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Toxin variation in Alpine salamander populations

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Abstract

Amphibian poisons provide protection against predators and against infections by micro-organisms. It might be expected that toxin composition will be driven by adaptation to local predators and pathogens. Nevertheless, studies on geographic variation in toxins and its underlying causes are sparse.

In this thesis, toxin variation and the possible reasons for this variation were studied in four populations of the alpine salamander (*Salamandra atra*) in the Dinaric Alps. The hypothesis was that salamanders would secrete higher amounts of toxins and more samandarine in populations with higher predation pressure. In populations with higher infection risk, higher concentrations of samandarone were expected. Toxin samples were collected in the field and analysed using Ultra Performance Liquid Chromatography – tandem Mass Spectrometry. Local predator communities were characterized from literature sources to estimate predation pressure. Soil samples were collected for 1) cultivation of soil bacteria and 2) DNA-sequencing of soil fungi. This way, the abundance and diversity of micro-organisms to which *S. atra* is exposed per population could be estimated.

Considerable among-population variation in toxin composition was detected. Toxin variation did not match variation in predation pressure, although it was found that body size and poison gland size were larger in populations with snake predators. Toxins contained more samandarone in populations with higher infection risks, however, contributions of samandarone were far lower than expected from literature. Males and females did not differ in toxin composition.

In conclusion, there was only limited support for the hypothesis that toxin variation is driven by variation in predation pressure and infection risk. This is one of few studies to look at the underlying reasons for toxin variation, and the first one to incorporate the effect of microbial variation in the environment.

Keywords: amphibian toxins, geographic variation, samandarines, predation pressure, infection risk, *Salamandra atra*,

Samenvatting

Het gif van amfibieën heeft een dubbele verdedigingsfunctie: bescherming tegen predatoren én tegen infecties door micro-organismen. Men kan dan ook verwachten dat gif-samenstelling een resultaat is van adaptatie aan lokale predatoren en pathogenen. Onderzoek op geografische variatie in gif-samenstelling, en de oorzaken hiervan, zijn echter schaars.

Deze thesis onderzocht variatie in gif-samenstelling, en de onderliggende oorzaken, tussen vier populaties van de alpenlandsalamander (*Salamandra atra*) in de Dinarische Alpen. De hypothese was dat salamanders meer gif zouden afscheiden, en specifiek hogere concentraties van samandarine, in populaties met een hoge predatiedruk. In populaties met een hoger infectierisico werden hogere concentraties van samandarone verwacht.

Gifstalen werden verzameld in het veld en geanalyseerd d.m.v. vloeistofchromatografie met massa spectrometrie. Plaatselijke predator-gemeenschappen werden gekarakteriseerd o.b.v. een

literatuuronderzoek. Bodemstalen werden verzameld voor 1) het cultiveren van bacteriën en 2) DNA-sequencing van bodemschimmels. Op die manier werd de relatieve abundantie en diversiteit van micro-organismen ingeschat per locatie.
Aanzienlijke variatie in gif-samenstelling werd waargenomen tussen de populaties. Deze variatie kwam echter niet overeen met variatie in predatiedruk, al bleek dat lichaamsgrootte en kliergrootte groter waren in populaties waar salamander-etende slangen aanwezig waren. Gif bevatte meer samandarone in populaties met een hogere densiteit aan bacteriën en een hogere proportie van parasitaire fungi, maar de concentraties van samandarone waren veel lager dan verwacht o.b.v. literatuurgegevens. Er was geen verschil in gif-samenstelling tussen de geslachten.
Er is dus slechts beperkte ondersteuning voor de hypothese dat variatie in gif-samenstelling wordt veroorzaakt door variatie in predatiedruk en infectierisico. Dit is één van de weinige studies die keek naar de onderliggende redenen voor gif-variatie, en de eerste studie die de invloed van microbiële variatie in de omgeving onderzocht.

Sleutelwoorden: amfibieën-gif, geografische variatie, samandarines, predatiedruk, infectierisico, *Salamandra atra*.

Summary in layman's terms

Amphibians produce poisons to defend themselves against predators and against infections by micro-organisms. It can be expected that their poisons will consist of those toxins that are most effective in defending against the predators and micro-organisms in their environment As a result, populations in different environments with a different number of predators and/or parasites should also differ in toxin composition.
In this thesis, I looked at variation in toxin composition among populations of the alpine salamander (*Salamandra atra*) in the Dinaric Alps, and what causes this variation. In environments with more predators, I expected salamanders to produce more toxins and have higher concentrations of one specific compound: samandarine. In environments with more pathogens present, I expected toxins with a higher concentration of another compound: samandarone.
Toxins were collected in the field and analysed in the lab. The number of potential predators in each population was estimated based on literature data. Soil samples were taken to measure the density of bacteria in the soil and the amount of parasitic fungi.
I found considerable variation in toxin composition among populations. Salamanders did not produce more toxins or more samandarine in populations with more predators, but salamanders seemed to be larger in populations with more snakes present. In environments with more bacteria and more parasitic fungi, salamander toxins had higher concentrations of samandarone. Males and females did not differ in toxin composition.

To conclude, there is only limited support for the hypothesis that differences in the number of predators and/or pathogens among populations will cause differences in toxin composition.

1. Introduction

1.1. Poisons of amphibians

1.1.1. General

The poisonous nature of amphibians has been known to mankind for a very long time. The Roman naturalist Pliny the Elder considered the salamander the "*most wicked of all venomous animals*", believing it could kill entire tribes with its poison (Cilliers & Retrief, 2000). Because of their toxic skin secretions, many amphibians were associated with witchcraft and black magic during the Middle Ages, as illustrated in Shakespeare's Macbeth, in which the witches throw "the eyes of a newt and the toe of a frog" in their boiling cauldron (Crump, 2015). The skin secretions of frogs from the genus *Phyllobates* are used by several South-American tribes to poison their darts for hunting (Daly et al., 1987; Crump, 2015).

Nowadays it is known that most amphibians do indeed produce poisonous or at least noxious skin secretions (Daly, 1995). Poisonous species can be found in all major groups, namely: Anura (frogs and toads), Caudata (salamanders and newts) and Gymnophiona (caecilians). Toxins are secreted by cutaneous granular glands (Daly et al., 1987; Clarke, 1997), which are considered a shared character of all adult amphibians (Daly et al., 1987). Often, the granular glands will cluster together and form larger gland-like skin structures, e.g. the parotoid glands posterior of the eyes in Bufonidae and some Salamandridae (Brodie & Smatresk, 1990; Clarke, 1997).

A wide diversity of bioactive molecules are found within the skin secretions of amphibians (Daly et al., 1987; Daly, 1995; Clarke, 1997). Even within a single species, the poison may vary substantially in composition (Clarke, 1997; Mebs & Pogoda, 2005: Saporito et al., 2007; Saporito et al., 2009; Bókony et al., 2016). Amphibian toxins are classified into four major categories: biogenic amines, bufodienolides, peptides/proteins and alkaloids (Daly et al., 1987; Clarke, 1997). For the alkaloids alone, more than 800 different molecules (from 20 structural classes) have been detected (Daly et al., 2005; Saporito et al., 2012). The distribution and the relative importance of each of these categories can differ remarkably within one group of amphibians (Hopkins & Migabo, 2010).

The origin of amphibian toxins is not always fully understood (Daly, 1995). Some toxins, like the biogenic amines and peptides/proteins, are synthesized by the animal itself (Daly et al., 1987; Clarke, 1997; Bokóny et al., 2016). Other toxins, like most alkaloids, are obtained from dietary sources such as mites, ants or beetles (Santos et al., 2003; Saporito et al., 2007; Saporito et al., 2012). The process of taking up, accumulating, storing and (potentially) modifying toxins originating from other organisms is called sequestration (Saporito et al., 2012). Anura that sequester alkaloids from their diet are found within the Dendrobatidae, Mantellidae, Bufonidae and Myobatrachidae, and are commonly referred to as the poison frogs (Saporito et al., 2009). The origin of the bufodienolides is not known, but they might

be endogenously produced from cholesterol (Daly et al., 1987). Skin microflora might further modify the secreted toxins (Clarke, 1997).

1.1.2. Functions of amphibian poisons

Amphibian toxins have a two-fold defensive function: they protect against both predators and microbial infections (Clarke, 1997; Macfoy et al., 2005).

Many compounds found in the skin secretions of amphibians induce adverse effects, such as neurotoxicity, cardiotoxicity or vasoconstriction, that may clearly harm or kill potential predators (Clarke, 1997). There are numerous reports of both vertebrate and invertebrate predators (and even humans) dying after consuming or mouthing poisonous amphibians (Brodie, 1968; Daly et al., 1987; Brodie et al., 1991; Mobley & Stidham, 2000). Other toxins will have non-lethal but still unpleasant effects including paralysis, involuntary opening of the mouth and vomiting (Brodie 1968; Brodie et al., 1991), which might give a poisonous prey the opportunity to escape from its predator and thus contribute to its survival. Noxious skin secretions might also make the amphibian unpalatable, either due to a bad taste or a burning sensation in the mouth (Brodie, 1968; Gray et al., 2010; Hopkins & Migabo, 2010; Williams et al., 2010). For example, the bitter taste of many non-toxic alkaloids of Dendrobatidae will repel predators (Saporito et al., 2007). Tarantulas capture both toxic and non-toxic frogs with the same probability, but release the toxic frogs (*Dendrobates auratus*) after initial contact more frequently (Gray et al., 2010). The higher the concentration of tetrodotoxin (TTX) within the skin secretion of rough-skinned newts (the garter snake *Taricha granulosa*) the higher the probability of getting rejected by their natural predators (*Thamnophis sirtalis*) (Williams et al., 2010). Often the presence of toxic skin secretions is associated with aposematic warning signals, like the bright colours in many poison frogs or the unken-reflex in fire-bellied toads (*Bombina* sp.) and some newts (e.g. *Taricha* sp.) (Brodie, 1968; Daly et al., 1987; Hopkins et al., 2010; Saporito et al., 2012). Toxins present in the skin secretions of an amphibian will thus clearly increase its survival, by either deterring, harming or even killing potential predators.

Toxic skin secretions might also protect amphibians from microbial infections. The wet and mostly unprotected skin of amphibians offers the perfect substrate for the growth of micro-organisms (Habermehl & Preusser, 1969). In addition, amphibians often inhabit environments with high loads of potential pathogens (Clarke, 1997), like rotting logs, forest litter layers, shallow ponds and so on. A defence mechanism against micro-organisms is thus essential to not succumb to infections. Toxic skin secretions can play an important role in this defence. Both crude poisons and individual toxins of several species of amphibians have been proven to inhibit the growth of a broad range of micro-organisms (Habermehl & Preusser, 1969; Preusser et al., 1975; Daly et al., 1987; Macfoy et al., 2005; Mina et al., 2015). However, both the strength and the specificity of antimicrobial activity may vary considerably among toxins (Preusser et al., 1975; Macfoy et al., 2005), populations (Mina et al., 2015) and species

(Woodhams et al., 2007). The importance of toxins in the defence against micro-organisms is illustrated by the fact that fire salamanders (*Salamandra salamandra*) completely deprived of their skin secretions succumb to infections within a few weeks, unless they were kept in sterile environments (Habermehl & Preusser, 1969).

It has been suggested that the large diversity of alkaloids found within single species, e.g. multiple toxins from the same structural class, enables amphibians to protect themselves against a broader range of predators and pathogens, compared to having only a small array of toxins (Clarke, 1997).

Despite the apparent benefits, being toxic comes at a cost. Both the synthesis of endogenous toxins and the uptake and modification of exogenous toxins are energetically demanding processes (Longson & Joss, 2006). For example, fire salamanders might face a trade-off between using cholesterol as precursor for their toxins (Habermehl & Haaf, 1968; Habermehl, 1971) or investing it in other body functions (e.g. cell membranes, steroid hormones, …). Production of toxins might also decrease the growth rate and delay the metamorphosis of larvae (Longson & Joss, 2006; Bókony et al., 2016). Amphibians that sequester toxins from their diet might pay the cost of toxicity by having a more limited choice of prey (Santos et al., 2003). Thus, it can be assumed that, depending on the costs and benefits, different environments and/or situations might have different optimal levels of toxicity (Longson & Joss, 2006).

1.2. Variation in toxin composition

As mentioned before, the composition of amphibian poisons can show remarkable variation even within a single species (Clarke, 1997; Bókony et al., 2016). Differences in toxin composition (the type, number and amount of different compounds) among populations and between sexes will cause differences in toxicity (Brodie et al., 2002; Saporito et al., 2012) and/or antimicrobial activity (Woodhams et al., 2007; Mina et al., 2015). However, the underlying reasons for such variation within species is often unclear (Bókony et al., 2016).

Many poison frogs sequester alkaloids from dietary sources. Geographic variation in alkaloid composition within species thus mainly reflects geographic variation in the availability of arthropod prey (Daly et al., 2007; Saporito et al., 2012; Mina et al., 2015). Adjacent populations often have more similar alkaloid profiles, probably due to the presence of the same arthropod species (Saporito et al., 2007; Saporito et al., 2012). Variation in toxin composition within the same population over seasons and over years has also been observed, and can be explained by temporal variation in the abundance and diversity of arthropod prey due to e.g. disturbance, forest succession or seasonal variation in activity of insects (Daly et al., 2007; Saporito et al., 2007).

A study on the strawberry poison frog (*Oophage pumilio*) also found differences in alkaloid composition between males and females (Saporito et al., 2009). This may be caused by differences in diet. Compared

to males, female poison frogs consume more arthropods, and also have larger home-ranges (Saporito et al., 2009). This probably results in females exploiting a larger diversity of arthropod prey, and thus sequestering a larger number and larger diversity of alkaloids. It has also been suggested that female *Taricha*-newts are more toxic than males as some kind of maternal investment to protect their eggs (Stokes et al., 2011).

Variation in toxin composition might also be driven by variation in predation pressure. Since higher toxicity indeed increases the survival probability in a confrontation with a predator (Gray et al., 2010; Williams et al., 2010), selection should favour higher concentrations of toxins, especially of those toxins that increase toxicity. However, natural selection will only favour high toxicity when the increase in survival probability compensates for the costs associated with the sequestration/production of these toxins (Longson & Joss, 2006). Where predation pressure is high, higher levels of toxicity are needed to increase survival probability. Where predation pressure is low, amphibians are less likely to invest in costly chemical defences.

Nevertheless, the relation between toxicity and predation pressure has rarely been studied, nor on amphibians nor in general. Recently, Dreher et al. (2015) showed a positive correlation between attack rate on clay models and conspicuousness in different populations of *O. pumilio*. Since conspicuousness signals toxicity in this species, it can be hypothesized that populations with a higher predation pressure undergo selection for both higher conspicuousness and toxicity. Another study found no significant effect of predation pressure on the amount or diversity of toxins in toad-tadpoles (*Bufo bufo*) in ponds over a large geographic range (Bókony et al., 2016). Poison variation can also be driven by an evolutionary arms race with one specific predator. Pacific newts (genus *Taricha*) secrete poisons containing tetrodotoxin (TTX), a potent toxin that blocks the sodium channels of the nerve cells (Williams et al., 2010). Garter snakes (*Thamnophis sirtalis*), the only natural predators of these newts, are resistant against TTX to some extent. TTX-resistance in *T. sirtalis* is high in populations where poisonous newts are present, but low in populations where newts are absent or non-poisonous (Brodie, 1968; Brodie et al., 2002). TTX-concentrations in the skin of newts were spatially correlated with TTX-resistance in snakes (Brodie et al., 2002; Hanifin et al., 2008), suggesting that in populations with more resistant snakes selection is favouring higher TTX-concentrations and vice versa. It should however be noted that in one third of the populations there was a mismatch between TTX-levels and TTX-resistance (Hanifin et al., 2008) and it has recently been suggested that predation by other species might still be a selective pressure (Stokes et al., 2015). Even though the evolutionary arms race between *Taricha*-newts and *T. sirtalis* is well documented, more attention has been given to the predator's side of the story and less to the evolution of the poisonous prey. More research is required to study the changes in toxin composition and toxicity as response to predation, preferably in a broader range of species.

If amphibian toxins do indeed play a major role in the defence against microbial infections, it can be expected that variation in infection risk is another reason for variation in toxin composition. A good question might be whether environments with high densities of pathogenic micro-organisms select for skin secretions with potent antimicrobial activity (Bókony et al., 2016)? Unfortunately, no studies have tackled this issue.

In conclusion, apart from studies on poison frogs and their diet, research on geographic variation in toxin composition and the underlying reasons for this variation has been sparse (notable examples, see above in text). In particular the hypothesis that toxin composition reflects adaptation to local predators and/or pathogens has rarely been investigated and is therefore the central focus of my thesis. This question is especially interesting to understand which factors drive the evolution of toxins and toxin diversity. Establishing such geographic variation in toxin composition might also contribute to other research topics on amphibians, such as susceptibility to diseases like chytridiomycosis (Lötters et al., 2012).

1.3. The alpine salamander (*Salamandra atra*)

1.3.1. General description

This study focusses on the alpine salamander (*Salamandra atra* Laurenti 1768). The alpine salamander is an uniform black coloured salamander (with the exception of the partially yellow *S. atra aurorae* and *S. atra pasubiensis*) with a body length up to 15 cm, tail included (Arnold & Ovenden, 2002). It is a poisonous salamander, characterized by large parotoid glans posterior of the eyes, a ribbed appearance and a double row of dorsal glands starting behind the head and extending towards the tip of the tail (Brodie & Smatresk, 1990; Arnold & Ovenden, 2002). Adult males are usually smaller and more slender than adult females (Luiselli et al., 2001; Helfer et al., 2012), and can be distinguished from females by their swollen cloaca (Luiselli et al., 2001).

Salamandra atra has a nocturnal and secretive lifestyle. This is due to the fact that it 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 salamander only emerges during the night if weather conditions are favourable (Arnold & Ovenden, 2002; Helfer et al., 2012). It is a strictly terrestrial species, more so than the related fire salamander (*S. salamandra*). Even for its reproduction it does not depend on water, since it is a viviparous species with the females giving birth to completely metamorphosed young (Helfer et al., 2012; Jeran et al., 2011; Vences et al., 2014).

Salamandra atra consists of four subspecies: the uniform black *S. atra atra* and *S. atra prenjensis* (from mountain Prenj in Bosnia and Herzegovina), and the partially black and partially yellow *S. atra aurorae* and *S. atra pasubiensis* (both from the Italian Alps - Riberon et al., 2001; Andreone et al., 2009; Vences

et al., 2014). The validity of some of these subspecies is still under discussion and *S. atra aurorae* is sometimes even considered a separate species (Andreone et al., 2009; Jeran et al., 2011).

Salamandra atra has an almost continuous distribution in the European Alps and can be found from France/Swiss to Austria (Riberon et al., 2001; Arnold & Ovenden, 2002; Andreone et al., 2009; Jeran et al., 2011). Several isolated populations also occur in the Dinaric Alps, more specific in montane regions in Slovenia, Croatia, Bosnia and Herzegovina, Montenegro, Kosovo, and Albania (Andreone et al., 2009; Jeran et al., 2011). It occurs typically in montane regions between 800 and 2000 meters a.s.l. (Andreone et al., 2009), where it can be found in mixed deciduous-coniferous forests, but also in alpine meadows, pastures or rocky grasslands above the tree-line (Andreone et al., 2009; Jeran et al., 2011; Šunje et al., 2014).

1.3.2. Samandarines

Samandarines are bioactive substances that were first discovered in the skin secretions of fire salamanders and alpine salamanders (Habermehl, 1962; Habermehl & Spiteller, 1967; Daly et al., 1999; Mebs & Pogoda, 2005), but their presence was recently confirmed in secretions of other species of the family Salamandridae, such as *S. lanzai*, *Lyciasalamandra billae* and *Triturus cristatus* (Vences et al., 2014). In contrast to alkaloids secreted by other amphibians, samandarines are not sequestered from dietary sources. Samandarines are synthesized by the salamander itself, probably via biochemical pathways starting from cholesterol (Habermehl, 1971; Mebs & Pogoda, 2005), and therefore constitute a unique class of steroidal alkaloids (for structural formulas see figure 8.1 in appendix), that are found nowhere else in nature but in the skin secretions of Salamandridae (Daly et al., 1999). The endogenous origin of samandarines is supported by the fact that fire salamanders still secrete samandarines after several generations in captivity (Daly, 1995) while poison frogs bred in captivity are alkaloid-free (Santos et al., 2003).

Samandarines are neurotoxins with nerve-blocking activity targeting the central nervous system. They have strong local anaesthetic effects, can cause convulsions and seem to increase blood pressure. Poisoned animals generally die due to respiratory paralysis (Habermehl, 1971; Daly et al., 1999; Mebs & Pogoda, 2005). The toxicity of most compounds is not known. Samandarine, the major compound in the skin secretions of *S. atra*, has a lethal dose of about 70 µg for mice (Daly et al., 1999). For comparison, the lethal dose of several other amphibian toxins are given in table 1.1. Other samandarines, such as samandarone, are generally considered less toxic (Daly et al., 1993; Daly et al., 2005).

Table 1.1: Toxicity (here expressed as the lethal dose for mice) of several amphibian toxins. For each toxin the taxon in which it occurs is given. Cyanide is included for comparison. Sources: Sheehy & May (1968), Daly et al. (2005) and the "Material Safety Data Sheet Tetrodotoxin ACC# 01139" (Acros Organics N.V).

Toxins	Taxon	Lethal dose for mouse
Samandarine	*Salamandra* sp.	70 µg
Batrachotoxins	*Phyllobates* sp.	0.1 µg
Pumiliotoxins 307A and 323A **Allopumiliotoxin 267A**	Bufonidae, Dendrobatidae, Mantellidae, Myobatrachidae	50 µg
Tetrodotoxin	*Taricha* sp.	± 6 µg
Cyanide	/	± 152 µg

Besides their harmful effect on potential predators, samandarines to some extent also show antimicrobial activity. Both the crude secretions (Habermehl & Preusser, 1969), as well as some isolated compounds (samandarine, samandarone and samandaridine) (Preusser et al., 1975), inhibit the growth of several gram-positive and –negative bacteria and fungi. From the individual compounds tested, samandarone worked against the broadest range of micro-organisms and showed the strongest antimicrobial activity (Preusser et al., 1975). It caused larger zones of inhibition on agar plates and completely inhibited the growth of yeast cells from concentrations as low as $2*10^{-6}$ moles per mL (Preusser et al., 1975). Nevertheless, even samandarone is less potent than most antibiotics used nowadays.

Apart from two studies (Mebs & Pogoda, 2005; Vences et al., 2014), there has been no recent work on samandarines. Older literature mainly focussed on describing the structures of these alkaloids (Habermehl, 1962; Habermehl & Spiteller, 1967; Habermehl & Preusser, 1969; Preusser et al., 1975), but the toxins of *Salamandra* have never been studied in an ecological context, and barely in an evolutionary context. Variation in toxin composition is also largely ignored in most studies. Habermehl (1971) already noticed differences in the skin secretions of two subspecies of fire salamanders, and Vences et al. (2014) recently discovered differences in alkaloid composition among species of the Salamandridae. Mebs & Pogoda (2005) noted strong interindividual variation in the amount of samandarone and samandarine in fire salamanders, but no studies have really looked at differences among populations of the same species.

1.4. Research questions

The research question of my thesis consists of two parts:

1) Is there variation in toxin composition among different populations of *S. atra* in the Dinaric Alps?
2) Can this variation be explained by differences in predation pressure and/or infection risk among populations? In other words, does toxin composition reflect adaptation to local threats?

Salamandra atra is a suitable study model to answer these questions. As explained above, it has a wide distribution and occurs in a broad range of habitats. It can be expected that populations will vary greatly in the presence of predators and pathogens, which should be reflected in the composition of their toxins. Populations of *S. atra* in the Balkan are isolated from each other, and without gene flow strong local adaptation can be expected. Last but not least, most previous studies on geographic variation of toxins focused on poison frogs, who sequester alkaloids from their diet (Saporito et al., 2012), and on *Taricha*-newts, where the origin of the toxins is still ambiguous (Hanifin et al., 2008; Bucciarelli et al., 2016). To my knowledge, there is only one other study that looked at geographic variation in endogenously produced toxins (Bókony et al., 2016).

To study toxin variation, secretions of the parotoid glands were collected from four different populations within the Dinaric Alps over a broad geographic range (see further for description of sites). These toxins were analysed using Ultra Performance Liquid Chromatography – tandem Mass Spectrometry (UPLC-MS/MS). Local predator communities were characterised from literature sources, and soil samples were taken to determine the abundance and diversity of pathogenic micro-organisms in each habitat. Special attention was given to samandarine and samandarone. Samandarine is the major toxin in the secretions of *S. atra* and is generally considered the most toxic compound (Daly et al., 1993; Daly et al., 2005). Samandarone is the compound with the strongest and broadest antimicrobial activity (Preusser et al., 1975).

In populations with higher predation pressure, it was hypothesized that salamanders would secrete more toxins, and especially more samandarine, in order to be more toxic for predators. In populations where salamanders are exposed to higher abundances and higher diversity of micro-organisms, higher concentrations of samandarone (the compound with the strongest and broadest antimicrobial activity) were expected. Last but not least, I will also look at differences in toxin composition between males and females. In *S. atra*, females are philopatric while males often disperse to other populations (Helfer et al., 2012). Due to this male-biased dispersal, it is likely that males are more prone to predation (Stokes et al., 2011). Therefore, in line with the example above, I expected males to secrete higher amounts of toxins and higher relative concentrations of samandarine. Due to this dispersal, male salamanders might

also be exposed to a larger diversity of micro-organisms (Scantlebury et al., 2010), and selection might thus favour higher concentrations of samandarone in males.

To summarize, my hypotheses are as follows:

1) In populations with higher predation pressure, salamanders will secrete higher amounts of toxins and secretions will contain higher concentrations of samandarine.
2) In populations with a higher infection risk, salamander secretions will have higher concentrations of samandarone.
3) Males will secrete more samandarine and samandarone, and higher amounts of toxins, compared to females.

2. Material and methods

2.1 Fieldwork

2.1.1. Locations

In this study, four populations of *Salamandra atra prejnensis* (Mikšić, 1969) were sampled: one population in Croatia, two populations in Bosnia and Herzegovina and one population in Montenegro (see figure 2.1). All populations are within the Dinaric Alps, but nonetheless differ considerably in both abiotic and biotic environmental conditions. One constant factor however, is the presence of rocky limestone and dolomitic karst (Redžić et al., 2010), resulting in an overabundance of crevices and holes, providing shelter for the animals during unfavourable conditions (Helfer et al., 2012).

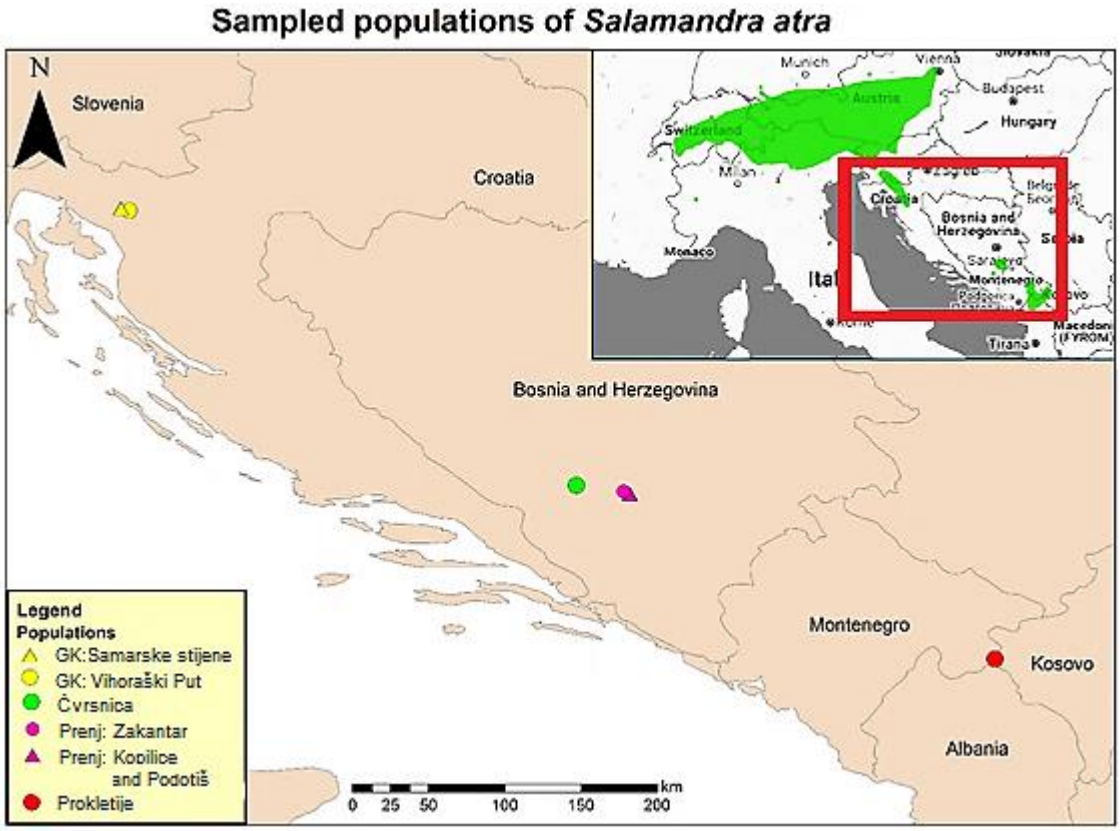

Figure 2.1: Map of locations where toxin samples of *Salamandra atra* were collected from June to September 2016. Symbols with the same colours are locations within the same population. Top-right: Map with the distribution of *S. atra* in Europe (MOL, 2017). Red square indicates the sampled areas.

The Croatian population is situated in the mountainous region of Gorski Kotar in north-western Croatia, more specifically in the nature reserve of Bijele and Samarske stijene, at the following locations: Vihoraški Put (45°13'41.6"N, 14°57'17.0"E) and Samarske stijene (45°14'30.1"N, 14°56'13.3"E). Samples were taken between 28/6/16 and 1/7/16 and on 8/9/16. The population of Gorski Kotar is characterized by mixed deciduous-coniferous forest, mainly beech (*Fagus sylvatica*) and fir (*Abies*

alba), with an undergrowth consisting mostly of ferns. Rocks are overgrown with thick layers of mosses and the soil is covered with plant litter and logs (figure 2.2a). The population was located at altitudes between 1000 and 1200 meter.

The first sampled Bosnian population of *S. atra* is located at mountain Prenj in southern Bosnia and Herzegovina. In this area, *S. atra* can be found between 1650 and 2000 meters a.s.l. (Šunje et al., 2014), in a zone that consists of rocky alpine grasslands with occasionally some groups of small mugo pines (*Pinus mugo*) in combination with Bosnian pines (*Pinus heldreichii*) (figure 2.2b). Fieldwork on Prenj was done at the following locations: Kopilice and Podotiš (43°33'24.3"N, 17°54'36.6"E) from 7/7/16 to 8/7/16 and Zakantar (43°34'20.6"N, 17°52'16.3"E) at 27/7/16.

The second sampled Bosnian population is found at mountain Čvrsnica (43°36'30.2"N, 17°35'40.6"E), in similar habitats and at similar altitudes as on Prenj (figure 2.2c). Sampling at Čvrsnica was done at 23/7/16 and 26/8/16. Due to neotectonic uplifting of the relief, there has been a deep incision of the Neretva river, resulting in a low river valley (95-278 a.s.l) (Lepirica, 2008; Redžić et al., 2010), separating the two adjacent mountains.

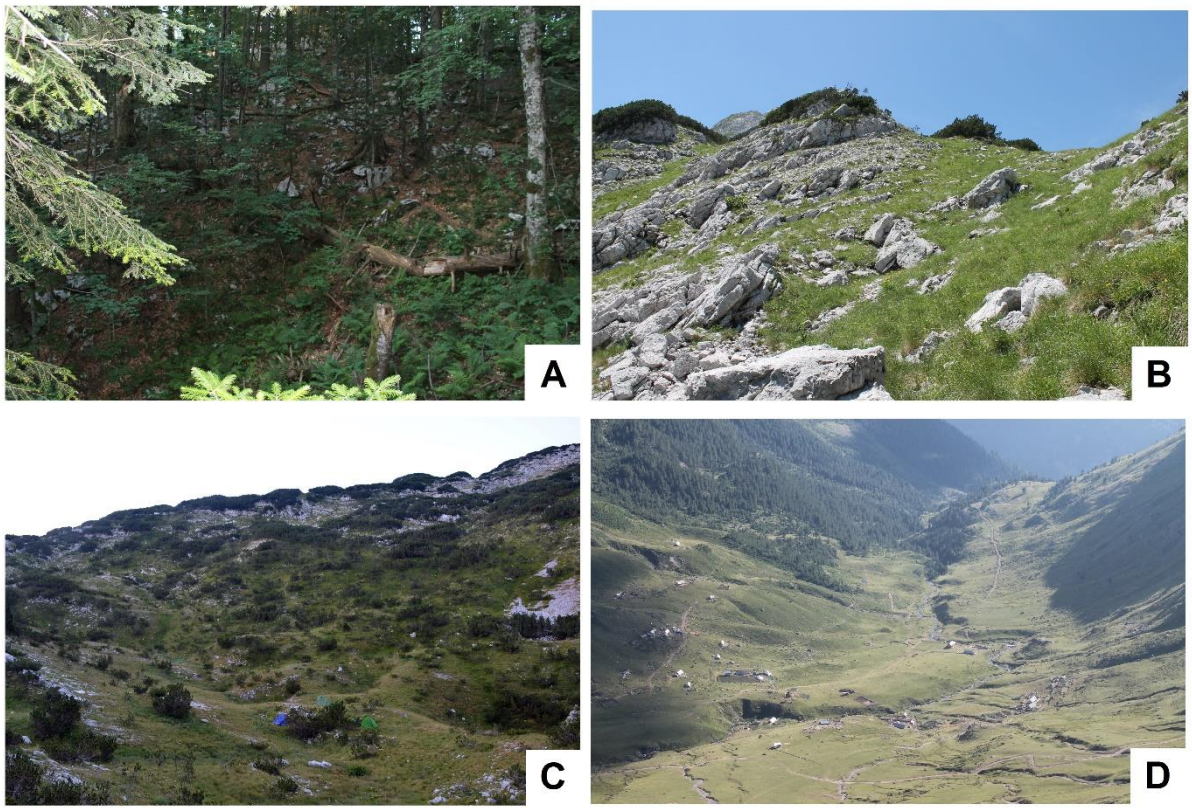

Figure 2.2: Typical habitat of *Salamandra atra* in the populations included in this study. A) Gorski Kotar (Croatia), B) Prenj (Bosnia & Herzegovina), C) Čvrsnica (Bosnia and Herzegovina) and D) Prokletije (Montenegro). Pictures belong to Šunje, E. (A, B & D) and Zimić, A. (C).

The last population of *S. atra* sampled in this study lives in the Bogićevića massif (42°35'05.0"N, 20°03'57.0"E) in the southeast of Montenegro, close to the border of Kosovo and Albania, part of the National Park Prokletije. Salamanders were found in wet grass fields and/or fir forests with rocky underground at an altitude around 1790 meters. In contrast to the other populations, where no surface water was present, the salamanders of Prokletije were found next to a small mountain stream (see figure 2.2d). This was also the only population in this study where humans were resident in the immediate vicinity.

2.1.2. Toxin sampling

The procedure for toxin sampling was adapted from Vences et al. (2014). Animals were caught in the field during the night and transported in plastic boxes to the field lab. Before toxin collection, each animal was weighed with a balance (precision of 0.01 gram, Camry Electronic Ltd, Zhongshang, China) and head length and width were measured with an electronic digital caliper (precision of 0.01 mm, Conrad Electronic, Hirschau, Germany). Additionally, I measured the width and the length of the left parotoid gland as an indirect measure for the amount of toxin produced (Saporito et al., 2010; Jeckel et al., 2015). The sex of the animal was determined based on the morphology of the cloaca (Luiselli et al., 2001). Next, the left parotoid gland was gently squeezed and the released toxin was collected with a small piece of sterile gauze. Due to the sticky and mucous nature of the secretions, it was not possible to take the exact same amount of toxins from each salamander. Instead, I kept squeezing until the gland did not release any more toxin. This gauze was then stored in an empty 1.5 mL plastic Eppendorf tube.

After toxin collection, the salamanders were released back in the area where they were found. Since these salamander populations live on relatively remote locations and because fieldwork in most cases lasted for several days, toxin samples could not be stored in a fridge up until return from the fieldwork. However, I always tried to keep the toxin samples at temperatures as low as possible (e.g. out of the sun, in a cabin when possible). Upon returning from fieldwork, gasiform argon (MESSER, Sarajevo, Bosnia and Herzegovina) was immediately added to the Eppendorf tubes and samples were stored at 4°C. Since argon is heavier than air it formed a protective layer around the toxin on the gauze and thus prevented oxidation processes and degradation of toxin compounds.

2.1.3. Soil sampling

Soil samples were taken in order to measure bacterial densities and to detect potential fungal pathogens in the environment, and thus estimate infection risk for the salamanders. This study specifically used soil samples since *S. atra* is a strictly terrestrial species (Jeran et al., 2011; Helfer et al., 2012) that spends a large proportion of its life underground (in crevices, burrows of mammals, coarse woody debris, …) (Gautier & Miaud, 2003; Helfer et al., 2012). Therefore, *S. atra* is almost continuously exposed to soil micro-organisms.

Soil samples were always collected at the end of a fieldwork session to minimize sample degradation. Soil samples were taken from all different micro-habitats in which salamanders were found during the toxin collection, with special attention to the crevices and holes they use as shelter during the day. Soil samples were taken in duplicate: one series of samples for bacterial cultivation, one series of samples for genomic analysis. Before and after each sample, our equipment was sterilized by cleaning it with 70% ethanol and heating it over an open flame of a camping stove. Soil samples were stored in 1.5 mL plastic Eppendorf tubes. In order to protect microbial DNA from degradation, LifeGuard® Soil Preservation Solution (MO BIO Laboratories, Inc., Carlsbad, USA) was directly added to the genomics-samples. After fieldwork, soil samples were stored at 4°C.

2.2. Lab work

2.2.1. Toxin analysis

Toxins were extracted from the gauzes by adding 400 µL 50% methanol solution to the Eppendorf tubes. After 30 minutes incubation, 200 µL of the 50% methanol solution was transferred to a spin filter that was centrifuged for 15 minutes at 7000 rpm in an Eppendorf centrifuge 5810R (Eppendorf AG, Hamburg, Germany). After filtration, the liquid was transferred to inserts for Ultra Performance Liquid Chromatography – tandem Mass Spectrometry (UPLC-MS/MS).

An ACQUITY UPLC system coupled to an ACQUITY TQD mass spectrometer (Waters, Milford, USA) was used to measure the samples. The column used was an ACQUITY UPLC® BEH C18 1.7µm, 2.1 X 100mm column. Solvent A= H2O + 0.1% of FA and solvent B= CAN + 0.1% FA. For every run, 6 µL solution was injected in 'partial loop' modus. All measurements were done in positive electrospray mode (ES+). Following conditions were set up: capillary = 1.78-1.80 kV, source temperature = 120 °C, desolvation temperature = 400°C. Nitrogen gas was used as desolvation gas and argon as collision gas.

Ultra Performance Liquid Chromatography – tandem Mass Spectrometry (UPLC-MS/MS) is widely used to separate and analyse compounds of complex organic mixtures with a very high specificity (McMaster, 2005; Pitt, 2009). Previous analyses on toxins from *Salamandra* species used gas chromatography – mass spectrometry (GC-MS) (Mebs & Pogoda, 2005; Vences et al., 2014). UPLC-MS/MS is able to analyse a much broader range of organic molecules (e.g. molecules not volatile enough to be separated by gas chromatography, higher thermostability, …) and has an increased sensitivity and better selectivity (McMaster, 2005; Alder et al., 2006).

Initially, mass spectra were recorded following thr full-scan mode. Peaks were then selected in the resulting mass spectrum that corresponded to the molecular weight of the samandarines known from the literature (Habermehl & Spiteller, 1967), an approach also followed by Vences et al. (2014). These first analyses however showed that full-scans were less efficient in differentiating between peaks from

compounds with a similar structure. Due to the high similarity between different samandarines, several compounds will be broken down to fragments with the same molecular weight as other compounds. These different compounds might then appear as one peak. As a consequence, Multiple Reaction Monitoring (MRM) was used to analyse the composition of the toxin samples. MRM will detect specific compounds based on the specific ion of this compound and one or more of its fragment ions.

Thanks to this precursor-fragment transition, the MRM method tends to be more specific, and therefore more suited to analyse the alkaloid-composition of the samples than the full-scan method. The selected MRM-settings are represented in table 8.1. in appendix. An example of a MRM-scan for each compound is given in figure 8.2 in appendix. Note that there are more described samandarines and derivatives (see Habermehl & Spiteller, 1969; Daly et al., 2005), but not all samandarines were detected in the first trail runs, meaning they were either not present or only present at concentrations too low for detection. For samanol two different peaks were found in each chromatogram, but due to not having any reference standards, I was not able to unambiguously designate one peak as samanol. Hence, the two peaks will be referred to as samanol and samanol2. No reference standards were used for verification and calibration, since these are not commercially available for samandarines.

2.2.2. Test degradation of toxins

Since most samples were stored for several weeks before analysis, a small experiment was conducted to check whether degradation of the toxins occurred. Nine Bosnian salamanders, kept in captivity at the University of Antwerp, were sampled and their toxins were analysed at three different times: 1) on the day the salamanders were sampled, 2) after five days of storage and 3) after thirty days of storage.

In order to check whether my method of storing salamander toxins was efficient, degradation of toxins was compared among three different treatments: 1) with argon at 4°C , 2) without argon at 4°C and 3) without argon at room temperature (20°C). Three salamanders were randomly assigned to each treatment. Analysis of the toxins with UPLC-MS/MS was conform the previous described method.

Salamanders were kept in plastic boxes (45x65x25 cm) covered in soil with branches, stones and moss as enrichment. Each box housed 5 to 6 individuals. Since animals were close to hibernation and were thus relatively inactive during this period, they were only fed once a week ad libitum with crickets, mealworms and earthworms. Temperature varied between 12-17°C and humidity around 85-95 %. Salamanders were checked daily and soil was changed every three weeks.

2.2.3. Cultivation of soil samples

Half of the soil samples were cultivated in order to estimate the bacterial densities in the soil in the four populations. Three different growth media were used: Tryptic soy broth (TSA), on which a broad range of heterotrophic bacteria are able to grow (Atlas, 2010), and MacConkey and Slanetz-Bartley media,

which are more selective for respectively coliform bacteria and Enterococci (Atlas, 2010). Cultures were incubated at two different temperatures: 35°C, to estimate the number of potential pathogens, and 15°C, in order to estimate the total bacterial load.

For each sample, 0.25 gram of soil was transferred to a 15 mL BD Falcon™ conical tube. Then 2.25 mL PBS (Phosphate buffered saline) was added and the tube was vortexed. Next, a series of tenfold dilutions was made, ranging from 10^0 to 10^{-4} times the original concentration, using PBS as diluent. Of each dilution 200 µL was transferred to an agar plate and spread over the surface of the plate. Each dilution was transferred to agar plates of all three types of growth media. The plates were then incubated at 35°C for one night. Afterwards, a second series of cultures was made following the same protocol, but only using TSA agar plates and at an incubation temperature of 15°C for two days. *Salamandra atra* is an ectotherm occurring in cold alpine environments. Nevertheless, the cultivation at 35°C has the advantage that many bacteria occurring on the skin of amphibians (such as *Pseudomonas* sp., see Bettin & Greven, 1986) show optimal growth at higher temperatures (Pasmans, personal communication).

After incubation, I selected for each series of agar plates the lowest dilution on which the number of colonies was countable. After counting colonies, formula 1 was used to calculate the original concentrations of Colony Forming Units (CFU's) in the soil samples.

$$Bacterial\ densities\ \left(\frac{CFU's}{g}\right) = \frac{counts * dilution * 5}{0.25\ g\ soil} \qquad (1)$$

2.2.4. Identification of soil fungi

DNA extraction and PCR

To isolate genomic DNA from the soil samples, a Powersoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, USA) was used. Following the accompanying protocol, 100 µL DNA-solution was obtained from 0.25 grams of each soil sample. DNA-solutions were stored at -20°C. One negative control was produced by following the same steps of the protocol but without any soil, in order to account for lab-contamination.

For the PCR, the protocol of the Phusion® High-Fidelity PCR kit (New England Biolabs Inc., Ipswich, England) was followed. 1 µL DNA was used as template in a 25 µL PCR-mix containing 16.3 µL nuclease free water, 5 µL Phusion® GC-buffer, 0.5 µL deoxynucleotide solution mix, 1 µL forward primers, 1 µL reverse primers and 0.2 µL Phusion® DNA Polymerase.

The following primers specific for fungal ribosomal RNA genes (White et al., 1990) were used: ITS1 5'- CTTGGTCATTTAGAGGAAGTAA -3' and ITS2 5'- GCTGCGTTCTTCATCGATGC -3'

(Integrated DNA Technologies, Inc., Leuven, Belgium). These primers were modified for Ilumina sequencing by adding multiplexing barcodes as in Smith & Peay (2014).

A C1000 Touch™ Thermal Cycler (Bio-Rad, Hercules, USA) was used. PCR conditions were as follows: initial denaturation at 98°C for 30s, an extra denaturation step at 98°C for 30s, annealing at 55°C for 30s and extension at 72°C for 30s. The last three steps were repeated 34 times, after which an extra-long extension step for 10 minutes was added. Both a negative control (PCR mix without DNA) and a positive control (DNA sample that was amplified successful in a previous study) were also produced.

Success of the PCR was verified by agarose gel-electrophoresis. For this, 5 µL of the PCR-amplicon was loaded on a 1.5 % agarose gel, adding also 1 µL Midori Green Direct (Nippon Genetics Europe, Düren, Germany) and 1 µL Gel Loading Dye, Orange (6X) (New England Biolabs Inc., Ipswich, England). Electrophoresis conditions were as follows: 45 minutes at 110 V. The gel was then checked by placing it in a FastGene® FAS Digi Imaging System (Nippon Genetics Europe, Düren, Germany).

For unsuccessful PCRs, the following solutions were applied: 1) Redo the DNA extraction, 2) make the DNA sample more pure by DNA ethanol precipitation and 3) increase the number of cycles to 40.

Next generation sequencing

To prepare the amplified DNA-samples for sequencing, PCR amplicons were purified and their concentrations were normalized using the SequalPrep™ Normalization Plate Kit (Invitrogen Corporation, Carlsbad, USA). PCR amplicons were then pooled into one DNA library, which was loaded on a 1.5 % agarose gel (110 V for 30 minutes). However, no band was visible on the agarose gel, thus I assumed DNA-concentrations of the library were too low. In order to increase the concentration of the library, an extra washing step was added following the protocol of the QAIquick® Gel Extraction Kit (Qiagen, Venlo, the Netherlands), but here the DNA library was distributed over five spin filters. After adding the eluent (Buffer EB), the flow-through of the five spin filters were pooled in order to create a new library. To check the success of this procedure, once again a gel electrophoresis was done, with positive results this time. The DNA-band was cut out of the gel in order to separate the amplified DNA from any remaining primers. DNA was then again washed and cleaned following the protocol of the QIAquick® Gel Extraction Kit (Qiagen, Venlo, the Netherlands). Note that I always waited five minutes after adding Buffer PE or Buffer EB to the spin filters before centrifuging.

As an extra check, the DNA library was quantified by the means of a qPCR, using the KAPA Library Quantification Kit Illumina® Platforms (KAPA Biosystems, Wilmington, USA). A C1000 Touch™ Thermal Cycler (Bio-Rad, Hercules, USA) was used with addition of a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, USA). PCR conditions were as described in the protocol of

the KAPA Library Kit Illumina® Platforms (Initial denaturation at 95°C for 5 min, denaturation step at 95°C for 30s, annealing/extension/data acquisition at 60°C for 45s. 35 cycles. Melt curve analysis at 65-95°C).

The DNA-library was prepared using a MiSeq® v2 Reagent Kit (Illumina Inc., San Diego, USA). The diluted and denaturised product was then sequenced on an Illumina MiSeq™ using default v2 chemistry for 300 cycles in the forward direction, in presence of a 10% spike of PhiX Control v3 (Illumina Inc., San Diego, USA) as an internal control and to increase cluster diversity.

Analysis of fungal ITS sequences

Sequences were analysed following the UPARSE fungal pipeline described in Edgar (2013) and Smith & Peay (2014) in order to reduce the amount of artefacts, like singletons (sequences for which there is only one read, and thus have a high change of representing a PCR error) or chimeras (sequences that were merged during PCR).

Sequences were first trimmed to a length of 250 bp and quality filtered according to a maximum estimated error of 0.5 %. If these trimmed sequences still contained the reverse primers, these were removed and replaced by 'N's in order to keep a total length of 250 bp (in order to avoid problems with clustering). Before clustering, the samples were de-replicated, and sorted by size. After removing singletons, 26 389 sequences were retained. These were clustered into Operational Taxonomic Units (OTUs) based on a similarity of 97%. An OTU is a group of sequences that corresponds to one taxonomic clade or monophyletic group (Edgar, 2013). Sequences were clustered into 4377 OTUs of which 3873 were non-chimeric. Chimeras were removed by comparing them to a database of known fungal OTUs (in this case the UNITE database of ITS1 sequences). Previously added 'N's were thereafter removed. Next, an OTU table is constructed and OTUs are blasted against the UNITE database. Non-fungal OTUs were removed from the dataset, resulting eventually in 2661 fungal OTUs.

OTUs were assigned to a certain lifestyle if they matched genera with a known lifestyle, as found in Tedersoo et al. (2014). The following lifestyles were recognized: animal parasite, ectomycorrhizal, lichen, mycoparasite, plant pathogen, saprotroph-brownrot, saprotroph-filamentous, saprotroph-whiterot and saprotroph-yeast.

2.2.5. Predation pressure

Estimating predation pressure is notoriously difficult. In the timeframe available, it was not possible to perform the exhaustive population monitoring that is required to assess predation pressure at each of the locations. In studies of lizards, clay or plasticine models are sometimes placed in the natural habitat for a while, and attack rate on this models is used as an indicator for predation rate (Vervust et al., 2007; Bateman et al., 2016). However, a main point of criticism on this method is that while it may be

appropriate in case of visually hunting predators (like birds), it might be less fitting for predators that rely on olfactory cues or heat signatures (such as snakes) (Bateman et al., 2016).

Instead, I opted to review the literature on the presence of species that potentially predate on *S. atra* in the four populations. I have chosen here to only include predators that have been confirmed to predate on *S. atra* (either in these or other populations), or other species from the same genus. Apart from literature, local biologists were contacted for information on the presence of certain species.

2.3. Data analysis

The software program R version 3.2.3. (Ihaka, R. & Gentleman, R., University of Auckland, New Zealand) was used for statistical analyses unless mentioned otherwise.

2.3.1. Toxins

Juveniles were removed from the dataset, since only 10 of the 149 sampled salamanders were juveniles, and in one population (Čvrsnica) no juveniles were found at all. MassLynx 4.1. (Waters Corporation, Milford, USA) was used to calculate peak surface areas of the alkaloids in the toxin samples. Peaks with a surface area lower than 30 were excluded from the dataset, since it could not be said with absolute certainty whether such low peaks represented actual toxins or were just random noise. Toxin data were analysed in two different ways.

The first method is used as an indication for the quantity of secreted toxins. As explained earlier, the parotoid glands were squeezed until the salamanders did not release any more toxins. The entire content of a parotoid gland was thus emptied and collected, and always dissolved in the same amount of 50% methanol. The higher the amount of secretions collected, the higher the concentration of toxins injected in the UPLC-MS/MS, which will result in higher peaks with a larger surface area on the chromatogram (figure 2.3). The sum of the surface areas of all compounds per sample can therefore be used as an indicator for the total amount of toxins secreted by an individual.

To test for differences between populations and sexes, I performed a two-way ANCOVA with parotoid width as a covariate. Parotoid width was included as covariate in order to correct for body size, since it is possible that larger salamanders will secrete more toxins (Saporito et al., 2010; Jeckel et al., 2015). Not all salamanders were weighed, but parotoid width was positively correlated with body mass (see further) and could therefore be used as a substitute for body size.

The second method of toxin data analyses was used to get an idea about the presence of the individual toxins relative to each other. In order to calculate this relative 'contribution' the peak surface area of the particular compound was taken and divided by the total surface area of all peaks from that sample (figure

2.3). Since this proportion does not really represent the biological concentration of compounds in the skin secretions, I will refer to this as the 'relative contributions'.

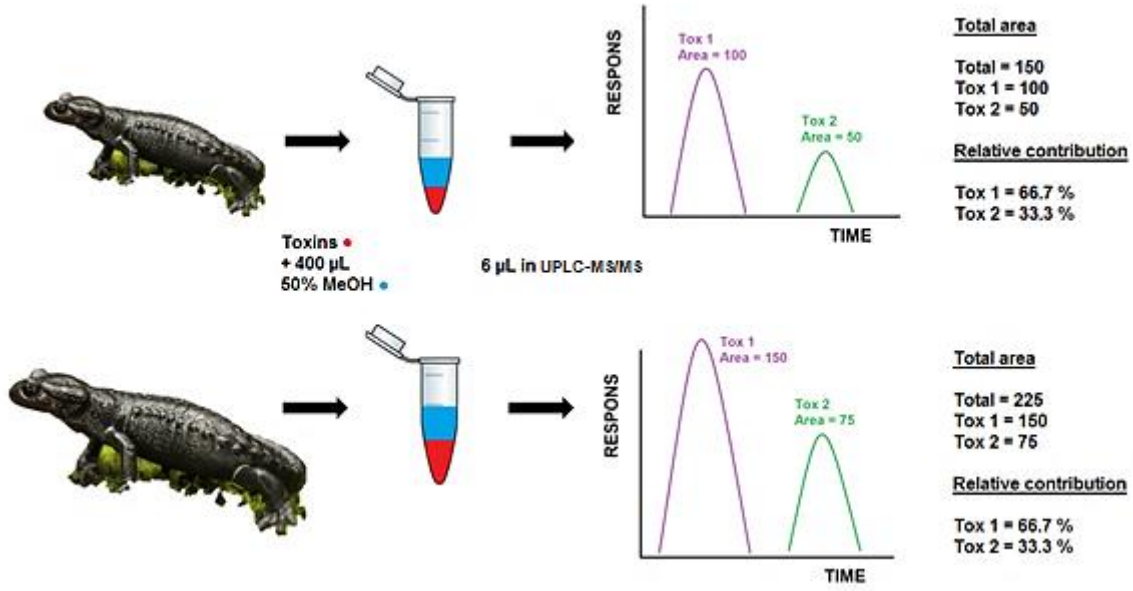

Figure 2.3: Procedure for toxin analysis. Parotoid glands from salamanders were squeezed until they did not release any more toxins. All released poison (red) was then collected, and back in the lab dissolved in 400 µL 50 % methanol (blue). After extraction, 6 µL solution was injected in the UPLC-MS/MS. Chromatograms could be processed in two different ways: 1) the sum was taken of the surface areas of all peaks to calculate the total peak surface area (e.g. top: total area = 150). This total area was then used as an indicator for the total amount of toxins secreted by salamanders. 2) The contribution of each compound relative to the total surface area was calculated (e.g. top: tox 1 = 100/150 = 66.7 %). As illustrated, it can be expected that larger salamanders secrete more toxins, resulting in larger peaks. Relative contributions are however independent from size.

Relative contributions were subjected to a Principal Component Analysis (PCA) to test which compounds were most responsible for toxin variation. Principal components with an eigenvalue higher than 1 were used for further statistical analyses. Individual variables were considered important if their loadings on the principal component was higher than 0.300 or lower than -0.300. To test for differences in toxin composition among populations and between sexes, I performed separate two-way ANOVAs on the principal components with an eigenvalue higher than 1. In order to see if the populations did differ significantly in *overall* toxin composition, an Analysis of Similarities (ANOSIM) was used (vegan package in R). This approach is often taken in studies on variation in alkaloid composition among populations of poison frogs (Saporito et al., 2007; Saporito et al., 2009; Mina et al., 2015). An ANOSIM returns a test statistic R which varies between -1 and 1. If R equals 0, there is no difference between within group and between group similarities. If $R > 0$, the within group similarity is larger than the between group similarity. Pairwise ANOSIMs are not possible with the vegan package, so in order to see which populations were more similar, I did separate one-way ANOSIMs for each pair of populations.

Differences between sexes were also tested with a one-way ANOSIM. The ANOSIMs were performed on Aitchison dissimilarity matrices.

I performed separate two-way ANOVAs to test for differences in the relative contribution of samandarine and samandarone between populations and sexes, since these compounds are believed to be respectively the most toxic and the most antimicrobial compound. In addition, I also performed separate two-way ANOVAs for samandaridine and samandenone, since these two compounds were quite abundant in the skin secretions (table 8.2 in appendix).

In case the ANOVAs or ANCOVAs indicated significant differences, a Tukey's HSD test was used to see which specific pairs of populations differed significantly.

2.3.2. Test degradation of toxins

Degradation of toxins was analysed using a general mixed model, with peak area surface of each compound (log-transformed) as response variable and time as continuous covariate. Type of toxin and treatment were included as fixed factors and animal ID as a random factor. I also included interactions between time and type of toxins, to see whether different toxins degrade at different rates, and between time and treatment.

2.3.3. Morphology

Covariation in morphological measurements was analysed using a Principal Component Analysis (PCA). The following morphological measurements were included in the PCA: parotoid width and length, head length and width and body mass. Principal components with an eigenvalue higher than 1 were used for further statistical analyses. Individual variables were considered important if their loading on the principal component was higher than 0.300 or lower than -0.300.

In order to test for size differences among populations and between sexes, a two-way ANOVA was used including the principal components (with an eigenvalue higher than 1) as response variable and population and sex as fixed factors. Ten salamanders from Gorski Kotar were excluded from the dataset, since head width, head length and body mass were not known for these individuals.

2.3.4. Bacterial concentrations

To test whether populations differed in bacterial density in the soil, general mixed models were used with bacterial densities (expressed as number of CFUs per gram soil) as response variable, population as fixed factor and microhabitat as random factor. The models were stepwise reduced by eliminating the least significant term. Bacterial density was in all models log-transformed to obtain normally distributed data. I ran separate models for the data obtained on each medium. For the TSA-media incubation temperature (35°C or 15°C) was included as an additional fixed factor.

2.3.5. Soil fungi

Reads from unidentified OTUs (NA) were removed from the dataset. For each sample the number of reads of animal parasites was expressed proportional to the total number of reads in that sample. In a large number of samples, there were no reads of animal parasites, but this might be due to their low abundance. To take this detection limit into account, zeros were replaced by lowest possible number of reads detected in that sample, calculated as 1/(total number of reads in that sample). Data were log-transformed to obtain normality. A general mixed model was used, with population as a fixed factor and microhabitat as a random factor.

The total number of fungal species (normal distribution) was also compared among populations using a similar general mixed model, but the total number of reads were included as a continuous covariate to account for the possibility that a higher number of species might be due to a higher number of reads in a sample.

Additional ANOSIMs were performed to test how similar the populations were in soil fungi communities, both for the complete dataset, as well as for each pair of populations separately similar to the approach followed for the toxin data. Since the abundance of OTUs in each population are considered count data, ANOSIMs were done based on Bray-Curtis dissimilarity matrices.

A Spearman's rank correlation test was used to check whether dissimilarity in toxin composition between populations was correlated with dissimilarity in soil fungi community between populations. With other words: are populations with similar toxin composition exposed to similar soil fungi communities?

3. Results

3.1. Toxins

3.1.1. Descriptive statistics

Toxins were collected from a total of 139 salamanders: 24 individuals from Gorski Kotar (HRV), 25 from Čvrsnica (BIH), 44 from Prokletije (MNE) and 46 from Prenj (BIH). In Gorski Kotar and on Prenj an equal number of males and females were sampled, where on Čvrsnica more females were found (64%) and in Prokletije more males (only 43% females).

All 8 toxins shown in figure 8.1 and table 8.1. in appendix were found in the samples. Other toxins known from literature (Habermehl & Spiteller, 1967) were not found in the first test runs, during which a random selection of samples from each population were taken, and were thus not further investigated. All 8 toxins were found in all populations, but not necessary in all individuals. Samandiol, samanol and samandarone were not detected in respectively 2, 12 and 16 samples.

Samandarine was the major compound in the skin secretions of alpine salamanders, with a minimum relative contribution of 46.5 % and a maximum of 86.7 %. Samandenone (3.4 to 23.7 %) and samandaridine (1.8 to 20.8 %) were the second and third most abundant compounds. Samandarone (0 to 0.14 %) and samanol (0 to 0.44 %) were the least abundant compounds. An overview of average relative contributions of each compound is given in table 8.2. in appendix.

3.1.2. Total amount of toxins

The total amount of secreted toxins, expressed as the sum of all peak surface areas, differed significantly among populations ($F_{3,130} = 9.74$; $p < 0.001$), but not between sexes ($F_{1,130} = 1.11$; $p = 0.29$). Interaction between population and sex was not significant ($F_{1,127} = 1.03$; $p = 0.38$). As shown by a Tukey's HSD test, salamanders from Gorski Kotar secreted significantly less toxins compared to other populations (all $p < 0.05$) (figure 3.1). There was a significant interaction between population and parotoid width ($F_{3,130} = 5.27$; $p = 0.002$). In most populations the amount of secreted toxins increased with parotoid width, except on Prenj, where peak surface area decreased by 56% per millimetre increase in parotoid width (figure 3.2).

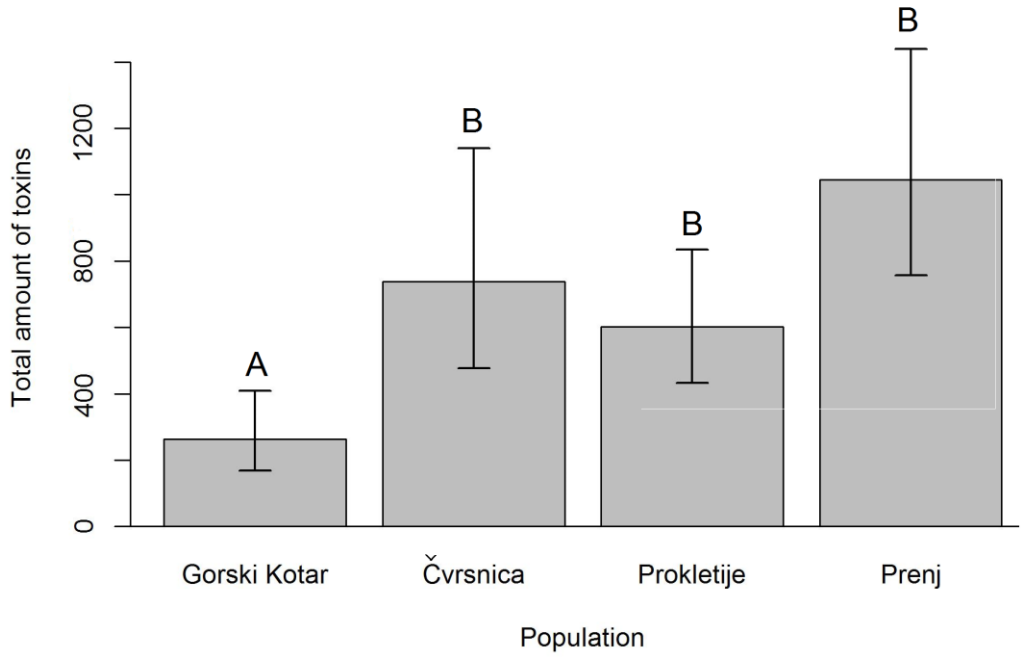

Figure 3.1: Average total amount of toxins secreted by individual salamanders (*Salamandra atra*) in four different populations. Total amount of toxins was calculated as the sum of the peak surface areas of all compounds per sample (divided by 1000). Different letters indicate significant differences at $p < 0.05$ as shown by a Tukey's HSD test. Error bars represent 95% confidence intervals. Sample sizes were as follows: Gorski Kotar (24), Čvrsnica (25), Prokletije (44) and Prenj (46). Females (70) and males (69).

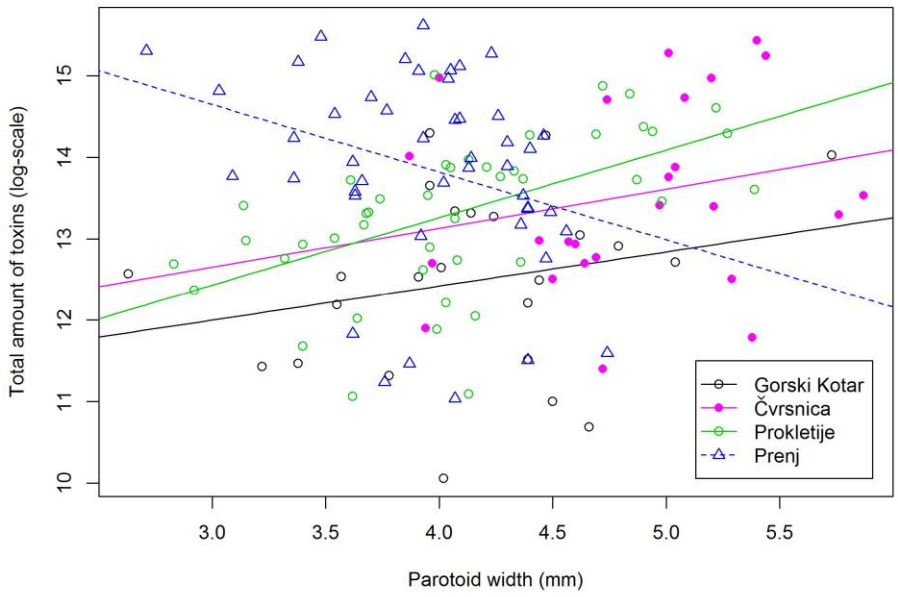

Figure 3.2: Association between parotoid width (mm) and the total amount of toxins secreted (indicated by the total peak surface area) per population. Peak surface areas were log-transformed. Significant differences between slopes are indicated by different line-types. Sample sizes were as follows: Gorski Kotar (24), Čvrsnica (25), Prokletije (44) and Prenj (46). Females (70) and males (69).

3.1.3. Relative contributions

Populations of *S. atra* differed significantly in toxin composition (ANOSIM R = 0.376; $p < 0.001$). The largest dissimilarity was found between Gorski Kotar and Čvrsnica, while Prokletije and Čvrsnica were the most similar (table 3.1). Male and female salamanders did not differ significantly in toxin composition (ANOSIM R = 0.05; p = 0.09).

The first three components of the PCA were significant (eigenvalue > 1), and explained together 73 % of the variance in toxin composition (table 3.2). Higher scores on PC1 (explaining 36 % of the total variance) reflected higher relative contributions of ecomytrin, samandenone and samanine, but lower relative contributions of samandiol and samandarine. PC2 explained 21 % of the total variance. Positive scores on PC2 reflected higher contributions of samandaridine, while negative scores reflected higher relative contributions of samanine, samanol and samanol2. See figure 3.3 for a visualisation of the first two principal components. Higher scores on PC3 (explaining 15 % of the total variance) reflected higher relative contributions of ecomytrin, but lower contributions of samandaridine, samandarone and samanol2.

Table 3.1: Dissimilarities in toxin composition among populations of *Salamandra atra* indicated by the ANOSIM R statistic. R-values > 1 indicate that the similarity within sites is larger than the similarity between sites. All differences were statistical significant ($p < 0.05$). Sample sizes were as follows: Gorski Kotar (24), Čvrsnica (25), Prokletije (44) and Prenj (46). Females (70) and males (69).

	Gorski Kotar (HRV)	**Čvrsnica (BIH)**	**Prokletije (MNE)**	**Prenj (BIH)**
Gorski Kotar (HRV)	-			
Čvrsnica (BIR)	R = 0.536	-		
Prokletije (MNE)	R = 0.362	R = 0.330	-	
Prenj (BIH)	R = 0.401	R = 0.512	R = 0.348	-

PC1-scores differed significantly among populations ($F_{3, 131} = 41.86$; $p < 0.001$) but not between sexes ($F_{1,131} = 1.48$; p = 0.23). Gorski Kotar and Čvrsnica has significantly higher PC1-scores (Tukey's HSD: all $p < 0.001$), and thus higher relative contributions of samanine, ecomytrin and samandenone than Prokletije and Prenj, where higher contributions of samandiol and samandarine were present (figure 3.4a). Interaction between population and sex was not significant ($F_{3,131} = 0.68$; p = 0.56).

Table 3.2: Results of the Principal Component Analysis of the toxin compositions of the salamanders. The first three components (PC1, PC2 and PC3) are given. For every component, the eigenvalue and the proportion of variance explained by this component is given, as well as the loadings of the individual variables. Individual compounds were only considered important if they had a loading higher than 0.300 or lower than -0.300. These loadings are indicated in bold.

	PC1	PC2	PC3
Eigenvalue	1.81	1.39	1.17
Proportion of total variance	0.36	0.21	0.15
Ecomytrin	**0.393**	0.115	**0.459**
Samandaridine		**0.442**	**-0.526**
Samandenone	**0.501**	0.166	
Samandiol	**-0.351**	-0.259	0.127
Samandarine	**-0.512**	-0.136	0.131
Samandarone		0.115	**-0.488**
Samanine	**0.406**	**-0.384**	
Samanol	0.101	**-0.616**	-0.185
Samanol2	0.164	**-0.372**	**-0.451**

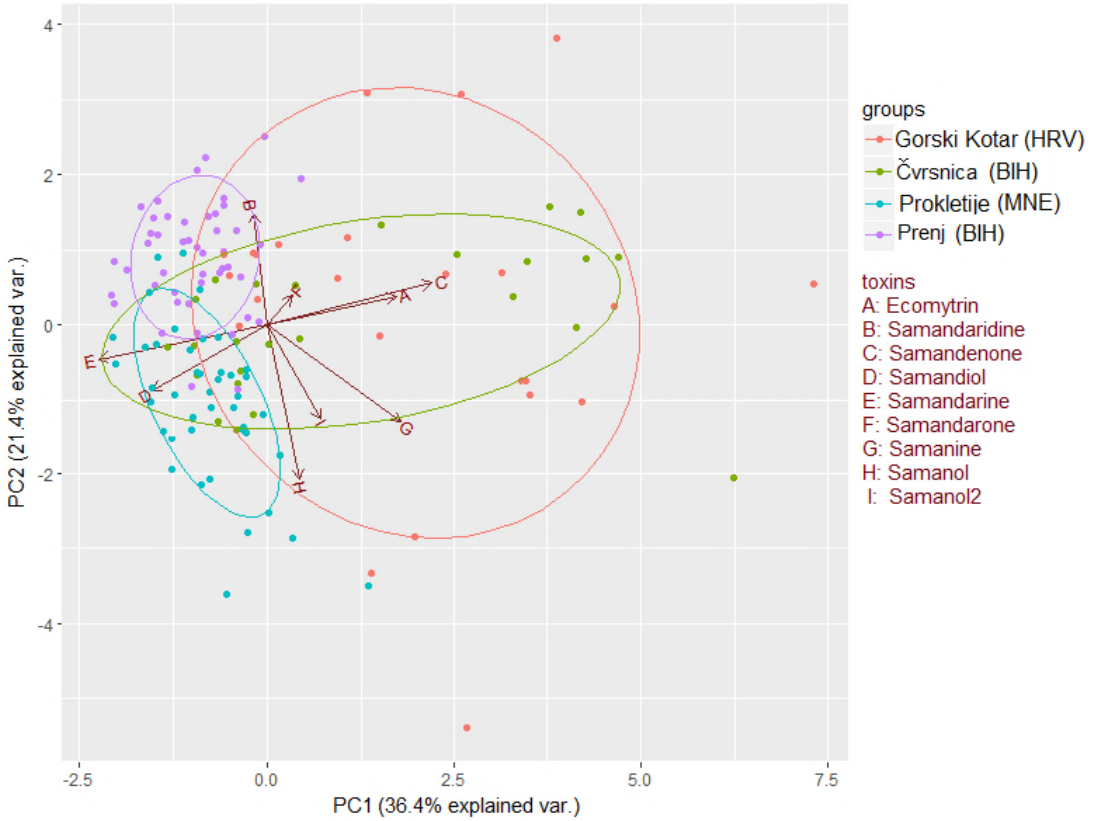

Figure 3.3: Visualisation of the Principal Component Analysis of the toxin composition. The first two principal components are shown (for their meanings, see table 3.2. Different letters represent different toxins, and the direction of the arrows indicate the correlations between their relative contributions. Different colours indicate samples from different populations. Ellipsoids represent normal data ellipses (probability of 68%) for each population. Sample sizes were as follows: Gorski Kotar (24), Čvrsnica (25), Prokletije (44) and Prenj (46).

There was a significant difference in PC3-scores among populations ($F_{3,131}$ = 14.39; p < 0.001) and between sexes ($F_{1,131}$ = 9.12; p = 0.003). Čvrsnica and Prokletije had significantly higher PC3-scores, and thus lower relative contributions of samandarone, than Prokletije and Prenj (figure 3.4b). Males had higher scores than females (figure 3.4c). Interaction between population and sex was not significant ($F_{3,131}$ = 1.43; p = 0.24).

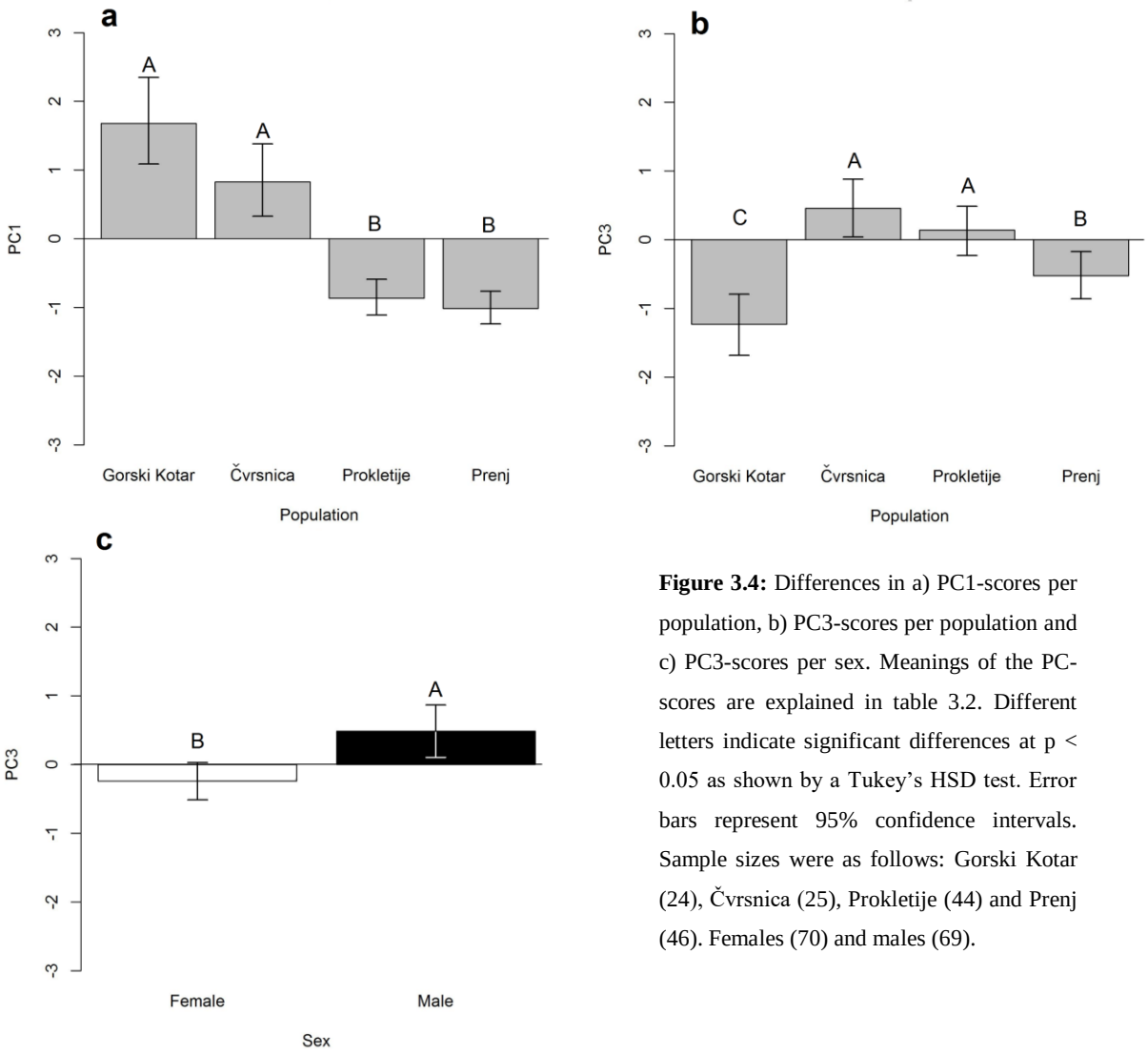

Figure 3.4: Differences in a) PC1-scores per population, b) PC3-scores per population and c) PC3-scores per sex. Meanings of the PC-scores are explained in table 3.2. Different letters indicate significant differences at p < 0.05 as shown by a Tukey's HSD test. Error bars represent 95% confidence intervals. Sample sizes were as follows: Gorski Kotar (24), Čvrsnica (25), Prokletije (44) and Prenj (46). Females (70) and males (69).

For the PC2-scores, I found a significant interaction between sex and population ($F_{3,131}$ = 6.76; p < 0.001). Females of the Gorski Kotar population scored higher on this axis than males (Tukey's HSD: p < 0.001), while no differences between the sexes were found in the other populations. Overall, scores for Gorski Kotar and Prenj were high compared to those for Prokletije and Čvrsnica (population effect: $F_{3,131}$ = 25.29; p < 0.001; figure 3.5). Gorski Kotar and Prenj had the highest scores and thus the highest relative contributions of samandaridine, while Prokletije and Čvrsnica had lower scores and higher relative contributions of samanine, samanol and samanol2.

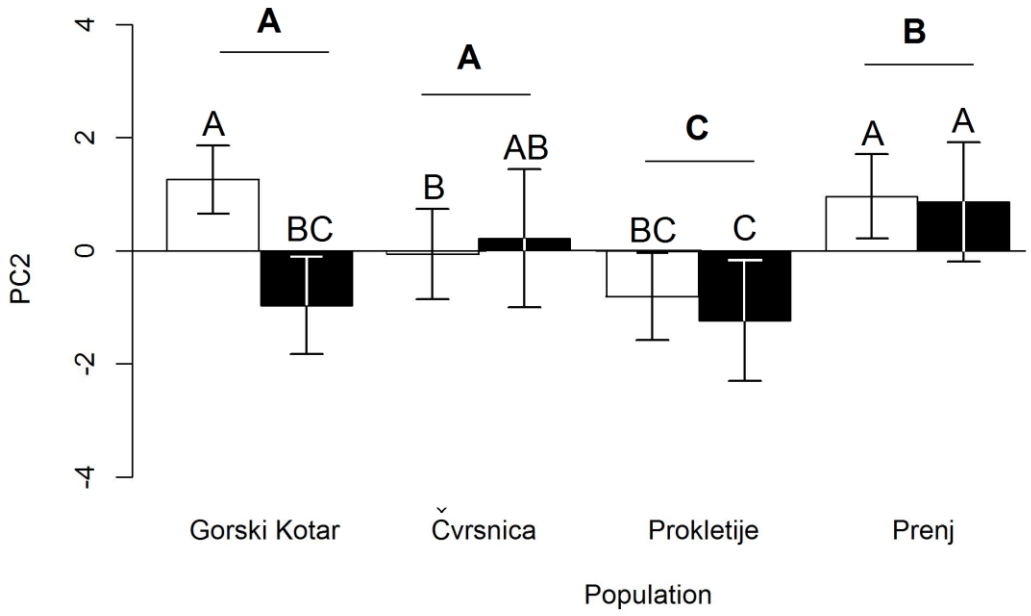

Figure 3.5: Differences in PC2-scores per population and per sex. White bars = females, black bars = males. Meanings of the PC-scores are explained in table 3.2. Different letters indicate significant differences at $p < 0.05$ as shown by a Tukey's HSD test. Error bars represent 95% confidence intervals.

Populations differed statistically in the relative contributions of samandarine ($F_{3,131} = 41.25$; $p < 0.001$), samandarone ($F_{3,114} = 3.21$; $p = 0.03$) and samandenone ($F_{3,134} = 55.74$; $p < 0.001$). A Tukey's HSD test showed that all populations differed from one another in samandarine (all $p < 0.05$), with the highest relative contributions of samandarine found in Prokletije and the lowest in Gorski Kotar (figure 3.6a). Salamander toxins from Gorski Kotar had significantly higher relative contributions of samandarone compared to Prokletije (Tukey's HSD: $p = 0.03$) and Prenj (Tukey's HSD: $p = 0.04$) (figure 3.6b). Significantly higher relative contributions of samandenone were found in Gorski Kotar and Čvrsnica (Tukey's HSD: all $p < 0.05$) (figure 3.6c) There were no differences between the sexes, neither for samandarine ($F_{1,131} = 0.25$; $p = 0.62$), nor for samandarone ($F_{1,114} = 3.60$; $p = 0.06$) or samandenone ($F_{1,134} = 0.0072$; $p = 0.93$).There were no significant interactions, nor for samandarine ($F_{3,131} = 1.15$; $p = 0.33$), samandarone ($F_{3,114} = 1.80$; $p = 0.15$) or samandenone ($F_{3,131} = 1.99$; $p = 0.12$)

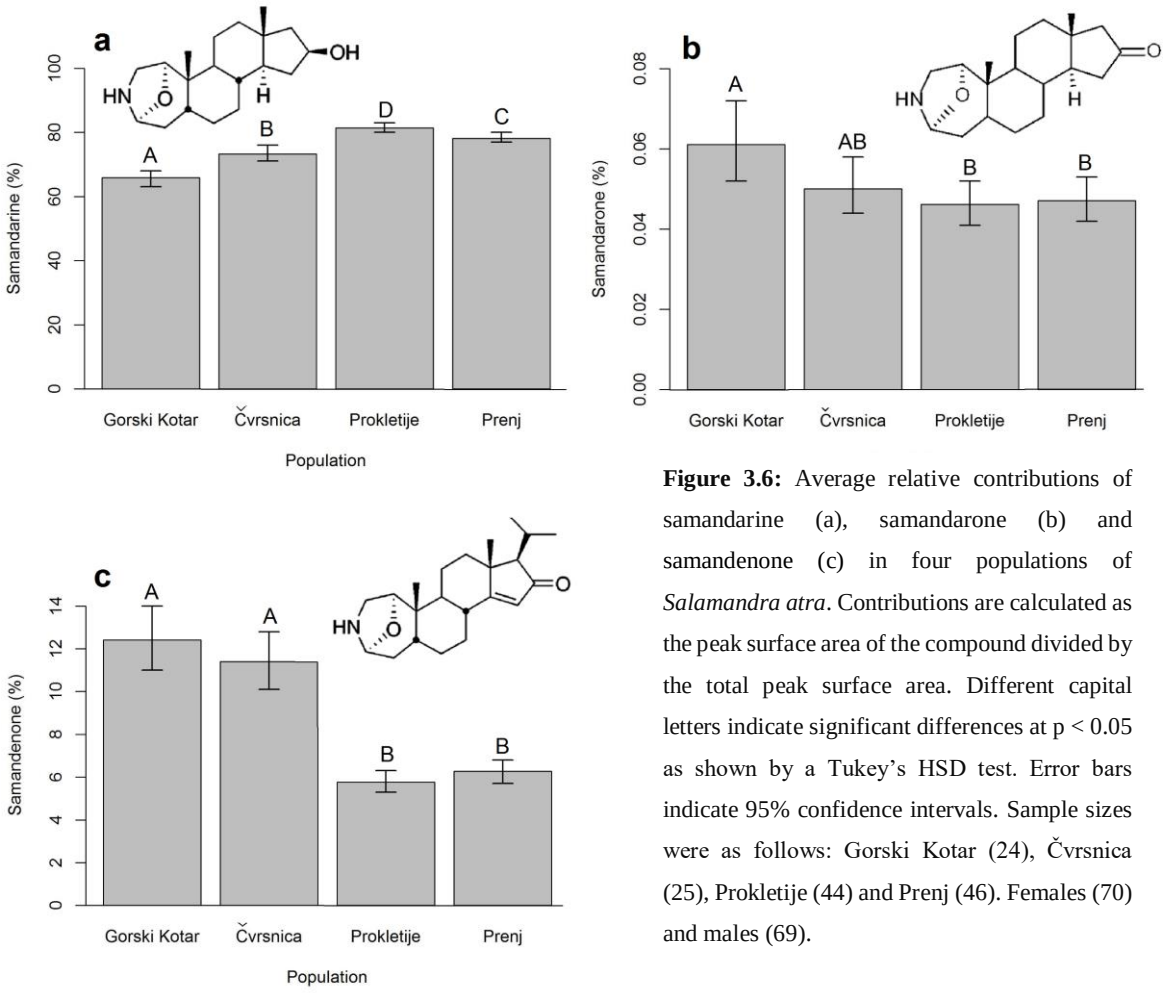

Figure 3.6: Average relative contributions of samandarine (a), samandarone (b) and samandenone (c) in four populations of *Salamandra atra*. Contributions are calculated as the peak surface area of the compound divided by the total peak surface area. Different capital letters indicate significant differences at $p < 0.05$ as shown by a Tukey's HSD test. Error bars indicate 95% confidence intervals. Sample sizes were as follows: Gorski Kotar (24), Čvrsnica (25), Prokletije (44) and Prenj (46). Females (70) and males (69).

For samandaridine, there was a significant interaction between population and sex ($F_{3,131} = 6.02$; $p < 0.001$). There were significant differences between sexes in Gorski Kotar (with females having higher relative contributions of samandaridine), but not in the other populations (figure 3.7). Populations did differ significantly in relative contributions of samandaridine ($F_{3,131} = 59.46$; $p < 0.001$). Gorski Kotar and Prenj had significantly higher contributions of samandaridine compared to Čvrsnica and Prokletije (Tukey's HSD: all $p < 0.05$).

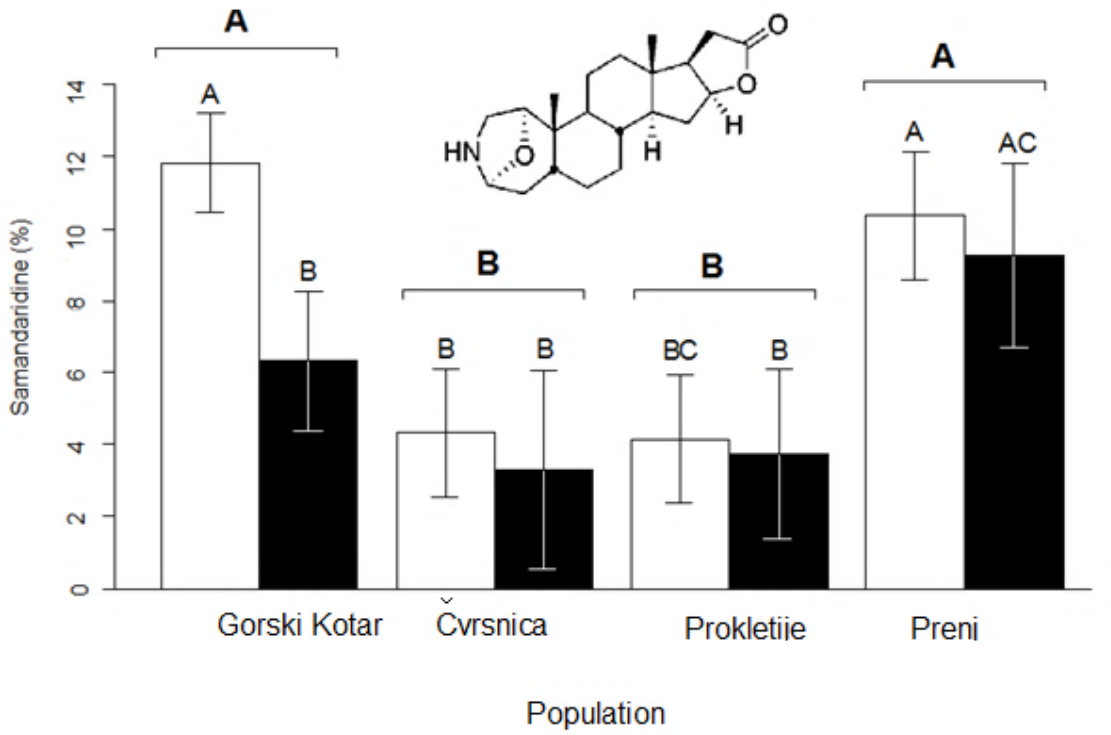

Figure 3.7: Average relative contributions of samandaridine in four populations of *Salamandra atra*. Contributions are calculated as the peak surface area of the compound divided by the total peak surface area. White = females and black = males. Different capital letters indicate significant differences at $p < 0.05$ as shown by a Tukey's HSD test. Error bars indicate 95% confidence intervals.

3.2. Degradation of toxins

Peak surface area decreased significantly with time of storage (slope = -0.021; $F_{1,240} = 12.21$; $p < 0.001$), but there was a significant interaction between time and treatment ($F_{2,240} = 11.14$; $p < 0.001$). The room temperature – treatment had a significantly steeper negative slope compared to the argon + fridge and fridge treatment (t-value = -2.40; p = 0.02), but there was no difference between the argon + fridge and fridge treatment (t-value = 1.48; p = 0.14). In the argon + fridge and fridge treatment there was a decrease of respectively 0.0208 and 0.0078 log-units peak surface area per day, while in the room temperature treatment peak surface area decreased with 0.0422 log-units per day.

There was no significant interaction between time of storage and the type of toxin ($F_{9,240} = 0.64$; p = 0.76). Different compounds thus degrade at the same rate.

3.3. Morphology

Morphology measures were taken from 129 individual salamanders. The number of individuals per population was the same as for the toxin data, but 10 salamanders from Gorski Kotar (5 males and 5 females) were excluded from the dataset due to incomplete data. Parotoid gland length varied from 7.5 to 11.38 mm and parotoid width from 2.63 to 5.87 mm. Body mass varied from 3.76 to 13.44 gram for males and from 6.1 to 15.38 gram for females. Descriptive statistics of the morphological variables for

each population are given in table 8.3 in appendix. The first two components of the PCA together explained 74 % of the total variance, but only PC1 had an eigenvalue higher than 1.

PC1 (explaining 58 % of the variance) showed a positive correlation between parotoid length, parotoid width, head length, head width and body mass (see table 3.3). Higher values on PC1 reflected smaller values for all variables. With other words, larger animals scored lower on PC1. The second component (PC2, explaining 16 % of the variance) reflected a contrast between width and length, but is not further discussed since the eigenvalue was lower than 1.

Table 3.3: Results of the Principal Component Analysis of the morphology of the salamanders. The first two components (PC1 and PC2) are given. For every component, the eigenvalue and the proportion of variance explained by this component is given, as well as the the loadings of the individual variables. Individual variables were only considered important if they had a loading higher than 0.300 or lower than -0.300. These loadings are indicated in bold.

	PC1	PC2
Eigenvalue	1.70	0.89
Proportion of total variance	0.58	0.16
Parotoid width	**-0.447**	**-0.587**
Parotoid length	**-0.379**	0.210
Head width	**-0.496**	-0.215
Head length	**-0.380**	**0.752**
Body mass	**-0.517**	

Before performing a two-way ANOVA, PC1-scores were multiplied with a factor of -1 to have higher values reflect larger animals. PC1-scores differed significantly among populations ($F_{3,125} = 10.73$; $p < 0.001$) and between sexes ($F_{1,125} = 9.92$; $p = 0.002$). The highest PC1-scores, and thus the largest animals, were found in Čvrsnica, while the lowest PC1-scores, and thus the smallest animals came from Gorski Kotar. Males and juveniles had lower PC1-scores, and were thus smaller, than female salamanders (figure 3.8).

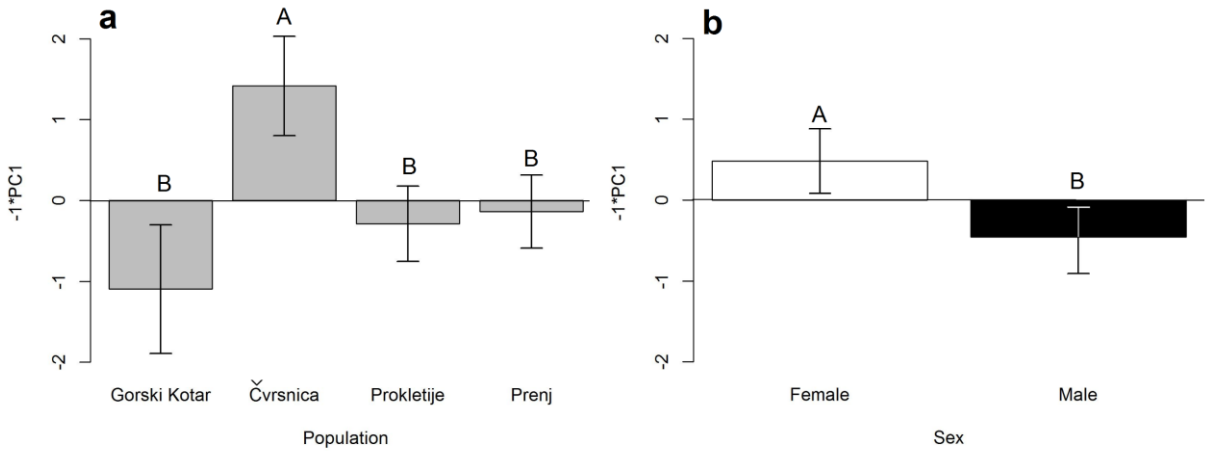

Figure 3.8: Average PC1-scores per population (A) and per sex (B). Higher values reflect larger animals, lower values reflect smaller animals. PC1-scores were transformed with a factor of -1. Different letters indicate significant differences at $p < 0.05$ as shown by a Tukey's HSD test. Error bars are 95 % confidence intervals. Sample sizes were as follows: Gorski Kotar (14), Čvrsnica (25), Prokletije (44) and Prenj (46). Females (65) and males (64).

There was a significant effect of body mass on parotoid width ($F_{1,120} = 89.11$; $p < 0.001$; figure 3.9a), but neither the interaction between body mass and population ($F_{3,120} = 0.3995$; $p = 0.7536$) nor the interaction between body mass and sex ($F_{1,120} = 0.9184$; $p = 0.3398$) was significant, meaning that the association between mass and parotoid width did not differ among populations or between sexes. Parotoid width increased with 0.130 millimetres per gram increase in body mass. Parotoid width also differed significantly among populations ($F_{3,120} = 10.13$; $p < 0.001$; figure 3.9a) with salamanders from Čvrsnica having the largest gland widths.

Parotoid length was also significantly related to body mass ($F_{1,120} = 36.67$; $p < 0.001$; figure 3.9b), with parotoid length increasing with 0.170 millimetres per gram increase in body mass. There were no significant interactions between body mass and population ($F_{3,120} = 1.17$; $p = 0.325$) or between body mass and sex ($F_{1,120} = 0.949$; $p = 0.332$). Parotoid length did not differ significantly among populations ($F_{3,130} = 1.50$; $p = 0.218$).

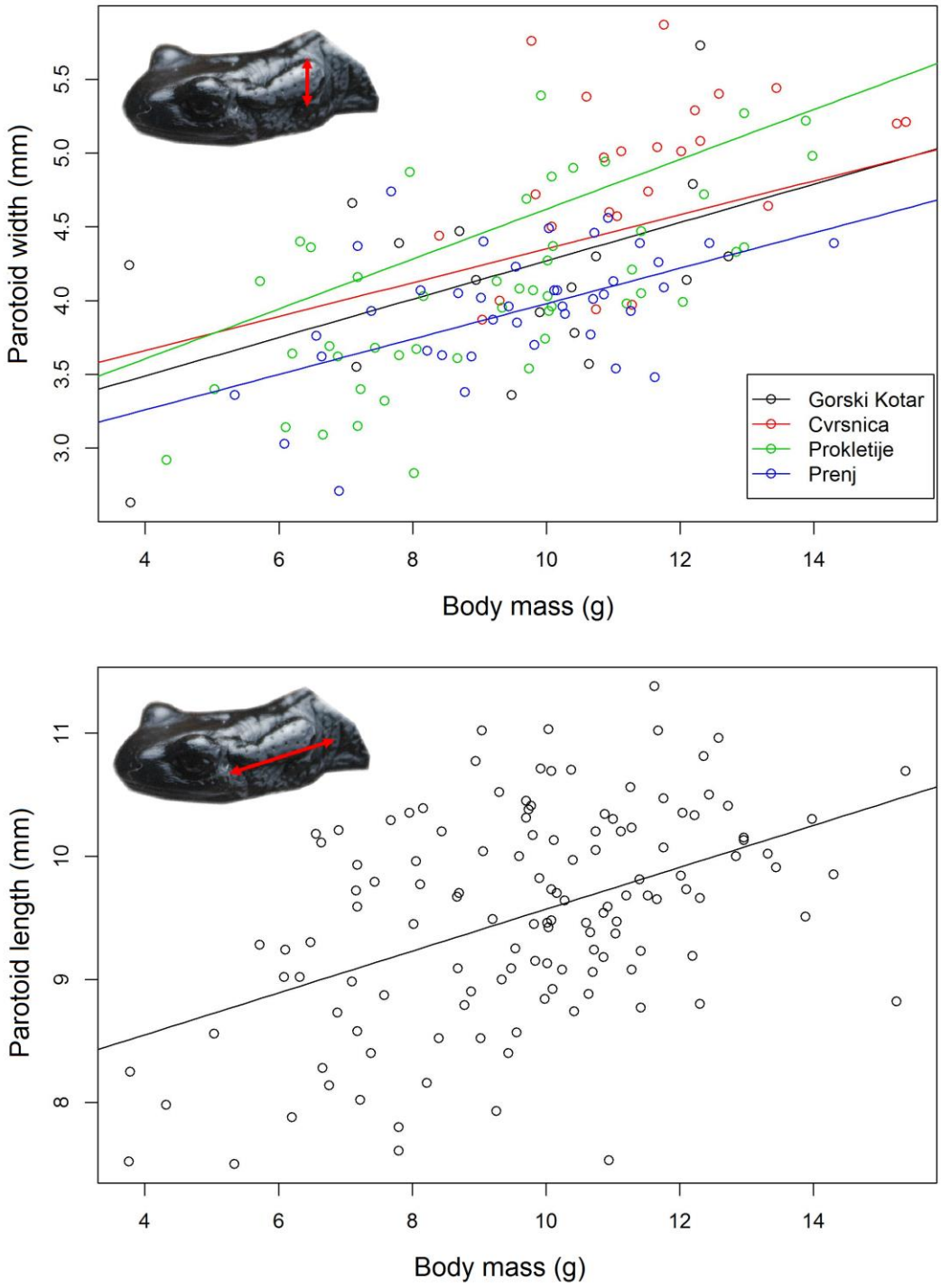

Figure 3.9: a) relation between the width of the parotoid gland (in mm) and body mass (g) and b) relation between the length of the parotoid gland (mm) and body mass (g) of *Salamandra atra*. Since parotoid width differed significantly among populations, separate regression lines were given for each population in graph a, although the slope of the relation did not differ among populations. Parotoid length did not differ among populations, hence why here the general regression line is given. Picture belongs to Šunje, E.

3.4. Bacterial densities

A total of 30 soil samples were cultivated: 5 from Gorski Kotar, 5 from Čvrsnica, 12 from Prokletije and 8 from Prenj. There was large variation in the estimated densities of CFUs in the soil. The number of CFUs per gram soil varied from $4*10^3$ to $42*10^6$ on the TSA-media and 4*10 to $7*10^5$ on the MacConkey media. No colonies were detected on the Slanetz-Bartely media. While no in-depth identifications of colonies were done, several colonies were suspected to be *Pseudomonas* sp., *Aeromonas* sp. and *Bacillus* sp. Descriptive statistics are presented in table 8.4 in appendix.

The number of CFUs that grew from one gram soil did not differ among populations regardless of whether they were cultured on TSA-media ($F_{3,55} = 0.560$; $p = 0.65$) or the MacConkey media ($F_{3,25} = 0.968$; $p = 0.42$). Significantly more colonies grew on cold-incubated (15°C) than warm-incubated (35°C) TSA-media ($F_{1,53} = 37.072$; $p < 0.001$)

3.5. Soil fungi

A total of 2661 fungal OTUs were identified in 37 soil samples (10 from Gorski Kotar, 7 from Čvrsnica, 12 from Prokletije and 8 from Prenj). Most OTUs (71 %) could not be identified to the level of the genus or lower. Most fungi were saprotroph-filamentous (15 %) or ectomycorrhizal (6 %). Less than 1 % of the OTUs were identified as animal parasites (figure 3.10). A small proportion of the OTUs could be identified but could not be 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 not known). For the distribution of lifestyles per population, see figure 8.3 in appendix. The three most abundant OTUs were identified as plant pathogens (*Cylindrocarpon* sp., *Neonectria* sp. and *Gibberella avenacea*). The proportion of parasite reads was low, with a maximum of 5.35 % of the total reads. A significantly higher proportion of parasite reads were found in Gorski Kotar and Prokletije compared to Prenj and Čvrsnica ($F_{3,33} = 3.16$; $p = 0.04$; figure 3.11). There was no significant difference in the total number of identified fungal species ($F_{3,30} = 0.518$; $p = 0.67$), but the total number of species increased significantly with number of reads ($F_{1,34} = 9.307$; $p = 0.004$). Descriptive statistics are included in table 8.4 (appendix). For a list of identified animal parasites, see table 8.5 (appendix).

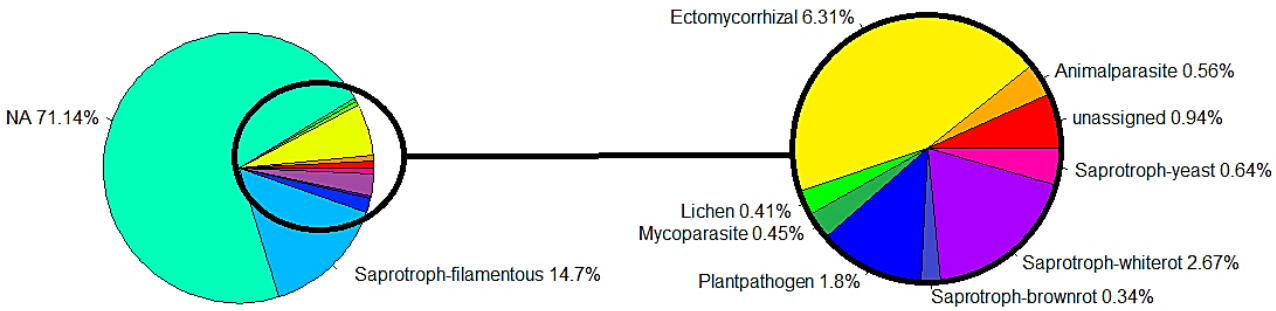

Figure 3.10: Distribution of lifestyles of identified fungal OTUs. Assignment of lifestyles to genera was based on the database of Tedersoo et al. (2014). NA = OTU could not be identified to the genus level and could thus not be linked to a particular lifestyle. Unassigned species were species that could be identified, but were not assigned to a lifestyle because either their lifestyle is not known, the lifestyle is not conserved at the genus level or the genus is not included in the database of Tedersoo et al. (2014). For the distribution of lifestyles per location, see figure 8.3 in appendix.

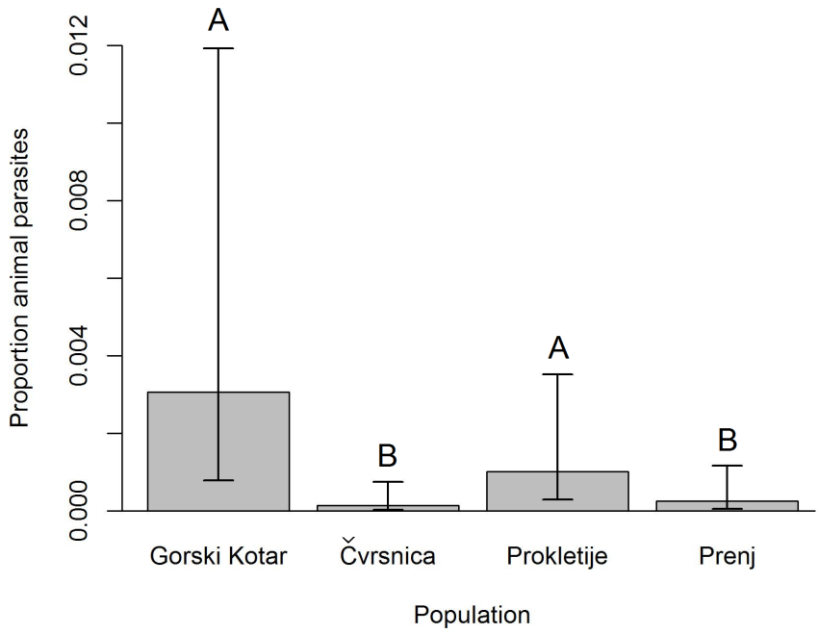

Figure 3.11: Average proportion of reads that were identified as animal parasites per population. Error bars represent 95% confidence intervals. Different letters indicate significant differences at $p < 0.05$ as shown by Tukey's HSD test. Sample sizes were as follows: Gorski Kotar (10), Čvrsnica (7), Prokletije (12) and Prenj (8).

Populations did differ significantly in their soil fungi communities (ANOSIM R = 0.2813; p = 0.001). All populations did differ significantly from each other, expect Prenj and Čvrsnica (ANOSIM R = 0.083; p = 0.08). Dissimilarities between populations can be found in table 3.5.

Table 3.5: Dissimilarities between populations of *Salamandra atra* in soil fungi communities, indicated by the ANOSIM R statistic. R-values > 1 indicate that the similarity between sites is smaller than the similarity within sites. Statistical differences ($p < 0.05$) are indicated in bold.

	Gorski Kotar (HRV)	**Čvrsnica (BIH)**	**Prokletije (MNE)**	**Prenj (BIH)**
Gorski Kotar (HRV)	-			
Čvrsnica (BIH)	**0.274**	-		
Prokletije (MNE)	**0.235**	**0.208**	-	
Prenj (BIH)	**0.495**	0.083	**0.388**	-

There was no significant correlation between the dissimilarity in soil fungi community and dissimilarity in toxin composition between populations ($r_s = 0.0286$; p = 1; figure 3.12).

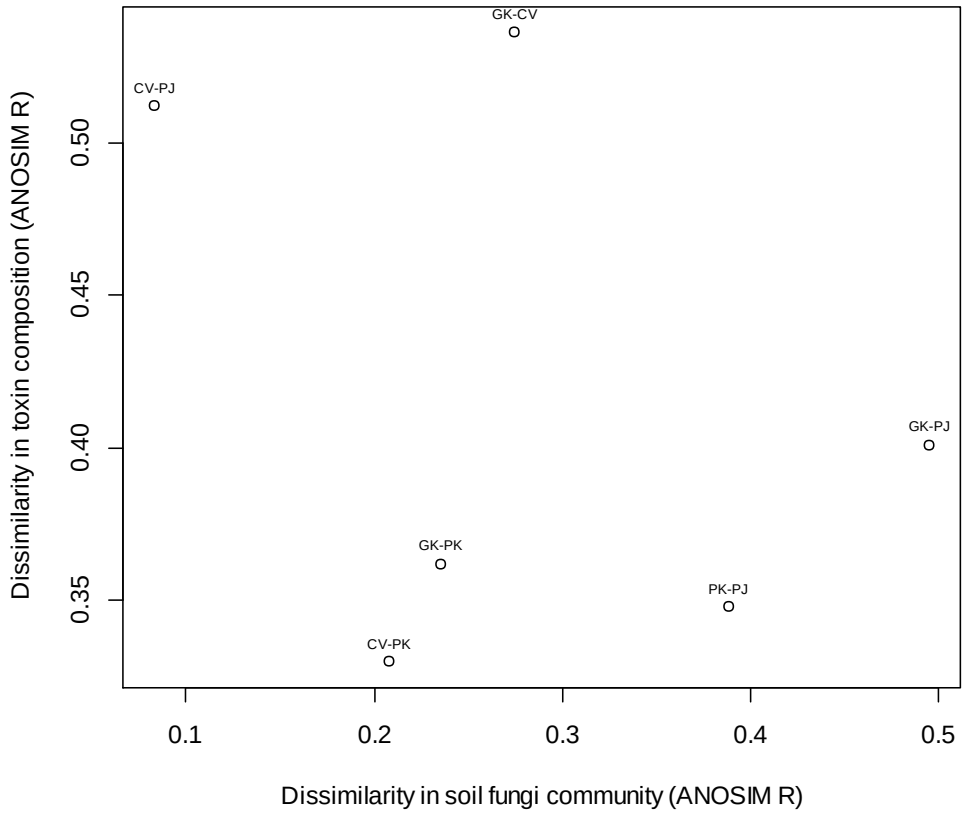

Figure 3.12: Correlation between dissimilarity in toxin composition and dissimilarity in soil fungi community between each pair of populations (both expressed as the ANOSIM R). GK = Gorski Kotar (HRV), CV = Čvrsnica (BIH), PK = Prokletije (MNE) and PJ = Prenj (BIH). Correlation was not significant ($r_s = 0.0286$; p = 1)

3.6. Predation pressure

Table 3.6. presents a list of potential predators of *S. atra*, in sympatry with the studied populations. From the eleven identified predators, only two (*Natrix natrix* and *Vipera berus*) have been reported to predate on *S. atra* (Luiselli et al., 1995; Luiselli et al., 1997; Luiselli et al., 2005). The other species are predators of either *S. salamandra* or *S. infraimmaculata*. However, since *N. natrix* feeds on both *S. atra* and *S. salamandra* (Velo-Antón & Buckley, 2015) and since fire salamanders have higher concentrations of samandarine than alpine salamanders (Daly et al., 1999) it seems safe to assume that predators of *S. salamandra* are also able to predate on *S. atra*.

I distinguished between species whose presence is confirmed (indicated by a C in table 3.6), either by literature, personal observations during the field work or communication with local biologists, and species whose presence is possible (indicated by a P). Possible means that the species is present in the area based on distribution data of the IUCN or New Atlas of Amphibians and Reptiles of Europe (Sillero et al., 2014), but whose presence is uncertain at the sampled locations. If literature data or local biologists claimed a certain species was not present in that location, or it was very unlikely this species was present there (habitat requirements, altitude, …), it was considered not present (0) even if the location fell within its predicted range.

Gorski Kotar had the largest number of potential predators (8) and Prenj the lowest number (4). However, if only the confirmed species are taken into account, there is no difference between Gorski Kotar, Čvrsnica and Prokletije (all three confirmed species). Prenj had only two predator with confirmed presence (*N. natrix* and *Sus scrofa*). Presence of *V. berus* is confirmed on Čvrsnica and in Prokletije, and possible in Gorski Kotar. *Natrix natrix* is present on Čvrsnica, but was also observed on Prenj at the same altitude as *S. atra* (personal observation).

Table 3.6: Distribution of potential predators of *Salamandra atra*. Species indicated in bold have been confirmed to feed on *S. atra*. Other species have been reported to feed on salamanders from the same genus. C = Presence confirmed, either by literature, personal observations (P.O.) or observation by local biologists (comm.), P = Possible presence, meaning that based on distribution data of the IUCN (2016) or the New Atlas of Amphibians and Reptiles of Europe (Sillero et al., 2014) this species can be expected in the area of the population, but there are no explicit recordings of the species at the sampled locations. 0 = Not present. Sources for the distribution of the species are given in the last column. Sources that confirm the presence of *Salamandra* in the diet of these species are indicated by numbers in superscript next to the name, with: 1= Böhme et al. (2013); 2 = Carretero & Rosell (1999); 3 = Irizar et al. (2004); 4 = Luiselli et al. (1995); 5 = Luiselli et al. (1997); 6 = Luiselli et al. (2005); 7 = Velo-Antón & Buckley (2015).

	Gorski Kotar (HRV)	Čvrsnica (BIH)	Prokletije (MNE)	Prenj (BIH)	Source
Reptiles					
***Natrix natrix*[5,6,7]**	0	C	0	C	Merdan, comm. Sillero et al. (2014) P.O. Šunje et al. (2014)
***Natrix tessellata*[1]** ***Natrix tessellata*[1]**	0	0	0	0	IUCN (2016) Sillero et al. (2014) Šunje et al. (2014)
***Vipera berus*[4]**	P	C	C	0	Jelić et al. (2013) Sillero et al. (2014) Šunje et al. (2014) Zagora, comm.
Birds					
***Buteo buteo*[7]**	C	P	P	P	IUCN (2016) Lukač (2007) P.O. Šunje, comm.
***Strix aluco*[7]**	C	P	P	P	Lukač (2007) IUCN (2016) Šunje, comm.
Mammals					
***Erinaceus sp.*[7]**	P	0	0	0	Đurović, comm Šunje, comm.
***Lutra lutra*[7]**	0	0	P	0	
***Meles meles*[7]**	P	0	0	0	Đurović, comm. Kusak et al. (2009) Šunje, comm.
***Mustella putorius*[7]**	P	0	0	0	Đurović, comm. Šunje, comm Trbović 2016
***Rattus* sp.[7]**	P	0	0	0	Đurović, comm. Slavica et al. (2010) Šunje, comm
***Sus scrofa*[2,3,7]**	C	C	C	C	Kullak et al. (2009) P.O. Šunje, comm.
Possible	5	2	3	3	
Confirmed	3	3	3	2	
Total	8	5	6	4	

4. Discussion

Table 4.1 gives an overview of the results of this study, including the toxin data, the morphology, the microbial data and the predation pressure data. I did not attempt to do statistical analyses to see whether e.g. relative contribution of certain toxins was correlated with predation pressure or bacterial density, since data was only available for four populations, which might be insufficient for such statistical analyses.

Table 4.1: Overview of the results for all four populations of *Salamandra atra* included in this study. Significant differences between populations are indicated in bold. For predation, no statistical tests were performed to compare populations. The total amount of toxins was expressed as the sum of all the peak surface areas from the chromatogram per sample. Relative contributions were expressed as the peak surface area of that compound divided by the total peak surface area of that sample. The bacterial densities were expressed as the number of colony forming units (CFUs) per gram soil. The abundance of parasitic fungi was calculated as the proportion of animal parasite reads. Predation pressure was estimated by the number of potential predators.

	Gorski Kotar (HRV)	**Čvrsnica (BIH)**	**Prokletije (MNE)**	**Prenj (BIH)**
Total amount of toxins	**Low**	Medium	Medium	High
Samandarine (%)	**Low**	**Medium**	**High**	**Medium**
Samandarone (%)	**High**	Low	Low	Low
Samandenone (%)	**High**	**High**	Low	Low
Samandaridine (%)	**High**	Low	Low	**High**
Morphology	Small	**Large**	Medium	Medium
Bacterial density (CFUs/g)	High	Low	Low	Medium
Fungi	**High**	Low	**Medium**	Low
Predation	High	Medium	Medium	Low

4.1. Variation in toxin composition

As was expected, my results showed that toxin composition varied among the four studied populations of *S. atra* in the Dinaric Alps. Geographic variation in toxins has been demonstrated before in the common toad (*B. bufo*, Bókony et al., 2016), South-American redbelly toads (*Melanophryniscus* sp., Daly et al., 2007), several species of poison frogs (Saporito et al., 2007; Saporito et al., 2012; Mina et al., 2015) and Pacific newts (*Taricha* sp., Brodie et al., 2002; Hanifin et al., 2008; Stokes et al., 2015). Redbelly toads and poison frogs sequester alkaloids from dietary sources (Saporito et al., 2012) and the origin of TTX in *Taricha*-newts is still ambiguous (Stokes et al., 2015; Bucciarelli et al., 2016), meaning that this is currently one of two studies that demonstrated geographic variation in endogenously produced toxins (the other being Bókony et al., 2016).

Similarities in toxin composition among populations were not related to geographic distance (table 3.1). Surprisingly, the second largest difference in toxin composition was found between salamanders from Čvrsnica and Prenj, despite the small geographic distance between these two populations. Čvrsnica and Prokletije were the most similar, despite the large geographic distance. This is in sharp contrast with previous studies on amongst others *O. pumilio* (Saporito et al., 2007; Saporito et al., 2012), *Melanophryniscus simplex* (Grant et al., 2012) and *Taricha*-newts (Hanifin et al., 2008) where variation among populations was related to geographic distance. In poison frogs, such similarity in alkaloid profiles between adjacent populations has been explained by similarity in diets, due to the presence of the same arthropod species (Saporito et al., 2007).

The lack of correlation between geographic distance and toxin composition in this study can be attributed to the fragmented distribution of *S. atra* in the Balkan peninsula (see figure 2.1). The Dinaric populations of *S. atra* have been isolated from each other since the end of the last ice age, during which the cold-adapted salamanders had to retreat to higher altitudes (Razpet et al., 2016). Adaptation to local conditions might have led to variation in toxin composition, although Čvrsnica and Prenj are very similar in habitat, altitude, climate (Šunje et al., 2014) and soil fungi communities (own results: table 3.5). In addition, gene flow between these fragments, if any, is presumably low. Razpet et al. (2016) found genetic signatures of migration between different (sub)populations on Prenj and between populations in Croatia, but no evidence of gene flow over larger geographic distances. Such isolation might also lead to differences among populations solely due to random genetic drift. Whether migration occurs between Čvrsnica and Prenj is not known, but it is very unlikely due to the deep river valley (much lower than the minimum elevation on which *S. atra* is found) between the two mountains (Lepirica, 2008; Redžić et al., 2010). It is not yet known how the sampled populations of *S. atra* are genetically related to each other, but this is currently under investigation in another study. As soon as the genetic differentiation of these populations is fully understood, it will be possible to relate toxin variation to genetic variation in *S. atra*.

As shown by a Principal Component Analysis (table 3.2., figure 3.4 and figure 3.5) secretions of salamanders from Gorski Kotar and Čvrsnica had higher relative contributions of ecomytrin, samandenone and samanine, but lower relative contributions of samandarine and samandiol, compared to salamanders from Prenj or Prokletije. Čvrsnica and Prokletije had lower relative contributions of samandarone than Gorski Kotar and Prenj. Salamanders from Prenj and Gorski Kotar had higher relative contributions of samandaridine, but lower contributions of samanine and samanol. Unfortunately, for many of the above compounds, nothing is known of their biological activity, their toxicity or antimicrobial activity, making it difficult to interpret these differences in an ecological and evolutionary context. A next step in this research should be the isolation of the individual samandarines from crude secretions. After isolation, the toxicity and antimicrobial activity of each compound can be tested. The

results explained above could then be more easily matched to patterns of predation and infection risk. Unfortunately, such extensive research was not possible within the limited timeframe of this thesis.

Apart from genetic differentiation, differences in predation pressure and infection risk, another explanation for variation in toxin composition might be differences in age structure among the populations. Variation in the quantity and diversity of toxins with age has been observed for both endogenously produced (Hayes et al., 2009) and sequestered toxins (Jeckel et al., 2015). If the four populations of *S. atra* differ in age structure, this could explain at least partially the variation in toxin composition.

As seen by the size of the normal data ellipses on figure 3.3, variation within Čvrsnica and Gorski Kotar was much larger than the variation within the other two populations. While toxins were collected in Prokletije and on Prenj within a relatively short time frame (all in July), Čvrsnica and Gorski Kotar were sampled in both June/July and September. The large variation within these populations might thus be due to temporal variation in toxin composition from June to September. Seasonal variation in toxin composition is known in many poison frogs, as a result of seasonal changes in arthropod sources (Daly et al., 2007; Saporito et al., 2007; Saporito et al., 2012). Both fire salamanders and *Taricha*-newts show remarkable variation in alkaloid-concentrations over time for reasons not yet known (Mebs & Pogoda, 2005; Bucciarelli et al., 2016). It is possible that the availability of cholesterol, the presumed precursor for the samandarines (Habermehl & Haaf, 1968; Habermehl, 1971), changes over time in *S. atra*. For example, depending on the stage in the reproductive cycle, cholesterol might be more invested in the synthesis of sex hormones instead of toxins. Another reason might be that *S. atra* goes into hibernation after September (Šunje & Lelo, 2010), meaning that cholesterol is at that point more likely to be involved in energy storage (Fitzpatrick, 1976) than the production of toxins.

4.2. Total amount of toxins and predation pressure

It was expected that there would be a positive association between toxicity and predation pressure among populations. However, the total amount of secreted toxins did not match the predicted predation pressure in the studied populations (table 4.1). While salamanders from Gorski Kotar were estimated to be under the highest predation pressure, they secreted the lowest amount of toxins. The number of potential predators on Prenj is low, yet salamanders from this population secreted the highest amount of toxins.

Previous studies yielded mixed results about the relation between predation pressure and toxicity. Dreher et al. (2015) showed that in a range of *O. pumilio* populations, there was a correlation between attack rate of birds on clay frog models and conspicuousness of skin colours (which is correlated with toxicity) of local frogs. Bókony et al. (2016) found no effect of predation risk on chemical defences of *B. bufo* tadpoles in 27 ponds in Austria and Hungary.

The mismatch between predation pressure and the total amount of toxins produced could be a result of the decrease in peak surface area over time as observed in the degradation-experiment. Due to logistic problems, the time period between collection and analysis of the samples from Gorski Kotar was much longer than those of other populations. This longer waiting time probably resulted in more severe degradation, explaining why salamanders from Gorski Kotar seemingly produced less toxins. Analysing toxin samples shortly after collection in the field and/or alternative methods of storing toxins should minimize degradation, but were not possible for this study.

There is however an alternative explanation. In populations with more potential predators (Gorski Kotar), salamanders will be attacked more frequently and will therefore secrete their toxins more often (Saporito et al., 2009). Since it might take some time to synthesize alkaloids, as shown in Mebs & Pogoda (2005), salamanders under a higher attack rate might have continuously lower amounts of toxins in their parotoid glands. Where less predators are present (Prenj), salamanders will be attacked less frequently and can thus accumulate more toxins over a longer timespan (Saporito et al., 2009). Under this assumption, predation pressure and the amount of toxins secreted perfectly match each other (table 4.1). To account for this, salamanders of different populations should be taken to the lab, where the parotoid glands should be emptied and after a fixed period for resynthesizing, fresh toxin samples could be taken for further analyses.

More reliable indicators for the total amount of toxins secreted by each salamander are parotoid size and body size. The larger the parotoid glands of an individual, the larger its capacity to store toxins (Jeckel et al., 2015). Previous research (Saporito et al., 2010) and my own results (figure 3.9) showed a positive correlation between body size/mass and the size and numbers of the poison glands. Saporito et al. (2010) however showed a positive allometric correlation between body size and poison gland size in the poison frog *O. pumilio*, while the correlation found here was negative allometric. This might be due to the fact that Saporito et al. (2010) used snout-vent length of *O. pumilio* as a measure for body size, while I used body mass of salamanders. Since this are the only two studies to correlate body size with poison gland size in amphibians, it is possible that growth rate of parotoid glands differs between anura and caudata. The negative allometric correlation might also indicate that *S. atra* invests less in toxin production, which might be the case if predation on this species is low.

The prediction that larger salamanders with larger parotoid glands can store more toxins is supported by the findings of Jeckel et al. (2015). This study proved that larger Brazilian red-belly toads produced larger quantities of alkaloids and bufotenine. My own results showed that the total amount of toxins secreted was positively correlated with parotoid width in 3 of the 4 populations (figure 3.2), but should be interpreted carefully due to the degradation of the samples. Nevertheless, it can be expected that larger individuals are more toxic than smaller ones (Arnold, 1982). The largest salamanders were found in Čvrsnica (medium predation pressure), and the smallest salamanders in Gorski Kotar (high predation

pressure) (figure 3.8a), meaning that body size, and by extent toxicity, is not related to the number of potential predators. However, it is interesting that although the number of potential predators in Čvrsnica was low, it is the only population in which both confirmed predators of *S. atra*, the snakes *V. berus* and *N. natrix* (Luiselli et al., 1995; Luiselli et al., 1997; Luiselli et al., 2005), are certainly present (table 3.6). In Gorski Kotar, where salamanders are small, only one of these two species is *possibly* present (Jelić et al., 2013) but not confirmed in the specific area and habitat where *S. atra* lives. Salamanders have an intermediate body size in Prokletije and on Prenj, where only one snake species has been confirmed. It can be hypothesized that evolution to larger body size and thus higher amounts of toxins might mainly be driven by the presence of these predatory snakes. Apart from higher toxicity, larger bodies will also make the prey harder to swallow for snake predators, which might also contribute to their survival (Arnold, 1982).

However, the observed differences in body size among these populations also correspond to Bergmann's rule, which claims that animals in colder environments (e.g. higher latitude and/or elevation) are larger, as an adaptation to minimize heat loss or to increase fasting endurance (Ashton, 2002; Adams & Church, 2007). *Salamandra atra* was indeed larger at higher elevations (Čvrsnica and Prenj: 1650-2000 meter a.s.l.) and smaller at lower elevations (Gorski Kotar: around 1000 meter a.s.l.) (figure 3.8a). Whether Bergmann's rule is valid for ectotherm animals like salamanders is still a point of discussion (Ashton, 2002; Adams & Church, 2007), but it is worth considering that this might be an additional driver for the evolution of body size (and thus the quantity of toxins), perhaps in combination with predation pressure.

Differences in body size among these populations might also be a consequence of differences in age structure. Salamanders, like all amphibians, show indeterminate growth, meaning that they keep growing their entire life, although at a decreasing rate (Halliday & Verrell, 1988; Hasumi 2010). The fact that salamanders from Čvrsnica are larger, might be a result of a higher proportion of old individuals in this population. Age structure itself can be influenced by predation pressure, e.g. where predation pressure is high, survival is low and the population will have a lower proportion of old individuals. Indeed, in Gorski Kotar, where the highest number of potential predators is present, the smallest, and maybe youngest, salamanders are found. Whether the studied populations really differ in age structure, and whether this is indeed influenced by predation pressure, should be investigated in further research.

4.3. Toxin variation and predation pressure

Since all compounds degraded at the same rate, relative contributions of the individual compounds should not change even after a long period of storing the samples. Nevertheless, even the relative contributions of samandarine do not match the estimated predation pressure (table 4.1). Salamanders from Gorski Kotar were estimated to be under the highest predation pressure, yet they had the lowest relative contributions of samandarine in their toxins compared to other populations (figure 3.4a; figure

3.6a). The highest relative contributions of samandarine were found in Prokletije, where the number of potential predators was only moderate. There are several explanations for this apparent lack of correlation.

First of all, the list of potential predators might be incomplete or inaccurate. Many of the predators listed in table 3.6 are in fact predators of *S. salamandra*, and it has not been proven that they feed on other *Salamandra* species as well. On the other hand, it is possible that several (local) species do predate on *S. atra*, but were just never observed doing it. The number of potential predators per population can thus be both over- and underestimated Nevertheless, there is a general trend that species richness declines with elevation (Rahbek, 1995), and as a consequence the number of potential predators should also decrease with elevation. In that case, Gorski Kotar is still expected to have the highest number of predators and Prenj the lowest. Stokes et al. (2015) found indeed that *Taricha*-newts at higher elevations had lower TTX-concentrations in the skin compared to conspecifics at lower elevations, and suspected this might have been due to a decrease in snake diversity with altitude.

Salamandra atra is prey to *V. berus* (Luiselli et al., 1995) and *N. natrix* (Luiselli et al., 1997; Luiselli et al., 2005) in the Italian Alps, but this does not guarantee that these snakes will also predate on *S. atra* in the Dinaric Alps. It has been speculated that snakes only include salamanders in their diet in montane regions where the density of lizards is low (Luiselli et al., 1995; Luiselli et al., 2005). In addition, Bonato & Fracasso (2003) found that densities of *S. atra aurorae* where much higher in open areas than in thick wood areas. Especially in the population of Gorski Kotar, a forest habitat around 1000 meters a.s.l., snakes might prefer other abundant prey, like lizards or rodents. The three other populations were found at higher altitudes and in more open alpine grasslands, where salamanders might be the only abundant prey present, and might thus suffer from a higher predation rate. It should however be noted that lizard densities on Čvrsnica and Prenj are relatively high (Šunje, personal communication) but the slow salamanders occurring in high densities might still present an easy food source. Predator density as well will influence predation pressure on *S. atra*. A single species of predator on Prenj might be far more abundant than several species together in Gorski Kotar for all we know.

A second possibility is that samandarine, despite being the major toxin, might not be the most toxic compound after all. While samandarone and other samandarines are often referred to as less toxic (Daly et al., 1993; Daly et al., 1995), I did not find any reliable data on their LD_{50}-values. Interestingly, many (sub)species of *Salamandra* are missing samandarine in their skin secretions (Habermehl, 1971; Vences et al., 2014). In fact, samandarine is only present at high concentrations in *S. atra* and *S. lanzai* (Vences et al., 2014). This might indicate that samandarine only plays a minor role in the toxicity of the salamanders, although it should also be noted that it is not known whether all *Salamandra*-species are equally toxic or not.

Lastly, higher toxicity can also be a consequence of an evolutionary arms race with a specific predator. Evolution of TTX-resistance in garter snakes (*T. sirtalis*) has driven the evolution of high TTX-levels in the skin secretions of *Taricha*-newts (Brodie et al., 2002; Hanifin et al., 2008). In two third of the studied populations, there was a close association between TTX-resistance of snakes and TTX-levels of newts (Hanifin et al., 2008). It is currently not known whether *S. atra* is in such an evolutionary arms race with a toxin-resistant predator. It should be investigated whether *V. berus* and *N. natrix* show geographic variation in their ability to eat *S. atra* and whether this relates to toxicity.

To summarize, since neither the total amount of toxins nor the relative contributions of samandarine correlates with the estimated number of potential predators, there seems to be no support for the hypothesis that predation pressure drives the evolution of higher toxicity. A more reliable estimation of predation pressure is however necessary. Further research should start with confirming whether the species in table 3.6 predate on *S. atra* or not, and if so, how the proportion of salamanders in their diet varies among populations.

The relative contributions of samandenone and samandaridine do not perfectly match the predicted predation pressure either. Both compounds have high contributions in samples from Gorski Kotar (figure 3.6c and 3.7), where predation pressure is high, which would imply a major role in the toxicity of the skin secretions. On the other hand, samandenone and samandaridine have also quite large contributions in populations where the number of potential predators is low or medium. Investigating the toxicity of these two compounds would help interpreting these results more clearly. Even if these compounds are indeed less toxic as mentioned in literature (Daly et al., 1993; Daly et al., 1995), it is possible that they are more involved in repelling the predator instead of killing it by e.g. causing a bitter taste. Repelling a predator will also increase the survival probability of a prey (Brodie, 1968; Gray et al., 2010; Hopkins & Migabo, 2010; Williams et al., 2010).

4.4. Toxin variation and infection risk

As seen on figure 3.12, populations that were more similar in toxin composition were not more similar in the soil fungi communities to which they were exposed. Such association would however only be expected if toxin composition was solely driven by defence against infections. In such case selection would only favour those compounds and concentrations in a population necessary to protect against the specific set of pathogens present in that environment. This is of course not the case, since toxin composition will also undergo selection for defence against predators. Besides, both samandarine and samandarone seem to act against a broad range of micro-organisms (Preusser et al., 1975), meaning that selection does not necessarily need to favour specific compounds that act against specific micro-organisms.

Gorski Kotar was the population with the highest proportion of parasitic fungi (figure 3.11) and the highest bacterial densities per gram soil (appendix table 8.4), although these differences in bacterial densities were not significant. This is in line with previous research that found lower bacterial counts and lower fungal abundances in the soil with increasing altitude (Margesin et al., 2008; Wang et al., 2015). Salamanders from Gorski Kotar were thus exposed to the highest infection risk, and incidentally had the highest relative contributions of samandarone (figure 3.4b; figure 3.6b). The other three populations, where the bacterial densities and the proportions of parasitic fungi were low or medium (table 4.1), had much lower relative contributions of samandarone. This seems to confirm the prediction that salamanders from environments with a higher infection risk invest more in defence against micro-organisms (Bókony et al. 2015). This is, to my knowledge, the first study investigating the link between toxin variation and variation in pathogen load.

It was demonstrated by Mina et al. (2015) that variation in alkaloid composition among populations of *O. pumilio* resulted in geographic variation in antimicrobial activity of the skin secretions. However, whether the poisons from salamanders in Gorski Kotar do indeed have higher antimicrobial activity than those from other populations remains to be proven.

While Preusser et al. (1975) showed that samandarone had stronger and broader antimicrobial activity compared to samandarine and samandaridine, many more alkaloids are present in the skin secretions of *S. atra* (Habermehl & Spiteller, 1967; Habermehl, 1971; Daly et al., 2005) and have the potential of also being antibiotic or antifungal. Samandenone had high relative contributions in Gorski Kotar, the environment with the highest infection risk, but also on Prenj, where infection risk was low. Samandaridine seems to perfectly fit the patterns of bacterial densities, but strangely this compound does only inhibit the growth of some fungi and is not antibiotic at all (Preusser et al., 1975). It is known that crude poisons of *S. salamandra* inhibited the growth of micro-organisms more strongly than samandarine alone (Habermehl & Preusser, 1969). Synergistic interactions between individual compounds might increase the antimicrobial efficiency of the total mixture (Rollins-Smith et al., 2005; Woodhams et al., 2007; Mina et al., 2015), something that was not taken into account in this study. Further studies should test for differences in the antimicrobial activity of crude secretions from all four populations, and then try to relate these to differences in composition.

Surprisingly, samandarone was only present in very low amounts (mean relative contribution of 0.06 % in Gorski Kotar) and even absent in 16 samples. These low relative contributions were also found in the fresh samples of the captive animals, which proofs that they are not the result of degradation. Given these low concentrations, it is doubtful whether samandarone can really play a major role in the defence against micro-organisms. Samandarone is generally considered the second most abundant compound of *Salamandra*-secretions (Habermehl, 1971). Previous studies found relatively high concentrations of

samandarone, sometimes even higher than those of samandarine (Mebs & Pogoda, 2005; Vences et al., 2014). There are three possible explanations for this finding.

Firstly, the two most recent studies on *Salamandra*-toxins (Mebs & Pogoda, 2005; Vences et al., 2014) used gas chromatography – mass spectrometry (GC-MS). This study however made use of UPLC-MS/MS, which has a much higher sensitivity and better selectivity than GC-MS (McMaster, 2005; Alder et al., 2006). Due to the high structural similarity between these alkaloids, it is possible that peaks from several compounds overlap in the full-scan mode during GC-MS. Such overlap between samandarone and other compounds might have led to overestimation of samandarone-concentrations in the past. The use of multiple reaction monitoring (MRM) in UPLC-MS/MS makes the detection of specific compounds more accurate (Alder et al., 2006; Freue & Borchers, 2012), and overlap is minimized.

Another possibility is that my study underestimated the concentration of samandarone. It could be that samandarone ionizes more difficult than other compounds. A lower degree of ionization will result in a reduced MS-signal. The MS-signal of samandarone might also be reduced by a process called ion suppression. Other compounds present in the sample might compete with samandarone during the ionisation process and "suppress" the ionization of samandarone (Pitt, 2009) resulting in an underestimation when quantified solemnly based on arbitrary integration units. The use of internal standards should make it possible to test whether this was the case or not. Unfortunately, internal standards for these salamander alkaloids are not commercially available. Nevertheless, even if the low MS-signals of samandarone correspond to much higher concentrations, the differences among populations will not change.

Last but not least, it has been suggested that samandaridine and samandenone are precursors for several other samandarines, such as samandarone (Habermehl, 1971). Relative contributions of samandaridine and samandenone were relatively high. It can be hypothesized that, if one of these compounds is indeed the precursor to samandarone, they only get converted to samandarone right before or after secretion, which might explain the low contributions of samandarone.

Susceptibility to the amphibian-killing disease chytridiomycosis, caused by the fungi *Batrachochytrium dendrobatidis* or *B. salamandrivorans* (Martel et al., 2013), varies among species (Woodhams et al., 2007; Pasmans et al., 2013) and even among populations of the same species (Tobler & Schmidt, 2010). Such variation in susceptibility is often attributed to variation in the antimicrobial activity of the skin secretions (Rollins-Smith et al., 2005; Woodhams et al., 2007; Pasmans et al., 2013). Studying variation in antimicrobial activity and by extent composition of amphibian toxins could help assessing which populations and/or species are more vulnerable and require additional protection (Tobler & Schmidt, 2010). It is interesting to note that infection by chytridiomycosis has not been reported yet for *S. atra* (Lötters et al., 2012; Bd-maps, 2017) despite the presence of the fungus in its areal (Bd-map, 2017). The

congeneric *S. salamandra* is almost at the brink of local extinction in north-western Europe due to mass mortality caused by chytridiomycosis (Martel et al., 2013). Whether *S. atra* is more resistant to infection by this fungus and whether this is caused by differences in toxin composition is currently not known.

4.5. Differences between sexes

It was expected that males would secrete higher amounts of toxins and have higher relative contributions of samandarine and samandarone, since they are more prone to predation and infection due to their higher mobility (Helfer et al., 2012). None of these predictions were confirmed. Sexes did differ from each other in the second and third component of the PCA (table 3.2, figure 3.4c and figure 3.5) and in relative contributions of samandaridine (figure 3.7), but these results are difficult to explain without more information on the effect, toxicity and antimicrobial activity of these compounds. Nevertheless, male and female salamanders did not differ from each other in *overall* toxin composition according to an ANOSIM.

It is possible that attack rate does not differ between sexes. Due to the fact that male *S. atra* disperse to other populations and females are philopatric (Helfer et al., 2012), it is safe to assume that males are exposed to a higher degree of predation. However, female *S. atra* are larger than males (Luiselli et al., 2001; Helfer et al., 2012, own results: figure 3.8b). The larger females might be more easily detected by predators (Arnold, 1982) especially against the bright background of the dolomitic karst. In addition, due to their large size, the amount of retreats that can be used for hiding are more limited for females than for the smaller males (Arnold, 1982). Considering this, predation pressure on both sexes might be more or less equal, and selection will not favour increased toxicity in one sex.

Due to the fact that males and females share most of their genome, they will have a correlated response to selection. If selection favours higher toxicity in males, the daughters of the selected males will also have higher toxicity. Selection on toxicity in one sex thus influences toxicity in the other sex. Such mutual selection might explain the lack of sex differences.

Differences between sexes in toxin composition were also not found in *Mantella bernhardi* (Daly et al., 2008), *Melanophryniscus moreirae* (Jeckel et al., 2015), *Taricha* sp. (Stokes et al., 2011) and most importantly *S. salamandra* (Mebs & Pogoda, 2005). In fact, sex differences in toxin composition were only found in one study on *O. pumilio* (Saporito et al., 2009). Later research has suggested that these differences were not due to differences in predation pressure, but a result of female strawberry poison frogs on average being older than males, and thus having accumulated more toxins over their longer lifespan (Jeckel et al., 2015).

4.6. Peptides?

It has been noticed that the poisons of the Salamandridae also contain peptides and proteins (Habermehl & Spiteller, 1969; Habermehl, 1971; Bettin & Greven, 1986). While it has been suggested that some of these peptides show haemolytic activity (Habermehl, 1971), these compounds have not been identified yet. No information can be found on their concentration and number, and nothing else is known about their antimicrobial activity and toxicity to my knowledge. Antimicrobial peptides are found within the skin secretions of a broad range of amphibians (Daly, 1995; Clarke, 1997; Rollins-Smith et al., 2005) and it is plausible that they will also have an antipredator function. How these peptides contribute to toxicity and antimicrobial defence, and whether their concentrations also vary among populations should be included in further studies on *S. atra* or related species.

5. Conclusion

The research question of this thesis consisted of two different parts. In the first part, it was asked whether variation in toxin composition exists among populations of the alpine salamander (*Salamandra atra*) or not. In the second part the underlying reasons for such variation, if any, were examined. My thesis is one of the few studies that tried to explain toxin variation, and to my knowledge, the first one to link microbial variation in the environment to toxin variation.

The first part of the question can be answered positively. It was indeed demonstrated that populations of *S. atra* differed considerably in both the total amount of toxins secreted as well as the composition of the toxins. However, for the second part, I did not find any support for the hypothesis that salamanders produced more toxins, or toxins with more samandarine, in populations where more potential predators were present. Body size, and by extent gland size, seemed to be associated with the presence of snake predators. Samandarone-contributions were linked to infection risk (bacterial densities and proportion of parasitic fungi), however, relative contributions of samandarone detected were probably too low to play a major role in defence against micro-organisms. Variation in toxin composition was not related to geographic distance or similarities in soil fungi community. No differences were found between male and female salamanders. In conclusion, there is only ample support for my hypothesis that toxin composition is driven by local predation pressure and microbial infection risk.

More data on the toxicity and microbial activity of other compounds, especially samandaridine and samandenone, are necessary and might shed new light on these findings. More reliable estimators of predation pressure are also recommended for better interpretation.

This study focused on variation in toxin composition, but I suggest that further research should follow a reversed approach: first study variation in toxicity and antimicrobial activity of salamander poisons among populations, and then relate this to variation in composition. The fact that *S. atra* shows

geographic variation in toxin composition also provides a good framework for further ecological and evolutionary studies.

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8. Appendix

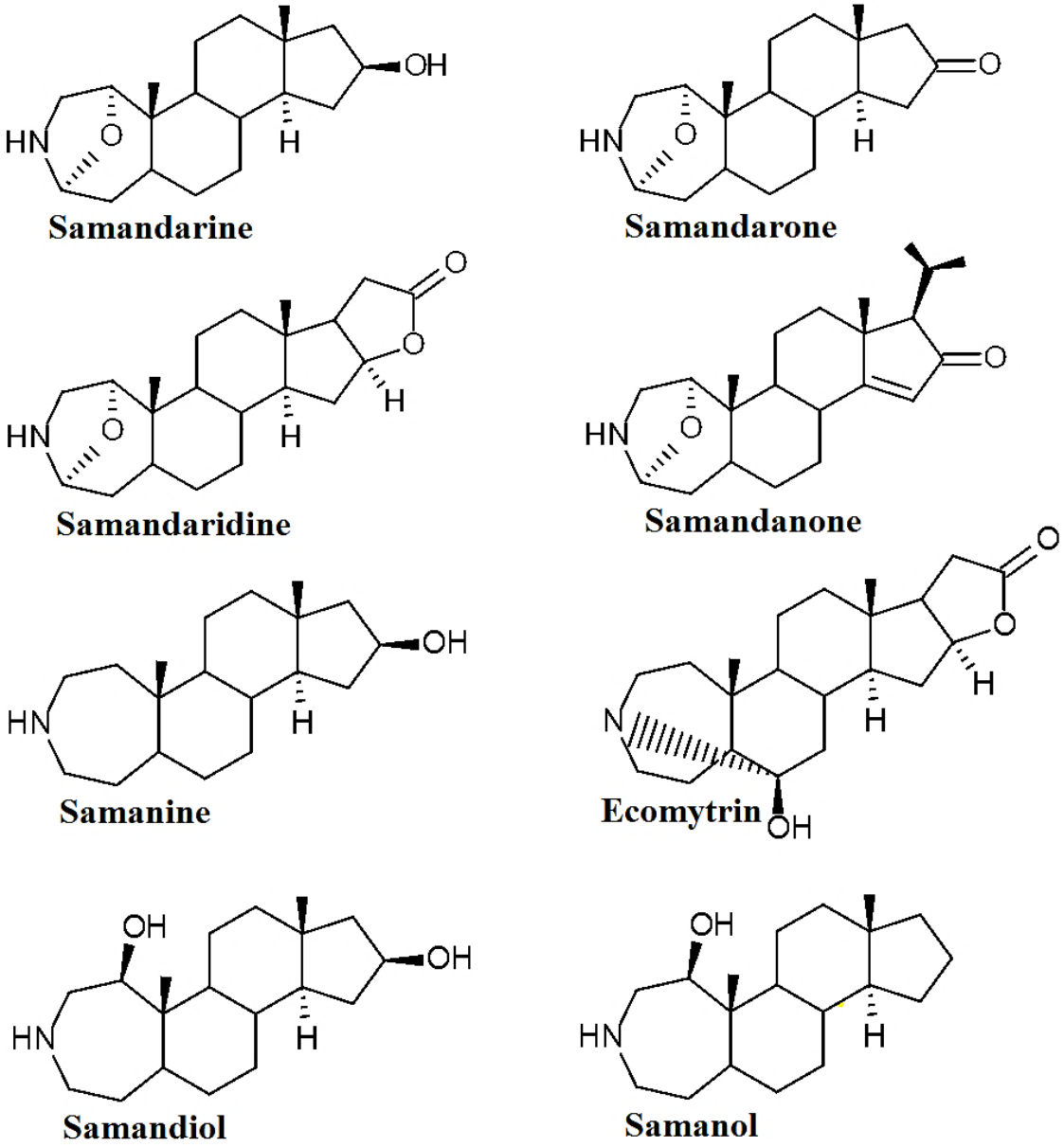

Figure 8.1: Structures of the samandarines considered in this study. Ecomytrin is more often referred to as isocycloneosamandaridine in literature. Structures after Habermehl & Spiteller (1967) and Daly et al. (1999).

Table 8.1: Settings selected for the Multiple Reaction Monitoring (MRM) during liquid chromatography – mass spectrometry (LC – MS) of 9 samandarines in the poison of *Salamandra atra*. For samanol two different peaks were found in each chromatogram, but due to not having any internal standards, I was not able to unambiguously designate one peak as samanol. Hence, the two peaks will be referred to as samanol and samanol2.

Compound	Transition	Dwell time (s)	Cone (V)	Collision energy (V)
Ecomytrin	346.50 > 306.00	0.028	18.0	33.0
Samandaridine	346.00 > 259.00	0.028	18.0	33.0
Samandanone	344.50 > 304.00	0.028	18.0	33.0
Samandiol	308.00 > 247.00	0.028	18.0	33.0
Samandarine	306.00 > 201.00	0.028	18.0	33.0
Samandarone	304.00 > 220.00	0.028	18.0	33.0
Samanine	292.50 > 292.50	0.028	18.0	33.0
Samanol	292.00 > 189.00	0.028	18.0	33.0
Samanol2	292.00 > 175.00	0.028	18.0	33.0

Table 8.2: Average relative contributions of the toxin compounds in the secretions of *Salamandra atra* per population and sex + 95 % CI. Relative contributions were expressed as the peak surface area of that compound divided by the total peak area surface of that sample. Sample sizes were as follows: Gorski Kotar (24), Čvrsnica (25), Prokletije (44) and Prenj (46). Females (70) and males (69).

	Gorski Kotar (HRV)	Čvrsnica (BIH)	Prokletije (MNE)	Prenj (BIH)	Sex	
					F	M
Ecomytrin (%)	4.0 [3.6; 4.4]	5.0 [4.5; 5.5]	3.4 [3.2; 3.7]	3.3 [3.0; 3.5]	3.6 [3.4; 3.9]	3.8 [3.5; 4.0]
Samandaridine (%)	9.1 [8.0; 10.2]	4.0 [2.9; 5.0]	3.9 [3.1; 4.7]	9.8 [9.0; 10.6]	7.5 [6.6; 8.5]	4.7 [5.1; 6.9]
Samandenone (%)	12.4 [11.0; 14.0]	11.4 [10.1; 12.8]	5.8 [5.3; 6.3]	6.3 [5.7; 6.8]	7.9 [7.1; 8.8]	7.4 [6.6; 8.2]
Samandiol (%)	0.115 [0.107; 0.123]	0.117 [0.110; 0.125]	0.134 [0.129; 0.140]	0.124 [0.118; 0.130]	0.125 [0.120; 0.130]	0.125 [0.120; 0.130]
Samandarine (%)	65.9 [63.2; 68.4]	73.3 [70.9; 75.5]	81.3 [79.8; 82.8]	78.1 [76.5; 79.6]	76.2 [74.4; 78.0]	76.5 [74.7; 78.3]
Samandarone (%)	0.061 [0.052; 0.071]	0.050 [0.044; 0.058]	0.046 [0.041; 0.052]	0.047 [0.042; 0.053]	0.053 [0.048; 0.058]	0.046 [0.042; 0.051]
Samanine (%)	5.6 [4.4; 7.1]	3.8 [3.1; 4.8]	4.7 [3.9; 5.6]	1.9 [1.6; 2.3]	3.1 [2.6; 3.7]	3.9 [3.3; 4.6]
Samanol (%)	0.061 [0.047; 0.079]	0.043 [0.034; 0.054]	0.086 [0.071; 0.104]	0.033 [0.027; 0.040]	0.048 [0.040; 0.057]	0.057 [0.048; 0.069]
Samanol2 (%)	0.24 [0.21; 0.27]	0.20 [0.17; 0.23]	0.18 [0.16; 0.20]	0.15 [0.13; 0.17]	0.19 [0.17; 0.21]	0.18 [0.16; 0.20]

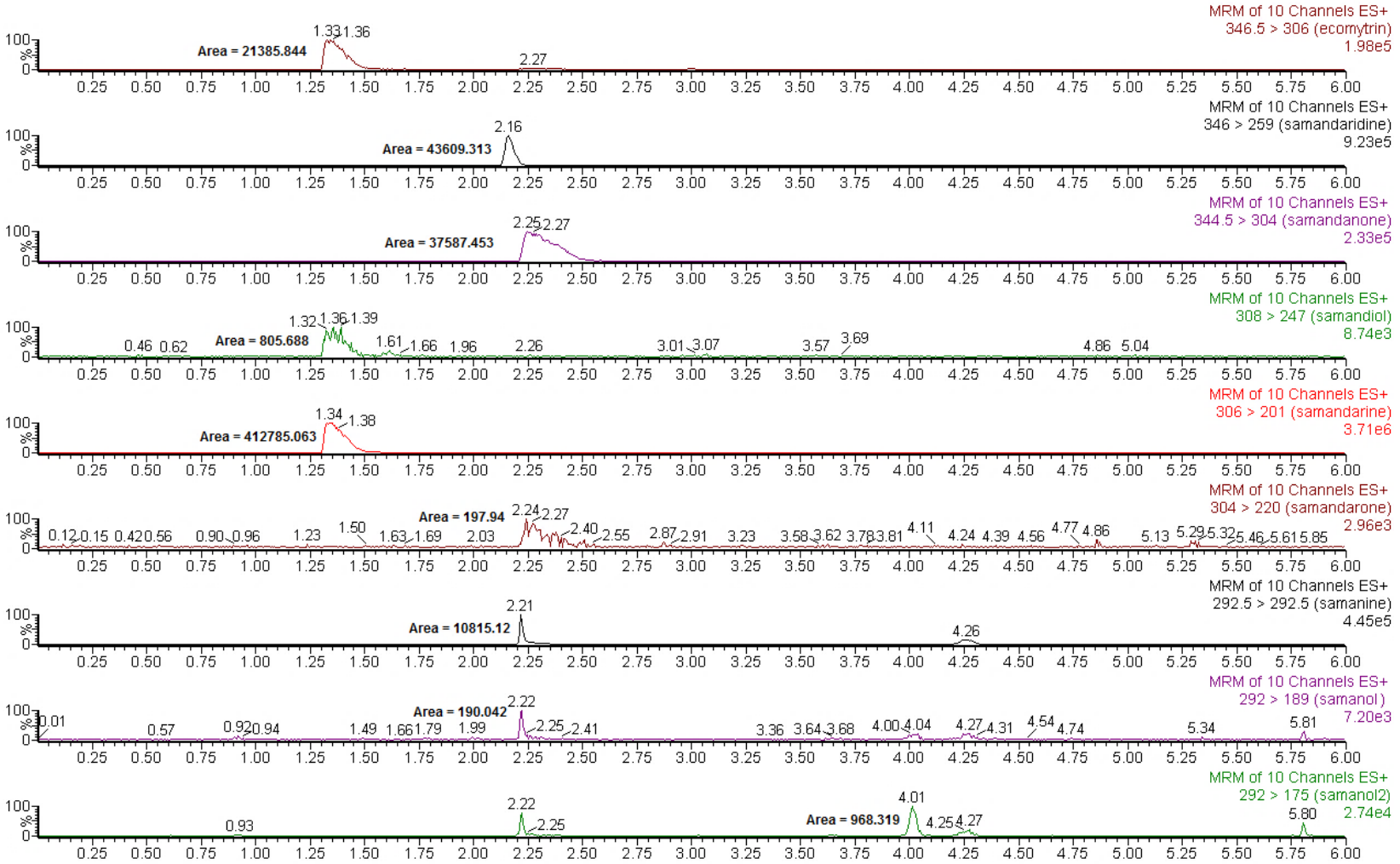

Figure 8.2: Mass spectra of samandarines included in this study. Multiple reaction monitoring (MRM)-scans are given for ecomytrin, samandaridine, samandarone, samandiol, samandarine, samandarone, samanine, samanol and samanol2. X-axis represents the retention time. Transitions on which the MRM-scans are based are given next to the names (top-right of each spectrum). Peak surface areas are given next to the selected peak of the compounds.

Table 8.3: Descriptive statistics of the morphology of *Salamandra atra* per population and per sex + 95 % CI. Gorski Kotar (14), Čvrsnica (25), Prokletije (44) and Prenj (46). Females (65) and males (64).

	Gorski Kotar (HRV)	Čvrsnica (BIH)	Prokletije (MNE)	Prenj (BIH)	Sex	
					F	M
Parotoid width (mm)	4.13 [3.84; 4.41]	4.85 [4.63; 5.07]	4.08 [3.92; 4.25]	3.95 [3.78; 4.11]	4.33 [4.18; 4.49]	4.04 [3.88; 4.20]
Parotoid length (mm)	9.03 [8.60; 9.46]	9.76 [9.43; 10.09]	9.53 [9.28; 9.78]	9.58 [9.33; 9.82]	9.64 [9744; 9.85]	9.42 [9.21; 9.63]
Head width (mm)	12.19 [11.51; 12.86]	13.99 [13.46; 14.51]	12.81 [12.41; 13.21]	12.68 [12.29; 13.07]	13.21 [12.87; 13.55]	12.62 [12.27; 12.97]
Head length (mm)	15.78 [14.91; 16.67]	18.19 [17.51; 18.87]	17.45 [16.94; 17.97]	18.17 [17.67; 18.67]	17.84 [17.39; 18.30]	17.46 [17.00; 17.92]
Body mass (g)	8.89 [7.79; 9.99]	11.37 [10.51; 12.22]	9.14 [8.50; 9.78]	9.68 [9.05; 10.31]	10.60 [10.08; 11.11]	8.84 [8.31; 9.37]

Table 8.4: Mean values with 95% confidence interval for microbial data per population, obtained from the mixed models. CFU stands for Colony Forming Unit. TSA stands for Tryptic soy broth medium. The proportion of parasite reads, number of fungal species and animal parasite species were obtained through next-generation sequencing of soil samples. Sample sizes were as follows: Sample sizes were as follows: Gorski Kotar (bacteria 5/ fungi 10), Čvrsnica (bacteria 5/ fungi 7), Prokletije (bacteria 12/ fungi 12) and Prenj (bacteria 8/ fungi 8).

	Gorski Kotar (HRV)	Čvrsnica (BIH)	Prokletije (MNE)	Prenj (BIH)
CFU/g on TSA at 37°C	$116 * 10^3$ $[38; 357]*10^3$	$105 * 10^3$ $[31; 360]*10^3$	$67 * 10^3$ $[30; 147]*10^3$	$110 * 10^3$ $[39; 312]*10^3$
CFU/g on TSA at 15°C	$6.9 * 10^6$ $[1.1; 17.8]*10^6$	$3.5 * 10^6$ $[0.2; 9.3]*10^6$	$5.1 * 10^6$ $[2.1; 9.5]*10^6$	$6.1* 10^6$ $[1.1; 16.1]*10^6$
CFU/g on Mac-Conkey	$105 * 10^3$ $[15; 718]*10^3$	$15 * 10^3$ $[2; 120]*10^3$	$15 * 10^3$ $[4; 62]*10^3$	$26 *10^3$ $[4; 167]*10^3$
Proportion parasite reads	0.0031 [0.0008; 0.0119]	0.00015 [0.00003; 0.00076]	0.0010 [0.0003; 0.0035]	0.0003 [0.0001; 0.0012]
Number of fungal species	78 [61; 96]	83 [62; 104]	77 [61; 92]	73 [53; 95]
Number of animal parasite species	2.90 [0.23; 4.78]	0.71 [0; 1.85]	1.58 [0;4]	0.50 [0; 1.83]

Table 8.5. Identified animal pathogens, including their normal hosts and their occurrence in the different populations. Numbers between brackets indicate the number of identified species of this genus, e.g. *Pochonia* (2) means that there were two unidentified *Pochonia* species detected.

Species	Host	Population				References
		Gorski Kotar	Čvrsnica	Prokletije	Prenj	
Ascosphaera apis	Insects – mostly bees			X	X	Qin et al. (2006)
***Beauveria* sp.**	Insects	X	X	X	X	Goettel et al. (2005); Rehner et al. (2011).
Engydotium album	Humans	X				Macêdo et al. (2007)
Haptocillium balanoides	Nematodes	X		X		Zare & Gams (2001)
Isaria farisona	Insects	X				Zimmerman (2008)
Lecanicillium psalliotae	Insects and nematodes	X			X	Zare & Gams (2001)
***Pochonia* sp. (2)**	Nematodes, rotifers, insects, molluscs	X		X		Stadler et al. (2003).
Tolypocladium album	Insects, rotifers and fungi	X		X		Quandt et al. (2014)
***Tolypocladium* sp. (3)**	Insects, rotifers and fungi	X	X	X		Quandt et al. (2014)
Trichosporon coremmiforme	On skin of humans and animals, opportunistic				X	Middelhoven et al. (2004)
***Trichosporon* sp.**	On skin of humans and animals	X		X		Middelhoven et al. (2004)

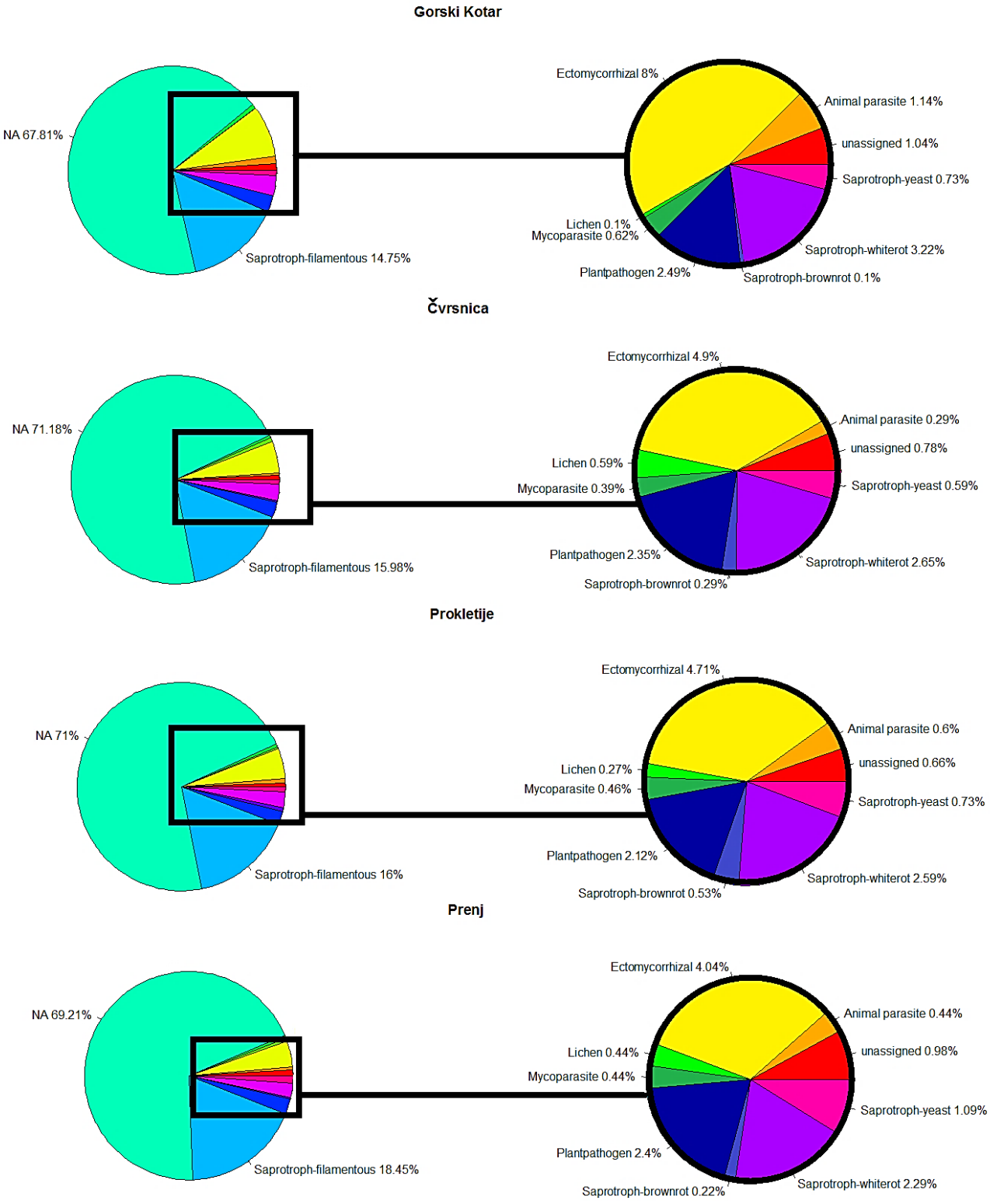

Figure 8.3: Distribution of lifestyles of identified fungal OTUs per population. Assignment of lifestyles to genera was based on the database of Tedersoo et al. (2014). NA = OTU could not be identified to the genus level and thus could not be linked to a lifestyle. Unassigned species were species that could be identified, but were not assigned to a lifestyle because either their lifestyle is not known, the lifestyle is not conserved at the genus level or the genus is not included in the database of Tedersoo et al. (2014).