Toxin variation among salamander populations : discussing potential causes and future directions

Running title: Toxin variation in the alpine salamander

TOXIN VARIATION AMONG SALAMANDER POPULATIONS: DISCUSSING POTENTIAL CAUSES AND FUTURE DIRECTIONS

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ABSTRACT

Amphibians produce defensive chemicals which provide protection against both predators and infections. Within species, populations can differ considerably in the composition and amount of these chemical defenses. Studying intraspecific variation in toxins and linking it to environmental variables may help us to identify the selective drivers of toxin evolution, such as predation pressure and infection risk. Recently, there has been a renewed interest in the unique toxins produced by salamanders from the genus *Salamandra*: the samandarines. Despite this attention, intraspecific variation has largely been ignored within *Salamandra*-species. The aim of this study was to investigate whether geographic variation in profiles of samandarines exists, by sampling four populations of *Salamandra atra* over its range in the Dinaric Alps. In addition, we preliminary explored whether potential variation could be explained by predation (counting the number of snake species) and infection risk (cultivation and genomic analyses of collected soil samples). Salamanders from the four populations differed in toxin composition and in the size of their poison glands, although not in overall toxin quantity. Nor predation nor infection risk could explain this variation, as populations barely differed in these variables. Sampling over a much broader geographic range, using better estimators for predation and infection risk, will contribute to an improved understanding of how environment may shape variation in chemical defenses. Nevertheless, as the four populations of *S. atra* did differ in their toxin profiles, we propose that this species provides an interesting opportunity for further ecological and evolutionary studies on amphibian toxins.

Keywords: amphibian toxins, geographic variation, poison glands, *Salamandra atra*, samandarines

INTRODUCTION

The poisonous nature of amphibians has been known since ancient times (Retief & Cilliers 2000). Modern research has confirmed that many amphibian species produce or sequester defensive chemicals or toxins (Bokony *et al.* 2019), which include biogenic amines, bufodienolides, peptides/proteins and alkaloids (Daly *et al.* 1987; Daly 1995; Clarke 1997; Daly *et al.* 2005). Some of these toxins induce adverse effects that may repel, harm or even kill potential predators (Brodie 1968; Brodie *et al.* 1991; Gray *et al.* 2010; Hopkins & Migabo 2010; Williams *et al.* 2010; Murray *et al.* 2016), and therefore protect amphibians against predation. Others are known to inhibit the growth of micro-organisms (Habermehl & Preusser 1969; Preusser *et al.* 1975; Macfoy *et al.* 2005; Woodhams *et al.* 2007; Mina *et al.* 2015; Calhoun *et al.* 2017; Hovey *et al.* 2018; Johnson *et al.* 2018) and may thus protect against parasitic infections.

For a limited number of species, there is evidence that chemical defenses exhibit geographical variation (Clarke 1997; Brodie *et al.* 2002; Saporito *et al.* 2012; Bokony *et al.* 2019), often leading to interpopulation differences in toxicity (Brodie *et al.* 2002; Saporito *et al.* 2012; Bolton *et al.* 2017) and/or antimicrobial activity (Tennessen *et al.* 2009; Mina *et al.* 2015; Hovey *et al.* 2018) of the skin secretions. In species that sequester toxins from their diet (e.g. poison frogs), such geographic variation in chemical defenses may merely reflect differences in local arthropod prey community composition (Daly *et al.* 2007; Saporito *et al.* 2007; Daly *et al.* 2008; Saporito *et al.* 2012). In other cases, geographic variation in chemical defenses is believed to result from changes in the ratio between costs and benefits of toxin production (Longson & Joss 2006; Blennerhassett *et al.* 2019). This ratio may depend on the environment, leading to selection for different toxin profiles among populations (Longson & Joss 2006; Bókony *et al.* 2016; Üveges *et al.* 2017). For example, toxin profiles may reflect local predation pressure (Brodie *et al.* 2002; Hanifin *et al.* 2008; Dreher *et al.* 2015; Bókony *et al.* 2016; Hettyey *et al.* 2019). Selection should favor higher toxicity, but only in environments where predation pressure is high, whereas it should act against costly chemical defenses when predation is low (Longson & Joss 2006; Yotsu-Yamashita *et al.* 2012; Hettyey *et al.* 2019). Alternatively, infection risk in the environment might also play a role in shaping local toxin profiles (Tennessen *et al.* 2009; Bókony *et al.* 2016). Unfortunately, few studies so far have investigated how local predation pressure and local infection risk contribute to

84 geographic variation in chemical defenses (Brodie *et al.* 2002; Hanifin *et al.* 2008; Dreher *et al.* 2015;
85 Bókony *et al.* 2016).

86 The genus *Salamandra* may provide an excellent study system for evolutionary and ecological research
87 on amphibian toxins. *Salamandra* populations occupy a variety of habitats (Arnold & Ovenden 2002;
88 Jeran *et al.* 2011; Šunje *et al.* 2014) across Europe, Northern Africa and the Near East (Lüddecke *et al.*
89 2018). Several species or subspecies of *Salamandra* carry brightly colored patches that likely function in
90 aposematism (Sanchez *et al.*, 2018; Vences *et al*., 2014; but see Preißler *et al*., 2019). The bioactive
91 compounds within the skin secretions of *Salamandra* sp. are a group of steroid alkaloids called
92 samandarines (SAMs) (Habermehl 1962; Habermehl & Spiteller 1967; Habermehl 1971; Lüddecke *et al.*
93 2018). SAMs are neurotoxins with nerve-blocking activity targeting the central nervous system, causing
94 respiratory paralysis and convulsions (Habermehl 1971; Lüddecke *et al.* 2018; Knepper *et al.* 2019) and
95 they seem to be cytotoxic as well (von Byern *et al.* 2017). SAMs also show antimicrobial and antifungal
96 activity (Habermehl & Preusser 1969; Preusser *et al.* 1975; Lüddecke *et al.* 2018; Smith *et al.* 2018), and
97 it was demonstrated that *S. salamandra* individuals completely deprived of their skin secretions succumb
98 to infections within weeks, unless kept in a sterile environment (Habermehl & Preusser 1969). Older
99 literature refers to the compound samandarine as the most potent neurotoxin among the SAMs (Geßner &
100 Esser 1935a; Kellaway 1939; but see Becker 1986; Lüddecke *et al.* 2018), and samandarone as the
101 strongest inhibitor of microbial growth (Preusser *et al.* 1975). In contrast to many other amphibian
102 alkaloids, which are commonly sequestered from dietary sources, SAMs are synthesized by the
103 salamander itself, via biochemical pathways starting from cholesterol (Habermehl & Haaf 1968; Mebs &
104 Pogoda 2005; Lüddecke *et al.* 2018). The endogenous origin of SAMs is supported by the fact that fire
105 salamanders still secrete SAMs after several generations in captivity (Daly 1995), while e.g. captive-bred
106 poison frogs are alkaloid-free (Santos *et al.* 2003). In the last decennium, there has been a renewed
107 interest in SAMs, with recent publications on interspecific variation in SAM profiles (Vences *et al.* 2014),
108 changes in alkaloid profiles during development (Sanchez *et al.* 2018), the link between coloration and
109 toxicity (Preißler *et al.* 2019; Sanchez *et al.* 2019) and a new protocol for their isolation (Knepper *et al.*
110 2019). The antimicrobial activity of SAMs has also regained attention in the light of the threat imposed
111 by the chytrid fungus *Batrachochytrium salamandrivorans* (Knepper *et al.* 2019). While interspecific

variation in SAM profiles has been documented (Vences *et al.* 2014), intraspecific variation has largely been ignored so far.

Our study focuses on the Alpine salamander (*Salamandra atra* Laurenti, 1768). This species has a wide, continuous distribution within the European Alps (Arnold & Ovenden 2002; Jeran *et al.* 2011), but several more isolated populations exist within the Dinaric Alps which belong to a separate subspecies *Salamandra atra prenjensis* Mikšić, 1969 (Bonato *et al.* 2018; Šunje *et al.* 2019). *Salamandra atra* is a strictly terrestrial species that spends a large portion of its life hidden in crevices, under stones or logs, in burrows of mammals etc. (Gautier & Miaud 2003; Helfer *et al.* 2012).

The main goal of this study was to investigate whether populations of the alpine salamander *S. atra prenjensis* show geographic variation in SAM profiles, in order to provide a framework for further ecological and evolutionary research. In addition, we preliminarily explored the potential role of environmental infection risk and, to a lesser extent, predation pressure in explaining such geographic variation.

MATERIAL AND METHODS

Populations

Four populations of *S. atra prenjensis* were sampled from the end of June until the beginning of September 2016. All populations were located within the Dinaric Alps, but differed considerably in altitude and general habitat (see Figure 1). Gorski Kotar consists of mixed deciduous-coniferous forest with a dense undergrowth. The soil is covered in plant litter and logs. Mounts Čvrsnica and Prenj are characterized by rocky alpine grassland, with sparse aggregations of mugo pines. The habitat in Prokletije consisted of a mix of grass field and fir forest near a small mountain stream. One constant factor was the presence of rocky limestone outcrops and dolomitic karst in all populations, providing holes and crevices for salamanders as shelter.

Samandarines (SAMs)

Animals were hand caught in the field and transported to the field lab in plastic boxes. Depending on weather conditions, animals were either collected opportunistically or by actively looking underneath

stones and logs along (± 40 m) hiking trails. Before collection of gland secretions, each animal was weighed on an electronic scale (precision: 0.01 g, Camry Electronic Ltd, Zhongshang, China) and width and length of head and parotoid gland were taken with electronic digital calipers (precision: 0.01 mm, Conrad Electronic, Hirhau, Germany). Animals were sexed based on the morphology of the cloaca (Luiselli *et al.* 2001). Next, the left parotoid gland was gently squeezed and the secretion released was collected with a small piece of sterile gauze (HEKA Soft, Venray, Netherlands). Squeezing continued until the gland did no longer discharge any fluid. Once the entire content of the gland was collected the gauze was stored in an empty 1.5 mL plastic microcentrifuge tube. Due to the sticky and mucous nature of the secretion, we were unable to reliably quantify the exact volume of fluid released by the salamanders. Directly after each fieldwork session, gasiform argon (MASSER, Sarajevo, Bosnia and Herzegovina) was added to the microcentrifuge tubes to prevent oxidation of the compounds, and samples were stored at 4°C. Salamanders were released back at the site of capture upon completion of fieldwork. As small tail and toe clips were collected from each animal for a related genetic study (Šunje *et al.* unpublished), recapture of the same individuals could be avoided during subsequent field work sessions at the same site. A total of 139 samples of adults were collected and used for further analyses (populations' sample size and composition are given in Figure 1).

Samples were analyzed using Ultra Performance Liquid Chromatography – tandem Mass Spectrometry (UPLC-MS/MS). Details on the UPLC-MS/MS can be found in the supplementary material.

During a series of test runs, a random selection of samples from each population were scanned for the presence of SAMs (Habermehl 1962; Habermehl & Spiteller 1967; Habermehl 1971; Daly *et al.* 2005; Lüddecke *et al.* 2018) using Multiple Reaction Monitoring (MRM). Compound specific MRM-settings were selected based on literature data (Supplementary Table S1). We measured the abundance of eight compounds in each sample: ecomytrin, samandaridine, samandarine, samandarone, samandanone, samandiol, samanine and samanol. For samanol, two different peaks were found in each chromatogram. We could not determine which of these two peaks represented samanol, therefore we will refer to the respective substances as 'samanol' and 'samanol2'. Reference standards could not be obtained, as these are not commercially available (Knepper *et al.* 2019) making further verification and calibration impossible.

Chromatograms were analyzed using MassLynx 4.1. (Waters Corporation, Milford, USA). Mass spectrometry results in peak surface areas (pA) for each individual compound, which are proportional to its abundance in the gland secretions (Sanchez *et al.* 2018). To avoid random noise, only peaks with a surface area higher than 30 (arbitrary) units were used. An additional experiment with captive animals showed that SAMs, and thus peak surface areas, degrade over time (mixed-effect model: slope = -0.02 log-units per day; $F_{1,240} = 12.21$; $p < 0.001$; see supplementary material). To take this into account, we recalculated the 'original' peak surface area of all compounds using the observed degradation rate and the number of days between collecting and analyzing samples. Peak surface areas were expressed relative to the size of the parotoid gland (calculated as the surface area of an ellipse – pA/mm²) to avoid that potential differences in SAM quantities would simply be a result of larger salamanders being able to store more alkaloids (Sanchez *et al.* 2018).

Data was analyzed using R version 3.5.1. (Ihaka, R. & Gentleman, R., University of Auckland, New Zealand). An Analysis of Similarities (ANOSIM, *Vegan* package, Oksanen *et al.,* 2017), based on a Bray-Curtis dissimilarity matrix, was used to test for overall differences in SAM profiles both among populations and between sexes. Separate ANOSIMs were done to test similarities between each pair of populations. Next, we tested for a correlation between geographic distances (*geosphere* package, Hijmans, 2016) and SAM dissimilarities using a Mantel test based on Spearman's rank correlation coefficient (*Vegan* package, Oksanen *et al.,* 2017).

To test for differences in the total amount of SAMs (sum of pA of all compounds /mm² cf. Sanchez *et al.,* 2018) produced by salamanders, we used a general mixed model containing population and sex as fixed factors. Sampling period (early July, late July and September) was included as random effect. Similar mixed models were used to test for differences in the relative amounts of four individual SAMs: samandarine (most potent neurotoxin), samandarone (strongest antimicrobial effect), samandaridine and samandenone (due to their high abundance). Interactions were initially included, but removed in case of non-significance. Data were log-transformed to meet normality and heteroscedasticity assumptions. *Post-hoc* pairwise comparisons were performed with the *emmeans* package (Lenth *et al.* 2018).

Salamander morphology and SAMs

Differences in body weight were tested using a two-way ANOVA, including population and sex as fixed factors, as well as their interaction. An ANCOVA was used to test for differences in parotoid gland size (in mm²), including population and sex as fixed factors and body weight as covariate, as well as interactions between population*sex and population*weight. Nine salamanders from Gorski Kotar were excluded from the dataset, due to incomplete body measurements.

We also tested whether salamanders with relative larger glands would produce higher amounts of SAMs (expressed absolutely – pA), using a general mixed model, with parotoid gland size and population as fixed factors, and body weight as controlling covariable. Sample period was included as random effect. We also specifically tested whether the association between relative gland size and total SAM-secretion (pA) would differ among populations by including a population*gland size interaction. While snout-vent-length or total body length are often regarded as better indicators for body size, we did not measure either of these. Nevertheless, a previous study showed that body mass and length are positively correlated in Bosnian populations of *S. atra* (Šunje *et al*. 2019).

Infection risk

Infection risk was estimated by taking soil samples in each population and identify soil bacteria and potentially parasitic soil fungi. Since *S. atra* is a strictly terrestrial species (Jeran *et al.* 2011; Helfer *et al.* 2012) that spends a large portion of its life underground (Gautier & Miaud 2003; Helfer *et al.* 2012), it is mostly exposed to soil micro-organisms. Soil samples were scooped out with a metal spoon that was sterilized prior to each sampling, by cleaning it with 70% ethanol and heating it over the open flame of a camping stove. Soil samples were taken in duplicate, one series of samples for bacterial cultivation and one series of samples for genomic analysis, from all microhabitats in which salamanders were found during the fieldwork, with special attention to the rock crevices which are the openings of the burrows used as shelter (see Supplementary Table S2). Samples were stored in 1.5 mL plastic microcentrifuge tubes and LifeGuard® Soil Preservation Solution (MO BIO Laboratories, Inc., Carlsbad, USA) was added to the genomic samples to prevent degradation of microbial DNA. Samples were stored at 4°C. A total of 67 soil samples were collected.

219 The first series of soil samples (Gorski Kotar: 5; Čvrsnica: 5; Prenj: 8; Prokletije: 12) were cultivated in
220 order to compare bacterial densities in the soil among the four locations using the plate-count method. A
221 series of tenfold dilutions (ranging from 10^0 to 10^{-4}) using PBS as diluent was spread onto the surface of
222 agar plates. Three different growth media were used: Tryptic soy broth (TSA) (general), MacConkey
223 (MC) (coliform bacteria) and Slanetz-Bartley (Enterococci) media (Atlas 2010). Cultures were incubated
224 at 35°C. Nevertheless, *S. atra* is an ectotherm living in cold alpine environments. Thus, in order to check
225 bacterial growth under more natural temperatures, we incubated a second series of TSA-plates at 15°C.
226 After incubation, the number of colonies was counted and used to calculate the original concentrations of
227 Colony Forming Units (CFUs) per gram soil.

228 Genomic DNA for soil fungi identification was isolated from the second series of soil samples (Gorski
229 Kotar: 10; Čvrsnica: 7; Prenj: 8; Prokletije: 12) using a Powersoil® DNA Isolation Kit (MO BIO
230 Laboratories, Inc., Carlsbad, USA). Following the accompanying protocol, 100 µL DNA-solution was
231 obtained from 0.25 grams of each soil sample. Fungal DNA was then amplified by a PCR using modified
232 versions of the primers ITS1F and ITS2, which amplify the fungal internal transcribed spacer (ITS) 1
233 region (see Smith & Peay, 2014). Each sample was amplified with an ITS2 primer containing a unique
234 index sequence (Kozich *et al* .2013). PCR-amplicons were pooled in one DNA-library and sequenced on
235 an Illumina MiSeq™. See supplementary material for more details on the primers, PCR-protocol and
236 sequencing. Sequences were analyzed following the UPARSE fungal pipeline described in Edgar (2013)
237 and Smith and Peay (2014). After removing singletons, sequences were clustered into Operational
238 Taxonomic Units (OTUs) based on a similarity of 97%. Chimeras were removed. An OTU table was
239 constructed and OTUs were blasted against the UNITE database of ITS1 sequences (version 7.0) using
240 the BLAST algorithm with default settings. OTUs were assigned to a certain lifestyle according to
241 Tedersoo *et al.* (2014). The OTU-table was rarefied using the Rarefy-function in R (*GUniFrac* package,
242 Chen, 2018).

243 ANOSIMs based on Bray-Curtis dissimilarity matrices were performed to test the similarity of
244 populations in soil fungi communities. Results were visualized using non-metric multidimensional scaling
245 (NMDS). In order to test whether dissimilarity in soil fungi communities was related to dissimilarity in
246 SAM profiles, we used a Mantel test based on Spearman's rank correlation coefficient. A high number of

OTUs could not be identified (NA) (71% of the OTUs, representing 61% of the total post-pipeline reads). These unidentified OTUs were included in the ANOSIM and NMDS, but removed from further analyses.

For each sample we scored: 1) the number of parasite fungi species (parasite diversity), 2) the total number of fungi species (as a measure of overall fungi diversity) and 3) the number of parasite reads (as a relative measure of parasite abundance).

A series of general mixed models was used to test population differences in: the density of soil bacteria (separate models for all media), parasite and overall fungi diversity and parasite abundance. All models included population as a fixed factor, and microhabitat as random effect. As we incubated one series of TSA-plates at 35°C and a second series at 15°C, both temperature and a temperature*population interaction were included in this model as additional factors. For the fungal data, the total number of reads per sample was also included as covariate (log-transformed). Where appropriate, response variables were log-transformed to meet normality and heteroscedasticity assumptions.

Predation risk

Predation risk in each population was estimated by counting the number of snake species present at each location. There are several reasons why predation by snakes is likely a stronger selective pressure for SAM composition in *S. atra* than predation by mammals or birds. First of all, snakes, more specifically the grass snake (*Natrix natrix*) and the common viper (*Vipera berus*), are currently the only documented predators of *S. atra* (Luiselli *et al.* 1995; Luiselli *et al.* 1997; Luiselli *et al.* 2005; Mebert *et al.* 2017). While predation by Eurasian magpies (*Pica pica*) and Alpine choughs (*Pyrrhocorax graculus*) has been reported, Klewen (1991) noticed that these birds avoid consuming the toxic parts of the animal. It is suspected that rats (genus *Rattus*) show a similar behavior when consuming other *Salamandra* species (Pezaro *et al.* 2017). In addition, during many field work sessions, we never observed carcasses mutilated this way. Hence, predation by birds and mammals is less likely to be a selective pressure on SAM composition or quantity in our populations. Secondly, both *V. berus* and *N. natrix* are known to tolerate higher injected doses of SAMs compared to other vertebrates (Geßner & Möllenhoff 1932; Lüddecke *et al.* 2018), which might indicate their active role as *Salamandra*-predators. Last but not least, independent evolutionary arms races between predatory snakes and poisonous amphibians have been hypothesized to

occur worldwide (Brodie *et al.* 2002; Feldman *et al.* 2012), illustrating how important snake predation may be for the evolution of amphibian chemical defenses.

Field observations, literature data and especially communication with local herpetologists (experts in the field) were used to compile a list of (presumably) present snake species at each location. All experts have been actively working on mapping and monitoring projects of reptiles in their respective area, and hence, their scoring of snake presence/absence is based on multiple field sessions. Snakes were scored as present if they had been sighted within the sampled areas (by authors or the local experts) within the last ten years, or were confirmed to be present by literature data (range 2003 – 2015). Some species were scored as 'expected to be present', as their ecological requirements match the conditions of a particular habitat despite not being observed at this location yet. Species were scored as absent if they were neither sighted, the literature data did not provide support for their presence and if their ecology did not match the habitat of that location. This way, we tried to account for the possible presence of more secretive species and avoid false negatives. Presence or absence of species was scored for a broader surrounding of the sampling locations (± 100 m altitude difference).

Ethical statement

Permissions to sample wild salamanders were issued by the Ministry of Nature Environment and Nature Protection in Zagreb (nr: 517-07-1-1-1-16-4) for Croatia, the Federal Ministry of Environmental Protection and Tourism in Sarajevo (nr: 04-23-550/16 ZM) for Bosnia and Herzegovina and the Agency for Protection of Environment in Podgorica (nr: UPI-952/2) for Montenegro. Permissions to export Bosnian animals were issued by the Ministry of Foreign trade and Economic Relations in Sarajevo (certificate nr: BA-KZV-VZ-40/14). Sampling and housing of the salamanders were approved by the Ethical Committee of the University of X (ECD nr: 2016-64) and according to the local legislation.

RESULTS

Samandarines

Populations of *S. atra* differed significantly in overall SAM composition (ANOSIM R = 0.125; p = 0.001). The strongest disparity was found between Prokletije and Gorski Kotar, while Čvrsnica and Prenj, and Čvrsnica and Gorski Kotar exhibited high similarity (Table 1). Male and female salamanders did not

differ significantly in overall SAM composition (ANOSIM R = -0.008; p = 0.654). Geographically distant populations tended to differ more in SAM profiles, but the relationship was not significant (Mantel statistic r = 0.657; p = 0.08, see Supplementary Table S3 for geographical distances).

The total amount of SAMs secreted by salamanders did not vary significantly among the four populations, neither when expressed in absolute terms (pA; $F_{3,10}$ = 2.24; p = 0.15; Figure 2A) nor relative to gland size (pA/mm². $F_{3,134}$ = 1.86; p = 0.14; Figure 2B). No sex-differences were found ($F_{1,134}$ = 0.29; p = 0.59). Populations differed significantly in the quantity of samandarine ($F_{3,90}$ = 4.89; p = 0.003) and samandaridine ($F_{3,124}$ = 15.73; p < 0.001), marginally in samandenone ($F_{3,128}$ = 2.66; p = 0.05), but not in samandarone ($F_{3,2}$ = 2.90; p = 0.27). We refer to Figure 3 for the specific interpopulation differences in each compound as indicated by post-hoc Tukey's tests. Female salamanders secreted higher quantities of samandarone ($F_{1,133}$ = 4.15; p = 0.04) and tended to produce more samandaridine ($F_{1,133}$ = 3.63; p = 0.06) but no sex-differences were found in samandarine ($F_{1,133}$ = 0.50; p = 0.48) or samandenone ($F_{1,133}$ = 0.61; p = 0.44). There were no significant sex*population interactions for any of the variables (all p > 0.05).

Salamander morphology and SAMs

A summary of morphological variables per population is given in Supplementary Table S4. Both body weight and parotoid gland size differed among our studied populations. Our results indicated that salamanders from Čvrsnica were heavier ($F_{3,125}$=7.63; p < 0.001; Figure 4A) than conspecifics from the other three populations, and, even after controlling for body size, they had larger parotoid glands compared to salamanders from Prenj ($F_{3,124}$=5.44; p = 0.002). There was a trend towards larger glands in Čvrsnica compared to Gorski Kotar (post-hoc Tukey's test: p = 0.08) and Prokletije (post-hoc Tukey's test: p = 0.09) (Figure 4B). Salamanders from the other three populations differed in neither body weight or gland size from each other. Females were heavier than males ($F_{3,125}$=19.33; p < 0.001) but did not possess larger glands ($F_{1,124}$ = 0.14; p = 0.71). No significant interactions were found between sex and population (p > 0.05) or body weight and population ($F_{3,118}$ = 0.91; p = 0.44). Heavier salamanders had larger parotoid glands ($F_{1,124}$=62.92; p < 0.001).

Overall, heavier salamanders produced higher absolute amounts of SAMs (pA, $F_{1,121}$ = 7.61; p = 0.007). There was a significant interaction between population and parotoid gland size ($F_{3,121}$ = 4.26; p = 0.007).

In Prenj, salamanders with relatively smaller glands produced more SAMs, while there was no significant correlation in any other population (Figure 5).

Infection risk

Bacteria were cultivated from 30 soil samples. The number of CFUs grown from one gram soil did not differ among populations, regardless of whether they were cultivated on TSA-media ($F_{3,3}$ = 0.52; p = 0.70) or MacConkey media ($F_{3,2}$ = 0.97; p = 0.54). No colonies were detected on the Slanetz-Bartely media. A significantly higher number of CFUs was grown on the TSA-media at 15°C than at 35°C ($F_{1,40}$ = 33.5; p < 0.001). There was no significant interaction between population and temperature for the TSA-media ($F_{3,38}$ = 0.34; p = 0.80). We refer to Supplementary Table S6 for average densities per population and per medium.

A total of 2661 fungal OTUs were identified in 37 soil samples. Most fungi were saprotroph-filamentous (51%) or ectomycorrhizal (22%). Less than 2% of the OTUs were identified as animal parasites (see also Figure S1). A small proportion of the OTUs (3%) could be identified but not assigned to a particular lifestyle (meaning that either they were not in the database of Tedersoo *et al.* (2014), the lifestyle was not conserved at the genus level, or the lifestyle was unknown).

Populations differed in their soil fungi communities (ANOSIM R=0.23; p = 0.002, Figure S2), with the highest dissimilarity found between Gorski Kotar and Prenj (Table 1). The dissimilarities in soil fungi community did not correlate with dissimilarities in SAM profiles (Mantel statistic r = 0.09; p = 0.46).

Our four study sites did not differ significantly in fungi diversity ($F_{3,8}$ = 0.44; p = 0.73) or parasite abundance ($F_{3,7}$ = 2.45; p =0.15), but did differ significantly in the parasite diversity ($F_{3,6}$ = 5.70; p = 0.04). Samples from Gorski Kotar contained more parasite species compared to Prenj (post-hoc Tukey's test, p = 0.05) and Prokletije (post-hoc Tukey's test, p = 0.07).

Predation risk

Our enquiries indicate a lower number of predatory snake species at the study site of Prenj compared to the other sites (Table 2). We have confirmed sightings of only two species (*Natrix natrix* and *Vipera ammodytes*) for Prenj, and local experts (see Supplementary Table S5) consider the presence of a third

354 species (*Coronella austriaca*) highly likely. Between four and six snake species occur in the three other
355 study areas.

356 **DISCUSSION**

357 Our analyses revealed significant among-population variation in the overall SAM profiles secreted by *S.*
358 *atra* of the Dinaric Alps. Geographic variation in chemical defenses has been demonstrated before in
359 common toads *Bufo bufo* (Bókony *et al.* 2016; Bokony *et al.* 2019), poison frogs (Daly *et al.* 2007;
360 Saporito *et al.* 2007; Mina *et al.* 2015), northern leopard frogs *Rana pipiens* (Tennessen *et al.* 2009) and
361 several newt species (Brodie *et al.* 2002; Hanifin *et al.* 2008; Yotsu-Yamashita *et al.* 2012; Stokes *et al.*
362 2015; Johnson *et al.* 2018). However, to our knowledge, this study provides the first example of
363 geographic variation in endogenously produced alkaloids.

364 Similarities in SAM profiles between populations were not related to geographic distance. Previous
365 studies on poison frogs often found higher similarities in alkaloid profiles between geographically closer
366 populations, but this might be explained by a larger overlap in arthropod communities, the dietary source
367 of the alkaloids (Saporito *et al.* 2007; Saporito *et al.* 2012). *Salamandra atra* has a fragmented
368 distribution in the Balkan peninsula and the studied populations have been isolated from each other since
369 the end of the last ice age (Helfer *et al.* 2012). Gene flow over larger distances is presumably low or non-
370 existent (Razpet *et al.* 2016), and local adaptation and/or random genetic drift may therefore have led to
371 differences in SAM profiles. Dinaric populations of *S. atra* are genetically well differentiated with
372 moderate values of genetic diversity (Šunje *et al.* unpublished), but it is currently unknown how the
373 genetic divergence among the populations relates to the detected variation in SAM profiles. Populations
374 that were more similar in SAM profiles were not more similar in the soil fungi communities to which they
375 were exposed. Such association would, however, only be expected if SAM composition was solely driven
376 by environmental infection risk.

377 While our four populations did not differ in overall SAM production, we did find significant variation in
378 the relative amounts of individual SAMs. Our dataset of four populations is obviously too small to draw
379 firm conclusions on the drivers of geographic variation in SAM production and composition, and
380 sampling more populations is necessary to look deeper into some of the trends reported here.

Nevertheless, we hope that our data for the current populations will be useful for more elaborate comparisons (within *S. atra,* or within the genus *Salamandra*).

Overall SAM quantities did not differ among our four populations neither when expressed absolutely or relative to gland size. It is generally assumed that larger animals and/or animals with larger poison glands are able to store more toxins and are thus more poisonous (Saporito *et al.* 2010b; Jeckel *et al.* 2015; Blennerhassett *et al.* 2019 but see Maan & Cummings, 2012). Our own data indeed confirmed a positive correlation between body weight and the total amount of SAMs secreted. Nevertheless, salamander with relatively larger glands for their body weight did not produce larger amounts of SAMs. In Prenj, the association between SAM production and relative gland size was even negative, a result we are currently unable to explain. Overall body size and absolute gland size may therefore be better indicators of an individuals toxicity. It is, however, interesting that albeit populations differed in body weight, no corresponding differences in total amounts of SAMs were found.

It is possible that this lack of variation in overall SAM quantities can be attributed to a similar predation rate across our populations. Indeed, our predation risk assessment suggested similar predator diversities in most populations (4 or 5 species confirmed), with only Prenj having a relatively lower number of snake species (2 confirmed). Nevertheless, it is currently not clear to what extend this predator diversity captures predation pressure. Both *N. natrix* and *V. berus* show geographic variation in the frequency of amphibians in their diet (Luiselli *et al.* 1995; Luiselli *et al.* 2005). The frequency with which salamanders are consumed may also differ among the species listed in Table 2. Additional data on snake diet and density at each location will help us to obtain more robust estimations of predation pressure~~. However, we still believe our presence/absence data can be useful as a first indicator of predation pressure.~~

Relative amounts of samandarine were higher in Prenj compared to Gorski Kotar. This pattern is highly similar to the observed variation in the total amount of SAMs, which is not surprising given that samandarine is the major compound of *Salamandra* skin secretions (Habermehl, 1971; Mebs & Pogoda, 2005; Vences *et al.*, 2014). Samandarine is often referred to as the most potent of the SAMs (Geßner & Esser 1935a; Kellaway 1939), yet, it is more abundant in Prenj, where snake diversity is low, compared to Gorski Kotar. Previous studies have often found a positive association between measures of predation pressure and toxin concentrations. E.g. Hague *et al.* (2016) showed that Pacific newts (*Taricha*

409 *granulosa*) have lower concentrations of TTX in allopatry with their TTX-resistant snake predators, and
410 Bokony *et al.* (2019) suggested that toads in anthropogenic habitats invest in more potent toxins due to a
411 higher density of predators compared to natural habitats

412 One possible explanation may be that salamanders in sympatry with snakes get attacked more frequently,
413 and will have to release their secretions more often. It has been shown in fire salamanders (Mebs &
414 Pogoda 2005), but in other amphibians as well (Blennerhassett *et al.* 2019), that refilling the parotoid
415 glands takes some time. It is possible that, due to lower snake presence, Prenj salamanders may get
416 attacked less frequently, allowing them to accumulate higher amounts of samandarine (similar to Saporito
417 *et al.* 2010). But, this should also be reflected by higher quantities of all SAMs in Prenj, which we did not
418 find. Ideally, future research should take the animals to a lab environment, where parotoid glands could
419 be completely emptied, and SAMs could then be collected after a standardized amount of time.

420 Another possibility, as discussed earlier, is that snakes show geographic variation in the frequency of
421 amphibians in their diet. In particular, it has been observed that *N. natrix* and *V. berus* only consume *S.*
422 *atra* at higher altitudes where alternative prey (such as lizards and rodents) are less abundant (Luiselli *et*
423 *al.* 1995; Luiselli *et al.* 2005). Interestingly, a shift in prey preference with altitude could explain the
424 difference in samandarine between Prenj and the low-altitude population of Gorski Kotar, but this
425 remains to be confirmed. It is also possible that snakes at different locations and from different species
426 vary in their resilience against samandarine. As evolutionary arms races between toxic prey and resistant
427 snake predators are known to lead to geographic variation in toxin concentrations (Brodie *et al*. 2002;
428 Feldman et al. 2012), this could be an interesting avenue for further research. As mentioned before, more
429 robust estimators of predation pressure are necessary to fully understand our results.

430 Salamanders showed no population differences in the relative amount of samandarone. As our four
431 populations barely differed in 'infection risk', showing no significant differences in bacterial densities,
432 parasite abundance or overall fungi diversity, it may not be surprising that samandarone-levels do neither.
433 In fact, the only population-differences were found in parasite diversity, with Gorski Kotar scoring higher
434 than Prenj and Prokletije, which did not correspond to an increase in samandarone-levels. Nevertheless,
435 while we focus on bacteria and fungi in this study, amphibian toxins may also inhibit infection by other
436 agents such as protozoa, trematodes or ranavirus (Rivas *et al.* 2009; Calhoun *et al.* 2017; Johnson *et al.*

2018). A negative correlation between individual TTX-levels and parasite richness (including both micro- and macroparasites) in *Taricha*-newts was shown by Johnson *et al.* (2018). Hence, future studies should take into account how populations of *S. atra* differ in their exposure to a broader range of parasites. It is worth mentioning that we did not find any traces of *B. dendrobatidis* nor *B. salamandrivorans* in our soil samples, the chytrid fungi responsible for worldwide declines and extinctions of amphibian populations (Skerratt *et al.* 2007; Tobler & Schmidt 2010; Martel *et al.* 2013). This is consistent with previous screenings in Prenj and Čvrsnica (Šunje *et al.* 2018).

Surprisingly, samandarone was seemingly only present in very low amounts and even absent in 16 samples. Samandarone is generally considered as one of the major SAMs and often found in concentrations equal to or higher than samandarine (Habermehl 1971; Mebs & Pogoda 2005; Vences *et al.* 2014). Given these low amounts, one could doubt whether samandarone really plays an important role in defense against infections. It is possible that samandarone is less likely to ionize than other SAMs, e.g. due to ion suppression, which leads to a lower MS-signal (Pitt 2009) and thus an underestimation of the actual amount of samandarone. Since internal standards are not commercially available for these compounds (Knepper *et al.* 2019), we were not able to verify and correct for this. However, even if the low peak surface areas correspond to much higher biological concentrations, this would not change the observed differences among populations.

Differences in samandaridine and samandenone among populations are difficult to explain, as little is known about their biological activity. Older sources claim samandarine is the most potent SAM (Geßner & Esser 1935b; Kellaway 1939) but Becker (1986) suggested similar LD50-values for all SAMs, at least in lab mice. In addition, chemical defense in *S. atra* is likely a far more complicated story involving more elements than just the SAMs. Both skin microbiota (Bettin & Greven 1986; Becker & Harris 2010) and peptides present in the skin secretions (Woodhams *et al.* 2007; Smith *et al.* 2018) have been suggested to play a major role in amphibian defenses against pathogens. In *Salamandra* sp., this is supported by the fact that crude skin secretions show higher antimicrobial activity than individual SAMs (Habermehl & Preusser 1969; Preusser *et al.* 1975), and that denaturation of proteins in the skin secretions drastically reduces its effectiveness in killing chytrid spores (Smith *et al.* 2018). These peptides likely show hemolytic activity, and thus may also contribute to the toxicity against predators (Habermehl 1971; Lüddecke *et al.* 2018). A far better understanding of all compounds in *Salamandra* gland secretions and

466 their biological activity, both individually and in combination with each other, is needed and will benefit
467 future evolutionary and ecological studies on this genus.

468 **CONCLUSIONS AND FUTURE PROSPECTS**

469 Our study provides the first evidence of intraspecific variation in SAM profiles within a *Salamandra*
470 species and, consequently, the first study to document geographic variation in endogenously produced
471 alkaloids. In addition, we also preliminary explored whether such variation could be explained by either
472 predation risk or environmental infection risk. Our data suggests that this was not the case, as both
473 variables barely differed among our populations. We do, however, recognize that we only obtained crude
474 estimations of both predation and infection risk.

475 Nevertheless, we hope that our results may open the door for a lot of new research opportunities. Apart
476 from improving estimations of predation pressure and infection risk, future research could also look at the
477 role of other environmental factors in explaining geographic variation, such as intra- and interspecific
478 competition (Bókony *et al.* 2016), exposure to anthropogenic herbicides, pesticides or other pollutants
479 (Bokony *et al.* 2017; Bokony *et al.* 2019), and diet (Saporito *et al.* 2007; Daly *et al.* 2008). While outside
480 the scope of this paper, we also noticed sex-differences in the relative amounts of some SAMs. All of
481 these are interesting avenues for further research. Documenting and understanding toxin variation may
482 also help to assess the vulnerability of specific populations and/or species to emerging diseases, such as
483 chytridiomycosis. Especially given the renewed interest in SAMs the last few years (Vences *et al.* 2014;
484 Lüddecke *et al.* 2018; Sanchez *et al.* 2018; Knepper *et al.* 2019; Preißler *et al.* 2019), which will lead to
485 better understanding of their biological activity, we fully believe that *S. atra* and related species provide a
486 good framework for further ecological and evolutionary studies on amphibian toxins.

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FIGURE LEGENDS

Figure 1. Distribution range (orange) of *Salamandra atra prenjensis*. Stars (★) indicate location of the study sites. The average altitude (in meters), the sample size and the dates of sampling are given for each population, as well as a picture of the general habitat.

Figure 2. Total amount of samandarines (SAMs) in the parotoid secretions, both expressed in absolute amounts (A) and relative to gland size (B). Total amount of SAMs was calculated by taking the sum of the peak surface areas (pA) of all individual compounds within the chromatogram of an individual (Sanchez *et al.,* 2018). Error bars represent standard errors. No significant differences were found.

Figure 3. Differences in the quantity of samandarine (A), samandarone (B), samandaridine (C) and samandenone (D) among the four populations of *Salamandra atra.* SAM quantities are expressed as the peak surface area in the chromatogram (pA) relative to the size of the parotoid glands (mm^2). Error bars represent standard errors. Significance levels are indicated as follows: '.' $p < 0.1$, '*' $p < 0.05$, '**' $p < 0.01$, '***' $p < 0.001$

Figure 4. Morphological differences among populations in A) body weight and B) parotoid gland size (controlled for overall body weight). Parotoid gland size was calculated as the surface area of an ellipse using parotoid width and length. Error bars represent standard errors. Significance levels are indicated as follows: '.' $p < 0.1$, '*' $p < 0.05$, '**' $p < 0.01$, '***' $p < 0.001$

Figure 5. Association between relative parotoid gland size (here represented by residuals from a gland size – body weight regression) and the total amount of SAMs (sum of the peak surface areas of the individual compounds, log-transformed) secreted by *Salamandra atra* in each population. Solid lines indicate a significant association. Grey area represents the 95% confidence interval.

TABLES

Table 1. Dissimilarities in overall SAM profiles (left-bottom) and soil fungal community (top-right, italic) among populations of *Salamandra atra* indicated by the ANOSIM R statistic. R-values > 0 indicate that the dissimilarity between sites is larger than the dissimilarity within sites. If R = 0, within-group dissimilarity equals between-group dissimilarity. Significant differences ($p < 0.05$) are indicated with an asterisk (*).

	Čvrsnica (BIH)	**Gorski Kotar (HRV)**	**Prenj (BIH)**	**Prokletije (MNE)**
Čvrsnica (BIH)	-	*R = 0.311* *p = 0.007***	*R = 0.050* *p = 0.172*	*R = 0.158* *p = 0.062*
Gorski Kotar (HRV)	R = 0.009 p = 0.293	-	*R = 0.479* *p = 0.001***	*R = 0.202* *p = 0.006***
Prenj (BIH)	R = 0.072 p = 0.055	R = 0.165 p = 0.002**	-	*R = 0.267* *p = 0.007***
Prokletije (MNE)	R = 0.157 p = 0.004**	R = 0.257 p = 0.001**	R = 0.085 p = 0.001**	-

763 **Table 2.** Presence or absence of snake species per population, based on literature data, field observations and
764 communication with local experts. The presence of species is scored for a broader surrounding of the sampling
765 locations (± 100 m altitude difference). "+" - the species is confirmed by field work (of authors or local experts)
766 within the last ten year and literature data; "?" – the species is expected to occur in the area of interest according to
767 local experts, based on a match between habitat conditions and the species' ecological requirements, but not yet
768 observed. Species were scored as absent if they were neither sighted, the literature data did not provide support for
769 their presence and if their ecology did not match the habitat of that location. The complete list of local experts and
770 affiliations is given in Supplementary Table S5.

	Gorski Kotar	**Prenj**	**Čvrsnica**	**Prokletije**
References	1-4	1, 5-6	1, 5-6	1, 7-10
Coronella austriaca	+	?	?	+
Natrix natrix	+	+	+	+
Natrix tesselata	?			
Vipera ammodytes	+	+	+	+
Vipera berus	+		+	+
Vipera ursinii			+	+
Zamenis longissimus	?			
TOT # SPECIES	4 (6)	2 (3)	4 (5)	5

771 1 - Sillero *et al.* (2014) 2 - Jelić *et al.* (2013) 3 - Jelić *et al.* (2015) 4 – Lauš, B. (personal communication) 5- Šunje
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773 communication) 9 – Zagora, V. (personal communication) 10 – Ajtić, R. (personal communication).
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