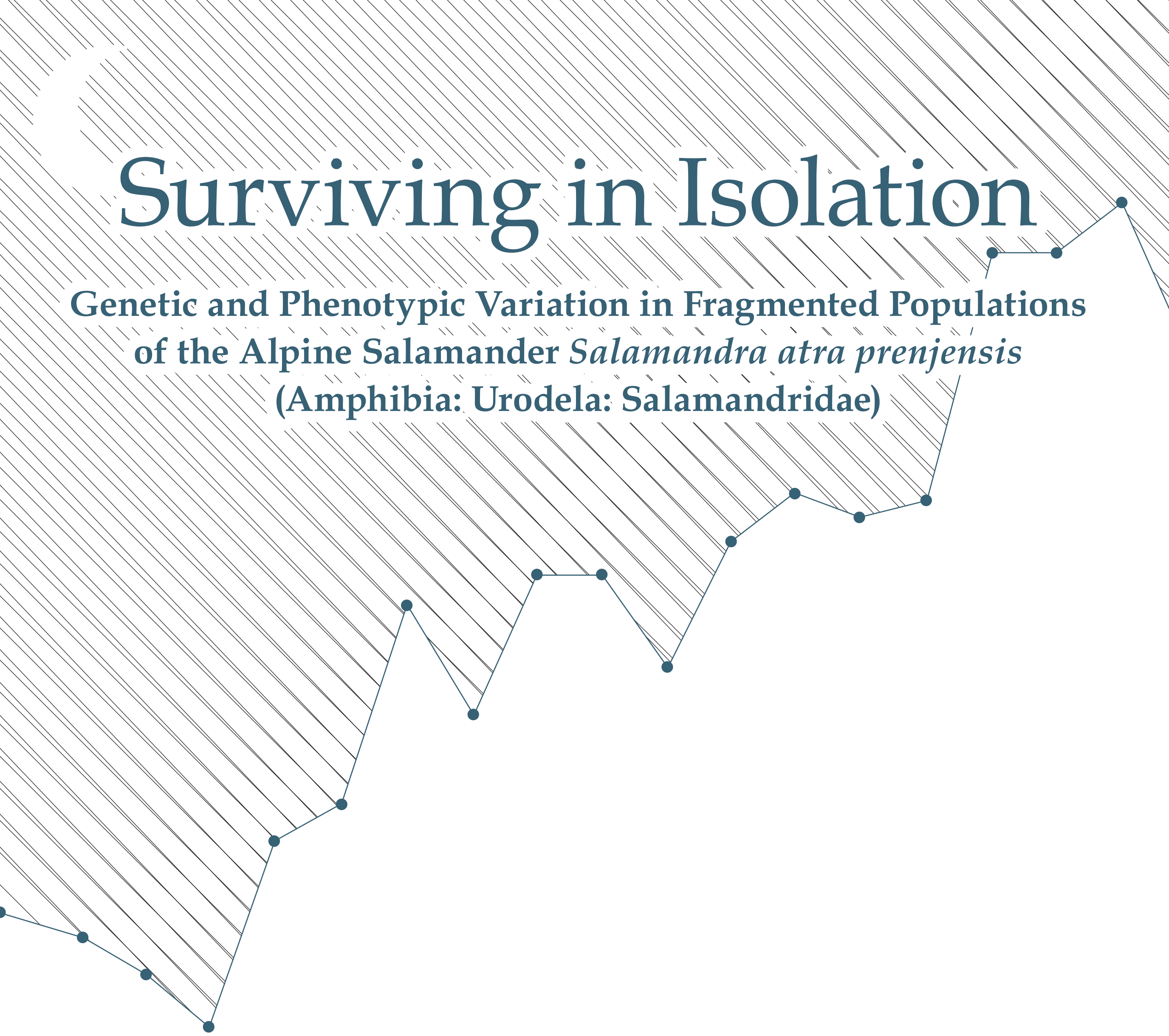
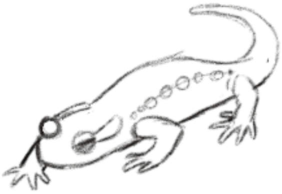


Surviving in Isolation

Genetic and Phenotypic Variation in Fragmented Populations of the Alpine Salamander *Salamandra atra prenjensis* (Amphibia: Urodela: Salamandridae)



Dissertation for the degree of doctor in Science (Biology) under the joint collaboration of the University of Antwerp and the University of Sarajevo to be defended by **Emina Šunje**

Promotors:
Prof. dr. Raoul Van Damme
Prof. dr. Lada Lukić Bilela

Faculty of Science
Department of Biology
Antwerp, 2022

Universiteit Antwerpen

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The
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Illustrations of salamanders on the cover page, chapter title pages, and throughout the thesis are made by **Alen Burazerović.**

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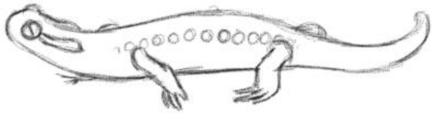

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Foreword

It is a funny anecdote about how all of this started. At the third year of my Biology studies, after the final exam for the course of 'Animal behaviour', I got a 'C' although I was missing a single point for a 'B' grade. Clearly, I tried to convince the former professor, Suvad Lelo that giving me one more point is not a big deal for him but it is for me. After some negotiations he gave me the task to write a small research paper on any subject related to zoology and, if it was good work, I would get a 'B'.

At that time, I was assisting many volunteering actions to rebuild the small hut "Jezerce" on mountain Prenj that was destroyed during the Bosnian war (1992 – 1996). So, for the topic of my research paper, I decided to compile a checklist of amphibians from this area as I had many encounters with different species up there. At the next building action I was ready for my research task. During a break from the hard work on the hut, I was exploring the nearby pond in search of amphibians. My quest caught the attention of Kenan Muftić, a passionate naturalist participating in the reconstruction, who approached me and asked me what I was searching for. After explaining him what was all about, he asked me if I knew about the existence of the unusual Alpine salamander living on mt. Prenj. As I did not, he promised that he would show me these animals although it required waking up at 4 am and hiking another hour away from the hut. I am not sure whether it was the full moon that night, the *rakija* that had been passed on around the fire or the excitement to meet these critters, but I had troubles sleeping that evening and was impatiently waiting for the clock hands to show 04:00 am. Finally, when the moment arrived, Kenan, as promised, walked me onto the plateau of Prenj where I saw the 'small dragons' for the first time. Many individuals were lying hidden in the grass, near rocks and boulders – it was love at first sight – so small, yet so attractive and mysterious… indeed, the absolute rulers of this harsh environment.

This finding did not only ensure me a 'B' for the course of 'Animal Behaviour', but it had completely enchanted me. In the following years, my passion for nature and alpinism kept me returning to the magnificent mountain Prenj, so I decided to devote my bachelor thesis to the study of the curious creatures I had met. My bachelor thesis turned out to be the foundation work for my master thesis, for which I continued studying the genetic structure of the Alpine salamanders from mt. Prenj, also as a contribution to their conservation. I got hooked by the work and this encouraged me to apply for a conservation grant to continue studying alpine salamanders, and this is how, in 2013, I obtained my first Rufford grant. At the early stage of the project, I was filled with enormous enthusiasm but I lacked experience in ecological field-work researches. Moreover, Dinaric populations of Alpine salamanders are challenging to study because they are, in most cases, difficult to get to and are also known for their elusive behaviour. Just reaching the plateaus of the high mountains in the Dinarides requires several hours of hiking on difficult terrain with a bunch of equipment carried on the back. Working often in wet, foggy conditions and late-night hours, spices up the overall experience.

Nevertheless, in collaboration with the Biodiversity Association 'Hyla' (Croatia) and a small local team, we successfully completed the first Rufford project. I gained ample experience; I learned details about the activity patterns, environmental preferences and population structure of Alpine salamanders in the central Dinarides (Prenj and Čvrsnica). This allowed expanding the project regionally (Northern and Southern Dinarides, 2015). By the end of the sampling season in 2015, I collected a variety of ecological data and samples included in this thesis. Soon after, in 2016, the project benefited from the crucial collaboration with the University of Antwerp through an Erasmus grant (Joint Penta EU SEE). It is in this period (2016 – 2018) that the main research ideas of this PhD were born and tested.

As any research enterprise, this study on the biology of the little Dinaric dragons is unfinished. The research on Alpine salamanders does not end with this thesis, actually, it seems just starting now as this PhD divulged interesting avenues for future research.

In my personal view, the most valuable outcome of this work is that it provides valuable insights for defining decisive conservation strategies that may insure the survival of Alpine salamanders in the Dinarides in the current time of climate change and biodiversity loss.

I hope you will enjoy!

To mom and dad,

Who always believed in me

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Summary

Intraspecific variation is essential for securing the adaptability and viability of natural populations, which also explains why it is particularly relevant from a conservation perspective. Understanding whether, how, and how fast organisms can adjust to environmental changes is more timely than ever considering the global environmental crisis of today (climate change, habitat change, biodiversity loss). Because of their particular physiological and life-history traits (permeable skin, low capacity of physiological buffering, relatively: low vagility, slow maturation, long generation time), terrestrial salamanders are especially threatened by environmental changes. Among salamanders, the Alpine salamander (*Salamandra atra*) is of particular concern; it is associated with cold climates (Alps, Dinarides) and has a viviparous reproductive mode accompanied by a very long gestation and low fecundity. In the Dinarides, the species is further threatened by the fragmented nature of its distribution. Spread across the 650 km long Dinaric Arc, isolated populations now survive on or near mountain tops, as relics of a more continuously distributed Pleistocene population. Given their latitudinal and altitudinal spread, it seems likely that these isolated 'sky-island' populations will experience divergent conditions (climate, structural habitat) and hence will exhibit geographical variation in various biological aspects (morphology, behaviour, physiology, diet). Studying geographic variation in naturally fragmented populations, such as *S. atra*, may help to understand aspects of population adaptability that is relevant both from a fundamental and applicative perspective.

In this PhD dissertation, I studied the geographic variation among populations of the Alpine salamander but particularly focused on the fragmented, Dinaric populations. I explored the potential role of the environment as the driver of variation and studied the impact of climatic change on specie's distribution.

Firstly, I investigated genetic variation that confirmed the unique evolutionary potential of Dinaric specimens in relation to other *S. atra* populations along the specie's range. I unravelled the evolutionary relationships among four Dinaric population fragments and discovered two refugia in the Dinarides that secured survival of Alpine salamanders during the Pleistocene glaciations. Moreover, I provide evidence that Dinaric populations where connected in the past and that mt. Prenj was the main diversification centre of Alpine salamanders.

Secondly, I studied morphological variation in two populations of each subspecies: *Salamandra atra atra* (Alps) and *Salamandra atra prenjensis* (Dinarides). I found morphological differentiation between the Alpine and Dinaric populations where former individuals, compared to the latter, are slightly longer with more costal grooves, while Dinaric specimens have wider heads and jaws compared to their northern relatives. I also found sexual dimorphism in traits associated with dispersal and reproduction.

Further, I studied the diet variation in four Dinaric population fragments also in relation to prey availability. All populations have an equally wide dietary span that is among the largest reported for terrestrial salamanders, while the amount of ingested prey is rather low compared to other salamander species. The most consumed prey taxa are the same across populations. Although younger individuals fed more than adults, the quantity of consumed prey did not differ among populations.

Following, I investigated the toxin variation in four Dinaric population fragments and preliminary explored whether potential variation could be explained by predation and infection risk. I found that the populations of salamanders differed in toxin composition. However, the quantity of produced toxins was the same across populations. Males and females did not differ in toxin composition nor produced quantity. I could not explain the toxin variation neither by predation nor by infection risk as populations barely differed in these variables despite substantial differences in their habitats.

I also studied the variation of mass-specific water loss rate (EWL) in two distant Dinaric populations using an experimental approach under controlled laboratory conditions (temperature and humidity). The results of this study suggest that individuals originating from a drier environment are more resistant to water evaporation, allowing them to be more active aboveground. The ecological relevance of the detected water loss rate suggests that risk of desiccation is the key factor determining the window of activity in the Alpine salamander especially in the drier site, therefore, it may have also driven the detected variation in EWL.

Finally, I use multiple modelling techniques coupled with numerous climate model projections to assess the impact of climate change on specie's distribution. Within the next 50 years, the predicted loss of climatically suitable land for *S. atra* in the Alps is between 27 - 53% of Alpine territory; ample decrease of climatic suitability is predicted in the eastern and southern Alps while areas in the North-Western Alps remain suitable. Predictions for the Dinarides are more striking; the anticipated loss of suitable land is between 52 - 87% of Dinaric territory where the remaining land quality, necessary for specie's occurrence, becomes considerably poor.

Given the registered variation on multiple biological levels, *S. atra* signals adaptive potential. However, it remains unclear whether it will secure survival taking into consideration the detected amplitude of ongoing climate changes. Overall, my PhD dissertation provides insights considering the biology of *S.atra* and delivers necessary knowledge to ensure the long-term conservation of Alpine salamanders.

Sažetak

Intraspecifična varijacija osigurava adaptaciju i održivost prirodnih populacija, što ujedno objašnjava i njen značaj u konzervacijskom kontekstu. Razumijevanje da li, kako i koliko brzo organizmi mogu da se prilagode promjenama okoliša je značajnije nego ikad s obzirom na današnju globalnu ekološku krizu (klimatske promjene, degradacije staništa, gubitak biodiverziteta). Zbog posebnih bioloških karakteristika (propusna i tanka koža, niska vagilnost, sporo sazrijevanje, dugo generacijsko vrijeme), kopneni daždevnjaci su osjetljivi promjenama okoline. Među daždevnjacima, alpski daždevnjak (*Salamandra atra*) je posebno ugrožen. Pojava vrste je uvjetovana hladnom klimom (Alpi, Dinaridi) te ove životinje imaju viviparan način razmnožavanja praćen veoma dugom gestacijom i niskim fekunditetom. U Dinaridima, vrsta je dodatno ugrožena fragmentiranom prirodom svoje rasprostranjenosti. Izolirane populacije alpskog daždevnjaka opstaju duž kompletnog Dinarskog luka, gdje se mogu naći neposredno ispod najviših planinskih vrhova kao glacijalni relikti nekadašnje široko rasprostranjene pleistocenske populacije. S obzirom na široko longitudinalno i latitudinalno rasprostranjenje, za pretpostaviti je da su izolirane populacije 'nebeskih otoka' vjerovatno izložene različitim uvjetima (klima, stanište) koji mogu izazvati geografsku varijaciju na različitim biološkim instancama (morfologija, ponašanje, fiziologija, prehrana). Izučavanje geografske varijacije u prirodno fragmentiranim populacijama, kao što je *S. atra*, može pomoći razumjevanju adaptacijskih mehanizama populacija koji su relevantni kako iz fundamentalne, tako i aplikativne perspektive.

U ovoj doktorskoj disertaciji proučavala sam geografsku varijaciju među populacijama alpskog daždevnjaka, fokusirajući se posebno na fragmentirane, dinarske populacije. Istraživala sam potencijalnu ulogu okoliša kao pokretača varijacije te uticaj klimatskih promjena na distribuciju vrste.

Istraživanje genetičke varijacije potvrdilo je jedinstveni evolucijski potencijal dinarskih primjeraka u odnosu na druge populacije *S. atra* duž areala vrste. Razriješila sam evolutivne odnose između četiri fragmentirane dinarske populacije te otkrila dva refugijuma u Dinaridima koji su osigurali opstanak alpskih daždevnjaka tokom pleistocenskih glacijacija. Također, prezentiram dokaze da su dinarske populacije bile povezane u prošlosti i da je planina Prenj predstavljala glavni centar diversifikacije alpskih daždevnjaka.

Analizirala sam i morfološku varijaciju populacija podvrste: *Salamandra atra atra* (Alpe) i *Salamandra atra prenjensis* (Dinaridi). U odnosu na Dinarske jedinke, Alpske jedinke su nešto duže sa većim brojem rebarnih žlijebova, dok dinarski primjerci imaju šire glave i čeljusti. Registrovala sam i seksualni dimorfizam u morfološkim osobinama povezanim sa rasprostranjenjem i razmnožavanjem.

Dalje, proučavala sam varijaciju u prehrani između četiri fragmentirane dinarske populacije također u odnosu na dostupnost plijena. Populacije imaju jednako raznoliku ishranu. Stepen raznovrsnosti ishrane je među najvećim registrovanim za kopnene daždevnjake, dok je količina ingestiranog plijena prilično niska u poređenju s drugim vrstama daždevnjaka. Najzastupljeniji taksoni plijena su isti među populacijama. Iako se mlađe jedinke hrane više od odraslih, količina konzumiranog plijena se ne razlikuje među populacijama.

Slijedi istraživanje varijacije toksina gdje sam pored kvantitativne i kvalitativne analize toksičnih supstanci koje ove životinje luče, preliminarno i istražila da li varijacija toksina može biti objašnjena predacijom i rizikom od infekcije. Istraživanje je pokazalo da se populacije daždevnjaka razlikuju prema sastavu toksina ali ne i prema količini proizvedenih. Nisam našla spolni dimorfizam u sastavu toksina niti u proizvedenoj količini. Predacijski i infektivni rizik nisu mogli objasniti varijaciju toksina jer se pokazalo da se ovi pritisci ne razlikuju znatno među populacijama uprkos značajnim razlikama u njihovim staništima.

Istražila sam i maseno-specifični stepen isparavanja vode (EWL) iz tijela daždevnjaka. EWL varijaciju sam pratila kod jedinki porijeklom iz dvije udaljene dinarske populacije koristeći eksperimentalni pristup u kontrolisanim laboratorijskim uslovima (temperatura i vlažnost). Rezultati ove studije sugerišu da su primjerci porijeklom iz sušnijeg habitata otporniji na isparavanje vode, što im omogućava prolongiranu aktivnost iznad zemlje. Ekološki značaj registrovane EWL varijacije sugeriše da je, kod *S. atra*, rizik od isušivanja ključni faktor koji definiše dužinu trajanja aktivnosti (iznad zemlje). Budući da je populacija sušnijeg lokaliteta izložena većem riziku od isušivanja, moguće je da je upravo taj pritisak pokretač registrovane (EWL) varijacije.

Na koncu, koristila sam veći broj tehnika modeliranja te brojne projekcije klimatskih modela kako bih procijenila uticaj klimatskih promjena na distribuciju vrste. U narednih 50 godina, predviđeni gubitak klimatski pogodnog staništa za *S. atra* u Alpama je između 27 - 53% alpske teritorije; u istočnim i južnim Alpama predviđa se veliko smanjenje klimatske pogodnosti, dok područja u sjeverozapadnim Alpama ostaju pogodna. Predikcije za Dinaride su upečatljivije; predviđeni gubitak odgovarajućeg staništa je procjenjen između 52 - 87% dinarske teritorije gdje preostali kvalitet staništa, neophodan za pojavu vrste, postaje znatno lošiji.

Budući da populacije *S. atra* ispoljavaju varijaciju na većem broju bioloških instanci, to signalizira i adaptivni potencijal vrste. Međutim, ostaje nejasno da li će adaptivni potencijal osigurati opstanak s obzirom na otkrivenu amplitudu tekućih klimatskih promjena.

Ova doktorska disertacija pruža uvid u biologiju vrste te neophodno znanje kako bi se osiguralo dugoročno očuvanje populacija alpskih daždevnjaka.

Samenvatting

Intraspecifieke variatie is essentieel voor het waarborgen van het aanpassingsvermogen en de levensvatbaarheid van natuurlijke populaties, wat ook verklaart waarom het bijzonder relevant is vanuit het oogpunt van instandhouding. Begrijpen of, hoedat en hoe snel organismen zich kunnen aanpassen aan veranderingen in het milieu, is actueler dan ooit gezien de wereldwijde milieucrisis van vandaag (klimaatverandering, verandering van leefgebied, verlies van biodiversiteit). Vanwege hun specifieke fysiologische en levenshistorische eigenschappen (permeabele huid, lage capaciteit van fysiologische buffering, relatief: lage vagiliteit, langzame rijping, lange generatietijd), worden terrestrische salamanders vooral bedreigd door veranderingen in de omgeving.

Onder salamanders is de Alpensalamander (*Salamandra atra*) van bijzonder belang; het wordt geassocieerd met koude klimaten (Alpen, Dinarides) en heeft een levendbarende reproductieve modus die gepaard gaat met een zeer lange zwangerschap en lage vruchtbaarheid. In de Dinarides wordt de soort verder bedreigd door de gefragmenteerde aard van zijn verspreiding. Verspreid over de 650 km lange Dinarische Boog, overleven geïsoleerde populaties nu op of nabij bergtoppen, als overblijfselen van een meer continu verspreide Pleistocene populatie. Gezien hun breedte- en hoogtespreiding, lijkt het waarschijnlijk dat deze geïsoleerde 'hemeleiland'-populaties uiteenlopende omstandigheden (klimaat, structurele habitat) zullen ervaren en dus geografische variatie zullen vertonen in verschillende biologische aspecten (morfologie, gedrag, fysiologie, dieet). Het bestuderen van geografische variatie in natuurlijk gefragmenteerde populaties, zoals *S. atra*, kan helpen om aspecten van het aanpassingsvermogen van de populatie te begrijpen die zowel vanuit een fundamenteel als een toepassingsperspectief relevant zijn.

In dit proefschrift heb ik de geografische variatie tussen populaties van de Alpensalamander bestudeerd, maar met name gefocust op de gefragmenteerde, Dinarische populaties. Ik onderzocht de mogelijke rol van de omgeving als aanjager van variatie en bestudeerde de impact van klimaatverandering op de verspreiding van soorten.

Ten eerste heb ik genetische variatie onderzocht die het unieke evolutionaire potentieel van Dinarische exemplaren bevestigde in relatie tot andere *S. atra* - populaties in het verspreidingsgebied van de soort. Ik ontrafel de evolutionaire relaties tussen vier Dinarische populatiefragmenten en ontdekte twee refugia in de Dinarides die de overleving van Alpensalamanders tijdens de Pleistocene ijstijden verzekerden. Bovendien lever ik bewijs dat Dinarische populaties in het verleden met elkaar verbonden waren en dat berg Prenj was het belangrijkste diversificatiecentrum van Alpensalamanders.

Ten tweede heb ik de morfologische variatie bestudeerd in twee populaties van elke ondersoort: *Salamandra atra atra* (Alpen) en *Salamandra atra prenjensis* (Dinarides). Ik vond morfologische differentiatie tussen de Alpen- en Dinarische populaties waar voormalige individuen, in vergelijking met de laatste, iets langer zijn met meer ribbengroeven, terwijl Dinarische exemplaren bredere koppen en kaken hebben in vergelijking met hun noordelijke verwanten. Ik vond ook seksueel dimorfisme in eigenschappen die verband houden met verspreiding en voortplanting.

Verder heb ik de dieetvariatie in vier Dinarische populatiefragmenten bestudeerd, ook in relatie tot de beschikbaarheid van prooien. Alle populaties hebben een even groot voedingsbereik dat tot de grootste behoort voor terrestrische salamanders, terwijl de hoeveelheid ingenomen prooi vrij laag is in vergelijking met andere salamandersoorten. De meest geconsumeerde prooitype zijn hetzelfde over populaties. Hoewel jongere individuen meer voedden dan volwassenen, verschilde de hoeveelheid geconsumeerde prooi niet tussen populaties.

Vervolgens heb ik de toxinevariatie in vier Dinarische populatiefragmenten onderzocht en voorlopig onderzocht of mogelijke variatie verklaard kon worden door predatie en infectierisico. Ik ontdekte dat de populaties salamanders verschilden in toxinesamenstelling. De hoeveelheid geproduceerde toxines was echter hetzelfde in alle populaties. Mannetjes en vrouwtjes verschilden niet in toxinesamenstelling of geproduceerde hoeveelheid. Ik kon de toxinevariatie noch door predatie, noch door infectierisico verklaren, aangezien populaties nauwelijks verschilden in deze variabelen ondanks aanzienlijke verschillen in hun leefgebieden.

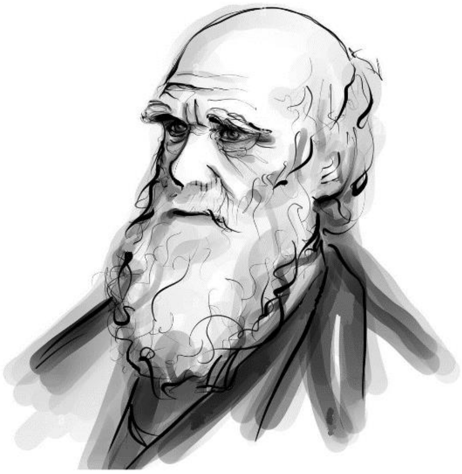
Ik heb ook de variatie van massaspecifieke waterverliessnelheid (EWL) in twee verre Dinarische populaties bestudeerd met behulp van een experimentele benadering onder gecontroleerde laboratoriumomstandigheden (temperatuur en vochtigheid). De resultaten van dit onderzoek suggereren dat individuen afkomstig uit een drogere omgeving beter bestand zijn tegen verdamping van water, waardoor ze bovengronds actiever kunnen zijn. De ecologische relevantie van de gedetecteerde waterverliessnelheid suggereert dat het risico op uitdroging de belangrijkste factor is die het activiteitsvenster van de Alpensalamander bepaalt, vooral op de drogere locatie, en daarom kan het ook de gedetecteerde variatie in EWL hebben veroorzaakt.

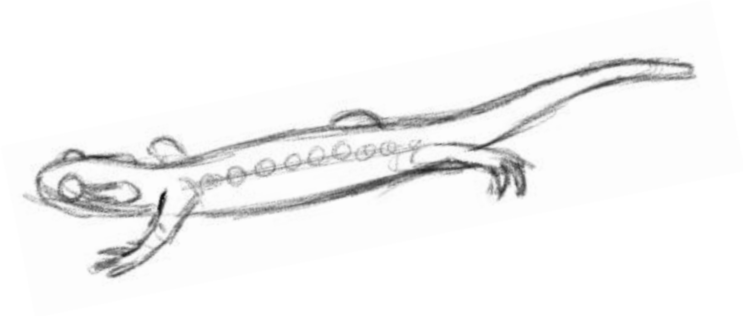
Ten slotte gebruik ik meerdere modelleringstechnieken in combinatie met talrijke projecties van klimaatmodellen om de impact van klimaatverandering op de verspreiding van soorten te beoordelen. Het voorspelde verlies van klimatologisch geschikt land voor *S. atra* in de Alpen ligt tussen 27 en 53% van het Alpengebied; in de oostelijke en zuidelijke Alpen wordt een ruime afname van de klimatologische geschiktheid voorspeld, terwijl gebieden in de noordwestelijke Alpen geschikt blijven. Voorspellingen voor de Dinarides zijn opvallender; het verwachte verlies aan geschikt land ligt tussen 52 en 87% van het Dinarische grondgebied, waar de resterende landkwaliteit, die nodig is voor het voorkomen van de soort, aanzienlijk slecht wordt.

Gezien de geregistreerde variatie op meerdere biologische niveaus, signaleert *S. atra* adaptief potentieel. Het blijft echter onduidelijk of het zal overleven, rekening houdend met de gedetecteerde amplitude van aanhoudende klimaatveranderingen. Over het algemeen biedt mijn proefschrift inzicht in de biologie van *S. atra* en levert het de nodige kennis om de instandhouding op lange termijn van (Dinarische) populaties van Alpensalamanders te garanderen.

‘It is not the strongest of the
species that survive, nor the
most intelligent, but the one
most responsive to change’

Charles Darwin





General introduction

Biological variation

Variation pervades all levels of biological organization: it occurs within and among individuals, populations, species, communities and ecosystems. Differences among species and higher taxa have long time been considered evidence for a Divine Plan and have been used by scholars starting with Plato and Aristotle to bring order in the living world. In sharp contrast, lower-level variation has long been considered a 'nuisance'. In Plato's typological essentialism, for instance, differences among individuals within species were deemed unintended and meaningless departures from the essence, the species' 'Form' (Mayr 1982, Vasil'eva 2003). It was not until Darwin and Wallace (1858) that inter-individual variation was recognized as the quintessence of evolution, and central to the whole of biology.

More recently, against a background of worldwide habitat fragmentation and climate change, it has become painfully evident that intraspecific variation is playing a key role in the adaptability and viability of natural populations (Mimura et al. 2006); understanding whether, how, and how fast organisms can adjust to environmental changes is now more timely than ever.

The central topic of this thesis is the study of geographic variation among populations of the Alpine salamander (*Salamandra atra* Laurenti 1768). As the future of this species is particularly periled by climate changes and fragmentation, any well thought-through conservation action plan will need to have roots in evolutionary biology. Therefore, this thesis contributes both to the fundamental and applicative context of the study of geographic variation.

The study of geographic variation

Documenting and sorting out variation is the core of taxonomy and biological classification. Starting with Carl Linnaeus (1735) and for the next 100 years or so, this biological discipline was mostly concerned with describing variation among species. However, among the earliest systematists, the zoologist E.J.C. Esper and the botanist J.F Ehrhart have already pointed out the importance of describing the variation below the species level and even proposed specific nomenclatural rules for the purpose (Vinarski 2015). Unfortunately, Espers' and Erharts' observations were generally ignored because many taxonomists wished to preserve the purity of the simple binomial system (genus-species). However, as biological collections were growing worldwide, so did the evidence for intraspecific geographic variation.

By the first half of the 19th century German biologists had noticed that species exhibit predictable, gradual phenotypic variation with latitude. The first to notice this was Gloger (1833) who showed that within many bird species, heavily pigmented individuals are more common in cold humid environments at higher latitudes. Following, Bergmann (1847) noticed that within mammal species, populations at higher latitudes consist of larger individuals. These observations are among the first clues that revealed a systematic link between environmental and phenotypic variation; however, not Gloger nor Bergman, disclosed the actual cause of this link.

In the early 1850s, geographic forms of the same species started to be viewed as an important intermediate stage between insignificant local variants and true species; only then, geographic variation started to be recognized as an important phenomenon. Undoubtedly, accumulated evidence of intraspecific geographic variation catalysed the birth of a new paradigm - the theory of evolution by means of natural selection (Darwin and Wallace 1858). More specifically, Charles Darwin and Alfred Russel Wallace realised that geographic variation, in absence of true time series, was the smallest scale at which the effects of natural selection were evident. Shortly after the delivery of the theory of evolution, intraspecific geographic variation became also the central aspect of speciation theories such as the allopatric/vicariant theory (Wagner 1868)[1], Kleinschmidt's 'nonspeciation' theory and Lotsy's hybridization theory (Mallet 2013). In parallel, naturalists such as Henry Bates, Elliott L. Coues and Karl Jordan started to use the trinomial nomenclature to formalize the existence of subspecies as distinct geographic forms. In other words, the 'subspecies' became a valid taxon representing incipient species (Mallet 2013). Clearly, after 1859, intraspecific variation, from a meaningless nuisance, became a central aspect of biology.

To conclude, the phenomenon of geographic variation played a central role in the birth and development of several concepts in both evolutionary biology and ecology (Thorpe 1987). Until today, the study of geographic variation continues to inspire researchers because it serves as a window into the evolutionary history and onto the potential future of species.

The adaptive nature of geographic variation

Geographic variation concerns differences in the average phenotype of two or more extant populations and may reflect genetic differences, phenotypic plasticity or a combination of both.

Differences in the genetic make-up can arise through local adaptation, but also by chance (genetic drift).

Local adaptation is a process that causes each local population to evolve traits that provide fitness advantage under the local environmental conditions (Kawecki and Erbert 2004). Natural selection is the underlying force of local adaptation. However, spatial variation in the nature or direction of selection will result in local adaptation and geographic variation only when gene flow among populations is restricted. This is generic in the case of physically isolated

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[1] According to Sulloway (1979), there is a controversy as to whether Darwin recognized a true geographical-based model of speciation in his first editions of the 'Origin of Species' (1859-1866)

populations, but may also occur in parapatry (Mayr 1970). Examples of local adaptation are numerous. For instance, in many species of brown frogs (*Rana spp.*), individuals from colder habitats will grow faster at low temperatures than individuals from warmer habitats (Skelly 2004, Ficetola and De Bernardi 2005, Oriazola et al., 2010, Richter-Boix et al., 2010). Evolutionary arms races provide an example in which a biotic component of the environment may drive local adaptation. For instance, Pacific newts (*Taricha granulosa*) evolve to become more toxic when sympatric with predatory garter snakes (*Thamnophis sirtalis*) but in turn, the snakes grow less sensitive to the newts' toxins (Brodie and Brodie 1990). Variation in the level, direction or target of sexual selection may also generate geographic variation, as seen for e.g. in Peter's dwarf frog (*Engystomops petersi*), where the high variation in the advertisement calls of males results from differences in female preferences for signal complexity (Boul 2007).

Nevertheless, attributing any instance of geographic variation to differential natural (or sexual) selection would represent a bad case of pan-adaptationism (Gould and Lewontin 1979). This is especially true for species consisting of smaller and isolated populations where genetic drift may also be an important source of geographic variation. As known, new populations may arise from small groups of colonizers with atypical allele frequencies ('founder-effect'), or may acquire deviant allele frequencies after the colonisation event, by random fixation (Mayer 1970). Such 'chance' effect appears less attractive to functional biologists, so examples of geographic variation by genetic drift are less prominent in the literature. However, one convincing case is the geographic variation in advertisement calls produced by Ornate chorus frogs (*Microhyla fissipes*; Lee et al., 2016).

Of course, natural selection and random drift may act to produce geographic variation at the same time (Mayer 1970). This is nicely illustrated in the Portuguese fire salamander (*Salamandra salamandra gallaica*) of the Iberian

Peninsula (Velo-Antón et al. 2012). Island populations of this subspecies carry the genetic marks characteristic of founding events and genetic drift, but the rapid, parallel shift from a larviparous (mainland) to viviparous reproductive mode (island) is most probably the result of natural selection, driven by a dearth of water bodies on the islands (Velo-Antón et al. 2012).

Geographic variation can also arise through non-genetic, plastic changes in the phenotype, a phenomenon described as phenotypic plasticity. Phenotypic plasticity, as a major mechanism of response to environmental variability, describes the capacity of the same individual organisms to exhibit different characteristics under varied environmental conditions (Pigliucci 2005). Phenotypic plasticity may be under opposing selective pressures so that a specific plastic trait may be beneficial in one context but harmful in another; hence, the adaptive nature of this phenomenon is not straightforward (Sih 2004). Inducible defences, in which individuals of prey species acquire a different, defensive phenotype when confronted with predator cues, offer a straightforward example of phenotypic plasticity. In the Hokkaido frog (*Rana pirica*), for instance, bodies of individual tadpoles will change in less than a week's time from the typical slender form to a more bulgy, hard to swallow shape in the presence of chemical cues from the predatory Ezo salamander (*Hynobius retardatus*) (Kishida and Nishimura 2006). Geographic variation in tadpole body shape could thus be simply due to the presence/absence of the predatory salamander (but see further, Kishida et al. 2007).

Finally, geographic variation can result from the interaction between the genotype and the environment (G x E). This will occur whenever populations have individuals with different reaction norms, i.e. when they hold genotypes that differ in the way that they respond to changes in the environment (Pigliucci 2001). For instance, continuing the story of the Hokkaido frog (*R. pirica*), the ability of mounting an inducible defence is reduced in insular populations that are allopatric to the predatory Ezo salamander (*H. retardatus*) (Kishida et al. 2007). This nicely illustrates that phenotypic plasticity itself may also have its genetic base, and therefore can be subject to evolutionary forces such as genetic drift and natural selection. Another fascinating example of a G x E interaction causing geographic variation is the plasticity of larval respiratory phenotypes in the fire salamander (*Salamandra salamandra*). Larvae of this species inhabiting ponds tend to have larger gills than conspecifics living in streams, likely to help them cope with poor oxygen conditions. The trait is 'partially plastic' in the sense that gill size will decrease in pond larvae that are transferred to streams, but will not increase in stream larvae that are moved to ponds. Sabino-Pinto et al (2019) suggest that the genetic basis for gill plasticity may have been eroded or selected against in stream-dwelling populations. Such examples illustrate that G x E interactions are actually a prerequisite for local adaptation (Kawecki and Erbert 2004).

Interestingly, the way in which geographic variation is maintained can also change over time. For instance, in genetic assimilation, differences between populations A and B may be initially due to phenotypic plasticity. Populations A and B may be at opposite extremes of the same reaction norm. However, selection (or genetic drift) may eliminate plasticity in the trait in population B, fixing the trait at one end of the norm. Now geographic variation is, in essence, a consequence of genetic differences between A and B (West-Eberhard 2003). Examples of plasticity preceding adaptation in wild populations are rare, but the study of Corl et al. (2018) provides an interesting case. Side-blotched lizards (*Uta stansburiana*) exhibit geographic variation in body colouration, being much darker when living on lava fields than when living on white sands. Independent of their place of origin, individual lizards will grow darker when experimentally kept on lava for a year, and paler when kept on white sand. However, side-blotched lizards native to the lava fields are darker than white-sand lizards artificially kept on lava, even after a year. This is consistent with a ‘plasticity-first’ scenario: phenotypic plasticity allowed side-blotched lizards to colonize the lava fields in the first place, and then natural selection further increased pigmentation in the lava population by inducing relevant genetic changes.

Geographic variation and conservation biology

The Earth is currently losing biodiversity at such a rate that scholars have started speaking of a sixth wave of mass extinction (Barnosky et al. 2011). Human-driven change in land use is one of the prime causes of this worldwide loss of populations and species. Deforestation, intensive agricultural activities and urbanisation have decimated and fragmented natural ecosystems and have driven many organisms back into relatively small remnants of their original biotope, where they have to survive in smaller populations (Novacek 2001).

These small, isolated populations typically exhibit reduced genetic diversity (e.g heterozygosity) and are more vulnerable to stochastic events (genetic, demographic, environmental) propelling them into a downward spiral that may lead to local extinction (Frankham et al., 2003). Emptied habitat patches may later be colonised by individuals dispersing from other populations, but this may come at the cost of loss of local adaptedness (Mayer 1970). On the long term, the erosion of genetic diversity in small populations may jeopardize their adaptability (i.e., the ability to respond to environmental changes by means of genetic adaptation; Bijlsma and Loeschcke 2012). In a time in which the environment is being altered fast in multiple ways due to human activities (e.g. climate change, pollution, invasive species), species adaptability is especially worrisome. A logic and popular mitigation strategy to elevate genetic diversity is to increase the size of the gene pool again, e.g. by connecting habitat fragments with corridors to encourage dispersal (gene flow), or by stocking depauperate populations with fresh individuals from nearby populations (Allendorf and

Luikart 2007). However, such policy may not be appropriate when the populations have lived isolated from one another for longer times (Jamieson et al 2018).

For instance, in Europe, many animal and plant populations survived the Pleistocene glatiations in disparate southern refugia and remained isolated from each other ever since (Hewitt 2000). Over such time frames, local adaptation seems plausible, and reconnecting populations may actually destroy adaptive gene complexes, leading to lower survival probabilities (Frankham et al., 2012). Species conservation plans thus require information on the partitioning of the (relevant) genetic variation; whether it is intra- or interpopulational. This explains why it is specifically important to know the evolutionary history of metapopulations, their past and current connectedness, and degree of local adaptation (Frankham et al., 2012, Jamieson et al 2018).

Naturally fragmented populations can be considered as natural laboratories for studying consequences of isolation on an evolutionary time scale (Baeckens and Van Damme 2020); moreover, they may offer a preview of what may happen to anthropogenically shattered populations. Studying geographic variation in naturally fragmented populations on islands, mountain tops ('sky-islands'), or in natural relict habitats, may help to understand aspects of adaptability that are relevant for their survival. So far, the adaptability of fragmented populations has been mostly studied in species with short generation time such as plants, insects (Cheptou et al 2017), fish (e.g Willoughby et al., 2018) or lizards (e.g Herrel et al., 2008) and much less so in long-lived, slow breeding species with limited dispersal abilities. In such organisms, the long-term effects of fragmentation may be better understood by comparing populations that have become isolated through natural events, in more ancient times (Cheptou et al. 2017). The study of geographic variation, especially in long-lived organisms, is crucial for both the fundamental and conservation aspects presented previously.

Salamanders (*Salamandra* Garsault, 1764)

The family Salamandridae Goldfuss, 1820 (salamandrids) currently comprises 130 species of tailed amphibians grouped in 21 genera (Amphibia Web, 02.12.2022). Members of most genera inhabit Europe, northern Africa or Asia; only the genera *Notophthalmus* and *Taricha* are endemic to North America. The family consist of true salamanders (subfamily Salamandrinae; 4 genera), newts (subfamily Pleurodelinae; 16 genera) and other salamandrids (subfamily Salamandrininae; 1 genus).

Within true salamanders, the genus *Salamandra* Garsault, 1764 contains six species (Steinfartz et al., 2000). The only species that is present throughout most of Europe is the fire salamander (*S. salamandra*); the other species have much more restricted distributional ranges (Figure 1). For example, the Corsican fire salamander (*S. corsica)*, as its name already announces, is endemic to the island of Corsica. Two species are associated with cold alpine climates: Lanza's alpine salamander (*S. lanzai*) and the Alpine salamander (*S. atra*). The former occurs only in a small area of the Cottian Alps along the border between Italy and France; *Salamandra atra*, on the other hand, is found throughout both the Alps and the Dinarides. From the two remaining species, the North African fire salamander (*S. algira*) inhabits northern Morocco and Algeria, while the Near Eastern fire salamander (*S. infraimmaculata*) is found in Iran, Iraq, Turkey, Syria, Lebanon, and Israel. For distinctive historical reasons, two of the *Salamandra* species (*S. algira* and *S. atra*) have a relatively wide, but highly disjunct distribution (Steinfartz et al., 2000, Figure 1) , which has prompted the description of a large number of subspecies (depending on the author, a total of 21-25 subspecies are recognized within *Salamandra*; Seidel and Gerhard 2016). Accordingly, recent studies have demonstrated substantial variation among populations of *Salamandra*-species (inter- and intraspecifically), both at the genetic (e.g Burgon et al., 2021 Rodriguez et al., 2017, Bonato et al, 2018, Degani

2016, Vörös et al 2017) and phenotypic level (e.g in morphology: Degani 2017, Bouzid et al 2017, Seidel and Gerhard 2016 & reproductive biology: Steinfartz et al. 2007, Velo-Antón et al., 2012). The degree of differentiation at the behavioural, physiological and ecological level has received less attention.

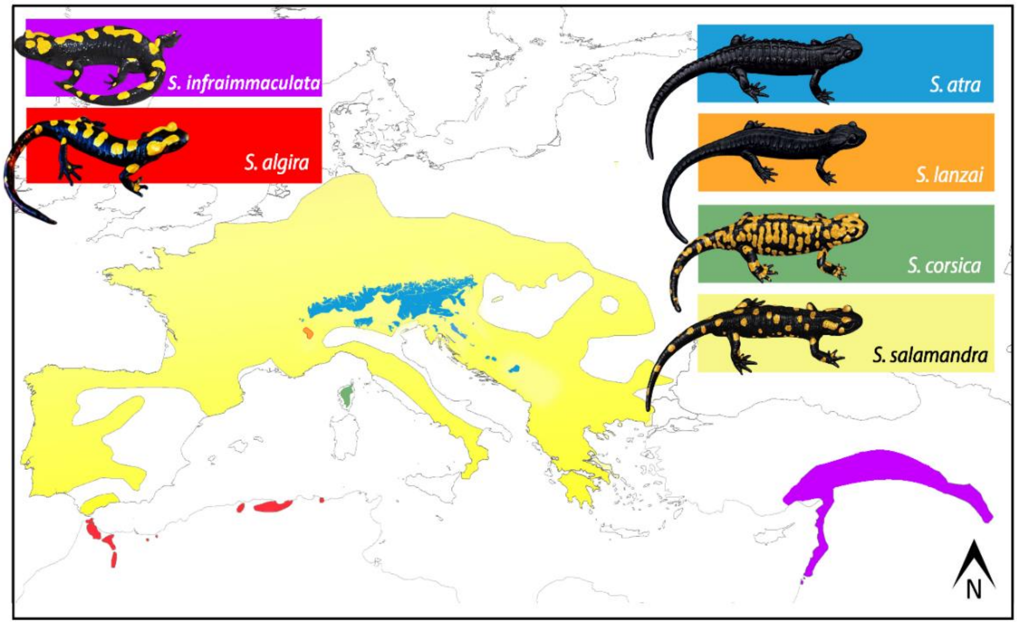

Figure 1. Distribution of the six species within the genus *Salamandra* Garsault, 1764; the distribution range of each is downloaded from IUCN (www.iucn.org; the range of *S. atra* is corrected following Bonato et al. 2018, that of *S. infraimmaculata* following Candan 2022). The pictures of the four European species (right) are taken from Speybroeck et al., 2016 while the pictures of *S. infraimmaculata* and *S. algira* are from W. Beukema.

Although terrestrial for most or all of their life cycle, *Salamandra* species (hereby after referred as salamanders), like most amphibians, have retained a highly water-permeable skin. The risk of dehydration through bodily evaporation narrows their habitat tolerance, restricting their distributional range and window of activity to places and times of relatively high environmental humidity and lower temperatures (Catenazzi 2016). Moreover, as ectotherms, they have a low capacity of physiological buffering. This explains why salamanders are typically found in moist woodlands and subalpine meadows, spending much of their time in burrows underneath logs or stones, shelters from which they emerge only on cold and damp occasions.

Another salient feature of the skin of salamanders is that it invariably comprises a series of exocrine glands that produce substantial amounts of a poisonous, milky liquid. These glands occur over the entire body, but are especially notable along the costal grooves on the flanks of the animals (the 'glandular knobs'), while the most prominent among them are located behind their eyes (the 'parotoid glands'; Figure 2). The toxins have a dual role in the life of a salamander; aside serving as a defence mechanism against predation, they also show strong antimicrobial activity protecting the salamanders from various infections (Lüddecke et al., 2018). Another mechanism that increases survival in salamanders is their ability to regenerate parts of their body if amputated (Seidel and Gerhard 2016).

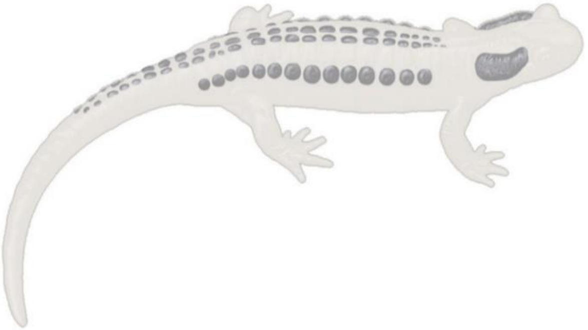

Figure 2. Although poisonous glands are present over the entire body of salamanders, the most visible are darkened in this figure (taken from Speybroeck et al., 2016). The continuous double line of gland pores on the centre of the back is absent in *S. lanzai* but present in other species. The glands on the flank are the glandular knobs, while the most prominent, large glands behind the eyes are the parotoid glands.

Salamanders have a relatively low vagility (Ficetola et al., 2012); females are generally philopatric while males seem to disperse more, presumably due to reasons related to territoriality and reproduction (Helfer et. al 2012). Salamanders differ in their reproductive mode in the sense that they are either larviparous, or fully viviparous. Larviparous populations bear live young that go through an aquatic larval stage. Only the cold adapted *S. lanzai* and *S. atra* are exclusively viviparous and produce fully metamorphosed young (without gills but developed lungs) that are directly capable of surviving on land. Two species (*S. salamandra* and *S. algira*) combine both types of reproduction: some populations being viviparous, others larviparous (Dinis and Velo-Antón, 2015, Velo-Antón et al. 2012, Beukema et al., 2010). Occasionally, the same can happen in *S. corsica* (Nöellert and Nöellert 1992; Miaud et al 2009). This illustrates the flexibility of salamanders' reproductive biology where, even within the same species, the reproductive modes and accompanying phases are governed by environmental conditions and habitat availability. Because they are restricted to colder environments, salamanders have a lowered metabolism which is why they generally mature very slowly; the fact that they are fully inactive during a substantial part of the year, due to hibernation or estivation, also prolongs their maturation time which explains why they do not start reproducing until the age of 2-5 years (Klewen 1991). Salamanders mostly exhibit low fecundity and a long generation time (Klewen 1991). For instance, populations living at high altitudes, associated with alpine climates, have a gestation period lasting up to four years (Klewen 1991, Sparreboom 2014). Individuals may live up to 20 years in the wild and to 40 years in captivity (Seidel and Gerhard 2016).

Salamanders take up a central role in ecosystem functioning. As mid-level predators they influence ecosystem processes and community composition by supplying tertiary consumers with high quality and rare nutrients. Moreover, salamanders connect energy and matter between aquatic and terrestrial landscapes and contribute to soil dynamics through their use of underground shelters (Davic and Welsh 2004). Although research examining the effect of salamanders on soil ecosystem structure is restricted mainly to North America, a recent European study revealed the impact of fire salamanders (*S. salamandra*) on leaf litter decomposition (Laking et al., 2021). More precisely, Laking et al. (2021) found that *S. salamandra* reduces the decomposition of 'poor quality (oak) litter' while the degradation of 'high quality (maple) litter' is not affected by their presence; this is mainly due a top-down effect that fire salamanders have on both invertebrate communities and the leaf litter microbiome. The increased retention of litter mass may be especially relevant for buffering climate changes (Laking et al., 2021).

Due to their permeable skin, narrow habitat tolerances, relatively low vagility, slow maturation and low fecundity (sometimes including a bimodal development - aquatic and terrestrial), salamanders respond faster than other species to environmental changes, hence are valuable as indicator and sentinel species (see Welsh and Droege 2001, Davic and Welsh 2004). For instance, Soto-Rojas et al. (2017) have demonstrated that the number morphological abnormalities (missing or extra limbs, toes gills, fused toes) in the Michoacan stream salamander, *Ambystoma ordinarium*, correlates with physical indicators of habitat quality. Neutrophil:lymphocyte ratios in hellbender (giant) salamanders, *Cryptobranchus alleganiensis*, covary with water quality measures such as conductivity, pH and levels of dissolved oxygen (Litmer et al. 2020). On a longer, but still ecological time scale, demographic and evolutionary changes have also been described. For instance, population sizes of long-toed salamanders, *Ambystoma macrodactylum*, decreased with the incidence of wildfires, especially so in isolated populations and human-managed forests (Hossack et al. 2013).

Accordingly, the special features of salamanders make them relevant indicators of biodiversity integrity (Davic and Welsh 2004).

Salamanders, as many other amphibians, are most threatened by habitat loss (along with fragmentation) and pollution, climate change and infection risk (Parra et al 2007). Emerging amphibian diseases, as the ones caused by *Batrachochytridium dendrobatidis (Bd)* and *Batrachochytridium salamandrivorans (Bsal)* can wipe out salamander populations in 'a blink of an eye', causing 'over-night' extinctions (*Bd*: Bosch and MartÌnez-Solano 2006; *Bsal*: Martel et al 2013). The salamanders' elusive behaviour imperils research, which also explains why there are many gaps in our understanding of their biology that further compromise conservation strategies and restoration plans. Even the distribution boundaries of some salamander species are still uncertain (See for instance Speybrouck et al. (2016) and Cikovac and Ljubisavljević (2020) on *S. atra*; Seidel and Gerhard (2016) on *S. algira;* and Candan (2022) on *S. infraimmaculata*). Moreover, the evolutionary potential of many populations is masked due to the unresolved evolutionary history and unknown extent of connectivity with other populations. Debate on the exact delimitation of species, and the validity of certain subspecies further hinders the establishment of conservation legislation (the 'no name – no conservation' hurdle, Parra et al. 2007). Based on the latest IUCN red list (2022, www.iucnredlist.org) only the populations of *S.corsica* is stable, while populations of all other species (*S. salamandra*, *S. algira*, *S. infraimmaculata*, *S. lanzai* and *S. atra*) are declining. Considering their conservation status, three species (*S. salamandra*, *S. corsica* and *S. atra*) are recognized as Least Concern (LC), *S. infraimmaculata* is Near-threatened (NT), *S. algira* is vulnerable (VU), while *S. lanza*i is Critically Endagered (CT).

The species under focus

This PhD focuses on *Salamandra atra* Laurenti, 1768, a true salamander that is widely distributed across the inner Alps (from western Switzerland to eastern Austria) where it occurs in large and contiguous populations (Klewen 1988). The species is also present in fragments along the southern Prealps (from the western Orobie mts. in Italy to the eastern Slovenian Prealps), and the Dinarides (fom mt. Hrušica in Slovenia, southwards to Prokletije mts. In Albania) where populations inhabit so-called ‘sky-islands’ (Bonato et al 2018, Figure 3). In the Alps, the species can be found over a wide altitudinal range (420-2500 m a.s.l.), but peripheral populations in the Prealps are usually restricted to the upper highlands (1000 - 2200 m a.s.l). Compared to their northern conspecifics, the fragmented populations from the central and southern Dinarides occupy a very narrow vertical range (1650 – 2000 m a.s.l; Speybroeck et al 2016).

Alpine salamanders are elusive creatures with peculiar life history traits; the activity season is strictly dependent on the environmental conditions but generally lasts 4 - 5 months (May – September). The rest of the year the animals hibernate below the surface in holes, crevices and fissures in the karst (Klewen 1988). Even during the summer, activity is restricted to colder and damp hours with no direct sunlight (air temperature from 4 - 13°C, relative air humidity generally around 85%, Klewen 1988). Unfavourable summer times are spent in natural karst cavities, in micromammal burrows or under logs (Klewen 1988). Salamanders leave their shelters mainly to feed and reproduce. When active aboveground, they are often found in high densities in a wide variety of habitats starting from woodlands, over meadows to rocky karstic habitats above the tree line (Klewen 1988).

The Alpine salamander is a generalist, mainly consuming insects but also spiders, centipedes, snails, worms and similar prey; Fachbach et al., 1975). Since salamanders are poisonous, most predators avoid them. However, they are registered in the diet of the common viper (*Vipera berus*) and the grass snake (*Natrix natrix;* Luiselli et al., 1995; Luiselli et al., 1997). Some birds, like the Eaurasian magpie (*Pica pica*), Alpine chough (*Phyrrhocorax graculus*), and tawny owl (*Strix aluco*) are known to predate on Alpine salamanders but in such a way that they remove the poisonous glands prior to ingestion (Klewen 1988).

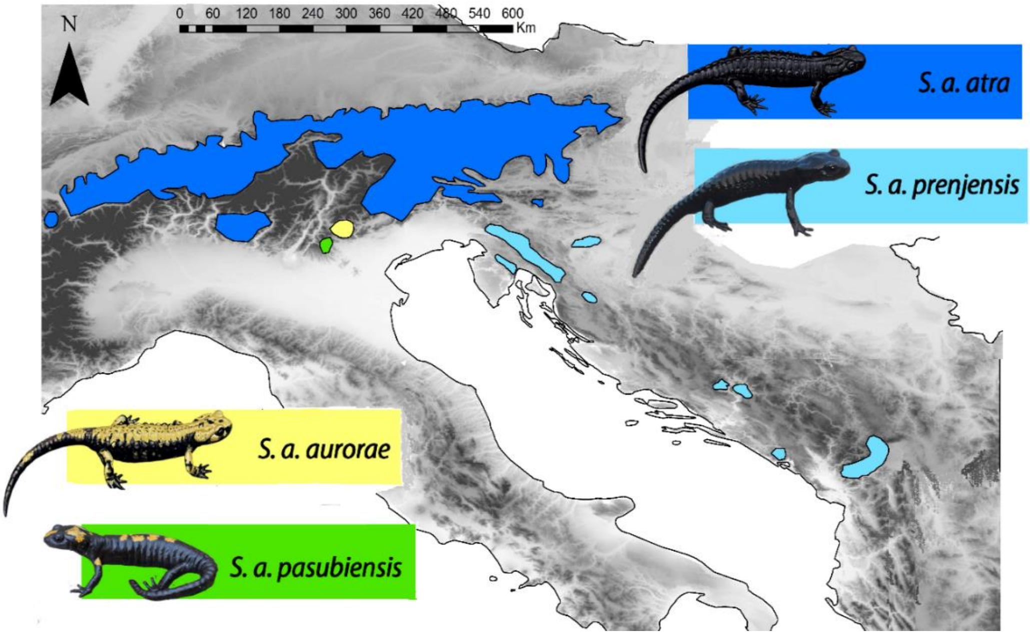

Figure 3. Distribution of the Alpine salamander (*Salamandra atra*) and accompanying subspecies. The status of *S. a. prenjensis* is doubtful by some authors while some populations in the Southern Alps, traditionally accepted as *S. atra atra* (as in this figure), belong to several lineages that await formal description (see text for details). Depicted distribution modified from Bonato et al., (2018). *S. a. prenjensis* photo E. Šunje, other pictures of subspecies are taken from Speybroeck et al., 2016.

Despite the Alpine salamanders' high site fidelity, dispersal does occur and is mainly driven by males and juveniles; dispersal distances can be quite long (up to 6km, based on the findings of Helfer et al., 2011 in Razpet et al., 2016) motivated by reproduction and occupation of new territories (Helfer et al., 2011). Depending on the altitude, adults reach maturity between 2-4 years of age. Although the sexual dimorphism in morphological features of *S. atra* is poorly studied (but see Kalezić et al., 2000), the only external trait differentiating males from females seems to be the shape of the cloaca that is swollen in the former compared to the latter (Luiselli et al., 2001).

Alpine salamanders mate multiple times during almost the entire duration of the activity season. The reproductive behaviour is characterized by five steps (Klewen 1991). In the course of mating, males release packages of sperm (spermatophores) that are taken up by females (Klewen 1991). Females can store sperm in spermathecae (tubular glands in the roof of the cloaca), and therefore fertilization can occur several months, or even years, after mating (Häfeli 1971, Greven 2003). The gestation period varies depending on the environmental conditions (Klewen 1988). In environments characterized by longer winters and colder temperatures followed by a prolonged snow cover, gestation can last up to four years while in milder environments it concludes after two years (Klewen 1988, Luiselli et al. 2001, Graven 2003). Interestingly, during gestation, developing fetuses feed on sibling eggs (oophagy) and it may even occur that they continue feeding on developing siblings (adelphophagy – intrauterine cannibalism); such gestational characteristics are very rare in amphibians and may be exclusive to *S. atra* (Vitt and Caldwell 2009). Upon gestation, females give birth to fully developed individuals (i.e., they are viviparous). Females can carry one, or, more often, two larvae that develop independently from each other (one in each of the two uteri), which explains why birth is often not synchronized (*pers. obs.*, Luiselli et al. 2001).

Salamandra atra has three recognized and one debated subspecies (Klewen 1988, Speybroeck et al., 2016). Of the former, the nominal subspecies, *Salamandra atra atra* Laurenti, 1768 occurs throughout the inner, northern Alps. The other two widely accepted subspecies are endemic to very small areas in the Italian Prealps: *Salamandra a. aurorae* Trevisian, 1982 and the relatively recently discovered, *Salamandra a. pasubiensis* Bonato and Steinfartz, 2005. Subspecies are described based on genetic characteristics and morphological (mainly chromatic) features. The nominal subspecies is completely melanistic (black), while the other two subspecies differ in the amount and distribution pattern of yellow patches on the body (Speybroeck et al., 2016, Figure 3). The subspecies *S. atra aurorae* has been considered a separate species by some (Joger 1986, Dubois and Raffaelli 2009). The validity of a fourth subspecies, *Salamandra atra prenjensis* Mikšić, 1969, encompassing the populations from the Dinaric region, is still debated (Klewen, 1988; Grossenbacher, 1994; Bonato and Steinfartz, 2005, Dubois and Raffaëlli, 2009; Speybroeck et al., 2010, Helfer 2010). Recent molecular studies show that the Dinaric population is genetically differentiated from the populations in the inner Alps and the Prealps (Helfer, 2010; Razpet et al., 2016; Bonato et al., 2018), but there are no clear morphological features distinguishing the alleged *S. a. prenjensis* subspecies from the nominal one. Molecular studies revealed how the quaternary glaciations affected the evolutionary history of *S. atra*. During the Last Glacial Maximum (LGM), the species was absent from the inner Alps but more widely distributed along the Dinarides (Razpet et al 2016). In the Prealpine region, the distribution of *S. atra* underwent repeated alternating phases of contraction, extinction and recolonization (Bonato et al 2018). Interestingly, the fragmented populations from the Italian Prealps show a very high genetic differentiation among each other and also from Alpine and Dinaric populations, suggesting that they have evolved independently over a considerable stretch of time. This happened probably because they survived near the very border of the Pleistocene ice cap covering the Alps, in non-glaciated areas where populations were disconnected between themselves by glaciers (Bonato et al 2018). Whereas many species postglacially recolonized northern Europe from refugia in the

Balkan region (Hewitt 1999), *S. atra* is believed to have re-expanded its distribution from a refugium in eastern Austria, and several other smaller 'nunatak' glacial islands in the Alps (Helfer 2010, Doufresness 2021). The aforementioned molecular studies on *S. atra* populations opened new questions concerning the evolutionary history of other population fragments such as the ones in the Dolomites, Venetian Prealps, Carnic Alps and along the Dinarides. To the present day, *S. atra* is treated as Least Concerned (LC), but it is clear that each conservation unit (minimum six, tentatively defined by Bonato et al 2018), harbouring a different evolutionary potential, should have an appropriate conservation status. The same issues, presented previously at the genus level, hinder the delivery of effective conservation strategies also for *S. atra*.

The study system

This thesis focuses on Dinaric populations of *Salamandra atra*, described as *Salamandra atra prenjensis* Mikšić 1969. The Dinarides stretch from the Julian Alps in the north (eastern Italy, south-east Slovenia) 650km southwards to the massif of Prokletije (Albania). Several population fragments of the Alpine salamander can be found along this mountain chain (Figure 4). In the northern Dinarides, populations are found in the mountains of Hrušica and Menešija, on the Snežnik plateau and the adjacent area of Gorski Kotar (Razpet et al 2016, Jelić et al., 2013). In the surrounding area, small population pockets are found in Žumberak (Jeran et al., 2011) and Istria (Ćićarija and Učka; Jelić et al., 2013). The southernmost population in this northern part of the range is in Mala Kapela (National park Plitvice; Jelić et al., 2013). From this point southwards, there is a big distribution gap after which population fragments appear again in the central Dinarides, specifically, on the mountains of Čvrsnica and Prenj that are separated by the deep canyon of the river Neretva (Šunje and Lelo, 2008, Šunje and Lelo 2010).

The distribution continues in the southern Dinarides on mt. Orjen and extends to the big massif of mt. Prokletije (Krizmanić 1997, Figure 4).

In how far these scattered populations are interconnected is still unclear, especially in the northern Dinarides. The population on mt. Orjen was found very recently, more precisely in 2018 (Cikovac and Ljubisavljević 2020), which confirms predictions by Klewen (1988) and Džukić (1991) considering undiscovered populations along the Dinarides, and further illustrates our incomplete awareness of the species' occurrence. Cikovac and Ljubisavljević (2020) list several additional potential areas of occurrence, yet to be investigated. Interestingly, two individuals from mt. Treskavica (Bosnia and Herzegovina – B&H) caught by Othmar Reiser in 1878, are preserved at the National Museum of B&H but neither the extensive field work by Mikšić (1969) nor my personal expeditions (2013 – 2016) managed to confirm the presence of the species on this mountain, possibly signifying a local extinction.

The molecular analyses by Helfer (2010), Razpet et al. (2016) and Bonato et al. (2018) included a small number of individuals from Dinaric populations and these were found to belong to a separate lineage, distinct from the Alpine populations. However, due to the under-sampling of the Dinaric populations, their exact relationships were unknown at the beginning of this PhD study. Interestingly, both Helfer (2010) and Razpet (2016) found Dinaric haplotypes in the Julian Alps (area of Pokljuka and Krn), suggesting that there are possible contact zones between the Dinaric lineage and *S. atra atra*.

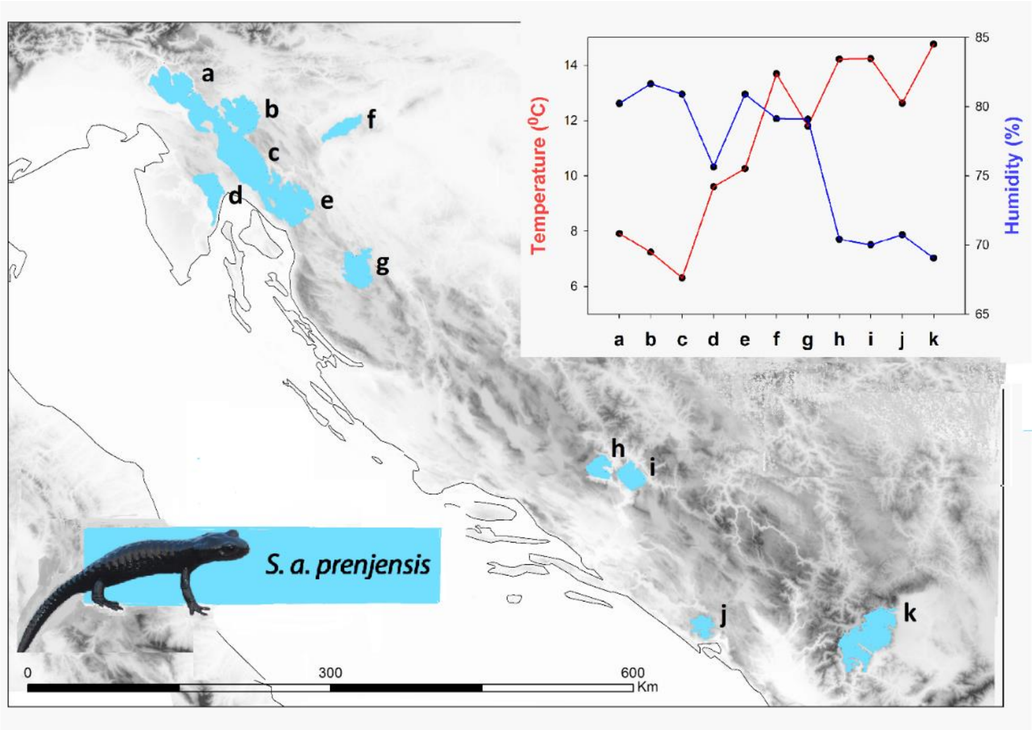

Figure 4. The Dinaric Arc and the distribution of *Salamandra atra prenjensis* (from north to south: a – k). Occurrence areas are in light blue as follows: a – mt. Hrušica, b – mt. Menešija, c – mt. Snežnik, d – Istria (mts. Ćićarija and Učka), e – Gorski Kotar, f – mt. Žumberak, g – Mala Kapela, h – mt. Čvrsnica, i – mt. Prenj, j – mt. Orjen, k – mt. Prokletije. From north to south, the temperature has an increasing trend, in contrast to the decreasing humidity. The average (annual) values for air temperature (°C) and relative humidity (%), are taken from: www.fao.org/aquastat/en/climate-info-tool/ .

As Dinaric populations are scattered over a wide latitudinal range, their habitat characteristics also differ (Figure 5). Populations from the northern Dinarides are mostly found in mixed deciduous-coniferous forests above 800m of altitude; the soil of these forests is covered with dense fern, logs and dry leaves. The populations from the central Dinarides and mt. Orjen are found exclusively above the tree line (cca 1650 – 2000 m) on open rocky grasslands, with sparse aggregations of mugo pines (*Pinus mugo*). The habitat in the Prokletije massif (ca 1700 - 2500 m) is the most variable as it contains coniferous forests, grass fields and rocky habitats beneath the highest peaks; it is also differs from the other habitats along the range because it contains small water streams.

Besides these differences in structural habitat characteristics, the respective populations also face different climatic conditions. More specifically, from north to south, the humidity decreases opposite to the increasing temperature gradient (Figure 4) characterizing the Northern Dinarides as colder and more humid compared to the warmer and drier conditions present in the central and southern Dinarides.

Considering the presented environmental differences, it is expected that these isolated salamander populations are under different selective pressures and that they exhibit geographic variation in several aspects of their biology such as: genetics, morphology, behaviour, physiology and ecology. The geographic variation among these populations is completely unexplored which also hampers the delivery of proper conservation strategies (as previously argued: p16 and p19).

Figure 5. The variety of habitats inhabited by *Salamandra atra prenjensis*. Pictures refer to mountain areas (locations) analysed throughout this PhD. Northern Dinarides: A, B – Gorski Kotar; Central Dinarides: E, F – Mt. Čvrsnica & G, H - Mt. Prenj; Southern Dinarides: C, D - Mt. Prokletije. Gorski Kotar is completely forested, while the habitats of the Central Dinarides are similar (agglomerations of *Pinus mugo* connected by alpine meadows and stony surfaces). The habitat of Prokletije is the only that includes small water streams and human settlements aside from forests, alpine meadows and rocky substrates. Photo: E. Šunje.

Thesis outline

In this thesis I explore geographic variation in fragmented populations of Alpine salamanders and study the adaptive nature of the phenomenon.

I begin the thesis with a phylogenetic study (chapter 2) in which I research the evolutionary history of *S. atra* also by studying patterns of genetic variation (mtDNA and microsatellites). This study includes many populations from the entire species' range (DNA sequences from public repositories) but focuses on four isolated populations that are well spread throughout the Dinarides (sampled throughout the PhD). I aim to discover evolutionary relationships among populations of *S. atra* and detect refugia that secured the survival of Dinaric populations during the ice age. To better understand evolutionary relationships and conservation needs, I estimate the genetic differentiation among populations and indices of genetic diversity (within populations).

In chapter 3 I explore the morphological variation within and among populations of Alpine salamanders using 14 morphometric characters. To remediate the intraspecific fuzzy taxonomy, I am primary interested in detecting characters that differentiate the nominal *Salamandra atra atra* population from the putative *Salamandra atra prenjensis*; however, I also explore the morphological variation between sexes. In this study I include two populations per subspecies (*S. atra atra* and *S. atra prenjensis*) and apply a thorough statistical approach to data analysis in order to identify existing morphologic groups and understand the patterns of trait variation among populations and between subspecies and sexes.

Further, in chapter 4, I explore the diet variation in four (Dinaric) population fragments and study the among-population variation in niche dimensions. Besides studying the variation in the prey consumption (from stomach flushes), I also study the variation in prey availability (from pitfall traps and netting); more precisely, I study the composition of potential and realized prey communities among populations and try to understand if salamanders consume prey taxa in proportion to their availability in the habitat. I also explore whether the diet, both in composition and quantity, differs among populations, sexes and age classes. Finally, I analyse the relative contribution of intra- and interindividual variation to niche width (within population).

In chapter 5 I aim to understand if predation pressure or infection risk are drivers of species' toxicity; I collect toxin samples from four isolated Dinaric populations of Alpine salamanders by squeezing the parotoid glands; for each sampled individual I also record relevant morphological measurements (parotoid gland size, body weight). In each population, I estimate the predation pressure by counting present snake species, and estimate the infection risk using genomic analysis and cultivation of soil samples. I test if toxins (amount and composition) and morphological traits differ among populations and between sexes and discuss if such variation could be explained by predation pressure and infection risk.

In chapter 6 I study the geographic variation in water loss rate in two Dinaric populations of Alpine salamanders originating from distant environments that differ considerably in thermal and hydric characteristics. Here, I take an experimental approach under controlled laboratory conditions and study how temperature and humidity influence water loss in Alpine salamanders, and the behavioural mechanisms they use for its control. More precisely, I measure the water loss of animals in restricted and unrestricted experimental settings under several treatments (different combinations of temperature and humidity). In the restricted setting the salamanders are deprived to control for water loss, while in the unrestricted setting they can control water loss behaviourally; in this latter, for each treatment, I also follow the time the animals invested: (i) aboveground, (ii) hiding, (iii) on moss. To understand the role of desiccation risk as a driver of water loss resistance, I am specifically interested to reveal whether populations differ in rates of water loss and behavioural patterns. To define the ecological relevance of the variation in water loss rate, I used the relationships between water loss rate, temperature and humidity obtained from the restricted experimental setting to estimate water loss rates under realistic field conditions.

In the last thesis chapter (chapter 7), I study how the predicted future climatic conditions (between: 2061-2080) will affect the specie’s distribution range. I use an advanced modelling approach relevant for concrete conservation actions.

Throughout this study, I: (i) deliver an accurate database of specie's occurrence based on available records in present time, (ii) apply multiple modelling techniques coupled with numerous climate model projections to asses the impact of climate change on species' distribution, (iii) conduct prediction analyses not only for the entire species but also for relevant infraspecific lineages, (iv) provide accessibility of model predictions to broader public. I translate findings from this work in concrete conservation actions.

Finally, in chapter 8 I link all the obtained results presented in chapters 2 to 7. From a fundamental point of view, I discuss the role of the environment in shaping the biology of *S. atra*, and give insights considering the ability of populations to adapt to local conditions. I describe perils and pitfalls of the applied methods that I used in this dissertation and in the broader context of evolutionary biology. Last but not least, I provide concrete and applicable information that are relevant for species' conservation.

Chapter 2

Genetic diversity and differentiation of alpine salamanders from the Dinarides – an evolutionary perspective with insights for species conservation

Emina Šunje, Ana Bermejo Zuazu , Raoul Van Damme, Thierry Backeljau, Naris Pojskić , Lada Lukić Bilela and Belma Kalamujić Stroil

Šunje, E., Zvazu, B. A., Van Damme, R., Backeljau, T., Pojskić, N., Lukić Bilela, L. Kalamujić Storil, B. (2021): Genetic diversity and differentiation of alpine salamanders from the Dinarides – an evolutionary perspective with insights for species conservation. Salamandra 57, 75-88.

Abstract. The fragmented population of alpine salamanders from the Dinaric Alps belongs to a distinct evolutionary lineage known as the subspecies *Salamandra atra prenjensis*. However, the phylogenetic relationships within this lineage are unknown since previous studies did not comprehensively include Dinaric population fragments. In this study, six microsatellite loci and two mtDNA markers (cytochrome b and D-loop) are used to examine the genetic structure, phylogeography and phylogenetic position of several population fragments of alpine salamanders along the Dinarides. The results are in line with a 'refugia within refugia' model of evolution as described for many other taxa in the Balkans. During Pleistocene glaciations, *Salamandra atra* persisted in at least two Dinaric refugia: an old one in the Prenj mountain, and a more recent one in Gorski Kotar, whereas evidence of colonization have been found for the populations of the Čvrsnica and Prokletije mountains. Moreover, the patterns of genetic diversity are discussed from both an evolutionary and conservation perspective. In addition, our dataset was analyzed with available (published) sequences of *Salamandra atra* from its entire distribution range; the results of these latter analyses provide a state-of-the-art review of the evolutionary history of the species overall.

Introduction

In the current epoch of the sixth mass extinction, characterized by a widespread and rapid decline of biodiversity (Ripple et al. 2017), phylogenetic, phylogeographic and population genetic studies are becoming increasingly important in the context of conservation biology and species management (Frankham et al. 2002).

The current geographical patterns of genetic variation in many species are a result of climatic and geomorphological changes that happened throughout the Earth's evolution (Lomolino & Heaney 2004). Probably the most famous example of such events are the climatic oscillations in the Pleistocene that caused a series of alternated contractions and expansions of distributional ranges (Hewitt 1999, 2000, 2004). Traditionally, European species were thought to have survived the Pleistocene glaciations in a few large, continuous areas in the extreme south of Europe – an idea largely bolstered by paleoclimatic reconstructions and palynological research (see Olalde et al. 2002). This explains why many studies (e.g. Taberlet et al.1998, Petit et al. 2003) emphasized the role of glacial refugia in the Iberian, Apennine and Balkan peninsulas on the current distribution patterns of species. More recently, however, phylogeographic analyses have accumulated evidence that many species may have found shelter in multiple small, isolated patches with milder climatic conditions (so-called 'refugia within refugia', Gómez & Lunt 2007). This scenario is more in line with the observed level of genetic variation and population sub-structuring in a wide variety of organisms, including amphibians, from the Apennine (e.g., Canestrelli & Nascetti 2008), the Iberian (e.g., Martinez-Solano et al. 2006) and the Balkan Peninsula (e.g., Sotiropoulos et al. 2007). Refugia are habitats with space and time dimensions that operate on evolutionary time-scales and have facilitated the survival of species under changing conditions for millennia (Keppel et al. 2012). Consequently, identifying refugia, not only plays an important role in understanding the evolutionary history of the world's biota, but could even contribute to protecting it against future climate change (Keppel et al. 2012). Furthermore, the high genetic diversity of populations that thrive in areas that acted as refugia during environmental instabilities, might represents the overall survival potential of a species, hence they may be the key for long-term species persistence (Conner & Hartl 2004).

In this study, we focus on the alpine salamander, *Salamandra atra*, a species endemic to the Alps, and the Dinarides (Fig. 1). Four subspecies have been described: (i) the completely melanistic (black) nominal subspecies, *S. a. atra* Laurenti, 1768 from the Alps; two subspecies inhabiting the Italian Prealps, i.e. (ii) *S. a. aurorae* Trevisian, 1982 and (iii) *Salamandra atra pasubiensis* Bonato & Steinfartz, 2005, that are differentiated by the amount and pattern of yellow patches on the body (see Bonato et al. 2018); and the fourth subspecies, also completely melanistic, (iv) *Salamandra atra prenjensis* Mikšić, 1969, which consists of several isolated population fragments in the Dinarides. However, the validity of *prenjensis* has been debated for a long time (Klewen 1988, Grossenbacher 1994, Guex & Grossenbacher 2003, Bonato & Steinfartz 2005, Dubois & Raffaëlli 2009, Speybroeck et al. 2010), although recent (evolutionary) studies support its separate taxonomic status (Helfer 2010, Razpet et al. 2016, Bonato et al. 2018). Morphometric comparisons between selected populations of alpine salamanders suggest that *prenjensis* could have a larger head and jaws compared to the nominal subspecies (Šunje et al. 2019), and, allegedly, it has a different arrangement of the palatal teeth (Mikšić 1969, Krizmanić 1997). Although the contact zones between *S. a. atra* and *S. a. prenjensis* have not been defined, both are found in the Slovenian Alps (Helfer 2010, Razpet et al. 2016). From there, the subspecies' distribution extends towards the south, in the Dinarides, where it becomes very patchy. Population fragments have been found in the mountain group area of Trnovski gozd (Slovenia: Trnovski gozd, Hrušica, Menešija, Nanos) and Snežnik (Slovenia, Razpet et al. 2016), Gorski Kotar, Žumberak, Istria and Plitvice (Croatia, Jelić et al. 2012), the mountains Prenj and Čvrsnica (Bosnia and Herzegovina, Šunje & Lelo 2010) and in the massive of Prokletije (Montenegro/Kosovo/Albania, Krizmanić 1997). Very recently, a new population fragment was discovered on Mt. Orjen (Montenegro, Cikovac & Ljubisavljević 2020, Fig. 1).

Dinaric samples of *S. atra* have been included in previous phylogenetic studies on alpine salamanders: from the initial works of Ribéron et al. (2001, 2004) and Bonato & Steinfartz (2005), up to the more recent studies of Helfer (2010) and Bonato et al. (2018). However, the number of sampled specimens was invariably small and sampling was restricted to one or two locations in the Dinarides, hence the phylogenetic and phylogeographic relationships among population fragments remained obscure. Hereby we present the first comprehensive study which includes most of the known population fragments of alpine salamanders in the Dinarides. Using both microsatellite and mitochondrial (mt) DNA markers, we describe the evolutionary relationships, genetic structure and levels of differentiation among and within these isolated population fragments. Further, as many taxa in the Balkans evolved following a 'refugia within refugia' scenario (e.g., *Ichtyosaura alpestris*, Sotiropoulos et al. 2007; *Dinaromys bogdanovi*, Kryštufek et al. 2007; *Vipera ammodytes*, Ursenbacher et al. 2008), and few experienced bottlenecks (e.g., *Pinus nigra*, Naydenov et al. 2017), we explore the possibility that such events occurred in the Dinaric population of *S. atra*. Considering the 'refugia within refugia' scenario, we expect a strong genetic subdivision among population fragments due to their isolation in separate refugia. This study contributes to a better understanding of the effects of past climatic oscillations on species confined to the mountainous habitats in the Dinarides, and provides relevant inputs to identify conservation units within the population of *S. atra* along the Dinaric arc.

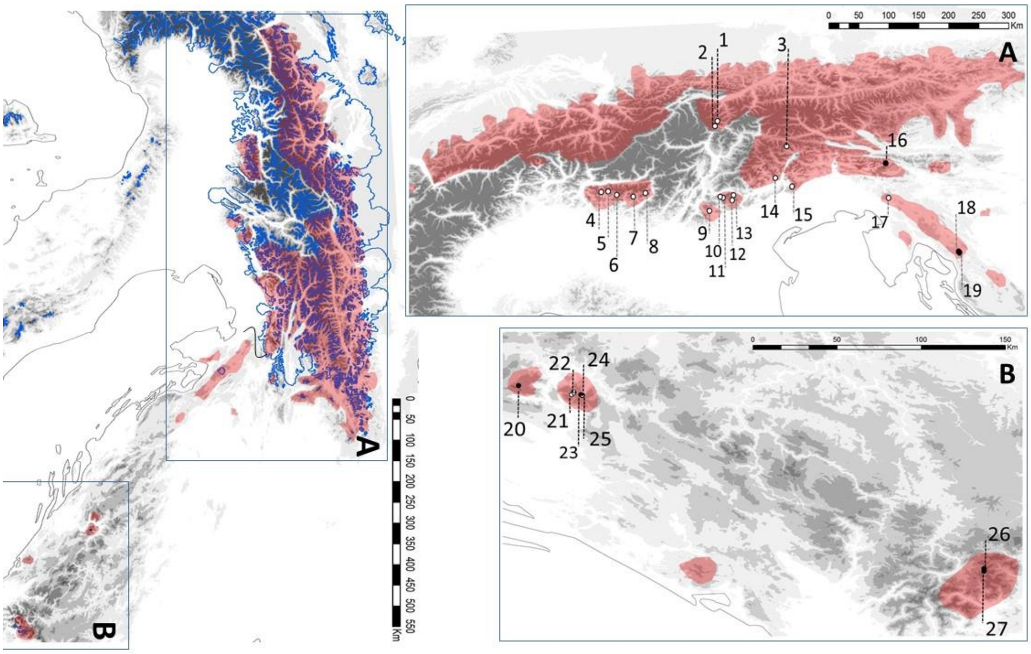

Figure 1. Distribution of *Salamandra atra* with sampling sites (modified from Bonato et al. 2018). Bordeaux polygons represent the known occurrence areas of *S. atra*, and the light blue lines are margins of the glaciers at their maximum extent during the last glacial period (LGM, in the beggining of the Pleistocene, Ehlers & Gibbard 2004); the grey color variation reflects altitude values, with higher altitudes being darker; the curve represents the border between the Dinarides and the Alps. **A** – Alpine, Prealpine, and North-Dinaric part of the distribution range. **B** – Distribution in the Central (Mt. Čvrsnica and Mt. Prenj) and Southern (Mt. Orjen and Prokletije Mts.) Dinarides;. Full symbols are sampling sites from this study; empty symbols respresent sequences retrieved from public repositories (see text for details); code 22 (in grey) is both, sampled in this study and in Bonato et al. 2018.

Material and methods

Sampling

Samples were collected in July and August 2016. We searched for individuals in suitable habitats focusing on the mountain areas in the Dinarides where *S. atra* has been confirmed: from north to south - Gorski Kotar (Samarske stijene and Vihoraški put), Mt. Čvrsnica (Pločno), Mt. Prenj (Podotiš, Kopilice and Zakantar) and Mt. Prokletije (Bogićevica and Gorazdevac). The sampling of individuals in Kredarica (Julian Alps, sampling site: Aljažev dom – see Table 1) was done in 2010 and is included in this study for comparative purposes (unpublished data). Individuals were found on the soil or within shelters during favorable weather conditions (late night, early-morning or after intense rainfall). Small tail clips were taken from a total of 95 individuals (Table 1). Clips were preserved in 96% ethanol.

DNA extraction

Total genomic DNA was extracted using the NucleoSpin® Tissue kit (Macherey-Nagel, Düren, Germany). DNA extraction from the samples of Kredarica was done in 2010 using the protocol from Taggart et al. (1992). All samples were brought to working concentrations by making 10-fold dilutions.

mtDNA markers amplification and sequencing

Two mitochondrial (mt) DNA markers were analyzed: the control region (*D-loop*) and cytochrome b gene (*cob*). Both regions were amplified and sequenced in two parts, using the Saat-H339-dloop/Saat-LPro-short (340 bp amplicon size) and L-Pro-ML/H-12S1-ML (820 bp) primers for *D-loop*, and the Sa_Cytb_12F/ Saat-H550 (560 bp) and Saat-L129/SaatCytb1046R (920 bp) primers for *cob*. All primers were designed by Bonato et al. (2018), except for L-Pro-ML/H-12S1-ML primer designed by Steinfartz et al. (2000). PCRs were performed in 25 µl volumes, following the protocols of Bonato et al. (2018). The PCR products were sent to Macrogen Europe (Amsterdam, The Netherlands) for Sanger sequencing (using the forward PCR primers). The sequences were inspected in Jalview 2.9.0b2 (Waterhouse et al. 2009) and manually corrected when needed; sequences were concatenated in Notepad (Microsoft), aligned using ClustalW (Thomson et al. 1994) and trimmed in Bioedit v5.09 (Hall 1999) to harmonize their length across the alignment. The protein-coding *cob* fragment was translated using the Translate tool on the ExPASy server (Gasteiger et al. 2003) to check for open reading frames. The obtained sequences were submitted to the GenBank under the accession numbers (Acc. No.) MN255339 - MN255350 and MN255326 - MN255337 (*cob* and *D-loop* respectively). Haplotype combinations (concatenated *cob* and *D-loop*) are given in Table S2.1.

Microsatellites genotyping

Six nuclear microsatellite loci were amplified: SalE6, SalE7, SalE8, SalE12, SalE14 and SalE23 (Steinfartz et al. 2004) in two multiplex and one single reactions (Table S2.2). Each PCR reaction (total volume 10 µl) contained optimized concentrations of each primer pair (Table S2.2), 1 × PCR buffer (20 mM Tris-HCl (pH 8.0), 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% (v/v) Nonidet P40, 0.5% (v/v) Tween 20, 50% (v/v) glycerol), 2 mM $MgCl_2$, 0.25 mM dNTPs, 1 U TaqNovaHS Polymerase (Blirt, DNA Gdansk, Poland) and 1 µl of DNA. All PCR reactions were done in Veriti Thermocycler (Applied Biosystems) following the thermal protocols given in Table S2.2. Genotyping was performed on the Genetic Analyzer 3500 Series (Applied Biosystems). PCR products from Mix 2 and the single reaction (SalE14) were pooled (1 µl each) and jointly genotyped. Allele scoring was done in GeneMapper v.5 using Liz 500 Size Standard (Applied Biosystems).

Phylogeographic analysis

DnaSP 6.0 (Rozas et al. 2017) was used to access the concatenated mitochondrial sequence haplotypes (*cob* and *D-loop*). These haplotypes were analyzed together with 24 other haplotypes (concatenated *cob* + *D-loop*) retrieved from published sequences (mainly Bonato et al. 2018, but also Vences et al. 2014, and Steinfartz et al. 2000 for some *cob* sequences; see Table S2.1) from 118 individuals distributed over 19 sites across the species distribution range (Fig. 1, Table 1; the haplotypes of these individuals were provided by B. Crestanello, pers. comm. - Table S2.1). In total, 164 individuals were included in phylogenetic and phylogeographic analysis (Table 1).

To test for phylogenetic congruence, we performed a partition homogeneity test (Farris et al. 1995) in Paup 4.0a (Swofford 2002) with 1,000 replicates and heuristic search using the TBR branch swapping algorithm. Partitioning of the *cob* gene fragment (on 1st, 2nd and 3rd codon position) was done in DAMBE (Xia & Xie 2001), whereas the non-protein coding *D-loop* fragment was not partitioned. DAMBE was also used to conduct a substitution saturation test (Xia et al. 2003) on the two mtDNA gene fragments.

To build phylogenetic trees, sequences of *D-loop* and *cob* from *Salamandra corsica* Savi, 1838 (Acc. No.: MF043388) and *S. lanzai* Nascetti, Andreone, Capula & Bullini, 1988 (Acc. No.: MF043391) were used as outgroups (following Bonato et al. 2018). Phylogenetic relationships were estimated using maximum likelihood (ML) and Bayesian inference (BI). ML analyses were conducted in RaxMl v. 8.2 (Stamatakis 2014) accessed through the Cipres Science Getaway (Miller et al. 2013) using the GTRGAMMA model with 1,000 bootstraps. BI analyses were done in MrBayes v. 3.1.2 (Ronquist & Huelsenbeck 2003) using the best-fit nucleotide substitution model that was selected on the partitioned datasets and *D-loop* by the corrected Akaike Information Criterion (Aicc) implemented in JModeltest 2.1.7 (Darriba et al. 2012) as suggested by Burnham & Anderson (2004). The analysis was run twice for 2 x 10^6 generations, with four parallel chains and sampled every 500 generations.

The McMc convergence was checked every 1,000 generations by the output of standard deviation of split frequencies. The resulting tree was constructed from 6,000 trees sampled from the posterior distribution, once the first 2000 trees were excluded as burn-in (25%). Trees were visualized and edited with FigTree v. 1.3.1 (Rambaut and Drummond 2009).

Sequence divergence and geographical distribution of the concatenated haplotypes (removed invariable sites) were analyzed using unrooted median-joining networks (Bandelt et al. 1999) constructed with Popart (http://popart.otago.ac.nz).

Genetic distances among detected lineages

Arlequin 3.5 (Excoffier & Lischer 2010) was used to calculate the uncorrected pairwise genetic distances (pi; simple p distances) for the concatenated mtDNA sequences among the detected lineages of *S. atra* from the previous analysis. The analysis were done under 10,000 permutations and the significance level of 0.05.

Inferring the number of populations

Using Structure 2.3.4 (Pritchard et al. 2010), the microsatellites were analyzed to group the sampled individuals of *S. atra* in clusters (K) which, hereby after, we define as populations. Following Bonato et al. (2018), exploratory analyses, with K values between two and nine (the total number of sampling sites) were conducted with five simulations (100,000 iterations, 25,000 burn-in) implementing different combinations of ancestry models (admixture vs. no admixture) and allele frequency models (correlated vs. independent). The final analysis was performed under the admixture model and assuming correlated allele frequencies among populations (following Razpet et al. 2016). The admixture model was chosen due to its flexibility (Pritchard et al. 2010), while the correlated allele frequency model was chosen because previous results showed genetic similarity between Dinaric population fragments (Helfer 2010, Razpet et al. 2016, Bonato et al. 2018). The analyses were run without using the information on sampling sites, and for K values from 2 to 10, running 20 simulations (500,000 iterations, 125,000 burn-in). The most likely number of clusters was estimated by means of likelihood ratios (Evanno et al. 2005) in Structure Harvester (Earl & von Holdt 2012).

Using Genetix 4.05 (Belkhir et al. 1996-2004), we performed a factorial correspondence analysis (FCA) on sampled individuals also to reveal potential population clusters and their genetic variation.

Genetic variation within populations

Populations are defined as inferred from the previous analysis[2].

Microsatellite data: The data were checked for genotyping errors with Micro-checker 2.2.3 (Van Oosterhout et al. 2004). Further analyses were performed in Arlequin. Deviation from Hardy-Weinberg equilibrium (HWE) was tested using the exact test (Guo & Thompson 1992). A linkage disequilibrium test was run between pairs of microsatellite loci for each population with a likelihood-ratio test (Excoffier & Slatkin, 1998; with 10,000 permutations). Levels of significance (alpha = 0.01, see Wigginton et al. 2005), were adjusted using the standard Bonferroni correction. For each population, observed and expected heterozygosity, mean number of alleles per locus and inbreeding coefficients (Fis; were tested using 10,000 permutations of alleles between individuals within each population). The PopGenReport R package (Adamack & Gruber 2014) was used to assess the mean allelic richness of each population adjusted for sample

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[2] Please note that the meaning of the term "population" changes somewhat during the course of this chapter. In the introduction, the pockets of *S. atra* in the Dinarides are called "population fragments" of a larger *S. atra* "population" that occupies the whole region of the Dinaric Alps. Later, after the structure analysis, the term "population" refers to genetically distinct groups of individuals found at the respective sampling areas and mountains.

size (using a rarefication procedure). The number of private alleles per population was obtained using the StrataG R package (Archer et al. 2016).

mtDNA data: For the concatenated sequences, the number of (private) haplotypes, haplotype diversity, number of polymorphic sites, and nucleotide diversity were computed in Arlequin.

Genetic differentiation among populations

The overall genetic variation was partitioned into within and among population components with an analysis of molecular variance (AMOVA, Excoffier et al. 1992) using Arlequin 3.5 (Excoffier & Lischer 2010); populations (as after Structure) were treated as one single group of data (one species, i.e. *S. atra*). The degree of among population divergence was estimated by the pairwise Fst (microsatellite data, Weir & Cockerhiam 1984). Differentiation among mtDNA sequences was generated by correcting for multiple hits with the method of Tamura & Nei (1993) because this is the alternative for the HKY + I best fit evolutionary model (unavailable in Arlequin) that is detected within the *cob* gene (also following Bonato et al. 2018). The significance of the differentiation values was assessed after 10000 random permutations (Excoffier et al. 1992) in Arlequin. A Mantel test was conducted using ade4 R package (Dray & Dufour 2007) with 9,999 permutations to evaluate whether the level of genetic differentiation among populations is correlated with their geographic distance.

Demographic tests and bottleneck

DnaSP 6.0 (Rozas et al. 2017) was used to compute Strobeck's S, Fu and Li's statistics and Tajima D test, for which we specified the coding (*cob*) and non-coding (*D-loop*) part of the sequence. The Tajima D test, combined with Fu and Li's statistics, test the hypothesis of range expansions, while Strobeck's statistics is relevant for the estimation of the number of existing haplotypes in relation to the number of recorded ones.

The bottleneck hypothesis was tested for the inferred populations, using the one-tailed Wilcoxon test for heterozygosity excess implemented in Bottleneck 1.2.02 (Piry et al. 1999) under different mutation models (infinite allele - IAM, two-phased - TPM, stepwise mutation – SMM). The TPM model was tested with a variance of 14 and a proportion of SMM between 70 and 95% (Piry et al. 1999).

Results

Phylogenetic and phylogeographic relationships

To obtain a final consensus sequence including outgroups, we had to trim our concatenated sequences in 10 bp (the first two of *cob* and the last eight from the *D-loop*). The final sequence included 1,657 bp (*cob* = 962 bp, *D-loop* = 695 bp). To achieve the same length, sequences of Bonato et al. (2018) had to be also trimmed, which caused several of their haplotypes to collapse with ours (Table S2.1). Finally, 32 distinct haplotypes were generated (Table S2.1). The partition homogeneity test revealed no significant incongruency between *cob* and *D-loop* (p = 1). The substitution saturation test showed that *cob* partitions and *D-loop* are suitable for phylogenetic analysis. jModelTest revealed that *cob* evolution is best described under the models TPM2, HKY, HKY+I (1^{st}, 2^{nd} and 3^{rd} partition respectively), while the *D-loop* evolution is under the TPM2UF + G model. The standard deviation of the split frequencies after 2×10^6 generations was 0.009 which is a good indication of convergence (Ronquist et al. 2011).

The clusters in the phylogenetic trees follow the ones in the haplotype network (Fig. 2) corresponding to those previously revealed by Bonato et al. 2018. The BI and Ml revealed the same tree topology, suggesting a basal split between the Orobie population and all the others, and an unresolved polytomy between individuals from the Dolomites (H25), the populations in the Venetian Prealps (H24 – Belluno and Cansiglio) and the clade uniting the populations assigned to the four described subspecies (Fig. 2). The latter clade separated populations from the Pasubio and Dinarides on one side, and the populations of Sette Comuni and the Alps on the other, although the monophyly of the first group is not well supported (branch values 0.84/49 – Fig. 2). In conclusion, phylogenetic analysis revealed the existence of seven lineages, two of them being represented by single haplotypes (H24 and H25 respectively; Table 1, Fig. 2).

Table 1. Dataset details and detected haplotypes (Hap) – Codes are as in Fig. 1; codes from 17 to 27 are located in the Dinarides; bold codes are samples from this study; others are retrieved from public repositories (see text for details). The number of *S. atra* individuals included in the analyses of microsatellites (μs) and concatenated - *cob* + *D-loop* - dataset (mt) is given in the respective columns; "/" - no data. Code 22 is both: * - sampled in this study, and also ° - taken from Bonato et al. (2018). Hap codes are as in Fig. 2. At code 23, heteroplasmy (H6 + H12) was detected in one sample, hence four sequences were generated although only three individuals were sequenced. B&H – Bosnia and Herzegovina

Code	Country/ Sampling area	Sampling site	μs	mt	Hap (number of individuals)	Lineage
1	Italy / Breonie	Pflerschtal	/	6	H18 (1), H19 (1), H20 (4)	*S.a.atra*
2	Italy / Breonie	Ridnauntal	/	6	H19 (5), H20 (1)	*S.a.atra*
3	Italy / Dolomites	Valle Fiscalina	/	1	H25 (1)	H25
4	Italy / Orobie	Val Tronella	/	1	H29 (1)	Orobie
5	Italy / Orobie	Val Terzera	/	8	H30 (4), H31 (4)	Orobie
6	Italy / Orobie	Laghi Gemelli	/	8	H27 (6), H28 (1), H32 (1)	Orobie
7	Italy / Orobie	Pizzo della Presolana	/	1	H27 (1)	Orobie
8	Italy / Orobie	Val di Scalve	/	9	H26 (1), H27 (8)	Orobie
9	Italy / Pasubio	Val Fontana d'Oro	/	13	H14 (9), H15 (4)	*S.a.pasubiensis*
10	Italy / Sette Comuni	Bosco del Dosso	/	16	H21 (15), H23 (1)	*S.a.aurorae*
11	Italy / Sette Comuni	Monte Fossetta	/	1	H21 (1)	*S.a.aurorae*
12	Italy / Sette Comuni	Val di Nos	/	13	H21 (11), H22 (2)	*S.a.aurorae*
13	Italy / Sette Comuni	Val Postesina	/	1	H21 (1)	*S.a.aurorae*
14	Italy / Belluno	Schiara-Val dell'Ardo	/	15	H24 (15)	H24
15	Italy / Cansiglio	Pian di Landro-Baldassare	/	3	H24 (3)	H24
16	Slovenia / Kredarica	Aljažev dom	8	8	H16 (1), H17 (7)	*S.a.atra*
17	Slovenia / Trnovski Gozd	Trnovski Gozd	/	1	H12 (1)	*S.a.prenjensis*
18	Croatia / Gorski Kotar	Samarske stijene	7	4	H1 (1), H2 (1), H3 (1), H4 (1)	*S.a.prenjensis*
19	Croatia / Gorski Kotar	Vihoraški put	10	5	H4 (4), H5 (1)	*S.a.prenjensis*
20	B&H / Čvrsnica	Pločno	20	9	H8 (8), H12 (1)	*S.a.prenjensis*
21	B&H / Prenj	Soplje	/	7	H11 (2), H12 (4), H13 (1)	*S.a.prenjensis*
22	B&H / Prenj	Zakantar	7	3*+ 3°	H9 (1°), H10 (1°) H12 (2*), H13 (1°+1*)	*S.a.prenjensis*
23	B&H / Prenj	Kopilice	7	3	H6 (1), H12 (3)	*S.a.prenjensis*
24	B&H / Prenj	Podotiš	11	4	H11 (1), H13 (3)	*S.a.prenjensis*
25	B&H / Prenj	Sedlo	/	5	H11 (1), H12 (3), H13 (1)	*S.a.prenjensis*
26	Montenegro / Prokletije	Bogićevica	22	9	H7 (9)	*S.a.prenjensis*
27	Montenegro / Prokletije	Gorazdevac	3	1	H7 (1)	*S.a.prenjensis*

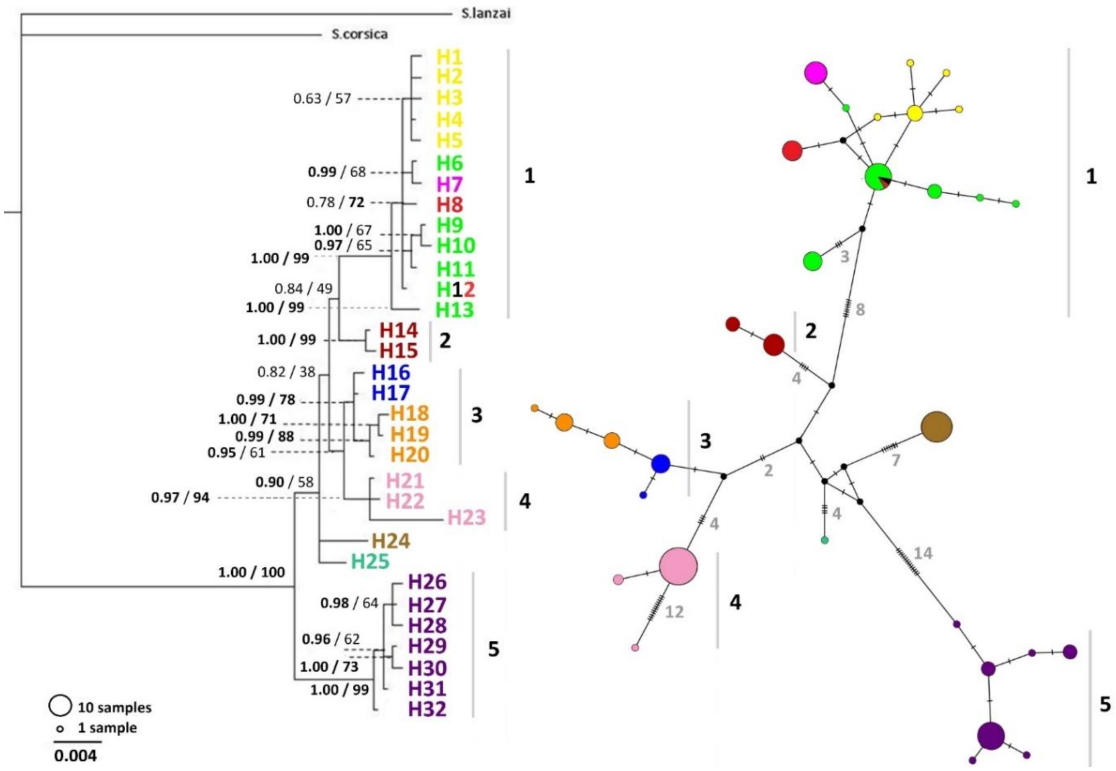

Figure 2. Phylogenetic tree and Median-joining (MJ) network of the 32 haplotypes (H) based on the concatenated *cob* and *D-loop* markers in 164 individuals of *Salamandra atra* (46 samples from this study, and 118 from public repositories) from 27 sampling sites across the species range. Bayesian posterior probabilities (PP) and ML bootstrap values (in percentages from 1000 replications) are showed on the tree nodes separated by a slash ("/"); PP ≥ 0.90 and ML values ≥ 0.70 are bolded (considered high support values). In the MJ, the size of the haplotype (pie chart) is proportional to the number of samples in which it was found (as in the figure legend). The colors of haplotypes represent the sampling areas as in Table 1. The analysis revealed seven distinct lineages : 1 - *S. a. prenjensis* (Dinaric clade, from north to south: black – Trnovski Gozd, yellow - Gorski Kotar, red – Čvrsnica, green – Prenj, pink - Prokletije), 2 - *S. a. pasubiensis* (Pasubio clade), 3 - *S. a. atra* (Alpine clade), 4 - *S. a. aurorae* (Sette Comuni clade), 5 – the Orobie clade potentially belonging to a new, yet undescribed subspecies; two lineages are revealed by a single haplotype (H24 - brown, H25 - turquoise). The dashes along the branches of the MJ network represent the minimum number of substitutions between haplotypes that is also indicated by a (grey) number if different from one. Black circles in the MJ network represent missing haplotypes

Genetic distances among detected lineages

According to the detected lineages from the previous analysis, we merged the sequences from the Dinarides (Trnovski Gozd, Gorski Kotar, Čvrsnica, Prenj and Prokletije - sampling areas as in Table 1) in a single group of data named *S.atra prenjensis*; the sequences from Kredarica (Julian Alps, code 16 in Fig.1) were merged to the sequences from Breonie (codes 1 and 2 in Fig. 1) as these are part of the same lineage (*S. a. atra,* Table 1). Following the same principle we defined the other groups representing the inferred lineages (*S. a. aurorae*, *S. a. pasubiensis*, Venetian Prealps – H24, Dolomites – H25, Orobie, Table 1). The analysis of p-distances showed that all detected lineages are significantly distant among each other (Table 2) except H25 which differs only from the Orobian lineage ($p = 0.03$, Table 2); H24 does not also differ from H25. Taking into consideration that only one individual represents the lineage H25 in the Dolomites (code 3, Table 1) the estimated distance between this and other lineages (Table 2) must be taken with precaution.

Summary statistics for samples originating from this study

A total of 95 individuals of *S. atra* were typed for six microsatellite loci (Table 1). All loci were polymorphic, with seven to 12 alleles per locus (mean = 8.7, Table S2.2). No null alleles, allelic dropout, or stuttering were detected for any locus. Raw microsatellite data are given in Table S2.3.

The alignment of the concatenated (consensus) mtDNA sequences had a length of 1667 bp (964 bp *cob* + 703 bp *D-loop*) and comprised 46 individuals from nine sampling sites (Table 1). Within the *D-loop*, heteroplasmy was observed (506 bp, C-T) in one individual from Prenj (sampled in Kopilice), therefore the two alternative copies of this sequence were kept (Accession numbers: MN255332 and MN255334). The Dinaric samples involved 13 haplotypes (Fig. 2), with 28 polymorphic nucleotide positions (1.6%; including nine transitions, four transversions, one indel and 14 substitutions [0.8%]).

Table 2. Uncorrected p distances (pi) among the detected *Salamandra atra* lineages; H24 – Venetian Prealps (Belluno and Cansiglio), H25 – Dolomites, as in Table 1. Above the diagonal is the average number (avg. nb) of pi between lineages; diagonal elements: avg. nb of pi within the lineage; below the diagonal are the p – values for estimates between the lineages (significant ones are bolded)

	Orobie	*S. atra prenjensis*	*S. atra atra*	*S. atra aurorae*	*S. atra pasubiensis*	**H25**	**H24**
Orobie	1.28	27.29	23.05	20.45	22.20	22.00	25.00
S. atra prenjensis	**0.00**	3.03	15.53	16.19	14.53	16.22	16.44
S. atra atra	**0.00**	**0.00**	1.18	6.50	9.36	9.05	13.05
S. atra aurorae	**0.00**	**0.00**	**0.00**	0.90	11.76	11.45	15.45
S. atra pasubiensis	**0.00**	**0.00**	**0.00**	**0.00**	0.46	10.31	14.31
H25	**0.03**	1.00	1.00	0.06	0.08	0.00	12.00
H24	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	1.00	0.00

Inferring the number of populations

All exploratory model combinations performed in Structure proposed five clusters as it did the final analysis (Fig. 3, Fig. S2.1). Although each cluster (population) mainly corresponds to a separate mountain area (from north to south: Kredarica (Julian Alps), Gorski Kotar, Mt. Čvrsnica, Mt. Prenj, Mt. Prokletije), they are not completely differentiated because some individuals in each population, resemble individuals from others with a high probability (especially noted in Gorski Kotar, Čvrsnica [1st sample] and Prokletije, Fig. 3).

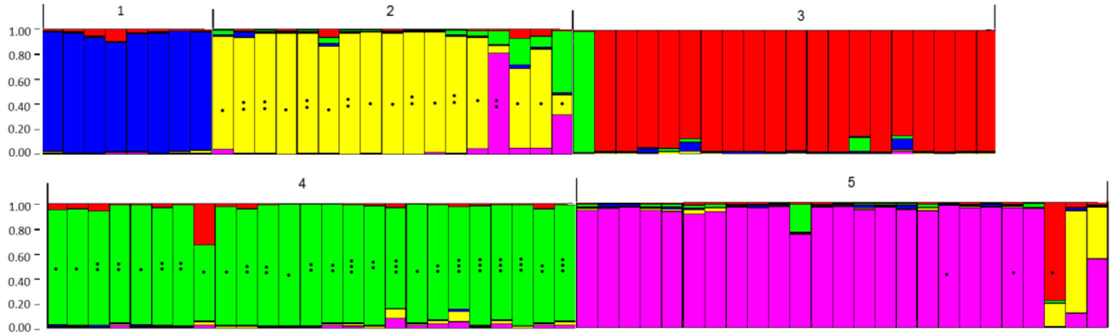

Figure 3. Results of the Structure assignment of 95 individuals of *Salamandra atra* (from this study) based on six microsatellite loci; clusters (populations) are divided by tick black lines as follows: 1 – Kredarica, 2 – Gorski Kotar (. – Vihoraški put, .. – Samarske stijene), 3 - Čvrsnica, 4 – Prenj (. – Kopilice, .. – Podotiš, ... – Zakantar), 5 – Prokletije (no dot – Bogićevica, . – Gorazdevac,). Compared to Kredarica population (nb. 1, in the Julian Alps), the Dinaric ones (nbs: 2-5) are less differentiated, as certain number of individuals in each population resemble individuals from others.

The first FCA axis revealed two main groups: the first is more variable and contains only the samples from the Julian Alps (Kredarica), and the second groups all the Dinaric samples. Within the latter group, four fairly distinct clusters can be recognized, each corresponding to a separate mountain area (Fig. 4). The combination of the three FCA axes accounted for 24.02% of the total variation. No clear substructure was detected among individuals of the same mountain area (clustering of individuals from different sampling sites within the same mountain area, Fig. S2.2).

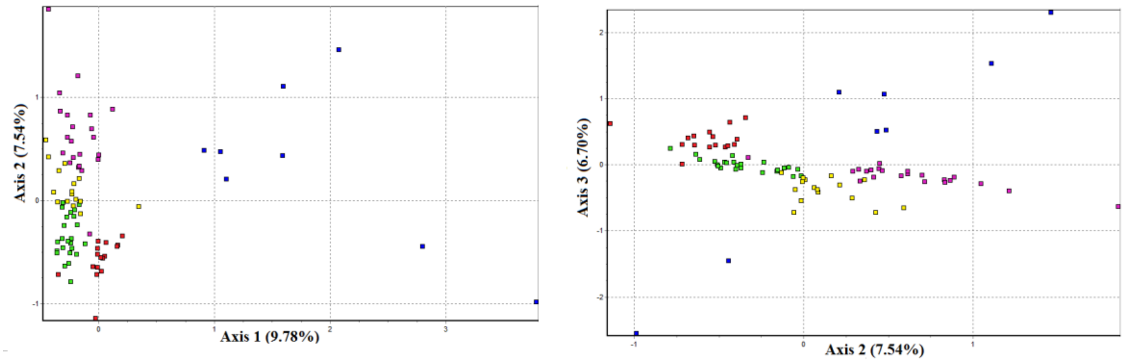

Figure 4. Factorial Correspondence Analysis (FCA) ordination along the first three axes of 95 individuals of *Salamandra atra* from four sampling areas in the Dinarides (following Table 1) and one in the Julian Alps (Kredarica), based on six microsatellite loci. Each label corresponds to a sampling area (from north to south): blue – Kredarica, yellow – Gorski Kotar, red – Čvrsnica, green – Prenj, pink – Prokletije. Axis 1 separates the Dinaric individuals on one side, and the ones from Kredarica on the other; Axis 3 separates Gorski Kotar (Northern Dinarides) from the other sampling areas in the Dinarides (Central and Southern - see also Fig. S2.2).

Genetic variation within populations

Estimated diversity indices for both microsatellites and mtDNA are shown in Table 3. Only the population of Prokletije deviated from HWE for the locus SalE7 ($p=0.0001$, after Bonferroni correction). No deviations from linkage equilibrium were detected.

The observed microsatellite heterozygosity for Dinaric specimens varied between 0.31 and 0.39, but was nearly twice as high in the Alpine population (Kredarica; Table 3). The highest and only significant ($p = 0.009$) inbreeding coefficient (Fis) was recorded in Gorski Kotar (0.19, Table 3).

The mtDNA diversity (pS, Hap, pHap, Hd in Table 3) was higher in Prenj and Gorski Kotar, and lower in Prokletije, Čvrsnica and Kredarica. Only one (private) haplotype was found in the population of Prokletije. The highest number of haplotypes (N = 5, all private) was observed in Gorski Kotar, but the highest nucleotide diversity - in Prenj (Nd = 0.18, Table 3).

Genetic differentiation among populations

With the microsatellite data, the among-population differentiation accounted for a significant 35.09% of the total variation (Amova, $p < 0.001$). Pairwise Fst values ranged from 0.28 (between Gorski Kotar and Prenj) to 0.46 (between Prokletije and Čvrsnica) and were all significant ($p < 0.001$, Table 4).

With the mtDNA data, the among-population differentiation accounted for a significant 87% of the total variation (Amova, $p < 0.001$). Pairwise mtDNA values (Tamura & Nei 1993) between populations ranged from 0.43 to 0.99, and were all significant ($p < 0.001$, Table 4). The strongest differentiation was observed between Kredarica and all other populations; within the Dinarides, mtDNA showed the highest differentiation between Čvrsnica and Prokletije (0.94) and the lowest between Prenj and Gorski Kotar (0.43; Table 4) which is concordant to previously reported pairwise Fst values.

The Mantel tests revealed no correlation b etween genetic differentiations and geographic distances ($p_{(microsatellites)} = 0.9$, $p_{(mtDNA)} = 0.6$).

Table 3. Genetic diversity from microsatellite data (µs - in grey) and mtDNA haplotypes (concatenated *cob* and *D-loop* – in white) in the populations (Pop) of *S. atra* from this study; N number of individuals (total µs - N = 95, total mtDNA - N = 46), Ho - observed heterozygosity, He - expected heterozygosity, mA – mean number of alleles, R – allelic richness, pA – number of private alleles, Fis – inbreeding coefficient (bold if significant); pS – number of polymorphic sites, Hap – number of haplotypes, pHap – number of private haplotypes, Hd – haplotype diversity, Nd - nucleotide diversity (averaged over the two gene fragments). Numbers in brackets are standard deviations

Pop	N	Ho	He	mA	R	pA	Fis	N	pS	Hap	pHa	Hd	Nd (%)
Kredarica	8	0.58 (0.25)	0.58 (0.19)	3.83 (0.98)	3.60	10	-0.002	8	1	2	2	0.25	0.02 (0.02)
Gorski Kotar	17	0.31 (0.21)	0.39 (0.26)	3.83 (1.47)	2.93	7	**0.19**	9	5	5	5	0.72 (0.16)	0.05 (0.05)
Prenj	25	0.39 (0.28)	0.36 (0.28)	3.50 (2.81)	2.78	4	-0.07	10	7	4	3	0.71	0.18 (0.11)
Čvrsnica	20	0.31 (0.32)	0.30 (0.27)	3.33 (1.75)	2.40	3	-0.03	9	2	2	1	0.22	0.03 (0.03)
Prokletije	25	0.33 (0.35)	0.37 (0.28)	3.83 (1.72)	2.72	5	0.09	10	0	1	1	0	0

Table 4. Pairwise divergence between populations of *S. atra* from this study; pairwise Fst for microsatellite loci is above the diagonal, and differentiation among mtDNA loci (*cob* + *D-loop*) is below the diagonal (TAMURA & NEI 1993, see text for details); all values (for both microsatellite and mtDNA) revealed significant ($p < 0.001$)

	Kredarica	Gorski Kotar	Prenj	Čvrsnica	Prokletije
Kredarica	/	0.30	0.36	0.33	0.33
Gorski Kotar	0.96	/	0.28	0.41	0.30
Prenj	0.88	0.43	/	0.35	0.35
Čvrsnica	0.98	0.78	0.53	/	0.46
Prokletije	0.99	0.88	0.61	0.94	/

Demographic tests and bottleneck

The Tajima D test could not reject neutral mtDNA sequence evolution (coding region: D = -0.65, p > 0.10; synonymous sites: D = -0.16, p > 0.10; nonsynonymous sites: D = -0.24, p > 0.10; silent sites: D = -0.24, p > 0.10), and thus did not provide evidence of range expansions. The results of the Tajima D test were in line with those of Fu and Li's statistic, which neither could reject neutrality (D = -1.27, P > 0.10; F = -1.24, P > 0.10). Strobeck's S statistic predicted a higher number of haplotypes than it has been observed (S = 1, P = 0.00). The bottleneck tests were unable to demonstrate that such events occurred in our populations (Iam: p ≥ 0.50, Smm: p ≥ 0.96, Tpm: p ≥ 0.44).

Discussion

Phylogenetic and phylogeographic relationships - taxonomic implications

All studied populations in the Dinarides belong to a unique evolutionary lineage (Fig. 2). Individuals from the Julian Alps (Kredarica) belong to *S. a. atra* (Table 1, Fig. 2) as it could have been anticipated from the high differentiation values between this and all the Dinaric populations (mtDNA, Table 4), and its high heterozygosity (Table 3) that are characteristic to this lineage when compared to all the others (Helfer 2010, Razpet et al. 2016, Bonato et al. 2018). This study confirmed already observed trends considering the evolutionary separation of the Dinaric population and the delineation of the commonly accepted subspecies: *S. a. aurorae, S. a. pasubiensis* and *S. a. atra* (Helfer 2010, Bonato et al, 2018). Moreover, the study also confirmed the evolutionary separation of the Orobian population of alpine salamanders and the populations from the Venetian Prealps and Dolomites (H24 and H25 respectively, Table 1, see also Helfer 2010, Helfer et al. 2011 and Bonato et al. 2018). However, as our phylogenetic results were inferred from mtDNA data, conclusions need further corroboration, since in *S. atra*, nuclear markers may generate different tree topologies (see Bonato et al. 2018, Ribéron et al. 2004 vs. Helfer 2010). Considering our Dinaric specimens, by reconfirming their separate evolutionary history and by presenting their significant genetic distance from other lineages (Table 2), we justify the sub-specific taxon *S. a. prenjensis* Mikšić 1969 as already proposed by previous authors (Helfer 2010, Razpet et al. 2016, Bonato et al. 2018, Šunje et al. 2019).

In order to fill the remaining gaps in our knowledge about the complex evolutionary history of *S.atra,* we highlight the importance to study other population fragments that are not included in this study; primarily the ones in the Prealps and Dinarides (Fig. 1, see also Helfer 2010).

Inference of populations

Both Structure and FCA showed that the specimens from this study belong to five populations that mainly correspond to sampled mountain areas. Populations are well differentiated but not completely, which is especially visible within the Dinaric clusters where in almost each, some individuals resemble more members from other populations than members of their own (Fig. 3). Considering the large distance among the population fragments and the strong geographic barriers between them (Fig. 1), it is improbable that their resemblance is attributable to gene flow, so we could only speculate that other evolutionary processes are responsible for such observations. A lack of gene flow is also confirmed by high and significant differentiation values (Table 4 and Amova). However, the incomplete differentiation among the Dinaric specimens suggests a past connection between the populations and/or a common origin. The population of Kredarica, revealed the most homogenous (Fig. 3) and distantly related (Fig. 4) compared to all Dinaric specimens (*S. a. prenjensis* lineage – Table 1, Fig. 2) which is explained by the fact that it belongs to another lineage (*S. a. atra* – Table 1, Fig. 2).

Distribution of genetic diversity

The observed heterozygosity calculated for the Prokletije population (Ho = 0.33) is twice as high as the one reported by Razpet et al. (2016; Ho = 0.17) probably because the latter study contained a small number of individuals from this location, two of which were intrauterine larvae related to a third sample (mother). The observed heterozygosity in Prenj was in line with previous observations (Razpet et al. 2016, Bonato et al. 2018: Ho = 0.36 and 0.37 vs. Ho = 0.39 in Table 3). The microsatellite genetic diversity indices were similar in all the Dinaric populations, whereas for mtDNA, the levels of genetic diversity are higher in Gorski Kotar and Prenj, and lower in Čvrsnica and Prokletije (Table 2). On the other hand, the heterozygosity indices for Kredarica, were almost twice as high as in the Dinaric populations (despite a considerably lower sample size) but, the mtDNA diversity was lower as in Čvrsnica and Prokletije (probably due to a considerable lower sample size - Tables 1 and 3). A high heterozygosity in Kredarica suggests gene flow that may be a consequence of a "leading edge" range expansion characteristic for other populations of *S. atra atra* across the northern and central Alps (Helfer 2010). Just like Razpet et al. (2016) we were unable to detect any signs of bottlenecks.

'Refugia within refugia': a plausible historical scenario for the *prenjensis* lineage

The 'refugia within refugia' hypothesis describes survival patterns in multiple glacial refugia, each within a larger refugial area (Gómez & Lunt 2007). The Balkan refugial area was mostly free of ice during the last glacial maxima (LGM, Fig. 1) but many glaciers persisted during other phases of the Pleistocene at higher altitudes (generally above 1,300 m a.s.l, Cvijić 1899, Hughes et al. 2011, Lepirica 2013, Žebre & Stepišnik 2015), which explains why multiple refugia must have characterized the Balkans. Our results suggest that during the Pleistocene, Dinaric populations of alpine salamanders thrived in at least two refugia: an ancestral in Mt. Prenj and, a more recent, in Gorski Kotar. The diversity pattern in Prenj (the highest Nd, high Hd with many pHap, Table 3) and its central position in the MJ network (Fig. 2), suggest that it represented the main diversification center of alpine salamanders (following Avise 2000). The fact that the lowest mtDNA differentiation is found between Prenj and all other populations (Table 4) supports this idea. Moreover, Razpet et al. (2016) showed that Mt. Prenj is home to the oldest populations of alpine salamanders in the Dinarides (according the estimated time since decline). Gorski Kotar, on the other hand, was another diversification center of alpine salamanders in the Dinarides, consequently by the high mtDNA diversity that is found thereby, which resemblances the detected pattern found at Prenj (Table 3). However, the diversification from Gorski Kotar, seems to be a more recent event which is revealed by the low nucleotide diversity and a star like phylogeographic pattern that constitutes of a homogeneous assemblage of very similar haplotypes (Fig. 2, following Avise 2000). Hence, Prenj and Gorski Kotar, as alpine salamanders' refugia, might not have co-existed. Furthermore, the central haplotype in Prenj (H12) is the most frequent in the '*prenjensis*' clade (Fig. 2, Table 1), signaling that it is a very old haplotype that was probably distributed over the entire Dinaric range and persisted in big populations but was lost in others during fragmentations. This ancestral haplotype is separated only by a single mutational step from the central haplotype in Gorski Kotar (H4, Fig. 2), suggesting that alpine salamanders from Gorski Kotar originate from Prenj. Nevertheless, the high numbers of private haplotypes found in Prenj and Gorski Kotar are also evidence of a long-term population establishment (see Haydar 2011), which supports our conclusion that these areas acted as refugia for *S.atra*.

In contrast, a lower mtDNA diversity, including less private haplotypes, is found in Čvrsnica and Prokletije (Table 3), signaling that these populations were established more recently (following Austerlitz et al. 1997 and Excoffier & Petit 2009). Considering Čvrsnica, these results are rather unexpected, as this mountain is neighboring Prenj. Although several areas of both mountains were glaciated in the middle Pleistocene (after the LGM; Mt. Čvrsnica - above 1,200 m a.s.l [Stepišnik et al. 2016], Mt. Prenj - above 1,500 m [Lepirica 2008]) the population of Prenj has a significantly higher mtDNA diversity compared to Čvrsnica. We suspect that the relatively larger glacier on Čvrsnica (ca. 200 km^2, see also Miličević 2013) hindered the population stability more than did the glacier on Mt. Prenj. This is especially plausible given that the survival of individuals on Mt. Čvrsnica was thwarted by several barriers: on the east, by the deep canyon of the river Neretva (at the foothill of Mt. Čvrsnica), on the west - by the glaciers Svinjača and Dugo polje (also on Mt. Čvrsnica, Stepišnik et al. 2016), and on the south by the glacier of Mt. Čabulja (south border of Mt. Čvrsnica, see Prskalo 2008). Moreover, the highest differentiation (both Fst and mtDNA) found between Čvrsnica and Prokletije (the southernmost population; Table 4) confirms no dispersal from Mt. Čvrsnica southwards probably due to the aforementioned barriers. The missing (haplotype) link between Čvrsnica and both, Gorski Kotar and Prenj (separated by a single mutational step, Fig. 4) suggests that the stabilization of Čvrsnica occurred from another population (not sampled/not existing) from which dispersal was probably bidirectional, i.e from north (Gorski Kotar) to south (Prenj) and vice versa (Fig. 2). Considering Prokletije, the thereby registered deviation from HWE, supports the idea that this area was colonized, as founding events are likely to create persistent non-equilibrium structures (Boileau & Hebert 1991). Individuals that were part of this founding event likely originated from Prenj since the only haplotype registered in Prokletije (H7, Fig. 4) is divided by a single step mutation from the Prenj haplotype (H6, Fig. 2 and Table 1). We conclude that our results are in line with the 'refugia within refugia' hypothesis that was suggested for several other species in the Balkans (Kryštufek et al. 2007, Sotiropoulos et al. 2007, Ursenbacher et al. 2008, Surina et al. 2011)[3].

Conservation insights for the *prenjensis* lineage

A major conservation task is to define the proper distribution range of *prenjensis*. Strobeck's S statistic and the newly discovered population on Mt. Orjen (Cikovac & Ljubisavljević 2020) suggest that there are populations of alpine salamanders in the Dinarides that are yet to be discovered (see also Klewen 1988). Therefore, field investigations of potential occurrence areas (see in Cikovac & Ljubisavljević 2020) are needed to clarify the distribution range of *prenjensis*. The northernmost locations where the *prenjensis* is registered are in the Southern Prealps (Kobarid - Krn, Helfer 2010 and Loibl Pass, Šunje et al. 2019). Since these two areas are relatively close to our sampling site of Kredarica (ca. 20 and 30 km of air distance respectively), several contact zones between *S. a. atra* and *S. a. prenjensis* may be present in the wider areas connecting the three sites. The identification of contact zones is, not only important from an evolutionary perspective and the study of speciation processes (Wollenberg Valero et al. 2019), but it is relevant in a conservation context (Allendorf et al. 2001), since conservation efforts should be preferably oriented towards populations where there is no risk of genetic swamping with other lineages. Genetic swamping causes outbreeding depression (Frankham et al. 2011), that, at it's extreme form, could be detrimental

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[3] The exsistence of another refugium south-east from Mt. Prenj (area nowadays coinciding with the mountain massifs of eastern B&H adjacent to the massifs of north and central Montenegro) is considered unlikely. During the Pleistocene glaciations this area was covered by conjoined ice caps; in Montenegro, the ice cap had a maximum surface of 1500km^2 (11% of the total surface area of Montenegro nowadays) (Hughes et al., 2011). This big ice conglomerate persisted during multiple Pleistocene glaciations (Hughes et al., 2011) making it highly improbable that salamander populations survived locally. In addition, the presence of deep river canyons would have thwarted dispersal events. This may also explain why salamanders are not registered in these areas so far. Nevertheless, we cannot exclude the possibility that unsampled populations (as the one of mt. Orjen or other (undiscovered) populations (Cikovac and Ljubisavljević 2020)) originate from other refugia than the ones detected here.

for the population (Rhymer & Simberloff 1996). The levels of genetic diversity of the Dinaric populations in this study suggest that these are not of immediate conservation concern, although the significant level of inbreeding in the population of Gorski Kotar (Fis, Table 3), signals a potential threat to its health and must be monitored. The significant differentiation among populations, indicates that each should be treated as a separate conservation unit. As the population of Prenj harbors substantial genetic diversity and no signs of inbreeding (Table 3), we believe that it is in conservation interest to focus the efforts on its preservation on a long term run. Another argument supporting this idea is that Prenj was a refugium for *S.atra* during past environmental perturbances, hence might also offer the best chances for survival under upcoming climatic change (for the rationale see Keppel et al. 2012).

Chapter 3

Morphometric characteristics of Alpine salamanders; a support for subspecies validation and conservation?[4]

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Abstract. The subspecies concept is not only useful to assess the evolutionary history of species and therefore describe their evolutionary potential, but it also has corollaries for defining conservation units and their management. Within Alpine salamanders, the subspecies status of *Salamandra atra prenjensis*, isolated in the Dinarides from its nominal subspecies *Salamandra atra atra* that occurs in the Alps, has been under debate. To remediate this fuzzy taxonomy, the present study investigates 14 morphological traits of Alpine salamanders originating from Austria and from Bosnia and Herzegovina (B&H). Multivariate analyses support a geographical structuring of morphological variation and the differentiation between the Dinaric (B&H) and Alpine (Austrian) populations. Within populations, a different correlation pattern among traits is registered, reflecting the distinct genetic architecture of multivariate phenotypes. This morphometric study supports recent molecular evidences of a strong differentiation between the Dinaric and Alpine populations and pleads in favour of the separate subspecies status, although a wider sampling of other populations and the inclusion of additional characters would be necessary to reinforce this conclusion. The recognition of *Salamandra atra prenjensis* as a distinct subspecies would highly contribute to the better conservation of this emblematic salamander.

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[4] The reader will notice that in this chapter, the validity of the *prenjensis* subspecies is still treated as uncertain. This is because at the time this paper was submitted for publication, the genetic data described in the chapter 2 were not yet available.

Introduction

Populations of the same species inhabiting distinct environments are under distinct selective pressures that, in absence of gene flow among populations, might lead to genetic differentiation resulting in speciation on the long term (Mayr, 1970). Populations of the same species that followed different evolutionary trajectories, are considered to be distinct Evolutionary Significant Units (ESU, Ryder, 1986). The concept is supported if molecular diversification exists: ESU's should be reciprocally monophyletic in mtDNA or nDNA based phylogenies and show significant divergence of allele frequencies on neutral nuclear markers (Moritz, 1994). The ESU concept offers an alternative to the subspecies concept in the conservation biological framework (Zachos, 2016). The definition of the subspecies differed among authors. Mayr (1969) argued that besides the geographic subdivision of populations, subspecies must also differ morphologically from each other in order to be recognized as such, while Avise and Ball (1990) defined subspecies as phylogenetically distinguishable and reproductively compatible populations without emphasizing morphological differentiation (Zachos, 2016). Nowadays, to be defined as a subspecies, taxa should be delimited using the combination of both genetic and morphological data (Guerra-Garcia 2008). The subspecies concept is useful for investigating the evolutionary history of species and describing their evolutionary potential. Moreover, subspecies are considered conservation units that may require specific attention, protection and management (Zachos, 2016). Indeed, one of the most important indicator of biodiversity trends, the Red lists of IUCN (The International Union for Conservation of Nature), recognize separate conservation status at the subspecies level (red list assessment criteria - IUCN, 2012). A recent taxonomic study on the Japanese gecko *Goniurosaurus kuroiwae,* for instance, identified a new subspecies (*G. k. toyamai*) that subsequently was accredited the status of 'Critically endangered' (compared to the other subspecies, which remain considered 'Endangered and Vulnerable', Ota 2000).

The Alpine salamander (*Salamandra atra* Laurenti, 1768), a species distributed over the Alpine and Dinaric regions, is another example where a fully resolved taxonomy on the level of subspecies, could help guiding conservation efforts. At the moment, three subspecies are recognized and commonly accepted, based on clear morphological (mainly chromatic) characteristics: the completely melanistic nominal subspecies, *Salamandra atra atra* (Laurenti, 1768), and two subspecies inhabiting the Italian Prealps, *Salamandra a. aurorae* (Trevisian, 1982) and *Salamandra a. pasubiensis* (Bonato and Steinfartz, 2005), that are differentiated by the amount and distribution pattern of yellow patches present on the body (Speybroeck et al., 2016). The validity of a fourth subspecies, *Salamandra atra prenjensis* (Mikšić, 1969), that encompasses the populations from the Dinaric region, is still debated (Klewen, 1988; Grossenbacher, 1994; Bonato and Steinfartz, 2005; Andreone et al., 2009).

The IUCN conservation status for the species *Salamandra atra* is evaluated as Least Concern, and only the subspecies *S. a. aurorae* qualifies for listing as Critically Endangered due to its small extent of occurrence and the degradation of its habitat (Andreone et al., 2009). Because the Dinaric populations of *S. atra* have received relatively little scientific attention, it is as yet unclear whether *S. atra prenjensis* constitutes a genuine subspecies. This lack of insight is thwarting the development of adequate conservation and management strategies.

On a molecular basis, recent studies (Helfer 2010; Helfer et al. 2011; Razpet et al. 2016, Bonato et al., 2018) support a phylogenetic differentiation between the *S. atra* populations from the Northern Alps and the Dinarides (contrarily to Bonato and Steinfartz, 2005 and previous phylogenetic studies), thus fulfilling Avise and Ball (1990) main criteria for the subspecies status and ESU recognition. Nonetheless, it remains unclear if there is morphological differentiation between these two divergent melanistic populations, as required according to Mayr's (1969) criteria, to accredit them as distinct subspecies.

To remediate this fuzzy taxonomy, the present study investigated morphological characteristics of the putative *S. a. prenjensis* in comparison to the nominal subspecies. We tested 14 traits and size for differences between populations of Alpine salamanders, accounting for potential differences between sexes. Correlation among traits was tested within populations to explore the genetic architecture of multivariate phenotypes. Multivariate analyses were conducted to determine the strength of morphological differentiation between Alpine and Dinaric populations.

Material and methods

Study species

The Alpine salamander is a fully terrestrial, viviparous urodele whose distribution covers the whole northern Alps range (from western Switzerland to eastern Austria) and some isolated areas in the Dinaric regions (Gasc 1997). In the Northern Alps, the species has a nearly continuous distribution, with a wide altitudinal range (from 433 up to 2.800 m a.s.l), thus occupying various ecosystems such as humid forests, meadows, pastures and rocky habitats above the tree line (Klewen 1988). In the Dinaric area, the distribution is highly fragmented and salamanders mostly occur on meadows above the tree line (fig. 1). *Salamandra atra* is a poisonous salamander, characterized by large parotid glands posterior to the eyes, a ribbed appearance and a double row of dorsal (costal) glands - glandular knobs - starting behind the head and extending towards the tip of the tail (Figure S3.1; Speybroeck et al., 2016). Compared to the nominal Alpine subspecies, Mikšić (1969) describes *prenjensis* individuals as shorter in length and brighter in black coloration with vomero-palatal teeth arranged in an S-shaped configuration, in contrary to the parallel configuration present in the nominal subspecies.

Sampling locations

Individuals of *S. a. prenjensis* were sampled on two different mountains in Bosnia and Herzegovina (B&H): Mt. Prenj and Mt. Čvrsnica (fig. 1), that are divided by the canyon of the river Neretva – a wide, deep and fast flowing river that separates these two mountains because of which they are assumed to shelter two isolated subpopulations of salamanders. A total of 124 individuals (71 females - F and 53 males - M) were sampled at five distinct locations on Mt. Prenj, and 59 individuals (F=25, M=34) at one location on Mt. Čvrsnica (Table S3.1). Sampling took place from June to September 2013, on altitudes between 1700 - 1850 m a.s.l, on open Alpine meadows dappled with *Pinus mugo*. Measurements were done in the field where individuals were shortly kept in boxes (a maximum of five individuals per box of 12x7x5 cm size). After measuring, each individual was released at the location of capture. Individuals of *S. a. atra* were sampled on two different mountain regions in Austria from mid-August to early September 2013 (fig. 1). In the Tappenkarsee area (Radstädter Tauern – Kleinarltal; Salzburg) 39 salamanders (F=16, M=23) were collected at two distinct altitudes (on ca. 1400 and 1600 m a.s.l respectively), while at Bluntautal area (Nördlichen Kalkalpen – Golling; Salzburg) 23 individuals (F=15, M=8; table S3.1) were sampled from one location at lower altitude (ca. 900 m a.s.l – table S3.1). All Austrian individuals were caught in mixed forest ecosystems dominated by *Fagus sylvatica*. Upon capture, Austrian animals were housed at the laboratory of the University of Salzburg where they were measured in October 2013, just before their release at their place of capture.

Following Cronin (2006), in this study we use the term „population" (pop) to distinguish individuals from B&H and Austria respectively, and the term "subpopulation" (subpop) referring to individuals from Mt. Prenj, Mt. Čvrsnica, Bluntautal and Tappenkarsee (table S3.1).

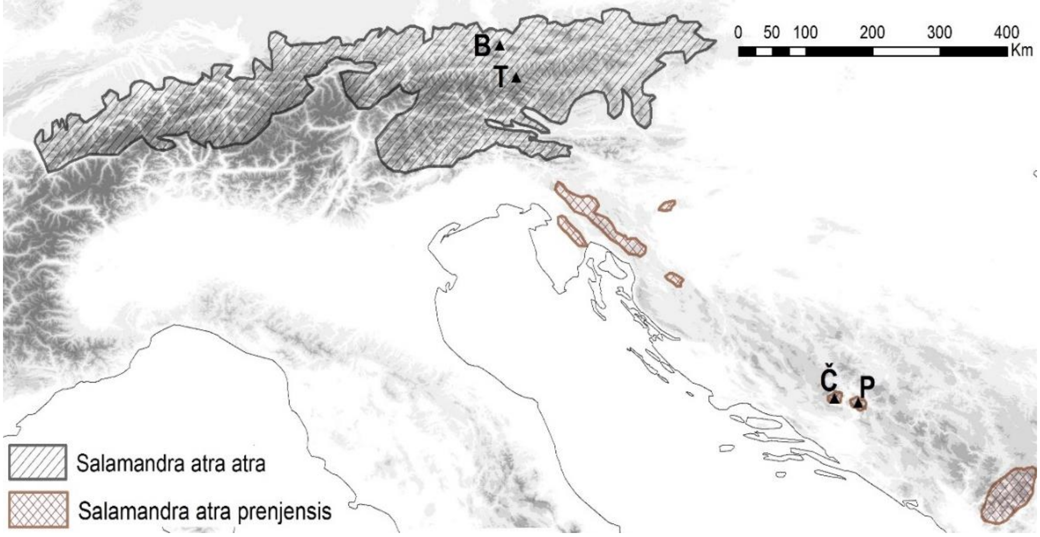

Figure 1. Continuous range of *S.atra atra* (Alps) and fragmented range of *S. atra prenjensis* (Dinarides) showing sampling locations (B – Bluntautal, T – Tappenkarsee, Č – Mt. Čvrsnica, P – Mt. Prenj). Occurrence areas from Andreone et al. (2009) are modified using the *gbif* database and the following references: Džukić, Kalezić, Krizmanić 2003, Jelić et al. 2012, Jeran, Đurić, Žganec, 2011, Kalezić et al. 2000, Krizmanić 1997, Razpet et al. 2016, Šunje and Lelo 2010.

Morphometric measurements

Twelve morphometric characters were measured for each specimen using a caliper of 0.01 mm accuracy (table 1, and Figure S3.1). Two additional traits were noted: Body mass (bm) was determined on a digital scale of 0.01 g precision, and number of costal grooves (cg) was estimated by counting the number of unpaired glandular knobs between the fore and hind limbs that also touched limbs posterior and anterior margins. As a standardization procedure, measurements were only taken from the right body side of the animals. To prevent potential transfer of amphibian pathogens, after measuring each B&H individual, we thoroughly washed our hands' with a fungicide (SEMIDERM®) and rinsed with water. Austrian individuals were handled with gloves, changed between each individual. Only sexually mature individuals were included in our analyses. Sex was determined by examining the shape of the cloaca (Luiselli et al. 2001). Individuals from the Austrian population were involved in other studies concerning chemical ecology and ethology. To prevent the possibility that the sedation of animals might have altered the outcome of these studies, we did not use any sedative prior to taking the measurements. To keep handling conditions as similar as possible between the two regions, sedation was neither applied in B&H individuals.

Statistical analyses

All statistical analyses were performed in R studio 1.0 (R studio team, 2016).

Preliminary analysis

As measurements were taken by distinct researchers (four in B&H, and two in Austria - one of which measured 49% of B&H samples – table S3.1), we evaluated the potential effect of the researcher on the results, by calculating *inter-researcher* repeatability between the two persons that measured the Austrian individuals, and also by assessing the *intra-researcher* repeatability to ensure that the variation within researchers was of the same magnitude as their between variation. Six individuals were measured by each researcher on all traits (except *body mass*); one of them measured once more all the traits of five of these individuals, whereas the other measured twice all traits for six new individuals. Inter- and intra-repeatability for each trait was assessed using variances of repeated measurements defined as random effects in linear mixed models; variances (residual variance and variances between: samples, researchers and samples measured by same researchers) were used to calculate the inter and intra class correlation coefficient (R or ICC) which indicates the repeatability between and within researchers: the higher the value, the more reliable are the measurements (Nakagawa and Schielzeth, 2010). All researchers within B&H were strictly monitored while taking the measurements by the common researcher that took measurements in both populations; a PCA analysis did not reveal any researcher effect on the measurements (Fig.S3.2).

We used the Shapiro-Wilk test to check whether the variables considered were normally distributed. All traits were normally distributed except number of costal grooves (*cg*), which followed a Poisson distribution.

Differences between populations and sexes

Following Berner's (2011) recommendation, the independent size metric size correction (ISMSC) method was used to standardize traits for size. Each trait (cg excluded) was divided by its overall mean to decouple variances from measurement scale and means ("*new trait*"). Differences were tested using generalized linear mixed models (GLMM), known to be more flexible for unbalanced designs (Logan, 2010). To test whether populations and sexes differ in size free morphological traits (relative to snout-to-vent-length – svl), each "*new trait*" was tested using a full model including *population* and *sex* (and their interaction) as fixed effects, *subpopulation* nested within *population* as random effect and *svl* as a covariate (except when testing differences in *svl/mean*). REML (Residual maximum likelihood) was specified to get unbiased estimates of the variances and a more precise estimation of degrees of freedom. Non-significant predictors were removed in a stepwise mode.

To test for differences based on a more general morphological feature such as size, we performed a Principal Component Analysis (PCA) on the raw traits (original traits – not transformed) matrix (*cg* excluded). The first component (PC1) is treated as size (Berner, 2011). When testing for differences, *population* and *sex* (and their interaction) were set up as fixed effects, and *subpopulation* nested within *population* as random effect (specifying REML).

The function „glmer“ (package lme4, Bates et al., 2015), was used for testing differences in the number of costal grooves (specifying Poisson distributed data) using the same model described for size (*svl* was not included as a covariate because it did not correlate with cg, r=0.06, P=0.33).

Correlation patterns among traits within populations

To explore the genetic architecture of multivariate phenotypes we assessed the correlations among traits independently within the two populations (see Berner, 2011). For each population, each trait (cg and *svl* excluded) was divided with its respective mean and then regressed against the respective *svl*. The residuals of each regression were considered *size-corrected traits* that were used to generate the correlation matrices. As *svl* was used to correct traits, we excluded it from the matrix. In these matrices the log number of costal grooves was included (log10 values of cg approached to normal distribution). Correlation values and their significance were assessed using the Bonferroni correction for multiple tests, in the R package psych (Revelle, 2017). To test if the correlation pattern among traits differs significantly in the two populations, the two correlation matrices were tested using the Steiger test (Steiger, 1980 - package psych).

Multivariate analysis

For the multivariate analysis (cluster and Mantel test) an *overall size corrected trait matrix* was prepared. This time, all traits (*cg* and *svl* excluded) were divided with the overall mean for that trait and regressed against *svl*. The residuals of the regressions were considered *overall size-corrected traits*. These traits were combined with log10 of *cg* (closer to normal distribution) and used to run these tests. As *svl* was used to correct traits, we excluded it from the matrix. To be able to conduct the canonical discriminating analysis (CDA), clustering and Mantel test we had to remove 20 individuals from these analysis because they had one or more missing data. All excluded individuals originated from B&H.

Detecting discriminating traits and the number of morphological groups

To assess if the matrix of traits differs between the two populations and to access the main discriminating traits between them, we conducted a canonical discriminating analysis using raw traits. The matrix of raw traits was tested between populations using a type II manova test (Pillai test statistic; total length was excluded due to multicolinearity).

To identify the number of distinct morphological groups we performed a clustering analysis on the *overall size-corrected traits* matrix (cantered and scaled) using the PAM algorithm (Partioning Around Medoids: R package: fpc - Hennig, 2015) that also reports the average silhouette width (sw) which is a measure of the strength of the clusters structure (Rousseeuw, 1987).

Assessing the relationship between morphological and geographical distance

We computed a matrix of geographical distances among all four subpopulations (using geographical coordinates) and a matrix of morphologic distances among them (using the *overall size-corrected traits*). The correlation between these two matrices of distances was assessed with a Mantel test (9999 permutations).

Results

Preliminary analyses

Intra-repeatability ICC values were very high (≥ 0.84 – Table S3.2) for all observed traits, and inter-repeatability values were in the same range, except for cg (ICC=0.7) and clo (ICC=0.5). These results suggest that morphological analyses presented hereafter are not compromised by a researcher effect.

Differences between populations and sexes

The GLMM showed that the populations differed on eight (57%) size free morphology traits (relative to *svl*: from now on *trait*$_{rel}$ - table 1). The limbs (*front*$_{rel}$ *and hind*$_{rel}$), and distance between them (*distance-between-limbs*$_{rel}$) *head-length*$_{rel}$, *body-mass*$_{rel}$ and *svl/mean* did not differ between the populations (table 1). Except *distance-between-limbs*$_{rel}$, all other relative traits that differed between sexes (*tail*$_{rel}$, *cloaca*$_{rel}$, *total length*$_{rel}$, *front-limb*$_{rel}$, *hind-limb*$_{rel}$, *hand*$_{rel}$, *foot*$_{rel}$) had higher values in males; neither population exhibited sexual shape dimorphism in *head*$_{rel}$ traits or *jaw*$_{rel}$ length, nor *svl/mean* (table 1). The only trait that showed an interaction between population and sex was *body-mass*$_{rel}$ (F=4.84, P=0.03); separate general linear models on each population subset revealed sexual dimorphism in *body-mass*$_{rel}$ in Austria (F=16.13, df = 1, P<0.001), not in B&H (F=3.1, df = 1, P=0.08).

The first PCA component (PC1 - size), explained 82.6% of the variation (eigenvalue=6.7). Neither populations (F=0.73, p=0.47), nor sexes (F=1.56, p=0.21) differed in size.

Populations differed in the number of costal grooves (Chi=5.7, P=0.02), but no differences between sexes were detected (Chi sq=0.19, P=0.66). The number of individuals within each subpopulation that has the respective number of costal grooves in presented in Table S3.3.

None of the tested interactions (pop:sex) in the models (for size and cg) were significant.

Correlation patterns among traits within populations

The correlation pattern among traits showed to significantly differ between the two populations (Chi sq=276.06, P<0.000). In B&H, a higher correlation among traits was registered (table 2). The *head-width*$_{rel}$, *jaw*$_{rel}$, *tail-length*$_{rel}$, *cloaca*$_{rel}$, appeared to be significantly correlated among other traits in B&H pop, whereas they show no significant correlation among traits in the Austrian pop. The *distance-between-limbs*$_{rel}$ and the *number of costal grooves* are independent from other traits in both populations (except in Austria where *distance-between-limbs*$_{rel}$ shows a significant correlation with *body-mass*$_{rel}$). Extremity traits (*hind*$_{rel}$ and *front-limbs*$_{rel}$, *hand*$_{rel}$, *foot*$_{rel}$) showed to be auto-correlated in both populations (except between *front-limb*$_{rel}$ and *hand*$_{rel}$ in Austrian pop).

Table 1. Differences between populations and sexes on size-free morphology traits (for the trait svl, differences refer to svl/mean - see text for details). The mean (m; median for cg) and range are given for absolute traits. ab.- abbreviation, nb. – number, dist. – distance. P values: 0.000 -***, 0.001 -**, 0.01 -*.

Morphological trait	**ab.**	♂	**Austria (N = 31)**	♀	**Austria (N = 31)**	♂	**B&H (N = 87)**	♀	**B&H (N = 96)**	**P pop**	**P sex**	**P pop*sex**
		m	range	m	range	m	range	m	range			
Body mass (g)	bm	6.54	4.56 – 8.24	7.50	4.3 – 10.9	7.47	3.10 – 12.00	7.11	3.00 – 12.50	0.82	0.11	*
total length (cm)	tot	11.53	10.6 – 12.8	11.46	10.4 – 13.1	11.83	8.73 - 14.45	11.57	8.58 – 13.72	*	*	0.65
snout to vent length (cm)	svl	6.84	6.25 – 7.37	6.89	6.23 – 7.96	6.78	5.00 – 8.06	6.66	5.08 – 8.12	0.34	0.98	0.64
head width (cm)	hw	1.13	0.99 – 1.30	1.17	0.99 – 1.30	1.30	0.88 – 1.53	1.29	1.02 – 1.60	**	0.68	0.21
tail length (cm)	ta	4.69	4.15 – 5.62	4.57	3.92 – 5.35	5.06	3.64 – 6.57	4.91	3.50 – 6.32	*	*	0.65
cloaca length (cm)	clo	0.63	0.44 – 0.85	0.53	0.37 – 0.81	0.71	0.49 – 1.05	0.66	0.42 - 0.96	***	***	0.11
head length (cm)	hl	1.36	1.26 – 1.54	1.35	1.18 – 1.51	1.42	1.07 – 1.89	1.41	1.13 – 1.72	0.07	0.71	0.48
jaw length (cm)	jw	1.08	0.98 – 1.18	1.07	0.84 – 1.23	1.22	0.95 – 1.41	1.20	0.93 – 1.52	**	0.37	0.96
front limb length (cm)	fll	1.21	1.01 – 1.45	1.10	0.91 – 1.26	1.29	0.94 – 1.66	1.21	0.88 – 1.55	0.06	***	0.52
hind limb length (cm)	hll	1.30	1.09 - 1.56	1.21	0.75 – 1.44	1.40	1.09 – 1.81	1.35	0.95 – 2.08	0.14	***	0.26
hand length (cm)	hnd	0.79	0.62 – 0.94	0.74	0.56 – 0.89	0.86	0.54 – 1.08	0.79	0.52 – 1.33	***	***	0.84
foot length (cm)	ft	0.95	0.76 – 1.18	0.86	0.65 – 0.97	1.07	0.78 – 1.78	0.98	0.66 – 1.28	*	***	0.84
nb. costal grooves	cg	12	10 - 15	12	10 - 14	11	9 - 14	11	9 - 14	*	0.19	0.20
dist. between limbs (cm)	dbl	3.46	2.98– 3.99	3.66	3.14 – 4.24	3.51	2.32 – 4.35	3.54	2.45 – 4.75	0.81	**	0.27

Table 2. Correlation values (below the diagonal) and their significance (above the diagonal) between size-free morphology trait pairs for B&H population (upper values) and Austrian population (lower values). 0.001 -**, 0.01-*, (abbreviation of traits correspond to table 1).

	hl	hw	jw	bm	tot	ta	clo	dbl	fll	hll	hnd	ft	cg
hl		*	*	1.00	0.17	0.17	1.00	1.00	0.59	**	**	1.00	1.00
		1.00	1.00	1.00	0.26	0.26	1.00	1.00	1.00	1.00	1.00	0.32	1.00
hw	0.28		**	**	**	**	*	1.00	**	**	*	**	1.00
	0.11		0.78	**	1.00	1.00	1.00	0.10	0.10	1.00	1.00	1.00	1.00
jw	0.30	0.40		1.00	**	**	**	0.45	**	*	*	**	1.00
	0.22	0.32		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
bm	0.14	0.43	0.32		**	**	0.09	1.00	0.47	1.00	0.17	*	1.00
	-0.13	0.59	0.13		1.00	1.00	1.00	**	1.00	1.00	1.00	0.83	1.00
tot	0.24	0.38	0.47	0.37		**	**	1.00	**	**	**	**	1.00
	0.37	-0.08	0.09	0.02		**	1.00	1.00	1.00	1.00	0.07	0.06	1.00
ta	0.24	0.38	0.47	0.37	1.00		**	1.00	**	**	**	**	1.00
	0.37	-0.08	0.09	0.02	1.00		1.00	1.00	1.00	1.00	0.07	0.06	1.00
clo	0.02	0.27	0.36	0.25	0.41	0.41		1.00	**	1.00	1.00	*	1.00
	0.29	0.24	0.15	-0.19	0.12	0.12		1.00	1.00	***	**	1.00	1.00
dbl	0.19	0.06	0.21	0.06	0.05	0.05	0.02		0.81	1.00	1.00	1.00	1.00
	-0.08	0.39	0.00	0.58	-0.13	-0.13	-0.28		0.66	1.00	**	1.00	1.00
fll	0.21	0.33	0.37	0.21	0.42	0.42	0.32	0.20		**	**	**	1.00
	0.14	-0.40	0.05	-0.26	0.23	0.23	0.01	-0.33		**	0.14	**	1.00
hll	0.44	0.32	0.28	0.14	0.32	0.32	0.24	0.25	0.52		**	**	1.00
	0.09	-0.26	0.09	-0.05	0.18	0.18	0.10	-0.26	0.56		*	*	1.00
hnd	0.31	0.30	0.30	0.24	0.45	0.45	0.23	0.10	0.42	0.31		**	1.00
	0.15	-0.26	0.02	-0.05	0.41	0.41	-0.08	-0.13	0.39	0.46		**	1.00
ft	0.16	0.32	0.32	0.29	0.31	0.31	0.28	0.06	0.31	0.33	0.39		1.00
	0.36	-0.26	0.05	-0.32	0.42	0.42	0.17	-0.18	0.53	0.44	0.65		1.00
cg	-0.13	-0.06	0.00	0.01	-0.10	-0.10	0.07	-0.08	-0.14	-0.09	-0.15	0.00	
	-0.13	-0.09	0.03	0.01	-0.03	-0.03	0.06	0.05	0.04	0.22	0.10	-0.10	

Detecting discriminating traits and the number of morphological groups

The type II manova test revealed that the matrix of raw traits significantly differs between the two populations (F=20.7, Df=13, P<0.001; fig. 2A). The vectors length of the CDA revealed that the main discriminating traits between the two populations are the head and jaw width and the number of costal grooves (fig. 2B). Austrian individuals have more costal grooves compared to B&H individuals, whereas B&H individuals have wider heads and jaws compared to Austrian ones.

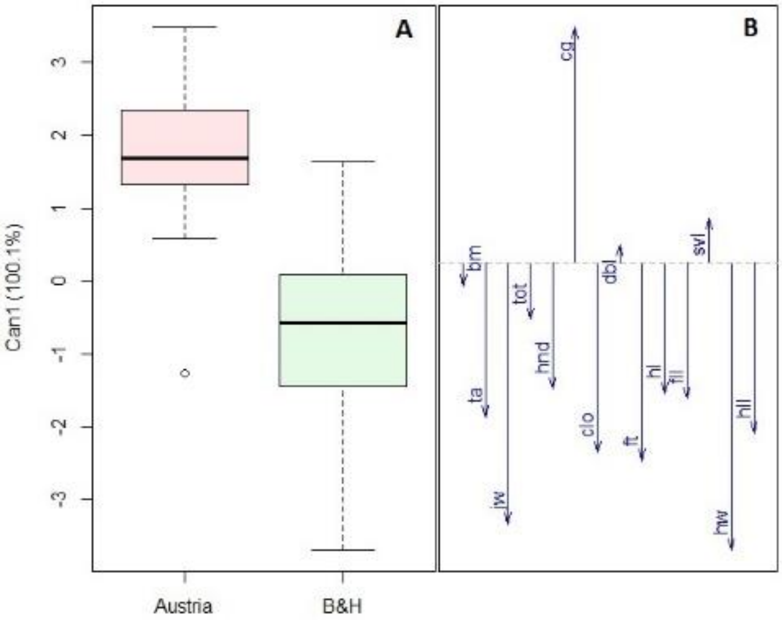

Figure 2. Canonical Discriminant analyses (CDA): A - Austrian and B&H individuals differ significantly from each other (type II manova test - see text for details); B - Austrians individuals have a higher number of costal grooves (cg) whereas B&H individuals have wider heads (hw) and jaws (jw). Besides a higher number of cg, Austrian individuals are slightly bigger in standard length (svl) and distance between legs (dbl) when compared to B&H samples. For full names of traits, see table 1.

The clustering analysis revealed two clusters of an average silhouette width (sw) of 0.22, indicating an overall weak data structuring (Rousseeuw, 1987). Cluster 1 contained 95% of Austrian and 32% of B&H individuals (sw = 0.29); cluster 2 contained 68% of B&H and 5% of Austrian individuals (sw=0.15; fig. 3). The two clustering components (equal to PCA components - PC1 and PC2) explained 51.58% of point variability.

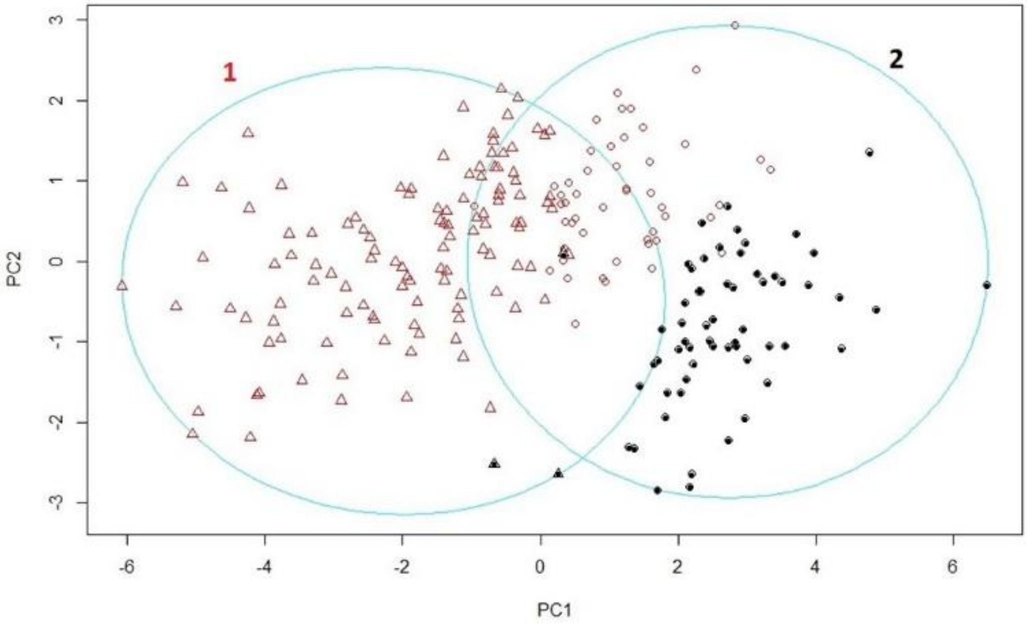

Figure 3. The populations grouped in two distinct clusters: Cluster 1 (triangles) contains 68% of B&H and 5% of Austrian individuals. Cluster 2 (circles) encloses 95% of Austrian and 32% of B&H individuals. Red and empty shapes are samples from B&H; black and filled shapes are Austrian. The ellipses represent the 95% confidence interval.

Assessing the relationship between morphological and geographical distance

The Mantel test revealed that geographical distance explained variation in morphological distance (r=0.14, P=0.0004).

Discussion

Analysis of individual traits showed that the most pronounced differences between the two populations are in the *hand*$_{rel}$, *head-width*$_{rel}$ and *jaw*$_{rel}$ length (table 1, due to lower ICC, the significance of the *cloaca*$_{rel}$ is not interpreted)[5]. A wider head with longer jaws is registered in B&H individuals, thus the wider vomero-palathal teeth described in Dinaric specimens (when compared to Alpine once, see Mikšić 1969 and Krizmanić 1997), could be a fallout of this observation. The reported differences in the vomero-palathal teeth probably reflect ecological differences in habitat and food availability as it is showed that in some fish (e.g. see Kumari et al., 2009, Yasphal et al., 2009) and amphibian species (Smith and Skulason, 1996) several mouth structures show significant adaptive modifications associated with the food and feeding ecology. The analysis of vomero-palatal teeth was not conducted in this study because it requires an invasive method of quantification.

S*ize* did not differ between the two populations nor sexes, suggesting that in their environments, similar evolutionary forces act on this trait. Size changes would influence the area/volume body ratio which would further affect main physiological functions such as the heat and water loss along with metabolic rate (Ivanović and Kalezić 2009). This might explain the lack of registered differences in size. A male biased sexual shape dimorphism was detected mainly on the extremity traits (relative to *svl*) which are particularly important in male combats, reproductive phases (e.g, ventral amplexus), and migration events that are characteristic to males (Helfer 2012).

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[5] Salamanders from the two populations were measured during different seasons (Austrian individuals were measured at the end of the reproductive period while Bosnian individuals were measured throughout it). As the reproductive period may influence the morphology of the cloaca in salamanders (e.g. see Norris et al, 1985), the detected differences in the size of the cloaca should be carefully interpreted by the reader due to the probable effect of reproductive season on cloaca size.

Austrian individuals tend to have more costal grooves compared to B&H pop (table 1, fig 2B). This trait can be used as a proxy to determinate the highly variable number of vertebrae in salamanders and newts (Litvinchuk and Borkin 2003). The number of vertebra is habitat dependent and the elongation of the trunk could be related to an increased importance to fossoriality (burrowing, Arntzen et al., 2015). It is noted that amphibians that use their head to burrow (such as *S. atra* - pers. obs.), have longer trunks since the digging in amphibian and reptile species is powered by the force of the axial skeleton (Kley and Kearney, 2008). The Dinaric habitat is characterized by lots of crevices, holes and passages in the limestone rocks that serve as shelters for the salamanders.

In contrary, the forested Alpine environment is less abundant of these structures and in such habitat, salamanders might increase burrowing to provide dwelling. Interestingly, a considerable number of individuals in the Austrian forests were found under stones and tree logs, whereas B&H individuals were rarely spotted under such.

Correlation among population level traits can arise by drift and can have an important effect on the evolution of populations and the divergence of lineages (Goodnight, 1989). Lande (1983) states that the evolution of population means is constrained by the correlation among traits. Indeed, our results show that the correlation pattern between the two populations differs mostly among traits which mean differs between the populations ($head\ width_{rel}$, jaw_{rel}, $tail_{rel}$, $cloaca_{rel}$, $hand_{rel}$ and $foot_{rel}$, table 2). Correlation patterns must be taken into account when studying the consequences of selection acting at the population level. Assuming selection acts on one trait, the mean of a second trait that is correlated with the trait under selection, may also change as a result of genetic correlations, leading to stronger differentiation. The populations are different evolutionary lineages (Helfer 2010, Bonato et al., 2008), thus their evolutionary potential also differs. In this context, different genetic interactions within populations can provide a pleiotropic effect of genes that can affect different traits at the population level. The Mantel test proved geographical structuring of morphological variation that is lineage specific. Ecological interactions that are specific to each environment (geographical dependent), additionally showed to be heritable at the group level, thus reinforcing the genetic differentiation of populations (Goodnight 1989). The different correlation patterns among salamanders' phenotype as well as the Mantel test, suggests that the observed differentiation is caused by both: a different genetic architecture of the populations and a different environment, thus leaving much space for evolutionary forces to act and shape these populations in different directions. Clustering and CDA also detected morphological differentiation and main traits that discriminate the populations; as in the analysis of individual traits, head width and jaw length together with the number of costal grooves appeared to be the most important traits diversifying the two lineages. The clustering showed that Austrian individuals separate well in the multivariate space from B&H individuals even in the cluster 2 which contains specimens from both populations (fig. 3).

Nonetheless, can we really claim that the registered differences are big enough to classify *S.a prenjensis* as a separate subspecies?

Zachos (2006) points out a distinction between the subspecies *taxa* and subspecies *category*. If subspecies represent evolutionarily distinct portions of a species, they are considered to be *subspecies taxa*, whereas the classification of a number of these portions at the same level would represent a *subspecies category*. Following this definition, *S. a. prenjensis* should be considered as a subspecies *taxa* since its populations represent real biological entities with distinct evolutionary history (Helfer 2010, Bonato et al. 2008). The morphological differentiation revealed in this study provides an additional argument for such classification.

Additional analysis

Although the reality of a *subspecies category* is doubtful (Zachos, 2006), the „75% rule" can be used for delimitations in this context (Amadon 1949). The rule states that 75% of individuals of group "A" must lie outside the 99% of the range of variation of group "B", for the trait(s) under consideration. Our results did not support the 75% rule (as 68% of B&H individuals were included in a separate cluster) thus more data must be collected to valorise the ssp. *prenjensis* as a *subspecies category* using this criteria. These data must include more characters that were not taken in consideration in this study (e.g the size and shape of vomero-palatal teeth) and morphometric measurements from other Dinaric and Alpine populations together with measurements from *S. a. aurorae* and *S. a. pasubiensis*.

To avoid invasive methods for the quantification of the size and shape of vomero-palatal teeth, the examination of the holotype and paratypes would also be required (ICZN, 1999). The subspecies *Salamandra atra atra* was described by Laurenti (1768) were the holotype is a figure representation. As stated in Bonato et al. (2018) the *locus typicus* of the holotype is not clear since both the upper valley of the Adige (Alto Adige/Südtirol) and the Loibl Pass are mentioned. Mertens and Müller (1928) intentionally considered the Loibl Pass as the single type locality of *S. atra* but without designating a neotype which is incorrect based on ICZN rules (ICZN, 1999). Aditionally, recent genetic analyses showed that the Loibl Pass corresponds to the contact zone between the Dinaric and the northern Alps lineages (two out of 12 analysed individuals had a Dinaric haplotype; Helfer, unpublished data).

The subspecies *S. atra prenjensis* was described by Mikšić (1969) who designated the holotype as one female individual from Prenj Mountain (*locus typicus*: Hercegovina: Prenj - area between Otiš and Vjetrena brda summit at around 1800 m asl). Mikšić did not define the museum collection ID number of this individual but mentioned that it was deposited at the National Museum of B&H with 21 additional individuals from Prenj (Otiš – Vjetrena brda). We reviewed the herpetological collection of the National Museum of B&H in Sarajevo and did not manage to find the holotype or any sample from the original series described by Mikšić (1969) which has probably been lost during the partial destructions of the museum building during the fall of Yugoslavia (1991-1999).

The above presented issues stress the need for the designation of both, *S. atra atra* and *S. atra prenjensis* neotypes.

Final conclusion

The hereby observed differences between the two populations cannot be neglected considering the conservative morphology of salamanders (Wells 2007). In regard to traits that did not show differences between the populations, it is noted that different lineages may accrue over time different *phenogenetic* mechanism(s) for such traits, as a response to a strong natural selection (Weiss 2007). The distinct evolutionary potential of the Alpine and Dinaric population must therefore be preserved, as it provides the key for species survival. The need for a separate conservation approach within *S. atra* population was also recognized in Bonato et al (2018). The first preservation step would be to assign appropriate conservation statuses to both populations. The Alpine salamanders are considered Least Concern (Andreone et al., 2009), but Dinaric populations are more vulnerable due to their peculiar and severely fragmented distribution (fig.1) restricted to lower latitudes and higher altitudes, where it is known that amphibian populations are less resilient (Morrison and Hero, 2003).

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Chapter 4

Patterns of variation in dietary composition among four populations of alpine salamanders (*Salamandra atra prenjensis*)

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Abstract. In this paper we studied the diet in four allopatric populations of alpine salamanders in the Dinarides (*Salamandra atra prenjensis*). Food consumption was assessed by stomach flushing while food availability by pitfall traps and netting. We aimed to: (i) assess the realized dietary niche, (ii) investigate prey preferences, (ii) explore individual specialization, clustering and nestedness. All populations have an equally wide dietary span that is among the largest reported for terrestrial salamanders. On the other hand, the amount of ingested prey is rather low compared to other salamander species; the quantity of consumed prey did not differ among populations but younger individuals fed more than adults. Food composition somewhat differed among populations but not among sex/age classes. In all four populations, the bulk of diet consisted of beetles, spiders, snails and millipedes; except for beetles, such prey was also preffered together with centipedes and isopods. For most of the prey categories, the direction of the electivity indices was the same across populations. In none of the populations a nested pattern in the interindividual subdivision of dietary resources was registered. However, indications for individual specialization and modularity were observed disclosing that the broad niche of populations is composed of smaller individual niches that cluster along the dietary axis. Overall, the four populations have very similar structural characteristics of the dietary niche and there is little evidence for local dietary differentiation probably due to the absence of drivers for change.

Introduction

In this time of unprecedented biodiversity decline and fragmentation of natural habitats, studies of whether and how isolated populations survive and adapt are more timely than ever. Isolation, especially of smaller populations, is likely to decrease the viability of populations due to the effects of demographic and environmental stochasticity coupled with genetic deterioration (Soulé, 1987). But isolation could also facilitate plastic responses or adaptation to local conditions (DeWitt and Scheiner, 2004). At the level of the community, fragmentation may impinge on ecological interactions between species, e.g. disturbing and/or rebuilding relationships between plants and pollinator, hosts and parasites, and predator and prey species (Legrand et al., 2017).

Studies of anthropogenically fragmented ecosystems have revealed several clear-cut examples of ecological and surprisingly fast evolutionary changes in natural populations and communities (for a review see Cheptou et al., 2017), although the literature on the subject is somewhat biased in favour of plants and arthropod species. Effects on long-lived, slow-breeding species may have remained unnoticed due to the relatively small number of generations born since the unset of fragmentation. In such organisms, the long-term effects of isolation may be better understood by comparing populations that have become isolated through natural events, in more ancient times (Cheptou et al., 2017). Comparison of populations inhabiting distinct islands, mountain tops, or natural relict habitats, for instance, may allow observing the consequences of isolation on an evolutionary time scale.

One important element that can change in the wake of geographic isolation is a species' trophic biology. Low food availability in habitat patches may drive top predator species to extinction (Duffy, 2003; Henle et al., 2004; Ryser et al., 2019), with cascading effects on meso-predator populations (Prugh et al., 2009; Beasley et al., 2013), and lower trophic levels (Pace et al., 1999). Consumer species may adjust to isolation-induced changes in the composition of the predator, prey and competitor community by shifting prey use (Nour et al., 1989) and preferences (Briggs and Smith, 1989; Lillywhite, Pfaller and Sheehy, 2015), or by becoming more picky (Yassin et al., 2016) or more catholic (Van Valen, 1965; Munoz-Lazo et al., 2019), with ensuing effects on niche width and positioning.

In a seminal contribution to niche evolution theory, Van Valen (1965) suggested that wider dietary niches in island versus mainland bird populations are achieved by increased between-individual niche variation. The idea was supported by subsequent theoretical work (Roughgarden, 1972; Roughgarden, 1974), but empirical studies in the following two decades downplayed the role of individual specialisation (reviewed in Bolnick et al., 2003), and many later ecological studies have simply ignored intra-population differences in diet (Shine and Wall, 2007; Bolnick et al., 2011). However, Bolnick and colleagues (Bolnick et al., 2003) established that individual dietary specialisation is a wide-spread phenomenon with important ecological, evolutionary and conservation implications, thus prompting a renewed interest in the subject (e.g. Araújo, Layman and Bolnick, 2011). Accordingly, empirical studies comparing the diet at both inter- and intra-population levels are critical to improve our understanding on the trophic niche and its plasticity, especially in geographically isolated populations over a wide latitudinal range.

In this paper, we examine and compare the dietary biology of four isolated populations of alpine salamanders in the Dinarides (*Salamandra atra prenjensis* Mikšić, 1969). Specifically, we describe variation in food availability and consumption within populations and examine among-population variation in niche dimensions. We compare our findings to the recently published data for a *S. atra* population from the Italian Dolomites (Roner et al., 2020). We also explore the relative contribution of intra- and interindividual variation to niche width in the four populations.

Material and Methods

Study species

The alpine salamander (*Salamandra atra)* is a cold-adapted terrestrial amphibian that occurs throughout the Alpine arc (from France to Austria), but a number of isolated populations are known to exist further south, in the Dinaric Alps (from Slovenia to Albania, Šunje and Lelo, 2010). Although the historical phylogeography of the species is not fully resolved, the populations inhabiting the European Alps and Dinaric Alps are thought to have been isolated from one another since the end of alpine orogeny (Riberon et al. 2001) and constitute different subspecies, namely the nominal *S. a. atra* (European Alps) and *S. a. prenjensis* (Dinaric Alps) (Šunje et al., 2019; Šunje et al., 2021). *S. a. prenjensis* may have survived the last glacial period as a continuous population in the plains of the far south-east of the Dinaric region; after that, the warming of the climate has probably forced the *prenjensis* to migrate onto the isolated mountain islands on which they survive until today (Razpet et al., 2016; Šunje et al., 2021).

Field work - assessing diet and prey availability

We sampled four isolated populations of *S. a. prenjensis* over a period of four years (2014 – 2017; see Table S4.1 for details). Although all of them are situated within the Dinaric Alps, the study sites differ considerably in both abiotic and biotic characteristics as they are located within a 600km long distribution range (Fig. S4.1). The first site is situated at 1000m a.s.l. in the region of Gorski Kotar (Croatia), in a forest dominated by beech (*Fagus sylvatica*) and fir (*Abies alba*) with dense undergrowth of ferns. The two study sites in Bosnia and Herzegovina, mt. Prenj and mt. Čvrsnica, consist of rocky alpine grasslands, dappled with thickets of small bog pine (*Pinus mugo*) and are situated between 1700 and 1800m a.s.l. The fourth study site, located between 1750 and 2000m a.s.l. on mt. Prokletije (Bogićevica, Montenegro), is characterised by alpine grasslands and coniferous forests (mainly Norway spruce, *Picea abies*) on a rocky substrate. Prokletije is the only site that includes streams and human settlements (figure S3.1).

Salamanders were collected while active (mostly during the night or early in the morning) in their natural environment. In order to prevent multiple stomach flushing of the same individual during a single fieldwork occasion, we started with the stomach flushing procedure only after collecting all the individuals. Individuals smaller than 3 cm snout-vent length (*svl*) were not collected because they might be damaged by the procedure. Adults (*svl* >7cm) were sexed based on the morphology of the cloaca (Luiselli et al., 2001); most of the unsexed individuals were subadults (*svl*: 6 - 7cm*)* while individuals of *svl* ≤ 5cm were considered juveniles. Stomach flushing was performed within two hours after capturing all individuals, following procedures outlined in Andreone, De Michelis and Clima (1999) with the following modifications. One field assistant secured the animal, holding it while wearing paper gloves to gain grip on the slippery skin. The salamander's mouth was then opened by forcing the end of a dental excavator between the upper and lower jaws. The sharp end of the instrument was coated with rubber to prevent damaging the salamander's mouth parts. As soon as the mouth was opened, a second field assistant carefully intubated the animal's pharynx and stomach with a tube (25 mm length, 0.81 mm outer diameter) cut from an intravenous cannula. The tube's ending had been softened by shortly (<1 s) exposing it to a flame to prevent damaging the digestive tract while intubating. With the tube in place, a 2.5 ml water dose was injected into the stomach using a 5 ml syringe connected to the other side of the tube. This caused ejection of the stomach contents, which were collected on a plastic tray (30 x 15 cm). Prey remains were collected from the tray using forceps and stored in 1.5 ml plastic tubes containing 70% ethanol. Only after flushing all collected salamanders, they were returned to shelters (rock cracks, underneath stones, holes) within the capture location. Before releasing the salamanders, we sprayed them with water to eliminate any possible effects of dehydration caused by the handling procedure. Since same locations were visited several times (Table S4.1), we cannot exclude the possibility that some individuals were flushed more than once during multiple field work occasions; however, this would not significantly affect our observations.

Prey availability within the natural environment of the salamanders was assessed using pitfall traps and netting. Pitfall traps consisted of plastic containers of varied sizes (250-800 cm^3) that were buried into the substrate leaving the top edge levelled with the soil surface. A mesh was put over the top of the containers to prevent salamanders getting trapped, while allowing ground-dwelling invertebrates falling in. The containers were half-filled with ethylene-glycol, a viscous liquid that prevents escape and aids preservation of invertebrate catches. A sloped plastic top was placed above each pitfall to prevent flooding of the traps by rainfall. Pitfall traps were buried in and left in the salamanders' habitat for 21 days. At the end of this period, their contents were poured into plastic containers using a funnel.

The netting was done by forcibly swaying entomological nets (45 cm diameter) through the vegetation of the study sites. Arthropods and other invertebrates that were knocked of the plants were retrieved from the net and transferred to containers holding an ethylene-glycol solution. Information on the exact timing of sampling events, and their intensity, can be found in table S3.1. Back in the lab, prey taxa were identified and assigned to one of 25 major taxonomic classes (Table 1).

Statistical analyses

For each study site, and for the entire dataset, we calculated the Gini-Simpson diversity index (1-D; Jost, 2006), the Shannon-Weaver diversity index (H'; Shannon, 1948) and the Smith's index (FT; Smith, 1982), which measures niche breadth while taking resource availability into account. The calculation of these indices did not include the unknown prey (prey that we were not able to identify).

We used *t*-tests to compare the Shannon-Weaver diversity index between study sites and to compare arthropod communities caught by netting or in traps to those consumed by the salamanders (due to multiple group comparisons, the *P* values where corrected with the Bonferroni correction (see chapter 9 in Gardener, 2014).

To test whether salamanders consumed prey classes in proportion to their availability in the habitat (as estimated by the pitfall trapping and netting), we calculated Ivlev's electivity indexes (Ivlev, 1961) and their approximate 95% confidence intervals (Strauss, 1979). Confidence intervals that considerably crossed the zero value or were outside the range of -1 to 2 were omitted.

The number of prey items retrieved per stomach was compared among study sites and sex/age classes (male adult, female adult, juvenile, subadult), using generalized linear models (GLzM) and assuming the dependent variable having a Poisson loglinear distribution (no evidence of over-dispersion was found in the dataset).

To compare diet composition among populations, sex and age classes, we used analysis of similarity (ANOSIM) based on the Bray-Curtis distance (Clarke, 1993) and indicator species analysis (De Cáceres, Legendre and Moretti, 2010) to identify which main prey taxa was associated to the diets of these groups. Jaccard's dissimilarity coefficient (Jaccard, 1901) was calculated to compare the composition of potential and realized prey communities among sites. These analyses were run with the 'vegan' package (Oksanen et al., 2019) and the 'indicspecies' package (De Cáceres and Legendre, 2009) in R (ver. 3.6.2, 2020) using the R studio interface (R Core and RStudio Team).

Indices of individual specialisation were calculated using the 'RInSp' package (Zaccarelli, Bolnick and Mancinelli, 2013). In particular, we calculated Araújo's

E-index, which takes a value of zero in absence of inter-individual niche variation and tends towards one as differences among individuals within a population increase (Araújo, 2008). The observed index (E_{obs}) was compared to 10 000 E-values (E_{null}), obtained by Monte Carlo resampling the data. Inter-individual niche variation was considered significant if the proportion of Monte Carlo simulated E-values greater than E_{obs} was <0.05 (Araújo, Layman and Bolnick 2011). We also calculated the IS index (under 10.000 replicates), a measure of individual specialization given as the average pairwise overlap between all individuals in a population (Bolnick et al., 2003).

Within each population, we also checked for nestedness and modularity. In dietary analyses, nestedness occurs when 'specialist' individuals eat a subset of items in the diet of 'generalist' individuals. It is indexed here by NODF (nestedness metric based on overlap and decreasing fill, Almeida-Neto et al., 2008). Larger values of NODF indicate stronger nestedness while smaller values suggest clustering. The significance of NODF was assessed using the 'falcon' package (Beckett, Boulton and Williams, 2014). The degree of modularity was assessed for each of the four populations by calculating C_{ws}, the weighted clustering index (Barrat et al., 2004; Araújo et al. 2008). Diet modularity in a population increases if distinct subsets of individuals specialize on particular prey items.

Results

Pooled data

Of all collected individuals, about half of them did not return any prey after stomach flushing, but we obtained at least one prey item from a total of 264 salamanders. We retrieved a total of 827 prey items from the stomachs, belonging to 26 prey categories (including "unknown" - prey items that could not be identified). Across populations, Coleoptera (*n* = 121; relative abundance = 14.6%), Araneae (81; 9.8%), Gastropoda (71; 8.6%), Diplopoda (67, 8.1%) and Lepidoptera (49; 7.1%) were the most frequently ingested prey taxa (fig. 1 and table 1.); most Lepidoptera were larvae. The diversity of prey items in the diet was high (Shannon-Weaver index H'=2.74; Gini-Simpson-index 1-D=0.92). Across populations, Smith's FT reached the maximum value of 1.

Among the 1812 invertebrates caught in our pitfalls, Coleoptera (22.3% of all items), Diptera (15.8%), Hymenoptera (15.7%), Orthoptera (10.6%) and Araneae (7.9%) were the most common (table 2, fig. 1). The diversity of the potential prey community (H' ± SD = 2.36 ± 0.022; 1-D=0.87 ± 0.003) was below that of the consumed prey community (H' ± SD = 2.73 ± 0.028; 1-D=0.92 ± 0.003; $t_{1.25}$=10.2, *P*=0.04).

Lumping diet and prey availability data across populations, *S. a. prenjensis* ingested Araneae, Chilopoda, Diplopoda, Gastropoda and Isopoda more frequently than could be expected from the presence of these taxa in the environment (Ivlev index, fig. 2). In contrast, Acarina, Diptera, Hymenoptera and Orthoptera were consumed less than proportionate to their abundance (fig. 2).

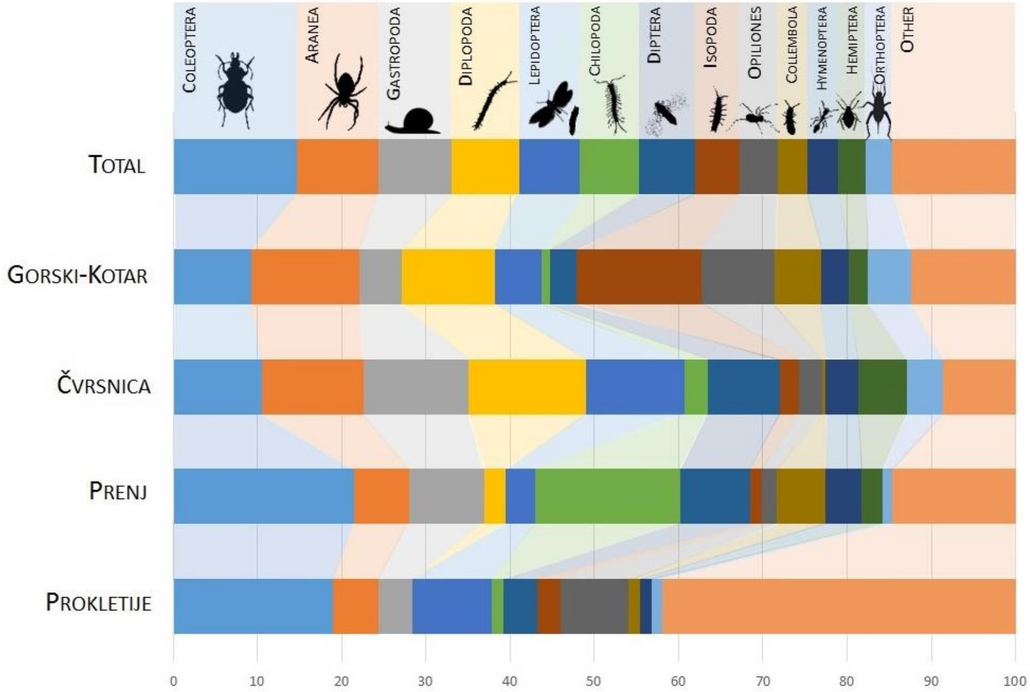

Figure 1. Taxonomic diversity of prey taxa ingested by *Salamandra atra prenjensis*. The graph shows the relative contribution of the 13 invertebrate taxa most frequently observed in stomach flushes, for the four populations studied separately and combined. The 'other' category includes prey items that could not be identified and rare prey (relative contribution per population is ≤ 2.2% or prey items are found only in one population).

Table 1. Taxonomic composition of the diet of *Salamandra atra prenjensis* in our four populations and combined (Total). Shown are the Number of prey items of different taxa retrieved in stomach flushes and the number of individual salamanders that had ingested at least one specimen of the taxon (between brackets). The unknown category refers to prey items that could not be determined.

	Gorski Kotar	Čvrsnica	Prenj	Prokletije	Total
Acarina	2 (2)	3 (3)	6 (2)	1 (1)	12 (8)
Araneae	28 (22)	31 (22)	18 (17)	4 (4)	81 (65)
Archaeognatha	3 (3)	0 (0)	1 (1)	0 (0)	4 (4)
Blattodea	1 (1)	1 (1)	0 (0)	0 (0)	2 (2)
Chilopoda	2 (1)	7 (4)	48 (28)	1 (1)	58 (34)
Collembola	12 (11)	1 (1)	16 (6)	1 (1)	30 (19)
Coleoptera	20 (14)	27 (19)	60 (26)	14 (7)	121 (66)
Dermaptera	3 (3)	0 (0)	0 (0)	0 (0)	3 (3)
Diplopoda	24 (21)	36 (17)	7 (7)	0 (0)	67 (45)
Diptera	7 (7)	22 (17)	23 (17)	3 (3)	55 (44)
Gastropoda	11 (7)	32 (20)	25 (16)	3 (3)	71 (46)
Hemiptera[6]	5 (5)	15 (9)	7 (7)	0 (0)	27 (21)
Heteroptera	0 (0)	0 (0)	0 (0)	1 (1)	1 (1)
Homoptera	2 (2)	3 (2)	1 (1)	1 (1)	7 (6)
Hymenoptera	7 (7)	10 (9)	12 (8)	1 (1)	30 (25)
Isopoda	32 (23)	6 (6)	4 (4)	2 (2)	44 (35)
Lepidoptera	12 (11)	30 (23)	10 (10)	7 (5)	59 (49)
Mecoptera	0 (0)	2 (2)	0 (0)	0 (0)	2 (2)
Myriapoda[7]	10 (10)	0 (0)	0 (0)	0 (0)	10 (10)
Neuroptera	0 (0)	0 (0)	3 (2)	0 (0)	3 (2)
Oligochaeta	4 (4)	1 (1)	1 (1)	1 (1)	7 (7)
Opiliones	19 (14)	7 (7)	5 (5)	6 (6)	37 (32)
Orthoptera	11 (10)	11 (10)	3 (3)	1 (1)	26 (24)
Pseudoscorpionida	0 (0)	0 (0)	4 (4)	0 (0)	4 (4)
Thysanura	0 (0)	0 (0)	8 (4)	0 (0)	8 (4)
Unknown	2 (2)	12 (8)	17 (12)	27 (12)	58 (34)

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[6] The category Hemiptera refers to members of the order Hemiptera other than the aphids, scale insects, cicadas and leafhoppers (Homoptera) or the true bugs (Heteroptera).

[7] The category Myriapoda refers to members of the subphylum Myriapoda, other than centipedes (Chilopoda) and millipedes (Diplopoda).

Table 2. Prey availability in the four populations and combined (Total), as estimated by the number of prey of respective taxa caught in pitfall traps or in sweep nets. Most frequent prey is bolded.

	Gorski Kotar	**Čvrsnica**	**Prenj**	**Prokletije**	**Total**
Acarina	0	26	7	50	83
Aranea	**24**	**70**	**34**	**15**	**143**
Archaeognatha	4	2	0	0	6
Blattodea	0	0	1	0	1
Chilopoda	1	2	0	1	4
Coleoptera	**190**	**113**	**69**	**32**	**404**
Collembola	85	1	5	0	91
Dermaptera	3	1	0	0	4
Diplopoda	18	10	2	1	31
Diptera	**48**	**116**	**73**	**49**	**286**
Gastropoda	11	1	0	0	12
Hemiptera	0	7	28	0	35
Heteroptera	0	45	28	0	73
Homoptera	0	7	0	0	7
Hymenoptera	**4**	**143**	**103**	**34**	**284**
Isopoda	30	1	1	3	35
Lepidoptera	0	9	36	0	45
Mecoptera	0	0	0	0	0
Myriapoda	16	0	0	1	17
Neuroptera	0	0	0	0	0
Oligochaeta	0	0	0	0	0
Opiliones	26	21	2	3	52
Orthoptera	**119**	**59**	**14**	**0**	**192**
Pseudoscorpionida	0	2	0	2	4
Thysanura	0	0	3	0	3

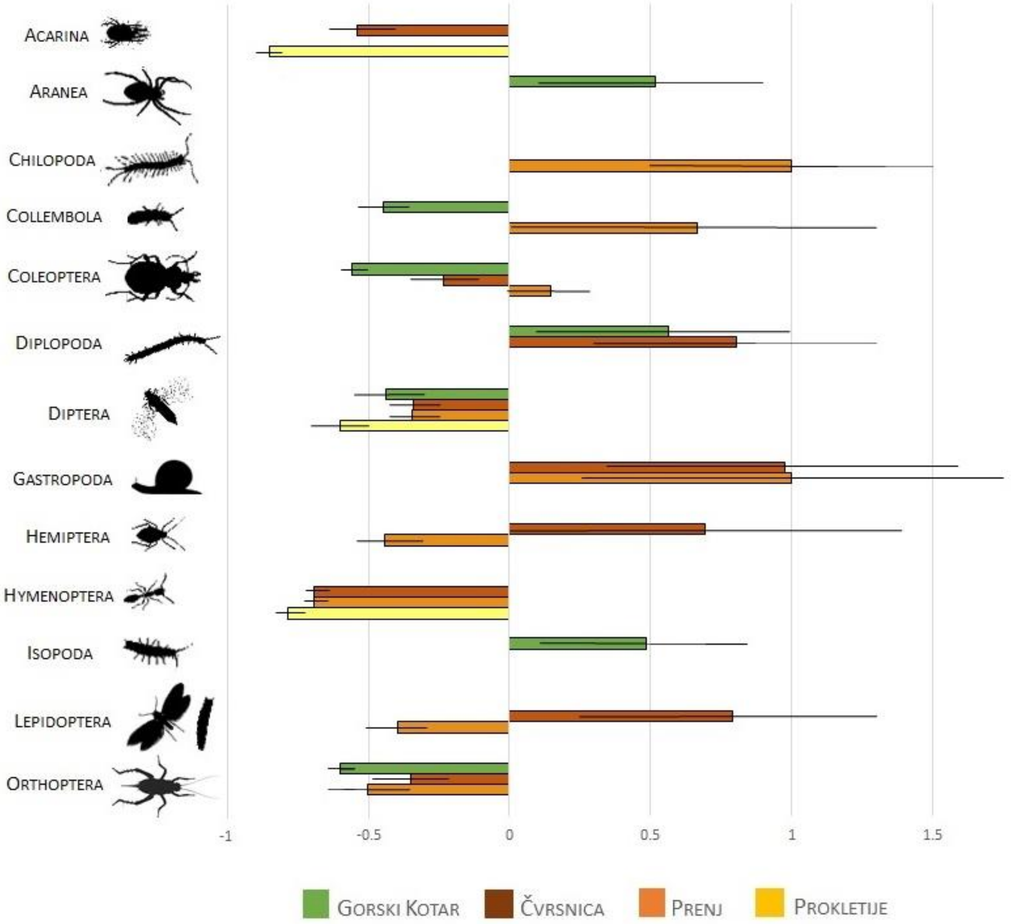

Figure 2. Ivlev index with confidence intervals across populations. Positive values indicate a strong preference while negative values show a weak preference towards respective prey items. For most of the prey categories, the direction of the electivity index was the same across populations.

Among populations comparison

After applying the Bonferroni correction, the diversity of consumed communities did not differ among populations (*P*=1 for all pairwise comparisons) nor did the diversity of available prey taxa (*P*=1 [Prenj vs Čvrsnica and Gorski Kotar & Čvrsnica vs Prokletije], *P*=0.63 [Prenj vs Prokletije], *P*=0.61 [Čvrsnica vs Gorski Kotar], *P*=0.96 [Prokletije vs Gorski Kotar], fig. 3). Within populations, the diversity of consumed and available prey, is not significantly different (no Bonferroni correction; *P*=0.07 [Prenj], *P*=0.15 [Prokletije], *P*=0.11 [Čvrsnica]) except in Gorski Kotar where the diversity of consumed prey revealed higher than the diversity of potential prey although on border of significance (*P*=0.04).

The number of prey items retrieved per stomach did not differ among populations (GLzM: Chi^2_3, *P*=0.23), but juveniles (mean number of prey ± SE = 5.42 ± 0.58, *n* =12) and subadult individuals (3.49 ± 0.37, *n*=77) tended to have larger numbers of prey in their stomach than adult males (2.74 ± 0.25, *n* =80) and females (2.88 ± 0.21, *n* =95; GLzM:Chi^2_3, *P*<0.001; see also fig. 5).

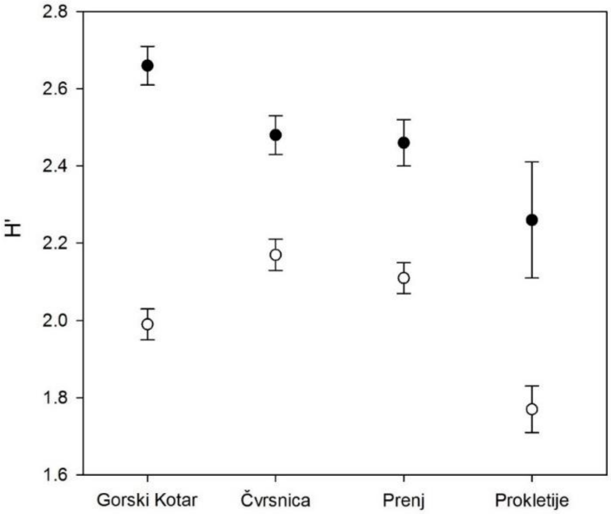

Figure 3. Shannon-Weaver diversity index (± standard deviation) for the prey community consumed by *Salamandra atra prenjensis* (closed symbols) and caught in the pitfall traps (open symbols) at the four study sites (top: consumed community; bottom: trap community).

The four populations differed somewhat in dietary composition (ANOSIM, *r* -index=0.064, *P*=0.0001). Prey taxa significantly associated with particular populations were Myriapoda (*P*=0.005) and Isopoda (*P*=0.002) for Gorski Kotar; Diplopoda (*P*=0.05) for Čvrsnica; Chilopoda (*P*<0.0001) for Prenj and Opiliones

(*P*=0.03) for Prokletije. We found no effect of sex/age class on diet composition (ANOSIM, *r* -index<0.0001, *P*=0.48). The Jaccard-dissimilarity coefficients of the prey communities available to, and consumed by the *S. a. prenjensis* populations at our four study sites varied between 0.31 and 0.83 (fig. 4). The consumed and available prey communities were more similar between themselves than with one another (fig. 4). They only exception was the prey community available to the salamanders of Gorski Kotar, that appeared more similar to the consumed communities than to the communities available at the three other study sites (fig. 4). The available and the consumed prey community were slightly more dissimilar for the Prokletije population (0.76) than for the other populations (0.67-0.69; see fig. 4).

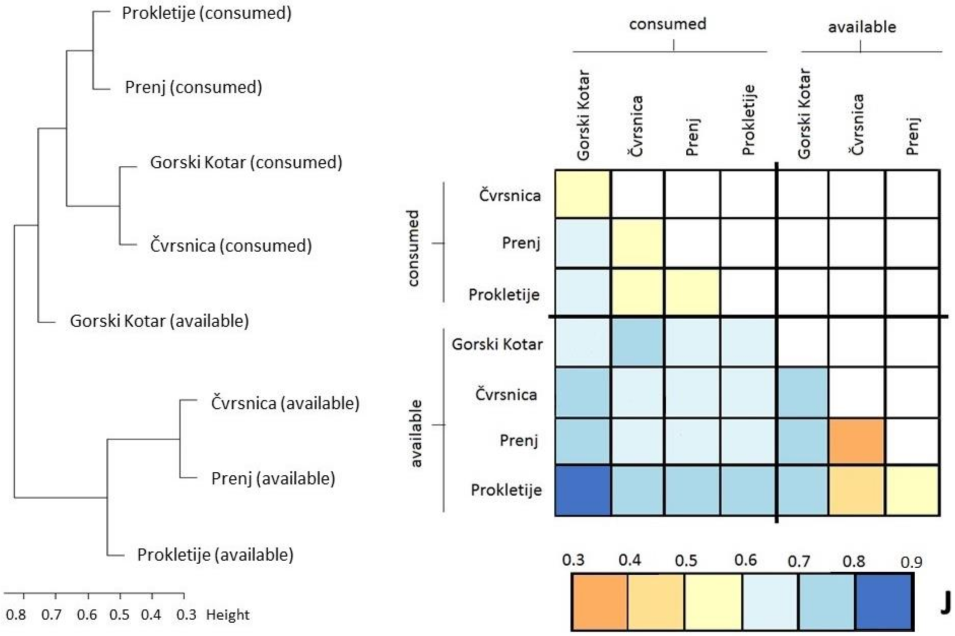

Figure 4. Comparison of the taxonomic composition of the prey communities that were available to, or consumed by *Salamandra atra prenjensis*. The colour matrix (right) shows pairwise Jaccard dissimilarities (orange – more similar; blue – more different). The dendrogram (left) was obtained using the complete linkage hierarchical clustering method on the Jaccard distances.

Individual specialisation and clustering

E-values for three of the four populations fell in the higher 5% tail obtained by Montecarlo resampling of the data (table 3), indicating some degree of individual specialisation. Only for Gorski Kotar the observed E-value fell just in the upper 6% of simulated E-values. Accordingly, the average IS-values were higher than expected under the assumption of random prey intake (table 3, all $P<0.05$).

We found no evidence for nestedness (i.e. specialists taking a particular subsets of the prey eaten by generalists); for all four populations and for the combined data, NODF-values were low (table 3) and well within the range of what could be expected when individuals were taking prey stochastically from a shared prey distribution (all $P>0.96$).

We did find strong indications for modularity. In all four populations and in the total dataset, C_{ws}-values were higher than expected under the assumption of individuals sampling randomly from the prey population (table 3), suggesting some degree of clustering. Inspection of the network plots (fig. 5) indicated that clusters are formed by small groups of individuals that share a prey type that is rarely eaten by other individuals in the population; members of a given cluster often consumed only that particular prey type. Clusters of individuals, eating specific prey taxa in each population, are highlighted in the network plots (fig. 5).

Table 3. Diversity indices and indices of individual specialisation, degree of clustering and nestedness for the four populations and for the lumped data (Total). Simpson's, Shannon-Weaver's and Smith's diversity indices are presented with 95% confidence intervals between brackets. Individual specialisation is portrayed by the IS index and Araújo's degree of variation (E_{obs}: observed values; E_{null}: average null values obtained from Montecarlo resampling the data, N=10.000 replicates; E_{adj}: values rescaled such that $E_{adj}=0$ when $E_{obs}=E_{null}$ and $E_{adj}=1$ with highest specialisation) and the probability that $E_{obs}>E_{null}$). Also shown are the weighted clustering coefficient C_{ws} and the proportion of Montecarlo simulated replicates that yielded higher C_{ws}-values, and an index of nestedness, NODF. *P* – p value.

	Gorski Kotar	**Čvrsnica**	**Prenj**	**Prokletije**	**Total**
Gini-Simpson diversity, 1-D	0.92 (0.91-0.93)	0.90 (0.89-0.92)	0.88 (0.86-0.90)	0.87 (0.81-0.94)	0.92 (0.91-0.93)
Shannon-Weaver diversity, H'	2.70 (2.61-2.80)	2.51 (2.42-2.60)	2.50 (2.39-2.62)	2.44 (2.17-2.71)	2.74 (2.68-2.80)
Smith's measure, FT	1 (0.98-1)	0.99 (0.97 – 1)	0.99 (0.97-1)	0.99 (0.94-1)	1 (0.98-1)
Araújo's index					
Observed values, E_{obs}	0.88	0.85	0.88	0.87	0.90
Null values, E_{null}	0.87	0.81	0.85	0.81	0.88
Scaled values, E_{adj}	0.12	0.21	0.23	0.31	0.17
P(E)	0.06	<0.001	<0.001	0.02	<0.001
IS index	0.19	0.23	0.19	0.22	0.16
P(IS)	0.02	<0.001	<0.001	0.01	<0.001
Clustering coefficient					
Cws	0.40	0.31	0.51	0.46	0.47
P(Cws)	0.005	<0.001	<0.001	0.003	<0.001
Nestedness, NODF	17.10	24.26	17.56	16.56	17.29
P(NODF)	(1)	(1)	(1)	(1)	(1)

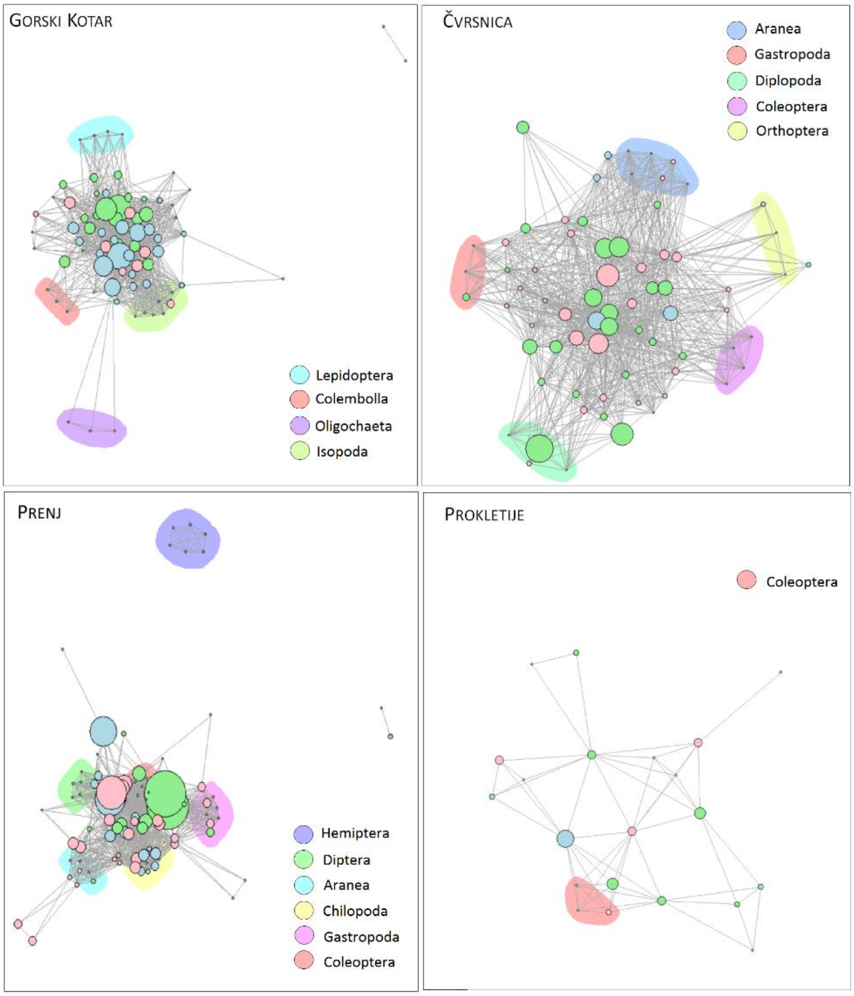

Figure 5. Network plot analysis. Each circle (node) represents an individual where the size is proportional to the number of ingested prey while the colour reveals the sex/age class as follows: Blue - males, pink - females, green - juveniles and subadults. The links (edges) among individuals are drawn from an individual overlap matrix (see RInSp; values < 0.1 are omitted). For each population, clusters of individuals specialized for a certain prey taxa are marked in colours; colours correspond to prey taxa as in the legend.

Discussion

With an average of 3.16 (± 0.55 SE, *n*=264) prey items per non-empty stomach, feeding intensity in our four populations of *S. atra* is similar to that reported for a conspecific population at 1850 m a.s.l. in north-eastern Italy (2.78 ± 0.75, *n* =45, Roner et al., 2020) and two populations in the Austrian Alps (1200 – 1700 m a.s.l: 2.59 ± 1.85, N=39, Fachbach, Kolossa and Ortner, 1975). Averages reported for other salamander species vary considerably. For *Salamandra salamandra* it ranges from 2.72 (for a population of in the Carpathians, 400 m a.s.l., Balogová et al., 2015); over 3.27 (population in Orşova, Romania, 110-130 m a.s.l., Ferenti et al., 2010); 4.66 (Baia de Arame, Romania, 330 m a.s.l., Ferenti, David and Nagy, 2010); 6.74 (Tismana, Romania, 300m, Ferenti, David and Nagy, 2010); and 14.83 (Ursului Valley, Romania, 300m, Lezau et al., 2010) up to 19.38 items per stomach (Gredos, Spain, 2000-2200 m a.s.l., Guerrero et al., 1989). Çiçek, Koyun and Tok (2017) found an average value of 4.95 items per stomach for a population of *Salamandra infraimmaculata* in eastern Anatolia (at 1400-1600 m a.s.l.). Polymeni, Radea and Papanayotou (2011) report an average of 16.8 prey items per stomach in a Greek insular population of *Lyciasalamandra luschani*. For a population of *Salamandrina perspicillata* in north-west Italy (900 m a.s.l.), Salvidio et al. (2012) obtained at an average value of 10.46 items for adults[8]. Alas, a direct comparison of feeding intensity across these salamander populations is difficult because different studies have used different methodologies and sampling periods. At first sight, there is no obvious correlation between feeding rate and altitude or habitat, and information on important drivers such as climatic condition and

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[8] Due to practical limitations, we were unable to provide information on prey volume or prey mass. This is an obvious shortcoming, since prey mass or volume is probably a better proxy for the relative importance of a certain prey taxon in terms of energy (e.g., IRI index - Cortes 1997). On the other hand, every prey item, big or small, needs to be captured individually and therefore the number of prey items is still likely to reflect the salamanders' prey choice.

food availability are not available, so the origin of the strong interpopulational differences in feeding rate remains unknown. Compared to feeding rates of other salamander species (previously reported), the feeding intensity found in *S. a. prenjensis* populations is generally lower; this may indicate that food is scarce or difficult to obtain. On the other hand, food intake may be directed by *S. atra*'s metabolic rate that is strongly influenced by temperature (amphibians' feeding intensity and metabolic rate generally decrease in colder conditions; Browne, 2009). Consequently, it would be worthwhile to check whether the feeding rate of *S. atra* is reflected in lower body condition and/or growth rates. Nevertheless, we cannot exclude the possibility that salamanders in our populations consumed fewer, but larger prey items.

While the diet of the *S. a. prenjensis* populations in this study seems relatively poor in quantity compared to other salamander populations, our results indicate that it is rich and diverse in terms of consumed invertebrate taxa. Effectively, as far as we know, the Simpson index of diversity (1-D) calculated across our four populations (0.92) is the highest recorded for any salamander population (e.g. *Salamandrina perspicillata*: 0.59 [Salvidio et al., 2012]; *S. salamandra*: 0.64 [Guerrero et al., 1990], 0.71 [Fachbach, Kolossa and Ortner, 1975], 0.82 [Lezau et al., 2010], 0.83 [Ferenti et al., 2010]; 0.84 [Balogová et al., 2015; Ferenti, David and Nagy, 2010], 0.87 [Fachbach, Kolossa and Ortner, 1975], 0.90 [Balogová et al., 2015]; *Lyciasalamandra luschani*: 0.88 [Polymeni, Radea and Papanayotou, 2011]). Interestingly, Simpson values for the diets of populations of *S. atra* in north-eastern Italy (0.86 [Roner et al., 2020]) and in Austria (0.85-0.86 [Fachbach, Kolossa and Ortner, 1975]) also ride relatively high. This paints *S. a. prenjensis* as a highly opportunistic predator that will feed on a wide range of prey. Even among terrestrial salamanders, a group renowned for its catholic feeding (Duellman and Trueb, 1994; Çiçek, Koyun and Tok, 2017), stand out for having such a wide dietary span. Ecological theory links a generalistic feeding style to unpredictable environments (Futuyma and Moreno, 1988), the availability of prey items requiring short handling times (relative to searching time) (Pyke, Pulliam and Charnov, 1977) and little interspecific competition (MacArthur and Levins, 1967). All these conditions seem to apply to *S. a. prenjensis* populations.

Beetles, spiders, snails and millipedes made up the bulk of the diet in the four *S. atra prenjensis* populations considered in this study. Very similar results were obtained for three other *S. atra* populations, in the Dolomites (Roner et al., 2020) and in the Styrian Prealps (Fachbach, Kolossa and Ortner, 1975). The species diet therefore consists primarily of terrestrial, slow herpetobiont arthropods, as could be expected on the basis of this salamander habitat use and foraging behaviour.

In spite of being catholic, the four *S. a. prenjensis* populations in our study did not consume prey taxa in accordance to their availability. For most of the prey categories, the direction of the electivity indices was the same across populations (fig. 2). Relatively slow and big herpetobionts, such as millipedes, snails, spiders and isopods were eaten more often than could be expected from their frequency of occurrence in the pitfall traps and sweep samples. Smaller, faster or flying arthropods, such as acari, grasshoppers, hymenopterans, and diptera obtained negative electivity indices. Roner et al. (2020) reported similar results for the *S. atra* population in Italy. It is tempting to attribute this bias in prey consumption to the relatively clumsy foraging style of *S. atra*, in which case significant electivity indices would reflect predatory (in)ability rather than actual preference or dislike. The apparent avoidance of beetles may be related to their relatively high agility, effective defence mechanisms, or low energetic profitability. As a cautionary note, electivity indices based on the comparison of prey items found in stomachs versus traps or nets must be interpreted carefully, because some taxa caught by the experimenters may not really be 'available' to the predator under study (e.g activity time of prey and salamanders may differ) while other taxa may elude the sampling effort (Lechowicz 1982, Peters 1995). Controlled behavioural experiments (e.g. using the 'cafeteria' set-up, Krebs, 1989) are required to properly investigate dietary preferences of *S. atra*.

Does the wide dietary niche of *S. atra* reflect general individual gluttony, or does it emerge as a population property, i.e. the sum of a large number, narrower and slightly divergent individual diets? Ever since Bolnick et al. (2003) revived the interest in the concept of niche variation (Van Valen, 1965), a substantial number of studies (reviewed in Araújo, Layman and Bolnick, 2011) have demonstrated individual dietary specialisation in a variety of species, including an Italian alpine salamander population (Roner et al., 2020) and several other salamander species (Costa et al., 2015; Salvidio et al., 2015). The significant E- and IS-values obtained here (table 3) also suggest individual dietary specialisation in the studied *S. a. prenjensis* populations. In general, individual specialisation is thought to arise because specimens within a population differ in the ability to apprehend, handle, or digest certain prey types, or have physiological requirements (Araújo, Layman and Bolnick, 2011), possibly mediated by intraspecific competition (Svanbäck and Bolnick, 2005). While these ideas are interesting, we consider it too early to speculate on the exact reasons for individual dietary specialisation in *S. atra*, basically because our dataset merely consists of 'snapshots': each individual salamander was stomach-flushed only once. As a consequence, low within-individual variation in diet composition could also result from the fact that an animal was foraging in a patch of habitat, or period of time particularly rich in some prey taxon (or on conspecific prey items clumped for some reason). Demonstrating real, habitual individual specialisation will require sampling the same individuals throughout the activity season.

We found no evidence for a nested pattern in the interindividual subdivision of dietary resources in the four studied *S. a. prenjensis* populations. Nestedness, in which specialist individuals consume a predictable subset of prey items of more generalistic individuals in the same population, has been described in a variety of vertebrates (e.g. Araújo et al., 2009; Pires et al., 2011; Santamaria et al., 2020), including the Italian population of *S. atra* studied by Roner et al. (2020). Nested diet niches arise when individuals in a population have the same rank preference, but differ in their willingness to include lower ranked prey types into their diet (the 'shared preference' model, Svanbäck and Bolnick, 2005). The absence of such nestedness could thus be due to the fact that individual salamanders differ in their intrinsic preference, encounter rates or ability to capture and handle particular prey taxa (the 'distinct preferences' model). This could be tested with standardized cafeteria experiments.

Instead, we did find indications for subsets of individuals specialising on specific subsets of the population's dietary niche, i.e. our results suggest a certain degree of modularity (Araújo et al., 2009). The modules found in the four populations typically consist of individuals of both sexes and different age classes (fig. 5), so they do not reflect sexual or ontogenetic variation in prey use. Modularity seems less wide-spread than nestedness (Pires et al., 2011), but has been described in the Italian population of *S. atra* (Roner et al., 2020). In theory, modularity could arise under condition of relaxed competition, where individuals specialize on some, particularly preferred prey items (the 'distinct preferences' model); or it could be the outcome of intraspecific competition for limited resources forcing individuals to focus on distinct prey types.

Overall, our analyses suggest that the broad niche of the four *S. atra* populations in this study is composed of smaller individual niches[9] that cluster along the dietary axis (figure 2 and option D in Araújo et al., 2008). However, as noted above, a more extensive data set with multiple observations per individual

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[9] As noted previously in the discussion, aspects of individual specialization were assesed by analyzing only a single stomach content per individual (one meal), therefore, the usage of the term 'individual niche' is probably overstated. Moreover, general conclusions related to individual specialization must be taken with caution.

predator is required to validate this conclusion. With the current data, the apparent individual specialisation and clustering could also be caused by individual salamanders that had been foraging in a specific microhabitat with an idiosyncratic prey community, or that had stumbled on aggregated group of the same prey species.

We found little evidence for local dietary differentiation. Although our study sites include widely diverging habitats (beech forest to alpine grasslands) along a substantial altitudinal gradient (900-2000m), the populations do not show extensive variation in the number of prey consumed, the composition and diversity of their diet, or the structural characteristics of their dietary niche. An analysis of similarity did reveal significant among-population variation in diet composition, but the R-index was relatively small, and the prey taxa that set apart the respective populations were generally not important in the sense that they were not consumed in large quantities.

However, in comparison with other species of salamanders, all four *S. a. prenjensis* populations have a highly diverse diet. Geographical variation in dietary niche does not seem very important in true salamanders in general (Fachbach, Kolossa and Ortner, 1975, Kuzmin, 1994, Lezau et al., 2010), with the possible exception of the most ecologically divergent populations of the widely distributed *S. salamandra* (Guerrero et al. 1990). Such low inter-populational variation could reflect an inability to respond to local conditions (which might then jeopardize survival of temporal environmental changes), or the mere absence of drivers for change. Considering the catholic dietary niche of the species, we tend towards the latter explanation.

Acknowledgments

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The sampling permit in Montenegro was issued by the Agency for Nature Protection in Podgorica (02, UPI-790/4); the sampling permits in Croatia were issued by the Ministry for the Protection of Nature and Environment (517-07-1-1-1-15-3) and by the

Ministry of Environmental Protection and Energy (517-07-1-1-1-16-4) both located in Zagreb. In Bosnia and Herzegovina the sampling permit was issued by the Federal Ministry of Environment and Tourism in Sarajevo (04-23-550/16 ZM).

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Chapter 5

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

Gilles De Meester, Emina Šunje[10], Els Prinsen, Erik Verbruggen, Raoul Van Damme

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

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[10] I contributed to this study by formulating the research question, developing the study design, procuring permits, leading the field work, obtaining the predation risk data from the literature and local experts, helping in analyzing the data, and in reporting. The lab work was done by GDM, under the supervision of EP and EV.

Introduction

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

For a limited number of species, there is evidence that chemical defenses exhibit geographical variation (Clarke 1997; Brodie *et al.* 2002; Saporito *et al.* 2012; Bokony *et al.* 2019), often leading to interpopulation differences in toxicity (Brodie *et al.* 2002; Saporito *et al.* 2012; Bolton *et al.* 2017) and/or antimicrobial activity (Tennessen *et al.* 2009; Mina *et al.* 2015; Hovey *et al.* 2018) of the skin secretions. In species that sequester toxins from their diet (e.g. poison frogs), such geographic variation in chemical defenses may merely reflect differences in local arthropod prey community composition (Daly *et al.* 2007; Saporito *et al.* 2007; Daly *et al.* 2008; Saporito *et al.* 2012). In other cases, geographic variation in chemical defenses is believed to result from changes in the ratio between costs and benefits of toxin production (Longson & Joss 2006; Blennerhassett *et al.* 2019). This ratio may depend on the environment, leading to selection for different toxin profiles among populations (Longson & Joss 2006; Bókony *et al.* 2016; Üveges *et al.* 2017). For example, toxin profiles may reflect local predation pressure (Brodie *et al.* 2002; Hanifin *et al.* 2008; Dreher *et al.* 2015; Bókony *et al.*

2016; Hettyey *et al.* 2019). Selection should favor higher toxicity, but only in environments where predation pressure is high, whereas it should act against costly chemical defenses when predation is low (Longson & Joss 2006; Yotsu-Yamashita *et al.* 2012; Hettyey *et al.* 2019). Alternatively, infection risk in the environment might also play a role in shaping local toxin profiles (Tennessen *et al.* 2009; Bókony *et al.* 2016). Unfortunately, few studies so far have investigated how local predation pressure and local infection risk contribute to geographic variation in chemical defenses (Brodie *et al.* 2002; Hanifin *et al.* 2008; Dreher *et al.* 2015; Bókony *et al.* 2016).

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

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

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

Material and methods

Populations

Four populations of *S. atra prenjensis* were sampled from the end of June until the beginning of September 2016. All populations were located within the Dinaric Alps, but differed considerably in altitude and general habitat (see Figure 1).

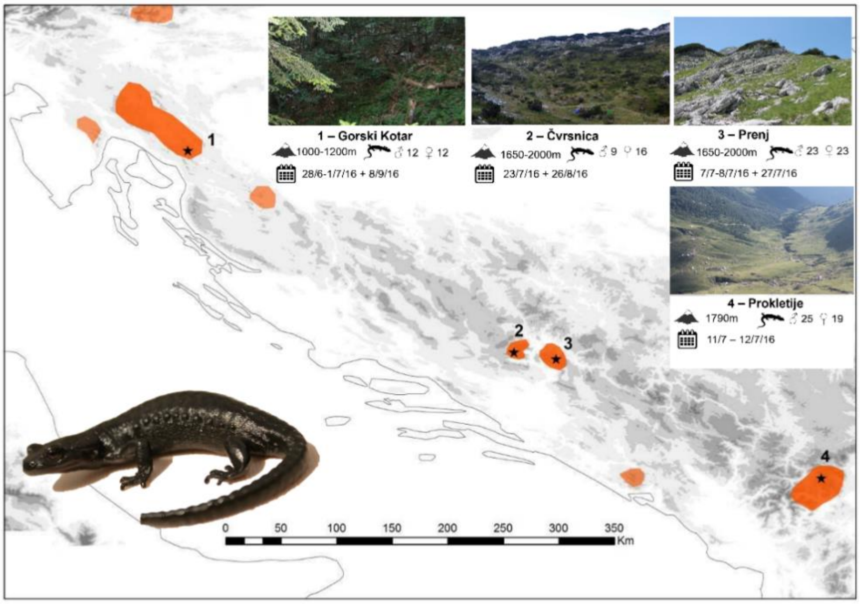

Figure 1. Distribution range (orange) of *Salamandra atra prenjensis*. Stars (★) indicate location of the study sites. The average altitude (in meters), the sample size and the dates of sampling are given for each population, as well as a picture of the general habitat.

Gorski Kotar consists of mixed deciduous-coniferous forest with a dense undergrowth. The soil is covered in plant litter and logs. Mounts Čvrsnica and Prenj are characterized by rocky alpine grassland, with sparse aggregations of mugo pines. The habitat in Prokletije consisted of a mix of grass field and fir forest near a small mountain stream. One constant factor was the presence of rocky limestone outcrops and dolomitic karst in all populations, providing holes and crevices for salamanders as shelter.

Samandarines (SAMs)

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

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

During a series of test runs, a random selection of samples from each population were scanned for the presence of SAMs (Habermehl 1962; Habermehl & Spiteller 1967; Habermehl 1971; Daly *et al.* 2005; Lüddecke *et al.* 2018) using Multiple Reaction Monitoring (MRM). Compound specific MRM-settings were selected based on literature data (Table S51). We measured the abundance of eight compounds in each sample: ecomytrin, samandaridine, samandarine, samandarone, samandanone, samandiol, samanine and samanol. For samanol, two different peaks were found in each chromatogram. We could not determine which of these two peaks represented samanol, therefore we will refer to the respective substances as 'samanol' and 'samanol2'. Reference standards could not be obtained, as these are not commercially available (Knepper *et al.* 2019) making further verification and calibration impossible.

Chromatograms were analyzed using MassLynx 4.1. (Waters Corporation, Milford, USA). Mass spectrometry results in peak surface areas (pA) for each individual compound, which are proportional to its abundance in the gland secretions (Sanchez *et al.* 2018). To avoid random noise, only peaks with a surface area higher than 30 (arbitrary) units were used. An additional experiment with captive animals showed that SAMs, and thus peak surface areas, degrade over time (mixed-effect model: slope = -0.02 log-units per day; $F_{1,240} = 12.21$; $p < 0.001$; see supplementary material). To take this into account, we recalculated the 'original' peak surface area of all compounds using the observed degradation rate and the number of days between collecting and analyzing samples. Peak surface areas were expressed relative to the size of the parotoid gland (calculated as the surface area of an ellipse – pA/mm^2) to avoid that potential differences in SAM quantities would simply be a result of larger salamanders being able to store more alkaloids (Sanchez *et al.* 2018).

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

To test for differences in the total amount of SAMs (sum of pA of all compounds /mm^2 cf. Sanchez *et al.*, 2018) produced by salamanders, we used a general mixed model containing population and sex as fixed factors. Sampling period (early July, late July and September) was included as random effect. Similar mixed models were used to test for differences in the relative amounts of four individual SAMs: samandarine (most potent neurotoxin), samandarone (strongest antimicrobial effect), samandaridine and samandenone (due to their high abundance). Interactions were initially included, but removed in case of non-significance. Data were log-transformed to meet normality and heteroscedasticity assumptions. *Post-hoc* pairwise comparisons were performed with the *emmeans* package (Lenth *et al.* 2018).

Salamander morphology and SAMs

Differences in body weight were tested using a two-way ANOVA, including population and sex as fixed factors, as well as their interaction. An ANCOVA was used to test for differences in parotoid gland size (in mm^2), including population and sex as fixed factors and body weight as covariate, as well as interactions between population*sex and population*weight. Nine salamanders from Gorski Kotar were excluded from the dataset, due to incomplete body measurements.

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

Infection risk

Infection risk was estimated by taking soil samples in each population and identify soil bacteria and potentially parasitic soil fungi. Since *S. atra* is a strictly terrestrial species (Jeran *et al.* 2011; Helfer *et al.* 2012) that spends a large portion of its life underground (Gautier & Miaud 2003; Helfer *et al.* 2012), it is mostly exposed to soil micro-organisms. Soil samples were scooped out with a metal spoon that was sterilized prior to each sampling, by cleaning it with 70% ethanol and heating it over the open flame of a camping stove. Soil samples were taken in duplicate, one series of samples for bacterial cultivation and one series of samples for genomic analysis, from all microhabitats in which salamanders were found during the fieldwork, with special attention to the rock crevices which are the openings of the burrows used as shelter (Table S5.2). Samples were stored in 1.5 mL plastic microcentrifuge tubes and LifeGuard® Soil Preservation Solution (MO BIO Laboratories, Inc., Carlsbad, USA) was added to the genomic samples to prevent degradation of microbial DNA. Samples were stored at 4°C. A total of 67 soil samples were collected.

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

Genomic DNA for soil fungi identification was isolated from the second series of soil samples (Gorski Kotar: 10; Čvrsnica: 7; Prenj: 8; Prokletije: 12) using a Powersoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, USA). Following the accompanying protocol, 100 µL DNA-solution was obtained from 0.25 grams of each soil sample. Fungal DNA was then amplified by a PCR using modified versions of the primers ITS1F and ITS2, which amplify the fungal internal transcribed spacer (ITS) 1 region (see Smith & Peay, 2014). Each sample was amplified with an ITS2 primer containing a unique index sequence (Kozich *et al* .2013). PCR-amplicons were pooled in one DNA-library and sequenced on an Illumina MiSeq™. See supplementary material for more details on the primers, PCR-protocol and sequencing. Sequences were analyzed following the UPARSE fungal pipeline described in Edgar (2013) and Smith and Peay (2014). After removing singletons, sequences were clustered into Operational Taxonomic Units (OTUs) based on a similarity of 97%. Chimeras were removed.

An OTU table was constructed and OTUs were blasted against the UNITE database of ITS1 sequences (version 7.0) using the BLAST algorithm with default settings. OTUs were assigned to a certain lifestyle according to Tedersoo *et al.* (2014). The OTU-table was rarefied using the Rarefy-function in R (*GUniFrac* package, Chen, 2018).

ANOSIMs based on Bray-Curtis dissimilarity matrices were performed to test the similarity of populations in soil fungi communities. Results were visualized using non-metric multidimensional scaling (NMDS). In order to test whether dissimilarity in soil fungi communities was related to dissimilarity in SAM profiles, we used a Mantel test based on Spearman's rank correlation coefficient. A high number of OTUs could not be identified (NA) (71% of the OTUs, representing 61% of the total post-pipeline reads). These unidentified OTUs were included in the ANOSIM and NMDS, but removed from further analyses.

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

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

Predation risk

Predation risk in each population was estimated by counting the number of snake species present at each location. There are several reasons why predation by snakes is likely a stronger selective pressure for SAM composition in *S. atra* than predation by mammals or birds. First of all, snakes, more specifically the grass snake (*Natrix natrix*) and the common viper (*Vipera berus*), are currently the only documented predators of *S. atra* (Anibaldi *et al.* 1995; Luiselli *et al.* 1997; Luiselli *et al.* 2005; Mebert *et al.* 2017). While predation by Eurasian magpies (*Pica pica*) and Alpine choughs (*Pyrrhocorax graculus*) has been reported, Klewen (1991) noticed that these birds avoid consuming the toxic parts of the animal. It is suspected that rats (genus *Rattus*) show a similar behavior when consuming other *Salamandra* species (Pezaro *et al.* 2017). In addition, during many field work sessions, we never observed carcasses mutilated this way. Hence, predation by birds and mammals is less likely to be a selective pressure on SAM composition or quantity in our populations. Secondly, both *V. berus* and *N. natrix* are known to tolerate higher injected doses of SAMs compared to other vertebrates (Geßner & Möllenhoff 1932; Lüddecke *et al.* 2018), which might indicate their active role as *Salamandra*-predators. Last but not least, independent evolutionary arms races between predatory snakes and poisonous amphibians have been hypothesized to occur worldwide (Brodie *et al.* 2002; Feldman *et al.* 2012), illustrating how important snake predation may be for the evolution of amphibian chemical defenses.

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

Ethical statement

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

Results

Samandarines

Populations of *S. atra* differed significantly in overall SAM composition (ANOSIM R = 0.125; p = 0.001). The strongest disparity was found between Prokletije and Gorski Kotar, while Čvrsnica and Prenj, and Čvrsnica and Gorski Kotar exhibited high similarity (Table 1). Male and female salamanders did not differ significantly in overall SAM composition (ANOSIM R = -0.008; p = 0.654).

Geographically distant populations tended to differ more in SAM profiles, but the relationship was not significant (Mantel statistic r = 0.657; p = 0.08, see Table S5.3 for geographical distances).

The total amount of SAMs secreted by salamanders did not vary significantly among the four populations, neither when expressed in absolute terms (pA; $F_{3,10} = 2.24$; p = 0.15; Figure 2A) nor relative to gland size (pA/mm². $F_{3,134} = 1.86$; p = 0.14; Figure 2B).

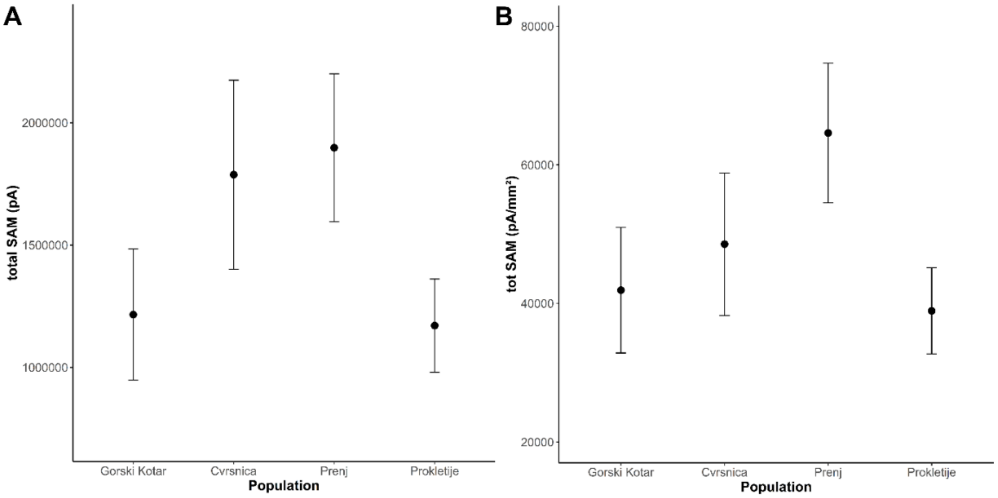

Figure 2. Total amount of samandarines (SAMs) in the parotoid secretions, both expressed in absolute amounts (A) and relative to gland size (B). Total amount of SAMs was calculated by taking the sum of the peak surface areas (pA) of all individual compounds within the chromatogram of an individual (Sanchez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

No sex-differences were found ($F_{1,134} = 0.29$; p = 0.59). Populations differed significantly in the quantity of samandarine ($F_{3,90} = 4.89$; p = 0.003) and samandaridine ($F_{3,124} = 15.73$; $p < 0.001$), marginally in samandenone ($F_{3,128} = 2.66$; p = 0.05), but not in samandarone ($F_{3,2} = 2.90$; p = 0.27).

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

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

We refer to Figure 3 for the specific interpopulation differences in each compound as indicated by post-hoc Tukey's tests. Female salamanders secreted higher quantities of samandarone ($F_{1,133} = 4.15$; $p = 0.04$) and tended to produce more samandaridine ($F_{1,133} = 3.63$; $p = 0.06$) but no sex-differences were found in samandarine ($F_{1,133} = 0.50$; $p = 0.48$) or samandenone ($F_{1,133} = 0.61$; $p = 0.44$). There were no significant sex*population interactions for any of the variables (all $p > 0.05$).

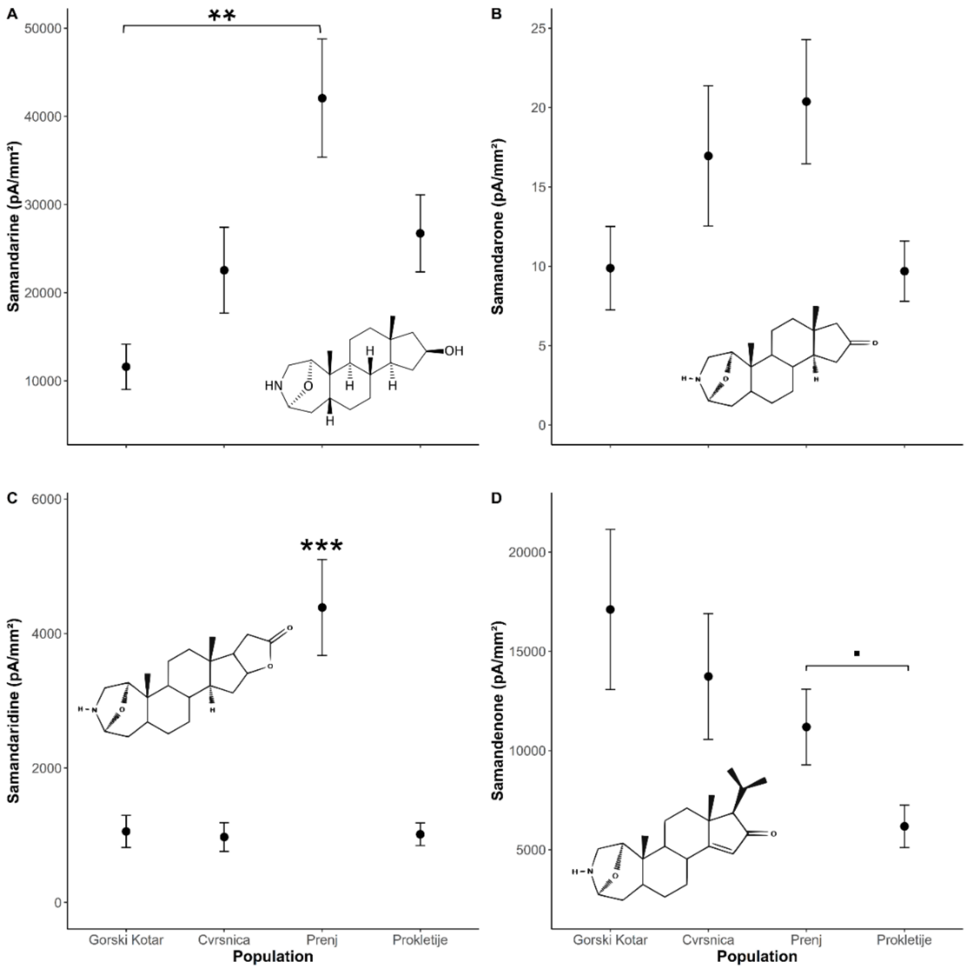

Figure 3. Differences in the quantity of samandarine (A), samandarone (B), samandaridine (C) and samandenone (D) among the four populations of *Salamandra atra.* SAM quantities are expressed as the peak surface area in the chromatogram (pA) relative to the size of the parotoid glands (mm²). Error bars represent standard errors. Significance levels are indicated as follows: '.' $p < 0.1$, '*' $p < 0.05$, '**' $p < 0.01$, '***' $p < 0.001$

Salamander morphology and SAMs

A summary of morphological variables per population is given in Table S5.4. Both body weight and parotoid gland size differed among our studied populations. Our results indicated that salamanders from Čvrsnica were heavier ($F_{3,125}$=7.63; $p < 0.001$; Figure 4A) than conspecifics from the other three populations, and, even after controlling for body size, they had larger parotoid glands compared to salamanders from Prenj ($F_{3,124}$=5.44; $p = 0.002$). There was a trend towards larger glands in Čvrsnica compared to Gorski Kotar (post-hoc Tukey's test: $p = 0.08$) and Prokletije (post-hoc Tukey's test: $p = 0.09$) (Figure 4B). Salamanders from the other three populations differed in neither body weight or gland size from each other. Females were heavier than males ($F_{3,125}$=19.33; $p < 0.001$) but did not possess larger glands ($F_{1,124}$ = 0.14; $p = 0.71$). No significant interactions were found between sex and population ($p > 0.05$) or body weight and population ($F_{3,118}$ = 0.91; $p = 0.44$). Heavier salamanders had larger parotoid glands ($F_{1,124}$=62.92; $p < 0.001$).

Overall, heavier salamanders produced higher absolute amounts of SAMs (pA, $F_{1,121}$ = 7.61; $p = 0.007$). There was a significant interaction between population and parotoid gland size ($F_{3,121}$ = 4.26; $p = 0.007$). In Prenj, salamanders with relatively smaller glands produced more SAMs, while there was no significant correlation in any other population (Figure 5).

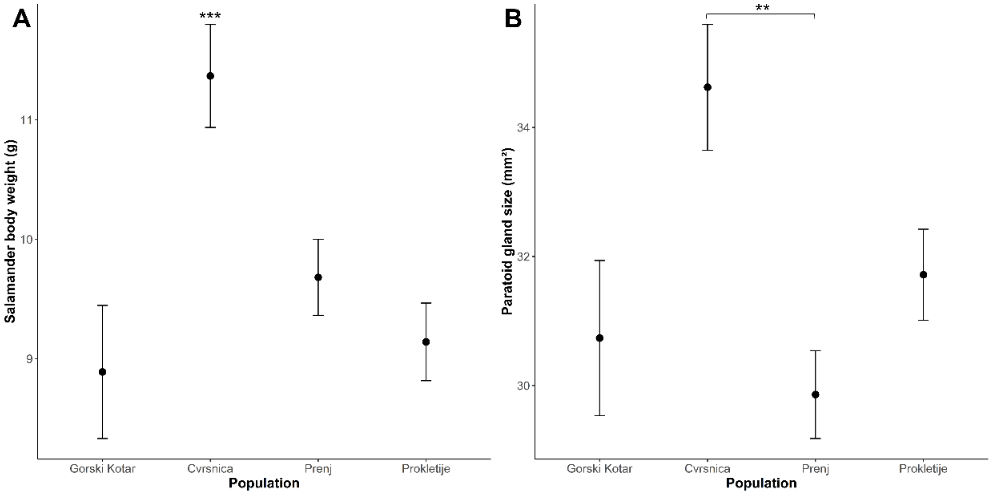

Figure 4. Morphological differences among populations in A) body weight and B) parotoid gland size (controlled for overall body weight). Parotoid gland size was calculated as the surface area of an ellipse using parotoid width and length. Error bars represent standard errors. Significance levels are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

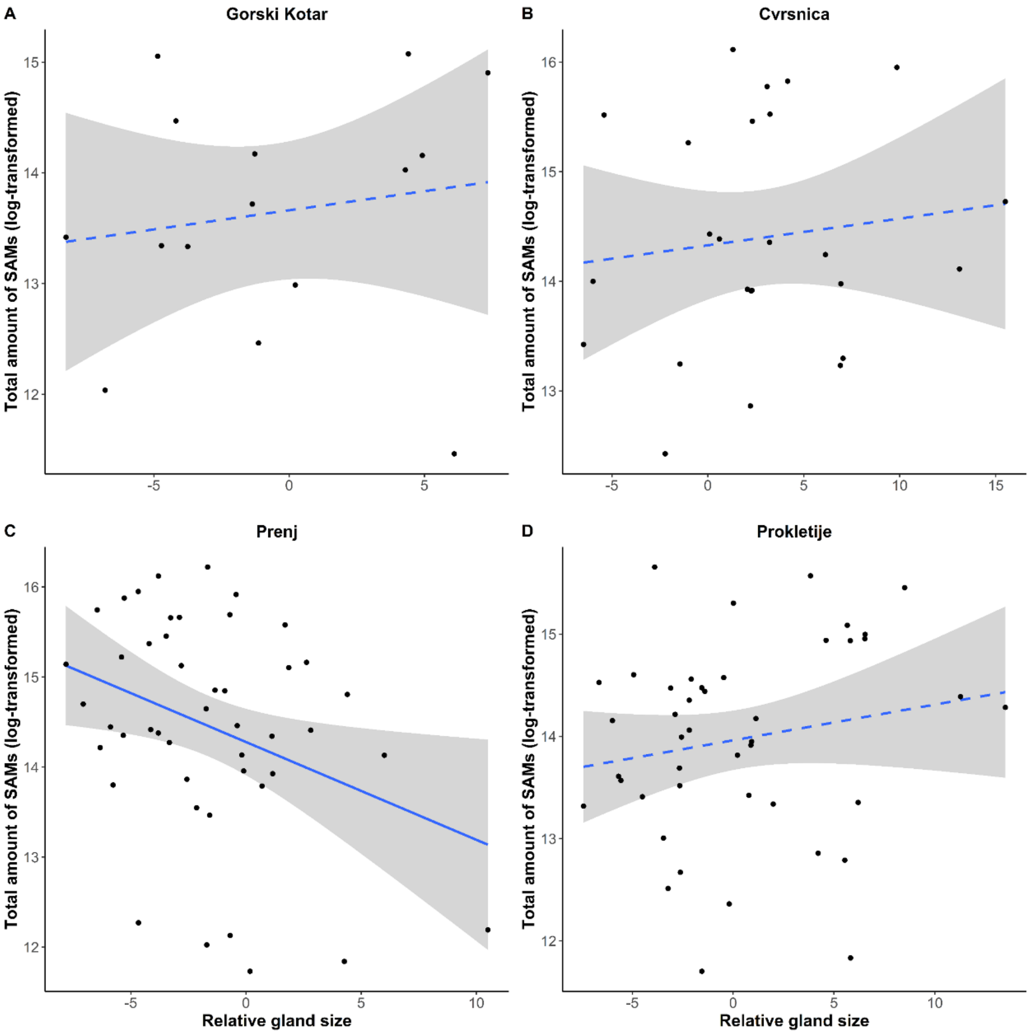

Figure 5. Association between relative parotoid gland size (here represented by residuals from a gland size – body weight regression) and the total amount of SAMs (sum of the peak surface areas of the individual compounds, log-transformed) secreted by *Salamandra atra* in each population. Solid lines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

Infection risk

Bacteria were cultivated from 30 soil samples. The number of CFUs grown from one gram soil did not differ among populations, regardless of whether they were cultivated on TSA-media ($F_{3,3} = 0.52$; $p = 0.70$) or MacConkey media ($F_{3,2} = 0.97$; $p = 0.54$). No colonies were detected on the Slanetz-Bartely media. A significantly higher number of CFUs was grown on the TSA-media at 15°C than at 35°C ($F_{1,40} = 33.5$; $p < 0.001$). There was no significant interaction between population and temperature for the TSA-media ($F_{3,38} = 0.34$; $p = 0.80$). We refer to Table S5.6 for average densities per population and per medium.

A total of 2661 fungal OTUs were identified in 37 soil samples. Most fungi were saprotroph-filamentous (51%) or ectomycorrhizal (22%). Less than 2% of the OTUs were identified as animal parasites (see also Figure S5.1). A small proportion of the OTUs (3%) could be identified but not assigned to a particular lifestyle (meaning that either they were not in the database of Tedersoo *et al.* (2014), the lifestyle was not conserved at the genus level, or the lifestyle was unknown).

Populations differed in their soil fungi communities (ANOSIM $R=0.23$; $p = 0.002$, Figure S5.2), with the highest dissimilarity found between Gorski Kotar and Prenj (Table 1). The dissimilarities in soil fungi community did not correlate with dissimilarities in SAM profiles (Mantel statistic $r = 0.09$; $p = 0.46$).

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

Predation risk

Our enquiries indicate a lower number of predatory snake species at the study site of Prenj compared to the other sites (Table 2). We have confirmed sightings of only two species (*Natrix natrix* and *Vipera ammodytes*) for Prenj, and local experts (see Table S5.5) consider the presence of a third species (*Coronella austriaca*) highly likely. Between four and six snake species occur in the three other study areas.

Table 2. Presence or absence of snake species per population, based on literature data, field observations and communication with local experts. The presence of species is scored for a broader surrounding of the sampling locations (± 100 m altitude difference). "+" - the species is confirmed by field work (of authors or local experts) within the last ten year and literature data; "?" – the species is expected to occur in the area of interest according to local experts, based on a match between habitat conditions and the species' ecological requirements, but not yet observed. Species were scored as absent if they were neither sighted, the literature data did not provide support for their presence and if their ecology did not match the habitat of that location. The complete list of local experts and affiliations is given in Table S5.5.

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

1 - Sillero *et al.* (2014) 2 - Jelić *et al.* (2013) 3 - Jelić *et al.* (2015) 4 – Lauš, B. 5- Šunje *et al.* (2014) 6 – Zimić, A. 7 - Džukic *et al.* (2003) 8 – Tomović, L. 9 – Zagora, V. 10 – Ajtić, R. 4 , 6, 8, 9, 10 - pers. comm.

Discussion

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

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

While our four populations did not differ in overall SAM production, we did find significant variation in the relative amounts of individual SAMs. Our dataset of four populations is obviously too small to draw firm conclusions on the drivers of geographic variation in SAM production and composition, and sampling more populations is necessary to look deeper into some of the trends reported here. Nevertheless, we hope that our data for the current populations will be useful for more elaborate comparisons (within *S. atra,* or within the genus *Salamandra*).

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

It is possible that this lack of variation in overall SAM quantities can be attributed to a similar predation rate across our populations. Indeed, our predation risk assessment suggested similar predator diversities in most populations (4 or 5 species confirmed), with only Prenj having a relatively lower number of snake species (2 confirmed). Nevertheless, it is currently not clear to what extend this predator diversity captures predation pressure. Both *N. natrix* and *V. berus* show geographic variation in the frequency of amphibians in their diet (Luiselli *et al.*

1995; Luiselli *et al.* 2005). The frequency with which salamanders are consumed may also differ among the species listed in Table 2. Additional data on snake diet and density at each location will help us to obtain more robust estimations of predation pressure~~.~~

Relative amounts of samandarine were higher in Prenj compared to Gorski Kotar. This pattern is highly similar to the observed variation in the total amount of SAMs, which is not surprising given that samandarine is the major compound of *Salamandra* skin secretions (Habermehl, 1971; Mebs & Pogoda, 2005; Vences *et al.*, 2014). Samandarine is often referred to as the most potent of the SAMs (Geßner & Esser 1935a; Kellaway 1939), yet, it is more abundant in Prenj, where snake diversity is low, compared to Gorski Kotar. Previous studies have often found a positive association between measures of predation pressure and toxin concentrations. E.g. Hague *et al.* (2016) showed that Pacific newts (*Taricha granulosa*) have lower concentrations of TTX in allopatry with their TTX-resistant snake predators, and Bokony *et al.* (2019) suggested that toads in anthropogenic habitats invest in more potent toxins due to a higher density of predators compared to natural habitats

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

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

Salamanders showed no population differences in the relative amount of samandarone. As our four populations barely differed in 'infection risk', showing no significant differences in bacterial densities, parasite abundance or overall fungi diversity, it may not be surprising that samandarone-levels do neither. In fact, the only population-differences were found in parasite diversity, with Gorski Kotar scoring higher than Prenj and Prokletije, which did not correspond to an increase in samandarone-levels. Nevertheless, while we focus on bacteria and fungi in this study, amphibian toxins may also inhibit infection by other agents such as protozoa, trematodes or ranavirus (Rivas *et al.* 2009; Calhoun *et al.* 2017; Johnson *et al.* 2018). A negative correlation between individual TTX-levels and parasite richness (including both micro- and macroparasites) in *Taricha*-newts was shown by Johnson *et al.* (2018). Hence, future studies should take into account how populations of *S. atra* differ in their exposure to a broader range of parasites. It is worth mentioning that we did not find any traces of *B. dendrobatidis* nor *B. salamandrivorans* in our soil samples, the chytrid fungi responsible for worldwide declines and extinctions of amphibian populations (Skerratt *et al.* 2007; Tobler & Schmidt 2010; Martel *et al.* 2013). This is consistent with previous screenings in Prenj and Čvrsnica (Šunje *et al.* 2018).

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

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

Conclusions and future prospects

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

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

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Chapter 6

Alpine salamanders (*Salamandra atra*, Amphibia: Salamandridae) from drier environments are more resistant to water loss compared to conspecifics from wetter habitats

Emina Šunje and Raoul Van Damme, *in prep*.

Abstract. Desiccation risk may limit the activity window and distribution of terrestrial amphibians. Interspecific studies suggest that amphibians adapt to dry conditions through anatomical, physiological and behavioural changes, but whether water resistance correlates with desiccation risk at the population level is unclear. Here, we report on two experiments in which we determined water loss rates under different combinations of temperature and humidity in the Alpine salamander, a species with disjunct distribution. The study involved individuals originating from a warm, humid habitat (Gorski Kotar [GK], Croatia) and individuals from a cold, dry habitat (Mt. Čvrsnica [CV], Bosnia and Herzegovina). In the first experiment, where animals could not control water loss behaviourally, GK salamanders exhibited higher water loss than CV salamanders, suggesting local adaptation or plasticity. In the second experiment, where salamanders were given the opportunity to behaviourally regulate water loss, individuals used this opportunity independent of their origin. Lab experiments revealed the relationship between water loss rate, temperature and humidity that we used to estimate water loss rates under realistic field conditions. This analysis indicates that desiccation risk may be a key factor determining the specie's activity window, and may have driven the increase in water loss resistance in the CV population.

Introduction

Resistance to water loss is a major physiological trait that influences the geographical distribution of many terrestrial plants and animals (e.g. Borchert, 1994; Chown *et al.*, 1995; Diaz *et al.*, 2013; Watling & Braga, 2015). With an integument missing any of the obvious structural adaptations for increased resistance (fur, scales, feathers), amphibians in particular should be prone to desiccation (Wygoda, 1992; Toledo & Jared, 1993). Nevertheless, amphibians survive in a wide range of habitats and climates, and several species have even conquered some of the world's driest habitats (e.g. Mayhew, 1968; Dayton & Fitzgerald, 2006).

Interspecific analyses suggest that amphibian skin itself can evolve in response to hydric conditions (e.g. Christensen, 1974; Grover, 2000; Watling & Braga, 2015; Titon *et al.*, 2010). Many species are also known to avoid desiccation through behavioural changes, by carefully adjusting activity windows, by selecting suitable microhabitats or by engaging in water conserving postures (e.g. Wells, 2007; Hillman *et al.* 2009; Tracy *et al.*, 2014; Vitt & Caldwell, 2009). Physiological changes (e.g hormonal or organic) to minimize water loss are also well documented (Vitt & Caldwell, 2009).

While among-species variation in physiology and behaviour in relation to hydric conditions is relatively well studied, the literature on within-species variability in these traits is scarce and inconsistent (Winters & Gifford, 2013). Geographic variation in water loss resistance (WLR) has been studied in a handful of frog species. In about half of those species, populations inhabiting drier habitats have developed increased WLR (Warburg, 1967; Canziani & Cannata, 1979; Rudin-Bitterli *et al.*, 2018; Geise & Linsenmair, 1988) while in others, WLR did not vary with the habitat dryness (Jameson 1966; Cruz-Piedrahita *et al.*, 2018). In some species of the toad genus *Rhinella*, populations inhabiting dry habitats even lose water more rapidly than populations from moist habitats (Prates & Navas, 2009;

Tingley *et al.*, 2012; Brusch *et al.*, 2019). However, in other *Rhinella* (Cruz-Piedrahita *et al.*, 2018) and toad species (Hoffman, 2014; Vimercati *et al.*, 2018), WLR is not correlated with habitat aridity. The results are equally disparate for salamanders. In plethodontine species, populations from drier habitats can exhibit both decreased (Gross, 1982; Winters & Gifford, 2013; Riddell & Sears, 2015) and increased (Riddell & Sears, 2015) rates of water loss. Of the Old World salamanders, only the Near Eastern fire salamander (*Salamandra infraimmaculata* Martens, 1885) has been studied in this respect; Israeli specimens from moist habitats lose water more rapidly than those from semi-arid conditions (Degani 1981; Degani, 1982). A literature review on intraspecific water economy of amphibians is given in Table S6.1.

The paucity and disparity of information on geographic variation in WLR is unfortunate; in the absence of long-term time series, variation among contemporary populations is probably the best proxy for studies into the consequences of current anthropogenic climate change (Valladares *et al.*, 2014). Only by acquiring data for a wider range of species can we hope to detect ecological or phylogenetic patterns in the evolutionary or phenotypic flexibility of WLR that will help us predict the effect of changes in aridity (e.g. see Peterman & Semlitsch, 2014; Riddell *et al.*, 2017).

Here, we study WLR in individual Alpine salamanders (*Salamandra atra prenjensis*) taken from two isolated populations living in different habitats along the Dinaric Alps. One population (Gorski Kotar, GK) inhabits a lowland forested area; the other population (Mt. Čvrsnica, CV) occupies mountain meadows situated above the treeline. The thermal and hydric conditions differ considerably between the sites, with the salamanders at GK enjoying warmer and more humid conditions than their conspecifics at CV (see Supporting information).

We compare WLR of individuals from both populations under different combinations of temperature and humidity. In addition, we examine whether salamanders from the two sites exhibit differences in behaviour that could compensate for differences in WLR. This is, to our knowledge, the first study in salamanders that looks at geographic variation in physiological and behavioural regulation of WLR rates.

Material and methods

Salamander sampling and maintenance

We studied alpine salamanders originating from two populations, one is situated in Gorski Kotar (GK, Northern Dinarides - 45.23°N, 14.95°E), and the other at Mt. Čvrsnica (CV, Central Dinarides - 43.64°N, 17.64°E); the two sites are approximately 350km of air distance apart (Fig. S6.1). The habitat of GK is characterized by mixed deciduous-coniferous forest, composed mainly of beech (*Fagus sylvatica*) and fir (*Abies alba*) trees with a dense understory of ferns and rotting logs. The habitat of CV consists of exposed, rocky alpine grasslands dappled with patches of dwarf mountain pine (*Pinus mugo*). Compared to GK, CV is significantly cooler and drier (see Supporting information and Fig S6.1).

In August-September 2016, we collected nine salamanders (four males, four females and one juvenile) from GK (1003 – 1172 m a.s.l) and 20 salamanders (eight males and twelve females) from CV (1663 – 1849 m a.s.l) and transported them to the University of Antwerp (Belgium). During the period of the experiments, each animal was kept in an individual box (home terraria – L 20cm × W 15cm × H 15cm) containing a wet cloth, a piece of stone and moist wood for shelter. Home terraria were kept in a climate chamber at a temperature between 8 – 10°C. Salamanders were checked daily. The cloth and box were washed every ten days, but faeces was removed from the box upon notice. The animals were fed two to three house crickets (*Acheta domesticus*) and an equal number of house fly larvae (*Musca domestica*) once a week but on different occasions. To provide the essential minerals and vitamins, the crickets and larvae were fed carrots, oranges and calcium before they were given to the salamanders.

Experiment 1: Evaporative water loss in restricted conditions

Experiment 1 was conducted on five individuals from GK and 20 individuals from CV. We monitored changes in body mass of these salamanders over 14 treatments (five different temperature values: 5°–10°–15°–20°–25°C, each combined with three humidity values: 50% – 70% – 90%). Results of the treatment on 10°C and 50% humidity were excluded from the analyses because of a failure in the humidity control system.

The experiments were conducted in a large climate chamber (L 2.25m × W 2m) at the laboratory of the Systemic Physiological and Ecotoxicological Research Group of the University of Antwerp. In the climate chamber, the temperature was set as desired, and the relative humidity was controlled by means of a purpose-build humidistat, connected to an Arduino UNO micro-controller board sporting a humidity sensor. The desired conditions of each treatment were set up two hours before the beginning of the experiment. During these two hours, both the temperature and relative humidity in the chamber were checked every 15min with a digital hygrometer and thermometer (Adorini Hygrometer Digital).

We placed seven empty plastic containers (L 70cm × W 40cm × H 30cm) in the climate chamber and divided each into four equal sections (L 35cm × W 20cm) using cardboard barriers. The terrariums were kept open, but the edge of their top was lined with wide tape to prevent the animals from escaping. We weighed each salamander (mass1) on a digital balance (Ohaus Scout Pro Electronic Balance - 600g, precision = 0.01g), then placed it into one of the sections. After one hour, we weighed the animals again (mass2) in the same order as in the beginning of the experiment. The change in body mass was taken as an estimate of the amount of bodily water lost through evaporation (evaporative water loss - EWL, see Warburg & Degani, 1979). Animals that urinated, defecated or shed skin during the experimental trails were excluded from the dataset. After each experimental trial, we returned the animals to their home terraria and sprayed them with water. The sequence of treatments was randomized and the animals were subjected to a maximum of two treatments per day, with a minimum of six hours break in-between successive trials to allow rehydration (Spight, 1967; Spotila, 1972).

Experiment 2: Evaporative water loss in unrestricted conditions and behavioural observations

In experiment 2, we logged the behaviour and evaporative water loss (EWL) of salamanders under 24 different treatments (eight temperature values: 5°–8°–10°–12°–15°–17°–20°–25°C, each combined with three humidity values: 50% – 70% – 90%). For this experiment, we followed nine salamanders from GK and 20 from CV. Temperature and humidity conditions were obtained as explained in experiment 1. This time, a total of eight plastic terrariums (L 70cm × W 40cm × H 30cm) were placed inside the climate chamber for the observation of salamander behaviour. The sides of the terrariums were screened with brown paper to prevent visual contact between individuals placed in neighboring terraria which could have affected their behaviour (each terraria contained one individual). The terrariums were kept open but the top edges were taped as notified in experiment 1. We covered the floor of the terraria with dry gravel (80%) and mulch (20%) and further enriched it with branches and stones. In one of the corners we positioned a piece of soil covered with moss (c 10cm × 15cm × 3cm). We assured that the piece of moss was always moist by spraying it with water whenever required. The animals in the terraria were filmed from above (camera types: Coolpix 885 Nikon and HD Everio JVC). The individuals were given two hours of time to accustom to the preset environmental conditions (treatments) before the filming, during which, temperature and humidity were followed as in experiment 1. Throughout the experiment, temperature and humidity were logged every minute with a USB data logger (PCE HT71N - 10). Two to five minutes before the onset of the experiment, each individual was weighed with a balance (Ohaus Scout Pro Electronic Balance - 600g, precision = 0.01g; mass1) and put back in the middle of the terrarium. Subsequently, its behaviour was filmed for 120 minutes. After this period, each individual was weighed again (mass2) in the same order as before the filming started. The difference between mass1 and mass2 was taken as an estimate of the EWL (similar to Warburg & Degani, 1979). After each experimental trail, we returned the animals to their home terraria and sprayed them with water. The sequence of the treatments was randomized and animals were given at least 48h of rest in-between successive trials.

From the videos, we quantified the duration of the following behaviours using the JWatcher software (Blumstein *et al.*, 2006): (i) time spent above ground (climbing, walking, resting on the ground and attached to the wall), (ii) time spent hiding (under moss and under branches), (iii) time spent on moss pad. Bouts of a certain behaviour were counted only if they lasted at least 3 seconds.

Data analysis

Throughout this paper, we express evaporative water loss relatively (relEWL) - per unit body mass (g water × gram body mass-1 × hr -1) to correct for size differences among salamanders. We used body mass rather than surface area, because the former is much easier to measure. Surface area and body mass are tightly interrelated (Santini et al. 2018), and within the size range considered here, can be used interchangeably. We converted each behaviour in percent of total movie time. To avoid the 'constant sum problem' (the sum of the duration variables is always 100, hence the individual variables are not allowed to vary independently), we followed Aitchison (1981, 1986) and expressed the raw percentages as logarithms of ratios, where the denominator is the geometric mean of the percentages in each sample. We replaced all zero values (behaviour did not occur) into 0.001 to avoid that the geometric mean would be zero in most cases. Each behaviour (expressed as log of ratios; Kucera & Malmgren, 1998) was treated as a dependent variable.

Analysis were conducted in R (R Core Team, 2020). Variation in the relEWL and behaviours was analysed using linear mixed models (package lmerTest: Kuznetsova et al., 2017). Temperature and humidity, as continuous variables, together with origin of population, were entered as fixed factors; the interaction among the three was also taken in consideration. Two random factors were included in each model: (i) individual code of the salamander nested in population, and (ii) code of the terrarium (its position in the climate chamber). In total, we fitted 16 models by dropping the interactions and main factors one by one, using a hierarchical approach. The model with the lowest AIC/BIC value was retained, and the significance of its variables was checked with function "anova" (Kuznetsova et al., 2017).

To test whether relEWL in experiment 2 was affected by the salamanders' behaviour, we compared a 'raw' with three 'behavioural' models. The 'raw' model predicted relEWL from the experimental temperature and humidity and their interaction; the 'behavioural models' in addition considered one of the three behavioural variables (active above ground, hiding, on moss) as an independent variable. Fit of the respective behavioural models was compared to that of the 'raw' model' using function "anova" (Kuznetsova *et al.*, 2017).

Ecological relevance

In an attempt to gauge the ecological relevance of the geographic variation in water loss resistance in our study species, for each population we used the equations obtained in experiment 1 to predicted water loss under field conditions. Monthly means for minimal, maximal and mean air temperature for

GK and CV were obtained from WorldClim 2.0 (30″ spatial resolution, 1970–2000 temporal range, Fick & Hijmans, 2017; see Supporting information for details) and monthly means for relative humidity were taken from the Aquastat database (10′ spatial resolution, 1961–1990 temporal range, New *et al*., 2002). From the monthly mean relative humidity and air temperatures, and using formula (2) in Eccel (2012), we calculated monthly averages of saturation pressure (eS) and then, using formula (9) in the same paper, average vapour pressure (e). Next, assuming that the diurnal variation in relative humidity is primarily due to eS, and not e (Eccel, 2012), we calculated relative humidity for each hour of an 'average' day per month. The hourly air temperature was calculated using a sine function described by formulas (2.2) and (2.3) in Campbell & Norman (1998), which only requires minimal and maximal air temperature. Finally, the hourly values of relative humidity and air temperature were fed into the equations obtained in experiment 1 to predict relative water loss on an hourly basis, for average days in the months May to September which covers the activity season of the study species.

Results

Both relEWL and the Aitchison-transformed behavioural variables tend to be normally distributed (Shapiro-Wilk statistics ≥ 0.90). Usage of the Aikake's (AIC) and Bayesian information criteria (BIC) result in the same models selected among those considered (Tables 1 and 2).

Experiment 1

Relative water loss in restricted conditions is best explained by the model lm9 (Table 1) that contains humidity (DenDf=318.3, F=913.0, $p<0.001$), population of origin (DenDf=340.4, F=124.0, $p<0.001$) and their interaction (DenDf=318.3, F=70.4, $p<0.001$). As expected, salamanders loose water at faster rates in drier conditions (releWL, Fig. 1). GK Individuals have higher relEWL than CV individuals at all temperature × humidity combinations, but the difference is most pronounced in drier set ups (Fig. 1).

Experiment 2

Relative water loss in unrestricted conditions is best explained by the model lm14 (Table 1) that contains only humidity (DenDf=639, F=59, p<0.001). Even when given the opportunity to behaviourally compensate, salamanders exhibit higher relEWL at lower humidity's (urelewl, Fig. 1). Mean values for relEWL in experiment 2 do not differ between populations (lm13 in Table 1, pop: DenDf=27.9, F=2, p=0.17; Fig. 1).

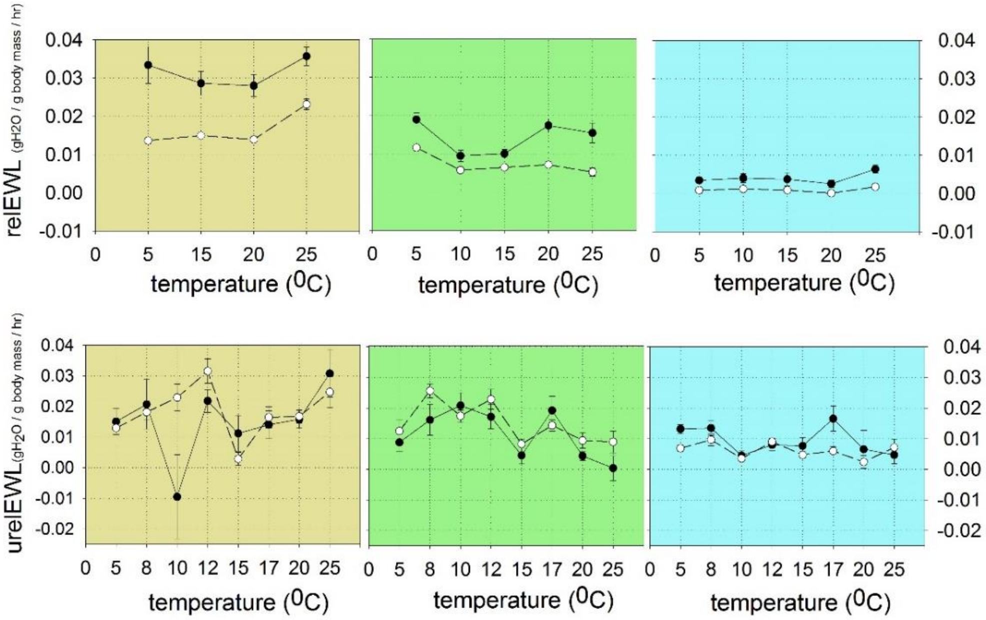

Figure 1. Relative salamander mass loss (g H_20 /g body mass/hr) through evaporation in experiment 1 (restricted conditions - rrelewl) and experiment 2 (unrestricted conditions - urelewl). Each humidity level is depicted with a different colour: 50% (yellow), 70% (green), 90% (blue). Filled symbols connected with a solid line show mean values for individuals from Gorski Kotar; white symbols and the dashed line are mean values for individuals from Čvrsnica. Bars illustarte standard errors.

In experiment 2, the salamanders change their behaviour according to the temperature and humidity conditions (Table 2). Salamanders from both populations reduce their activity (on ground) at higher temperatures (lm5 - DenDf=628.01, F=4.8, $p=0.03$) and lower humidity (lm5 - DenDf=627.8, F=52.7, $p<0.001$), with the effect of temperature being more pronounced at 70% and 90% humidity (lm5 - DenDf=628.5, F=30.5, $p<0.001$; Fig. 2). The animals are more often seen hiding in drier (lm7 - DenDf=631, F=48.2, $p<0.001$) and warmer (lm7 - DenDf=631.5, F=133.7, $p<0.001$; Fig. 2) conditions. The time spent on the moss pad may be explained by humidity (lm14 and lm13 - DenDf=641.7, F=9.0, $p=0.003$), although a model with equal AIC/BIC values is also reported (lm16, Table 2); another model suggests that it is also influenced by temperature (lm 15 - DenDf=642, F=4.8, $p=0.03$) where in warmer conditions, animals visit the pad more frequently (Fig. 2)

None of the favoured models contains a population × temperature or a population × humidity interaction, indicating that the effect of climatic conditions on behaviour is very similar for salamanders of different origin (Table 2). However, across the examined thermal and hydric conditions, salamanders from CV exhibit significantly higher aboveground activity (lm5 -DenDf=25.31, F=16.2, $p<0.001$) and are less often seen hiding (lm 7 - DenDf=25.34, F=13.15, $p=0.001$) than individuals from GK; populations make equal use of the moss pad (lm 13 and lm 16 - DenDf=27, F=1.3, $p=0.3$; Fig. 2). Adding the behavioural data (active aboveground, hiding, on moss) to the raw model of relEWL improves the fit significantly (aboveground activity: $Chi^2=87.4$, Df=4, $p<0.001$; hiding: $Chi^2=78.1$, Df=4, $p<0.001$). For any set of temperature and humidity conditions, animals that are more active aboveground (or are hiding less), loose notably more water than conspecifics with the opposite behaviour. The time spent on moss does not explain the variation in relEWL ($Chi^2=5.4$, Df=4, $p=0.24$), suggesting that this behaviour may be a good strategy to prevent relEWL.

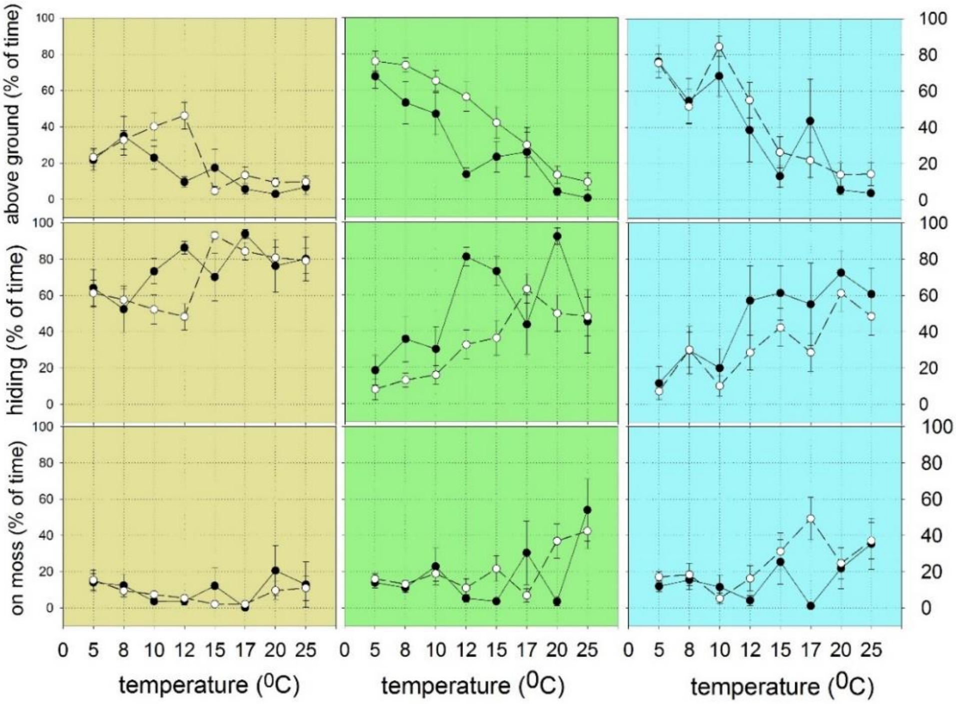

Figure 2. Salamander behaviour in experiment 2 expressed as percent (%) of total movie time (120 minutes). From up till down (% of time): above ground, hiding, on the moss pad. Each humidity level is depicted with a different colour: 50% (yellow), 70% (green), 90% (blue). Filled symbols connected with a solid line show mean values for individuals from Gorski Kotar; white symbols and the dashed line are mean values for individuals from Čvrsnica. Bars illustarte standard errors.

Ecological relevance

Feeding the equations obtained from experiment 1 with combinations of realistic environmental temperature and relative humidity (monthly database averages), reveals clear differences between the two study sites. In GK, exposed salamanders are predicted to lose a considerable amount of water during the daytime, but not between sunset and sunrise (Fig. 3 and Fig. S6.3). In CV, salamanders were predicted to lose water even during parts of the night (Fig. 3 and Fig. S6.3). In most circumstances, salamanders with a GK-type of water balance would lose more than twice as much water as salamanders with a CV-type of water balance during daytime (between sunrise and sunset, Fig. 3).

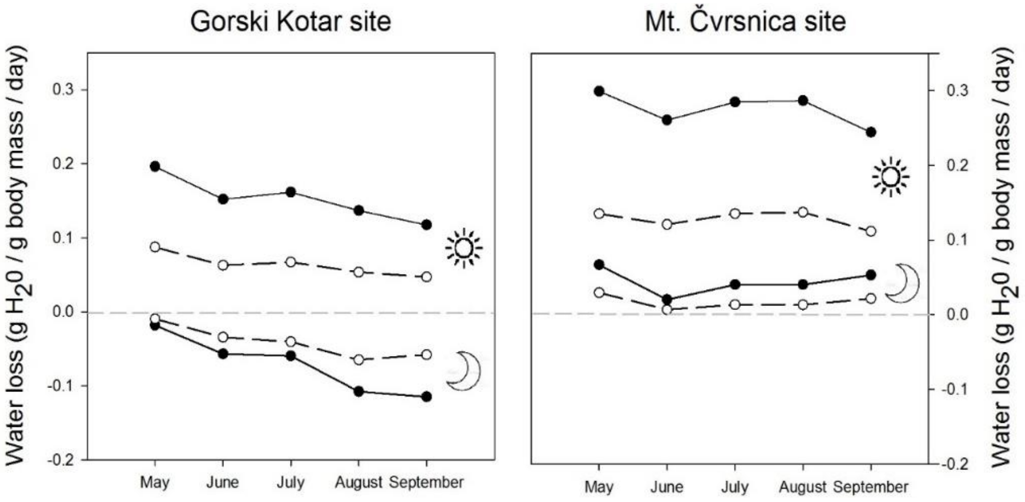

Figure 3. Expected water loss in salamanders originating from Gorski Kotar (black symbols, full lines) and Mt. Čvrsnica (white symbols, dashed lines) when exposed to average field conditions of temperature and humidity in months of salamanders' activity (x- axis) at both study sites. The relative amount of water exchanged with the environment (grams of water per gram of body mass) is shown for a full day (24hrs) but divided over the day period (hrs from sunrise to sunset - sun) and night (hrs from sunset to sunrise - moon). See Fig. S6.3 for more details.

Table 1 AIC and BIC values of the fitted models (lm) of relative evaporative water loss (relewl) in experiments 1 and 2. Pop – population, temp – temperature, hum – humidity. The asterix indicates the variables (and interactions) included in each model. The model of choice is bolded

MODEL	lm1	lm2	lm3	lm4	lm5	lm6	lm7	lm8	lm9	lm10	lm11	lm12	lm13	lm14	lm15	lm16
pop:temp:hum	*															
pop:temp	*	*		*		*				*						
pop:hum	*	*	*			*			*							
temp:hum	*	*	*	*	*			*								
pop	*	*	*	*	*	*	*		*	*		*	*			*
temp	*	*	*	*	*	*	*	*		*	*	*			*	
hum	*	*	*	*	*	*	*	*	*		*		*	*		
Experiment 1:																
AIC	-2641	-2661	-2680	-2616	-2635	-2675	-2651	-2605	**-2713**	-2244	-2621	-2262	-2670	-2640	-2239	-2278
BIC	-2598	-2623	-2646	-2582	-2604	-2640	-2624	-2578	**-2686**	-2217	-2598	-2239	-2646	-2621	-2219	-2259
Experiment 2:																
AIC	-3667	-3686	-3703	-3670	-3717	-3704	-3736	-3728	-3737	-3683	-3747	-3700	-3751	**-3763**	-3711	-3715
BIC	-3616	-3641	-3662	-3659	-3681	-3664	-3704	-3697	-3705	-3651	-3720	-3673	-3724	**-3740**	-3688	-3693

Table 2 AIC and BIC values of the fitted behavioral models (lm) in experiment 2. Pop – population, temp – temperature, hum – humidity. The asterix indicates the variables (and interactions) included in each model. A - “active above ground”, H - “hiding”, M - “on moss”. The model of choice is bolded; values similar to the model of choice are in grey colour - lm13 and lm16 (for “M”) omit differences between populations (DenDf=27.1, F=1.3, p=0.26; see text for detailes)

MODEL	lm1	lm2	lm3	lm4	lm5	lm6	lm7	lm8	lm9	lm10	lm11	lm12	lm13	lm14	lm15	lm16
pop:temp:hum	*															
pop:temp	*	*		*		*				*						
pop:hum	*	*	*			*			*							
temp:hum	*	*	*	*	*			*								
pop	*	*	*	*	*	*	*		*	*		*	*			*
temp	*	*	*	*	*	*	*	*		*	*	*			*	
hum	*	*	*	*	*	*	*	*	*		*		*	*		
A:																
AIC	4185	4174	4170	4167	**4163**	4191	4180	4175	4346	4203	4192	4199	4339	4350	4211	4351
BIC	4235	4219	4211	4208	**4199**	4232	4212	4206	4378	4235	4219	4226	4367	4373	4233	4374
H:																
AIC	4295	4284	4281	4277	4275	4282	**4273**	4285	4394	4313	4283	4310	4387	4397	4320	4416
BIC	4345	4329	4322	4318	4311	4323	**4304**	4317	4426	4345	4310	4337	4414	4419	4343	4439
M:																
AIC	3711	3700	3695	3692	3687	3695	3682	3686	3687	3686	3681	3681	3679	**3678**	3680	**3678**
BIC	3761	3745	3735	3733	3723	3736	3713	3717	3718	3718	3708	3708	3706	**3700**	3703	**3700**

Discussion

The results of experiment 1 show that salamanders from Mt Čvrsnica have reduced relEWL rates compared to those from the Gorski Kotar site. This finding is in line with the idea that amphibians from drier habitats should develop higher water loss resistance. The result is also in agreement with earlier observations on intraspecific variation in water loss rates in salamanders. Elevational differences in water loss resistance have been demonstrated in the lungless salamanders *Plethodon wehrley* (Gross, 1982), *P. montanus* (Winters & Gifford, 2013) and *P. metcalfi* and *P. teyahalee* (Riddell & Sears, 2015). In all four species, individuals from wet, cool high-elevation sites lost water more rapidly than individuals from dry, warm low-elevation sites. In the Near Eastern fire salamander, *Salamandra infraimmaculata*, individuals from an area with permanent water bodies exhibited increased water loss rates compared to individuals from two habitats which are dry in summer (Degani 1981; Degani, 1982). Overall, salamanders seem to behave more predictably in this matter than frogs and toads, in which specimens from populations exposed to drier, warmer conditions can have higher (Warburg, 1967; Canziani & Cannata, 1979; Rudin-Bitterli *et al.*, 2018; Geise & Linsenmair, 1988), similar (Jameson, 1966; Cruz-Piedrahita *et al.*, 2018; Cruz-Piedrahita *et al.*, 2018; Hoffman, 2014; Vimercati *et al.*, 2018) or even lower (Prates & Navas, 2009; Tingley *et al.*, 2012; Brusch *et al.*, 2019) water loss resistance than those from cooler, wetter habitats (see literature review in supplementary Table S6.1).

However, the observed differences in relewl rates between the two studied populations should be taken with caution. Firstly because all caveats relating to two-species comparisons (Garland & Adolph, 1994) apply to our study. Secondly, using the body mass for the standardization of ewl rates across individuals, may have introduced some error. Terrestrial salamanders are known to store considerable amounts of fluid in their bladder. In extreme cases, this urine may constitute up to 72% of the total body mass (Jørgensen 1997). Because we did not want to stress our animals, we did not try to remove any fluid present in the bladder before weighing them. Therefore, for some individuals, body mass may be overestimated. However, our study species *S. atra* does not often seem to store much fluid in its bladder (<10% body mass, personal observation). Also, individuals of both populations might have stored urine or not, so a systematic bias seems unlikely. Other possible mechanistic changes that may be responsible for the observed differences in water loss resistance in our study species remain an open question. These may involve other morphological changes than body size, such as alterations in the thickness, composition, texture and sculpturing of the skin (Toledo & Jared, 1993), or in subcutaneous vascularisation (Burggren & Vitalis, 2005). Further, it may implicate physiological modifications, e.g. variation in the amount or composition of mucous skin secretions produced by cutaneous glands (Lillywhite & Licht, 1975), size of the urinary bladder as water reservoir (Jørgensen 1997) or the activity of hormones that are known to influence skin perfusion (Bentley, 2002; Uchiyama & Norifural, 2005; Cooper, 2017). At a molecular level, differences in the density or activity of water channel proteins (aquaporines) in the plasma membrane of skin cells could be involved (Suzuki *et al.*, 2007).

It is also unclear whether the inter-populational variation in evaporative water loss in *S. atra* may have a genetic base or represents a case of phenotypic plasticity. Riddell and Sears (2015) have argued that variation in water loss resistance among populations of lungless salamanders *P. metcalfi* and *P. teyahalee* was likely to be plastic, because suitable habitats for the species were available along the elevational gradient from which the populations were sampled. In a recent paper, Riddell *et al.* (2019) have confirmed that in *P. metcalfi*, a differential, thermal dependent expression of the same gene (involved in angiogenesis) leads to phenotypes of high or low resistance to water loss. More precisely, a high temperature will downregulate the stem cell differentiation and vessel development while a lower temperature upregulates this genetic machinery. Such mechanism remains to be explored in alpine salamanders moreover because, compared to terrestrial salamanders in general (*Salamandra* Garsault, 1764), the lungless *Plethodon metcalfi* might develop a trade-off between respiration and skin permeability. To our knowledge, no other studies have addressed the issue of whether plasticity of genetic adaptation underlies geographic variation in water loss resistance in amphibians. The two populations of *S. atra* studied here are well isolated from one another, facilitating local genetic adaptation. Whether this condition has effectively prompted changes at the genetic level, awaits investigation.

The results of our second experiment demonstrate that *S. atra* salamanders adjust their behaviour when thermal and/or hydric conditions become unfavourable, by becoming inactive, but also by visiting the moist moss pad more frequently. These observations corroborate earlier findings that salamanders may avoid adverse climatic conditions by vertical movements within the soil (e.g. Vernberg, 1953; Caldwell & Jones, 1973), but also by horizontal shifts in microhabitats (Camp *et al.*, 2013; Farallo & Miles, 2016). Our results also indicate that these behavioural shifts will affect their evaporative water loss rates.

We note that, in experiment 1 we did not find a linear relationship between temperature and ewl rates (Fig. 1) which, at first, is contra intuitive. However, behavioural data reveal that, across treatments, salamanders are considerably more active at lower temperatures (till 10°C, Fig. 2). As activity significantly increases ewl rates (Chi2=87.4, Df=4, $p<0.001$, see results), the explanation for the not-linear relationship between temperature and ewl (Fig. 1) may be due the salamanders' behaviour since animals were probably more active during colder treatments in experiment 1 (as observed in experiment 2) that, in turn, caused a higher relewl at lower temperatures when compared to relewl rates registered during warmer treatments (Fig. 1).

We found no indication that individuals of the GK population (warm, wet habitat of origin) compensated behaviourally for their lower water loss resistance by visiting the moss pad more often. However, individuals from the CV population (cold, dry) were considerably more often seen active above ground, across temperature and humidity treatments, suggesting that their higher resistance to water loss may allow them to expand their activity window. Relaying our laboratory findings to (average) field conditions suggests that water balance may be a key factor influencing the activity of the Alpine salamander. At the GK study site, evaporative water loss is unimportant to the animals for most of the night, but becomes an issue soon after sunrise (Fig. 3 and Fig S6.3). At the CV study site, salamanders will lose water throughout much of the day but also during night (Fig. 3 and Fig S6.3), and it is tempting to view that the registered higher water loss resistance of CV salamanders might indicate a genetic adaptation or a plastic adjustment to these dry conditions. By how much climatic settings, through their effects on water loss, are restricting activity of Alpine salamanders at CV would best be evaluated by calculating an activity window, the time slot during which salamanders can be active aboveground without drying out too much. However, this would require hourly data on humidity and temperature, on a daily basis, and we do not have this information available at the time. In addition, humidity and temperature may exhibit considerable spatial variability, especially in structurally complex habitats as our study sites. Salamanders are likely to exploit this thermal and hydric heterogeneity to increase their opportunities for activity.

We believe that our results have important messages for the species distribution modelling community. Several recent studies have employed mechanistic models to predict the distribution of a species along a geographical range (see

Kearney *et al.*, 2008; Lyons & Kozak, 2020 for examples on terrestrial amphibians). These models are typically fed with species-specific data on physiology, morphology and behaviour and with rather broad-scaled climatic data, often obtained from global or regional climate databases. However, our results suggest that vital physiological characteristics (such as water loss resistance) may exhibit within-species variation and that animals such as salamanders may exploit microclimates at a scale (of centimetres) that will not be captured by standard climate databases.

Overall, our result indicate that within *S. atra* there is variation in this vital trait that is especially relevant in the current time of global climate change.

Acknowledgements

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Chapter 7

A multiple modelling approach for the conservation of the alpine salamander in the face of climate change

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* Mirza Čengić and Šunje Emina should be considered joint first author

Abstract. Species Distribution Models (SDMs) are a widely used tool to understand how species respond to climate change. In this study, we used intraspecific SDMs to model climate change impacts on two lineages of alpine salamanders that show a strong phylogeographic structure (*Salamandra atra atra* from the Alps and *Salamandra atra prenjensis* from the Dinarides); we also included one species-level model (*Salamandra atra* as an undifferentiated entity). Predictions incorporated uncertainties arising from occurrence precision and modelling choices by using species' occurrences from a carefully curated database and by applying eight modelling techniques combined with 24 GCMs and two RCP scenarios. We used model consensus layers to define the impact of climate change on each modelling unit. Projections of climate change impact differed between species and intraspecific approach and were more pronounced for predictions in current time than in the future scenarios. Compared to species models, *prenjensis* models predicted a larger climatic extent in the Dinarides. In contrast, the species and *atra atra* models did not differ considerably. The predicted decrease of climatically suitable land for the species in the Dinarides is between 51 - 87% and in the Alps between 24 - 53%. This study delivers important conservation insights formulated into concrete conservation actions. Results of this study are made available to broader public via a web application delivering species distribution maps throughout an interactive interface.

Introduction

Climate change threatens the persistence of many populations of plants and animals (Deutsch et al., 2008), and habitat specialists with relatively low dispersal ability are expected to be particularly vulnerable (Mestre, 2018). Understanding how the ranges of such species respond to the changing climate is crucial for their conservation (Bellard et al., 2012; Pacifici et al., 2015). Species distribution models (SDMs) are widely used to assess species' response to present or future climate (Elith et al., 2010; Hijmans & Graham, 2006) and subsequently to inform conservation efforts (Muscatello et al., 2021; Villero et al., 2017).

Traditionally, SDMs assume that each species responds to the environment as an undifferentiated entity, hence ignoring the fact that populations within species may adjust to local environments through phenotypic plasticity or local adaptation (Bocedi et al., 2013; Hällfors et al., 2016; Smith et al., 2019; Valladares et al., 2014). Therefore, models that consider species as single modelling entities are unlikely to capture adequately their distribution if populations within species vary in their climatic tolerance (Smith et al., 2019). Consequently, multiple studies have argued that infraspecific variability should be incorporated in SDM studies where there is evidence (or reasonable expectation) of variation in environmental responses among populations of a species (Collart et al., 2020; Hällfors et al., 2016; Homburg et al., 2014; Marcer et al., 2016; Smith et al., 2019; Z. Zhang et al., 2021). For instance, if different infraspecific units (e.g., lineages, subspecies) are expected to differ in their response to climate change, separate niche models for each unit should be constructed and their results combined to accurately predict the species' range (Smith et al., 2019).

Here we focus on the alpine salamander (*Salamandra atra*), a completely terrestrial amphibian endemic to the Alps and Dinarides. Populations of alpine salamanders are more abundant and connected throughout the Northern Alps compared to the Southern Alps and Dinarides (Bonato et al., 2018; Fig 1). Within the species, several infraspecific lineages exist: *Salamandra atra atra*, resides in the Northern Alps while two other (*S. a. aurorae* and *S. a. pasubensis*) live in the Southern (Italian) Alps. Several phylogenetic studies evidence that the Southern Alps harbour additional lineages that await formal description (Bonato et al., 2018; Helfer, 2010; Helfer et al., 2011; Riberon et al., 2004). The fourth lineage, *S. a. prenjensis*, is found in the Dinarides (Šunje et al., 2021).

Worldwide, the geographic area covering the distribution of alpine salamanders is predicted to be the most affected by climate change in the twenty-first century (Giorgi, 2006); predictions report an immense decrease in precipitation, followed by 20% warmer temperatures than the global average (Giorgi, 2006; Lionello & Scarascia, 2018). As epigeic activity of *S. atra* (feeding, mating) is conditioned by high air humidity (ca 85%) and colder temperatures (4 - 13°C) (Klewen, 1986), it is likely that climate change will considerably affect these animals, especially taking in consideration their low dispersal abilities (see Rabitsch et al. (2010) and Schlumprecht et al. (2010) in Feldmeier et al. (2020)). Predictions of climate change impact on Italian batrachofauna report a range decrease between 36-95% for *S. atra* (within Italian territory), categorizing the species as one of the most threatened by climate warming (D'Amen et al., 2011). However, although it is suspected to threaten survival, the impact of the changing climate on other populations along the species' range, remains unknown (IUCN, 2022).

In the face of climate change, this study aims developing and applying a multiple modelling approach for the conservation of the alpine salamander throughout its distribution range This implies: (i) delivering an accurate database of species' occurrence, based on available records in present time, (ii) applying multiple modelling techniques coupled with numerous climate model projections to assess the impact of climate change on species' distribution, (iii) conducting prediction analyses not only for the entire species but also for relevant infraspecific lineages, (iv) providing accessibility of model predictions to broader public. We translate findings from this work in concrete conservation actions.

Material and methods

Species occurrence data

We compiled a database containing occurrence points of alpine salamanders, including our own fieldwork data along with literature and public database records. We collected data from all published records of *S. atra* occurrence in the Southern Alps (Bonato et al., 2018) and Dinarides (Appendix S1) along with personal observations (hereafter, Bonato & Sunje database). We also extracted species occurrence data from two major databases, namely: the iNaturalist app (California Academy of Sciences and the National Geographic Society - https://www.inaturalist.org/) and Alpensalamander database (www.alpensalamander.eu, Meikl et al., 2010 - obtained by courtesy of Magdalena Meikl). Moreover, we included data from the herpetological collection of the Natural History Museum Vienna (NHMW, courtesy of Dr. Silke Schweiger) and the Global Biodiversity Information Facility (GBIF, https://www.gbif.org). The composite database consisted of a total of 5415 occurrences; we screened the database and removed uncertain and erroneous records based on an explicit suite of strict criteria of validation to assess the reliability of the species identification, and the accuracy of reported localities (criteria listed in Table S1). The number of valid and excluded data from each database, along with the most frequent exclusion criteria is given in Fig. S1. Occurrence points with accuracy > 500 m were excluded to match the resolution of climate data (ca. 1km x 1km) and, moreover, because the reliability of SDMs can be compromised by the precision of geographical position (Bloom et al., 2018; Newbold, 2010). After the validation and removal of double data (i.e., identical coordinates originating from two source databases), we remained with a total of 2235 species occurrences that we used for our analysis (Fig. 1, Appendix S6).

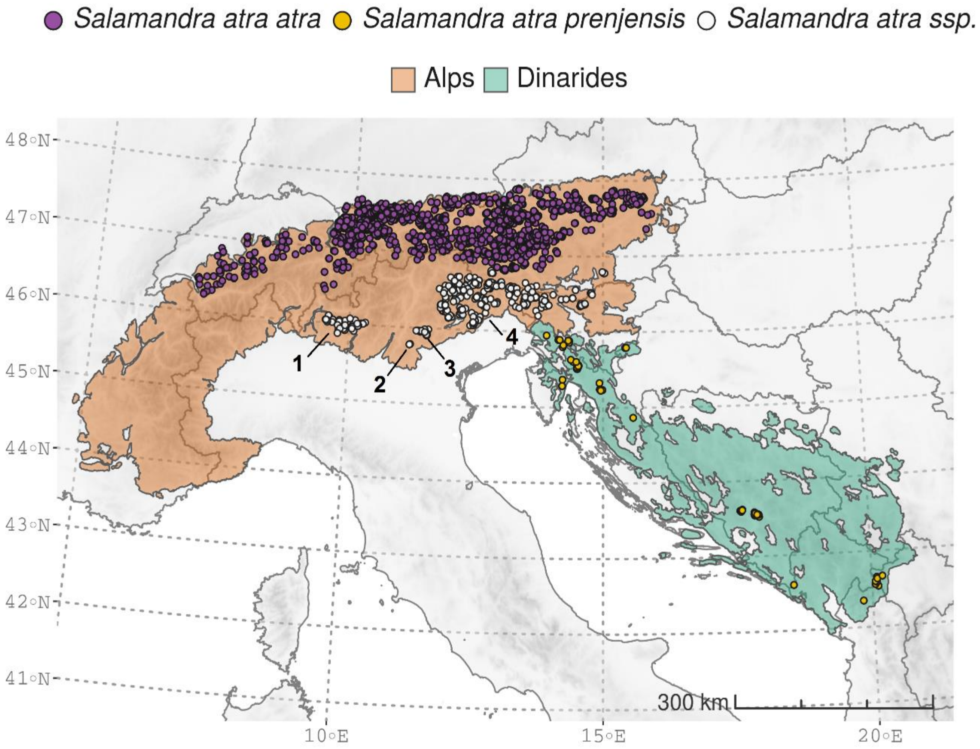


Figure 1: Occurrence points of *S. atra* used in this study. Populations marked in purple belong to the infraspecific lineage *S. atra atra,* those in yellow to *S.a. prenjensis*, and those in white to other lineages (1 – Orobian lineage, 2 – *S. a. pasubiensis*, 3 – *S. a. aurorae*, 4 – multiple lineages in the South-Eastern Alps). The boundaries of the Alps (in brown) and the Dinarides (in green) are also indicated (after Snethlage et al., 2022).

Defining units for distribution modelling

To assign each point in the database to the corresponding infraspecific lineage of alpine salamanders, we used existing data from genetic screenings followed by a reasonable principle of interpolation according to which: if a population is located in between two that belong to a single lineage (genetically identified), we considered that population most probably belonging to the same lineage. The genetic characterization of multiple populations in the Northern Alps showed that they belong to the *S. a. atra* lineage, a genetically homogenous group with no clear geographical structure (Riberon et al., 2001; Helfer, 2010). On the other hand, genetic analyses of population fragments well scattered throughout the Dinarides, revealed that they belong to a distinct infraspecific lineage - *S. a. prenjensis*. The strong genetic differentiation found among these populations suggests a lack of gene flow in the present time, but unveils their connection in the past (Razpet et al., 2016, Šunje et al., 2021). Genetic analysis of population fragments in the Southern Alps showed that this part of the species' range is inhabited by various lineages well differentiated from *S. atra atra* and *S. atra prenjensis* (Riberon et al., 2004, Helfer 2010, Helfer et al., 2011, Bonato et al., 2018); besides the already recognized *S. a. pasubiensis* and *S. a. aurorae*, there is evidence for a distinct lineage in the Orobic mountains (the "Orobian" lineage, Helfer et al., 2011; Bonato et al., 2018) while parts of the South-Eastern Alps shelter a few lineages with unclear phylogenetic position and undefined distribution ranges (Helfer, 2010; Bonato et al., 2018; Fig 1).

We performed SDM analysis for the entire species (using all *S. atra* occurrences as a single modelling unit) and we also performed separate SDM analysis for *S. atra atra* and *S. atra prenjensis* as major lineages with a non-negligible geographic extent (see Species Data in Appendix S6). We chose not to consider lineages in the Southern Alps for separate SDM analyses because their extent of occurrence is too narrow or undefined; moreover, we did not take into consideration populations from the South - Eastern Alps that are bordering with the Dinarides due to evidence of possible contact zones between *S. a. atra* and *S. a. prenjensis* (Razpet et al., 2016; Šunje et al., 2019). Data points that were not considered for infraspecific models represent 15% of the entire dataset (a total of 335 points - white coloured in Fig. 1).

Climate variables

We obtained the data for current and future climate conditions from the CHELSA v1.2 dataset that contains 19 bioclimatic variables given at 1 km spatial resolution. These variables describe different aspects of temperature and precipitation, many of which are considered relevant for species and biodiversity modelling (Karger et al., 2017). We used bioclimatic variables based on monthly temperature and precipitation for the 1979–2013 period to describe the current climate and predicted bioclimatic variables for the 2061-2080 period (from here on referred to as 2070) to describe the future climate.

From the full set of 19 bioclimatic variables, we removed variables bio8-9 and bio16-17 (climate described by wettest/driest quarter of year). Which quarter of the year is the wettest/driest varied depending on the location of the grid cell, and including these variables would introduce artificial spatial structure (see Appendix S4). To reduce the effects of multicollinearity between the climate variables, which may lead to erroneous parameter estimation (Dormann et al., 2013), we then selected only the variables with a variance inflation factor (VIF) value smaller than three (VIF < 3) (Zuur et al., 2010). We calculated VIF values with the R package 'usdm' (Naimi et al., 2014) using the current climate variable values extracted from the locations of the species occurrence points, since VIF variable exclusion based on occurrence data yields models with higher performance (Čengić et al., 2020). After the VIF-based exclusion procedure, five variables remained which were used for model fitting (Table S4), namely, bio4 (temperature seasonality), bio6 (minimum temperature of the coldest month), bio7 (temperature annual range), bio13 (precipitation of the wettest month), and bio15 (precipitation seasonality).

Niche overlap

To assess whether the infraspecific lineages occupy differing climatic niches, also in relation to the entire species, we calculated the overlap between the sets of climate niche hypervolumes, using the method proposed by Blonder et al. (2014). We estimated climate niche hypervolumes based on climate variables used for model fitting; values were extracted from occurrence points. We standardized variable values prior to the hypervolume calculation by subtracting the mean and dividing by the standard deviation (Blonder et al., 2014). We used R package "hypervolume" version 2.0.12 to construct the hypervolumes (Blonder et al., 2014), using the Gaussian kernel method (Blonder et al., 2018). We repeated the construction of hypervolumes 30 times, where each iteration was based on a different random seed, to avoid the stochasticity that can arise when a single hypervolume overlap is calculated (Blonder et al., 2014). We quantified pairwise niche overlap among modelling units, by calculating the Jaccard similarity index (Jost, 2006) and the centroid distance among the hypervolumes, following the best practices outlined by Mammola (2019).

Species distribution modelling

Assessments of climate change impacts on biodiversity using SDMs can be sensitive to the uncertainties originating from the choice of modelling techniques, Representative Concentration Pathways (RCPs, i.e., greenhouse gas concentration trajectories), and General Circulation Models (GCMs, i.e., climate models) (Sanderson et al., 2015; Thuiller et al., 2019). Hence, using multiple modelling techniques, RCPs, and GCMs has been recommended as a best practice (Thuiller et al., 2019). Following these recommendations, we used eight individual modelling techniques to link occurence data to the climate variables, namely Generalized Linear Model (GLM), Generalized Boosted Model (GBM), Random Forest (RF), Artificial Neural Network (ANN), Maximum Entropy (MAXENT), Generalized Additive Model (GAM), Classification Tree Analysis

(CTA), and Multiple Adaptive Regression Splines (MARS). We projected future climate under two RCPs adopted by the IPCC, namely the RCP2.6 and RCP8.5 scenarios (Meinshausen et al., 2011). To account for uncertainties arising from the choice of different GCMs, we modelled the climate response under both RCP scenarios using 24 different GCMs (Table S3).

We fitted individual models for three modelling units, namely the two intraspecific models and the generic species model (See species data in Appendix S6). To avoid pseudoreplication effects (Hargrove & Pickering, 1992), we subsampled occurrence points to keep one point per 1 km^2 grid cell of the bioclimatic data set. After subsampling, we were left with a total of 1,786 occurrence points for the species model, 1,437 occurrence points for the *atra* model, and 59 occurrence points for the *prenjensis* model; these numbers directly reflect the number of occupied grid cells by the species and the respective intraspecific lineages. We created 1,000 randomly selected pseudo-absences within the processing extent for each of the modelling units, where we defined the processing extent as the rectangular extent of ecoregions (Olson et al., 2001) that intersect with the occurrence points of the alpine salamander (see Appendix S2 for additional info). We used a fixed number of 1,000 pseudo-absences, as the number of pseudo-absences used for model fitting does not show a strong effect on model performance (Čengić et al., 2020). We used equal weighting of occurrences and pseudo-absences in model fitting (Barbet-Massin et al., 2012). We evaluated the predictive performance of models using a cross-validation approach, where we used 70% of data for model fitting, and the remaining 30% for model evaluation. We quantified the model performance using the True Skill Statistic value (TSS). We used R package biomod2 to implement the described species distribution modelling procedure (Thuiller et al., 2016).

Climate suitability

To assess the impact of climate change on the distribution of alpine salamanders, we used projected suitability layers which we converted into binary climate suitabilities (presence/absence) by binarizing continuous projections using a threshold that maximizes TSS (Liu et al., 2016). More precisely, for each modelling unit, we transformed predicted suitabilities across 8 projections (for 8 modelling techniques) for current climate, and across 192 projections (8 modelling techniques and 24 GCMs) for each future RCP scenario, into binary predictions (presence/absence maps). Then, for each grid cell, we summed the binary predictions and divided that sum by the number of model projections. The resulting model consensus score can range between 0 and 1; for grid cells where the value is 1, 100% of models agreed that the taxon considered was present; if the value is 0, 0% of models agree that grid cell is projected as present. We constrained the raster projections to the polygonal outline of mountain regions inhabited by the alpine salamander, i.e. the Alps and Dinarides (Fig 1), taken from Snethlage et al. (2022). The polygonal outlines of the Alpine and Dinaric mountain regions are 174,990 km^2 and 82,728 km^2, respectively.

We used binarized projections to quantify the extent of suitable climate and to quantify changes in the extent of suitable climate between current and future climatic conditions. For each lineage, we quantified separately the extent of suitable climate in the mountain region that it occupies (using mountain region definition based on Snethlage et al. (2022); Fig. 1) while for the species model, we present separate quantifications for the Alpine and Dinaric region to allow comparison with infraspecific models. To this end, for each possible combination of modelling unit, modelling technique, GCM, and RCP scenario, we estimated the size of suitable climate space as the area that grid cells projected as suitable to occupy. To minimize the raster distortions and consequently distortions in the calculation of grid cell area, we calculated the extent of suitable climate (in km^2)

using a local UTM33N projection. Changes in the extent of suitable climate between current and future climatic conditions were quantified using pairwise differences for each modelling technique, across 24 GCMs (relative changes in km^2 expressed as %). We repeated this procedure for each modelling unit and RCP scenario ending up with 192 estimates (8 modelling techniques and 24 GCMs) of changes in the extent of suitable climate.

Results

Niche overlap and model performance

The climatic niche of *S. atra atra* is relatively similar to the one estimated for the entire species (mean value ± standard deviations - jaccard: 0.74 ± 0.0036; centroid: 0.15 ± 0.00074). Indifference, the climatic niche of *S. a. prenjensis* is much more different from the species climatic niche (jaccard: 0.07 ± 0.0036; centroid: 1.38 ± 0.0025) and even more different from the niche of *S. atra atra* (jaccard: 0.02 ± 0.00026; centroid: 1.52 ± 0.0014).

Models for all units recorded high performance measured with average TSS value in cross-validation across modelling techniques (TSS>0.91). However, infraspecific models showed higher performance than species-level models (Table 1). On average, the best performing models were *prenjensis* models, with a mean TSS value of 0.96.

Table 1: Predictive performance of models for three modelling units in current climate conditions, averaged across modelling techniques (n = 8). TSS - True Skills Statistics: mean value ± standard deviation.

Modelling unit	**TSS**
Salamandra atra	0.916 ± 0.020
Salamandra atra atra	0.930 ± 0.036
Salamandra atra prenjensis	0.955 ± 0.017

Suitability under current and future climate conditions

The model consensus maps showing climate suitability for the species and infraspecific lineages under current and future climate conditions are presented in Figure 2. Interactive maps are available for visualization and download via a web application (https://atra-climate.shinyapps.io/atra-climate/; see Appendix S6).

Under current climatic conditions, the species and *S. atra atra* models, recognized large parts of the Alps as highly suitable for species' occurrence (consensus value = 1, Fig. 2 – a, g panels), while the *prenjensis* models overall did not consider the Alpine region as highly suitable (Fig. 2d). Species and *atra* models predicted limited suitability in the central Alps (area separating *S. atra atra* from the lineages in the Southern Alps) and the western Alps (consensus value = 1, Fig 2 – a,g panels). Indifference from species and *atra* models, *prenjensis* models identified large areas of high suitability in the North-Western Dinarides (Fig. 2d). Although with different proportions, all modelling units predicted highly suitable areas in the Central and Southern Dinarides (*prenjensis* models gave biggest predictions, Fig 2d). All models agree that future conditions will significantly decrease the extent of climatically suitable areas not only for the entire species, but also for each of the two major lineages (Fig. 3, Fig 4, Table 2). Precisely, species and *atra* models, reveal ample decrease of suitability in the eastern and southern Alps from center to periphery while areas in the NW Alps remain suitable (Fig. 2). Future predictions reveal a considerable decrease of species' range in the Dinarides (Fig. 3, Fig. 4) accompanied by poor climatic suitability that is especially worrisome under RCP 8.5 where this mountain chain becomes no longer suitable for the species (Table 2).

Table 2: Percentage of the distribution range recognized as highly suitable (HS, consensus score =1) and suitable (S, consensus score ≥ 0.5) after the consensus model projections. Values for *S. atra* are given relative to the total area of Alps and Dinarides combined (after Snethlage et al., 2022), and also in relation to Alps (A) and Dinarides (D) alone; values for *S. atra atra* and *S. atra prenjensis* are given only relative to the mountain region they occupy (Alps and Dinarides respectively). Predictions of both RCPs lack HS grid cells (/)

	Current period (8 projections)		RCP 2.6 (192 projections)		RCP 8.5 (192 projections)	
Modelling unit	HS	S	HS	S	HS	S
S. atra	29.1 (A=41.4; D= 3.3)	45.1 (A=60.2; D= 13.3)	/	35.9 (A=51.2; D=3.8)	/	19. 6 (A=28.9, D= 0)
S. atra atra	38.1	60.4	/	47.1	/	25.1
S. atra prenjensis	5.1	25.9	/	7.4	/	0.1

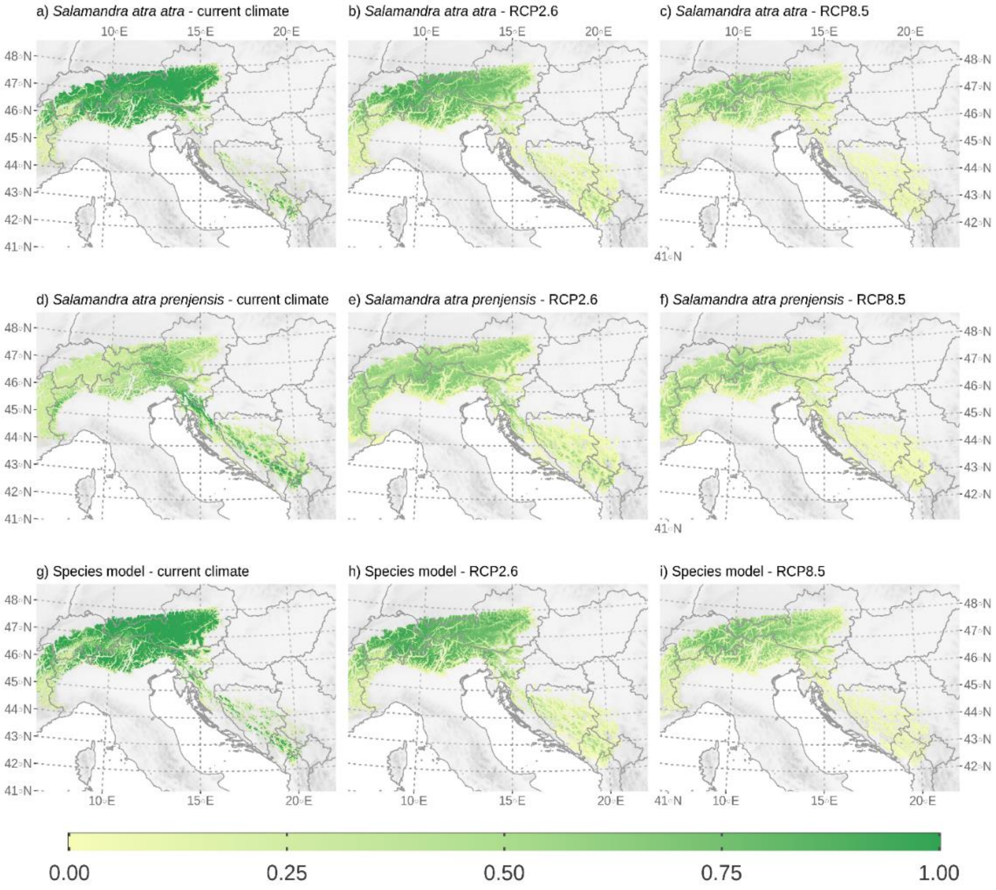


Figure 2: Maps showing current and future climate suitability (2070) for species and infraspecific models (atra and prenjensis). Climate suitability values represent the degree of model consensus; value of 1 means that 100% of binarized model projections predicted grid-cell as suitable, while value of 0 means that 0% of binarized model projections predicted grid-cell as suitable. Model consensus values are derived from 8 model projections for current climate (across 8 modelling techniques), and 192 model projections for future climate (across 8 modelling techniques and 24 GCMs). Map shows only projected values inside of the mountain regions for Alps and Dinarides (after Snethlage et al., 2022).

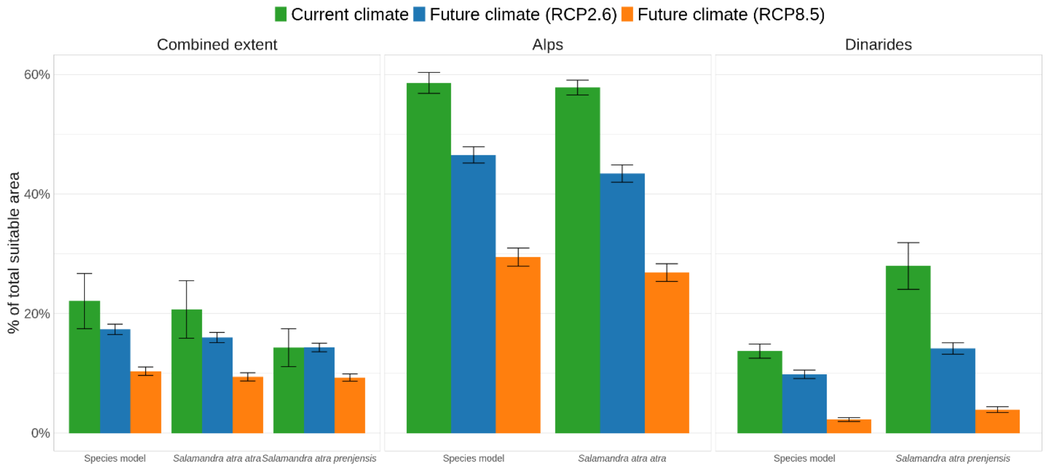


Figure 3: Projected extent of suitable climate for each modelling unit expressed in % of total area within each mountain region (Alps, Dinarides); Combined extent refers to the total extent of both mountain regions (Alps + Dinarides). The mean values are inferred from each model. Error bars show standard error of the mean, across modelling techniques and GCMs.

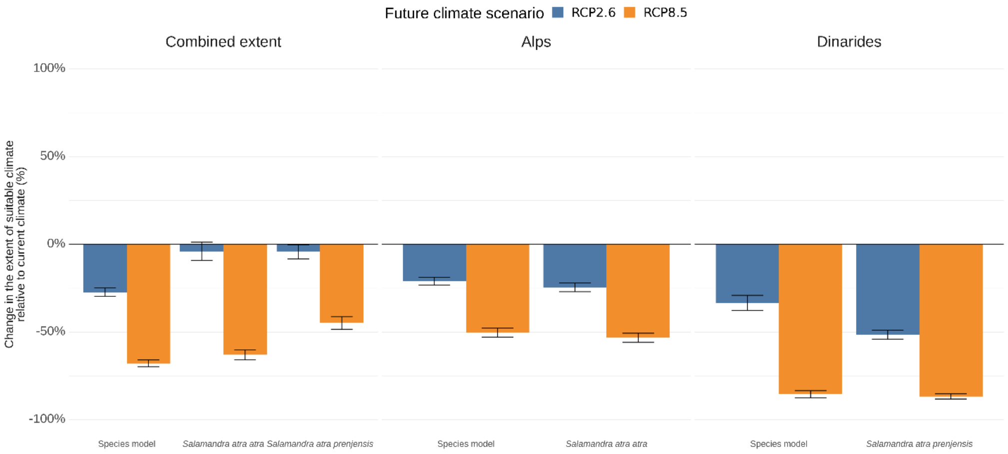


Figure 4: Changes in the extent of suitable climate in future climatic scenarios, relative to the extent of suitable climate in current conditions. Bars show mean value of changes, expressed as percent change compared to the current extent of suitable climate. Error bars show standard error of the mean, across modelling techniques and GCMs. Values are presented separately for the mountain regions of the Alps and Dinarides, and across the combined extent of both mountain regions (Alps + Dinarides), for all three modelling units.

Discussion

Our study attempts to estimate the impact of climate change on populations of *S. atra* along its range, by using an elaborate modelling approach where we addressed the uncertainties arising from imprecise occurrence data, choice of modelling techniques, climate scenarios, and modelling units. We relate our findings to existing studies and suggest concrete conservation actions.

Impact of climate change

All models reveal a considerable decrease in climate suitability throughout the entire range of alpine salamanders that is particularly visible in the Dinarides (Fig. 2, Fig. 3). Precisely, in the next 60 years, between 51% and 87% of the currently suitable Dinaric territory may become unsuitable for *S. atra prenjensis* (Fig. 4) while the suitability of the remaining territory is predicted to be poor (Table 2). A similarly high loss of climatic suitability is reported for other Dinaric endemics adapted to high mountains. For instance, by 2080, the predicted habitat loss for *Cerastium dinaricum* is up to 70% (Kutnjak et al., 2014) while several hoverfly species (*Cheilosia sp.*) may completely vanish from the Dinarides (Radenković et al., 2017). These insights stress the urgency for setting up conservation measures not only for the *prenjensis*, but for Dinaric endemics in general (e.g, monitoring programs, assisted migrations, ex-situ reproduction).
Compared to the *prenjensis*, future predictions for the *S. atra atra* lineage are more promising which corroborates predictions for future survival of other species that inhabit both Alps and Dinarides such as *Cheilosia* sp. (Diptera; Radenković et al., 2017). However, the inevitable decrease of suitable climate in the Alps (from 24% - 53%, Fig. 4) is also reported for other cold-adapted species. For example, by 2050, the available habitat for *Primula farinosa* is predicted to decrease by 25% (Theodoridis et al., 2018), and for *Berardia subacaulis* even by 80% (Guerrina et al., 2016).

We emphasize that this study takes into consideration only the climatic aspect relevant for *S. atra*'s occurrence while omitting other factors that may be important for assessing species' climate change risk. Using only abiotic factors such as climate, enables the description of species' fundamental climatic niche and the assessment of climate impacts. However, other factors beyond climate shape species distribution in reality, ranging from topography (Austin & Van Niel, 2011), species' dispersal ability and dispersion barriers (Guisan & Thuiller, 2005), adaptation (Pearman et al., 2008), presence of species that are predatory or competing (Hirzel & Le Lay, 2008; Wisz et al., 2008), and stochastic events such as floods, fires, and landslides .

For example, to mitigate the drier and warmer climate, alpine salamanders may adapt and exploit microclimatic conditions of complex mountain habitats to its maximum. A study that used high-resolution climatic data (spatial resolution of 100 m), reflecting topographic complexity, to predict the impact of climate change on Swiss populations of *S. atra*, revealed that salamanders will migrate towards higher elevations and shift activity from southern, warmer slopes to cooler northern ones (Feldmeier et al., 2020). Moreover, acknowledging dispersal revealed an important predictor of climate change impact on *S. atra*. Concretely, the range decrease of Italian populations under B1 scenario of climate change is predicted to be 64% but, when dispersal is accounted for, predictions are downsized to 36% (D'Amen et al., 2011).

Using coarser resolution climate data like in our study (spatial resolution of 1 km) can obscure the effects of microclimate on habitat availability, especially in heterogeneous terrains such as mountainous areas (Connor et al., 2018; Suggitt et al., 2011). For example, a grid-cell with 1 km resolution that is projected as unsuitable, could in reality harbour multiple microclimatic sites that provide suitable (micro)habitats within the coarser unsuitable grid-cell. However, inclusion of such factors in SDMs is severely hindered by the lack of availability of high-resolution climatic data with a wide spatial coverage, as well as the spatial uncertainty in species data that may be larger than the spatial resolution of climate data. Nevertheless, even in the absence of high-resolution data that can be used to describe potential habitats with a greater accuracy, insights from SDMs at coarser spatial scale can be used to pinpoint populations along the species' distribution range that are under the biggest pressure from the changing climate

Additional limitation in our assessment on the impacts of climate change is using binarized model projections, where the thresholding process can introduce an additional source of uncertainty in model projections (Steen et al., 2017). While we did not look into the impacts of threshold selection on model results, we used an appropriate thresholding rule (Steen et al., 2017), and additionally, used a consensus model approach where we combined multiple binary projection to reduce potential biases.

Species models vs. intraspecific models

While species and intraspecific models had relatively high TSS values, the intraspecific models performed slightly better, matching observations from other SDM studies that accounted for infraspecific variation (Chardon et al., 2020; Lecocq et al., 2019; Oney et al., 2013; S.-N. Zhang & Kubota, 2021). Despite the similar TSS values, species and infraspecific models predicted differing spatial patterns and range size estimates. Among modelling units, *prenjensis* models differed most, which may be explained by the low niche overlap of *prenjensis* lineage with *atra* lineage and the species as a whole. Observed differences may also be due to the number of intraspecific occurrence points that often related to the distributional width, and/or the rarity of the species. Given that intraspecific lineages have a narrower distribution than the species itself, intraspecific models may as well capture a narrower niche (Oney et al., 2013). Thus, perceived differences between species and intraspecific models may arise because species-level models are unable to properly estimate the niche width of its intraspecific variants. This could be further exacerbated when comparing models built from intraspecific data with a different prevalence (Thuiller et al., 2008).

Nevertheless, in our case, differences among modelling units revealed larger for distribution predictions in current time than future (Fig. 3), where likely the large number of future projections led to smaller differences in this temporal context (192 vs. eight for the current period). Compared to our analyses, studies that modelled separately species and infraspecific units, reported more drastic discrepancies in the distribution projected by the two modelling targets, but indeed also used a limited number of projections to predict future distributions (Hällfors et al., 2016; Homburg et al., 2014; Marcer et al., 2016; Pearman et al., 2010, Oney et al., 2013 Benito Garzón et al., 2011). However, as the magnitude of climate change impact using SDMs cannot be anticipated between species and infraspecific units, our study pleads in favour of a separate modelling approach that is especially justified in cases where modelling units have a low (climatic) niche overlap.

Conservation insights

Results of this study may be used as a decision-support tool to inform conservation and field survey efforts (see Appendix S6). Consequently, this study is also relevant for other cold-adapted taxa with low dispersal that are endemic to the Alps and Dinarides (see e.g: Cikovac & Ljubisavljević, 2020; Komposch & Kreiner, 2018; Kutnjak et al., 2014; Surina et al., 2011).

Species distribution modelling has been confirmed as an effective tool for discovering new populations of both plant (Sarkinen et al., 2013; Williams et al., 2009) and animal species (Chunco et al., 2013; Mizsei et al., 2016; Rhoden et al., 2017). Consequently, we propose that our results, especially when combined with published historical data, can be useful for prioritizing areas for field research in the Dinarides where the distribution of the alpine salamander is yet unsolved (Cikovac & Ljubisavljević, 2020; Džukić, 1991; Klewen, 1991; Speybroeck et al., 2016). For instance, the species was found on mt Treskavica (central Dinarides) more than 140 years ago (Bolkay 1929). Since then, neither the extensive fieldwork of Mikšić and Klewen (Mikšić 1969, Klewen, 1986), nor personal field investigation of one of the authors (E. Š: 2013 – 2016) managed to confirm *S. atra* on this mountain. *Prenjensis* models show that mt. Treskavica is nowadays not highly suitable for the species (fragments of a total of only 5km^2 with consensus score = 1, Appendix S6), placing the area low on the list of priorities for field research all things considered. On the other hand, Forenbacher (2002) reported the occurrence of *S. atra* on mt. Velebit (Northern Dinarides) in 1971, and although such finding is nowadays considered doubtful by expert authors (Dušan Jelić, pers. comm.), our predictions consider the northern and southern areas of this mountain as, climatically, highly suitable for the species (consensus score = 1, Appendix S6) placing mt. Velebit high on the list of priorities for field research. Other highly suitable areas are detected in the Southern Dinarides (Fig. 1). However, as this part of Dinaric mountain region experienced extensive and long glaciations in the Pleistocene (Hughes et al., 2011), it is questionable whether it allowed the survival of alpine salamanders at the time or if it was successfully occupied afterwards. Nevertheless, as this region appears highly suitable for the species it may as well represent potential (re)introduction areas.

While acknowledging the fact that the species' distribution in the Dinarides is still unresolved, our results suggest that these populations are most threatened by climate change (Fig. 2, 3, 4), carrying the implication that some may disappear even before being discovered. Indeed, recent decades witness accelerated deglaciation in the Dinarides (Allard et al., 2021) that may be directly linked to the decline of alpine salamander populations through the pronounced "sky-island" effect which implies the inability of individuals for upslope migration (Kutnjak et al. 2014). In the Dinarides, *S. atra* already reached the upper limit of vertical distribution as specimens are restricted to the uppermost mountain areas from which they lack escape routes that would allow their migration to other "safe areas". Although they may now be using cooler northern slopes to buffer the warming climate (see Feldmeier et al., 2020), the ultimate survival strategy may be to retreat to underground systems (caves, pits) as it is known that salamanders colonize subterranean environments to avoid climate stress and exploit new ecological opportunities (Lunghi et al., 2015; Salvidio et al., 2017). However, long-term survival of alpine salamanders in underground environments remains unknown.

The current conservation status of *S. atra* is Least Concern (Andreone et al., 2008) and might be appropriate only for the *S. atra atra* lineage. As the different genetic backgrounds of each lineage of alpine salamanders may signal a different genetic potential for species' adaptation and survival (for the rationale see Hoban et al., 2021), we plead for a separate conservation status for all lineages of alpine salamanders. Results and following outputs of this work (particularly Appendix S6) can be relevant in defining individual conservation status.

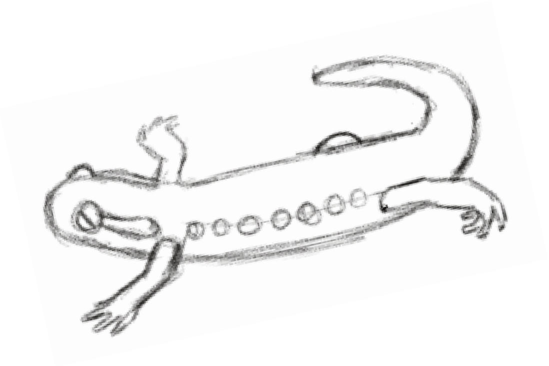
To this end we note that our findings suggest that assisted migrations of alpine salamanders may soon become the only possibility to preserve evolutionary significant units within *S. atra*. Given the fundamental limitations of SDM studies (Araújo & Peterson, 2012; Heikkinen et al., 2006; Jarnevich et al., 2015), introduction/relocation efforts must have additional input from approaches such as mechanistic models (Kearney & Porter, 2009) and individual/agent – based models (Kelle et al., 2013) that take in consideration the biotic aspects of species' distribution. However, such fine-tuned approaches require complex species-specific data that are often unavailable or hard to obtain (Hällfors et al., 2016).

Until additional studies are carried out, this study provides initial guidance for conservation actions not only for the alpine salamander, but also for other cold-adapted species with similar distribution patterns.

Acknowledgements

We thank Dr. Silke Schweiger from the Natural History Museum of Vienna (NHMW) and Magdalena Meikl for providing occurrence data of Alpine salamanders (NHMW and Alpensalamander databases).

General discussion



In this thesis, I used a combination of field observations, molecular analyses and experimental work to document geographic variation in genetics, morphology and physiology in Alpine salamanders (*Salamandra atra* Laurenti 1768). Moreover, I used species distribution modelling to assess the impact of climate change on specie's distribution. The thesis is particularly focused on the naturally fragmented *prenjensis* populations in the Dinaric Alps.

I will now discuss the major findings of my work, framing them in a general evolutionary biology context, but also examining their relevance for practical conservation purposes.

Geographic variation

I found evidence for among-population variation in genetics, morphology, toxin composition and evaporative water loss (chapters 2, 3, 5, 6). On the other hand, I did not find significant variation in the diet of *prenjensis* populations (chapter 4). In this section, I will provide insights on the origin of the studied variation and suggest future research avenues.

Genetic differentiation

Chapter 2 provides evidence that populations of alpine salamanders in the Dinarides were connected in the past. Likely, populations where widely distributed in the Pleistocene along the entire Dinaric Arc due to favorable conditions that ruled throughout the Balkan Peninsula during major glaciations in other parts of Europe (Allard et al, 2021). Indeed, while most of Europe was covered by ice sheets during the Last Glacial Maximum (LGM; 27 500 – 23 300 years ago), the Dinarides were not; the glacial maxima in the Dinaric Alps took place long time before the LGM (more precisely: 478 000 and 191 000 years ago; Allard et al., 2021). During LGM, the Dinaric landscape was composed of forest steppes, grasslands and mix of tundra (Ray and Adams, 2001) - habitats that may have secured the survival of alpine salamanders. Patterns identified by genetic analysis suggest that Dinaric populations responded to climatic oscillations in the Pleistocene primarily by elevational shifts rather than latitudinal shifts (chapter 2). This is also corroborated by the fact that Dinaric specimens did not colonize the Alps after the LGM (Bonato et al., 2018, Helfer 2010). Altitudinal range shifts caused strong genetic structure and differentiation also found in other Dinaric glacial relics such as the rocky bell - *Edraianthus serpyllifolius* (Surina et al., 2011), the Balkan snow vole - *Dynaromis bogdanovii* (Kryštufek et al., 2007), and the common European viper - *Vipera berus* (Ursenbacher et al., 2006). Besides altitudinal range shifts, the canyon formed by the Neretva river between mts. Čvrsnica and Prenj (Central Dinarids) probably also contributed to the strong genetic differentiation detected in chapter 2. This canyon is well known to plays an interesting and fickle role in the phylogeographic structuring of the Dinaric fauna. Our data suggest that it may have prevented northern *prenjensis* populations from moving further south. The canyon acted as a long-standing barrier producing marked phylogenetic structure also in other high altitude species, such as Balkan snow voles and meadow vipers (*Vipera ursinii*) (Kryštufek et al., 2007; Zinenko et al., 2015); however, no such effect was found in horned vipers (*Vipera ammodytes*) and Dalmatian wall lizards (*Podarcis melisellensis*) (Ursenbacher et al., 2008; Podnar et al., 2004) probably because these species occur within a relatively wider elevation range. Much as today, during unfavorable Pleistocene conditions, *S. atra* most likely survived in isolated habitats and differentiated through genetic drift and probably selection. However, the neutral markers used in our (and other) phylogeographic analyses are ill suited to infer the evolutionary mechanisms that produced the detected genetic structure (Cortazar-Chinarro et al., 2017). Dispersal and secondary contacts seemingly occurred during favorable conditions, further shaping specie's adaptive potential. The moderate levels of genetic diversity found in our *S. atra* populations may not be expected considering the effect that genetic drift causes in long-lasting, small and isolated populations (Frankahm et al., 2012). Nevertheless, the moderate values may be explained by *S. atra*'s a long generation time, which can cause a delayed genetic response to fragmentation masking the eroding effect of genetic drift as it showed to be the case for the Florida skink -*Plestiodon reynoldsi* (McCoy et al., 2010) and some plant species (Dobeš et al., 2017) with a long generation time.

Morphology

Despite the relatively conservative nature of morphological evolution in salamanders (Wells, 2007), we detected intraspecific morphological variation in our study species (chapter 3). We found that relative to *S. atra atra* (Alpine populations), *S. atra prenjensis* (Dinaric populations) tends to have wider heads, longer jaws and fewer coastal grooves.

In amphibians, the plasticity in head shape and associated measurements is often linked with prey choice (Holomuzki and Collins 1987) and predation (Van Buskirk and Schmidt 2000, Wilson et al., 2005); however, the seemingly conservative feeding behavior of *S. atra* (chapter 4) questions diet as a potential driver of the detected variation in head/jaw size. Developmental differences may be involved although a recent study shows that variations in head morphology, often found between subspecies of fire salamanders (*S. salamandra*), constitute lineage specific traits independent to the type of reproductive mode that affects developmental dynamics (Alarcón – Rios et al., 2020). It remains unclear whether the detected morphological variation is due to ecological adjustments or genetic constraints. The detected difference in the number of costal grooves between the two lineages may reflect variation in the number of vertebra (costal grooves are a proxy for the number of trunk vertebra in tailed amphibians (Urodela), Litvinchuk and Borkin, 2003). The vertebrae number is considered a highly variable trait within many species of Urodela that is associated to climatic conditions (Arntzen et al., 2015; Ficetola et al., 2016). In *S.*

atra, winter precipitation and temperature diurnal range have a significant, negative relationship on the number of vertebra, causing inter-population differences in this trait (Ficetola et al., 2016). Arguably, due to reduced surface activity caused by the drier (and warmer) conditions, alpine salamanders may adopt longer trunks that facilitate passive burrowing and slinking through underground karst features (Arntzen et al., 2015). Besides climate, structural differences in habitat may also affect the vertebra number as in lacertid lizards (Lacertidae) where species from open habitats tend to have fewer vertebrae than lizards from cluttered habitats (Arnold, 1983, 1998, Van Damme and Vanhooydonck 2002). Although the relationship between habitat complexity and vertebra number has not yet been explored for terrestrial salamanders, in the context of our study, I note that the Dinaric habitat is an open space above the tree line, while the forested Alpine environment is more crowded by dense undergrowth of vegetation, rotten branches and similar elements. To facilitate the movement through these obstacles, *S. a. atra* in the Alps may have developed a longer trunk (i.e., increased spinal flexibility - see Mia Carter et al., 2021) when compared to Dinaric individuals explaining the higher number of costal grooves registered in the former population compared to the latter (chapter 3).

Nevertheless, as we did not specifically assess potential selective drivers (diet, predation, climate, habitat complexity) in the habitat of the studied populations (from chapter 3), we cannot explicitly discuss their relevance in producing the morphological variation reported thereby; hence, it remains unclear if recorded variation has an adaptive background.

Taxonomic implications

Regardless of whether it is adaptive or not, the detected morphological variation pleads in favor of recognizing *prenjensis* as a genuine subspecies and provides candidate traits for the description of alpine salamander lineages that await formal classification (as after Bonato et al., 2018 and chapter 2). I recommend to delve into this further by examining morphological variation in as many populations from different lineages. This could ideally be done using intelligence-based methods (see MacLeaod et al., 2010), geometric morphometric and photogrammetry (e.g see Alarcon-Rios et al., 2017; Chiari et al., 2008).

In the context of taxonomic research it is important to note that in chapter 3 I wrote that the original holotype and paratypes of *S. atra prenjensis* (that Sofija Mikšić used to describe the subspecies - Mikšić, 1970) had gone missing from the herpetological collection of the National Museum of B&H. However, since the publication of this work, Adnan Zimić, the newly appointed curator of the collection, has been able to recover the holotype along with 11 individuals of Mikšić's original series that had been misplaced in the ichthyological (fish) collection of the same museum. As a reference, and most important type specimen, the holotype justifies the usage of a taxon's name. Nowadays, the herpetological collection of the National Museum of B&H holds a total of 42 *prenjensis* individuals and allows a proper comparison of the taxon with other *S. atra* populations allowing to study morphological/anatomical features that would otherwise require sacrificing individuals (e.g the vomero-palatal teeth and number of vertebra – see chapter 3). On the other hand, I'd like to note my personal experience considering methodological aspects of measuring live individuals. Firstly, extracting measurements from pictures may be particularly challenging; light reflecting from the black and shiny skin of *S. atra* makes the species frustratingly difficult to photograph and complicates the definition of landmarks (pers. obs). Consequently, I would advise taking direct measurements from live individuals. But, as I had difficulties measuring animals in the field, mainly due to the slippery skin of *S. atra* and its ability to evade the holding grip, I am in favor of anesthetizing individuals prior to measuring them. I believe this approach minimize both the stress of individuals and sampling error. Using anesthetics such as MS-222 proved completely safe for *S. atra* if applied in recommended doses (pers. obs., see also Mitchell 2009).

When it comes to molecular taxonomy, the inclusion of RADseq data (restriction site-associated DNA sequencing that allows recovering thousands of polymorphic sites across individual genomes) proved to be especially efficient to infer shallow relationships in terrestrial salamanders (Rodríguez et al., 2017). It is worth mentioning that an ongoing study is using RADseq over a wide sampling area, to clarify *S. atra's* intraspecific phylogeny framed in a wider interspecific context (Vences, pers. comm). Only by such an eclectic approach it is possible to infer phylogenetic details necessary for drawing the big picture of species' evolution.

Diet

I did not find any substantial variation in the diet of *prenjensis* populations; the small detected differences concern the dietary composition (chapter 4) that may reflect prey availability, abundance and accessibility in the period of stomach flushing (see Roth, 1987) moreover influenced by microclimatic conditions. As salamanders may often have to "compromise" on different prey types, I consider it unrealistic to expect any strict adaptation to a certain prey type (apart from possible correlations between the morphology of the feeding apparatus and prey spectrum). However, I discovered that prey consumption was not in line with prey abundance in the field. As argued in chapter 4, this may reflect prey preference, (in)ability to catch some prey or simply errors in assessing prey consumption/availability. Identifying feeding preferences is best achieved by comparing data from natural diet and laboratory experiments. For instance, Freed (1982) showed that the green tree frog - *Hyla cinerea* in its natural habitat eats primarily larvae of moths (*Spodoptera*) and soldier beetles (*Chauliognathus*) but in the laboratory shows strong preferences towards houseflies *(Musca domestica),* which are not natural prey. To my knowledge, the only study that explored prey preferences in *S. atra* is the master thesis of M. Müller (2018 - unpublished). In a cafeteria experiment, alpine salamanders were offered isopods (Isopoda), millipede (Diplopoda) and larvae of beetles (Coleoptera) but did not show any specific preferences among these prey types (in terms of time spent with prey). However, salamanders are shown to be selective with regard to a variety of prey parameters such as size (Luthard 1981), movement pattern, orientation, and velocity (Finkenstadt and Ewert 1983; Himstedt 1967), prey density (Jaeger and Barnard, 1981; Takagi and Miyashita 2019) and prey nutritive value and digestibility (Jaeger and Rubin 1982). Considering the latter, a feeding ecology study of Lanza's salamander (*Salamandra lanzai*) demonstrated that pregnant individuals fed differently compared to non-pregnant females and males, implying the ability of terrestrial salamanders to assess prey nutritive value and digestibility (Andreone et al., 1999). In order to estimate potential prey preferences it would be interesting designing a cafeteria experiment where the prey would be sorted by size and movement speed in several categories while controlling for their movement pattern. If salamanders would prefer a certain prey in each of the category, this could signal preferences possibly concerning the prey nutritive value or digestibility. Lastly, it is important to note that the selectivity of prey in salamanders is partly influenced by visual and olfactory learning (Roth 1987) that may have a genetic component (Gibbons et al., 2005). Hence, testing learning ability (e.g. by increasing the encounter frequency with specific prey type), and heritability of salamanders to catch prey is of specific interest in this context.

Toxins

Considering the detected variation in steroid alkaloids (samandarines - SAMs) as toxic compounds of salamanders' poison (chapter 5), it is hard to discuss its origin due to the poor knowledge on the topic. Salamander toxins are mixtures of complex compounds and it is currently unknown how these elements (and/or their interaction products) evolve and in which context (Lüddecke et al., 2018). Moreover, we know very little on how these SAMs are synthetized - in which organs, through which biochemical pathways, controlled by which genes (if any)? In addition, the absolute functions of SAMs are not yet revealed (Lüddecke et al., 2018).

First of all, I would like to emphasize that we need more robust estimators of both predation pressure and infection risk (than in chapter 5) in order to understand the role of these factors in driving salamanders' toxin variation. For instance, snake diversity (as number of snake species, chapter 5) may not reflect predation pressure in salamanders' populations; here, snake densities, may be a better measure along with analysis of snakes' diet. Sampling environmental DNA (eDNA) from clay models imitating salamanders in the field (e.g by taking swabs from the models) may also help to reveal predation pressure on salamanders. Considering infection risk, our results from the bacterial culturing are quite crude as we did not identify bacteria to their taxon level. The same concerns our mycobiome analysis since the presence of the same fungi in the habitat of salamanders (in terms of fungal lifestyle diversity, chapter 5) can be considered ubiquitous and, necessary, soil compound. Consequently, the infection risk outputs (from chapter 5) should rather be considered preliminary. For the assessment of infection risk one must also follow far more complex interactions between pathogens (virulence, abundance), host and environmental factors. Due to financial and time limitations we did not have the opportunity to accurately study predation pressure and infection risk. However, I find that a proper assessment of these factors is necessary to understand the evolution of toxin variation in populations of salamanders.

Nevertheless, SAM variation may as well be shaped by selective drivers other than predation and infection risk. For instance, the volatile components of the SAMs may have a role in attracting mates (Lüddecke et al., 2018). In the fire salamander (*S. salamandra*) SAM synthesis appears to take place in ovaries and testes while larvae are entirely free of alkaloids (Mebs and Pogoda 2005). The fact that these substances may be produced in the reproductive organs and that they are synthetized in metamorphosed individuals, suggests a possible role in reproduction. Interestingly, in the bella moth (*Utetheisa ornatrix*) certain alkaloids are found to play multiple roles in reproduction by increasing the developmental success of fertilized eggs, and also indirectly by metabolizing into pheromones that have a role in attracting mates (Eisnar and Meinwald 1995). The head rubbing between salamanders during courtship (Klewen, 1991) may involve SAM secretion from the glands and stimulate reproduction. Aside the potential role of SAMs to lure mates, they may also deter competitors. Alpine salamanders are known for their territorial behavior so that they avoid shelters marked with feces of conspecifics of the same sex (Gautier and Miaud 2003). Specifically, scent marks are deposited on a substrate by rubbing them on the surface and also through urination and defecation (Gautier and Miaud 2003). Accordingly, the SAM alkaloids from the parotoid glands may be delivered by rubbing the substrate while the SAMs from the testes and liver (Mabs and Pogoda 2005) may be expelled through the urine or feces. Checking whether male salamanders avoid places marked with SAMs, and whether female salamanders are attracted to them, would help to understand the role of these substances in deterring competitors and/or attracting mates.

All in all, this would imply that intra- and inter- sexual selection could induce SAM variation for example in populations with a large number of reproducing males (increased intrasexual competition) and where female choice is strongly determined by local mate preferences. In this context, it is interesting to note that we observed sex differences in the relative amounts of some SAMs (chapter 5).

Besides sexual selection, another interesting avenue of SAM research concerns exploring the inducibility of these substances in relation to predator cues and pathogens as the synthesis of defensive chemicals in amphibians can be indeed triggered by these factors (see Hettyey et al., 2014 for review). For instance, Buciarelli et al., 2017 showed that in the California newt (*Taricha torosa*) concentrations of the defensive chemical - tetrodotoxin (TTX) are maintained at a baseline level that can quickly increase in response to perceived predation risk. Miele et al., 1998 showed that adult Oriental fire-bellied toads (*Bombina orientalis*) increase the production of skin peptides after experimental exposure to the bacterium *Aeromonas hydrophila*. Conversely, Mangoni et al. (2001) observed a sharp decrease in peptide synthesis in the edible frog (*Pelophylax* (*Rana) esculenta)* frogs kept in sterile water as compared to control animals in naturally bacterium-rich water. Similar experiments could reveal if SAM synthesis is plastic in *S. atra*. Combining such studies with transcriptome analyses (eg. see Czypionke et al., 2015) may help to detect potential genes involved in salamanders' toxicity, hence also mechanisms underlying toxin variation along with the adaptive value.

Moreover, it would be also interesting to test the relationship between SAMs and diet. Dietary resources already proved to be an important source of amphibian alkaloids (Saporito et al., 2007). *Salamandra atra* produces SAMs *de novo* using cholesterol as a precursor (Lüddecke et al. 2018) similarly as the common toad (*Bufo bufo*) tadpoles produce the toxic steroids bufadienolides (Bokony et al., 2016). It has been suggested that the structurally similar and toxic lucibufagins of fireflies are produced from dietary cholesterol (Eisner et al., 1978, Daly et al., 2008). To my knowledge there are no studies linking toxicity of *Salamandra* with dietary sources although salamanders are good candidates for such research (Sayitzky et al., 2012). If diet turns out significant in the context of SAM synthesis, the next step could be to test if alpine salamanders show dietary preferences in relation to the experienced environment. Specifically, do salamanders eat prey that makes them more poisonous if predation pressure is increased, or do they eat prey that makes them more attractive to females given an increased sexual selection? Considering the latter, it is interesting to mention the study of Jaeger et al., 1995 which showed that female red backed salamanders (*Plethodon cinereus*) prefer males that forage on termites rather than on ants.

Future studies exploring the relationship between infection risk and SAM variation may focus on the skin microbiota that has been suggested to play a major role in amphibian defenses against pathogens (Becker and Harris 2010). For instance, bacteria isolated from the parotoid gland of the cane toad (*Bufo marinus*) have the ability to biotransform steroid toxins (bufadienolides) that, in turn, may extend the host evolutionary potential (Hayes et al., 2009, see also Henry et al., 2021). Understanding the bacterial community on the skin of *S. atra*, their survival mode, interaction with infectious agents, and their ability to transform steroid alkaloids, along with the effect of these new substances against infections, is an exciting new line of research in this field.

Evolutionary research on toxin variation in salamanders is clearly in its infancy; we know next to nothing on its genetic and physiological basis, nor on its ecological or evolutionary significance.

Water loss rate

Temperature and precipitation can drive geographic variation in water loss rates of ectotherms (Tracy 1976, Tracy 1982, Ridell et al., 2019, Cox and Cox 2015). Although amphibian research is poor and inconsistent in this regard, salamanders from drier habitats seem to evaporate water slower than individuals from wet habitats, and our results are in line with this finding (chapter 6: *S. atra* population of Čvrsnica [dry environment] evaporate water slower compared to the population of Gorski Kotar [humid environment]). As in woodland salamanders, *Plethodon* species (Ridell and Sears 2015), an increased water loss resistance of individuals from Čvrsnica allows a prolonged above ground activity compared to individuals from Gorski Kotar. We can only speculate that the different hydric and thermal environment of the two studied populations caused this variation. One way to disguise the mechanism behind water loss rate is to conduct common garden experiments by rearing individuals originating from different habitats in a common environment and follow their water loss rate and activity patterns. Another way includes the detection of the genetic machinery behind water loss rate that combines reciprocal transfer experiments with transcriptome analysis. In these experiments, individuals from dry and wet habitats would be transferred to opposite environments where their transcriptome profile would be followed along with water loss rates and activity patterns (e.g for experimental design see Sabino-Pinto et al., 2019). If responses would be asymmetrical between transplanted individuals this would mean that the adjustments are not fully plastic and that some level of local adaptation has already occurred.

The survival value of detected variation

The studied populations are allopatric (chapter 2), they occupy different climatic niches (chapters 6 & 7) and live in structurally different habitats (chapters 3, 4, 5). These facts suggest that, at least, part of the detected variation is adaptive and aids survival.

Indeed, many studies in natural populations of animals recorded phenotypic changes associated to the environment (Merilä and Hendry 2014, Urban et al., 2013). Owed to the complexity of the research behind local adaptation (Merilä and Hendry 2014) it has been difficult to discern if these changes are due to plasticity or genetic adaptation moreover because both mechanisms can establish similar phenotypes and are not mutually exclusive (Sabino-Pinto et al., 2019). This also explains why proof of heritable adaptive changes remains limited in natural populations (Urban et al., 2013, Merilä and Hendry, 2014). In a meta-analysis, Urban et al., 2013 reviewed studies documenting empirical evidence of phenotypic or genetic differences in amphibians associated with climate change where the climate change factor was recorded along with the phenotypic response (Urban et al., 2013). From a total of 32 studies, evidence for climate-related trait variation was found for 50 amphibian species, the majority frogs, followed by toads, salamanders and newts. Most of the evidence included phenological changes in breeding and life-history traits (embryonic development, survival, growth) while few concerned changes in resistance to diseases prompted by climate change, along with changes of traits such as melanism, body length and body condition. According to the meta-analysis, amphibians have a high response to climate variation. Based on 69 estimates, the proportion of plastic responses was 70% (67% of which was considered adaptive) and the proportion of genetic response revealed equally high (65%), although existing genetic data were scarce compared to studies on plastic responses. In neither analyses (plastic/genetic responses), the effect of species proved significant, which suggests that amphibians in general are able to change their phenotype in response to environmental perturbations. Given the relatively high adaptive ability of amphibians to respond to environmental changes (Urban et al., 2013), here I will discuss the survival value of phenotypic changes observed in my studies regardless of the mechanism that produced them (phenotypic plasticity/genetic adaptation).

As discussed previously, the survival value of the recorded differences in morphological traits (head size and jaw & number of costal grooves – proxy for vertebra number) can be only speculated. However, both the head measurements and the number of vertebra seem to be genetically determined in salamanders (vertebra number: Highton 1960, Highton 1977, Jockusch 1997, Ficetola et al., 2016; head shape: Walls et al., 1993, Adams 2011, Matsunami 2015), hence also bear evolutionary potential. Therefore, I rule out the likelihood that such variation is maladaptive but cannot reject the possibility that it is neutral/random (e.g correlated with another trait under selection). However, the ability of morphological evolution seems to play an important role for the persistence of tailed amphibians under climate change (Ficetola et al 2016, Urban 2010) which suggests that the detected morphological variation in *S. atra* has also an important survival value.

Given the crude approach for obtaining estimates of both predation and infection risk in chapter 5, one still cannot exclude predation and infection risk as drivers of toxin variations. Moreover, taking into consideration other potential drivers (previously discussed) and the vital role these substances play in the life of salamanders (Lüddecke et al., 2018), it is probable that at least part of the detected variation in toxic compounds has an important survival value. Accordingly, as for morphological variation, I doubt that the detected toxin variation is maladaptive but cannot exclude that a portion of toxin variation might be also random (assuming selection acts on one substance that affects the mean of a second, neutral one).

The study of geographic variation in water loss rates (chapter 6) revealed a potentially higher selective pressure for water loss at mt. Čvrsnica when compared to Gorski Kotar (chapter 6); hence, it is tempting to attribute the increased water loss resistance of the individuals from Čvrsnica to genetic adaptation or a plastic adjustment to drier conditions. Consequently, the detected variation in water loss rate would indeed have an important survival value.

The studied levels of biological variations, regardless of the mechanism that produced them (phenotypic plasticity/ genetic adaptation) have a genetic background (regulatory or programmed). The fact that the studied populations show different patterns of genetic diversity (chapter 2), may also imply a different adaptive potential for expressing phenotypic plasticity or genetic adaptation. As pointed out, I assessed the genetic diversity using neutral markers. Although heterozygosity at neutral loci can reflect population's fitness (Willy et al., 2006, Szulkin et al., 2010, Reed and Frankham 2003, Leimu et al. 2006), there is no simple general relationship; hence, neutral genetic variation cannot be directly linked to genetic variation in adaptive traits (Teixeira and Huber, 2021, Holderegger et al. 2008). In order to link genetic diversity with survival, one must use markers directly targeted at specific genes or gene families known to be under selection (van Tienderen et al., 2002). Wide genome scans can also identify presumably neutral markers of potential adaptive significance (reviewed in Holderegger et al., 2008). For example, by studying the genetic differentiation in the moor frog (*Rana arvalis*), one STR locus was found to be linked to habitat characteristics. The locus is partially located inside an up-regulated thyroid receptor gene coordinating the expression of genes relevant for larval life-history traits (Richter-Boix et al., 2011). Hence, directional selection on genes coding larval life-history traits maintains the divergence in apparently neutral genomic regions.

Nevertheless, the genetic basis of most resource polymorphisms remains poorly understood.

Although the ‘plasticity-first evolution’ theory is controversial, there is evidence that phenotypic plasticity precedes and facilitates the origin and canalization of novel, heritable phenotypes (Levis and Pfennig 2019). Specifically, the theory suggests that plasticity promotes the storage and release of ‘cryptic genetic variation’ (expressed variation only under new conditions, Ledon-Rettig et al., 2014, Paaby et al., 2014). In other words, unexpressed genetic variation (e.g, alleles associated with environmentally triggered traits) accumulates as it is not exposed to, nor removed by selection (Van Dyken et al., 2010, Schlichting, 2008). When novel phenotypes are triggered by environmental change, the cryptic genetic variation behind these phenotypes becomes exposed to selection and influences the regulation of plastic traits (Scheiner et al., 2002). The expression of such variation increases when populations encounter novel environments (Lardies and Bozinovic 2008) leading to genetic accommodation (Moczek 2007).

Considering my study, I cannot know whether any of the genetic markers used in chapter 2 has any potential adaptive significance. More precisely, I cannot know whether they are linked to loci under current selection or if they may be linked to cryptic genetic diversity. The neutrality of the recorded genetic variation cannot also be excluded. However, given the aforementioned, there is a possibility that the unique genetic structure recorded in each of the studied population (chapter 2) may (in)directly affect some of the phenotypic variation. In the optimistic case, the fact that genetic variation is recorded can be considered beneficial in the context of current and future adaptive response. Nevertheless, I cannot exclude the effect of drift and inbreeding underlying the detected genetic variation that could, in this worse case, be detrimental. I consider it is unlikely that the genetic structure found in each population reflects, by default, detrimental variation; however, it is important to note that in Gorksi Kotar I detected an inbreeding signal (chapter 2) that is worthy further monitoring.

In many of the aspects considered in this thesis, *S. atra* exhibits considerable variation, suggesting that it is a 'flexible' species. Although variation was not detected in the diet study, this proposes that alpine salamanders are opportunistic predators with a generalistic feeding style (chapter 4). Such feeding flexibility may reflect the unpredictability of dynamic environments that it inhabits (see Futuyma and Moreno 1988). Catholic species such as *S. atra* are less likely to suffer from major changes in prey communities than specialist species (Colles et al., 2009). Further, *S. atra* can be considered flexible in terms of habitat use. It lives on a variety of habitats, including forested areas, different types of meadows, rocky/stony substrates below mountian tops (Klewen 1989, Geiger 2006, Lelo et al., 2008). Nevertheless, regardless of the habitat it occupies, *S. atra* is dependent on underground shelters including burrows, rock fissures, crags, crevises and caves (Klewen 1991). In many areas of the Northern Dinarides (Smrekovac, Brestice, Šverda, Ravna Gora) it has been reported in cave systems (from one to 10 individuals) rather then above ground (Grbac et al. 2006, Ozimec 2005, Reš 2005). On mt. Prenj (Central Dinarides), five individuals were found in a pit 52 m below surface (Bajraktarević and Muftić, pers. comm). Such findings are not surprising as salamanders are known to colonize subterranean environments in order to avoid climate stress, exploit new ecological opportunities and reduce competition (Salvidio et al. 2017, Lunghi et al., 2015).

From an optimistic point of view, the level of detected biological variation combined with *S. atra*'s 'flexible' lifestye, hints that populations are responsive to environmental changes which should aid survive putative alterations in their environment. But, do phenotypic adjustments occur fast enough to follow the pace and amplitude of ongoing environmental changes? Models predict a considerable drop in climatic suitability for *S. atra* along its range and especially worrying are the anticipations for the *prenjensis* lineage in the next 50 years (chapter 7). A series of field attempts in the past 50 years (Klewen 1986, Mikšić 1970, my personal fieldworks 2013-2017) failed to reconfirm the specie's presence on mt. Treskavica (Central Dinarides, see chapter 7 for details) and, this fact, in combination to our climatic predictions (chapter 7) suggest that extincion may have already commenced. Considering the ultimate strategy to mitigate climate change, one possibility may be that alpine salamanders will retreat in underground systems (caves, pits) where they will be slowly adjusting to these less fluctuating environments. However, climate warming may affect their reproductive rhythm as it is reported that *S. atra* may need a long exposure to colder temperatures for successful reproduction (Klewen 1991, Seidel and Gerhard 2016). It is clear that gaps in the specie's biology further hamper the answer to it's future.

To conclude, my thesis provides evidence that *S. atra* (*prenjensis*) bears adaptive potential that must be monitored because, without human intervention, it may be insufficient for long-term survival.

Conservation insights

In this section I will discuss the relevance of my work for conservation practice. I will discuss the need to protect the species and suggest practical ways to do so. *Salamandra atra* is especially vulnerable to fast climatic changes because it is cold-adapted, slow maturing species that hibernates substantial part of the year (chapter 1, chapter 7); it has relatively poor dispersal capacities (Helfer et al., 2012), adapts slowly to structural habitat changes (Geiger 2006) and is particularly sensitive to emerging amphibian diseases (Gilbert et al. 2020).

There are several arguments justifying the need to protect *S. atra*. Firstly, the alpine salamander has multiple roles in ecosystems. These animals occupy an important position in the food web, they are involved in soil dynamics and are excellent indicators of ecosystem health and functioning (chapter 1). Moreover, the alpine salamander has an outstanding research value for example, in the study of limb regeneration (Joven et al., 2019, Broecks and Kumar 2005), and as a source of new compounds (mainly extracted from the toxic skin) that may have a strong medical and pharmaceutical value such as the antioxidant peptide Salamandrin-I recently isolated from its cousin species, the fire salamander (Placido et al., 2020). Aside from such utilitarian functions, the alpine salamander has a cultural value as an emblematic species that can be found in many cultural images of several societies where it is also part of legends and myths (Seidel and Gerhard 2016). Moreover, the intrinsic value of the species stems from the fact that these animals are much older inhabitants of planet Earth compared to us, human beings. Due to its charismatic nature, the alpine salamander may serve as an umbrella species representing cold adapted taxa with low dispersal ability endemic to the Alps and Dinarides.

I will now consider the practical steps that are relevant to protect the species, particularly focusing on the *prenjensis* given its extinction risk (chapter 7).

Taxonomic recognition

An important step for conservation initiatives and the protection of the alpine salamander is to recognize the taxonomic position of each lineage (the 'no name – no conservation' hurdle, Parra et al. 2007). The evidence presented in chapter 2 shows that *prenjensis* is an evolutionary significant unit, independent from other lineages of alpine salamanders. The evolutionary uniqueness in combination with the specific morphological traits that describe *prenjensis* in relation to its nominal subspecies (*S. atra atra*, chapter 3) provide support for officially recognizing the *prenjensis* subunit. The fact that the *prenjensi*s inhabits a different climatic niche compared to *S. atra atra* (chapter 7) reinforces this statement. The recognition of the *prenjensis* subunit would also formalize its conservation status (red list assessment criteria – IUCN, 2012). The recognition of the subspecies and its conservation status are approved by the Amphibian Specialist Group (ASG), an independent working group within the International Union for Conservation of Nature and Natural resources (IUCN). A scientifically based report (such as this thesis), once delivered to ASG, may ensure the recognition of *prenjensis* and its conservation status since ASG provides the scientific foundation to inform effective global amphibian conservation (ASG Secretariat, 2018). The ASG works together with the Amphibian Ark and Amphibian Survival Aliance that further enable concrete action plans. These organizations are established by the partnership of several worldwide associations for nature conservation to ensure the survival of amphibians worldwide. Following this formalization step, both location-specific actions and taxon-specific actions are required to ensure the long-term survival of *prenjensis*.

Location specific actions

Considering the dramatic drop of climatic suitability that is predicted for the *prenjensis* and other cold adapted species in the Dinarides in the next 50 years (chapter 7), it would be important to start regional habitat monitoring programs.

Habitat monitoring includes both structural (e.g spatial distribution of ecosystems, vegetation) and qualitative analysis (e.g invasive species, pathogens) followed up by a frequent collection of environmental parameters (climatic data). Such approach may help, not only to understand the dynamics of climate change, but also to detect the most stable (micro)habitats (Lengyel et al., 2008). The regional monitoring of the Dinaric Arc could be justified using the *prenjensis* as an umbrella species representing other cold adapted taxa with low dispersal. Such monitoring seems most appropriate in several protected areas along the *prenjensis* range[11] (Figure 4, chapter 1) but should not exclude mt. Prenj, the only area along the distribution that is not protected by any legislation. I consider, mt. Prenj a location of special conservation interest. Firstly, this locality is home to the oldest and most stable population of alpine salamanders in the Balkans, established more than 300.000 years ago (Razpet et al., 2016). As a Pleistocene refugium and the center of diversification of other Dinaric populations (chapter 2), mt. Prenj may be an important refugium during ongoing climate changes (for the rationale see Keppel et al., 2012); future predictions sustain this reasoning (view predicted climatic suitability for mt. Prenj from Appendix S6 in chapter 7). Moreover, the population of mt. Prenj is characterized by the highest levels of genetic diversity (chapter 2) and although the used genetic markers are in principle neutral, this finding may hint important adaptive potential (as previously discussed). Consequently, to preserve the *prenjensis*, an important step would be to safeguard mt. Prenj. Besides, mt. Prenj is one of the biggest endemic centers in the Balkans and a hot spot of biodiversity (Redžić et al., 2010; Šunje et al., 2014). Studies on paleoendemics of Prenj confirm

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[11] from north to south: regional parks Notranjska and Snežnik (Slovenia); several areas in Croatia: nature park Žumberak, strict reserve Bijele and Samarske stijene in Gorski Kotar, National Parks Risnjak and Plitvice; nature parks Blidinje (Čvrsnica, B&H) and mt. Orjen (Montenegro); National parks Prokletije and Valbona (Montenegro, Kosovo, Albania)

that it secured the survival of many other species from the ice age until present day (e.g Orthoptera – Kenyeres et al., 2008; *Dinaromys bogdanovi* – Kryštufek et al, 2007; *Cerastium dinaricum* - Kutnjak et al., 2014, and many other plant endemics - Redžić et al., 2010). Moreover, due to its specific geology and its karstic landscapes, mt. Prenj meets the UNESCO criteria as World Natural Heritage site (Varišić, 2012). Accordingly, establishing a legal basis for the protection of mt. Prenj, at least on a national level, is of major importance.

Taxon-specific actions

Taxon-specific actions include demographic monitoring, the monitoring of genetic diversity, and disease monitoring. Besides monitoring programs, taxon specific action also include the delimitation of species' distribution boundaries, and may include ex-situ breeding programs and assisted migrations (translocations or the establishment of new populations in new localities).

Demographic monitoring

Demographic monitoring involves the assessment of population size, survival rate, natality/mortality ratio, recruitment and emigration, and other demographic and life history parameters (Begon et al., 2006). These analyses can provide key information on the natural dynamics of populations and how population size and structure may be affected by present and future processes (McCaffery et al., 2014). Although rigorous and extensive demographic programs are a prerequisite to active management, *Salamandra* species still suffer from a lack of surveys (IUCN - www.iucnredlist.org, Nichols & Williams 2006). In 2018, under the Rufford Foundation support[12], I started a demographic monitoring of two *prenjensis* populations in the Northern and Central Dinarides (Gorski Kotar and Prenj). The monitoring is established on a surface of ca 4000

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[12] https://www.rufford.org/projects/emina-sunje/establishment-of-the-regional-monitoring-program-for-the-dinaric-populations-of-alpine-salamanders/

m^2 on each locality and it is still ongoing. I use a combination of transect data (counting individuals on several transects) and a Capture-Mark-Recapture (CMR) study design that includes marking individuals with Passive Integrated Transponders (PIT tags, see Ryan et al., 2014). These surveys provide several parameters such as population size, survival rate and capture probability (e.g. see: transects – Dodd 1990, CMR - Burgstaller et al., 2021). Survival rate and capture probability are assessed for each sex separately using the CMR approach. During the survey, I photograph each marked individual and take its body mass and length – the necessary measurements to assess the individual body condition index that is followed through time if that individual is recaptured (Dodd 2009). I also take the GPS coordinate of each captured individual and, upon PIT tag insertion and recovery, I release each at the exact spot where it was captured. This allows establishing dispersal patterns (Dodd 2009). The transect method is completely safe for animals as it does not include any contact with the salamanders. On the other hand, the CMR technique is invasive as it requires a small chirurgical procedure to insert the PIT tags in the animals (Ryan et al., 2014). However, in my experience, the animals recover extremely fast (within an hour of the implant) and, so far, I did not notice adverse effects on the long term (see also Dodd 2009). The advantage of CMR is that the PIT tag gives a code specific to each individual and stays in the body of animals during their lifetime allowing long-term surveys indifference from any other marking technique for salamanders (Dodd 2009). To my knowledge, this is the longest demographic monitoring on *S. atra* and I hope that after five more years of survey (according to Luymes and Chow-Fraser 2019 monitoring programs should run at least for a decade), I will be able to deliver the information on population trends in *prenjensis*, that are so relevant for conservation management.

Monitoring of genetic diversity

Hoban (et al., 2020) have proposed to index a species' genetic diversity status on the basis of three, pragmatic indicators that are also applicable for policies under the Convention of Biological Diversity (CBD). The first indicator is the number of populations within species with an effective population size (Ne) above 500 compared to the number below 500 (Ne>500/Ne<500); Ne tells us the pace of genetic erosion in a population, i.e. if greater than 500, populations are considered big enough to maintain genetic diversity that would also secure the adaptive change (Jamieson and Allendorf, 2012). The second indicator is the proportion of distinct populations maintained within species. In other words, it is the number of genetically distinct, extant populations in relation to the number of extinct ones. This indicator takes into consideration historical data and is influenced by species' ability to shift distribution ranges under climate change. It may serve as a proxy to assess the speed of extinction and populations that are under the biggest threat (e.g based on the location of extant populations). The third indicator is the number of populations with a known DNA based status.

This thesis provides valuable knowledge for the metrics from Hoban et al., 2020. Microsatellite data (STRs) can be used to estimate the Ne of populations (e.g see Xu and Fu 2004, Thuillet et al., 2005), hence the STRs from chapter 2 can be useful in the context although we used only six loci, while for an unbiased estimation of Ne a minimum of 11 STRs is needed (Wang et al., 2021). For the second indicator, we provide a historical overview of the Dinaric populations by reviewing the species distribution (from the year 1885. till present day); we also pinpointed the potential extinction of one population (mt. Treskavica) and provide insights on the effects of climate change on specie's distribution (chapter 7). Nevertheless, the second metrics cannot be definitely assessed since the distribution in the Dinarides is still unresolved (chapter 7). Considering the third indicator, we provide genetic data for new populations of alpine salamanders in relation to existing ones. The latter revealed a high genetic differentiation among Dinaric populations (chapter 2), thus suggesting that each fragment should be treated as a separate conservation unit. Within *prenjensis*, each population is monophyletic (see Figure 2 in chapter 2) which supports this reasoning (see Moritz 1994). Moreover, maintaining genetic diversity requires maintaining populations throughout a species range, not just in small areas as each population contributes to future resilience (Hoban et al., 2021). The genetic assessment (chapter 2) also detected an inbreeding signal in the population of Gorski Kotar that may reduce the viability of offspring, as seen in other amphibian species (see Pabijan et al., 2019, Halverson et al., 2006, Ficetola et al. 2011). The importance of this signal must be evaluated with more genetic markers.

Disease monitoring

'*Salamandra*' species are declining due to amphibian pathogens that are rapidly spreading throughout Europe (the fungi: *Batrachochytrium dendrobatidis* – *Bd* and *B. salamandrivorans* - *B. sal*). Many populations of fire salamanders experienced fatal mortalities (*Bd*: Bosch and Martinez-Solano , 2006 ; *Bsal*: Martel et al 2013, see special issue Salamandra - Volume 56[3])). Setting up disease monitoring programs is essential to prevent pathogen spreading (Gilbert et al., 2020, see http://bsaleurope.com/). As all other *Salamandra* species (no complete data for *S. lanazi*), *S. atra* is susceptible to *Bsal* (Gilbert et al., 2020, EFSA, 2018) but fortunately, there are no official reports of infections in the wild as neither for *Bd* (see *Bd* maps, available at: amphibiaweb.org, see also Baitchman and Herman 2014). The setup of a disease monitoring program is explained in details in EFSA 2018. Shortly, there are two types of surveillance for pathogens detection in the wild: passive and active. Passive surveillance involves the collection of dead salamanders, which should be sent for disease diagnosis. All *Bsal* outbreaks in Belgium, Germany and the Netherlands were detected by passive surveillance

(EFSA 2018). On the other hand, active surveillance is the proactive collection of data on pathogen presence in amphibian populations to promote early detection for disease introduction. This form of surveillance involves visiting subpopulations of salamanders and screening for disease according to a clearly defined and harmonized sampling strategy involving the collection of skin swabs. Several European countries (Austria, Belgium, Croatia, Czech Republic, France, Germany, Portugal, Slovenia, Spain, Switzerland, the Netherlands and the UK) perform a non-systematic active surveillance of amphibian populations; the long-term monitoring actions in Czech Republic can be a great example of good practice (EFSA, 2018). This monitoring started in 2006 and involves four species of Urodela; for the Northern crested newt (*Triturus cristatus*), for instance, there are 30 permanently monitored sites across the entire Czech Republic. Each site is visited at least three times in the season during periods of reproduction and larval metamorphosis. Disease monitoring is coupled with demographic and habitat monitoring to estimate population sizes and structural changes in the habitat (EFSA 2018). Considering *prenjensis*, I note that I screened 145 individuals from Prenj and Čvrsnica (Central Dinarides) for *Bd* and three dead specimens for *Bsal*; analysis revealed no infections in these populations (Šunje et al., 2018). The finding is consistent with the results of infection risk analysis from chapter 5 that unveiled the absence of these pathogens in the soil at both locations. However, since an absence of pathogens on screened individuals does not automatically exclude the presence of disease (EFSA 2018), conducting both passive and active monitoring in *prenjensis* populations is necessary to prevent eventual over-night extinctions.

Last but not least, it is important to mention some of the preventive measures biologists should take in order to prevent the potential spread of pathogens when studying salamanders in the wild. During fieldwork we should avoid direct contact with the animals whenever we can, but, when needed, this should be done with disposable (powderless) gloves changed or thoroughly disinfected after handling each individual. The most effective disinfectants are given in Van Rooij et al., 2017. Other effective measures imply hygiene protocols that also include step-by-step procedures on the disinfection of working equipment, clothes, footwear and vehicles used prior to fieldwork (bsaleurope.com; EFSA 2018).

Delimitation of *prenjensis* distribution

In particular from a conservation perspective, gaps related to *prenjensis* distribution need to be filled in as soon as possible. The missing links in the *prenjensis* haplotype network (Figure2 in chapter 2) suggest the existence of undescribed populations in the Dinarides that may be extinct, or are extant but as yet undiscovered. Chapter 7 illustrates our embarrassingly poor knowledge considering the matter and hints at which areas should be prioritized when trying to find undocumented populations before they disappear. Taking into consideration the relatively bad predictions for the future of the *prenjensis*, we seem to be running out of time, which also stresses the urgency for setting up the previously proposed conservation measures (monitoring programs).

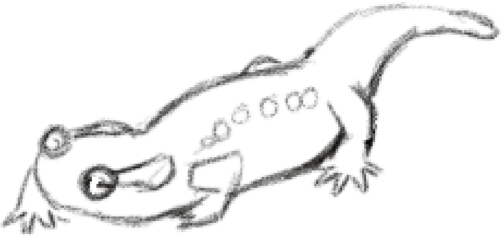
Captive breeding programs

A possible additional course of action could be to establish breeding populations from which individuals can be used to restock dwindling populations or empty habitat patches. Specifically, breeding animals may be used to enrich populations that have an effective population size (Ne) < 500 or, exhibit signs of inbreeding (see previously). Moreover, a stock of individuals would allow their introduction to new areas that might have been unsuitable since the last glaciations but became convenient with the course of climate change (e.g in the Southern Dinarides, see chapter 7).

The number of captive breeding programs and reintroduction of amphibian populations significantly increased in the past 10 years. However, reintroduction, although considered as high profile, remains a controversial intervention (Harding et al., 2016). The main difficulty is that the factors threatening amphibian populations are often persisting, reducing the likelihood of successful reintroduction in the long term (Harding et al., 2016). This explains why only a small number of amphibian captive breeding programs proceeded with reintroduction activities. However, from those that did, around 60% of species established self-sustaining populations in the wild (see Table 1 - Griffiths and Pavajeau 2008). This suggests a moderate response of amphibians to reintroductions. Identifying suitable areas for introduction using very precise modelling approaches that incorporate biotic aspects of the species to predict distribution (e.g mechanistic models - Kearney and Porter, 2009, individual/agent – based models - Akçakaya, et., 2008, remote sensing analysis – Ma et al., 2019) may increase the success of these programs. However, before any action, it must be assured that the species presence in new areas would not misbalance the ecosystem (e.g. through interspecific competition or by exterminating predators due to the toxic nature of salamanders). Moreover, findings from chapter 2 suggest that the long separation between population fragments led to the development of adaptive gene complexes that may become disrupted by randomly mixing individuals from all populations in a hypothetical translocation event (Frankahm and Ballou, 2012). This explains why, apart from identifying the most suitable locations for introduction, carefully designed studies should aim to increase the genetic (adaptive) potential of introduced individuals. These studies involve identifying and mixing the right specimens from different populations, which is a big challenge (Frankahm and Ballou, 2012). Studying mate choice among individuals from different populations may be useful when combined with research on the viability of hybrids between populations/lineages.

The establishment of breeding programs would not require a large infrastructure nor specific facility which makes the breeding program affordable, moreover because the initial stock of animals would be taken directly from nature. I have no doubts that the initiation of such programs is worthwhile. The establishment of new populations in new areas, given its neutral impact assessment and carefully designed research steps (aforementioned), would benefit the long-term preservation of the species.

Nevertheless, I find that the lack of basic research on the species biology is hampering broader understandings. In the context of breeding programs, we do not know much about the environmental triggers that are necessary for a successfully reproduction. For instance, the courtship behavior and reproductive cycle of *S. atra* is well understood (Klewen, 1991) but I was unable to find any information considering breeding success in captivity. Cracking this process is necessary for setting up breeding programs and successful species propagation that may secure a long-term preservation of the alpine salamander. Moreover, the underground life of the species remains a mystery. As pointed out throughout the thesis, the alpine salamander spends most of its life below the surface ground but we do not know much about this underground life (e.g hibernation rhythm, experienced fluctuations in abiotic factors, possible intra and interspecific interactions). Hence, I strongly encourage basic biological research involving more observations of the species in its natural habitat along with efforts to propagate the animals in captivity.



Supplement

Supplement overview

Figure S2.1 STRUCTURE anaylsis - different combinations of ancestry and allele frequency models

Figure S2.2 Factorial Correspondence Analysis (FCA) of microsatellite data from Dinaric individuals of *S. atra*

Table S2.1. Accession numbers (Acc. No) of the sequences (*cob* and *D-loop*) from public repositories (GenBank) used in this study

Table S2.2. PCR conditions for amplification of microsatellite loci

Table S2.3. Raw microsatellite data for 95 *S. atra* individuals sampled in the study.

Figure S3.1. Dorsal and ventral view of morphological traits

Figure S3.2. PCA analysis of B&H individuals (measurments from multiple observers)

Table S3.1. Number of males and females sampled in each location.

Table S3.2. Intra- and inter-class correlation coefficient (ICC/R) for the two observers

Table S3.3. Number of individuals from each subpopulation with respective number of costal grooves

S7.2 Processing extent

Figure S7.2. Spatial extent of ecoregions (Olson et al., 2001) where alpine salamanders occur

Table S7.2. Number of occurrence points for each modelling unit that are found in a respective ecoregion.

S7.3 Climate scenarios

Table S7.3. Overview of General Circulation Models (GCM) used in this study

S7.4 Removal of climate variables

Figure S7.3. Overview of temperature-related bioclimatic variables for the study extent.

Figure S7.4. Overview of precipitation-related bioclimatic variables for the study extent.

Figure S7.5: Spatial distribution of the month that is considered as the first month of the driest quarter.

Figure S7.6. Spatial distribution of the month that is considered as the first month of the wettest quarter.

Table S7.4. Values of the Variance Inflation Factor (VIF)

S.7.5 Project code

S.7.6 Digital Supplementary Information

APPENDIX CHAPTER 2

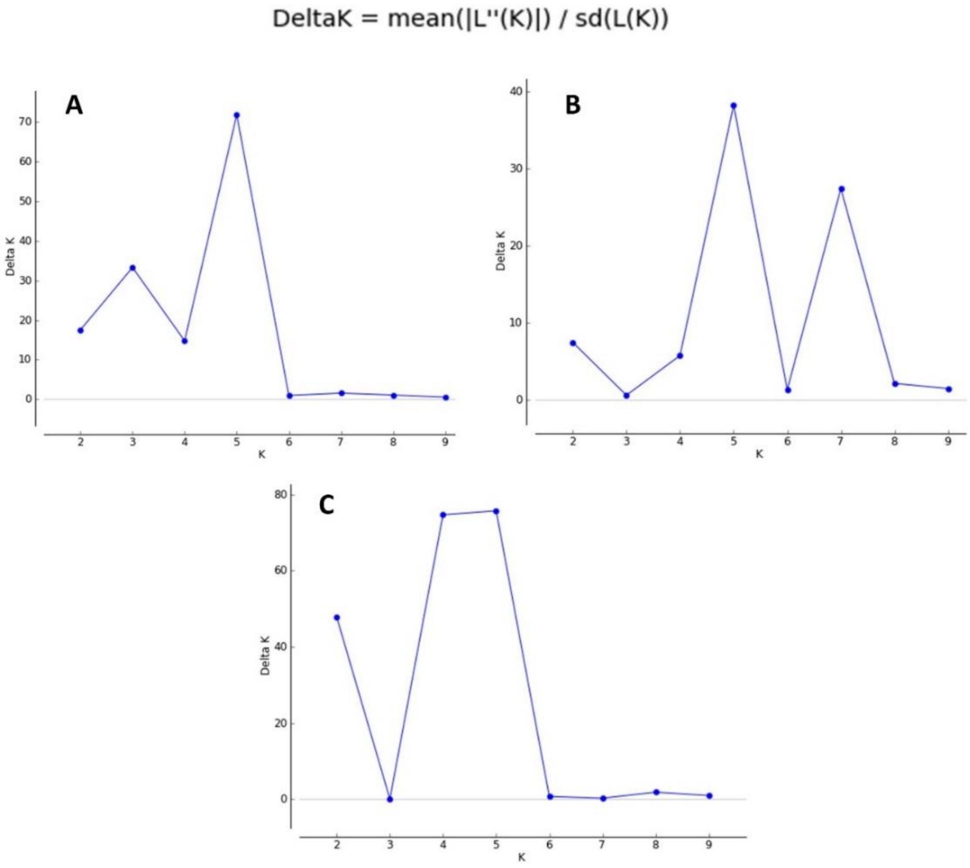


Figure S2.1 Structure exploratory anaylsis using different combinations of ancestry and allele frequency models (5 simulations, 100000 iterations, 25000 burn-in): A: admixture and independent; B: no admixture and correlated; C – no admixture and independent; x – axis: K number (2-9), y – axis – Delta K. All exploratory analysis suggested five clusters as it did the final analysis (see text for details).

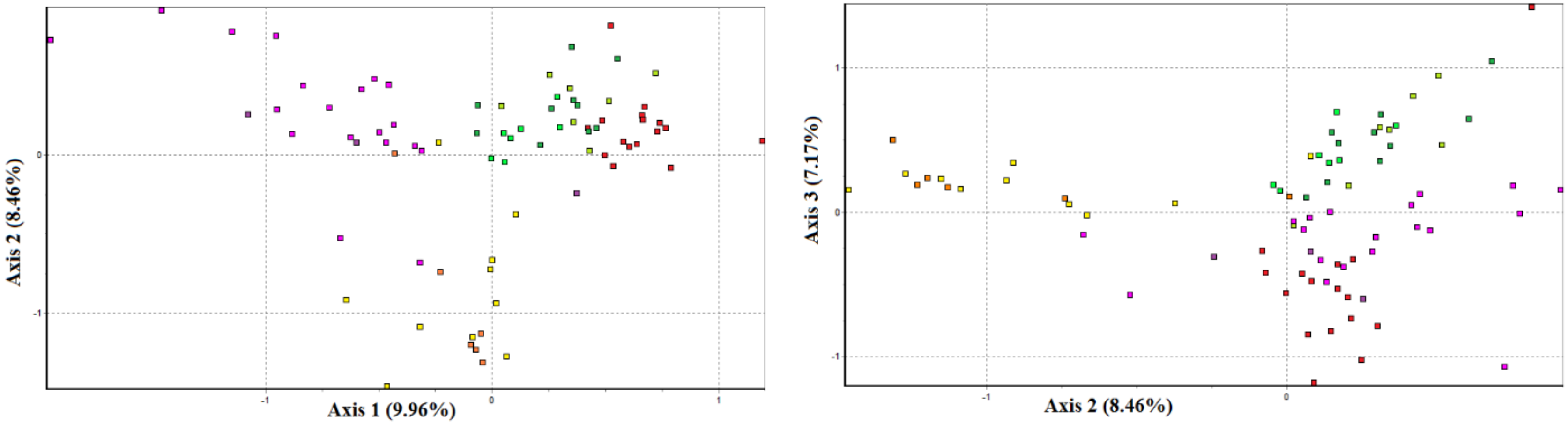


Figure S2.2. Factorial Correspondence Analysis (FCA) ordination along the first three axes of 67 individuals of *Salamandra atra* from the Dinarides based on six microsatellite loci. Each label corresponds to a sampling site (from north to south): Gorski Kotar [yellow – Vihoraški put, orange – Samarske stijene], Prenj [light green - Zakantar, dark green – Podotiš, green – Kopilice], Prokletije [pink – Bogićevica, violet – Gozdarevac], Čvrsnica – red. No clear clustering of sampling sites within the same sampling area is inferred. The individuals from Čvrsnica are included for a proper partitioning of variation among the Dianric population. Axis 2 separates well Gorski Kotar (Northern Dinarides) from the other sampling areas (Central and Southern Dinarides).

Table S2.1. Accession numbers (Acc. No) of the sequences (*cob* and *D-loop*) from public repositories (GenBank) used in this study (courtesy of Crestanello). Hap: Inferred haplotypes (as in Fig. 2 and Table 1). Codes of sampling locations are as in Fig. 1 and Table 1. Collapsed haplotypes from BONATO et al. (2018) are in bold. In the last column, if different, the reference for *cob* is separated from the reference of *D-loop* by a comma: α - BONATO et al. (2018), β - VENCES et al. (2014), γ - STEINFARTZ et al. (2000), δ - this study.

Hap	Code	Acc. No: cob	Acc. No: D-loop	Reference
H1	18	MN255339	MN255326	δ
H2	18	MN255340	MN255327	δ
H3	18	MN255341	MN255328	δ
H4	19	MN255342	MN255329	δ
H5	19	MN255343	MN255330	δ
H6	23	MN255345	MN255332	δ
H7	26, 27	MN255351	MN255338	δ
H8	20	MN255344	MN255331	δ
H9	22	MG968412	MG968391	α
H10	22	MG968413	MG968391	α
H11	24	MN255346	MN255333	δ
H11	21, 25	MG968412	MG968392	α
H12	20, 23, 22	MN255347	MN255334	δ
H12	21, 25	MG968415	MG968392	α
H12	17	MG968415	MG968393	α
H13	24, 22	MN255348	MN255335	δ
H13	21, 22, 25	MG968414	MG968394	α
H14	9	MG968416	AY628442.1	α, γ
H15	9	MG968417	AY628442.1	α, γ
H16	16	MN255349	MN255336	δ
H17	16	MN255350	MN255337	δ
H18	1	MG968419	MG968401	α
H19	1, 2	MG968411	MG968401	α
H20	1, 2	MG968418	MG968401	α
H20	1	MG968411	MG968390	α

H21	10, 11, 12, 13	MG968410	AY628440.1	α, γ
H22	12	MG968410	MG968399	α
H23	10	KF645910	KT335870	β, γ
H24	14, 15	MG968403	KF645583.1	α, β
H25	3	MG968402	MG968400	α
H26	8	MG968405	MG968397	α
H27	8, 7, 6	MG968406	MG968397	α
H28	6	MG968404	MG968397	α
H29	4	MG968409	MG968396	α
H30	5	MG968408	MG968395	α
H31	5	MG968407	MG968397	α
H32	6	MG968407	MG968398	α

Table S2.2. PCR conditions and characteristics of the microsatellite loci

Locus/Forward primer label	PCR option	Allelic range	Number of alleles	Primer conc. (µM)	PCR thermal profile
SalE6/HEX	MIX1	252-300	9	0.20	94 °C (120s) [94 °C (40s) – 60 °C (30 s) – 65 °C (40 s) x 35] 65 °C (300 s)
SalE8/HEX		135-157	7	0.10	
SalE12/TAMRA		160-194	12	0.10	
SalE7/HEX	MIX2	188-220	10	0.08	94 °C (120 s) [94 °C (40 s) – 56 °C (30 s) – 65 °C (40 s) x 35] 65 °C (300 s)
SalE23/HEX		257-323	7	0.20	
SalE14/TAMRA	Single	219-265	7	0.08	94 °C (120 s) [94 °C (40 s) – 64 °C (30 s) – 65 °C (40 s) x 35] 65 °C (300 s)

Table S2.3. Raw microsatellite data for 95 individuals sampled in this study. S. – sampling; K. – Kotar; st. - stijene

S. area	S. site	SalE8	SalE8	SalE6	SalE6	SalE12	SalE12	SalE7	SalE7	SalE23	SalE23	SalE14	SalE14
Čvrsnica	Pločno	149	149	268	268	168	168	194	198	299	303	225	225
Čvrsnica	Pločno	149	149	264	280	176	180	198	198	299	299	229	229
Čvrsnica	Pločno	149	149	264	280	176	180	198	198	299	299	229	229
Čvrsnica	Pločno	149	149	280	280	166	176	198	198	299	303	229	229
Čvrsnica	Pločno	149	149	264	264	176	186	198	198	299	303	229	229
Čvrsnica	Pločno	139	149	252	264	176	180	198	198	299	299	229	229
Čvrsnica	Pločno	149	149	264	280	176	176	198	198	299	299	229	229
Čvrsnica	Pločno	149	153	264	280	176	180	198	198	299	299	229	229
Čvrsnica	Pločno	149	153	280	280	176	180	198	198	299	299	229	229
Čvrsnica	Pločno	149	149	280	280	180	186	198	198	299	299	229	229
Čvrsnica	Pločno	149	149	264	280	176	180	198	198	299	303	229	229
Čvrsnica	Pločno	149	149	280	280	180	180	198	198	299	299	229	229
Čvrsnica	Pločno	149	149	264	280	180	186	198	198	299	299	229	229
Čvrsnica	Pločno	149	149	264	280	176	190	198	198	299	299	229	229
Čvrsnica	Pločno	149	149	264	280	176	186	198	198	299	299	229	229
Čvrsnica	Pločno	149	149	264	284	176	180	198	198	299	299	229	229
Čvrsnica	Pločno	149	149	264	280	176	176	198	198	299	299	229	229
Čvrsnica	Pločno	149	149	264	280	176	186	198	198	299	299	229	229
Čvrsnica	Pločno	149	149	280	280	176	176	198	198	299	303	229	229
Čvrsnica	Pločno	149	149	264	280	176	180	198	198	299	299	229	229
Prenj	Kopilice	149	149	254	264	180	180	194	198	299	299	225	225
Prenj	Kopilice	149	149	264	264	180	184	194	198	299	299	225	229
Prenj	Podotiš	149	149	264	268	186	190	194	194	299	299	225	229
Prenj	Podotiš	149	149	264	264	184	184	194	194	299	299	229	229
Prenj	Kopilice	149	149	264	268	188	192	194	194	299	299	229	229
Prenj	Podotiš	149	149	264	264	168	188	194	198	299	299	229	229

Prenj	Podotiš	149	149	264	264	172	190	194	194	299	299	229	229
Prenj	Kopilice	149	149	264	264	176	180	194	198	299	299	229	229
Prenj	Kopilice	149	149	264	264	168	190	194	198	299	303	225	229
Prenj	Podotiš	149	149	264	264	168	176	194	198	299	303	225	229
Prenj	Podotiš	149	149	264	264	172	188	194	194	299	299	229	229
Prenj	Kopilice	149	149	264	264	172	188	194	194	299	299	225	229
Prenj	Podotiš	149	149	264	268	168	180	194	194	299	299	225	225
Prenj	Podotiš	149	149	264	268	176	184	194	194	299	307	225	229
Prenj	Zakantar	149	149	264	264	176	188	194	204	299	307	225	229
Prenj	Podotiš	149	149	264	264	176	184	194	198	299	303	225	225
Prenj	Zakantar	149	149	264	264	176	176	194	194	299	307	229	229
Prenj	Kopilice	149	149	264	264	168	176	194	194	299	307	225	229
Prenj	Podotiš	149	149	264	264	176	180	194	194	299	307	225	229
Prenj	Zakantar	149	149	264	264	176	180	194	204	299	307	229	229
Prenj	Zakantar	149	149	264	264	172	176	194	194	299	299	229	229
Prenj	Zakantar	149	149	264	264	176	180	194	194	299	307	225	229
Prenj	Zakantar	149	149	264	264	168	176	194	194	299	299	225	229
Prenj	Podotiš	149	149	264	264	176	180	194	194	299	299	229	229
Prenj	Zakantar	149	149	264	264	176	176	194	194	299	307	225	229
Prokletije	Bogićevica	149	149	264	280	176	184	194	194	307	307	229	229
Prokletije	Bogićevica	149	149	284	284	160	168	194	194	307	307	229	229
Prokletije	Bogićevica	145	149	280	284	176	184	194	194	307	307	229	229
Prokletije	Bogićevica	149	149	264	280	176	180	194	194	307	307	229	229
Prokletije	Bogićevica	149	149	264	284	176	180	194	194	307	307	229	229
Prokletije	Bogićevica	149	149	264	280	176	176	194	194	307	307	229	229
Prokletije	Bogićevica	149	149	264	280	176	180	194	194	307	307	229	229
Prokletije	Bogićevica	145	149	264	280	180	180	194	194	307	307	229	229
Prokletije	Bogićevica	149	149	280	284	168	176	194	194	307	307	229	229
Prokletije	Bogićevica	145	145	280	280	160	184	194	194	307	307	219	229
Prokletije	Bogićevica	145	149	280	280	180	190	194	194	307	307	225	229

Prokletije	Bogićevica	149	149	280	280	180	180	194	194	307	307	229	229
Prokletije	Bogićevica	145	145	264	280	180	180	194	194	307	307	229	229
Prokletije	Bogićevica	149	157	272	280	168	176	194	194	307	307	229	229
Prokletije	Bogićevica	149	149	280	284	180	184	194	194	307	307	229	229
Prokletije	Bogićevica	135	157	280	284	184	188	194	194	307	307	229	229
Prokletije	Bogićevica	149	149	264	280	176	180	194	194	307	307	229	229
Prokletije	Gorazdevac	145	145	280	280	180	184	194	194	307	307	229	229
Prokletije	Bogićevica	145	149	264	280	176	180	194	194	307	307	229	229
Prokletije	Bogićevica	145	149	280	284	168	176	194	194	307	307	229	229
Prokletije	Gorazdevac	145	149	264	280	176	180	194	194	307	307	229	229
Prokletije	Bogićevica	149	149	280	280	168	188	194	194	307	307	229	229
Prokletije	Gorazdevac	149	149	264	280	176	180	192	198	299	299	229	229
Prokletije	Bogićevica	149	149	264	280	176	180	192	192	307	307	229	229
Prokletije	Bogićevica	145	149	280	280	180	180	192	192	307	307	229	229
Gorski K.	Vihoraški put	141	145	264	268	180	184	192	192	303	307	229	229
Gorski K.	Samarske st.	149	149	264	292	176	176	192	192	303	307	229	229
Gorski K.	Samarske st.	149	149	264	264	176	176	188	192	307	307	229	229
Gorski K.	Vihoraški put	149	149	264	264	176	176	192	192	303	311	229	229
Gorski K.	Samarske st.	149	149	264	264	176	176	188	192	307	307	229	229
Gorski K.	Vihoraški put	149	149	264	264	176	180	192	192	299	307	229	229
Gorski K.	Samarske st.	149	149	264	264	176	180	192	192	303	307	229	233
Gorski K.	Vihoraški put	149	149	264	264	180	184	192	192	307	311	229	229
Gorski K.	Vihoraški put	149	149	264	264	176	176	192	196	303	307	229	229
Gorski K.	Samarske st.	149	149	264	264	176	176	192	196	307	311	229	229
Gorski K.	Vihoraški put	145	149	264	264	176	184	192	192	311	311	229	229
Gorski K.	Samarske st.	149	149	264	264	176	188	192	192	311	315	229	229
Gorski K.	Vihoraški put	149	149	264	264	180	180	190	194	311	311	229	229
Gorski K.	Samarske st.	149	149	264	264	180	184	194	194	307	307	229	229
Gorski K.	Vihoraški put	149	149	264	264	176	176	194	198	307	315	229	229
Gorski K.	Vihoraški put	149	149	264	264	180	180	194	198	307	311	229	233

Gorski K.	Vihoraški put	149	149	264	268	176	176	194	194	307	307	229	229
Kredarica	Aljažev dom	149	149	280	284	176	176	212	212	307	323	229	241
Kredarica	Aljažev dom	149	149	284	292	176	194	198	212	257	299	235	265
Kredarica	Aljažev dom	149	153	284	284	176	186	212	220	299	303	229	229
Kredarica	Aljažev dom	149	149	280	280	176	176	212	212	299	323	229	229
Kredarica	Aljažev dom	149	149	280	300	176	180	202	212	299	307	229	235
Kredarica	Aljažev dom	149	149	284	292	176	194	198	212	299	307	235	235
Kredarica	Aljažev dom	149	153	284	300	180	180	212	212	307	323	229	241
Kredarica	Aljažev dom	149	149	284	284	176	176	212	220	303	307	229	229

APPENDIX CHAPTER 3

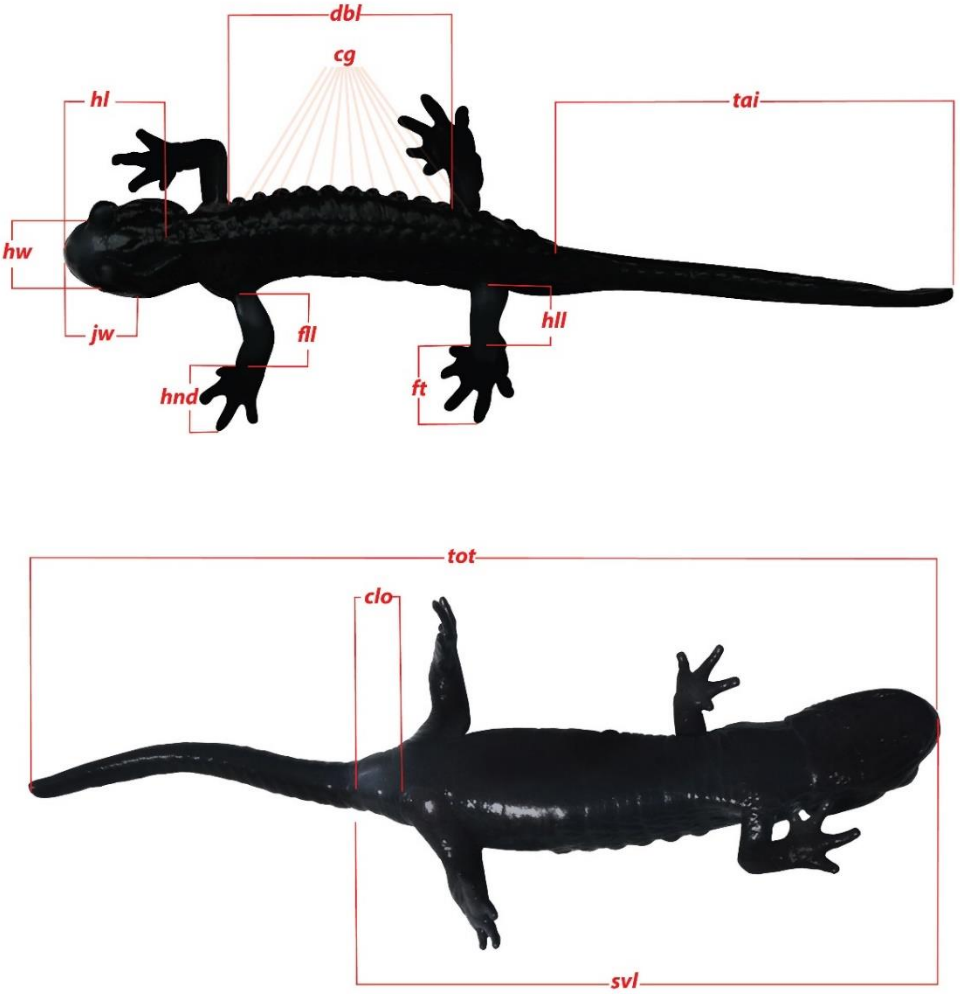


Figure S3.1. Dorsal and ventral view of observed morphological traits (full trait names are given in table 1 - see text)

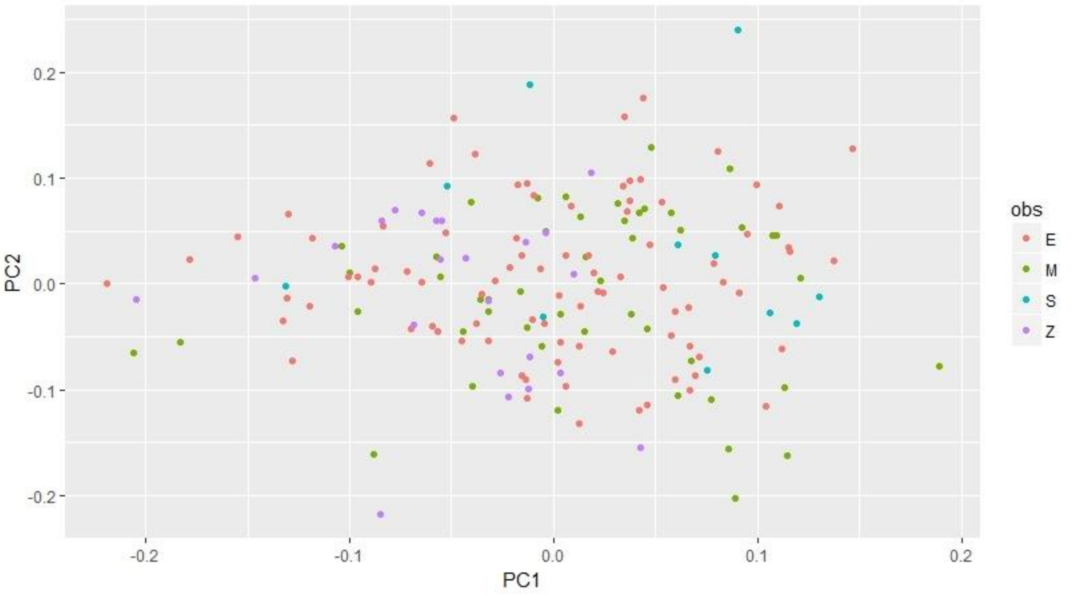


Figure S3.2. PCA analysis of B&H individuals taken by multiple observers suggests no observer effect on the measurments. Observer: E is the common observer of the two populations.

Table S3.1. Number of males (Nm) and females (Nf) sampled in each location. The total number of individuals measured by the common observer of both populations (Nob) is mentioned as indicative information. B&H - Bosnia and Herzegovina.

Population	Subpopulation	Location	Nm	Nf	Nob
B&H	Prenj	Kopilice	11	18	29
B&H	Prenj	Podotiš	23	26	32
B&H	Prenj	Zakantar	13	18	0
B&H	Prenj	Osobac	4	4	8
B&H	Prenj	Soplje	2	5	3
B&H	Čvrsnica	Ledeno jezero	34	25	16
Austria	Bluntautal	Nördlichen	8	15	3
Austria	Tappenkarsee	Radstädter Tauern	23	16	11

Table S3.2. Intra- and inter-class correlation coefficient (ICC/R) for the two observers (class), as assessed based on variances of linear mixed models of repeated measurments for all traits (see e.g. table 1 for full names).

Observer	clo	fll	cg	hl	hll	tot	hw	hnd	svl	jw	fl	ta	dbl
intra ICC	0.92	0.90	1	0.93	0.88	0.99	0.94	0.91	0.98	0.96	0.94	0.98	0.84
inter ICC	0.50	0.90	0.7	0.90	0.88	0.99	0.93	0.91	0.97	0.96	0.94	0.97	0.84

Table S3.3. Number of individuals from each subpopulaitons with the respective number of costal grooves (cg; column names reffer to the range for cg: 9 -15 – see Table 1)

Subpopulation	9	10	11	12	13	14	15
Bluntautal	0	2	4	9	5	2	1
Tappenkar	0	2	6	17	7	7	0
Čvrsnica	1	9	31	15	2	1	0
Prenj	7	36	47	16	11	6	0

APPENDIX CHAPTER 4

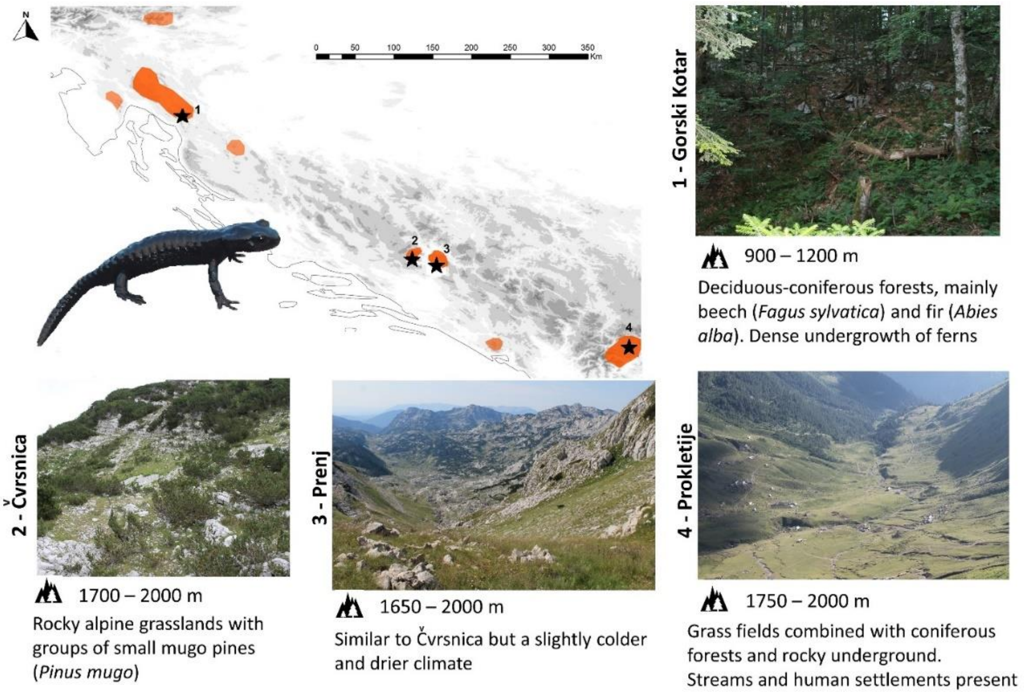


Figure S4.1. The Dinaric Arc and distribution of *Salamandra atra prenjenis* (orange polygons). The stars show the sampling locations (i.e, populations); the habitat of each population is photographed and described

Table S4.1. Field work details: *n* – total number of flushed individuals/pitfall traps. The number of pitfall traps is given for the date when these were positioned in the field (the content of each was collected after 21 days in the field).

Study site	**Date of field work**	***n***	***n* Pitfall traps**	**Net sampling**
Gorski Kotar ∑N=78	30.6.2016	5		
	8.9.2016	16		
	22.9.2016	4		
	27.7.2017	20	6	+
	28. – 29.8.2017	26	6	
	30.9.2017	4		
	1.10.2017	3		
Čvrsnica ∑N=72	24.8.2014	15	3	
	30.8.2014	14		
	20.7.2015	5		
	3. - 5. 8.2015	15		
	23.7.2016	14		
	25.8.2016	9		
	10.8.2017	0	4	+
Prenj ∑N=93	20 - 25. 8.2014	61	6	
	13. - 14. 7.2015	10		
	5. – 8. 9.2015	14		
	7. – 8. 7.2016	8	3	+
Prokletije ∑N=21	11. – 12. 7.2016	21	3	
	15.8.2016	0		+

APPENDIX CHAPTER 5

Supplementary methods

SAM analysis

Samples were analyzed using Ultra Performance Liquid Chromatography – tandem Mass Spectrometry (UPLC-MS/MS). Prior to analysis, SAMs were extracted by soaking the gauzes with 400 µL 50% methanol solution. After 30 minutes of extraction, 200 µL of the solution was filtered (Chromafil AO20/3 polyamide syringe filter 0.20µm, Ø3 mm, MACHEREY-NAGEL, Düren, Germany) by centrifugation (15 min, 7000 rpm, Eppendorf centrifuge 5810R, Eppendorf AG, Hamburg, Germany).

An ACQUITY UPLC system coupled to an ACQUITY TQD mass spectrometer (Waters, Milford, USA) was used to analyze the samples for the presence of alkaloids. We used an ACQUITY UPLC® BEH C18 1.7µm, 2.1 X 100mm column. Solvent A= H2O + 0.1% of FA and solvent B= AN + 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.

Testing SAM degradation

Since most samples were stored for several weeks before analysis, a small experiment was conducted to check whether degradation of SAMs occurred. Nine Bosnian salamanders were exported (Ministry of Foreign trade and Economic Relations, Sarajevo, certificate nr: BA-KZV-VZ-40/14) to and kept in captivity at the University of Anwerp (approved by Ethical Committee of the University of Antwerp, ECD nr: 2016-64). Salamanders were sampled according to previously described methods (see main text) and the collected secretions were analyzed 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 our method of storing secretions was efficient, degradation of SAMs 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 secretions with UPLC-MS/MS was conform the previous described method.

Salamanders were kept in plastic boxes (45x65x25 cm) covered with soil, branches, stones and moss as enrichment. Each box housed five to six individuals. Animals were fed once a week ad libitum with crickets and mealworms. Temperature varied between 12-17°C and humidity between 85-95 %. Salamanders were checked daily and soil was changed every three weeks.

Degradation of SAMs was analyzed using a general mixed model, with peak surface area (log-transformed) as response variable and time as continuous covariate. Type of SAM and treatment were included as fixed factors and animal ID as a random factor. Interactions between time and type of SAM were also added, to test whether different SAMs degraded at different rates, and between time and treatment.

Identification of soil fungi

To isolate genomic DNA from the other half of 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.

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 5X Phusion® GC-buffer, 0.5 µL deoxynucleotide solution mix (10 Mm), 1 µL forward primers (10 µM), 1 µL reverse primers (10 µM) and 0.2 µL Phusion® DNA Polymerase (2 U μL^{-1}). Modified versions of the primers ITS1F and ITS2, which amplify the fungal internal transcribed spacer (ITS) 1 region, were used as in Smith & Peay (2014). Each sample was amplified with an ITS2 primer containing a unique index sequence.

PCR was performed in a C1000 Touch™ Thermal Cycler (Bio-Rad, Hercules, USA). 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 a longer extension step for 10 minutes was added. Water samples were used as negative control. For negative PCRs, we repeated the extraction of DNA from these samples, additional purified them with ethanol precipitation and finally increased the number of cycles to 40. PCR amplicons were purified and their concentrations normalized using the SequalPrep™ Normalization Plate Kit (Invitrogen Corporation, Carlsbad, USA). PCR amplicons were then pooled together into one DNA-library which was separated from primers by gel-electrophoresis, and washed following the protocol of QIAquick® Gel Extraction Kit (Qiagen, Venlo, the Netherlands).

The DNA-library was prepared using a MiSeq® v2 Reagent Kit (Illumina Inc., San Diego, USA). The diluted and denaturized 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.

Supplementary results

SAM degradation

Peak surface area decreased significantly with time of storage (slope = -0.02 log-units per day; $F_{1,240} = 12.21$; $p < 0.001$), 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 other treatments (t-value = -2.40; p = 0.02). There was no significant interaction between time of storage and the type of SAM ($F_{9,240} = 0.64$; $p = 0.76$). Different compounds therefore degrade at the same rate.

Supplementary tables

Table S5.1. Settings selected for the Multiple Reaction Monitoring (MRM) during Ultraperformance Liquid Chromatography – tandem Mass Spectrometry (UPLC – MS/MS) of eight SAMs in the parotoid secretions of *Salamandra atra*. For samanol two different peaks were found in each chromatogram, but due to the lack of reference standards, we could not unambiguously designate one peak as samanol. Hence, the two peaks will be referred to as samanol and samanol2.

SAM	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 S5.2. Overview of microhabitats sampled in each population + the number of samples from which bacterial densities (Bact) or soil fungi communities (Fung) were estimated (see main text). Not all microhabitats were present at each location (indicated by a 0). Differences in sample size between Bact and Fung result from the fact that not all samples were suited for cultivation of bacteria.

MICROHABITAT	**Gorski Kotar**		**Čvrsnica**		**Prenj**		**Prokletije**	
	Bact	**Fung**	**Bact**	**Fung**	**Bact**	**Fung**	**Bact**	**Fung**
Rock ***(includes crevices leading to burrows)***	3	4	3	5	6	6	2	2
Plant litter	0	2	1	1	0	0	2	2
Wood ***(rotting tree trunks used as shelter)***	1	2	0	0	0	0	4	4
Moss	1	2	0	0	0	0	2	2
Ground ***(bare soil)***	0	0	1	1	2	2	2	2

Table S5.3. Geographical distances (in meters) between the four populations of *Salamandra atra* sampled in this study. Distances were calculated using the *geosphere* package in R (Hijmans, 2016). See also Figure 1 in main text.

	Čvrsnica (BIH)	**Gorski Kotar (HRV)**	**Prenj (BIH)**	**Prokletije (MNE)**
Čvrsnica (BIH)	-			
Gorski Kotar (HRV)	276 689	-		
Prenj (BIH)	26 087	299 779	-	
Prokletije (MNE)	230 932	504 449	205 995	-

Table S5.4. Morphological measurements of *Salamandra atra*. Mean values + SE are given per population and per sex. Sample sizes: Čvrsnica (25),Gorski Kotar (14), Prenj (46) and Prokletije (44). Females (65) and males (64).

	Gorski Kotar	**Čvrsnica**	**Prenj**	**Prokletije**	**Sex**	
					F	M
Parotoid width (mm)	4.14 ± 0.11	4.82 ± 0.11	3.95 ± 0.08	4.10 ± 0.08	4.37 ± 0.07	4.13 ± 0.07
Parotoid length (mm)	9.07 ± 0.17	9.72 ± 0.17	9.58 ± 0.12	9.55 ± 0.13	9.61 ± 0.10	9.34 ± 0.11
Head width (mm)	12.2± 0.3	13.9 ± 0.3	12.7 ± 0.2	12.8 ± 0.2	13.1 ± 0.2	12.7 ± 0.2
Head length (mm)	15.8 ± 0.4	18.1 ± 0.3	18.2 ± 0.3	17.5 ± 0.3	17.6 ± 0.2	17.2 ± 0.23
Body mass (g)	8.8 ± 0.5	11.1 ± 0.4	9.7 ± 0.3	9.2 ± 0.3	10.5 ± 0.3	8.9 ± 0.3

Table S5.5. List of herpetological experts + affiliations who were contacted to provide information about the presence/absence of snake species in each population (continued on next page for Prokletije).

Population	Local expert
Gorski Kotar	**Boris Lauš | Herpetologist** Association for Biodiversity Research: Hyla, Lipovac I 7, 10 000 Zagreb, Croatia boris.laus.pmf@gmail.com http://www.hhdhyla.hr
Prenj and Čvrsnica	**Adnan Zimić | Herpetologist** Natural History Museum & Herpetological Association in B&H Zmaja od Bosne 3 & Urijan dedina 137 71 000 Sarajevo, Bosnia and Hercegovina (B&H) adnan.zimic@gmail.com www.zemaljskimuzej.ba www.bhhuatra.com
Prokletije	**Prof. Dr Ljiljana Tomović | Herpetologist** Faculty of Biology (Animal morphology, systematics, and phylogeny) University of Belgrade Studentski trg 16 11 000 Belgrade, Serbia **lili@bio.bg.ac.rs** **Vernes Zagora | Herpetologist** Montenegrin Ecological Society Bulevar Sv. Petra Cetinjskog 73 81000 Podgorica, Montenegro vzagora92@gmail.com **Dr. Rastko Ajtić | Herpetologist** Institute for Nature Conservation of Serbia Dr Ivana Ribara 91 11 070 Belgrade, Serbia rastko@mail.ru

Table S5.6. Mean values + SE for microbial data per population. CFU stands for Colony Forming Unit. TSA stands for Tryptic soy broth medium. Fungi species were identified and counted through next-generation sequencing of soil samples. Sample sizes were as follows: Gorski Kotar (bacteria 5/ fungi 10), Čvrsnica (bacteria 5/ fungi 7), Prenj (bacteria 8/ fungi 8) and Prokletije (bacteria 12/ fungi 12).

	Gorski Kotar	**Čvrsnica**	**Prenj**	**Prokletije**
CFU/g on TSA at 37°C	1.1 ± 0.7 $*10^5$	1.1 ± 0.8 $*10^5$	1.7 ± 1.1 $*10^5$	0.6 ± 0.3 $*10^5$
CFU/g on TSA at 15°C	34.2 ± 37.5 $*10^5$	6.7 ± 7.5 $*10^5$	26.5 ± 24.9 $* 10^5$	14.7 ± 10.8 $* 10^5$
CFU/g on Mac-Conkey	0.9 ± 1.0 $*10^5$	0.1 ± 0.2 $*10^5$	0.4 ± 0.4 $*10^5$	0.1 ± 0.1 $* 10^5$
# parasite species per sample	2.33 ± 0.33	0.71 ± 0.38	0.25 ± 0.35	1.00 ± 0.28
# fungi species per sample	49.6 ± 7.4	61.0 ± 8.7	59.1 ± 8.8	47.8 ± 6.6
Total # parasite species	9	5	6	7
Total # fungi species	270	237	373	364

Supplementary figures

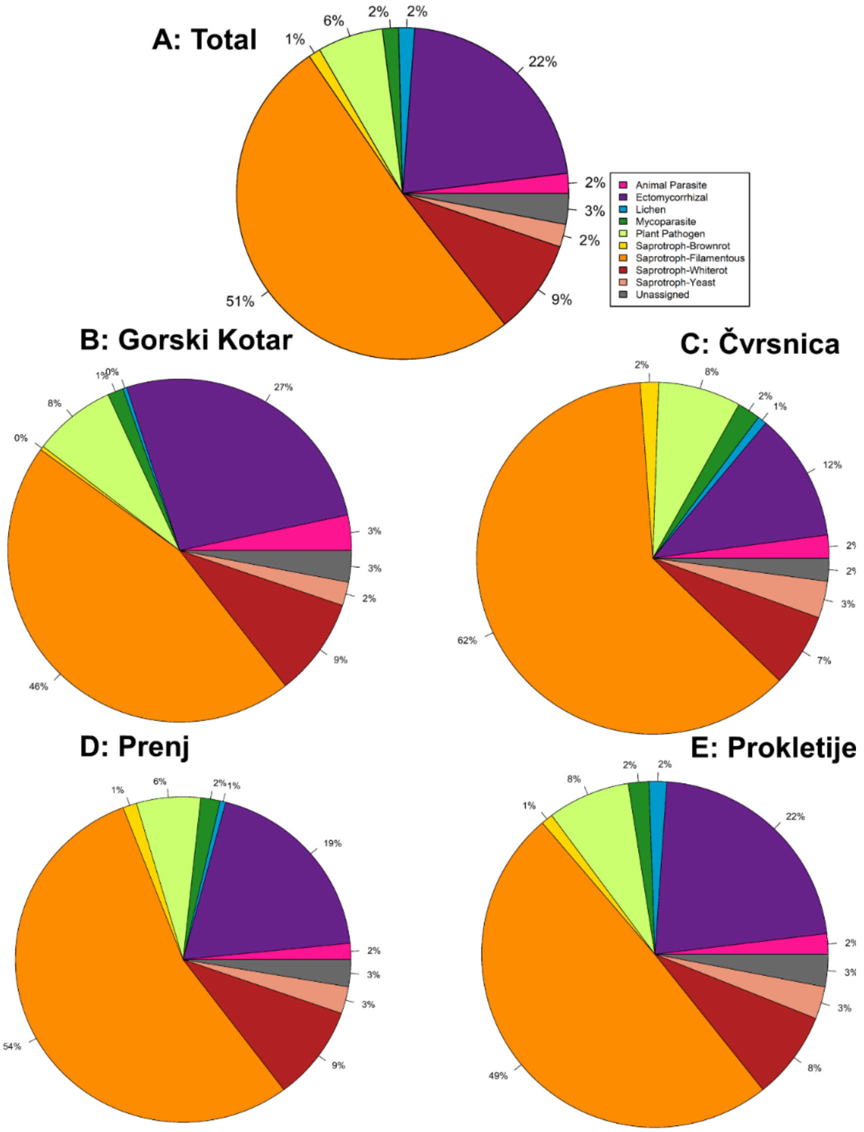


Figure S5.2. Distribution of lifestyles of identified fungal OTUs in general (Total) and per population. Assignment of lifestyles to genera was based on the database of Tedersoo *et al.* (2014). NA's were removed. Unassigned species were species that could be identified, but were not assigned to a lifestyle 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).

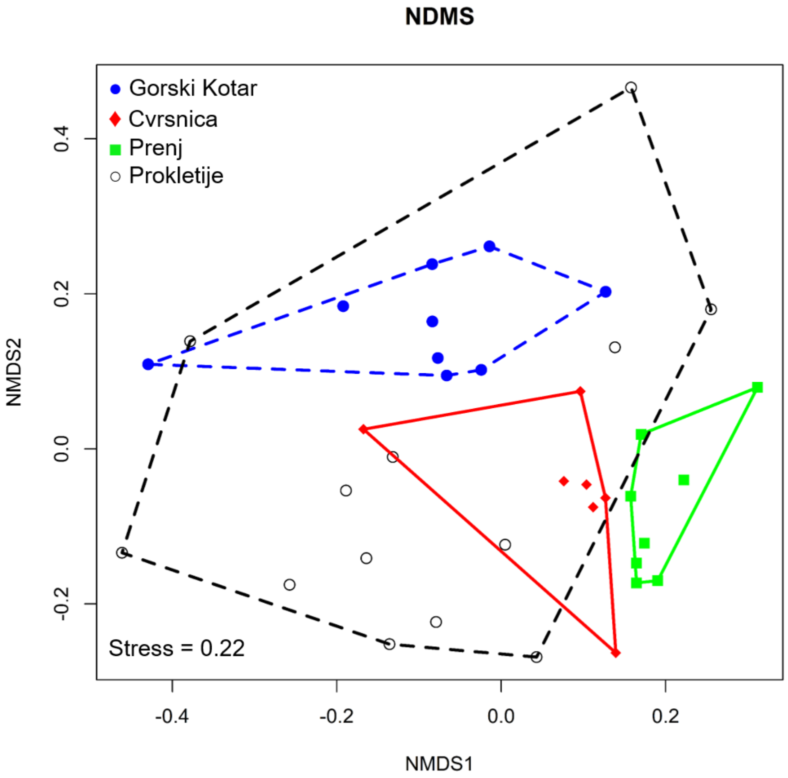


Figure S5.2. nMDS plot of soil fungi communities in the four populations, based on a Bray-Curtis dissimilarity matrix. Each point represent a different soil sample. Distance between points reflects the difference in soil fungi community. Convex hulls were drawn around all points of the same population.

APPENDIX CHAPTER 6

Supplementary material

Spatial data: environmental characteristics of the study sites

To characterize the environments of the two study sites (populations: Gorski Kotar - GK in the Northern Dinarides and mt Čvrsnica - CV in the Central Dinarides, Fig. S1) we used macroclimatic data of the average temperature (°C), water vapor pressure (kPa), precipitation (mm) and wind speed (m/s) from WorldClim ver. 2 (Fick & Hijmans, 2017). The data were extrapolated from a precise raster layer (1 arc second ~30 meter - NASA SRTMGL3, Farr & Kobrick, 2000), containing altitudinal information. Using this raster, we first delineated the area of GK and CV in ArcGis 10.0 with the mask tool and constructed polygons of size 50x65 km (for GK) and 25x20 km (for CV). Within these extracted areas, we selected all 1 arc second (30 x 30 m2) squares that fell within the altitudinal range of the species (for GK, above 850m asl and for CV above 1650m asl). Altitudinal ranges were defined from literature (Jelić *et al*, 2012, Šunje & Lelo, 2010) and matched the personal experience in the field. This was done using the reclassification and the raster to polygon tool of ArcGis 10.0. In this way we likely reconstructed the occurrence areas of the studied populations at both sites. The R-packages raster, sp (Bivand *et al.*, 2013) and sf (Pebesma & Bivand, 2005) were then used to extract average environmental data (for the period 1997-2000, Fick & Hijmans, 2017) for these occurence areas. Approximately a value for each variable of interest was extracted on a spatial resolution of ~1 km2 (30 seconds).

Surface activity in relation to habitat conditions

During a six year period (2014 – 2019), we conducted several field works in both the Northern and Central Dinarides (Gorski Kotar and two neighboring mts:

Prenj and Čvrsnica, respectively, Fig. S1). During each visit we collected environmental data (air temperature – T, and relative humidity - H) using a hand logger (Kestrel, 5000 Environmental Meter), and registered the number of salamanders that were active on ground.

Supplementary results

Spatial data: environmental characteristics of the study sites

The size of the polygons in ArcGis, determined the number of collected data points for each study site (populations). For each variable of interest in Gorski Kotar (1106 km2), we retrieved 1102 data points, and for Mt. Čvrsnica (area 133 km2) 166 points. Comparative plots of the environmental data show that, compared to GK, CV appears to be significantly colder and drier whereas the wind speed does not differ in the two populations (Fig S1). Based on these findings, we hypothesize that the selective pressure causing potential differences in evaporative water loss in the two populations is due to differences in air temperature and humidity.

Surface activity in relation to habitat conditions

Likewise the spatial data analysis, the air temperature and humidity values collected during field work, showed that Gorski Kotar (Northern Dinarides) is warmer and more humid compared to the Central Dinarides (mean values and standard deviations: Gorski Kotar - T=15.6 ±4 .2°C, H=81.8 ±9 .8%; Prenj and Čvrsnica combined – T=13.3 ± 2.6°C, H=76.6 ± 9.3%). Northern and Central Dinarides differed in both air temperature (T test: $t = 2.4$, $df = 47.1$, $p = 0.019$) and humidity (T test: $t = 2$, $df = 53$, $p = 0.048$), but they did not differ within the latter mountain group (Prenj vs Čvrsnica – T: $t = -0.59$, $df = 7.1$, $p = 0.6$; H: $t = 1.3$, $df = 12.3$, $p = 0.2$).

We plotted the number of registered individuals during each field work against the values of temperature and humidity recorded for that particular visit. When compared to the populations of the Central Dinarides, the individuals from Gorski Kotar were mostly active during moister (humidity >80%) occasions (Fig. S6.2) which matches the environmental conditions of their originating habitat (Fig. S6.1).

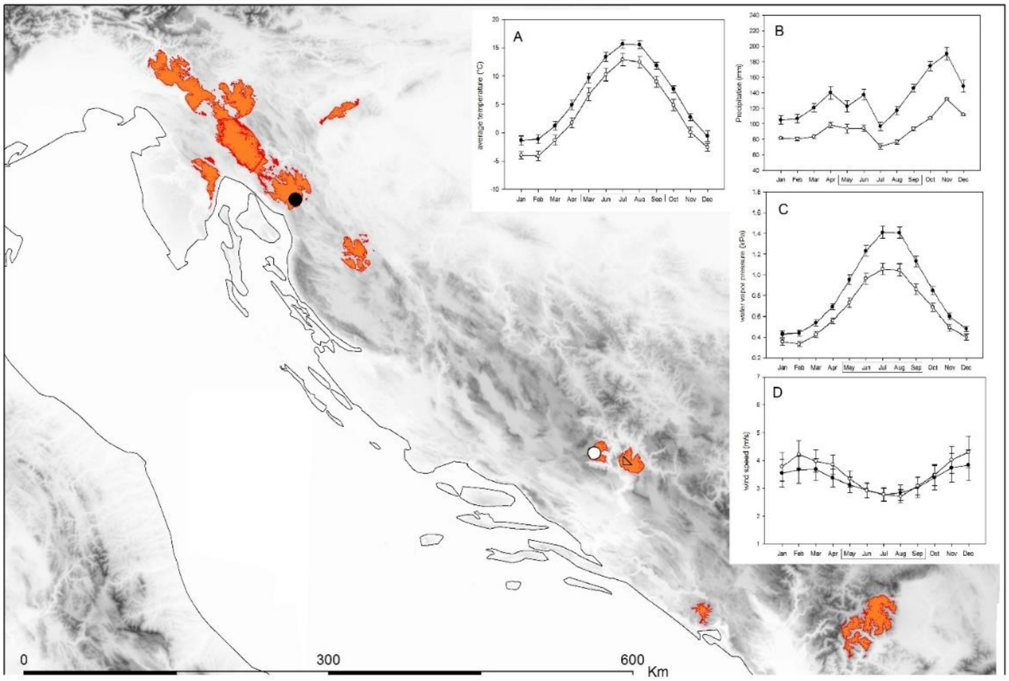


Figure S6.1. Coloured polygons show the distribution of *S. atra prenjensis* in the Dinarides. Sampling locations: black circle – Gorski Kotar (GK), white circle - mt. Čvrsnica (CV). Graphs show environmental variation in the studied populations (mean ± standard deviation) in the average temperature (A), precipitation (B), water vapor pressure (C) and wind speed (D) for each month of the year; squared months represent the rough period of salamanders' activity. CV is significantly cooler and drier when compared to GK. Empty triangle - mt. Prenj.

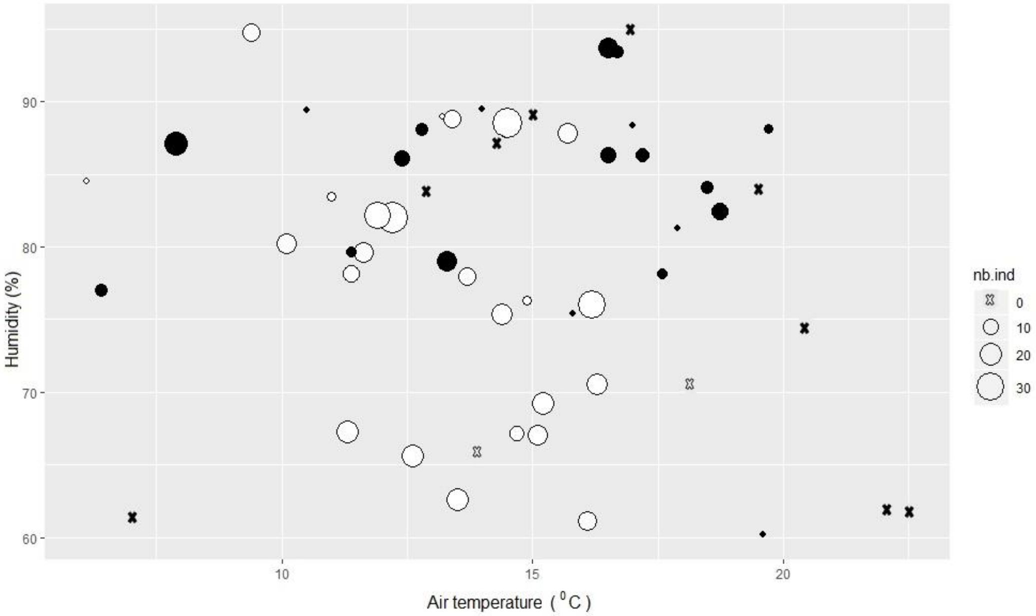


Figure S6.2. Sized scatterplot showing the number of registered salamanders in relation to temperature and humidity values during several field work occasions conducted in the Northern Dinarides (black circles) and Central Dinarides (white circles). The size of each circle is proportional to the number of registered individuals. Individuals from the Northern Dinarides prefer moister conditions for activity.

Figure S6.3. (on following page) - Daily fluctuations in air temperature, relative humidity and relative water lost by salamanders, as estimated for the two study sites and for average days in May, June, July, August and September. Hourly values for air temperature (red lines) were calculated from monthly minimum and maximum temperatures available in WorldClim (Fick & Hijmans 2017). Those for relative humidity (blue lines) were calculated from averages available in Aquastat (New *et al.*, 2002). The hourly estimates of water exchange for salamanders originating from GK (black symbols, full line) and CV (white symbols, dashed line) were obtained using the empirical equations relating water loss, temperature and humidity in experiment 1. The vertical lines in the graphs correspond to the time of sunrise and sunset.

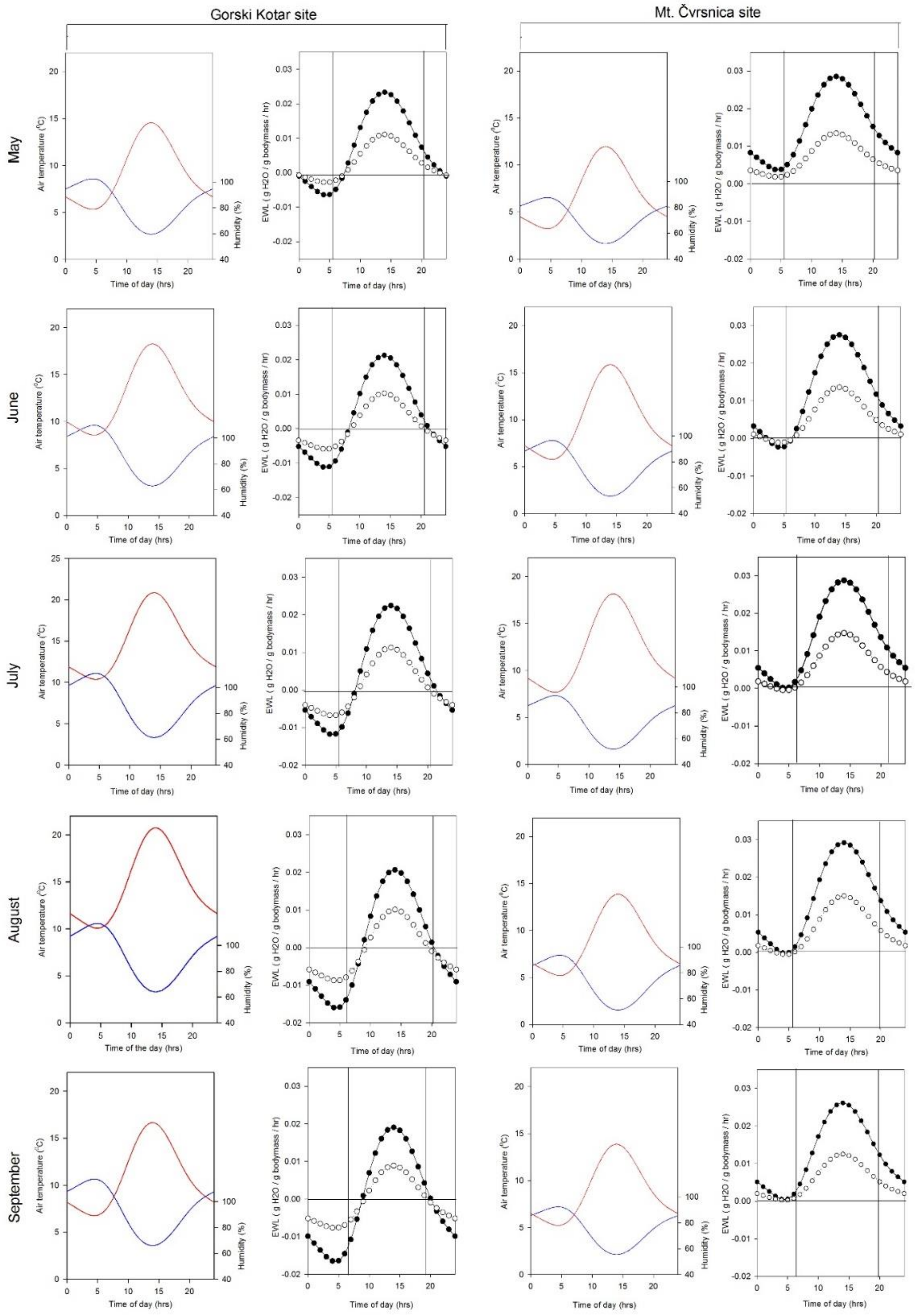

Gorski Kotar site
Mt. Čvrsnica site
May
June
July
August
September
Air temperature (°C)
Humidity (%)
EWL (g H2O / g bodymass / hr)
Time of day (hrs)
Time of the day (hrs)

Table S6.1: Literature review of intraspecific water economy in amphibians; in each study, populations originate from different hydric environments: D = dry, W = wet (e.g. D=W - water loss rate did not differ between populations inhabiting dry or wet environments). Tolerance to desiccation refers to survival or performance studies on dehydrated animals. NS = not significant, blanks = not studied. *S.* – *Salamandra*

Species	**Taxon**	**Water loss**	**Water uptake**	**Desiccation tolerance**	**Reference**
Hypsiboas	frog	D=W	D<W		Cruz-Piedrahita
Leptodactylus	frog	D=W	D>W		Cruz-Piedrahita
Hyla regilla	frog	D=W			Jameson, 1966
Ceratophrys ornata	frog	D<W	D<W		Canziani &
Hyla (Littoria)	frog	D<W			Warburg, 1967
Pseudophryne	frog	D<W	D<W	D>W	Rudin-Bitterli *et*
Hyperolius	frog	D<W			Geise &
Rhinella humboldti	toad	D=W	D>W		Cruz-Piedrahita
Rhinella granulosa	toad	D>W			Prates & Navas,
Rhinella granulosa	toad			D=W	Prates *et al.*, 2013
Bufo viridis	toad	D=W	D=W		Hofmann, 2014
Rhinella marina	toad	D>W			Brusch *et al.*, 2019
Rhinella marina	toad	D>W	D>W	D>W	Tingley *et al.*,
Rhinella marina	toad			D>W	Kosmala *et al.*,
Sclerophrys	toad	D=W	D=W	D>W	Vimercati *et al.*
Desmognathus	salamander			D>W	Camp *et al.* 2007
Plethodon	salamander	D<W	D=W		Winters &
Plethodon metcalfi	salamander	D<W			Riddell & Sears,
Plethodon	salamander	D>W			Riddell & Sears,
Plethodon wehrley	salamander	D<W		D>W	Gross, 1982
Ambystoma	salamander			D>W	Larson, 1971
S. infraimmaculata	salamander	D<W		D>W	Degani, 1981
S. infraimmaculata	salamander	D<W	D>W		Degani, 1982

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APPENDIX CHAPTER 7

Species occurrence data

List of references for species' occurrences (mainly) in the Dinarides (Bonato & Sunje database)

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Table S7.1: Criteria for validation of *Salamandra atra* occurrence points from the five databases. The last two arguments (N, O) are characteristic only to the databases specified in the parenthesis. Codes are as in Figure S1

Argument for exclusion	**Code**
the photos or the textual information provided are not compatible with the identity of *Salamandra atra*	A
the coordinates are obviously erroneous - far from the species range	B
It is georeferenced but the uncertainty (either declared or inferred) of the coordinates is > ±0.5 km (i.e. full uncertainty range > 1 km) or it is unknown	C
it is not georeferenced (i.e. coordinates are not given) and it is impossible to georeference the record with confidence (0.5 km) from the given indications of locality	D
the author of the record is known to have otherwise provided erroneous or inaccurate herpetological records	E
the author of the record has unknown expertise in species identification, and the identity cannot be confirmed from supporting evidence (no photographs, no details given on morphology or behavior)	F
the record has been explicitly considered dubious by another expert author	G
the record has been demonstrated erroneous by another expert author	H
There is otherwise no well-documented evidence (voucher samples, photograph, report of at least two [other] expert authors) for the occurrence of the species in that (mountain) area	I
Occurrence not based on actual sightings, but assumed due to climatic and structural similarities of the putative (mountain) area with other confirmed localities	J
record before 1921	K
the record states that coordinates have been displaced for conservation purposes	L
the uncertainty of the given coordinates is declared < 0.5 km or is unknown, but the coordinates correspond to unsuitable/improbable habitat (e.g.: city, highway, exact mountain peak)	M
the “coordinate” is “casual” (i naturalist; a casual record is a record that is more than 100 years old, lacks a photograph, location and date, i.e it is not eligible for research)	N
the “basis of record” (record’s nature: preserved specimen, human observation, fossil, literature data, living specimen, and similar) is marked as “invalid” (GBIF)	O

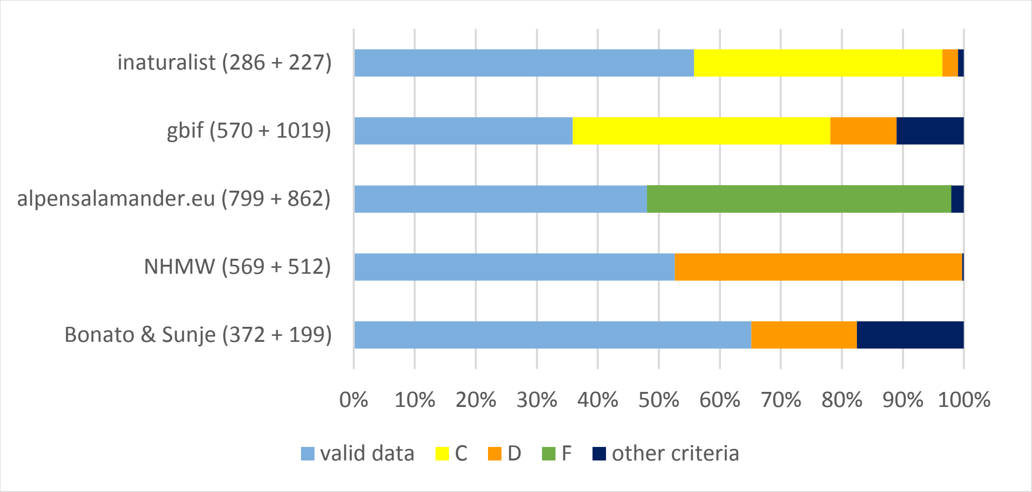

Figure S7.1: A total of 571 occurrences were taken from the personal database (Bonato & Sunje), including 347 previously assembled by Bonato et al. (2018), 184 recovered from the Dinarides (Appendix S1) and 38 additional original observations. The iNaturalist and Alpensalamander database yielded 513 and 1661 reliable occurrences, respectively. An additional 1081 occurrences were extracted from NHMW and 1589 more from GBIF. Percentage of (in)valid data in each of the five databases used to map the species occurrence; in parenthesis, the first number is the number of valid data points and the second is the number of excluded data points. Only valid data are considered for modelling. Presented data is prior to duplicate removal (see text for details). Discarded data are given for the most frequent exclusion criteria (C, D, F); "other criteria" are discarded data points based on arguments other then C, D, F (see Table S1). GBIF and iNaturalist revealed 1252 redundant occurrences (i.e., identical datapoints). After duplicate removal, we were left with 337 occurrence points from GBIF.

Processing extent

In the main text, we define the processing extent as the rectangular extent of ecoregions (Olson et al., 2001) that intersect with the occurrence points of the alpine salamander, which we used to sample pseudo-absences for Species Distribution Modeling. Occurrence points of the alpine salamander can be found in six different ecoregions, where three ecoregions (Illyrian deciduous forests, Pannonian mixed forests, and Po Basin Mixed forests) contain only a small number of points. In Table S7.2 we provide an overview of the number of occurrence points for each study unit belonging to various ecoregions. From these ecoregions we have derived a rectangular area (Figure S7.2, marked with a dashed line) which we used as processing extent, namely to sample pseudo-absences for Species Distribution Modeling.

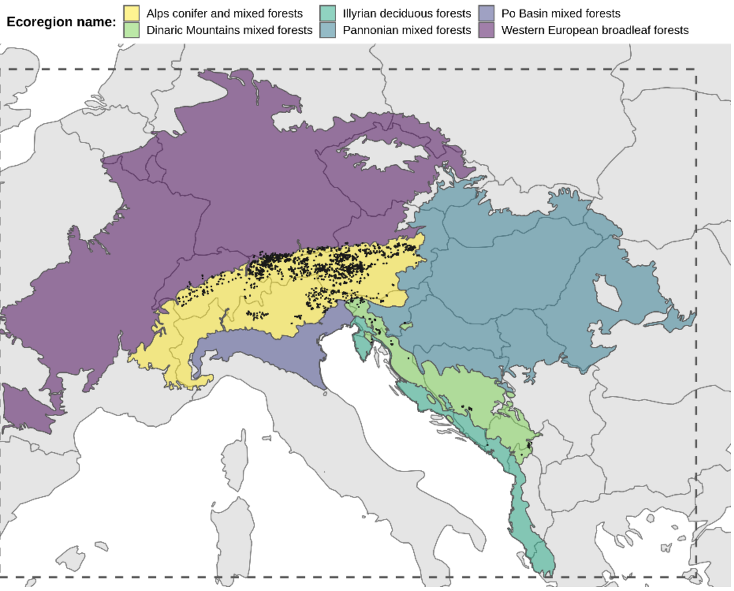

Figure S7.2: Spatial extent of ecoregions where alpine salamanders are found. Rectangular extent of these ecoregions, which we have used as a processing extent, is outlined with a dashed line. Data source for ecoregions is Olson et al., 2001.

Table S7.2: Number of occurrence points for each modelling unit that are found in a respective ecoregion.

Study unit / Ecoregion name	*Salamandra atra*	*S. atra atra*	*S. atra prenjensis*
Alps conifer and mixed forests	2041	1740	0
Dinaric Mountains mixed forests	101	0	72
Illyrian deciduous forests	1	0	1
Pannonian mixed forests	3	1	2
Po Basin Mixed forests	4	0	0
Western European broadleaf forests	80	80	0

Climate scenarios

CHELSA climate database v1.2 (Karger et al., 2017) provides outputs of multiple General Circulation Models (GCMs) to describe future climate. For future climate projections we used multiple GCMs to describe climate under Representative Concentration Pathway (RCP) scenarios RCP2.6 and RCP8.5. Names of GCMs included for each RCP scenario are given in Table S2.

Table S3: Overview of General Circulation Models (GCM) used in this study, and their availability under respective Representative Concentration Pathway (RCP) trajectories used to describe future climate scenarios.

General Circulation Model (GCM)	**RCP2.6**	**RCP8.5**
bcc-csm1-1	✓	✓
BNU-ESM	✓	✓
CanESM2	✓	✓
CCSM4	✓	✓
CESM1-CAM5	✓	✓
CNRM-CM5	✓	✓
CSIRO-Mk3-6-0	✓	✓
FGOALS-g2	✓	✓
FIO-ESM	✓	✓
GFDL-CM3	✓	✓
GFDL-ESM2G	✓	✓
GFDL-ESM2M	✓	✓
GISS-E2-H	✓	✓
GISS-E2-R	✓	✓
HadGEM2-AO	✓	✓
IPSL-CM5A-LR	✓	✓
IPSL-CM5A-MR	✓	✓
MIROC-ESM	✓	✓
MIROC-ESM-CHEM	✓	✓
MIROC5	✓	✓
MPI-ESM-LR	✓	✓
MPI-ESM-MR	✓	✓
MRI-CGCM3	✓	✓
NorESM1-M	✓	✓

Removal of climate variables

Bioclimatic variables are commonly used variables to describe climatic conditions for ecological applications (Figure S7.3-4). They are derived from monthly minimum, mean, and maximum temperature and precipitation values. Quarterly variables describe extreme or limiting environmental factors (temperature of the coldest and warmest months, and precipitation of the wettest and driest months). A quarter is defined as a period of three months (1/4 of the year). Variables that describe quarters can be therefore derived from one of twelve combinations of three months (Figure S7.5-6). Since these quarters may fall at different times of the year in different geographical regions, use of quarterly variables could introduce artificial spatial structuring.

Visual inspection of the bioclimatic variables for the study extent used in this work revealed that variables describing wettest and driest quarters do show sharply delineated spatial structure (Figure S7.3-4). More importantly, the delineation observed in these quarterly variables overlaps with the distribution of the modelling units, indicating that the three consecutive months included in the quarters differ between the Alps and Dinarides.

Given that this study assesses comparable differences in climate impacts when intraspecific differences between study units are considered, from the initial set of 19 bioclimatic variables we manually removed variables described by different months between the study region (bio8-9 and bio16-17). Further, we removed multicollinear variables that showed a Variance Inflation Factor larger than VIF=3. List of the remained variables and their VIF values are given in Table S4.

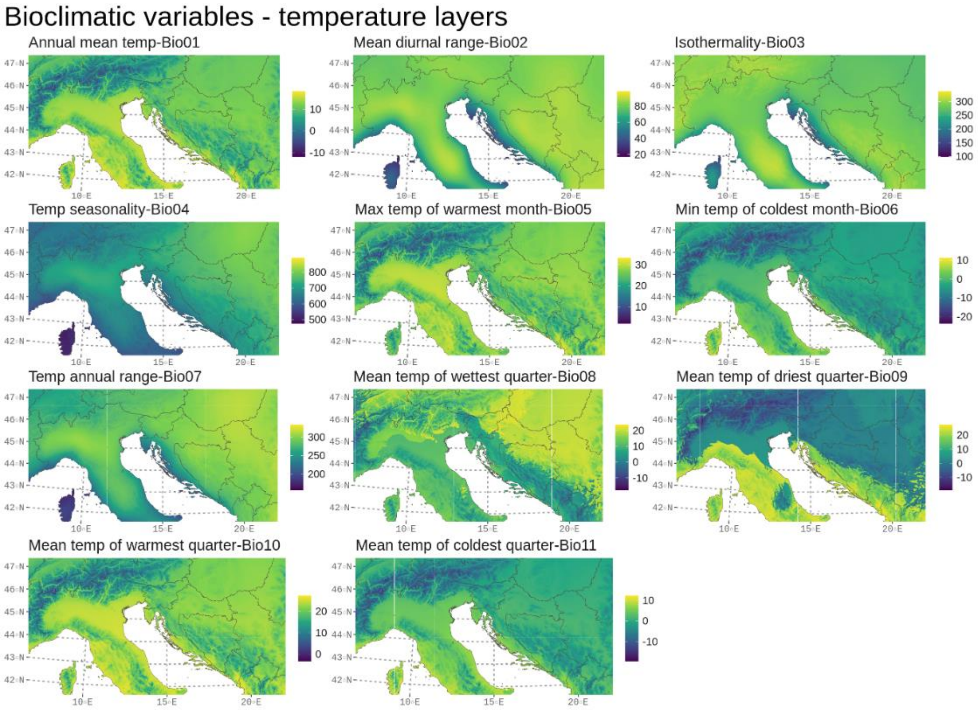

Figure S7.3: Overview of temperature-related bioclimatic variables for the study extent.

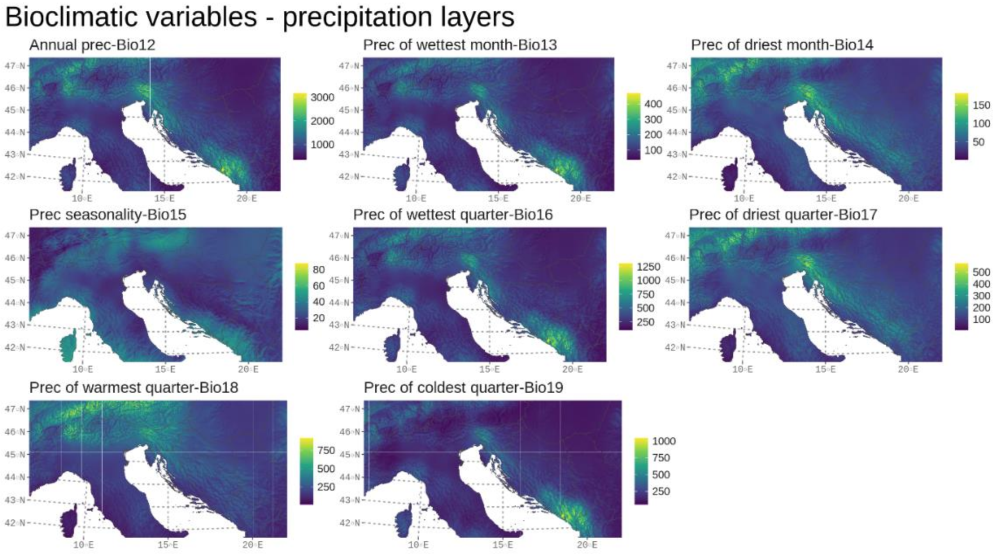

Figure S7.4: Overview of precipitation-related bioclimatic variables for the study extent.

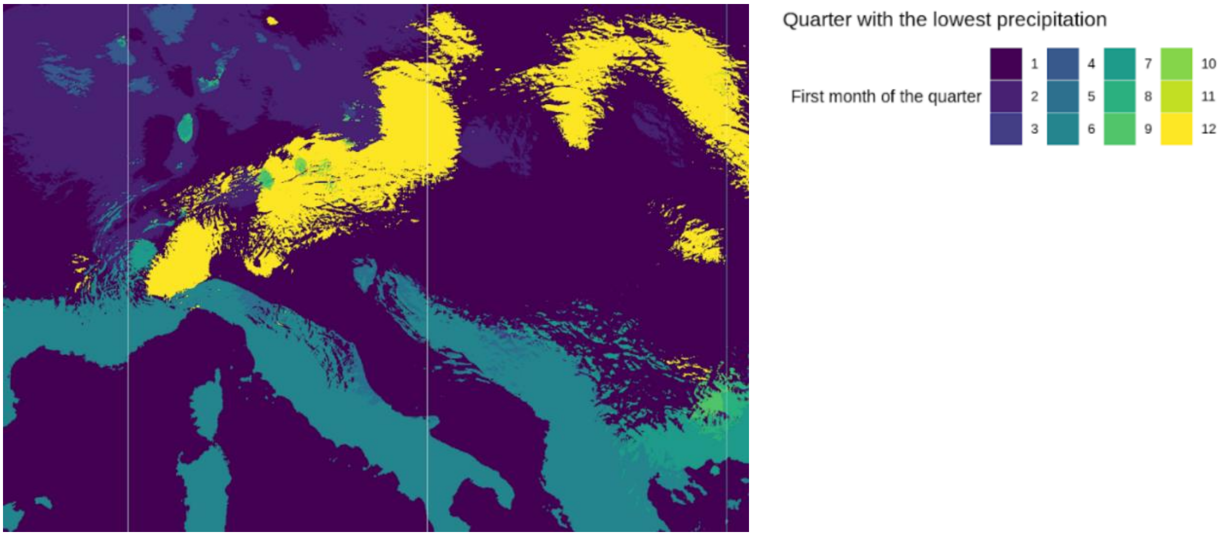

Figure S7.5: Map showing the spatial distribution of the month that is considered as the first month of the driest quarter. Month numbers in the legend correspond to the calendar month label.

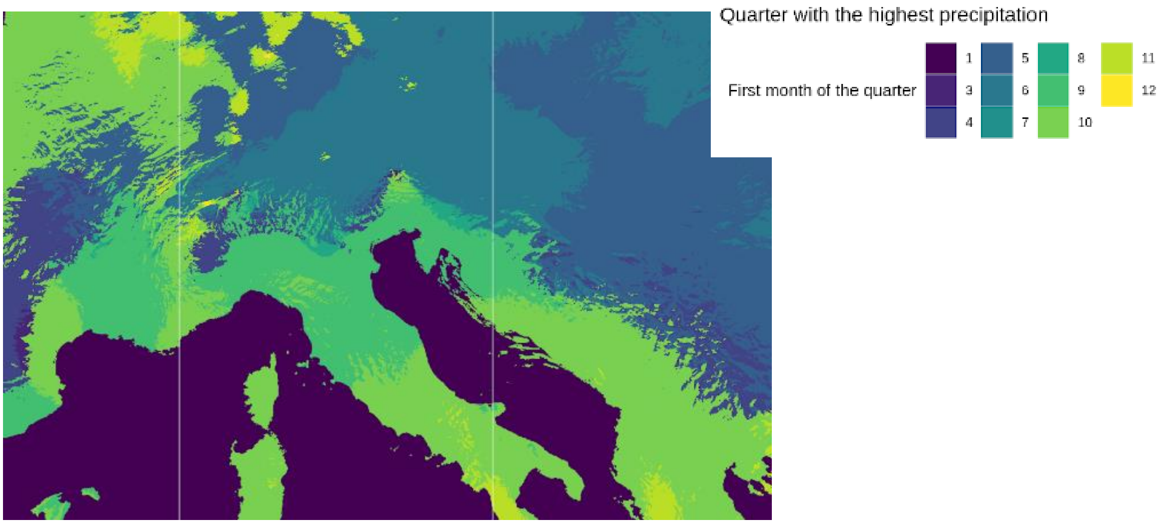

Figure S7.6: Map showing the spatial distribution of the month that is considered as the first month of the wettest quarter. Month numbers in the legend correspond to the calendar month label.

Table S7.4: Values of the Variance Inflation Factor (VIF) for variables that remained after the VIF-exclusion procedure. VIF=3 is the threshold used to remove collinear variables.

Variable name	**Variable shortname**	**Variance Inflation Factor (VIF)**
Temperature seasonality	bio4	1.362
Minimum temperature of the coldest month	bio6	1.242
Temperature annual range	bio7	1.234
Precipitation of the wettest month	bio13	1.106
Precipitation seasonality	bio15	1.221

Project code

Codes used to generate the results in this paper are provided in GitHub repository available at https://github.com/atra-climate/atra-climate. Analysis workflow is maintained with GNU Make, and users can reproduce the results of this paper by following the description available at the website.

Digital Supplementary Information

This paper is accompanied with a Shiny app where we provide main study outputs. It enables users to view and download species occurrence data used in this study, and to view and download the consensus model projections for each modelling units and time periods used in this study. For this purpose, Shiny app features individual tabs Species data and Consensus projections. Shiny app is available at https://atra-climate.shinyapps.io/atra-climate/.

Academic CV

Personal

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Interests	nature, mountaineering, sport climbing, traveling

Language skills

Bosnian	excellent, mother tongue
English	very good – fluent in speech and writing
Italian	very good – fluent in speech and writing
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Dutch	ik begrijp een betschije ☺

Academic education

2015 – 2022	Joint PhD at the University of Antwerp & University of Sarajevo (Faculty of Science, Department of Biology) **PhD thesis**: 'Surviving in isolation - genetic and phenotypic variation in fragmented populations of the Alpine salamander *Salamandra atra prenjensis* (Amphibia: Urodela: Salamandridae)'

2009 – 2010

Master in genetics at the University of Sarajevo (Faculty of Science, Department of Biology)
graduated *maxima cum laude* (weighted average of 97%)
Master thesis: 'Molecular markers as indicators of diversity of the black salamander from mountain Prenj - *Salamandra atra prenjensis* (Mikšić, 1969) for the needs of conservation genetics'

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Bachelor in genetics at the University of Sarajevo (Faculty of Science, Department of Biology)
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Publication list (English language)

2022

De Meester, G., Van Linden, L., Torfs, J., Pafilis, P., **Šunje, E**., Steenssens, D., Zulčić, T., Sassalos, T. & Van Damme, R. (2022). Learning with lacertids: studying the link between ecology and cognition within a comparative framework. Evolution, 2022: 1-22.

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2021	**Šunje, E.,** Stroil, B. K., Raffaëlli, J., Zimić, A., & Marquis, O. (2021). A revised phylogeny of Alpine newts unravels the evolutionary distinctiveness of the Bosnian alpine newt – *Ichthyosaura alpestris reiseri* (Werner, 1902). Amphibia-Reptilia, *42*(4), 481-490. **Šunje, E.,** Zvazu, B. A., Van Damme, R., Backeljau, T., Pojskić, N., Lukić Bilela, L. Kalamujić Storil, B. (2021). Genetic diversity and differentiation of alpine salamanders from the Dinarides – an evolutionary perspective with insights for species conservation. Salamandra 57(1):75-88.
2020	De Meester, G., **Šunje, E**., Prinsen, E., Verbruggen, E., Van Damme, R. (2020). Toxin variation among salamander populations: discussing potential causes and future directions. Integrative zoology, 16(3):336-353.
2019	**Šunje, E**., Van Damme, R., Dušan, J., Mueller, M., Škrijelj, R., Helfer, V. (2019). Morphometric characteristics of Alpine salamanders; a support for subspecies validation and conservation? Amphibia-Reptilia, 40(1): 78-89
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vulnerable Alpine salamander, *Salamandra atra prenjensis* (Amphibia: Caudata), is not associated with the presence of known amphibian pathogens. Salamandra: 54(1): 75-79.

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2018 **Šunje, E.,** Lelo, S., Jelić, D. (2018): Revizija distribucije i konzervacijskog statusa potočne žabe (*Rana graeca* Boulanger, 1891) u Bosni i Hercegovini. Prilozi fauni Bosne i Hercegovine 13, 87-100.

2016 Lelo, S., Zimić, A., **Šunje, E.** (2016): Crvena lista gmizavaca (Chordata: Vertebrata: Reptilia) Federacije Bosne i Hercegovine. Prilozi fauni Bosne i Hercegovine, 12: 34-42.

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Awards and Grants

2021	Grant for Science and Youth of Sarajevo Canton (‘*Immunoenzymatic detection of antibodies to SARS Cov-2 of selected species in the fauna of Bosnia and Herzegovina*’’). Ministry of Education, Science and Youth of Sarajevo Canton. Grant ID: 27-02-11-4375-2/21.
	3MT award by BH Futures Foundation for one of the best PhD presentations in three minutes time (3rd place). See video at https://www.youtube.com/watch?v=stZ43e7UHdc
2017	The Rufford Foundation Grant for the establishment of the regional monitoring program of *S. a. prenjensis* (3rd grant - ID: 24291B)
	Grant for Biodiversity preservation (‘*Meet and protect the unique biodiversity of Bosnia and Hercegovina*’). Federal Ministry of Environment and Tourism (Sarajevo). Grant ID: 04-23-1105-IV/16-65-1.
2015	The Rufford Foundation Grant for the establishment of a regional conservation approach for Dinaric populations of alpine salamanders (2nd grant - ID: 17459-2)
	Erasmus Mundus grant (JoinEUSEE Penta for South-East Europe students) for conducting part of PhD research at the University of Antwerp (Belgium)

SYNTHESIS grant (taxonomy research, AT- TAF-5061): Topic (I) - Taxonomical differentiation of the *brown frog* complex from Central and South-East Europe with focus on *Rana graeca*. National History Museum of Vienna (NHMW), Vienna (Austria).

SYNTHESIS grant (taxonomy research, HU-TAF-5057): Topic (II) - Classical and molecular systematics with aspects of population genetics of the *brown frog* complex from Central and South-East Europe with focus on *Rana graeca*. Hungarian Natural History Museum (HNHM), Laboratory for Molecular Taxonomy, Budapest (Hungary).

2013 Erasmus grant (SIGMA staff exchange program for research), topic: Morphology, feeding ecology and ethology of the alpine salamander (*Salamandra atra*). Faculty of Science, University of Salzburg (Austria).

The Rufford Foundation Grant for basic research of alpine salamander populations in Bosnia and Hercegovina (1st grant - ID: 12728-1)

2009 Award by the Federal ministry of Education and Science (B&H) for ‘exceptional and recognizable results and achievements in the course of studies’

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I will be completely honest. The PhD path was very hard for me. There were many, many, moments when I just wished to quit and leave everything behind. Coming from an Easter European educational background, I was faced with more challenges compared to an average Wester European PhD student. I had a very poor experience in data interpretation and literally no experience in data analysis. Moreover, I did not have a clear idea of what I was actually doing since the variable research topics I had collected data for, seemed impossible to be linked in a fluid and meaningful way.

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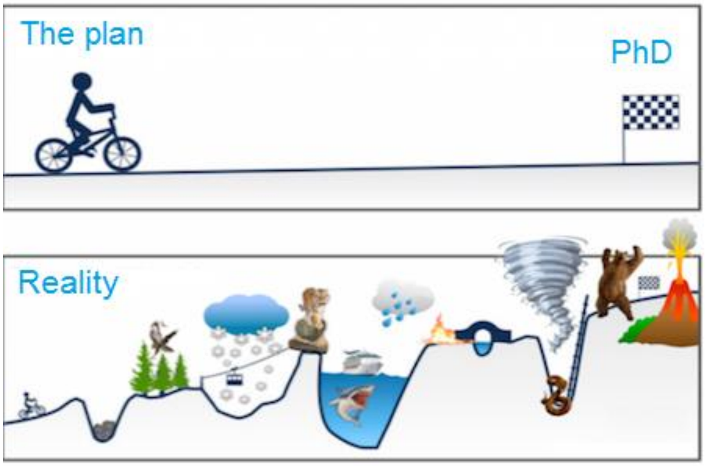

Figure 1. The PhD path I had in mind when I started working at Fumorph lab (upper panel) revealed the complete opposite to the experienced reality (lower panel).

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