrode (12 V, 7 Hz), therefore inducing the contraction of the muscles surrounding the granular glands and the release of the secretions (Siano, Gatti, et al., 2014). Each animal was then rinsed with Milli-Q water, the aqueous solutions were collected and

142 the specimens were released. Samples were pooled according to previous sexing, frozen and
143 lyophilized.

144 ***Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC)***

145 Samples were reconstituted in 0.1% (v/v) TFA, centrifuged, and injected for RP-HPLC analysis
146 on a Waters equipment (USA) using an Atlantis (Waters) C_{18} analytical column (5 µm, 4.6 mm x
147 150 mm). A linear gradient of 5-80% ACN: H_2O with 0.1% of TFA for 33 min at a flow rate of
148 0.8 ml min^{-1} was used. The eluates were monitored by their absorbance at 220 and 280 nm; 20 µl
149 of a 5 mg ml^{-1} solution of each sample was injected.

150 ***Thin Layer Chromatography (TLC) and Protein Quantification***

151 The samples were developed by TLC silica gel 60 F254 with two solvent mixtures: butanol : acetic
152 acid : water (55.6 : 22.2 : 22.2) and chloroform : methanol (95 : 5). Iodine (for organic compounds),
153 ninhydrin (for peptides) and Dragendorff reagent (for alkaloids) were used as developing agents.
154 For steroid determination, detection was performed using the Liebermann–Burchard reagent
155 (Burke, Diamondstone, Velapoldi, & Menis, 1974).

156 The bicinchoninic acid assay (BCA) was used for protein quantification. Fifty µl of each sample
157 were incubated with 1 ml of the BCA reagent at 60º C for 30 min. After cooling, absorbance was
158 measured at 562 nm in a Metrolab 1700 UV-Vis spectrophotometer. A calibration curve with
159 Bovine Serum Albumin (BSA) in the concentration range from 0.5 to 5 mg ml^{-1} was performed.

160 ***Mass Spectrometry Analysis***

161 Mass spectrometry analyses of both crude skin secretions were performed at Laboratorio de
162 Espectrometría de Masas (LEM), FBCB-UNL. The measurements were carried out using a
163 Shimadzu MALDI-TOF-MS instrument (Axima Performance ID Plus model) operated in linear
164 positive ion mode.

165 Prior to analysis, each extract was desalted and pre-concentrated using reverse-phase C_{18} pipette
166 tips (ZipTip®, Millipore). One microliter of the eluate was mixed 1:1 (v/v) with a saturated
167 solution of α-cyano-4-hydroxycinnamic acid (CHCA; 10 mg ml^{-1} in 50% acetonitrile/0.1% TFA)
168 and spotted onto a stainless-steel target plate. Samples were air-dried at room temperature and
169 analyzed in the 500–4500 m/z range. Calibration was performed using a standard peptide

calibration mix (Shimadzu Biotech), and each spectrum was acquired by averaging at least 500 laser shots. The instrument was operated under optimized parameters for peptide detection, enabling soft ionization with minimal fragmentation. Data acquisition and processing were carried out using the Shimadzu Biotech Launchpad software.

Cholinesterase Inhibition Assay

The *in vitro* inhibitory activity of the skin secretions of *O. asper* against both cholinesterases was evaluated by following a previously reported method based on Ellman's colorimetric assay (Ellman, Courtney, Andres, & Featherstone, 1961). The reaction was performed in a final volume of 100 µL with Electrophorus electricus acetylcholinesterase (eeAChE, 0.25 U ml-1) or recombinant human butyrylcholinesterase (rhBChE, 2 nM), respectively; 0.4 mM acetylthiocholine iodide (ATCI, Sigma-Aldrich) or S-butyrylthiocholine iodide (BTCI, Sigma-Aldrich); 0.33 mM 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB, Sigma-Aldrich) and the sample at 1250–156 µg ml^{-1}, in 0.1 M phosphate buffer (pH=8.0). The enzyme and sample solutions were mixed and incubated for 15 min (room temperature) and the reaction was started by the addition of the substrate and DTNB solutions. Absorbance at 405 nm was measured after 5 min in a 96-well microplate reader (Thermo Fisher FC Multiskan). For both enzymes, galantamine 200 µM was used as positive control.

DPPH Radical Scavenging Activity

Antioxidant activity was determined by the 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay according to the method of Memarpoor-Yazdi adjusted with a final volume of 250 µl (Memarpoor-Yazdi, Mahaki, & Zare-Zardini, 2013). Fifty µl of decreasing concentrations of the samples (1000–62.5 µg ml^{-1}) were mixed with 150 µl methanol and 50 µl DPPH (0.15 mM in methanol). The mixture was incubated for 30 min in the dark. Ascorbic acid 300 µM was used as positive control. The absorbance of the resulting solution was measured at 490 nm.

Inhibition of Lipid Peroxidation

The capacity of both samples to inhibit linoleic acid peroxidation was determined according to the method of Kim with modifications (J. S. Kim, 2018). A mixture solution containing 200 µL of the samples at a final concentration of 1 mg ml^{-1}, 200 µL of 2.5% (v/v) linoleic acid in anhydrous ethanol, 400 µL of 50 mM phosphate buffer (pH 7.0), and 200 µL Milli-Q H_2O was prepared in

Eppendorf tubes, sealed tightly with a silicon rubber cap and kept in darkness at 40 °C for 5 days. The lipid peroxidation inhibition was evaluated at 24 h intervals for 5 days, as follows: an aliquot of 20 µL of the mixture was mixed with a reaction solution containing 940 µL of 75% (v/v) ethanol, 20 µL of 30% (m/v) potassium thiocyanate and 20 µL of 20 mM ferrous sulfate in 3.5% (v/v) HCl. After 3 minutes of reaction, the absorbance was determined at 500 nm. Ascorbic acid 300 mM was used as positive control.

Fe^{2+} Chelating Activity

Fe^{2+} chelating activity was measured following a previously described method (He et al., 2012) with a final volume of 250 µl. Fifty µl of decreasing concentrations of the samples (1000–62.5 µg ml^{-1}) were premixed with 5 µl of ferrous sulfate solution (2 mM), and then 185 µl of distilled water was added and mixed vigorously. Then, 10 µl of ferrozine solution (5 mM) was added, mixed, and incubated at room temperature for 10 min. After incubation, the absorbance of the final solution was determined at 620 nm. EDTA 5 mM was used as positive control.

Brine shrimp (Artemia salina) Bioassay

Sea water was prepared following formulations and conditions from the maritime ordinance previously described (Argentina, 1998). One hundred mL of seawater previously oxygenated to achieve an oxygen saturation of 90% was placed in graduated cylinders with aeration. Then, 100 mg of *A. salina* cysts were added and incubated in the presence of visible light (750 lm), at 25 ± 1°C for 48 h. After that, aeration was stopped. The hatched brine shrimp nauplii, which remained in the middle section of the graduated cylinder, were collected. The toxic activity of both samples was evaluated according to a standardized method, optimized by the workgroup (R. Spinelli, Rietmann, Sanchis, Goicoechea, & Siano, 2024). Following the addition of 360 µL of decreasing concentrations of the samples (2500–312 µg ml^{-1}), 6 nauplii were placed in each well of the 96-well microtiter plates. The incubation was performed at 25°C in the absence of light for 24 h and then the dead nauplii were counted under a stereoscopic microscope. The median lethal concentration (LC50) was calculated using the R package BioRssay, based on Probit functions (Karunarathne, Pocquet, Labbe, & Milesi, 2022). Methanol and $K_2Cr_2O_7$ 408 µM were used as positive controls for 100% mortality.

Hemolytic Activity

The hemolytic activity was determined by a previously reported methodology (Siano, Humpola, Rey, Simonetta, & Tonarelli, 2011). Briefly, human erythrocytes were isolated from heparinized blood by centrifugation (1000 g for 10 min) after washing three times with saline solution. Erythrocyte solutions were prepared to a concentration of 0.4% (v/v) in isotonic saline solution. Eppendorf tubes containing 200 µl of erythrocyte solution were incubated at 37 °C for 60 min with 200 µl of decreasing concentrations of the samples (2500–312 µg ml^{-1}). After centrifugation at 1000 g for 5 min, the supernatant absorbance was measured in microplates at 405 nm. Lysis induced by 1% Triton X-100 was taken as 100%.

Data Analysis

All experiments were performed in triplicate (technical replicates). In all cases, the percentage of activity (enzyme inhibition, radical scavenging, metal chelation or hemolysis) was calculated as:

$$\% = \left[1 - \left(\frac{A_s - A_b}{A_0 - A_b}\right)\right] * 100$$

Where A_s is the absorbance of the sample, A_b is the absorbance of the blank (negative control) and A_0 is the absorbance of the control (no sample).

The creation of dose-response models, along with the IC_{50}/EC_{50} calculations, were performed using IC50 Studio, an open-source R Shiny app recently developed by our group (Sanchis, 2026). The data were fitted to standard sigmoidal models, and the uncertainty of the calculated parameters was estimated using bootstrap methods (200 iterations) with a 95% confidence interval.

RESULTS

After mild electrical stimulation, abundant skin secretions were obtained and later classified according to sex. The final samples were named Oa-M for the male and Oa-F for the female. A preliminary chemical and bioactivity characterization of the natural skin secretions was then performed, exploring the differences between male and female.

Chemical Composition

Bicinchoninic acid assay clearly established the presence of peptides, with percentages of proteins reaching 100% for both male and female samples. This predominantly peptidic nature was also

supported by the ninhydrin reaction, which showed strong positivity **(Figure 1c)**, confirming the presence of free amino groups.

General organic compounds were detected with iodine **(Figure 1b)** and UV light at 254 nm **(Figure 1a)** using a polar elution system (butanol : acetic acid : water). When TLC was carried out with a non-polar solvent system (chloroform : methanol), no migration was observed under 365 nm **(Figure 1e)**. The Liebermann–Burchard reaction showed no significant staining of migrated bands (Figure 1f), suggesting the absence of detectable lipophilic steroids. Similarly, the absence of coloration with Dragendorff's reagent **(Figure 1d)** suggested no detectable alkaloid content, further reinforcing the highly peptidic nature of the secretions.

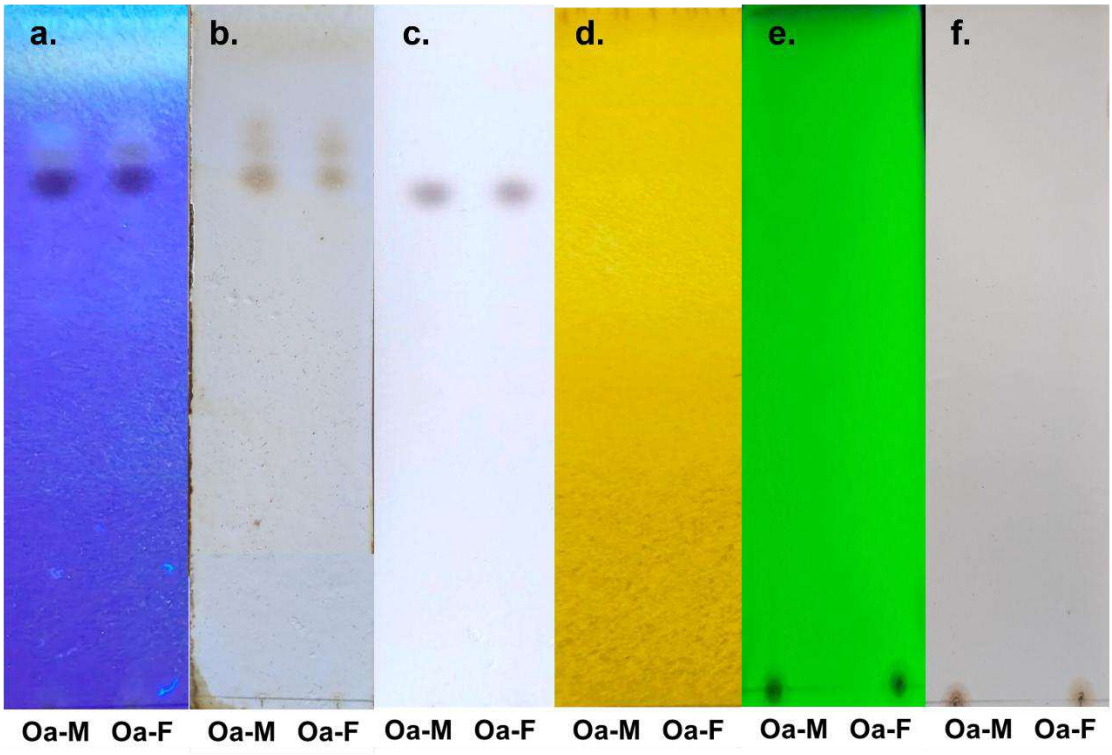


Figure 1. TLC analysis: (a) 254 nm; (b) iodine; (c) ninhydrin; (d) Dragendorff reagent; (e) 365 nm and (f) Liebermann–Burchard for male (Oa-M) and female (Oa-F) secretions of *O. asper*. Elution was carried out with butanol: acetic acid: water (55.6 : 22.2 : 22.2) for (a-d); and with chloroform: methanol (95 : 5) for (e-f).

RP-HPLC analysis provided further evidence of the peptide-rich composition of both male and female secretions of *O. asper* **(Figure 2)**. The chromatograms were quite similar for both sexes and demonstrated the presence of mostly hydrophilic compounds at 220 and 280 nm. Corresponding peaks showed quite similar retention times in both samples, but differences in their

intensities were observed, indicating variations in their relative analyte concentrations according to sex. Interestingly, the male specimens showed an additional peak at a retention time of 3.384 min both at 220 and 280 nm, absent in the female **(Figure 2A)**.

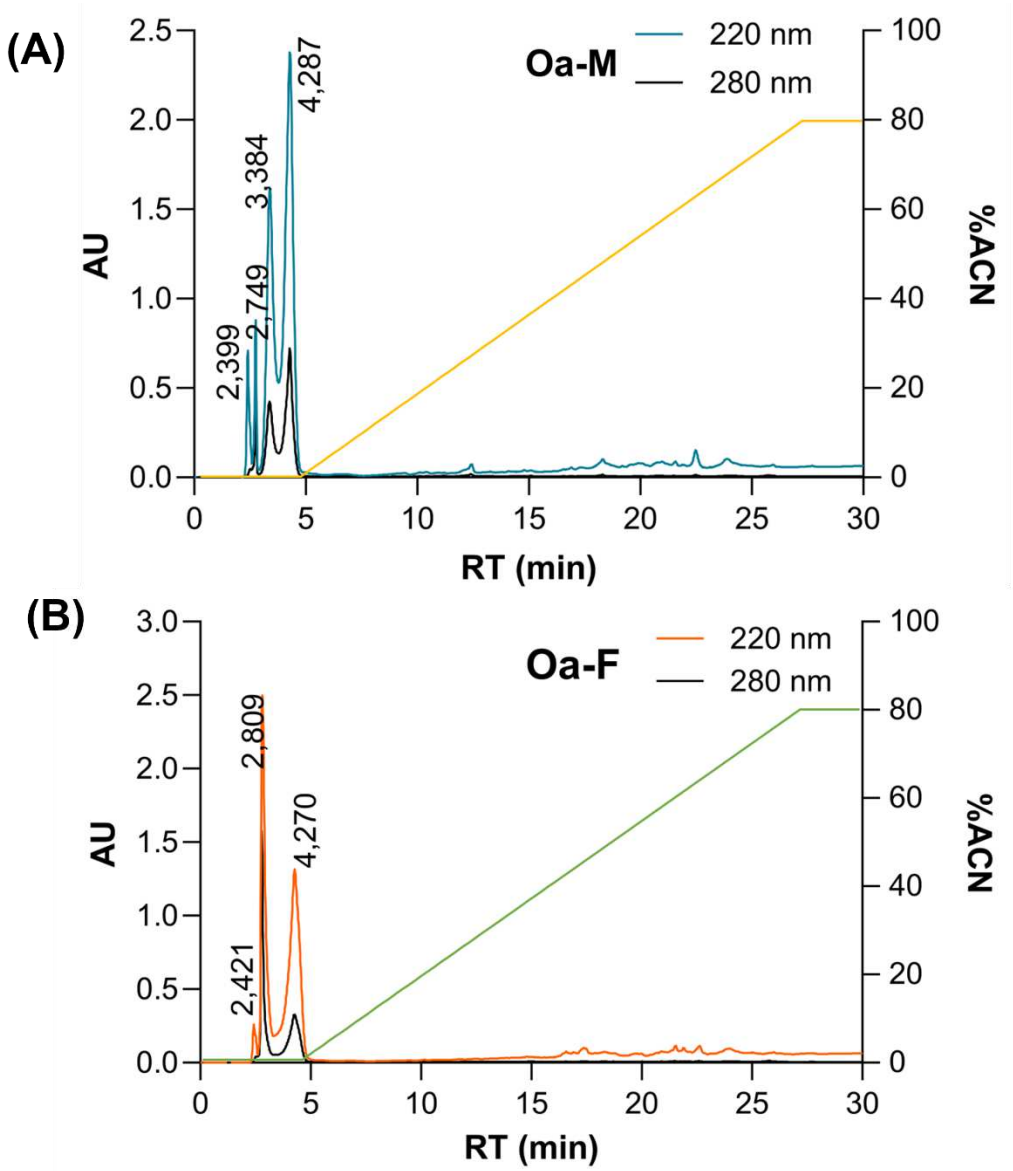


Figure 2. RP-HPLC analysis of the male (A) and female (B) samples of *O. asper*. C_{18} column, linear gradient (5 to 80 %) from solution A (0.1 % TFA in water) to solution B (0.1 % TFA in ACN) for 30 min. Detection: 220 and 280 nm.

Lastly, MALDI-TOF-MS analysis in positive linear mode revealed deeper sex-related differences in peptide profiles **(Figure 3).** The female secretion exhibited intense signals in the mid-to-high mass range (1435–3757 m/z), with prominent peaks at 1435.88, 1734.09, 1777.13, 1835.15, and 2075.03 m/z. In contrast, male secretions were enriched in low-mass components (1000–1165

m/z). Notably, some m/z values were shared between both sexes (e.g., 1436, 2076, and 3356 m/z), indicating the presence of conserved peptides. In addition, no major signals were detected at the lower end of the acquisition range (500–700 m/z).

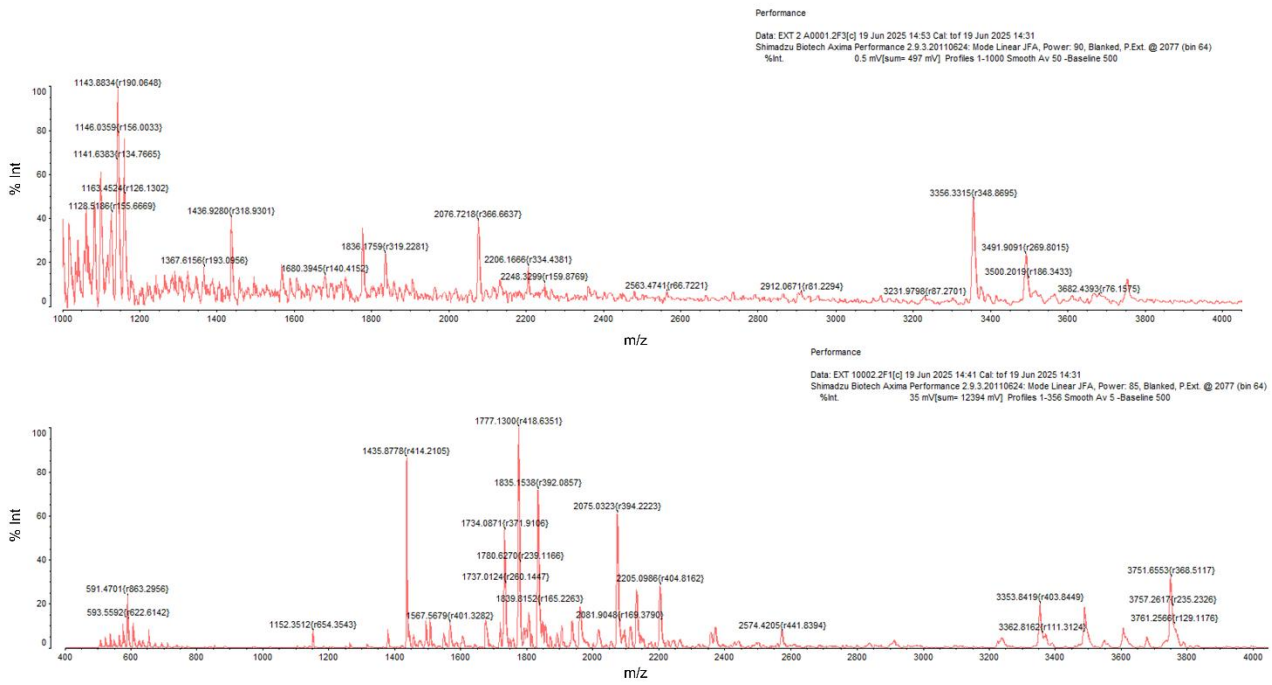


Figure 3. MALDI-TOF MS analysis of male (top) and female (bottom) secretions of *O. asper*.

Cholinesterase Inhibitory Activity

The inhibitory activity of *O. asper* secretions against AChE and BChE was evaluated following Ellman's methodology over the concentration range 156-1250 µg ml^{-1} (Ellman et al., 1961). **Figure 4** shows the enzyme inhibition variations with the concentration of the tested samples against both enzymes. In AChE (**Figure 4-A**), the highest percentage of inhibition of 40.2% was obtained at 1250 µg ml^{-1} for the male, while the female showed a 29.16% inhibition at the same concentration. The rest of the inhibition percentages showed a logical dose-response decrease for the lower concentrations, with the male remaining rather more active than the female.

For BChE (**Figure 4-B**), both samples demonstrated a slightly higher biological activity than with AChE. At the highest assayed concentration, the male sample of *O. asper* produced an inhibition of the BChE enzyme of 44%, somewhat higher than that produced by the female sample at the same concentration (35%). The inhibition percentages remained around 20% at the lowest concentration.

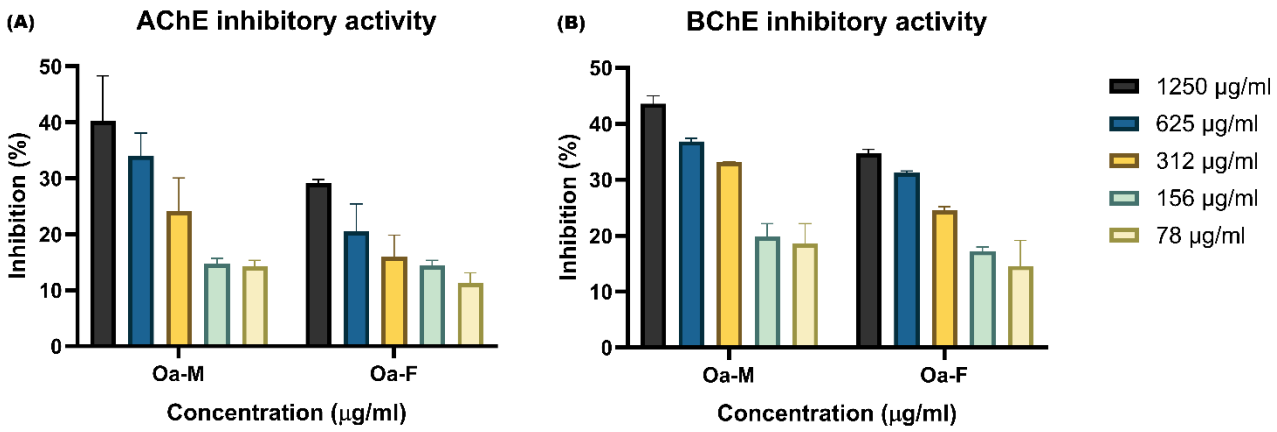


Figure 4. Inhibition of (A) AChE and (B) BChE by male (Oa-M) and female (Oa-F) secretions of *O. asper* vs. sample concentration. Bars express the mean ± SD (n=3).

Antioxidant and Metal Chelating Activities

The biological activity was expressed as EC_{50} values **(Table 1)**, defined as the concentration of sample that produces 50% of a certain activity, e.g. radical scavenging, metal chelation.

Table 1. Biological activities and toxicity (µg/ml) of male (Oa-M) and female (Oa-F) secretions of *O. asper*

Species	*Sample*	*DPPH*	*Fe^{2+} chelation*	*Toxicity*	
				LC50	*HC_{50}*
O. asper	**Oa-M**	48 ± 6	107 ± 3	1434 ± 133*	2340 ± 143*
	Oa-F	49 ± 7	156 ± 9	944 ± 79*	674 ± 1*

EC_{50}: effective concentration 50%; HC_{50}: hemolysis concentration 50%; LC50: lethal concentration 50%; *: significant difference between Oa-M and Oa-F.

DPPH Free Radical Scavenging Activity

The DPPH assay was used to evaluate the free radical scavenging capacity of *O. asper* skin secretions under the assay conditions. The antioxidant activity was expressed as EC_{50} values (**Table 1**). Both male and female secretions showed marked free radical scavenging ability even at low concentrations (<100 µg ml^{-1}, see **Figure S1**). In addition, we observed a quick color change when performing the DPPH bioassay due to a rapid capture of the free radical, indicating the presence of fast-acting antioxidant compounds. The EC50 values of the male and female secretions (48 and 49 µg ml-1, respectively) did not differ significantly (Table 1). The positive control, ascorbic acid, showed an EC50 of 7 µg ml-1.

316 *Inhibition of Lipid Peroxidation*

317 Both samples, Oa-M and Oa-F, effectively inhibited the lipid peroxidation reaction during 5 days
318 at 40 °C (see **Figure S2**), with an inhibition close to that of the positive control of ascorbic acid.
319 After only one day, both male and female secretions successfully prevented the reaction at a rate
320 of 67% and 65% respectively. After 2 days, lipid peroxidation was almost completely inhibited
321 (close to 100% inhibition), and this effect was maintained up to day 5.

322 *Fe^{2+} Metal Chelating Activity*

323 **Table 1** shows the results of the ferrozine-based colorimetric assay. Interestingly, both samples
324 demonstrated a strong ability to chelate Fe^{2+} ions, sequestering 100% of the ions at the highest
325 assayed concentration of 1000 μg ml^{-1}. This high activity was relatively maintained up to 250 μg
326 ml^{-1} **(Figure S3)**. The final EC_{50} values for Oa-M and Oa-F were, correspondingly, 107 and 156
327 μg ml^{-1}, the male being slightly more active.

328 ***Toxicity Assessment***

329 Toxicity assays were used to characterize the biological effects of the crude secretions of *O. asper*
330 in two experimental models, by in vivo and in vitro methodologies. Mortality against brine shrimp
331 (*Artemia salina*) and hemolytic activity against healthy human erythrocytes were assessed. LC50
332 and HC_{50} values, defined as the concentration required to achieve 50% of lethality or hemolysis,
333 respectively, were calculated **(see Table 1; Figures S4 and S5).**

334 Regarding the brine shrimp toxicity assay, the LC50 values for the male and female secretions
335 were 1434 and 944 μg ml^{-1}, respectively; the male being considerably less toxic (around 35%).
336 Both secretions showed slight to no toxic effect up to 625 μg ml^{-1} **(Figure S4)**. For the hemolytic
337 activity, the tendency was analogous: the studied secretion from the female turned out to be more
338 hemolytic than males (**Figure S5**). In this case, however, the difference in HC_{50} values was much
339 greater (around 70%): 2340 and 674 μg ml^{-1} for the males and females, respectively.

340 Notably, Oa-M and Oa-F showed toxicity in both assays at much higher concentrations than those
341 needed to achieve 50% antioxidant, chelating, and peroxidation inhibition activities.

342

DISCUSSION

Frog skin secretions are rich in peptides and proteins but can also contain alkaloids, steroids and amines (Daly et al., 2004). It is important to note that amphibian whole-skin extracts normally contain between 20 to 65% of total protein content, but this concentration can be higher for the skin secretions (Siano, Gatti, et al., 2014; R. Spinelli et al., 2021). *Rhinella arenarum* secretions contained 77 ± 1% of proteins, while *Hylarana erythraea* comprised around 50% of proteins, although peptides were the major constituent (Ong et al., 2022; R. Spinelli et al., 2022). In the present report, however, peptides and proteins appear to account for most of the compounds detected in the *O. asper* secretion, although other minor constituents (e.g., biogenic amines) cannot be excluded with the methods used. Closely-related *O. americanus* has been reported to contain several peptides with antimicrobial activity (Cancelarich et al., 2024); and the skin secretions from *O. cultripes* also yielded distinctive peptides, with common alkaloids/steroids as well. Alkaloid content in frog secretions is highly dependent on the feeding behavior, since most are sequestered from the diet, which is from small arthropods (Indriani, Karnjanapratum, Nirmal, & Nalinanon, 2023).

The predominance of polar compounds found by RP-HPLC at 220 and 280 nm is consistent with the presence of short or hydrophilic peptides with aromatic residues. This also correlates with the lack of migration observed in the chloroform : methanol (95:5) TLC system (**Figure 1e**), further reinforcing that the secretion components are predominantly polar and do not partition into non-polar solvents. Sex-related differences both in relative analyte concentrations (as inferred from the RP-HPLC intensities) and in specific peaks may reflect genuine compositional differences between males and females, although, since secretions were pooled by sex, the contribution of individual variation cannot be excluded. Although not much research has been conducted to elucidate sex-specific molecular differences among amphibians, there are a few reports about the presence of a single peptide in males which is absent in females, with pheromone-like activities. Splendipherin, isolated from the skin secretions of male *Rana splendida*, is a female-attracting aquatic sex pheromone; while LASP, found in *Leptodactylus fallax*, has a chemoattractive effect on other males, stimulating aggressive behaviors (King et al., 2005; Wabnitz et al., 2000).

The MALDI-TOF-MS mass profiles reinforce sex-associated variation. Male secretions, enriched in low-mass peptides, suggest a composition dominated by smaller peptides, which could include

signaling molecules such as the male-specific peptides with pheromone-like activities reported for other anurans, although this remains to be tested. On the other hand, the higher-mass peptides found in the female secretions are consistent with larger, potentially amphipathic peptides. Shared m/z values between both sexes (e.g., 1436, 2076, and 3356 m/z) may indicate the presence of conserved peptides with potential basal physiological or defensive roles. MALDI-TOF MS with a CHCA matrix is not suited to detecting low-molecular-mass compounds, since the acquisition range started at 500 m/z and matrix-derived signals dominate below approximately 700 m/z; nevertheless, the absence of major signals at the low end of the spectra is consistent with the TLC results. Some of the signals observed also appear to reflect peptide variants previously reported for *O. americanus* (Cancelarich et al., 2024); in particular, the peak at 1777.13 m/z in the female extract (**Figure 3**) is close to the expected mass of a truncated form of americanin-1 (NVXHEXGNTVDNAXRX) at 1778.6 m/z. However, small mass shifts and the detection of unique peaks not previously reported may also indicate variation; such differences may arise from environmental factors, local adaptation, or differential gene expression patterns associated with distinct habitats (Samgina et al., 2023). On the other hand, several signals in the 1400–1850 m/z region fall within the mass range of peptides previously identified in *O. cultripes*, such as HLPSLTPVVFHAR ([M+2H]2+ 737.45 m/z; expected [M+H]+ ≈ 1473.9) and HLPSLRQAKTREVHK ([M+3H]3+ 600.70 m/z; expected [M+H]+ ≈ 1800.0), although none of the observed peaks matches these values exactly; tandem MS sequencing will be required to determine whether homologous peptide sequences are present in both related species (Mailho-Fontana et al., 2017). These tentative matches are compatible with the hypothesis of shared or conserved peptide components within the genus *Odontophrynus* species, while also highlighting the importance of considering both inter and intra-species variability when characterizing natural peptide libraries.

The in vitro assays for both secretions of *O. asper* revealed significant bioactivities overall for both sexes in a broad range of evaluated targets, the male consistently showing equal or slightly superior activity. Compared to other amphibian secretions or extracts, the cholinesterase inhibition (~40%) of *O. asper* secretions is similar to that reported for *Rhinella arenarum* and higher than secretions from frogs belonging to Hylidae and Leptodactylidae families, such as *Boana pulchella* and *Leptodactylus luctator* (Siano, Gatti, et al., 2014; R. Spinelli et al., 2022). The antioxidant (EC_{50}: 48–49 μg mL^{-1}) and Fe^{2+} chelating activities (EC_{50}: 107–156 μg ml^{-1}) compare favorably

with those reported for other frog-derived skin secretions and fall within the range of isolated amphibian antioxidant peptides, such as antioxidins and andersonins (R. Spinelli et al., 2021; X. Yang, Wang, Zhang, Lee, & Zhang, 2016). Additionally, the lipid peroxidation inhibition (~90–100%) further supports the antioxidant activity observed in both secretions. Regarding toxicity, *O. asper* secretions displayed generally low toxicity against both human red blood cells and A. salina, with the male secretion being notably less toxic than the female one.

Evaluating cholinesterase inhibition is essential for understanding the biochemical and neurotoxic properties of amphibian skin secretions. Compounds that inhibit cholinesterases can induce a rapid accumulation of acetylcholine in a predator's synapses, causing paralysis, muscle spasms, and physiological distress (Pope & Brimijoin, 2018; Rodriguez-Ithurralde et al., 1983). Targeting both AChE and BChE provides a broader-spectrum neurotoxic defense, since different classes of predators have different AChE:BChE ratios in their nervous systems (Massoulie, Pezzementi, Bon, Krejci, & Vallette, 1993). Thus, a secretion capable of dual-cholinesterase inhibition could potentially affect a broader range of predators, although this remains to be tested using predator-derived enzymes and ecologically relevant doses. Nevertheless, there are scarce reports regarding the dual-cholinesterase inhibition of amphibian skin compounds. Spinelli et al. reported this activity for various amphibian extracts from four different families (Hylidae, Microhylidae, Leptodactylidae, and Bufonidae) (R. Spinelli et al., 2021; R. Spinelli et al., 2022). For AChE, *Rhinella arenarum* reached a 50% enzyme inhibition at 991 μg ml^{-1} (R. Spinelli et al., 2022), which is fairly comparable to the results here reported; and the administration of a *Rhinella diptycha* secretion to cockroaches in vivo showed a dose-dependent AChE inhibition (40% at the highest dose), which was interpreted as a direct anti-predator insecticidal defense (Leal et al., 2020). Concerning BChE, Siano et al. first described the inhibition activities of skin extracts and secretions of *L. luctator* (Leptodactylidae) and *B. pulchella* (Hylidae) (Siano, Gatti, et al., 2014). Remarkably, the natural secretions of *O. asper* here reported demonstrated a more powerful inhibition at the same concentration (40% at 1250 μg ml^{-1} compared to 17% and 20% for *L. luctator* and *B. pulchella*). In other reports, *R. arenarum* showed a remarkable IC_{50} value of 23 ± 1 μg ml^{-1}, while *Boana cordobae* and *Pseudis minuta* (Anura: Hylidae) exhibited comparable IC_{50} values of 392 ± 14 and 377 ± 1 μg ml^{-1}, respectively (R. Spinelli et al., 2022; R. Spinelli et al., 2019). Interestingly, they all showed similar or better inhibition properties against BChE than AChE, as did *O. asper*.

Since it is well established that amphibian skin is rich in bioactive peptides, there are also records of anti-cholinesterasic peptides isolated from the skin of several amphibian species, such as Hp-1935, Hp-1971, BcI-1003, BcI-4, among others (Sanchis, Spinelli, Aschemacher, Humpola, & Siano, 2020; Siano et al., 2017; Roque Spinelli, Humpola, Sanchís, de los Angeles Méndez, & Siano, 2022; R. Spinelli et al., 2026). An interesting finding is that isolated peptides or purified fractions tend to be more active against both cholinesterases than their respective crude extract or secretion; this could be attributed to a dilution effect present in the complete extract (Sanchis et al., 2020; R. Spinelli et al., 2022). In this sense, the results obtained for the skin secretions of *O. asper* could be a starting point for the isolation and characterization of bioactive ChE-inhibitory peptides from an underexplored species.

As for the antioxidant activities, both secretions showed a marked DPPH radical-scavenging capacity (EC_{50}: 48–49 μg mL^{-1}) with no significant differences between sexes, which could mean they share common antioxidant compounds in a similar concentration. These results are notable not only when comparing with the positive control, ascorbic acid (EC_{50}: 7 μg mL^{-1}) but also with other reports about the antioxidant activity of amphibian skin extracts. *O. asper* skin secretions proved to have better radical scavenging potential than extracts from eleven different species of Hylidae, Leptodactylidae and Microhylidae families, whose EC_{50} range from 176 to 928 μg ml^{-1} (R. Spinelli et al., 2021; R. Spinelli et al., 2019). This remarkable activity reflects the ecological pressures on amphibian skin: since it lacks protective structures and must maintain its integrity in both aquatic and terrestrial environments, amphibians (more than other vertebrates) require efficient mechanisms for protection against oxidative stress. In this sense, frogs and toads have evolved a strong antioxidant system composed of enzymes, small molecules, and antioxidant peptides (AOPs) (Demori et al., 2019). Among AOPs, pleurains, antioxidins, nigroains, and andersonins are among the most potent ROS scavengers. Several isolated peptides from these families showed an interesting DPPH radical scavenging activity in ranges of concentration from 4 to 80 μM (X. Chen et al., 2022; Liu et al., 2010; X. Wang et al., 2017; H. Yang et al., 2009). Although these values are not directly comparable, since O. asper secretions are complex mixtures and their activity is expressed on a mass basis, EC50 values of 48 and 49 μg ml-1 suggest that these secretions could also be a rich source of AOPs. Since aromatic residues such as Trp, Tyr and Phe are frequently implicated in the antioxidant activity of AOPs, it is important to note that both studied secretions exhibited prominent peaks in the RP-HPLC chromatograms at 280 nm,

potentially indicating the presence of peptides with these residues. Additionally, Yang et al. also reported the ability of antioxidins to rapidly scavenge DPPH, sequestering almost 100% of the free radicals within seconds (H. Yang et al., 2009); the same fast-acting behavior was observed for both secretions of *O. asper*.

The powerful radical-scavenging capacity of these secretions is mirrored in their ability to inhibit lipid peroxidation, another critical physiological mechanism for maintaining skin integrity. Lipid peroxidation is a chain reaction triggered by ROS that progressively degrades membrane phospholipids, ultimately compromising membrane integrity and cellular function. The continuous exposure of amphibian naked skin to environmental stressors can lead to extensive tissue damage through such reactions (S. Wang et al., 2022; Yin, Xu, & Porter, 2011). Many antioxidants with the ability to scavenge DPPH free radicals have also shown a high lipid peroxidation inhibition activity (N. Chen, Yang, Sun, Niu, & Liu, 2012; Nguyen, Laosinwattana, Teerarak, & Pilasombut, 2017). Particularly, some amphibian-derived peptides with proven radical scavenging activity, successfully inhibited linoleic acid peroxidation as well, such as the 21 AOPs isolated from *Limnonectes fragilis* (Yu et al., 2015). OS-LL11, an 11-amino acid antioxidant peptide from *Odorrana schmackeri* significantly decreased the levels of lipid peroxides in a cellular model with PAM 212 cells previously stimulated by UV-B or H_2O_2 (Xie et al., 2021). Ocellatin-K1(1–21) from *Leptodactylus vastus* reduced the malondialdehyde (MDA) content on mice hippocampus, an important by-product of hydroperoxides generated during lipid oxidation (Sousa et al., 2020). The powerful ability of skin secretions of *O. asper* to inhibit lipid peroxidation is consistent not only with the link between this activity and radical scavenging activity but also with the antioxidant potential of amphibian-derived peptides discussed above. For this bioactivity, no significant differences were observed between sexes, as was also the case for the DPPH assay, indicating similar antioxidant activity under the tested conditions.

Beyond radical scavenging, *O. asper* also demonstrated a remarkable ability to chelate Fe^{2+} ions (EC_{50}: 107 and 156 μg ml^{-1} for male and female, respectively). The slightly higher activity of the male secretion may reflect differences in secretion composition; since all specimens were collected at the same site, differences in environmental metal exposure seem less likely, although sex-related differences in microhabitat use cannot be ruled out. More generally, amphibians are known to tolerate heavy metals and persist in habitats contaminated with high concentrations of copper, zinc,

arsenic, and cadmium, among other metals (Adlassnig, 2013; T. H. Chen, Gross, & Karasov, 2009). Metal chelation serves a dual physiological role for the amphibian skin in this context: on one hand, sequestering redox-active metals limits oxidative damage to the skin (Dixon & Stockwell, 2014); on the other, it constitutes an effective antimicrobial strategy, given that iron is an essential nutrient for bacterial and fungal growth, and its sequestration can suppress pathogen proliferation at the skin surface (Cassat & Skaar, 2013; Hood & Skaar, 2012). One of the possible contributions to this bioactivity is attributed to AOPs present in the skin. Carboxyl and amino groups in the side chains of acidic (Glx, Asx) and basic (Lys, His, Arg) amino acids are thought to play an important role in chelating metal ions (Indriani et al., 2023; Saiga, Tanabe, & Nishimura, 2003).

The vast majority of reports on peptides with chelating activity are from food-derived peptides, mainly obtained through enzymatic hydrolysis. Goat milk casein exhibits Fe^{2+}-chelating activity with an EC_{50} of 262 μg ml^{-1}, whereas its hydrolysates show a significantly enhanced chelating capacity with an EC_{50} of approximately 48 μg ml^{-1}, highlighting the potential of peptides over intact proteins (Li et al., 2013). Chelating activity has also been reported for peptides derived from protein hydrolysates of spirulina and green beans (Carrasco-Castilla et al., 2012; N.-H. Kim et al., 2014).

Regarding amphibian skin, while there are no records about the metal chelating activity of isolated natural peptides, there is some evidence of the ferrous chelation of extracts from frogs and toads (Indriani et al., 2022; R. Spinelli et al., 2022). To our knowledge, the Fe^{2+}-chelating activity described here is higher than that reported for the frog-derived samples considered in the cited literature. This remarkable activity may be attributed, at least in part, to the presence of specific amino acid residues with known metal-binding potential. However, because the present study did not identify the individual peptides responsible for the activity, this possibility remains to be tested. For Odontophrynidae, several peptides reported from *O. cultripes* contain Glu, Asp, Lys, His, and Arg residues, and americanin-1, isolated from *O. americanus*, contains Glu, Asp, His, and Arg, all recognized contributors to metal chelation (Cancelarich et al., 2024; Walters, Esfandi, & Tsopmo, 2018). Altogether, our findings suggest that amphibian skin peptides may represent an underexplored source of metal-binding compounds.

Finally, toxicity studies revealed how these natural peptide mixtures of *O. asper* interact with biological models, which may be related to their membrane-disrupting capabilities and, potentially, to antimicrobial action and predator deterrence. Overall, for both sexes, the concentrations required to achieve toxic effects in both hemolysis and *A. salina* assays are much higher than the EC_{50} values observed for the antioxidant, chelating, and peroxidation inhibition activities. This difference in concentration ranges may suggest that the basal physiological functions necessary for daily skin maintenance and protection against oxidative stress could operate at concentrations that are not harmful to the amphibian's own tissues. However, these preliminary results should not be interpreted as evidence of a defined ecological or pharmacological selectivity window.

The *A. salina* and hemolysis assays showed consistent toxicity profiles, in line with previous work regarding the agreement between *A. salina* and MTT assay results, supporting brine shrimp assay as a reliable, cost-effective model for cytotoxicity screening in bioprospecting studies (R. Spinelli et al., 2024). Here, for both tests, the markedly higher toxicity observed in the female secretion (around 35% for brine shrimp and 70% for hemolysis bioassays) may be associated with differences in the composition of the secretions. The greater abundance of larger, amphipathic peptides suggested by the MS profiles in the female secretion (**Figure 3**) could be responsible for a toxic activity through membrane disruption. In this case, *A. salina* has permeable membranes sensitive to pore-forming peptides, and human erythrocytes are vulnerable to amphipathic α-helical peptides rich in cationic residues (Lys, Arg). Additionally, sex-specific ecological exposures (such as more prolonged time in terrestrial vs. aquatic environments during reproductive migration) might favor the expression of more cytolytic or membrane-active peptides in females as part of their defensive mechanism.

As for the reports on the toxicity of amphibian skin extracts, for the Bufonidae family, Nalbantsoy et al. and Kyriachenko et al. did not find any hemolytic activity against rabbit erythrocytes for extracts of *Bufo bufo*, *Bufo verrucosissimus*, *Bufotes variabilis* and *Bufo viridis* at the tested concentrations (Kyriachenko, 2020; Nalbantsoy, Karis, Yalcin, & Gocmen, 2016). More recently, Spinelli et al. characterized the toxicity of nine skin extracts from Leptodactylidae, Microhylidae, and Hylidae families against CHO-K1 cells and *A. salina* (R. Spinelli et al., 2024). To our

554 knowledge, the hemolytic and cytotoxic activities described in this article represent the first report
555 on the toxicity of skin secretions from a species of the Odontophrynidae family.

556 The ecological relevance of the evaluated activities depends on whether the concentrations tested
557 in vitro can be reached during a natural encounter. Amphibian skin secretions are released as a
558 concentrated solution on the body surface, therefore the local concentrations encountered by a
559 predator during seizing or ingestion may be considerably higher than the concentrations used in
560 the assays, particularly for small predators. Conversely, the concentrations required for 50%
561 radical scavenging (48–49 μg ml^{-1}) and Fe^{2+} chelation (107–156 μg ml^{-1}) were approximately 4-
562 to 50-fold lower than those producing toxic effects (674–2340 μg ml^{-1}), which is consistent with
563 these activities being readily attainable on the skin surface. However, secretion yield per individual
564 was not estimated in this study. Because each specimen released only small amounts of secretion
565 and samples were pooled by sex prior to lyophilization, any per-individual estimate would carry a
566 large relative error and was therefore not considered reliable enough to support quantitative
567 conclusions. In addition, in vitro concentrations do not account for absorption, distribution, or
568 detoxification in the predator. Future studies should include a larger number of individuals and
569 individual-level quantification of secretion yield, together with in vivo assays with natural
570 predators, to obtain representative values and establish the defensive relevance of the
571 cholinesterase-inhibitory and membrane-disrupting activities described here.

572 Overall, the present study provides an initial characterization of the chemical profiles and in vitro
573 bioactivities of *O. asper* skin secretions. The results reveal differences in the mass profiles of
574 pooled male and female samples and significant differences in toxicity, together with smaller,
575 apparent differences in cholinesterase inhibition and Fe^{2+} chelation, whereas DPPH radical-
576 scavenging activity and lipid peroxidation inhibition were broadly comparable under the
577 conditions tested. Because the analyses were performed on pooled crude secretions and the
578 individual active components were not identified, the molecular basis and ecological relevance of
579 these patterns remain unresolved. Further studies involving independent biological replicates,
580 peptide isolation and structural characterization, and functional assays are needed to determine
581 whether the observed differences are consistent across individuals and whether they are associated
582 with specific physiological or ecological functions.

583

CONCLUSIONS

This study provides a preliminary characterization of the chemical composition and in vitro bioactivities of skin secretions of *Odontophrynus asper*, with particular emphasis on differences between male and female secretions. Analytical profiling by TLC, RP-HPLC, and MALDI-TOF MS indicated a predominantly peptide-based composition in both sexes and revealed differences in the relative distribution of peptide components. Notably, male secretions are enriched in low-mass peptides (1000–1165 m/z), whereas female secretions feature a greater abundance of mid-to-high-mass components (1435–3757 m/z). These differences suggest sex-associated variation in the molecular profiles of the pooled secretions, although the identity and biological functions of the individual components remain to be established.

Both secretions exhibited multiple in vitro bioactivities associated with physiological and defense properties, including inhibition of acetyl- and butyrylcholinesterase (~40–44%), DPPH radical-scavenging activity (EC_{50} ~48–49 μg/mL), inhibition of lipid peroxidation, and potent Fe^{2+}-chelating activity (EC_{50}: 107–156 μg/mL). Male secretions showed greater potency in cholinesterase inhibition and metal chelation, while female secretions were significantly more toxic against *A. salina* and human red blood cells. These results suggest that sex-associated differences in peptide profiles can be accompanied by differences in specific biological activities.

The observed activities should be interpreted as properties of crude skin-secretory mixtures under in vitro experimental conditions and do not by themselves establish specific ecological functions or a multifunctional chemical-defense mechanism in vivo. Nevertheless, the combination of chemical profiling and biological assays identifies *O. asper* skin secretions as a previously undercharacterized source of bioactive components and provides a basis for further investigation. Future studies should focus on the isolation and structural characterization of the individual peptides responsible for the observed activities, as well as on determining whether the sex-associated differences identified here are consistently expressed across individuals, populations, and ecological contexts.

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STATEMENT AND DECLARATIONS

ETHICAL STATEMENT

The study was evaluated by the Ethics and Research Safety Committee of the Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Argentina (Acta 04/20, 6 March 2020; certificate CE2021-44-C2047, issued 4 August 2021). Amphibian collection and cutaneous secretion sampling were authorized by the Ministry of Environment and Climate Change of the Province of Santa Fe under Resolution No. 337/2021, dated 29 November 2021.

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COMPETING INTERESTS

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.

AUTHOR CONTRIBUTIONS

Álvaro J. Rietmann: research idea, manuscript, biological studies, statistical analyses. **Roque Spinelli**: research idea, manuscript. **Maria Veronica Humpola**, biological studies, statistical analyses , **Melina Erben**, biological studies **Javier A. López:** animal sampling and supervision, manuscript. **Álvaro S. Siano:** research idea, manuscript, supervision. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article and/or its supplementary information file. Any other datasets are available from the corresponding author on reasonable request.

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