

## Skin Mucus Metabolites in *Salamandra Infracinnata* from Various Habitats

Gad Degani<sup>1,2</sup>, Eliav Peretz<sup>1</sup> and Sanaa Musa<sup>1,2\*</sup>

<sup>1</sup>MIGAL – Galilee Research Institute, Kiryat Shmona, Israel.

<sup>2</sup>Faculty of Science and Technology, Tel-Hai Academic College, Kiryat Shmona, Israel.

### \*Correspondence:

Sanaa Musa, Migal- Galilee Research Institute, Faculty of Science and Technology, Tel-Hai Academic College, Kiryat Shmona, Israel.

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### ABSTRACT

We conducted an untargeted metabolomics analysis of the skin mucus of Near Eastern fire salamanders (*Salamandra infracinnata*) using LC-MS/MS. Samples were collected from salamanders living in three distinct locations along the southern border of their distribution in Israel: Kibbutz Sasa in the Upper Galilee and Manof in the Lower Galilee, both semi-arid mountainous regions, and Dan River, which has a constant year-round water supply with stable temperature. We identified and described 10 metabolites found in the mucus of *S. infracinnata* in all three habitats. These substances belong to various chemical groups, and we provide their chemical structures and secretion levels. Salamanders from Dan had a greater number of metabolites (5 out of 10) at higher concentrations compared to the Manof and Sasa populations. In addition, differences were observed in 6 mucus metabolites among the mountain populations. Findings support the hypothesis that the extreme habitat conditions in the southern range influence the quantity and composition of salamander mucus secretions.

### Keywords

*Salamandra*, Skin Mucus, *S. infracinnata*.

### Introduction

The genus *Salamandra* is widely distributed across Europe and extends into North Africa, where the species *S. algira* is found. It also reaches into the Middle East, where the species *S. infracinnata* has been identified [1]. The distinct salamander species in this genus, characterized by their black and yellow coloration patterns, have been thoroughly examined in numerous studies [1-4] describing the various color-pattern phenotypes of the yellow markings on the black dorsal surface of *S. infracinnata* in different habitats located at the southern extremity of its distribution within Israel.

All *Salamandra* species secrete a toxic substance known as *Salamandra* skin poison (SSP), which functions as a defense mechanism alongside the salamander's distinctive cautionary hues [1]. Most of the research on the mucus secretions on salamander skin has centered on *S. salamandra* from Europe [5]. SSP has been identified as consisting of steroidal alkaloids,

mainly samandarine, a saturated secondary amine, C<sub>19</sub>H<sub>31</sub>NO<sub>2</sub>, with a secondary hydroxy group. It is converted by chromic acid oxidation to the corresponding ketone, samandarone. The second oxygen atom is present as an ether bridge and there are two C-methyl groups [6]. Steroidal alkaloids exhibit the fundamental steroidal framework, featuring a nitrogen atom integrated into rings or side chains as an essential component of the compound's structure [7]. These compounds have a broad array of biological effects, and a subset has even been harnessed as medicinal agents, exemplified by the noteworthy drug abiraterone acetate, which is extensively employed in treating prostate cancer [7]. The realm of naturally occurring steroidal alkaloids, characterized by diverse structural arrangements, manifests a wide spectrum of biological functionalities. This makes them highly attractive to the fields of both natural chemistry and medicinal chemistry. In this comprehensive overview, structural categorization, isolation processes, and myriad biological activities are discussed [7].

The skin mucus of the Near Eastern fire salamander (*S. infracinnata*) was examined in three isolated populations located in different habitats at the southern border of this species'

distribution. The conditions in these habitats are relatively extreme when compared to the other species of the *Salamandra* genus [5]. Among the various metabolites, 891 were detected across all populations of *S. inframaculata*, whereas 119 metabolites were exclusive to salamanders from the Manof region (the population that lives under the most extreme conditions), 84 were unique to the Sasa population (located on the highest mountain in Israel), and only 7 of these metabolites were identified in Dan salamanders (an area where the conditions are constant year round with flowing water) [3,5]. The proportion of metabolites that were shared between the Dan–Manof and Dan–Sasa salamander groups (0.1 and 0.2%, respectively) was notably smaller compared to the similarity observed between the Manof and Sasa populations situated in the mountains (0.7%) [3,5].

Metabolites in the skin mucus of *S. inframaculata* were analyzed by [5]. Some of these substances had been previously described in salamanders and other organisms. Here we summarize a few of them. Samanine is the main steroidal alkaloid secreted by the fire salamander (*S. salamandra*) [7]. This compound is known to be highly toxic. Samandridine is another highly toxic alkaloid produced by the skin glands of various salamanders, including *S. salamandra* and *S. inframaculata* [5]. Olean-12-en-28-oic acid, found in *S. inframaculata*, is probably secreted from the skin as a protective substance; this natural product is also found in other organisms. L-pyroglutamic acid is the levo isomer of pyroglutamic acid, and is the biologically active enantiomer in humans; pyroglutamic acid is an intermediate in glutathione metabolism. Antillatoxin B is an unusual N-methyl homophenylalanine analog of the potent neurotoxin antillatoxin [8]. Nebrosteroid L, a 19-oxygenated steroid, has been evaluated for its anti-inflammatory activity in RAW 264.7 macrophages [9]. Cholic acid is an amphiphilic steroidal molecule which, along with chenodeoxycholic acid, is a major bile acid produced by the liver, where it is synthesized from cholesterol; cholic acid is used to treat bile acid synthesis disorder because it suppresses the production of toxic bile acid intermediates [10]. 5 $\alpha$ -Dihydroandrolone (also known as 5 $\alpha$ -DHN, dihydroandrolone, DHN, 5 $\alpha$ -dihydro-19-nortestosterone, or 5 $\alpha$ -estran-17 $\beta$ -ol-3-one) is a naturally occurring anabolic–androgenic steroid and a 5 $\alpha$ -reduced derivative of nandrolone (19-nortestosterone); it is a major metabolite of nandrolone. Its formation, via the enzyme 5 $\alpha$ -reductase, is analogous to that of dihydrotestosterone from testosterone [11].

This study serves as a follow-up study to [5], providing a more comprehensive description of the metabolites found in the mucus of *S. inframaculata*—a less studied species. In addition, secretion of these metabolites in the mucus of isolated populations of salamander found in different habitats with extreme ecological differences [3] is compared.

## Materials and Methods

### Sample Collection

The mucus covering the salamander skin was gathered from salamanders in three locations in Israel: Dan, Sasa, and Manof (Figure 1) as described in [5]. Sasa and Dan are 50 km apart, Sasa

and Manof 36 km, and Dan and Manof 86 km. Each salamander was held in one hand, and the slimy material was accumulated by gently pressing filter paper using the thumb. The mucus was incubated with 3 mL methanol for 10 min with a mild swirling motion. The samples were passed through a 0.22- $\mu$ m filter, and the fluid was dried under nitrogen flow and stored at -80 °C. These samples were later analyzed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), as depicted in Figure 2 [5]. The samples were freshly prepared by dissolving them to a concentration of 2 mg/mL in methanol. To ensure the quality of the data, a set of quality control (QC) procedures were carried out for untargeted metabolomics analysis. Small amounts (20  $\mu$ L) from each experimental sample were combined to form a pooled matrix, which was used as the QC sample, and this process was repeated four times. A control sample, containing only methanol, was also included [5].

### Metabolomics Instrumentation

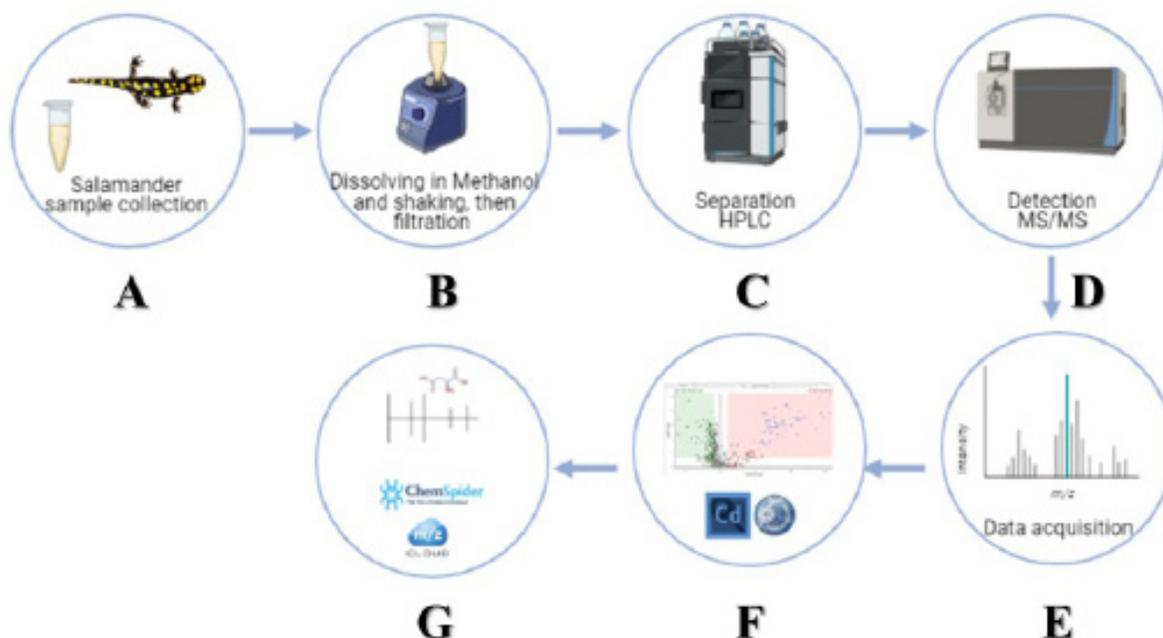
The analytical process involved injecting 5  $\mu$ L of the collected solutions into an ultra-high-performance LC (UHPLC) setup connected to a Dionex Ultimate 3000 photodiode array detector. This LC system was equipped with a reverse phase column (ZORBAX Eclipse Plus C18, 3.0 x 100 mm, 1.8  $\mu$ m) [5]. To maintain data integrity, the QC and control samples were injected first. They were then injected after every 10 samples and also at the end of the run. The mobile phase in the LC column consisted of: (A) deionized water (DDW) with 0.1% formic acid, and (B) acetonitrile with 0.1% formic acid. Gradient elution began with 2% of B, maintained for 1.5 min, then raised to 30% B over 2.5 min, followed by a 1-min increase to 40% B, and maintaining an isocratic flow at 40% B for 3 min. It was then further increased to 50% B over 6 min, and held there for 4 min. The composition was then raised to 95% B over 5 min and kept at 95% for an additional 9 min. Finally, it was returned to the initial 2% B over 2 min, and the column was equilibrated at 2% B for 4 min before the next injection [5]. Flow rate was 0.4 mL/min. A heated electrospray ionization source (HESI-II) was connected to a Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer (Thermo Scientific). The voltage applied to the ESI capillary was set at 3900 V. Both the capillary temperature and gas temperature were set at 350°C. Nitrogen gas (N<sub>2</sub>) was used. The settings for the mass spectrometer were configured as follows: the sheath gas flowed at a rate of 35 L/min, the auxiliary gas at 10 L/min, and the sweep gas at 1 L/min. For the analysis involving tandem mass spectrometry (MS<sup>2</sup>), the collision energy was adjusted to three levels: 15 eV, 50 eV, and 100 eV. The measurements of mass-to-charge ratio (m/z) were taken within the range of 67 to 1000 in both negative-ion and positive-ion modes. The measurements were taken with a high level of precision, indicated by a full width at half-maximum (FWHM) resolution of 70,000 at m/z 200. The accepted range for the mass accuracy was set at 5 ppm [5].

### Metabolomic Data Processing

Peak identification and quantification were carried out using Compound Discoverer 3.3 (version 3.1.0.305; Thermo Xcalibur). The automated integration of peak areas was manually reviewed



**Figure 1:** Three locations were chosen for mucus collection from salamanders. The accompanying illustration depicts a salamander excreting its toxic white mucus. The geographical distance between Sasa and Dan is 50 km, Sasa and Manof 36 km, and Dan and Manof 86 km. Salamanders in Sasa inhabit artificially created pits and caves for breeding, while those in Manof prefer man-made pools. In Dan, these salamanders are found in a river that flows consistently throughout the year [3,5].

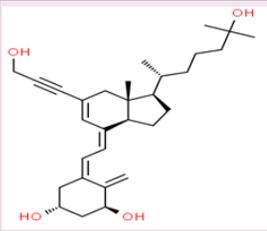
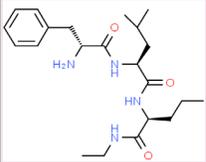
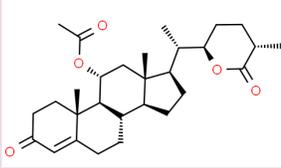
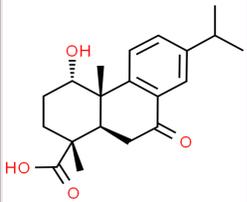
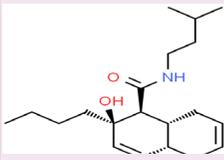
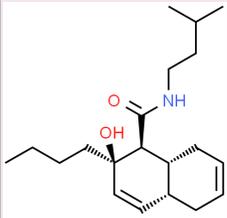
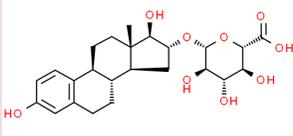
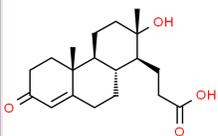
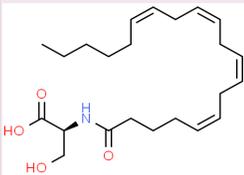
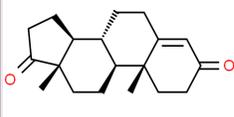


**Figure 2:** Study protocol [5]. Mucus samples were collected from both the back and head of the salamander and then treated with methanol to extract the compounds. Untargeted metabolomics analysis was conducted using HPLC to separate the extracted substances. MS/MS was conducted for substance identification. Compound Discoverer software was used to analyze the data and metabolites were identified by referring to the MzCloud and ChemSpider databases [5].

**Table 1:** Ten metabolites in the mucus of *S. infraimmaculata*. D, Dan; M, Manof; S: Sasa.

Compound name	Chemical formula	Calculated MW	$\Delta$ Mass [ppm]	Log <sup>2</sup> fold change			p-value		
				M / D	S / D	S / M	M / D	S / D	S / M
1alpha,25-Dihydroxy-11-(3-hydroxy-1-propynyl)-9,11-didehydrovitamin D3/1alpha,25-dihydroxy-11-(3-hydroxy-1-propynyl)-9,11-didehydrocholecalciferol	C <sub>30</sub> H <sub>44</sub> O <sub>4</sub>	468.32393	-0.06	-3.37	-4.61	-1.24	0.0043	0.0001	0.4244
Minabeolide-8	C <sub>29</sub> H <sub>42</sub> O <sub>5</sub>	470.3034	0.38	-0.22	-3.08	-2.86	0.9959	0.0005	0.0017
Taurochenodeoxycholic acid	C <sub>26</sub> H <sub>45</sub> NO <sub>6</sub> S	499.2972	0.89	-4.81	-1.39	3.42	0.0056	0.4123	0.0502
16alpha,17beta-Estriol 16-(beta-D-glucuronide)	C <sub>24</sub> H <sub>32</sub> O <sub>9</sub>	464.20514	1.09	-4.83	-3.76	1.08	0.0377	0.1161	0.6336
N-Arachidonoyl-L-serine	C <sub>23</sub> H <sub>37</sub> NO <sub>4</sub>	391.27168	-1.48	7.31	2.63	-4.68	0.0305	0.6931	0.1131
D-Phenylalanyl-L-leucyl-N-ethyl-L-norvalinamide	C <sub>22</sub> H <sub>36</sub> N <sub>2</sub> O <sub>3</sub>	404.27719	-3.84	-4.62	-5.46	-0.83	0.0015	0.0001	0.8157
Lophacinin D	C <sub>20</sub> H <sub>26</sub> O <sub>4</sub>	330.18262	-1.48	-1.23	-2.78	-1.56	0.4093	0.0002	0.0145
Caldorin	C <sub>19</sub> H <sub>31</sub> NO <sub>2</sub>	305.23501	-1.55	3.68	0.02	-3.66	0.0035	0.7714	0.0011
Testolic acid	C <sub>19</sub> H <sub>28</sub> O <sub>4</sub>	320.19882	0.17	0.28	-3.98	-4.26	0.6738	0.0000	0.0005
Androstenedione	C <sub>19</sub> H <sub>26</sub> O <sub>2</sub>	286.19302	-0.91	-0.79	-2.85	-2.06	0.3961	0.0129	0.3333

**Table 2:** Chemical structures of metabolites identified in the mucus of *S. infraimmaculata* (see Table 1).

Compound name	Chemical structure	Compound name	Chemical structure
1alpha,25-Dihydroxy-11-(3-hydroxy-1-propynyl)-9,11-didehydrovitamin D3/1alpha,25-dihydroxy-11-(3-hydroxy-1-propynyl)-9,11-didehydrocholecalciferol		D-Phenylalanyl-L-leucyl-N-ethyl-L-norvalinamide	
Minabeolide-8		Lophacinin D	
Taurochenodeoxycholic acid		Caldorin	
16-alpha-17-beta-Estriol 16-(beta-D-glucuronide)		Testolic acid	
N-Arachidonoyl-L-serine		Androstenedione	

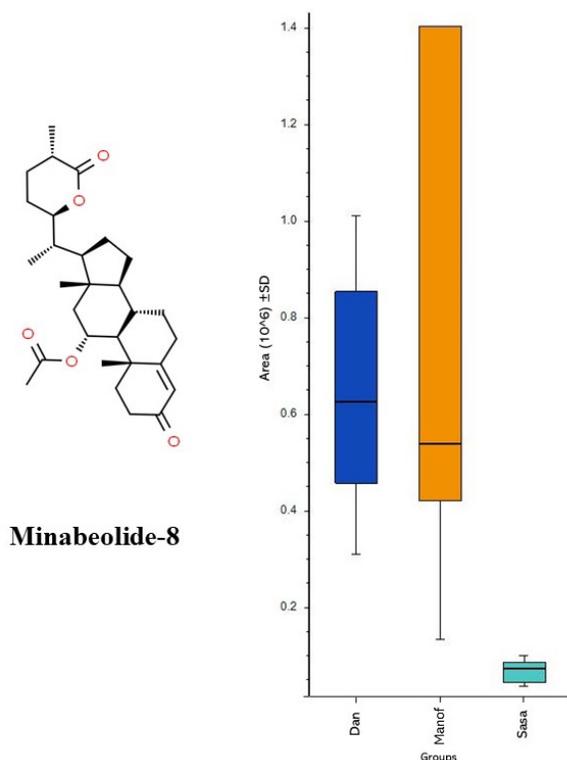
and adjusted where needed. Peak areas from each sample were standardized against the QC by eliminating areas with a relative standard deviation (RSD) exceeding 50% in the pooled QC samples. Instances where RSD < 50% were rectified using QC samples, following the procedure outlined in [12]. For certain compounds, determination of their identity relied on the MzCloud database through MS2 data (ensuring precise qualitative and relative quantitative outcomes), as well as the ChemSpider database employing high-resolution mass spectrometry (HRMS)) [4].

### Data Processing Calculations and Statistics

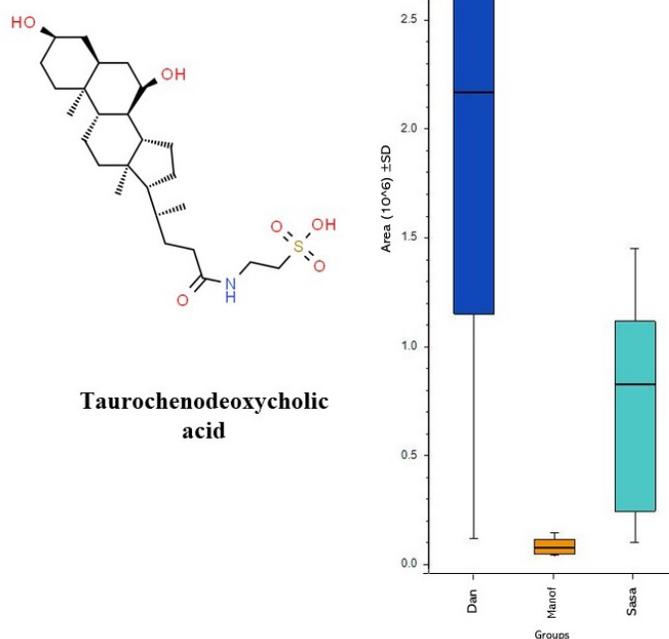
Regarding the LC-MS/MS outcomes, Compound Discoverer 3.3 software was used to compute the mean, SD, and RSD of peak areas. To identify noteworthy distinctions among groups (with a significance level of  $p < 0.05$ ), one-way analysis of variance was conducted.

### Results

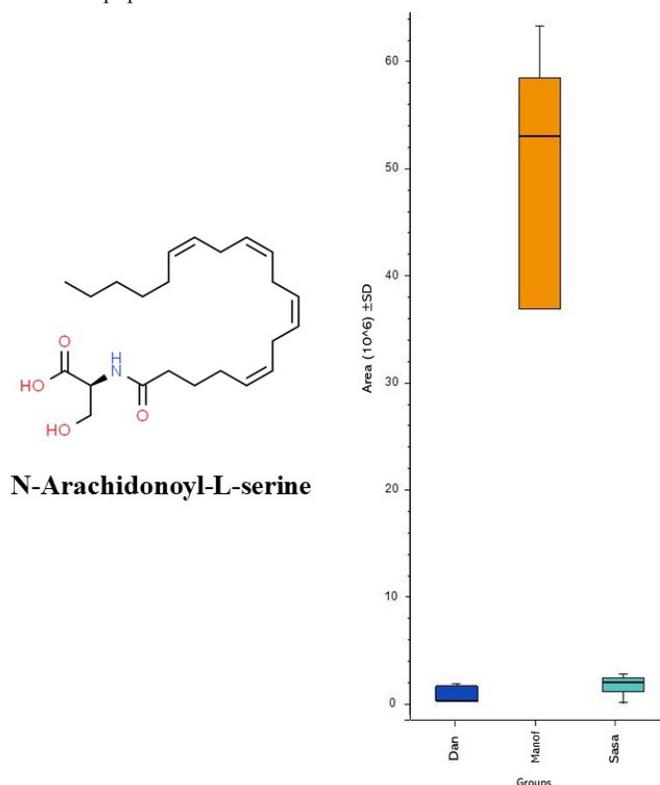
In this study, 10 new metabolites were characterized in the mucus of *S. inframaculata* collected from all three populations in northern Israel – Dan, Sasa, and Manof (Table 1). These substances belong to different groups and their chemical structures are provided in Table 2. However, their biological functions in the mucus remain unclear, although some of them have known biological functions in other contexts. The identified chemical groups to which these mucus metabolites belong include steroid lipids (estrogens), alkaloids (samaranines), withanolides, biogenic amines, polyketides, and fatty acid-based compounds, among others.



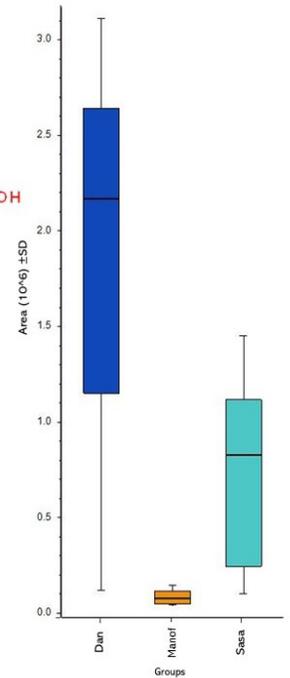
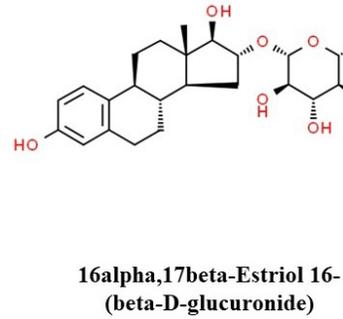
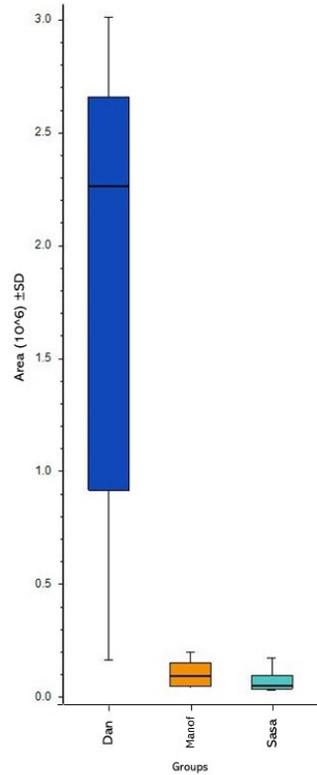
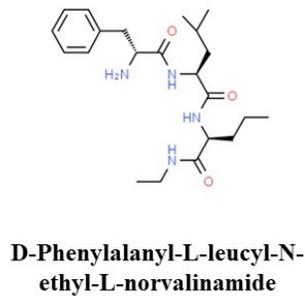
**Figure 3:** Comparison of normalized peak areas of minabeolide-8 detected in the mucus (mean ± SD) across the three distinct salamander populations.



**Figure 4:** Comparison of normalized peak areas of taurochenodeoxycholic acid detected in the mucus (mean ± SD) across the three distinct salamander populations.

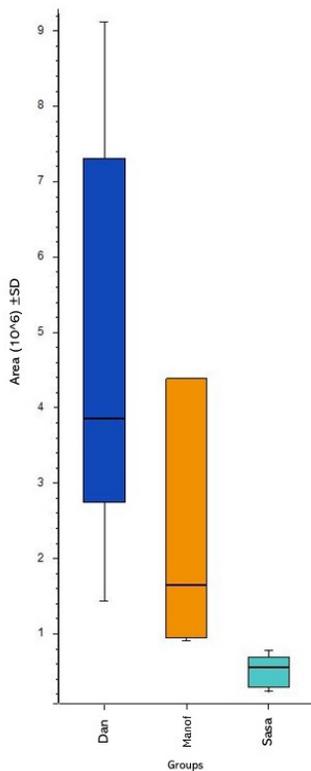
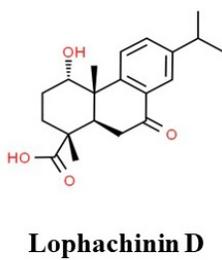


**Figure 5:** Comparison of normalized peak areas of N-arachidonoyl-L-serine detected in the mucus (mean ± SD) across the three distinct salamander populations.

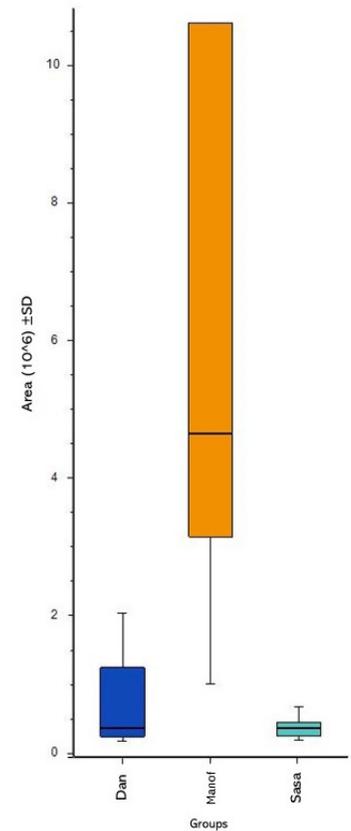
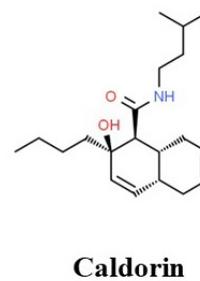


**Figure 8:** Comparison of normalized peak areas of 16alpha-17-beta-estriol 16-(beta-D-glucuronide) detected in the mucus (mean ± SD) across the three distinct salamander populations.

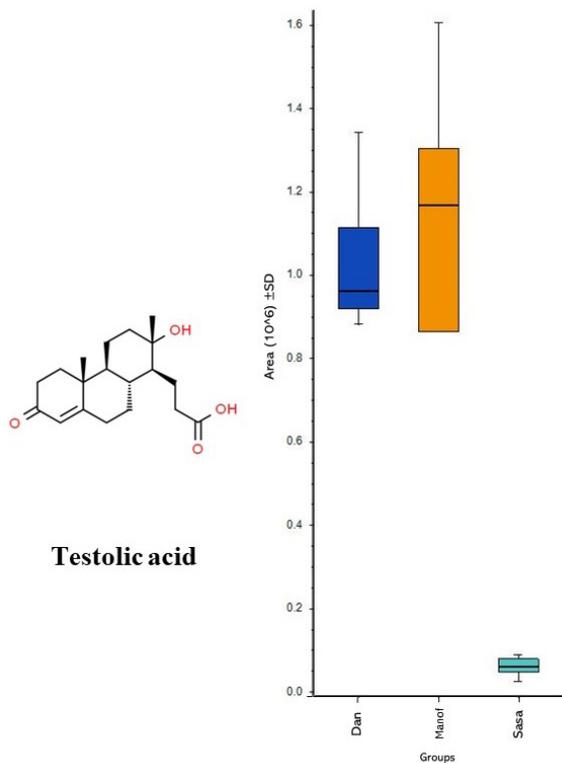
**Figure 6:** Comparison of normalized peak areas of D-phenylalanyl-L-leucyl-N-ethyl-L-norvalinamide detected in the mucus (mean ± standard deviation) across the three distinct salamander populations.



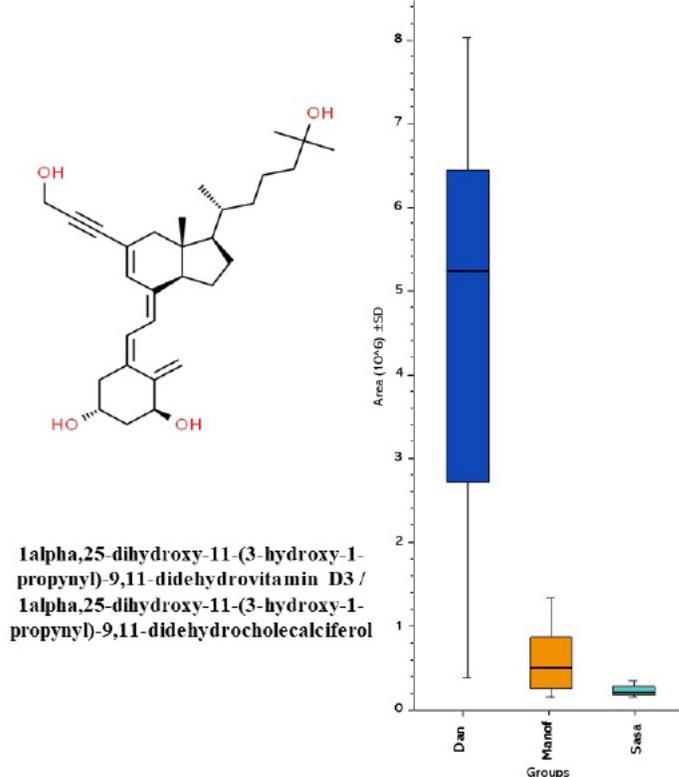
**Figure 7:** Comparison of normalized peak areas of lophachinin D detected in the mucus (mean ± SD) across the three distinct salamander populations.



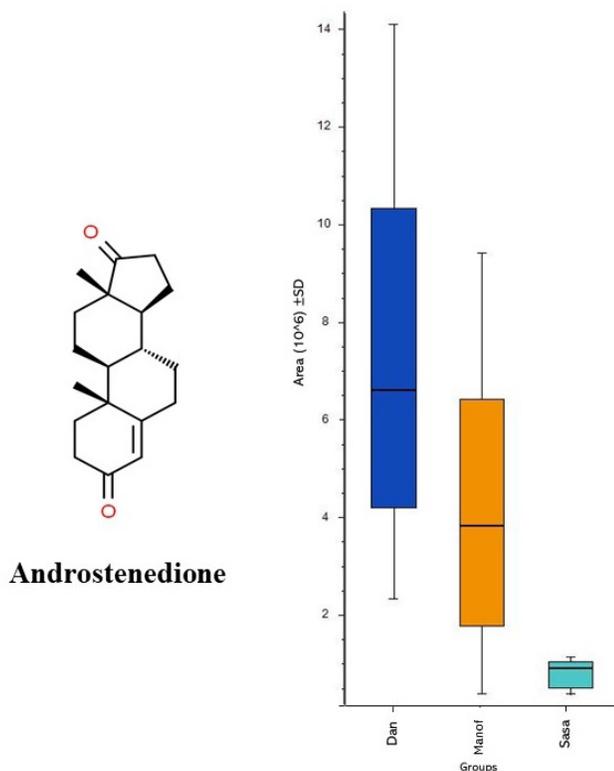
**Figure 9:** Comparison of normalized peak areas of caldorin detected in the mucus (mean ± SD) across the three distinct salamander populations.



**Figure 10:** Comparison of normalized peak areas of testolic acid detected in the mucus (mean  $\pm$  SD) across the three distinct salamander populations.



**Figure 12:** Comparison of normalized peak areas of 1-alpha,25-dihydroxy-11-(3-hydroxy-1-propynyl)-9,11-didehydrovitamin D3/ 1-alpha,25-dihydroxy-11-(3-hydroxy-1-propynyl)-9,11-didehydrocholecalciferol detected in the mucus (mean  $\pm$  SD) across the three distinct salamander populations.



**Figure 11:** Comparison of normalized peak areas of androstenedione detected in the mucosal lining (mean  $\pm$  SD) across the three distinct salamander populations.

The amounts and types of secreted metabolites varied among salamanders from the different habitats and regions. (Figures 3-12). Mucus composition of the salamanders from Dan differed from those of the other two populations, Sasa and Manof. In salamanders from Dan, a greater number of metabolites was found at higher concentrations than in the other populations: taurochenodeoxycholic acid (Figure 4), D-phenylalanyl-L-leucyl-N-ethyl-L-norvalinamide (Figure 6), lophachinin D (Figure 7), 16alpha,17beta-estriol 16-(beta-D-glucuronide) (Figure 8), and 1-alpha,25-dihydroxy-11-(3-hydroxy-1-propynyl)-9,11-didehydrovitamin D3 /1-alpha,25-dihydroxy-11-(3-hydroxy-1-propynyl)-9,11-didehydrocholecalciferol (Figure 12). A clear difference in mucus composition between the salamanders of the two mountain populations (Sasa and Manof) was found for the metabolites minabeolide-8 (Figure 3), taurochenodeoxycholic acid (Figure 4), N-arachidonoyl-L-serine (Figure 5), lophachinin D (Figure 7), 16alpha, 17beta-estriol 16-(beta-D-glucuronide) (Figure 8), caldorin (Figure 9), testolic acid (Figure 10), and androstenedione (Figure 11).

## Discussion

This study, which deals with the structure and secretion levels of metabolites in the mucus of *S. inframaculata*, a species found in isolated habitats at the southern border of its distribution, presents

interesting and novel biological phenomena. The mucus itself is a mixture of substances that have been described in European fire salamanders (*S. salamandra*) [1] and Near Eastern fire salamanders (*S. infraimmaculata*) [4]. In a previous work, 1112 different metabolites were found in the mucus of *S. infraimmaculata*, but only 14 of them were defined and their structure described [5]. In this article, the structure and relative area of another 10 metabolites that appeared in all three populations isolated in different habitats are described. Some of the metabolites are known, found in various organisms in the animal kingdom, whereas for others, the information is limited to type, i.e., steroidal alkaloids [10]. In most cases, the structure has been described in online databases such as the National Center for Biotechnology Information (NCBI). However, there have not been many studies comparing the substances in the mucus of different populations of salamanders living in different habitats, as discussed in this follow-up study for [5]. We found that in the habitat that is most different from the other two habitats (Dan—where the conditions are good and there is water year round), the composition of the mucus and the levels of its components differed compared to the other two habitats (two mountain populations where the summer is dry and hot, and water is not available) [2,3]. Differences between the mountain populations living under relatively extreme conditions and the population living under optimal conditions have also been found for other ancestral biological variables: morphological [4], biological [2,3,13], ecological, physiological [14-19] and genetic [20]. Another contribution of this work, together with other works, is that it enables establishing a connection between the habitats of *S. infraimmaculata*, the distribution of spots on its back, the ratio of black to yellow color [4] and the metabolites found in the mucus [5]. Aposematic bright colors and SSP function as defense mechanisms for the salamander [1]. The SSP metabolites play a crucial role in defending the animals, and the colors can manifest either through metabolic processes or by obtaining pigments from their diet [21]. *S. salamandra* displays significant color diversity and inhabits a wide range of environments. *S. infraimmaculata* shows a smaller variation in speckle dispersion and black-and-yellow hyaline than *S. salamandra* [4]. Color variations, habitat characteristics and food accessibility among adults from 25 different populations were studied by [21]. Here, we have no information on the differences in the adult salamanders' diets in the different habitats, but the great difference between the habitats could imply a difference in the food available to *S. infraimmaculata* [2].

Some of the metabolites detected in *S. infraimmaculata* mucus are steroid derivatives, which may be related to the sex hormones androgen and estrogen (Tables 1 and 2). The life cycle of *S. infraimmaculata* has been described in detail [22] and the mucus was collected during the reproductive period, when hormonal levels change and courtship and sexual behavior take place [18]. Gonadal steroid concentrations were assessed in the ovaries of *S. infraimmaculata* during their reproductive cycle in populations inhabiting a dry region in northern Israel [18]. Mature oocytes were only found during the winter and spring seasons, with peak numbers between December and April, following parturition [18]. As oocyte vitellogenesis and maturation occurred, the levels

of 17 beta-estradiol and testosterone increased. Furthermore, the concentrations of progesterone and 17-alpha-hydroxy progesterone appeared to be linked to the extent of vitellogenesis. Gravid (pregnant) females exhibited higher quantities of all four steroid hormones compared to non-gravid females [2,3,18].

Other metabolites found in the mucus of *S. infraimmaculata* had unclear biological function in this species, but have been described in other animals. For example, minabeolide-8, which is found in the marine invertebrates corals, significantly inhibits accumulation of the pro-inflammatory iNOS protein; it also effectively reduces the expression of COX-2 protein. This metabolite is also found in plants, a withanolide that serves as an anti-inflammatory agent (NCBI). Taurochenodeoxycholic acid is a bile acid that originates in the liver of various species, including humans. It is produced through the combination of chenodeoxycholic acid and taurine. It is released into the bile and subsequently into the intestinal tract. Under normal physiological conditions, it tends to carry an electrical charge, but it can also be converted into a crystalline form as its sodium salt. Its primary role is as a cleansing agent, aiding in the dissolution of fats in the small intestine. Interestingly, it is taken up through active transport in the terminal ileum [23,24]. Androstenedione is a naturally occurring steroid hormone produced in the adrenal glands and gonads, as well as a precursor to testosterone and other hormones in the body. It gained popularity as a dietary supplement in the 1990s and early 2000s due to claims that it could boost testosterone levels and provide various performance-enhancing and health benefits. Another interesting metabolite that was identified is N-arachidonoyl-L-serine, which is an endogenous arachidonoyl amide known as endocannabinoid anandamide. It has been found to activate the orphan G protein-coupled receptor GPR18 in a pertussis toxin-sensitive manner and produces antinociceptive and anti-inflammatory effects [25]. 1alpha, 25-Dihydroxy-11-(3-hydroxy-1-propynyl)-9, 11-didehydrovitamin D3, is a synthetic analog of vitamin D. It is also known as 1alpha, 25-dihydroxy-11-(3-hydroxy-1-propynyl)-9, 11-didehydrocholecalciferol. This compound is designed to mimic the biological activity of the active form of vitamin D in the body, known as 1,25-dihydroxyvitamin D3 (calcitriol). Calcitriol plays a crucial role in regulating calcium and phosphate metabolism and is essential for maintaining bone health and other physiological processes. Other substances discovered in the salamanders' mucus have been described in the literature in different animals with different uses for medicine [23,24].

Nevertheless, additional research is required to quantitate, isolate, and characterize some of these metabolites, as well as assess their biological activity in various assays. This is an ongoing topic of study in our laboratory.

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