Czech University of Life Sciences Prague

Faculty of Environmental Sciences

Department of Ecology

Master Thesis

Monitoring of the pathogen *Batrachochytrium salamandrivorans* within wild populations of Caudates in Croatia

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DIPLOMA THESIS ASSIGNMENT

Martina Ugrinovic

Engineering Ecology
Nature Conservation

Thesis title

Monitoring of the patogen Batrachochytrium salamandrivorans within wild populations of Caudates in Croatia

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Objectives of thesis

Chytridiomycosis is serious disease of amphibians, which causes damage and loss functionality of their skin. Disease agent are chytridiomycota fungi- Batrachochytrium dendrobatidis (Bd) and recently discovered species B. salamandrivorans (Bsal), which attacks caudate amphibians. In contrast with Bd, the Bsal makes a mass death in European caudata in wild (Netherland) and also in captivity (UK, Germany). The aim of the diploma thesis is monitoring the Bsal in selected populations of salamanders and newts in Croatia.

Methodology

As part of the fieldwork will be carried out non-destructive skin swabs from caught salamanders by standardized methodology, samples will be further analyzed by qPCR at a specialized clinic at VFU Brno. Field work will take place during the spring and summer 2019. Immediately after sampling, samples will be transported and analyzed for the presence of DNA of the pathogen.

The proposed extent of the thesis

30-40 stran + přílohy dle potřeby

Keywords

chytridiomycosis, amphibian conservation, Bsal, newts, salamanders

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Recommended information sources

Baláž V., Vojar J., Civiš P., Šandera M. & Rozínek R. 2014b: Chytridiomycosis risk among Central European amphibians based on surveillance data. Diseases of Aquatic Organisms 112: 1–8.

Baláž V., Vörös J., Civiš P., Vojar J., Hettyey A., Sós E., Dankovics R., Jehle R., Christiansen D. G., Clare F., Fisher M. C., Garner T. J. W. & Bielby J. 2014a: Assessing Risk and Guidance on Monitoring of Batrachochytrium dendrobatidis in Europe through Identification of Taxonomic Selectivity of Infection. Conservation Biology 28(1): 213–223.

Berger L., Speare R., Daszak P., Green D. E., Cunningham A. A., Goggin C. L., Slocombe R., Ragan M. A., Hyatt A. D., McDonald K. R., Hines H. B., Lips K. R., Marantelli G. & Parkes H. 1998: Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. Proceedings of the National Academy of Science USA 95: 9031–9036.

Fischer C. F., Garner T. W. J. & Walker S. F. 2009: Global Emergence of Batrachochytrium dendrobatidis and Amphibian Chytridiomycosis in Space, Time, and Host. The Annual Review of Microbiology, 63: 291–310.

Longcore J. E., Pessier A. P. & Nichols D. K. 1999: Batrachochytrium dendrobatidis gen et sp. nov., a chytrid pathogen to amphibians. Mycologia, 91(2): 219–227.

Martel A., Spitzen-van der Sluijs A., Blooi M., Bert W., Ducatelle R., Fisher M. C., Woeltjes A., Bosman W., Chiers K., Bossuyt F. & Pasmans F. 2013: Batrachochytrium salamandrivorans sp nov causes lethal chytridiomycosis in amphibians. Proceedings of the National Academy of Sciences USA 110(38): 15325–15329.

Sabino-Pinto J., Bletz M., Hendrix R., Perl R. G. B., Martel A., Pasmans F., Lötters S., Mutschmann F., Schmeller D. S., Schmidt B. R., Veith M., Wagner N., Vences M. & Steinfartz S. 2015: First detection of the emerging fungal pathogen Batrachochytrium salamandrivorans in Germany. Amphibia-Reptilia 36(4): 411–416.

Spitzen-van der Sluijs A., Spikmans F., Bosman W., de Zeeuw M., van der Meij T., Goverse E., Kik M., Pasmans F. & Martel A. 2013: Rapid enigmatic decline drives the fire salamander (Salamandra salamandra) to the edge of extinction in the Netherlands. Amphibia-Reptilia 34(2): 233–239.

Voyles J., Young S., Berger L., Campbell C., Voyles W. F., Dinudom A., Cook D., Webb R., Alford R. A., Skerratt L. F. & Speare R. 2009: Pathogenesis of Chytridiomycosis, a Cause of Catastrophic Amphibian Declines. Science, 326: 582–585.

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Dean

Prague on 26. 03. 2020

Declaration

I declare that I have worked on my master thesis titled "Monitoring of the pathogen *Batrachochytrium salamandrivorans* within wild populations of Caudates in Croatia" by myself and I have used only the sources mentioned at the end of the thesis. As the author of the master thesis, I declare that the thesis does not break copyrights of any their person.

In Prague 30st June 2020 ---------------------------------

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Abstract

Amphibians nowadays are the most endangered vertebrate group in the world. Considering the various threats that affect them, such as global climate change, pollution, invasive species, human alterations and emerging diseases, their population numbers are rapidly decreasing and causing many communities to become endangered or even extinct. One of the major threats is wildlife emerging diseases with **chytridiomycosis** as an impactful problem specific to amphibians worldwide. Induced by two pathogens, ***Batrachochytrium dendrobatidis*** **(*Bd*)** and ***Batrachochytrium salamandrivorans*** **(*Bsal*)**, the disease had successfully spread via human pet trade. Newly discovered pathogen *Bsal* (originating from Asia) has caused massive losses in western European wildlife Caudata populations and the spread is continuous. In view of having a more precise picture of the pathogen's distribution, we have conducted research on the premises of Croatia, as one of the susceptible and less monitored countries. By following standard protocols for non-invasive sampling and analysis of DNA samples, we have contributed to a better overview in the distribution of the disease and the health status of wild Caudates in these areas. Due to the current lack of *Bsal* presence found in Croatia, preventive measures will help to protect this highly endangered group against future infections.

Keywords: chytridiomycosis, amphibian conservation, *Bsal*, *Batrachochytrium dendrobatidis*, newts, salamanders

Abstrakt.

Obojživelníci jsou v současnosti nejohroženější skupinou obratlovců na světě. V důsledku mnoha ohrožujících příčin, jako např. globální klimatická změna, znečištění, vliv invazních druhů, změny v krajině či patogenů a nemocí, se jejich početní stavy snižují, některé druhy již prokazatelně vyhynuly. Jednou z nejnebezpečnějších příčin jsou zmíněné nemoci, zejména chytridiomykóza způsobená dvěma patogeny – ***Batrachochytrium dendrobatidis*** **(*Bd*)** and ***Batrachochytrium salamandrivorans*** **(*Bsal*)**, k jejichž rozšíření výrazně napomohl člověk v rámci mezinárodního obchodu s obojživelníky. Nedávno objevený patogen *Bsal* způsobil hromadné úhyny ocasatých obojživelníků v Evropě, kam byl zavlečen z Asie. Za účelem zmapování situace stran výskytu tohoto patogenu v Chorvatsku, zemi s vysokou diverzitou citlivých druhů a současně zemi dosud v rámci monitoring opomíjenou, byl proveden monitoring patogenu *Bsal* na vybraných lokalitách. Vzorky (stěry z pokožky) byly odebírány a následně analyzovány standardními technikami (pomocí PCR detekující přítomnost DNA patogenu). Výskyt patogenu naštěstí u sledovaných populací prokázán nebyl. O to důležitější je důsledně dodržovat opatření preventivního charakteru, aby se zamezilo zavlečení patogenu do nových oblastí.

Klíčová slova: chytridiomykóza, ochrana obojživelníků, *Bsal*, *Batrachochytrium dendrobatidis*, čolci, mloci

Content

1. Introduction

Currently, amphibians are the most endangered vertebrate group which is listed by IUCN Red List of threatened species, with over than 41% of endangered species over the world (IUCN, 2020). The causes of declines are various and considered to be consequences of the debated 6th mass extinction (Briggs, 2017; Ceballos et al., 2015). Climate change, pollution, habitat alteration, invasive species are just some of the leading threats to amphibian declines (Murray et al., 2011; Stuart et al., 2004). Furthermore, with increasing globalization and human development, new dangerous causes have occurred, such as overexploitation and emerging diseases (Fisher & Garner, 2007).

Amphibians have been affected by numerous emerging diseases responsible for their population declines and extinctions (Blaustein et al., 2018; Lips et al., 2006). One of them are ranaviruses, whose impact on amphibian populations was felt over five continents (Miller et al., 2011). The second, and probably even more severe disease, is chytridiomycosis, caused by two fungal pathogens *Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrivorans* (*Bsal*) (Lips, 2016).

Bd was described earlier in 1990s affecting the orders of Anura (frogs and toads) and Caudata (salamanders and newts) (Longcore et al., 1999), while *Bsal* is a newly described pathogen impacting only order of Caudata (Martel et al., 2013). Clinical symptoms for both fungi are anorexia, ataxia and abnormal posturing (Martel et al., 2013; Pessier, 2007). Since the impact of both fungi is located on keratinized skin, in the case of *Bd* it creates discoloration, thickening of the skin's superficial layer, accompanied by loss of skin breathing capability and, eventually, a blockage of osmoregulation resulting in death (Berger et al., 2005; Pessier, 2007). As for *Bsal*, the fugus also influences the skin, invading the deeper layers of epidermis. Typical signs are the creation of multifocal superficial erosions with deeper ulceration, following epidermis decay, and excessive skin shedding and thickening (Blooi et al., 2015a; Gray et al., 2015; Martel et al., 2013). Death can occur up to seven days or more, depending on the level of infection (Berger et al., 2016; Blooi et al., 2015a; Blooi et al., 2015b; Stegen et al., 2017; Van Rooij et al., 2015).

Since the discovery of *Bsal* is recent, the information about distribution and the consequences on amphibian communities are not the same as in case of *Bd*.

Nevertheless, its dispersal is quite efficient and extremely virulent to many Caudate species (Martel et al., 2014; Spitzen-van der Sluijs et al., 2016). Originating from Southeast Asia, it arrived in Europe most likely via pet trade (Laking et al., 2017; Martel et al., 2014). Starting in the Netherlands, it has spread over Belgium and Germany towards the newly discovered presence in wild amphibian populations in Spain (González et al., 2019; A. Martel et al., 2014; An Martel et al., 2020; Spitzen-Van Der Sluijs et al., 2013; Spitzen-van der Sluijs et al., 2016). So far, *Bsal* establishment in Europe was detected in wild populations of alpine newt (*Ichthyosaura alpestris*), smooth newt (*Lissotriton vulgaris*), fire salamander (*Salamandra salamandra*), and newly detected in palmate newt (*Lissotriton helveticus*) and marbled newt (*Triturus marmoratus*) (González et al., 2019; Martel et al., 2014; Martel et al., 2020; Spitzen-van der Sluijs et al., 2016). In captive collections *Bsal* was confirmed in Netherlands, Belgium, United Kingdom, Germany and Spain (Andrew A. Cunningham et al., 2015; Fitzpatrick, Pasmans, Martel, & Cunningham, 2018; More, Angel Miranda, Bicout, Bøtner, Butterworth, Calistri, Depner, Edwards, Garin-Bastuji, et al., 2018; Sabino-Pinto et al., 2015).

Since the disease has been officially listed by World Organization for Animal Health (OIE) as a dangerous threat towards amphibians worldwide, it is necessary to keep performing monitoring activities for detection and prevention of unaffected populations (More, Angel Miranda, Bicout, Bøtner, Butterworth, Calistri, Depner, Edwards, Garin-Bastuji, et al., 2018). In the case of *Bsal*, many areas have been neglected to monitor for pathogens, one such area is Croatia (Beukema et al., 2018; More, Angel Miranda, Bicout, Bøtner, Butterworth, Calistri, Depner, Edwards, Garin-Bastuji, et al., 2018). In the past, there have been just three projects conducted for the presence of *Bd* and only two for *Bsal* (Garner et al., 2005; González et al., 2019; More et al., 2018; Vörös & Jelić, 2011).

Considering the facts that Balkan Peninsula is a hotspot of European amphibian diversity with the presence of many susceptible species and that Croatia was marked as one of the susceptible countries by environmental conditions (Beukema et al., 2018; Džukić & Kalezić, 2004; Martel et al., 2014). Therefore, the main aim of my diploma thesis is to perform a monitoring of the presence of the new amphibian pathogen, *Bsal*, within wild populations of Caudates in Croatia.

2. Goals

The main aim of the diploma thesis is monitoring the pathogen *B. salamandrivorans* (*Bsal*) in selected wild populations of salamanders and newts in Croatia. To reach this main goal, it is necessary:

- To select a target species for the monitoring of the fungus in Croatia. The species should be a member of newt's family and simultaneously susceptible to *Bsal*.
- To select places for subsequent monitoring with the presence of target species.
- To perform field monitoring by taking skin samples and analyses of the pathogen presence by standard techniques (all samples by PCR, positives or equivocal results will be reanalysed by qPCR) and procedures in molecular laboratory.
- Based on results of analyses to suggest measures for conservation of target species and other susceptible amphibians in Croatia against the monitored pathogen.

3. Literature Review

To understand risk of amphibian pathogens we should know more about their ecology and distribution, therefore I am focusing within my literature review on general requirements and characteristics of amphibians together with major threats that are shaping their future. We will start with an overview of all threats on amphibians and working towards emerging diseases. Among the emerging diseases, we focus on the major emerging pathogens of amphibian communities, specifically on targeted *Bsal* pathogen.

3.1 Overview of Threats to Amphibians

In the past few decades, the rate of extinction has increased that the scientific world is debating if the Earth is facing a 6^{th} mass extinction (Briggs, 2017; Ceballos et al., 2015). Therefore, from the 1980's, there have been many conventions and conservation programs with the worldwide effort on collecting the data, making the monitoring of biodiversity and evolving measurements for its protection (Cardinale et al., 2012). In 1948, International Union of Conservation of Nature (IUCN) was established with a goal to lead the global efforts in the conservation actions of biodiversity of species and habitat with developed scientific knowledge and tools (IUCN, 2020). Since that time, developed and shared methods of monitoring contributed in creating todays world's most comprehensive data source on the global extinction risk of species (IUCN, 2020). Nowadays, the current situation reported by IUCN show the percentage of endangered vertebrate groups (see Figure 1). At present, the most threatened vertebrate group on IUCN Red List are amphibians with more than 40% of species threatened to extinction worldwide, meanwhile in Europe almost 23% of species are placed in IUCN categories from "Vulnerable (VU)" to "Critically endangered (CR)" (see Figure 2) (European Commission, 2020). Amphibians are severely affected by many threats, these current drivers are habitat loss, global climate change, pollution and invasive species (Murray et al., 2011; Stuart et al., 2004). At the same time, the amphibian trade and emerging infectious diseases are sharping its conservation concern (Fisher & Garner, 2007). In the following text, I am going to explain why the amphibians are the most affected taxa, and on a which way the above-mentioned global drivers affect them.

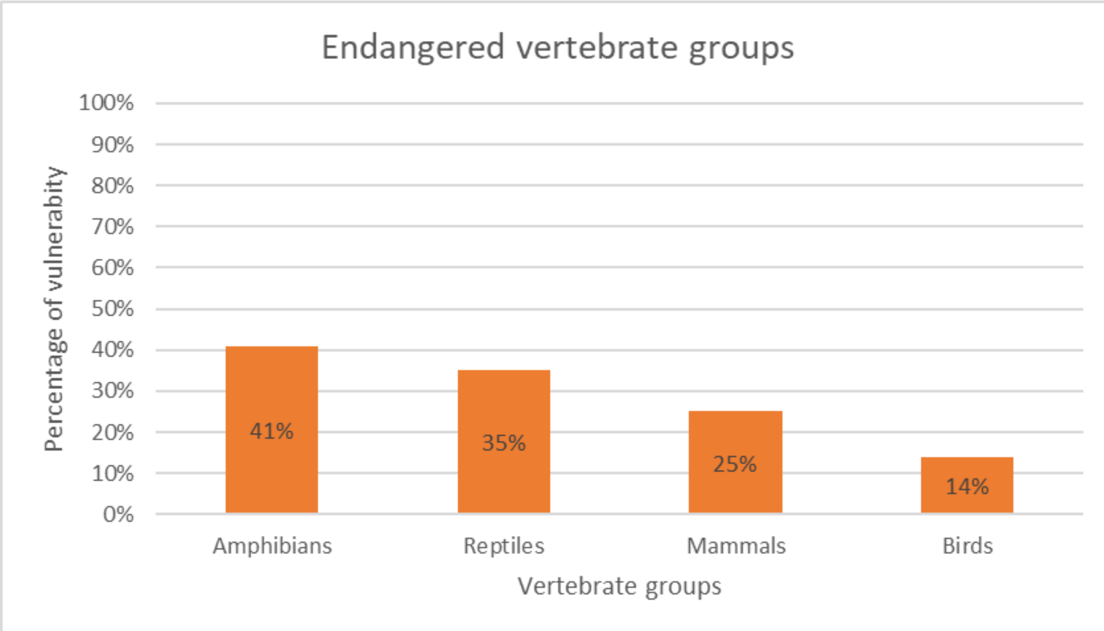

Figure 1: The most threatened vertebrate group on IUCN Red List. Data was adopted from IUCN, 2020.

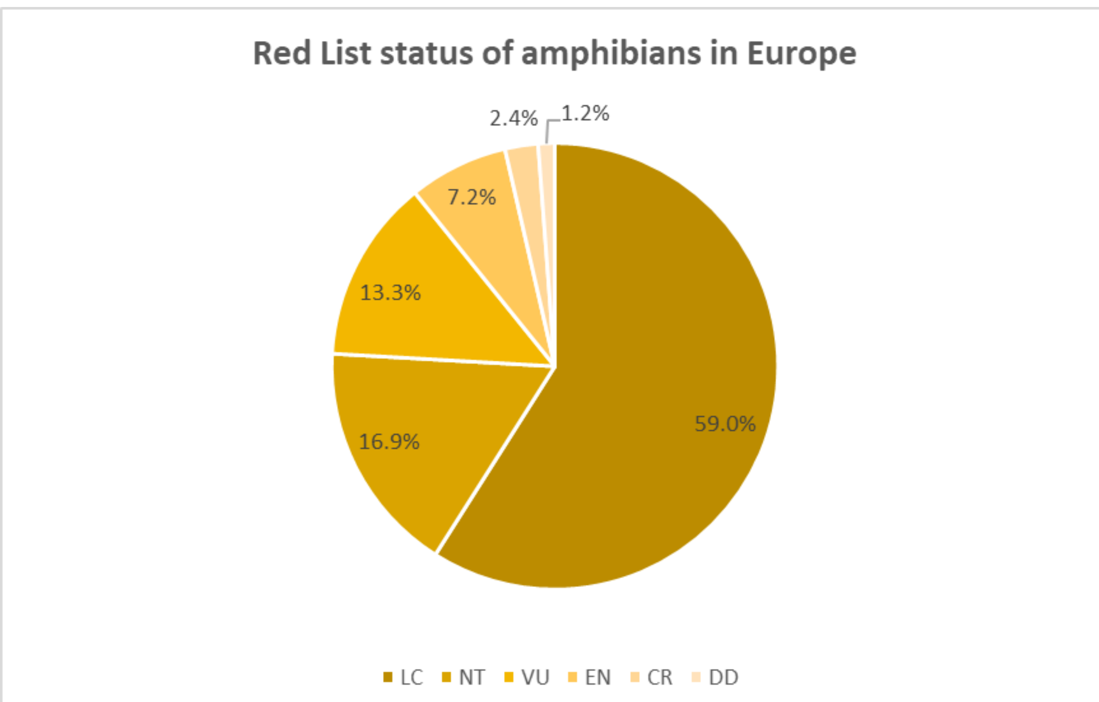

Figure 2: Red List status of amphibians in Europe. Last Concern (LC), Not Threatened (NT), Vulnerable (VU), Endangered (EN), Critically Endangered (CE), Data Deficit (DD). Data was adopted from European Commission, 2020.

3.1.1 Climate Change

Global climate change is a serious threat to biodiversity and various ecosystems; hence, it is considered to be one of the drivers for debated 6th mass extinction (Briggs, 2017). For the past 100 years, the global average temperature has been recorded to increase for 0,85°C, which caused diverse weather changes (IPCC, 2013). Consequences like global warming, increased UV radiation and extreme weather events (floods and droughts) have made various effects on wildlife and ecosystems (IPCC, 2007; Parmesan & Yohe, 2003). Furthermore, climate change is usually not working alone, but in relation with other threats such as: industrial threat (logging, agriculture, chemical etc.), overuse of food and energy, waste management, water management etc. Hence, together they are having synergetic and stronger effects on nature (IPCC, 2007).

As it has been stated above, the most threatened taxa by **climate change** are amphibians (Warren et al., 2013). Furthermore, their populations are easily susceptible to suffer from declines and extinctions since amphibians have very complex habitat requirements, are highly susceptible to weather changes and have low dispersal abilities (Blaustein et al., 2010; Warren et al., 2013). Atmospheric changes, mostly caused by pollution, can have a strong impact on lifelong adaptation. Moreover, the annual change in precipitation and temperature rise can leave an effect on physiology, behaviour, breeding phenology, amphibian reproductive success and the ecology of various species (Araújo et al., 2006; Blaustein et al., 2010; Hayes et al., 2010a). In case of the change in rain pattern, water bodies that serves as a reproduction site (especially in semi-arid regions) would decrease and with them, amphibians would not be able to have a proper reproduction. Hence, there are a lower number of individuals reproducing which leads to the proportion of successfully developed offspring being lower, thus the population size decreases (Araújo et al., 2006). In the event of significant weather changes and consequent population migration, populations may overcome the barriers due to the fragmented habitats. Hence, the populations are less likely to find new suitable habitat. Without connectivity between habitats, populations may suffer from less genetic variability, thus being less resistant towards other threats (Subba et al., 2018). Communities that are affected by weather changes (e.g. temperature and precipitation) and fragmentation would presumably suffer from the immunity decrease and therefore would be more susceptible for infective diseases (Araújo et al., 2006).

Besides being affected by precipitation and temperature changes, amphibians are also endangered by the increased levels of **UV-B radiation** (Hayes et al., 2010a; Hyla, 2020). Kiesecker et al. (2001) found out that during the drier period and reduced pond water levels, embryos are exposed to greater levels of UV-B radiation, which makes them more susceptible to infectious diseases. Furthermore, individuals, after being exposed to the combination of UV-B radiation and chemicals, would suffer from sublethal effects and lower hatching success (depending about the species) (Blaustein et al., 2003). Due to this synergetic effect of climate change and UV-B radiation, other factors like contaminants and pathogens tend to have a greater effectiveness (Blaustein et al., 2003; Cockell & Blaustein, 2001). By cause of atmospheric pollution, the global temperature increase, and rain patter will probably make an impact on the extinction of numerous species (Briggs, 2017).

3.1.2 Pollution

There are various forms of pollution, which affects air, water, soil, etc., but one with the greatest effect is air pollution, which causes precipitation and temperature changes (IPCC, 2007). Environmental pollutants, such as pesticide atrazine, are very dangerous since they have the ability to cause reproductive and development changes without further altering of the physiology or health of an animal (Thurman & Cromwell, 2000). Atrazine, as one of pollutants, is common pesticide in agriculture over the world, with possible dispersal of 1,000km even during the rainy weather (Thurman & Cromwell, 2000). The main characteristics of this pesticide are the feminization of male individuals and the hormonal stress which inhibits breeding development behaviour (Hayes et al., 2010a). In case of complete feminization, breeding occurs between two males which then result in viable eggs but with 100% male offspring such as in African clawed frogs (*Xenopus laevis*) (Gutierrez & Teem, 2006; Hayes et al., 2010b). Furthermore, males that are not “turned over” are unable to compete and have lower fertility since their male characteristic have gradually been decreased (feminized laryngeal structures, decreased breeding gland size, decreased mature sperm) (Murphy et al., 2006).

Additionally, atmospheric changes have diverse indirect effect on levels of environmental pollutants depending about their specifications (Hayes et al., 2010b). In some cases, with the increasement of atmospheric changes levels of pollutants reduce or vaporize into the atmosphere, while vice versa, other chemical in combination with temperature or precipitation just increase their impact on the environment (Hayes et al., 2010a). For example, the positive influence on degradation of chemical is with increased UV-B radiation which breaks the environmental pollutant into less harmful compounds and hence the impact reduces (Chen et al., 2009). Meanwhile, negative example would be with the atmospheric moisture such as fog, which forces the contaminants to stay put on the land, making their concentrations in nature higher (Sparling et al., 2001). Considering that amphibians in their life cycle go through the phase of metamorphose (which can be stimulated faster with the increasement of temperature), higher concentrations of environmental pollutants can present a serious threat. Since amphibian bodies are going through several changes, the immunity system is still in the development which makes the individuals more vulnerable (Rollins-Smith et al., 2011).

3.1.3 Habitat Loss and Alteration

Habitat modification and alteration is one of the most impactful threats on amphibians' communities (Hayes et al., 2010a). Since amphibians have various requirements (dominantly aquatic-terrestrial, terrestrial or aquatic) and they are dependent on migration from one habitat to another, loosing just one of these habitats would present a serious threat to amphibian life cycle (Dodd & Smith, 2003). Habitats are used not only for reproduction, but also as migration corridors, feeding sites and as a refuge's locations during different types of temperature extremes (Dodd & Smith, 2003).

Direct human alteration of amphibian's habitat is mostly altered by physical change of a habitat and we can divide it into; habitat destruction, habitat transformation and habitat fragmentation (AmphibiaWeb, 2020; Dodd & Smith, 2003). **Habitat destruction** is defined as the complete elimination of a local or regional ecosystem leading to the total loss of its former biological function (Dodd & Smith, 2003). Most obvious example would be the drainage of the original habitat and its **transformation** into any typical urban area (e.g. parking lot, housing development or agricultural development etc.). After the habitat is transformed, for example into agricultural field, the next threat can be habitat alteration defined as changes made to the environment that adversely affect ecosystem function, although not necessarily completely or permanently (Dodd & Smith, 2003). For example, destruction of habitat from grazing of livestock can present a serious problem for amphibians and organisms living in ponds or any kind of aquatic environments surrounded with an agricultural land. The last addressed habitat transformation here would be **fragmentation.** Fragmentation which creates a patchy habitat structure has both direct and indirect impact on amphibians due to human activity. Many previously large populations may be divided into numerous smaller populations which may be at great threat to other environmental factors (Wind, 1999). Natural metapopulations may be at higher risk from habitat fragmentation since their persistence depends on the biological corridors and connection between population (Dodd & Smith, 2003). If fragmentation of the landscape destroys these corridors, each small population can be at the greater risk of extinction (Dodd & Smith, 2003; Hanski, 1999; Wind, 1999).

As mentioned before, considering that amphibians have low dispersal abilities, the changes in habitat would present a serious problem for continuing their life cycle (Wind, 1999). Hence, populations would gradually lose genetic variability and with the time, the strength to resist climate change, physical change and invasive species alterations (Hanski, 1999; Wind, 1999). Other examples of human alteration threats that are influencing on amphibians' migrations and recruitment are: increased global urbanization, change of water regimes, agricultural modification, logging, etc (Dodd & Smith, 2003; Hayes et al., 2010a).

3.1.4 Overexploitation

The already threatened amphibian species are also endangered from many types of activities, such as: medicine, science, and food and trade which have the biggest impact (AmphibiaWeb, 2020). Amphibians are commonly used in trade all over the world. The range of species used in a trade is very wide, but the most wanted once are generally exotic species from the rainforests of Africa, South America, Central America and Southeast Asia (Rowley et al., 2016). Many species of Caudata from Southeast Asia are in great danger from the pet trade worldwide (Rowley et al., 2016). The main exporters of Asiatic Salamandridae species are: China (65%), Hong Kong

SAR (22%) and Japan (9%) (UNEP-WCMC, 2016). The biggest importer of wild-caught amphibians is United States of America (USA). In USA, from 2002–2004, the trade of more than 26 million individuals has been recorded legally together with the 127 non-native species, probably intended to be used in the pet trade (Jenkins et al., 2007; Schlaepfer et al., 2005).

According to report of UNEP-WCMC, (2016), in Europe, the biggest importers of live Asiatic Salamandridae are Germany (79%), the Czech Republic (18%) and Spain (3%). The problem of the amphibian trade is not just in exploiting the wildlife populations, but also the consequences that comes with it; the potential to spread and introduce invasive species and wildlife diseases more easily around the world (Fisher & Garner, 2007; Kriger & Hero, 2009).

3.1.5 Invasive Species

Spread of invasive species by humans can happen indirectly through modification of habitat (changed hydrology of freshwater sites or through built roads) which they further use as a pathway to the native habitat, or often through pet trade or trade for other purposes (D'Amore et al., 2010; Rowley et al., 2016; Stuart et al., 2004). The other potential driver of invasion is climate change impact, which forces the species to migrate, e.g. away from the equator and upward the mountains (Ficetola et al., 2007).

The reason why invasive species are out winning the native populations is because they have wider adaptation range and higher resistance to disturbances (such as pollution). Pollution is transforming the habitat, thus only species which can tolerate those changes can survive. Invasive species are typically able to adjust to alerted conditions better than native once (D'Amore et al., 2010; Ficetola et al., 2007).

Being able to persist in such conditions, gives them a chance to drive out the native population from their habitat with competitive performances. Competition is happening with respect to food or breeding sites (D'Amore et al., 2009; Hayes et al., 2010a). Such examples can be the American bullfrog (*Lithobates catesbeianus*) in Western United States and British Columbia, the cane toad (*Rhinella marina*) in Australia, and the Puerto Rican coqui (*Eleutherodactylus coqui*) that was accidently introduced to Hawaii in the late 1980's (AmphibiaWeb, 2020). Furthermore, amphibian eggs and aquatic larvae are especially at risk from the predation of invasive species (Kats & Ferrer, 2003). The example of such an invasion is the trout introduction through Sierra Nevada in USA for sport fishing, which decreased the mountain yellow–legged frog populations (*Rana muscosa*) (Knapp & Matthews, 2000). Additionally, invasive species can also present a risk due to their ability to bring a new pathogen to the healthy population while altering the native habitat (Hayes et al., 2010a).

3.2 Emerging Pathogens

One of the most influential drivers of today's amphibian declines are emerging diseases (Blaustein et al., 2018). Further in the chapter, I am going to present two main severe groups of amphibian pathogens – ranaviruses and chytrid fungi *Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrivorans* (*Bsal*) which are responsible for the disease chytridiomycosis.

3.2.1 Ranaviruses

Ranaviruses are one of the most well recognized emerging pathogens in the amphibian communities responsible for worldwide die offs (Miller et al., 2011). Moreover, ranaviruses along with chytridiomycosis, presents a serious threat to amphibian populations declines and extinctions (Rollins-Smith, 2017; Rosa et al., 2017). Ranaviruses are related to aquatic environment and affects amphibians (Anura and Caudata), fish and reptiles (Miller et al., 2011). The taxa have six species from which three main groups affecting amphibians are: *Ambystoma tigrinum virus* (ATV), *Frog virus 3* (FV3) and the common midwife toad virus (CMTV) (Price et al., 2017). They belong to the genus *Ranavirus*, the *Iridoviridae* family (double-stranded DNA viruses) and they are widely recognized in 72 amphibian species and in 14 families (Miller et al., 2011). Confirmed on five continents (North America, South America, Australia, Asia, and Europe), most of the recorded death incidents occurred in North America and Europe (Miller et al., 2011).

The level of infection generally depends about host specifics and on the species age range (Brunner et al., 2015). Furthermore, amphibians at all life stages are susceptible to this virus, but the most critical cases of infection are identified in larvae stages, while adults are more resistant and are known to carry infections that are asymptomatic (Brunner et al., 2015). Furthermore, some species in aquatic and terrestrial habitat can survive ranaviruses infection with only sublethal effects, hence acting as natural reservoirs for the disease (Gray et al., 2009). Additionally, reptiles and fish are also identified as potential reservoirs for amphibian type viruses (Brenes et al., 2014) .

Ranaviruses have the ability to persist in different environmental surroundings and to wait for the more favourable conditions and occurrence of susceptible host. When the susceptible host appears, transmission of ranaviruses happens via food, feces, water and handling (fomites) (Gray et al., 2009). In nature, viruses can overwinter between epidemics and to persist for a longer time in water and soil. Persistence time of free virions in water of 4°C can go up to weeks or months (Plumb & Zilberg, 1999). In the soil, the virus requires wet and cool conditions for its survival (Nazir et al., 2012).

In various species and life stages, the viruses cause following; necrosis and haemorrhage with consequent symptoms such as gross lesions (e.g. swelling, erythema), lethargy, lordosis, erratic swimming, anorexia, and buoyancy problems (Gray et al., 2009; Miller et al., 2011). Additionally, in both larvae and adults, extensive organ necrosis can be detected. The most affected organs are liver, spleen, kidney and intestines (Converse & Green, 2005). In some cases, clinical signs are not visible, and the animal just suddenly dies (Cunningham, 1996). Proper treatment of animals is still not developed. Iridoviral oral vaccine is being tested on fish and hopefully will give the further insight in development of vaccine for amphibians (Tamara et al., 2006). Currently the only possible support is to keep animal hydrated and to try to prevent the occurrence of secondary bacterial infection (Densmore & Green, 2007).

3.2.2 Chytridiomycosis

Chytridiomycosis is a disease responsible for severe declines and extinctions in amphibian communities over the world (Fisher et al., 2012; Lips, 2016). It is driven by two fungal pathogens, *Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrivorans* (*Bsal)*. It has been assumed that both pathogens are originating from Asia (Laking et al., 2017; Martel et al., 2014; O'Hanlon et al., 2018). While the identification of them took place in different time: *Bd* has been described in 1999, and *Bsal* in 2013 (Longcore et al., 1999; Martel, Spitzen-Van Der Sluijs, et al., 2013). As for their dispersion, humans have been stated as the main influencers (Martel et al., 2014). In this part of review, I am focusing on a description of ecology and distribution of both fungi. The parts related to diagnosis of the disease and proposed conservation measures are also included. Within each part, I deal with both fungi, focusing on differences between them. Much more attention is paid to *Bsal*, as it is target pathogen within my diploma thesis.

Taxonomy

Bd and *Bsal* are a chytrid fungi in the order *Rhizophydiales*, closely related to one another with the nearest saprobic non-pathogenic relative (*Homolaphlyctis polyrhiza*). *Bd* along with *Bsal*, belong to the Chytridiomycota, a "lower fungi" phylum (see in Figure 3). Such pristine microscopic fungi, with motile flagellated spores or known as zoospores, are distinguished by their non-micellar morphology. Chytrid fungi usually inhabit wetlands, such as wet soil or water, and are generally saprobic or parasitic on plants, algae, and invertebrate organisms (Longcore et al., 1999). However, from recent observations, chytrid fungi apparently can also be hosted on vertebrates' groups (amphibians and fish) (Liew et al., 2017; Longcore et al., 1999; Martel et al., 2013). The average estimation of separation of these two sister fungi, made using genetic coding, took place approximately 67.3 million years ago (Martel et al., 2014).

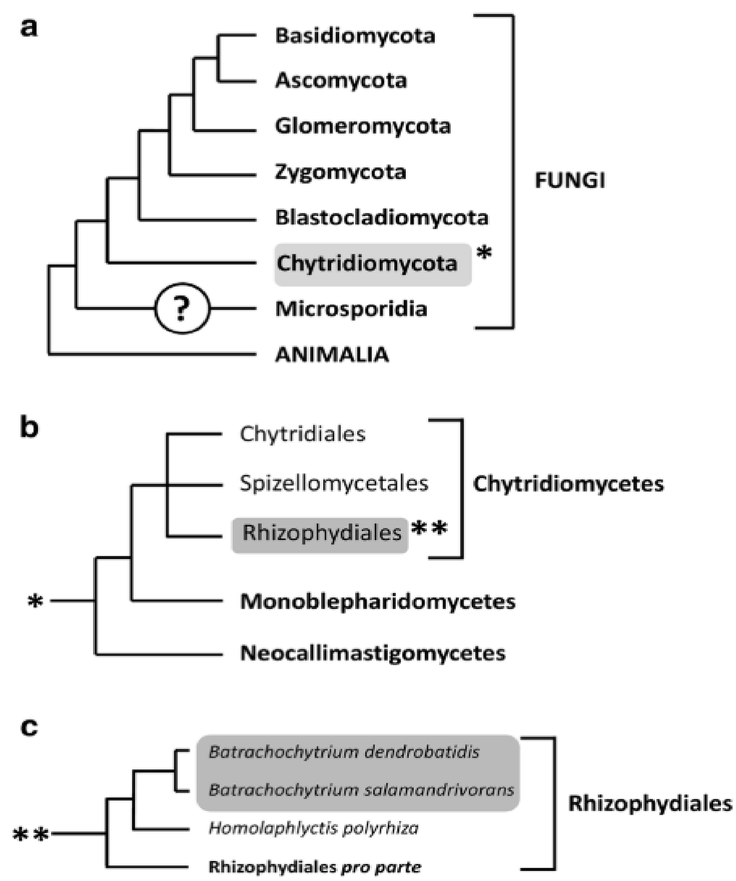

Figure 3: Cladogram tree taken from Van Rooij et al. (2015); showing the topology of the genus *Batrachochytrium* and the taxonomic position of *Bd* and *Bsal* is presented. The location of position is in (a) fungal kingdom, (b) the phylum Chytridiomycota and (c) the order of *Rhizophydiales*. The author has stated an uncertainty about position of Microsporidia and the unproportioned branch lengths in the view of genetic distance.

Morphology and Life Cycle

Bd and *Bsal* both reproduce asexually, and their life cycle consists of two main life stages; motile flagellated zoospores and reproductive body or zoosporangium (thallus), which is developing asexual zoospores (see in Figure 4) (Berger et al., 2005; Longcore et al., 1999; Piotrowski et al., 2004). With the consumption of its flagellum and formation of a cell wall, zoospores encyst themselves and afterwards form the germling with rhizoids. The germling evolves into an immature zoosporangium which after a certain time forms itself into the last stage of a cycle; mature zoosporangium. Zoosporangium can develop into a monocentric (predominant; single sporangium) or colonial form (less common, more than one sporangium). The mature zoosporangium with a develop discharged tube dissolves, and the new asexual zoospores are released out to repeat the cycle (Berger et al., 2005; Longcore et al., 1999).

Batrachochytrium dendrobatidis

Bd temperature gradient for growth in culture is from 10°C to 25°C and the optimum temperature is from 17°C to 25°C, pH 6–7 (Voyles et al., 2012). As mentioned, zoosporangia production may be monocentral (thallus contains only one sporangium) or colonial (thallus contains more sporangia). However, in case of *Bd*, mostly the monocentric type of zoosporangia prevails (Longcore et al., 1999). *Bd* zoospores are only in motile flagellated form (Longcore et al., 1999). Zoospores have an interesting survival feature. Under sterile conditions, they can survive in water and land for weeks to months (Johnson & Speare, 2003; Voyles et al., 2012). Such traits can be a problem considering that they can accumulate somewhere and become a potential environmental reservoir. *Bd* transmission between adult units occurs indirectly by mobile water zoospores (Longcore et al., 1999) or by direct contact such as during mating (Piotrowski et al., 2004).

Batrachochytrium salamandrivorans

Bsal temperature preferences are of wider and lower range than in *Bd*. The culture cycle is completed within five days at 15°C (Spitzen-Van Der Sluijs et al., 2013). The temperature range within which *Bsal* can grow is 5–25°C, but the most optimal temperature for the growth is 10–15°C. Mortality of *Bsal* is recorded on more than 25°C, until the discovery of *Bsal* presence in Vietnamese water ponds with temperature over than 26°C (Laking et al., 2017). This finding is suggesting that the temperature range of the disease may be greater than originally thought; further researches are required (Laking et al., 2017).

In the life cycle, *Bsal* varies from *Bd* in existence of an extra stage. The characteristics are the emergence of germ tubes from encysted zoospore which results in appearing of the novel sporangia and more abundant colonial thalli (see in Figure 4) (Spitzen-Van Der Sluijs et al., 2013). Regarding zoosporangia-generating thallus, as far as *Bsal* is concerned, it is mostly present in a monocentric form in which only one zoosporangium develops. In the case of colonial appearance, zoosporangia would be created along the inner septa (Longcore et al., 1999; Martel et al., 2013). Although the thalli of *Bsal* is predominantly monocentric, the number of colonial thalli is more abundant compared to *Bd* (Martel et al., 2013).

The spores produced can be in the usual form with flagella that allow them to be transferred to other hosts, or they can be in an encysted non-motile form with thick–walled and stronger resilience. After some time, non–motile spores develop resistance to predation by zooplanktons and are characterized by the ability to survive in filtered water for up to 30 days. And as they float, they can also be easily glued to new hosts or, for example, to the feet of waterfowl, which will then mediate their distribution into new, non-polluted habitats (Stegen et al., 2017). Sub-clinically infected salamanders and frogs can act as a passive carrier due to *Bsal* ability to persist on them over a few months (Stegen et al., 2017). Although the fungus response to non-amphibian hosts is not entirely certain, in the event that the disease can be transmitted via waterfowl, nature and wildlife could potentially be at double the risk of infection (see Figure 5) (Kolby et al., 2015). Furthermore, Kolby et al. (2015) stated that we should also not neglect the capabilities of chytrid fungus for aerial dispersal. When heavy rainfall occurs in the environment along with the onset of wind, it showers contaminated vegetation or animals housed in terrestrial or arboreal habitat. After such a vigorous wash, the shower allows the zoospores to move from their host (vegetation or animal) in the form of droplets, which are then distributed to further locations by wind. The droplet path and its relocation are influenced by wind speed and power, air temperature, and human mediations (Kolby et al., 2015).

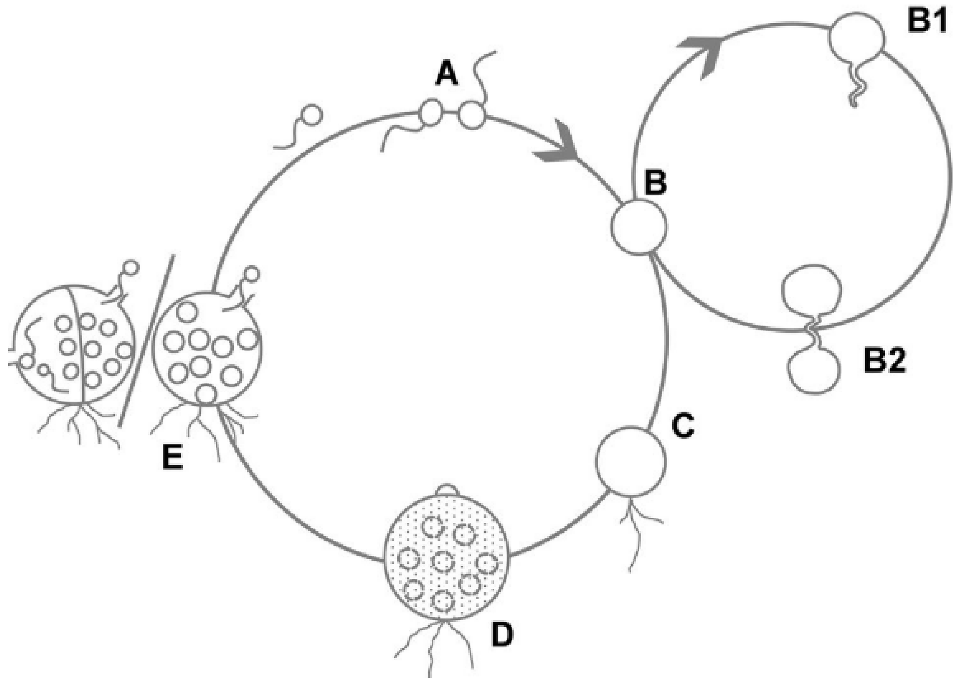

Figure 4: Life cycle of *Bd* and *Bsal* in a culture. *Bd l*ife cycle (A, C, D, E); *Bsal* life cycle (A, B, B1, B2, C, D, E). (A) flagellated zoospores; (B) encysted zoospore; (B1) germ tube development; (B2) transport of a cell contents into a novel thallus; (C) germling with rhizoids; (D) immature zoosporangium development; (E) on the right, mature zoosporangium with monocentric formation and one discharge tube; and on the left, zoosporangium colonial formation consisting of several sporangia having their own discharge tubes. This figure was adopted from Van Rooij et al. (2015).

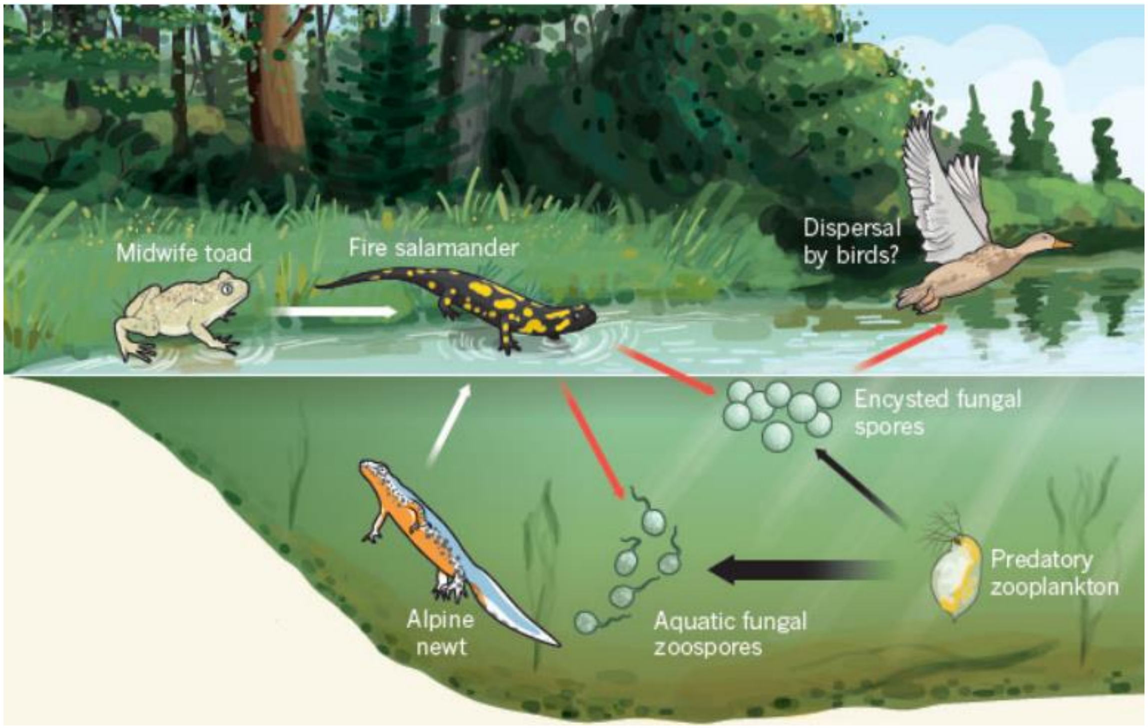

Figure 5: One variation of transmission path of *Bsal* in the environment. More detailed explanation described above. This picture was adopted from Stegen et al. (2017).

Pathogenesis and signs of the disease

Chytridiomycosis is a severe lethal disease with devastating mortality outcomes (Mutschmann, 2015). Because both of its fungi are localized to grow on keratinized skin, *Bd* was thought that it influences skin breathing functions (Berger et al., 2006; Longcore et al., 1999). Infection causes excessive shedding and thus diversely influences on exchange of metabolites, minerals, nutrients and electrolytes (Mutschmann, 2015). Clinical sings are anorexia, ataxia, abnormal posturing and death (Pessier, 2007). The difference between *Bd* and *Bsal* is that in *Bd*, hyperkeratosis and hyperplasia are common side effects on amphibian epidermis, meanwhile in *Bsal*, amphibian skin is primarily suffering from lesions and focal necrosis (Lee Berger et al., 2005; Martel et al., 2013).

Batrachochytrium dendrobatidis

When zoospores are lodged in keratinized skin, they induce hyperkeratosis, thickening of the skin's superficial layer, accompanied by loss of skin breathing capability and, eventually, a blockage of osmoregulation, until death (Berger et al., 2005). *Bd* is present in all forms of amphibians, yet it acts most deadly on metamorphosed individuals. In adults, they can be found on the outer keratinizing layer of the skin, and in tadpoles on the external parts around the mouth and eyes that undercome the depigmentation as the result of the infection (Hanlon et al., 2015).

Batrachochytrium salamandrivorans

When animals become infected with *Bsal*, there is superficial erosion and great ulceration throughout the body (see in Figure 6). First, infection affects keratinized epidermal cells, and the deeper layers of the epidermis are invaded. Over time, multifocal superficial erosions are created with even deeper ulceration, leading to epidermis decay, excessive skin shedding and thickening (Blooi et al., 2015b; Gray et al., 2015b; Martel et al., 2013). The clinical signs of infection are animals suffer from anorexia, nervosa, apathy (lack of feelings or interest), ataxia (not able to control muscles) and after a certain period, it ends with death. Martel et al. (2014) stated that mortality in an individual can occur very quickly (up to seven days) or more than a month (54 days) within the experimental conditions on an infected fire salamander (*Salamandra salamandra*). This means that the level of infection and virulence will depend on several conditions such as environmental temperatures, stage of disease development and host susceptibility (Berger et al., 2016; Blooi et al., 2015a; Blooi et al., 2015b; Stegen et al., 2017; Van Rooij et al., 2015).

Figure 6: *S. salamandra* completely covered with *Bsal* ulcerations and excessive skin shedding (photo credit = F. Pasmans). doi:10.1371/journal.ppat.1005251.g001.

Host defence against chytrid infection

There is a wide range of immune responses depending on the type and age of the amphibians. As far as infectiousness between larvae and adult animals is concerned, the infectious occurrence so far is predominantly in already metamorphosed animals. Specifically, when the amphibian enters the metamorphosis phase, the skin of the larvae begins to stratify under the control hormone triidodothyronine (T3), which plays a major role in the process of metamorphose (Thekkiniath et al., 2013). Triidodothyronine (T3), a corticosteroid hormone, secretes more than usual amounts of hormone levels during metamorphosis of the animal, which in return inhibits immunological defence and makes the animal more susceptible to chytrid infection. Also, young metamorphosed individuals who have just entered a new phase of life that their body needs to adjust too, are still in the process of developing immunological defences, and thus are more susceptible to infections because their immunity is weaken (Rollins-Smith et al., 2011; Thekkiniath et al., 2013).

As far as the immunity system in adult amphibians, the way in which immune protection functions against chytridiomycosis is still not clearly established but, researchers suggest following types of defence: antimicrobial peptides (AMP) secretion, amphibian metabolites secretion, lysosome immunological response, and host microbial community composition (microbiome). Since most of the studies were tested for *Bd* and the fact of lack of knowledge in case of *Bsal*, further researches are necessary to understand these processes better (Bates et al., 2019; Brucker et al., 2008; Pask et al., 2012; Rollins-Smith et al., 2011; Van Rooij et al., 2015).

Treatment

Even though chytridiomycosis is one of today's impactful amphibian diseases and more research is needed to understand its biology and pathology, some treatment trials have been conducted and the findings have shown a promising possibility to treat animals.

Batrachochytrium dendrobatidis

Bd was found to decrease under temperature, salt and antifungal treatments (Chatfield & Richards-Zawacki, 2011; Garner et al., 2009; Geiger et al., 2011; Tobler & Schmidt, 2010; White, 2006). The most effective treatment was with antifungal itraconazole which removed 100% of pathogen from tested individuals (Garner et al., 2009; Tobler & Schmidt, 2010). However, there is potential occurrence of consequent side effects such as depigmentation or even death in some species (Pessier & Mendelson, 2011). Hence, the treatment is not fully efficient (Garner et al., 2009). Additionally, Tobler and Schmidt, (2011) were arguing about genetic difference between populations and its influence of different variations of results of treatment. Another promising treatment is the higher temperature performed treatment (Chatfield & Richards-Zawacki, 2011; Geiger et al., 2011). Tested on both tadpoles and adult individuals, more than 90% of threated individuals were no longer infected with *Bd* (Chatfield & Richards-Zawacki, 2011; Geiger et al., 2011). Although this treatment has promising future, not all the subjected animals heal from the infection, especially considering the various range of specie's temperature preferences (Geiger et al., 2011; Pessier & Mendelson, 2011). Furthermore, the salt treatment in constructed pond was conducted by White, (2006) and it resulted in absence of *Bd* at least for six months. The only noticeable change was the growth rate which was lower in salty water compared to fresh water. Additionally, in case of this treatment, not all the species can survive the salty environment (White, 2006).

Batrachochytrium salamandrivorans

As for *Bsal*, exposure to fungus at high temperatures along with fungicidal treatments has proven to be one of the more effective ways of treating amphibians against disease. Exposure of the *S. salamandra* captured from the wild (n = 30) to a temperature of 25°C, over a 10–day period, resulted in complete elimination of the fungus in 26 specimens (see Figure 7) (Blooi et al., 2015a; Blooi et al., 2015b). Also, in another experimental treatment, the treatment of *S. salamandra* was tried at a slightly lower temperature in order to try to maintain the same effective results while reducing the temperature pressure on the animal. For *S. salamandra*, treatment at 20°C in duration of 10 days with polymyxin E and voriconazole fungicide application, resulted in complete elimination of *Bsal* infection under controlled laboratory conditions (Blooi et al., 2015b). Possible issues faced during this type of treatment are that different species have different temperature thresholds and at such high treatment temperatures (20–25°C) exposure to particular species may end up with fatal results.

Another possible and less explored treatment would be with hosts microbial community composition which has been proven to have an interaction with chytridiomycosis (Bates et al., 2019). Since this kind of treatment is not yet well explored, it’s not possible to fully discuss this topic. Further researches are needed.

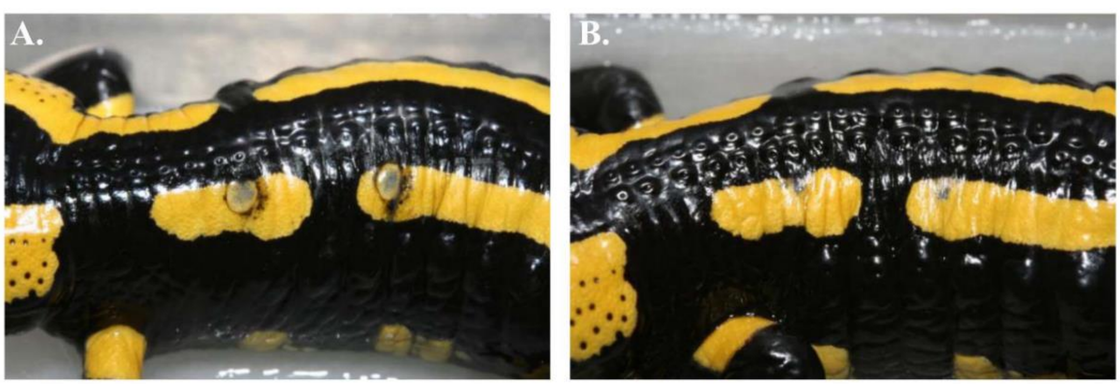

Figure 7: Treatment of a *S. salamandra* under 25°C over a 10–day period. (A) infected individual before the treatment with obvious signs of ulceration on skin; (B) recovered individual after the treatment. This figure was adopted from Blooi et al. (2015a).

Distribution and Evolution

Batrachochytrium dendrobatidis

Bd has been detected in Anura (frogs and toads) and Caudata (salamander and newts) of at least 520 species (Olson et al., 2013). Until 10 years ago, it was claimed that its roots probably originated in Africa, South America and Asia (Bai et al., 2010; Bai et al., 2012; Rachowicz et al., 2005). However, latest studies point to the theory of Asian endemism and further spread via amphibian trade, developing multiple panzootic lineages over the world (O'Hanlon et al., 2018).

The effect and dispersion of this highly infective disease are so powerful that many communities found themselves on the verge of extinction in a short time period (Lips et al., 2006; Longcore et al., 1999; Martel et al., 2014). The link between the temperature rise in warmer years, amphibian migration and the spread of pathogenic fungus *Bd* disease in altitude regions has been reported in rainforests in Costa Rica (Pounds et al., 1999; Pounds et al., 1997). Global distribution and species affected are presented in Figure 8.

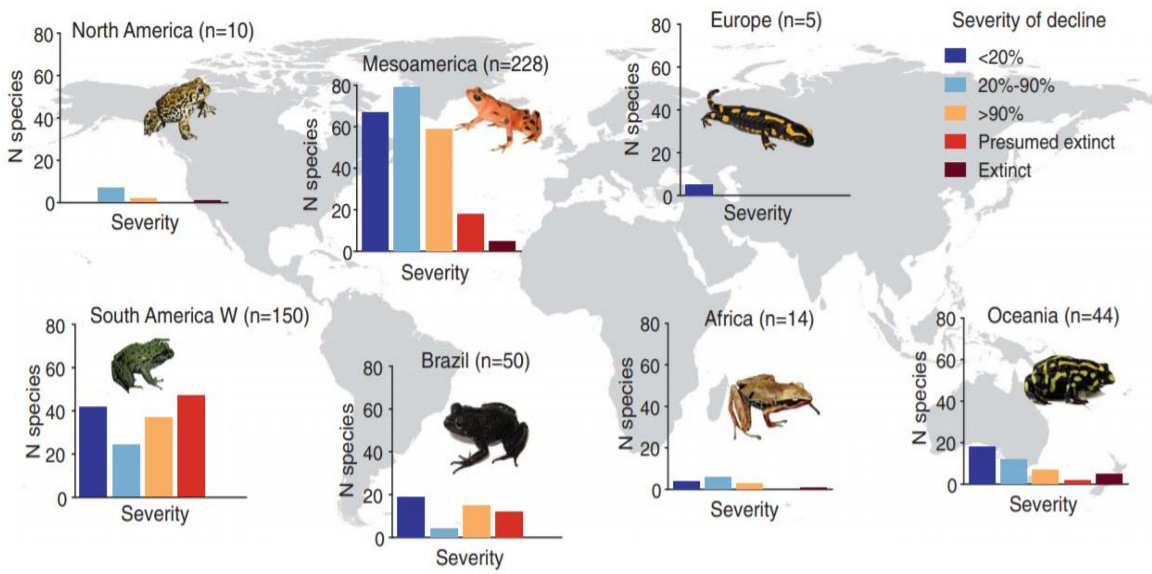

Figure 8: Global distribution and declines of the amphibian species infected with chytridiomycosis. Bar plots represent the declined species number (N), clustered by continental region and graded by severity of decline. Mesoamerica represents Central America, Mexico and Caribbean Islands together; Oceania represents Australia and New Zealand; Brazilian species are classified separately from all other species in South America (South America W); In Asia, there were no reported declines. *n*, represents total number of per region declines. [Photo credits (clockwise from top left): *Anaxyrus boreas*,C. Brown, U.S. Geological Survey; *Atelopus varius*,B.G.; *Salamandra salamandra*, D. Descouens, Wikimedia Commons; *Telmatobius sanborni*, I.D.l.R; *Cycloramphus boraceiensis*, L.F.T.; *Cardioglossa melanogaster*,M.H.; and *Pseudophryne corroboree*, C. Doughty]. The figure was adopted from Beukema et al. (2019).

Batrachochytrium salamandrivorans

In contrast to *Bd*, *Bsal* has a deadly effect just on members of Caudata order (Martel et al., 2013). However, some Anuran species, e.g. common midwife toad (*Alytes obstetricans*), can harbour the pathogen without getting any symptoms. Hence, they are identified as one of the potential vectors for this pathogen (Stegen et al., 2017).

Bsal is native in Asia and it has been spread through Europe by trade with amphibians (Laking et al., 2017; Martel et al., 2014). In Europe, the first massive declines in wild populations due this pathogen were observed in the Netherlands from which it further extended to Belgium and Germany (Martel et al., 2014; Spitzen-Van Der Sluijs et al., 2013; Spitzen-van der Sluijs et al., 2016). Additionally, recently studies on the spread in Spain have been found (González et al., 2019; Martel et al., 2020). In Europe, *Bsal* has been established in wild populations of alpine newt (*Ichthyosaura alpestris*), smooth newt (*Lissotriton vulgaris*), *S. salamandra,* palmate newt (*Lissotriton helveticus*) and marbled newt (*Triturus marmoratus*), although no significant population declines have been observed in *I. alpestris* (González et al., 2019; Martel et al., 2014; Spitzen-Van Der Sluijs et al., 2013; Spitzen-van der Sluijs et al., 2016; Stegen et al., 2017). In captive species, the disease was found in the Netherlands, Belgium, UK, Germany and Spain. Example of species in captivity naturally infected and died from *Bsal* pathogen are: *I. alpestris,* North African fire salamander (*Salamandra algira*), Corsican fire salamander (*Salamandra corsica*), near Eastern fire salamander (*Salamandra infraimmaculata*), *S. salamandra*, Macedonian crested newt (*Triturus macedonicus*) etc. (Cunningham et al., 2015; Fitzpatrick et al., 2018; More et al., 2018; Sabino-Pinto et al., 2015). The current distribution of *Bsal* in wild populations of Caudates can be found in Figure 9.

Figure 9: Allain and Duffus (2019) presented a figure with *Bsal* spread in Europe in wild amphibian populations. Orange colour indicates positive findings that are currently present in Netherlands, Germany, Belgium and Spain.

Further "possible" distribution was presented in study research of Beukema et al. (2018). Beukema et al. (2018) made the ensembles small niches model (ESMs) and stated the possible potential areas for further dispersal of *Bsal.* The areas with suitable conditions are: North – western Europe and Pyrenees, northern Apennines, Western and Dinaric Alps, as well as parts of the Caucasus mountain ranges. Thus, Croatia and its Dinaric Alps are possible locations (see Figure 10). As Croatia is the focused area of this thesis, the fact that presence is possible is part of the motivation for conducting this research. Furthermore, suitable location as well as susceptible species determines the risk within this region. Originally, Martel et al. (2014) describe susceptible species, many of which are present in Croatia such as *S. salamandra* and *I. alpestris.* Additionally, Spitzen-van der Sluijs et al. (2016) in their research state that another susceptible species could be *L. vulgaris*, which also inhabits Croatia.

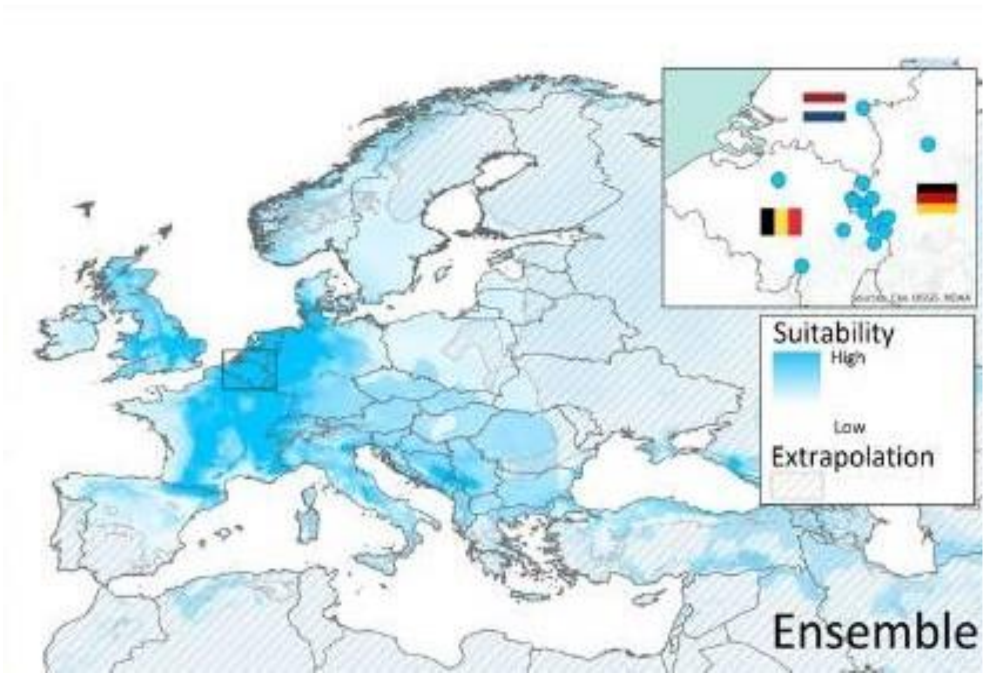

Figure 10: Relative habitat sustainability through Western Palearctic populations from invasive European hotspots (Belgium, Netherlands, Germany) using small niches model (ESMs), suggesting the possible suitable regions for *Bsal* colonization; North – western Europe and Pyrenees, northern Apennines, Western and Dinaric Alps, as well as parts of the Caucasus mountain ranges. Hatched areas represent regions with different climate conditions (extrapolation), not suitable for *Bsal* persistence. Dark blue represents higher suitability; lighter blue represents less suitable environment for *Bsal*. Croatia and its Dinaric Alps are among the optimum regions for *Bsal* expansion. This figure was adopted from Beukema et al. (2018).

3.3 Amphibians and Chytridiomycosis Research in Croatia

Within this chapter, I briefly focus on description of amphibians inhabiting Croatia and their susceptibility mainly to *Bsal* pathogen. In following part of the text, I am summarizing current knowledge about chytridiomycosis research in Croatia.

3.3.1 Amphibians of Croatia and their susceptibility to *Bsal*

Croatia is a relatively small country, with an area of only 56,538 km^2, divided into three bio-geographic regions: the continental, alpine and Mediterranean regions. Furthermore, the eastern part of Croatia contains some features of the bio-geographic region of Pannonia (Jelić et al., 2012). We can separate Croatia into two main regions by herpetological distribution; the continental-mountain region and the Mediterranean region (see Figure 11). In Croatia, there are 20 amphibian species arranged in two orders: Anura with 13 species, and Caudata with seven species. Largest families present in Croatia are: Ranidae (green and brown frogs); *Salamandridae* (salamanders and newts) (Hyla, 2020). Caudata order consists of following species: alpine newt (*I. alpestris)*, smooth newt (*L. vulgaris*), Italian crested newt (*Triturus carnifex*), Danube crested newt (*Triturus dobrogicus*), alpine salamander (*Salamandra atra*), fire salamander (*S. salamandra*) and proteus (*Proteus anguinus*). From these seven species, four are regionally endemic (*T. carnifex, T. dobrogicus, S. atra, P. anguinus*); and infections have been recorded for three of them (*I. alpestris, L. vulgaris* and *S. salamandra*) (A. Martel et al., 2014; Spitzen-van der Sluijs et al., 2016).

Figure 11: Division of Croatia by herpetological importance into the Continental-mountain (Kontinentalno-gorska regija) and Mediterranean region (Mediteranska regija). This figure was adopted from Jelić et al. (2012).

Since Croatia has suitable conditions and species, and the objective of this thesis is to check the presence of *Bsal,* I will further focus on *I. alpestris, L. vulgaris* and *S. salamandra* species and their susceptibility to *Bsal*. From these three species, *S. salamandra* is highly susceptible species to *Bsal* infection (Martel et al., 2014; Spitzen-Van Der Sluijs et al., 2013; Stegen et al., 2017). Considering its wide distribution in Europe and in Croatia, in case of pathogen presence, Croatian Caudata populations would be in severe danger of decline. Other two species, *I. alpestris* and *L. vulgaris* are also susceptible and furthermore are mentioned as the potential reservoir of chytrid fungi (Spitzen-van der Sluijs et al., 2016; Stegen et al., 2017).

A potential reservoir is defined as a carrier that "typically harbours the infectious agent without injury to itself and serves as a source from which other individuals can be infected" (Oludairo et al., 2017). Stegen et al. (2017) mention ***I. alpestris*** as potential natural reservoirs for *Bsal*. To prove this theory, they conducted an experiment on *I. alpestris* with small and large doses of *Bsal* in captivity. *I. alpestris* that has been tested under more intense doses of infection died in an average of three weeks, yet individuals exposed to decreased doses survived due to fungal clearing. Stegen et al. (2017) also showed that survival does not necessarily protect against future infection. Additionally, Spitzen-van der Sluijs et al. (2016) also pointed ***L. vulgaris*** as a potential reservoir for chytrid fungus. The problem with species that are a potential reservoir is that they are very common and widespread species with large home ranges that, during their wanderings, could come in direct contact with less common and more susceptible populations, thus infecting them (Spitzen-van der Sluijs et al., 2016). In the case of pathogens, especially chytrid fungus, amphibians could soon succumb to extinction or the drastic reduction of population size. Furthermore, in case of the existence of chytrid fungus; *S. salamandra, I. alpestris* and *L. vulgaris* as susceptible species and the potential reservoirs, would present a serious threat to more restricted and endemic populations.

Habitat requirements of Croatian Susceptible Caudata

Alpine newt (*Ichthyosaura alpestris*)

Their habitat extends from the mountainous regions to the lowlands and can be found generally along the water. They prefer humid, shaded areas close to the coniferous trees, mixed and deciduous forests, subalpine meadows, and pastureland (Arntzen et al., 2009). This species resides in numerous stagnant waters during the mating and larval stages such as shallow puddles, slow-moving streams, temporary pools and lakes, and sometimes in the ditches (Arntzen et al., 2009). In addition to the regular life cycle, there is also the possibility of hibernation in the larvae stage and the appearance of neotenic individuals (AmphibiaWeb, 2020; Vujica, 2018). Species can adapt to slightly modified habitats, with exception of large cultivated fields(Arntzen et al., 2009).

Smooth newt (*Lissotriton vulgaris*)

Typically, species inhabits forest zones in mid latitude belt with woodland habitats (deciduous, coniferous, mixed, dry forests and woodlands), with preference for humid habitats. Furthermore, for reproduction they require stagnant and shallow water bodies. In terrestrial habitat, it is possible to find them in gardens, forests, under the rocks and in rocks crevices etc. Highly adaptive species, hence, can be found also in gardens, meadows, parks, damp habitats and in rural and urban areas (Arntzen et al., 2020; BHHU-ATRA, 2018).

Fire salamander (*Salamandra salamandra*)

The species mostly inhabits cool deciduous forest with well shaded brooks and near the small rivers. Sometimes can be found in mixed or coniferous forests. Populations that live in the mountain areas prefer woodlands, glades and forest edges where they can hide in rocky places with dense bushes and herbaceous vegetation. Species prefer habitats covered with leaf-litter and moss. Females are generally ovoviviparous and releasing the developed larvae into the water (ponds, streams, still water) where they complete their metamorphosis. Species can tolerate modified habitats (AmphibiaWeb, 2020; Kuzmin et al., 2009; Velo-Antón et al., 2012).

3.3.2 Chytridiomycosis research in Croatia

Over the last 15 years, only four studies have been conducted to test the presence of chytrid fungi in Croatian amphibian populations. In case of ***Bd***, the presence was first tested on eight individuals of Italian agile frog (*Rana latastei*) species by Garner et al., (2005). The individuals were collected in low elevations of coastal area. All the tested animals were negative on *Bd*. Second sampling was performed by Vörös and Jelić, (2011) in Zagreb surroundings on 13 individuals of three species: yellow-bellied toad (*Bombina variegata*), *I. alpestris* and Edible frog (*Pelophylax esculentus*). All the samples resulted negative. The third survey analysed populations of wild *L. vulgaris* and *S. salamandra* both for *Bd* and ***Bsal***. The sampling was performed in wetland forest area (Crna Mlaka) and in a pond more southern in Croatia. This survey failed to detect presence of both fungi (González et al., 2019). There was only one more attempt of research for *Bsal* in which two species *(P. anguinus* and *S. salamandra*) from various parts of Croatia were tested. The number of collected individuals was 24 and all the samples resulted negative (More et al., 2018). More detail data can be seen in Table 1.

Region/Area	Year	Species tested	No.	*Bd*	*Bsal*	Published
Low elevations of coastal area	1994–2004	*R. latastei*	8	-		Garner et al. (2005)
The surroundings of Zagreb	2010	*B. variegata* *I. alpestris* *P. esculentus*	13	-		Vörös and Jelić (2011)
South western from Zagreb, Dalmatia	2016	*L. vulgaris* *S. salamandra*	32	-	-	Lastra González et al., (2019)
Dalmatia, Istria, Gorski Kotar, Zagreb	2015/2016	*P. anguinus* *S. salamandra*	24		-	More et al., (2018)

Table 1: The summary of chytridiomycosis research in Croatia.

From the data collected, it is evident that there is an absence of additional studies related to the presence of chytridiomycosis in Croatia, and the start of active surveillance is needed. Therefore, we have decided to perform second pilot study on *Bsal* surveillance in that country.

4. Methods

For proposition of data sampled, in this chapter we have conducted the following: Reasons for the chosen research area, description of sampled sites and species, the sampling procedure and steps, DNA extraction and statistical analyses.

4.1 Study Site Description

Croatian flora and fauna have a high biodiversity and regional endemism due to Croatian diverse climate regions which were suitable area for migration during glacial and interglacial periods (Jelić et al., 2012). In his research about potential *Bsal* further dispersal, Beukema et al. (2018) include Croatia in the prediction model for suitable areas.

Furthermore, since our targeted species had specific habitat requirements, we tried to focus on areas with those characteristics. For *I. alpestris* we focused on habitat that extend from the mountainous regions to the lowlands and can be found generally along the water. Preferably humid, shaded areas close to the coniferous trees, mixed and deciduous forests, subalpine meadows, and pasturelands. As for *L. vulgaris*, preferred habitat for the study was the one which inhabits forest zones in mid latitude belt with woodland habitats (deciduous, coniferous, mixed, dry forests and woodlands) and with preference for humid habitats. In case of *S. salamandra* species, we focused on locations with cool deciduous forest with well shaded brooks and near the small rivers. Since the species sometimes can be found in mixed or coniferous forests, we preferred that our study sites are composed of that kind of vegetation. Moreover, for populations that live in mountain areas, we tried to find the localities with woodlands and glades, favourably in forest edges. Additionally, *S. salamandra* prefers habitats covered with leaf-litter and moss, so that characteristic was preferred to be found in our study sites.

Since our sampling time was conducted during the summer and early autumn period during 2019, there was a high chance of possible migration in terrestrial habitat of most of the target species. Knowing this fact, we had to choose localities which were in higher altitudes with favourable conditions for the species (e.g. permanent water bodies). Furthermore, it was known that the neotenic communities are present in higher altitudes (they do not migrate in terrestrial habitat), which was an additional reason for choosing these sites. From 12 selected localities, in only 6 of them we found Caudata species and sampled them.

The chosen localities were selected considering the habitat requirements of susceptible species. Considering that the study was conducted in the summer season, the required condition was to select the sites that were in higher altitudes or are deep enough to not be affected by summer conditions outside (maintaining lower temperatures). Other requirements were; the general presence of a water body, humid shaded areas, mountain and lowland regions, woodland habitats with different tree composition (deciduous, coniferous, mixed, dry forests and woodlands). The sample locations can be seen on the map below (see Figure 12).

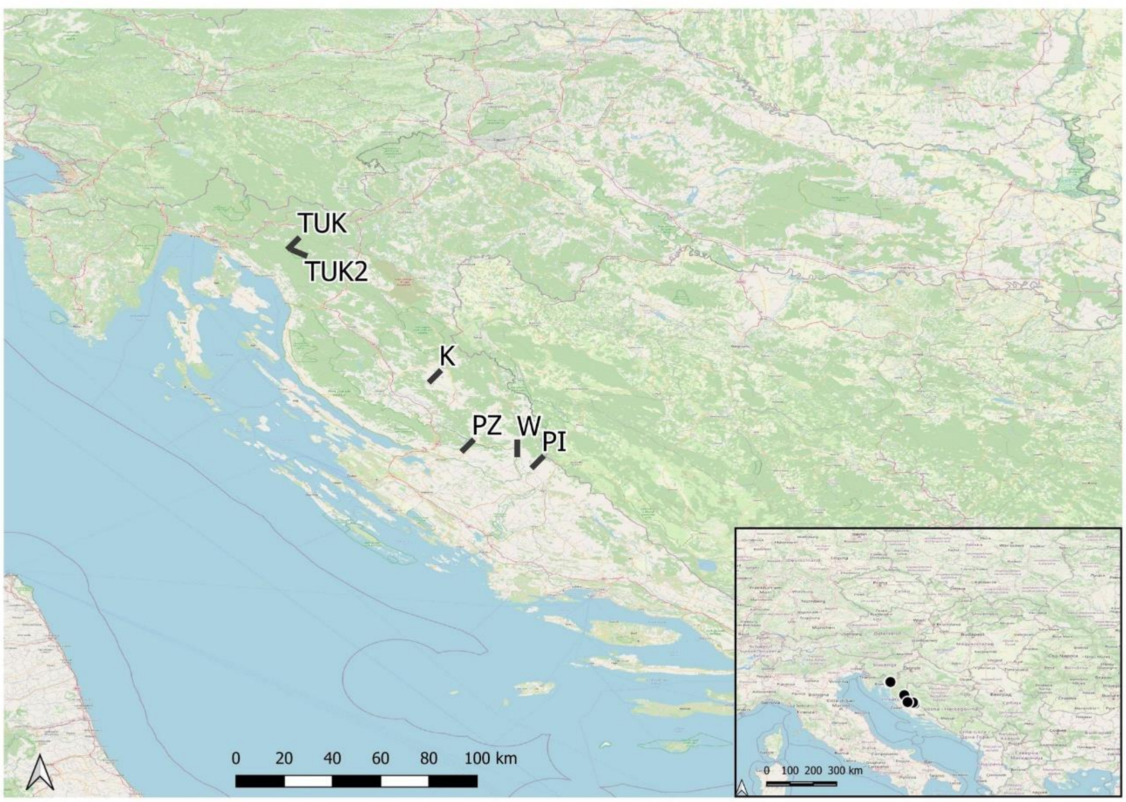

Figure 12: The map is presenting six localities with sampled populations. Information with full localities names and abbreviations (codes) that are presented on this map can be found in table 2. The map was made in QGIS.

a) Tuk (TUK)

Mountain pond with 2/3 surrounded with vegetation of dominantly grass and bushes, 1/3 was surrounded with mixed forest (coniferous and deciduous trees). Inside of a pond we noticed underwater grass with floating dead trees. The pond dimension was 20×15m, with depth of up to 2m (see Figure 13).

Species that were found and sampled: *I. alpestris*, 20 adult individuals.
Other species present: *Diptera* and *Ephemeroptera* larvae and other water insects.

Figure 13: Mountain pond at TUK study site. Photograph taken on 09/07/2019 by Martina Ugrinović.

b) Kod Kod Lovačke kuće Plana (TUK2)

Mountain pond fully surrounded with vegetation of mixed forest (coniferous and deciduous trees), grass community and bushes. We noted a noticeable quantity of fern and nettle around the pond. Inside of the pond, many thinner floating dead trees. The pond dimension was 5×6m, with depth of up to 1m (see Figure 14).

Species that were found and sampled; *I. alpestris*, 22 adult individuals.

Other species present: *Diptera* and *Ephemeroptera* larvae and other water insects.

Figure 14: Mountain pond at TUK2 study site. Photograph taken on 09/07/2019 by Martina Ugrinović.

c) **Pećine Izvori (PI)**

The lake is inside of the abyss, surrounded with stones. Hence, to reach it, first it is necessary to climb down the 50m rocky slope. The lake is surrounded with rocks across which the higher and smaller vegetation grows. Vegetation consists from bushes and thinner trees that are adapted to this kind of habitat. From the top until the bottom, vegetation is gradually decreasing until there are only rocks left close to the water. The lake dimension was 30×25m, with depth of up to 9m. Inside the lake, there is a water spring (see Figure 15).

Species that were found and sampled; *I. alpestris,* 2 adults and 11 subadults. Adults were present in high abundance on the bottom of the lake, while subadults were mostly present close to the surface and on the edges of the lake.

Other species present: water insects' larvae and leeches.

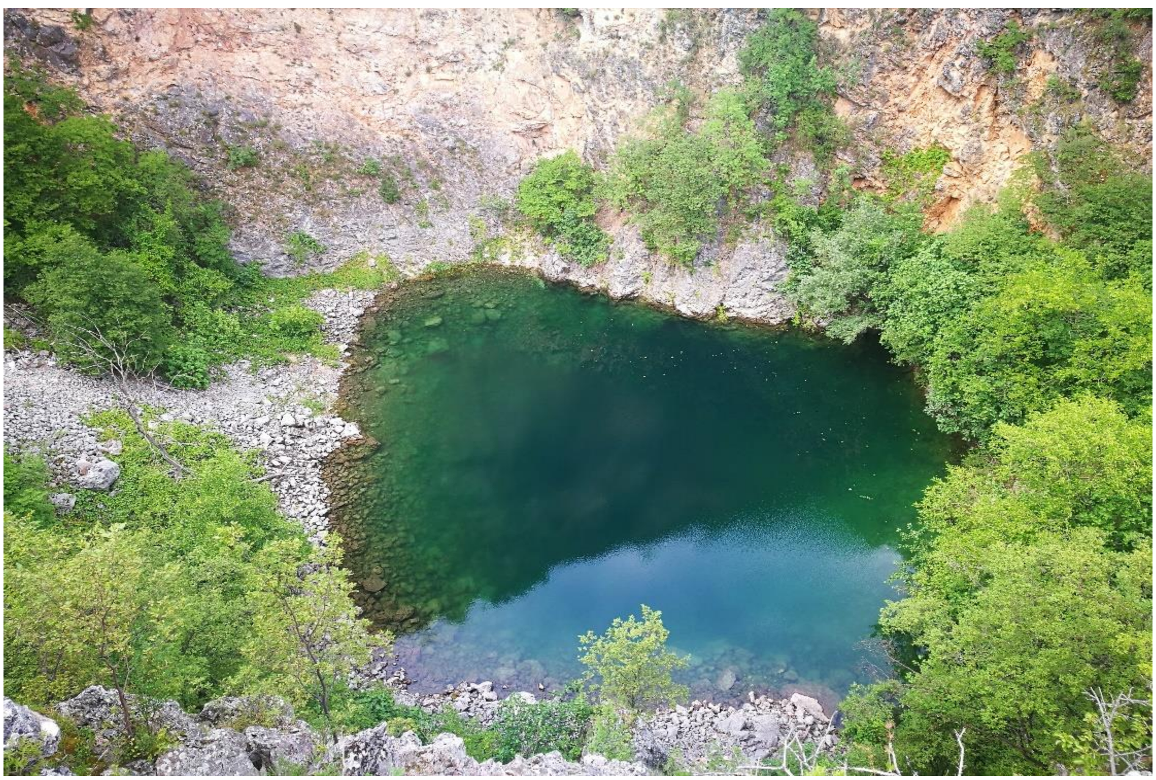

Figure 15: Lake at PI study site. Photograph taken on 13/07/2019 by Martina Ugrinović.

d) Bunar u Potplači (K)

The location was a well, transformed habitat in the middle of the meadow. The well-walls were low therefor the water was easily assessable. Surrounding was consisting only of grass vegetation. Surface of the water was covered with floating vegetation (see in Figure 16).

Species that were found and sampled; *I. alpestris,* six subadults individuals. The depth was unknown.

Other species were not present.

Figure 16: The well at K study site. Photograph taken on 15/07/2019 by Martina Ugrinović.

e) Bunar u Otriću (W)

The well was located close to a road. Because the walls were too tall, it was hard to access the water. We had to put overnight traps. The inside was with one bigger Mediterranean type of bush, and the surrounding was mostly grassy and bushy vegetation (see in Figure 17).

Species that were found and sampled; *I. alpestris,* 12 subadults individuals. Other species were not present.

Figure 17: The well at W study site. Photograph taken on 18/08/2019 by Dušan Jelić.

f) Lokva na Prezidu (PZ)

The pond was in the mountains of Dinaric region; hence, it was surrounded only with rocks and low grass communities. Additionally, pond had 50% of underwater grass. The pond dimension was 9×7m, with depth of up to 1m (see in Figure 18).

Species that were found and sampled; *I. alpestris,* 2 adults and 8 subadults.

Other species present: water insects' larvae.

Figure 18: Mountain pond at the Prezid study site. Photograph taken on 30/09/2019 by Dušan Jelić.

4.2 Field Sampling

Specific number of sites, species and individuals are shown in the results. Total of 12 localities were selected for this study. Among those 12 localities, six were without any presence of species, while the rest came to a total of 83 individuals of *I. alpestris* species, which were sampled during summer and early autumn of 2019. Additionally, for the presence and prevalence of *Bsal*, all swab in groups of four (24) samples were tested.

For sample collection we used pyramid-shaped crayfish traps, dip net and non-invasive sampling techniques. Trap was filled with meat that served as a bait and was left to overnight in the well. Pyramid-shaped crayfish traps should be installed properly, meaning they should not be harmful for amphibians which get trapped inside; the trap should be fixed to some stick or littoral vegetation so that part of the trap could always extend above the water surface (necessary oxygen supply) (see Figure 19) (Johnson & Barichivich, 2004).

Dip-netting is a very common sampling technique whit which we had captured individuals from the population and proceeded onto the non-invasive sampling with swabs (see Figure 16). Dip-netting strongly depends about the targeted species, environment type sampled and sweeping experience to not bury the animals (especially larvae) in an overabundance of mud and vegetation (Dodd, 2010).

As for non-invasive sampling techniques, we used tubed sterile dry swab tips (MW113) and conducted swabbing on their skin (see Figure 20). During this process, it is necessary to be careful and to follow the hygienic protocol for disease control (Boyle et al., 2004). While swabbing the animals, we were taking care to constantly change gloves for each individual and to use the disposable materials to avoid the possible transmission. Likewise, the captured animals were all tested at once (one by one) to be sure to avoid sampling of the same individual twice. The swabbing of each part of the body was done according to a standardized approach (Hyatt et al., 2007). Regions swabbed were those which provide the most probable signal in genetic evaluation such as: abdomen, hips, back, inner thighs, and between the toes of hind legs. Additionally, during sampling, we took care to track the visual signs of infection (lethargy or skin changes). The sampling was finished after we marked the eppendorfs and released the animals back to their habitat. In order to avoid the transmission of the disease to another localities, it is of major importance to properly dispose, clean and disinfect the used field equipment. The recommended procedure is to use ten 10% solution of bleach, autoclave and preheating at 60°C before the disposal. Since our base camp during the field work was with limited conditions, the sanitation step was done with 96% alcohol (used in field and in the camp) and the detergent washing. The operative samples were stored in a cooling device at about 4°C for future diagnosis. Transport of samples was made with a mini fridge and ice packs that kept the temperature consistent. After the arrival, samples were stored in the fridge at about 4°C. We attempted to collect about 20 samples per location as is suggested by Parrot et al. (2016).

Figure 19: Pyramid-shaped crayfish trap extending above the water surface and fixed to littoral vegetation. The picture was provided from doc.Ing. Jiří Vojar.

Figure 20: Non-invasive sampling at TUK2 site. Photograph was taken on 09/07/2019 by Matej Vucić.

4.3 Laboratory Analyses and Pathogen Detection

In case of infection with chytrid fungi, changes in behaviour as well as physical characteristic shifts are typical (Berger et al., 1998). Since the symptoms of chytrid fungi are similar to other diseases, it is necessary to be careful and extremely precise in the determination of the disease. In order to avoid misidentification, it is best to use the DNA test method for the precise detection of *Bsal* (Hyatt et al., 2007).

4.3.1 DNA Extraction

Collected samples are marked and properly stored in the laboratory fridge (4°C). Total genomic DNA from four individuals per locality together was extracted using the Blood and Tissue Kit by Qiagen protocol with the adjustment in: the third step the adding of proteinase K and overnight at 56°C incubator, we adjusted the overnighting to 2,5h of waiting before the fourth step; the last step the elution buffer, which was originally 200ul Buffer AE incubated at 15–25°C was adjusted to 100ul, preheated at 37°C and repeated. Samples were analysed by a standard PCR, using the primers STerF & STerR, described by Martel et al. (2013). Gel electrophoresis was completed to visualize the amplified target.

Multiple diagnostic techniques have been identified for detecting *Bsal* such as histology/histopathology, PCR, qPCR, and lateral flow technology (Thomas et al., 2018). In our study, considering the quantity and type of samples (many subadults), first we have conducted the standard PCR. In case of a positive or equivocal result in the population tested, the next step would be to perform the qPCR for each of the sample. Generally, qPCR is more rapid and precise method of testing which can detect and quantify pathogen in comparison with histology/histopathology, PCR and lateral flow technology. Furthermore, to operate and conduct the testing, no special skills are required. This method is faster, more precise and can analyse non-invasive collected samples (Blooi et al., 2013; Thomas et al., 2018). The presence for *Bsal* was tested on four samples, a relevant number of samples per population in order to maximize the area checked and to save the money. In case of positive findings, we would analyse each of the samples separately and make further analysis with qPCR. However, since our results were negative, further step with qPCR was not conducted.

5. Results

5.1 Species Collected and Results of Analyses

From six localities out of 12 chosen, we have sampled 83 individuals of *I. alpestris*. Throughout the collection of amphibians, we tried to follow the suggested number (20) of population sampled (Dodd, 2010). However, considering the season, size and accessibility of locations, we sometimes failed to fulfil the suggested quantity. During the sampling, we did not notice any visual sign of infection on the selected individuals. Table 2 contains specific information of each site (date, locality name, code, coordinates) and population sampled (species and number).

Overall, 83 samples were collected and tested in groups of four. All tested samples resulted in negative presence. In the case of presence, each sample from the populations would be individually tested.

Locality name	Code	Coordinates		No.	Date
Tuk, pond, Gorski Kotar	TUK	45.26778°N	14.91625°E	20	09/07/2019
Kod Lovačke kuće Plana, smaller pond, Gorski Kotar	TUK2	45.2534°N	14.93874°E	22	09/07/2019
Pećine Izvori, smaller lake, Pećine Plavno	PI	44.16537°N	16.14816°E	13	13/07/2019
Bunar u Potplači, the well, Krbavsko Polje	K	44.59745°N	15.63019°E	6	15/07/2019
Bunar u Otriću, the well, Otrić	W	44.22579°N	16.06668°E	12	18/08/2019
Lokva na Prezidu, smaller pond, Prezid	PZ	44.24783°N	15.79533°E	10	30/09/2019

Table 2: Sample sites information and number of individuals of *I. alpestris* sampled per site.

6. Discussion

6.1 Absence of *Bsal* in sampled populations

To test whether the concern that amphibian populations of Caudates in Croatia may be vulnerable to *Bsal*, we have conducted a project with sampling and DNA extraction for the detection of chytrid fungi that caused massive salamander die-offs across Europe. We tested all the swabs in groups of four, from dominantly mountain regions in southern Croatia, for *Bsal* presence. In our results, we have confirmed that there is no presence of *Bsal* in any of our sampled newt populations, which consisted only from *I. alpestris*.

Surveys on the presence of *Bsal* are still lacking all over the Balkan region, making it hard to describe and assume the potential distribution. A few studies have been conducted on the distribution of *Bd* in Balkan Peninsula (Garner et al., 2005; Vojar et al., 2017; Vörös et al., 2013; Vörös & Jelić, 2011). Vojar et al. (2017) performed the latest survey, which updates the distribution to include presence of the *Bd* pathogen in central and southern Montenegro, southern Albania and eastern Macedonia. Species tested were *B. variegata,* European tree frog (*Hyla arborea*)*, L. vulgaris, Pelophylax spp*., and *T. macedonicus,* which have high genetic variability and linages endemic to the areas tested (Vojar et al., 2017). Lastly, the overall *Bd* presence on Balkan Peninsula is known to be distributed in Romania, Montenegro, Albania and Macedonia. Considering the *Bd* distribution in countries surrounding Croatia (specifically Montenegro), it is a likely scenario that the disease will soon spread to Croatia as well if preventative measures are not enforced.

The survey was the fourth one to screen for a chytrid fungi, and the second precisely intended for *Bsal* in Croatia. The results justify the theory that *Bsal* is a new chytrid pathogen, which originates from Asia and it has spread over Northwest Europe resulting in extreme Caudata declines (Martel et al., 2014). The other regions and EU countries were less affected by *Bsal* impact, like Croatia. However, our results do not necessarily present the accurate situation of the populations health status in Croatia. If *Bsal* was distributed and it is present but in low prevalence, there can be a probability that we have missed it.

Although we believe that if *Bsal* is present in Croatia, but at such a low concentration it was simply chance that our selected samples were healthy, there is the possibility that other issues may have arose during both field and laboratory work. During fieldwork, two major factors could have influenced the present results and possible overlook of *Bsal*. First, sampling time was performed during the summer season, in which it is already more difficult to find amphibians in aquatic habitats. This is due to the fact that they have generally already migrated into their terrestrial habitats. Thus, it was difficult to collect the 20 proposed representative samples per population (Parrott et al., 2016). Additionally, since migration already occurred, there is a lower probability to sample adult individuals, which may provide a more accurate result. In our study, around 50% of collected samples are subadults. Another potential complication could have occurred while the samples were transported between localities as well as to Prague. The improvised cooling system may not have functioned as intended; hence the DNA sample may be degraded or contaminated.

Results of this thesis indicate good health of tested populations for fungal pathogen *Bsal*, yet it is also important to stress the need to continue monitoring and surveillance actions. Since Croatia is a highly susceptible country by the environmental conditions and composition of species, in case of the disease outbreak, the amphibian biodiversity would suffer from major population declines. One of the threats are to populations of species such as *I. alpestris*, which are highly abundant and have a larger home range that overlaps with other small-range and endemic populations. Since *I. alpestris* has the ability to persist with fungus and even clinically clear itself, it would pose a serious threat as a potential reservoir and vector for further transmission (Stegen et al., 2017).

The results of this thesis indicate the lack of detection of *Bsal* in Croatian Caudates communities. Nevertheless, further and more detailed research is necessary to be conducted on this area and other susceptible countries in Europe. Given that our sampled populations were lacking in number and proper age of individuals, we cannot conclude that the populations are in fact without *Bsal* infection. To perform the proper survey, it would be mandatory to include more individuals and populations all over the country. Additionally, to have more representative results, the survey should also include all susceptible species such as: *I. alpestris, L. vulgaris, S. salamandra*, and the other Caudates which are in data deficit (*T. cristatus, T. dobrogicus, S. atra, and P. anguinus*).

This study's focus was primely on presence of chytridiomycosis in Croatia, but to have a better overlook of its worldwide distribution, it is relevant to also mention numerous similar studies that were carried out across various continents. In 2016, Parrot et al. have screened 17 species for presence of *Bd* and *Bsal* which inhabit mountain regions (The Smoky Mountains, the Peruvian Andes and the Swiss Alps) in three continents (North America, South America, Europe). From 509 salamander samples and 192 samples that were previously untested, all resulted negative for *Bsal* presence. On the other hand, *Bd* in low prevalence was highly present in most of the samples (Parrott et al., 2016). Additionally, many more studies have been conducted on these and other continents.

In case of the native continent of chytrid, Asia, 11 countries (Indonesia, Japan, South Korea, China, Malaysia, Kyrgyzstan, Laos, The Philippines, India, Sri Lanka, and Vietnam) have reported presence of *Bd* fungus in their areas, but without a clear geographic pattern (Savage et al., 2011; Swei et al., 2011). As for *Bsal* distribution, a lack of surveys has been conducted on Asian continent (Wang et al., 2017). In China, only two studies have been performed to date (Wang et al., 2017; Zhu et al., 2014b). Zhu et al. (2014b) tested 13 provinces and two municipalities which resulted in no presence. Later, in 2017, Wang et al. conducted a more complex survey on the Qingham-Tibetan Plateau, since it is a one of the global biodiversity hotspots. With a non-invasive sampling method, they have collected 336 wild amphibians and 117 museum samples to additionally assess the historical prevalence. They did not find any positive sample which would point on presence of *Bd* or *Bsal* fungi in this area (Wang et al., 2017). The museum collection also failed to detect a presence (Wang et al., 2017). Another study was conducted in Japan, with the aim to provide the first data on the composition and diversity of cutaneous microbiome of Japanese giant salamander (*Andrias japonicus*) (Bletz et al., 2017a). Since some of the Asian species have the ability to clear themselves from chytridiomycosis infection, Bletz et al. (2017a) tested *A. japonicus* for *Bsal* presence and the results were negative. The argument was on the potential resistance of species (considering their microbiome composition) or that there was no presence from the start (Bletz et al., 2017a).

In case of North America, *Bsal* is still not distributed there, but it is considered as a potential threat (US Fish and Wildlife Service, 2016). Since around 50% of the world's wild salamander population inhabits the area of North America, the potential risk of fungus spreading quite high. The United States has taken preventive measures to limit the trade and the introduction of exotic animals into the country (Bales et al., 2015; Iwanowicz et al., 2017). Under the Lacey Act, 201 salamander species of 20 genera (including European species) have been declared as "injurious wildlife" to USA amphibian wildlife (US Fish and Wildlife Service, 2016). Furthermore, import and transport of restricted salamander species can be granted only for scientific, medical, educational or zoological purposes. Owners of prohibited animals are permitted to keep them, but in case of interstate transport they should follow the given restrictions (US Fish and Wildlife Service, 2016). Hypothetically, in the case of the introduction of a fungus with currently recorded transmission characteristics (propagation rates and host adaptations), the wild populations in America would be in imminent danger (Yap et al., 2015). Risk models for potential fungal transmission predict that the greatest possible northward expansion of *Bsal* would be from the United States' West Coast, the Southern United States, and Mexico's highlands (Richgels et al., 2016; Yap et al., 2015). Transmission would be aided by other variability such as the two widespread species in North America, the rough-skinned newt (*Taricha granulosa*) and the eastern newt (*Notophthalmus viridescens*). Both species have shown extreme susceptibility to *Bsal* in laboratory conditions (Martel et al., 2014) and with their sensitivity and overlap with larger known families susceptible to infection (*Salamandridae* and *Plethodontidae*), a new fatal epizootic would occur (Richgels et al., 2016; Yap et al., 2015). In 2014, along with *Bd* testing, Muletz et al. performed the first study to test for *Bsal* in 94 samples of 12 *Plethodon* salamander species, all of which resulted in no presence of *Bsal*. Later, in 2015, Bales et al. collected 91 sample of eastern hellbenders (*Cryptobranchus alleganiensis*) from eastern United States (New York, Pennsylvania, Ohio and Virginia) and tested them for *Bd* and *Bsal*. *Bsal* was absent from the whole sample collection, while 22 samples tested positive for *Bd* with low prevalence. Bales et al. (2015) stated the necessity of future studies, especially with a focus on aquatic or semi-aquatic species like those in Europe (e.g. *S. salamandra*). Additionally, it could be of crucial importance for studies to focus on vulnerable geographical regions such as coastal ports since those spots are high likely to support pet trade (Kolby, 2014).

Similar to USA, Canada has also conducted an assessment of threatened native species and is consequently in process of drawing out shot-term recommendations on the trade of Caudata species, generally from Asia and Europe (UNEP-WCMC, 2016). *T. granulosa* and *N. viridescens* have been identified as two most vulnerable native species considering their distribution range, habitat preferences, to what extent they are involved in the pet trade and their level of susceptibility to fungal infection (Stephen et al., 2015). Furthermore, European carp (*Cyprinus carpio*) and goldfish (*Carassius auratus*) have been addressed as potential "mechanical vectors" for *Bsal* by Canadian authorities since their shipments are often mixed with the import of Asiatic salamanders (Cooper, 2016). Canadian Food Inspection Agency has been regulating the import of these two species (UNEP-WCMC, 2016).

In Europe, the highest distribution spot is the Netherlands, Belgium and Germany (Spitzen-van der Sluijs et al., 2016). Bletz et al. (2017b) tested German wild species of *I. alpestris, L. vulgaris* and *T. cristatus* on the chytrid fungi and their connection to amphibian skin microbiota. From 273 samples, *Bd* was found in 15 of them while *Bsal* presence was negative. Bletz et al. (2017b) also argued that microbial function may play important role in amphibian resistance towards fungal pathogen and that more studies should be performed on this topic. Other studies with similar results were conducted in Austria, Czech Republic, Switzerland and Italy. Since 2011 in Austria, *Bd* presence was detected at 10 breeding spots, thus the survey on *Bsal* distribution followed during 2013 with a school project. *S.salamandra* and *S. atra* from 8 localities around Salzburg were tested and resulted in negative presence (Gimeno et al., 2015). Furthermore, in the neighbor country, Czech Republic, Baláž et al. (2018) collected 126 samples of wild Caudata species (*I. alpestris, L. vulgaris* and *S. salamandra*) and 198 samples of captive salamanders and screened them for *Bd* and *Bsal* distribution. Both wild and captive populations were completely free of *Bsal*, meanwhile *Bd* was detected in low prevalence at three individuals of wild *L. vulgaris* and in one individual of ribbed newt (*Pleurodeles waltl*) in a captive collection (Baláž et al., 2018). Similarly, in Italy, a survey was performed on wild and captive collections (Grasselli et al., 2019). All the samples showed the absence of *Bsal*, while *Bd* was present only in three wild individuals of *T. carnifex* and in one wild individual of *I. alpestris.*

All mentioned surveys in Europe indicate a generally good health of European populations of Caudates, except in the Netherlands, Belgium, Germany and newly identified Spain (González et al., 2019; Martel et al., 2020; Spitzen-van der Sluijs et al., 2016). However, these results should not be taken as a representative situation of disease distribution, since its known that *Bsal* can occur in a very small density in wild and captive populations (Sabino-Pinto et al., 2018; Yuan et al., 2018). Therefore, each population in Europe that is susceptible and vulnerable to *Bsal* is at high risk and should be tested. The presence of *Bsal* in captive populations represent a serious threat for further spread of the disease (Grasselli et al., 2019).

One of the major drivers for its spread is the increased globalization and anthropogenic activity and with that, the global animal trade rate (Van Rooij et al., 2015). Non-native pathogens are often introduced to new locations due to the lack of regulations on wildlife trade (Fisher & Garner, 2007). To prevent those cases, proper measures and regulates are necessary to implement and to follow (Wyler & Sheikh, 2013). The problem with establishing proper measures is that not all countries will uniformly follow the recommended regulations. For example, each country that participates in CITES (Convention of International Trade of Endangered Species), which established regulations and commitments, did not adopt those proposed measures in the same way, even though the measures should be equally followed by everyone. Examples of measures and preventive actions established in some of the European countries are: The Netherlands and Belgium where the first outbreaks were confirmed, the preventive measures have been established. Countries are establishing and supporting research with the focus on surveillance and monitoring of wild and captive (imported) animals together with development of biosafety protocols (Natuurpunt, 2020; UNEP-WCMC, 2016). Furthermore, Switzerland has established the preventive policy actions of all imported salamanders since 2015 (Schmidt, 2016). The Swiss CITES Management Authority (MA) has addresses potential problem of increased illegal trade if a ban of all imports keep ongoing. Hence, Swiss CITES MA are reconsidering of re-opening of import of certified, non-infected animals (UNEP-WCMC, 2016). On a higher level, the European Commission for Food and Safety has conducted legislations and directives for category of “other live animals” imports into EU countries; Animal health requirements for trade in and imports to the EU of “other” live animals with focus on key points of Directive 92/65/EEC: Harmonisation-the same import principles apply across the EU, preventing the entry of animals with infectious diseases, animal health requirements before authorising imports, organisation and competence of veterinary services, health certificates that all animals must have, conditions for certain infectious diseases; directive 91/496/EEC: mandatory veterinary check of animals entering the EU countries. In case the animal enters the EU with an EU national certificate, the given country on certificate needs to conduct the veterinary check (European Commission, 2020). Furthermore, the European Council in December of 2015 has recommended the following: restrictions on salamander trade, pre-imported screening of animals intended to be used in live animal trade, establishment of monitoring and surveillance programs, utilization of biosafety rules in field and captive collections, and the development of emergency action plans (UNEP-WCMC, 2016). Additionally, in adopting Recommendation No.176 on the prevention and control of the *Bsal* fungus, the Bern Convention Standing Committee addressed the need for the development of risk assessment and legislation to prevent, manage and control the introduction, release or escape of alien invasive species (including those which present a potential threat to native species in spreading a disease) (Secretariat of the CBD, 2010; UNEP-WCMC, 2016). Another developed prevention is the Early Warning System. It is of crucial importance to recognize the symptoms and declines early. Since it is impossible to analyse all the regions and populations, the Early Warning System was developed so even public science can participate (Stark et al., 2018). In case of encountering an amphibian, which was obviously not affected by transport or natural death, it is possible to contact one of the 8 hotlines in EU countries that have been established for this purpose (www.bsaleurope.com/laboratories/) (Stark et al., 2018). Thus, if a country did not develop the preventive systems, at least the people in science and pet trade should follow the rules of field and captive collections to try to prevent the spread by themselves. In wild populations, the following procedure is recommended: (i) tadpoles and adults should not be transmitted between 2 different populations; (ii) release programs should be strict and monitored to prevent any possible contamination of healthy populations; (iii) during the field sampling, disposable materials should be used (e.g. gloves and swabs) and changed after each performed sampling; (iiii) before leaving the site, boots and other equipment should be cleaned to avoid potential spreading of the diseases to another locations and healthy populations (Mutschmann, 2015). In the case of captive collection: (i) amphibians intended for pet trade should be placed in quarantine up to 8 weeks and tested for chytrid; (ii) and captive animals should not be released into nature (Mutschmann, 2015).

During the initial outbreak of *Bd*, preventive measures were not taken seriously, and it resulted in a global pandemic for amphibian biodiversity (Stuart et al., 2004; Voyles et al., 2012). On the other hand, since the occurrence of the second chytrid fungi (*Bsal*), the global response was quicker and more developed (Grant et al., 2017). However, the given examples of countries with developed prevention systems are mostly those which had already dealt with major Caudata declines. The rest of Europe, like Balkan, still lacks basic monitoring and surveillance programs for *Bsal* (Yap et al., 2017).

6.2 Threats to Amphibians in Croatia

In the field, we have observed possible drivers that could have an influence on our populations and with it, on the results. The effects of global climate change and pollution were not significantly influential since we have conducted our research in generally un-modified nature or with low presence of humans. However, the general problem as increasing of average temperature and consequent animal migration upwards, can present a future problem in populations that already inhabits higher altitudes (Pounds et al., 1999; Subba et al., 2018). Three of our study sites (TUK, TUK2, PZ) were located in mountain areas, where in case of unfavourable living conditions, populations will have no place to migrate.

Croatia has around 46% of area with karst topography which influences on its fauna biodiversity endemism and population isolation (Bonacci, 2003). Since in this kind of habitat it is common for the water levels to decline and for the springs to run dry, species must retreat underground and develop themselves as neotenic individuals (Bendik & Gluesenkamp, 2013). Exactly this situation was present in PI study site, where on 9m of depth close to the spring, we encountered a sizable population of neotenic species of *I. alpestris.*

One of the biggest problems in Croatia are drainage, agricultural transformation and the modification of river flow (Priroda Hrvatske, 2020; Žeželj, 2011). Such a human alteration was obvious at 2 study sites, which were modified into wells. The Bunar u Otriću site was highly modified and the well was at a distant of about 10m from the road, but the population was deep enough in the well to not directly be influenced by the traffic and air pollutants. On the other hand, the well in Potplača is a product of human modification of habitat, the location was centered in low inhabited location by humans and surrounded by a meadow ecosystem. The potential threat in this kind of habitat transformation is when the wells are not made or used accordingly to the water capacity. Overuse or shift in the water regime can change the habitat of the population living inside, thus influence on the connectivity between populations (Beja & Alcazar, 2003; Kupferberg et al., 2012). Furthermore, depending on the way of construction, populations can suffer from neoteny and fragmentation consequences if they are not able to migrate out from the well (to climb or to connect with other ponds and populations via underground channels) (Wind, 1999). Both of our localities (K and W) were easily to exit, meaning the studied communities should not be threatened by mentioned risks.

Since Croatia is not predominantly a wetland country, the number of major wetlands and water areas suitable for amphibian communities are small. Hence, those areas generally have a status as national park or nature park with special conservation and protection plans (e.g. nature parks Lonjsko polje and dolina Neretve; national parks Krka and Plitvička jezera, etc.) However, there are many smaller unmarked water bodies, especially alongside of the rivers, that are not being consistently maintained and are presumably drastically changed by humans (logging, agriculture, urbanization, etc.) (Kudeljnjak, 2009). For those kinds of habitats, unfortunately Croatia lacks information. The logging problem was noticeable in two of our study sites which were near by the forestry area. Currently the ponds are not directly affected by those activities, but considering the fast spread and careless actions in these industries, it could be a potential threat which could change the quality of the ponds and to reduce an available habitats for breeding (Hayes et al., 2010a; Semlitsch et al., 2009). It is also important to mention the lack of accessible tunnels for amphibian migration. In Croatia, this measure is still not fully applied, and the consequences of it (as the road killing) are presenting a serious problem for amphibian populations (Kudeljnjak, 2009). Exactly this issue is present at the W sight, where the well is too close to the main road and there are no mitigation measures carried out (migration tunnels and temporary or permanent fences).

In some other cases, human action had contributed to the development of suitable habitats. Sometimes, susceptible water surfaces succeed due to the transformation of habitat, especially within the aim to make a water trough for cattle. However, those water surfaces are becoming rarer and more threatened (Ćirović et al., 2008). The reason for their decline is conditioned by human activity and changing climatic conditions (e.g. lack of precipitation). Furthermore, as traditional cattle breeding disappears, water surfaces remain forgotten and enter the process of succession and ecosystem recovery (Ćirović et al., 2008). On our excursions, we did not come across extremely modified water bodies or those undergoing succession, but we did notice in PZ site a potential agricultural threat to the pond. It is not sure was the pond natural or artificially made, nevertheless, it was observed that the pond is highly used by the cattle for water. Without a future conservation and the management of the pond, such as division management for the water trough, this habitat could soon be destroyed by overgrazing and stepping from the cattle (Kudeljnjak, 2009).

Furthermore, on the excursion, we noticed another significant human factor, the over-stocking of invasive fish species, *C. carpio* and *C. auratus*. This upcoming trend is extremely dangerous towards amphibian populations, since fish eliminate the existing larvae. Hence, after some time, the population fails to persist (Arntzen W. et al., 2009). These alterations were noticed outside of our sampling sites, nevertheless it indicates a potential problem which needs to be further managed. As for the threat of overexploitation, the most endangered Croatian species in pet trade is *P. anguinus*. Since it is a regionally endemic species of underground habitats, it is highly interesting for collectors in illegal trade (HBSD, 2020). Regarding the species found in our localities, *I. alpestris*, only small amount of this species was detected in commercial pet trade (mostly from Ukrainian localities) (Arntzen et al., 2009).

The presence of logging, urbanization, agriculture etc. is affecting daily on amphibians and their habitats. We observed a potential threats and lack of conservation actions in studied locations. To maintain native local habitats, I suggest that amphibian conservation efforts will be the most effective with implementation of mitigation measures and conservation actions and projects. It is of major concern to not lose small water bodies, but to encourage people (especially local) to preserve them. Measures such as habitat conservation, reducing the impact on the river flow, implementation of migration tunnels and fences, preserving ponds from the cattle, elimination of invasive species etc., could all be beneficial for pond persistence. Likewise, it would be of great help to restore the ponds that were formerly used as water trough for cattle. In this way, favourable living conditions would be restored, and amphibians would have more

7. Conclusion

IUCN listed amphibians as the most threatened vertebrate group with 41% of endangered species worldwide. The reasons behind these declines are various threats such as: climate change, pollution, habitat loss and alteration, overexploitation, invasive species and emerging diseases.

One of the most threatening emerging wildlife diseases is chytridiomycosis caused by *Bd* and *Bsal* pathogens. Originating from Asia, by way of pet trade, the disease has spread over the world causing massive declines in amphibian populations. *Bd* was discovered earlier in 90's and has made a dreadful impact on Anura and Caudata of at least 520 species over the world. However, since *Bsal* was discovered in 2013, and the preventive management was slightly better given *Bd* 's experience, the new pathogen did not spread in the same amount.

Hotspot of declines in wild populations of Caudates due to *Bsal* pathogen was in Netherlands, Belgium and Germany. Considering the losses, those countries have developed and implemented various preventive measures and actions, while many other EU countries did not even establish basic monitoring surveillance. Furthermore, this lack of data for many EU countries have made the real *Bsal* distribution less accurate.

Since Croatia is one of those countries with a lack of monitoring and the suitable conditions for further spread, we have carried out the study within wild populations of Caudates. We have sampled six populations of *I. alpestris* in various kind of habitats (mixed forest, mountain regions and wells). Sampling was conducted following the hygienic protocol for disease control with non-invasive sampling techniques. DNA was collected with sterile dry swabs that were further placed in eppendorfs and stored in cooling device. To analyse samples, it was necessary to perform the isolation of DNA and to run samples in standard PCR.

The standard PCR analysis showed negative presence of *Bsal* fungal pathogen among sampled populations. With the analysis of skin swabbed samples from tested Caudata populations, it is proven that the Croatian Caudates communities lack of detection of *Bsal.* Further studies are necessary to conducted with more populations, better representative sample size and to include all susceptible and in data deficit species.

With this pilot study, we have carried out monitoring surveillance in Croatia, confirmed the absence of fungal pathogen *Bsal* and the good health of wild populations of Croatian Caudates. The study was necessary to conduct since Croatia contains all the requirements for *Bsal* potential spread and persistence. The study followed previous protocols standard for *Bsal*, with the expectations of negative presence.

The Balkan peninsula is generally a highly susceptible area for further spread; however, it lacks monitoring, basic surveillance and conservation actions. This lack of data representing Balkan regions could be improved with more developed monitoring systems to keep track of amphibian health. Additionally, more surveys should be performed to have a better overlook of this diseases spread. Following the development of *Bd* survey distribution would be a logical place to start.

Furthermore, as mentioned in the methodology, in the case of the developed surveillance system, it would be advised to conduct surveys on a higher number of localities and on a wider range of species. With understanding the effect in different species and their defence mechanisms, it would be easier to establish proper preventive management actions and health treatments. Moreover, besides getting more data on *Bsal* distribution, the implementation of conservation and action programs would also contribute to prevention of the poorly regulated animal trade.

Nowadays, amphibians' populations all over the world are facing various threats daily. As conservationists, one of our main goals is to try to minimize the effect of these threats and help to prevent the endangered populations from rapid declines and extinctions. In order to have precise data, the continuous and basic monitoring surveillance is necessary.

In the case of *Bsal*, knowing its accurate presence is a necessary and important step for having a better global picture of its distribution. Furthermore, with additionally education of public and developed emergency hot lines, the citizen science could contribute in recognizing the diseases in time. Considering amphibians are important bioindicator of natural health and key species for various ecosystems, their conservation and protection is of major importance.

8. References

AmphibiaWeb. (2020). AmphibiaWeb. Retrieved from University of California, Berkeley, CA, USA website: https://amphibiaweb.org/

Araújo, M. B., Thuiller, W., & Pearson, R. G. (2006). Climate warming and the decline of amphibians and reptiles in Europe. *Journal of Biogeography*, *33*(10), 1712–1728. https://doi.org/10.1111/j.1365-2699.2006.01482.x

Arntzen W., J., Denoël, M., Kuzmin, S., Ishchenko, V., Beja, P., Andreone, F., … Tejedo, M. (2009). Ichthyosaura alpestris. *The IUCN Red List of Threatened Species 2009*. https://doi.org/10.2305/IUCN.UK.2009.RLTS.T59472A11946568.en.

Arntzen W., J., Kuzmin, S., Beebee, T., Papenfuss, T., Sparreboom, M., Ugurtas, I. H., … Tuniyev, B. (2020). Lissotriton vulgaris. *The IUCN Red List of Threatened Species 2009*.

Bai, C., Garner, T. W. J., & Li, Y. (2010). First evidence of batrachochytrium dendrobatidis in China: Discovery of chytridiomycosis in introduced American bullfrogs and native amphibians in the Yunnan Province, China. *EcoHealth*, *7*(1), 127–134. https://doi.org/10.1007/s10393-010-0307-0

Bai, C., Liu, X., Fisher, M. C., Garner, T. W. J., & Li, Y. (2012). Global and endemic Asian lineages of the emerging pathogenic fungus Batrachochytrium dendrobatidis widely infect amphibians in China. *Diversity and Distributions*, *18*(3), 307–318. https://doi.org/10.1111/j.1472-4642.2011.00878.x

Baláž, V., Solský, M., Lastra González, D., Havlíková, B., Gallego Zamorano, J., González Sevilleja, C., … Vojar, J. (2018). First survey of the pathogenic fungus Batrachochytrium salamandrivorans in wild and captive amphibians in the Czech Republic. *Salamandra*, *54*(1), 87–91.

Bales, E. K., Hyman, O. J., Loudon, A. H., Harris, R. N., Lipps, G., Chapman, E., … Terrell, K. A. (2015). Pathogenic chytrid fungus Batrachochytrium dendrobatidis, but not B. salamandrivorans, detected on eastern hellbenders. *PLoS ONE*, *10*(2), 1–9. https://doi.org/10.1371/journal.pone.0116405

Bates, K. A., Shelton, J. M. G., Mercier, V. L., Hopkins, K. P., Harrison, X. A., Petrovan, S. O., & Fisher, M. C. (2019). Captivity and Infection by the Fungal Pathogen Batrachochytrium salamandrivorans Perturb the Amphibian Skin Microbiome. *Frontiers in Microbiology*, *10*(August), 1–13. https://doi.org/10.3389/fmicb.2019.01834

Beja, P., & Alcazar, R. (2003). Conservation of Mediterranean temporary ponds under agricultural intensification: An evaluation using amphibians. *Biological Conservation*, *114*(3), 317–326. https://doi.org/10.1016/S0006-3207(03)00051-X

Bendik, N. F., & Gluesenkamp, A. G. (2013). Body length shrinkage in an endangered amphibian is associated with drought. *Journal of Zoology*, *290*(1), 35–41. https://doi.org/10.1111/jzo.12009

Berger, L., Speare, R., Daszak, P., Green, D. E., Cunningham, A. A., Goggin, C. L., … McDonald, K. R. (2006). Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *PNAS*, *95*(15), 9031–9036.

https://doi.org/10.1073/pnas.95.15.9031

Berger, Lee, Hyatt, A. D., Speare, R., & Longcore, J. E. (2005). Life cycle stages of the amphibian chytrid Batrachochytrium dendrobatidis. *Diseases of Aquatic Organisms*, *68*(1), 51–63. https://doi.org/10.3354/dao068051

Berger, Lee, Roberts, A. A., Voyles, J., Longcore, J. E., Murray, K. A., & Skerratt, L. F. (2016). History and recent progress on chytridiomycosis in amphibians. *Fungal Ecology*, *19*(February), 89–99. https://doi.org/10.1016/j.funeco.2015.09.007

Berger, Lee, Speare, R., Daszak, P., Green, D. E., Cunningham, A. A., Goggin, C. L., … Parkes, H. (1998). Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(15), 9031–9036. https://doi.org/10.1073/pnas.95.15.9031

Beukema, W., Martel, A., Nguyen, T. T., Goka, K., Schmeller, D. S., Yuan, Z., … Pasmans, F. (2018). Environmental context and differences between native and invasive observed niches of Batrachochytrium salamandrivorans affect invasion risk assessments in the Western Palaearctic. *Diversity and Distributions*, *24*(12), 1788–1801. https://doi.org/10.1111/ddi.12795

BHHU-ATRA. (2018). Mali vodenjak (triton). Retrieved from Bosansko-Hercegovačko Herpetološko Udruženje Atra website: http://bhhuatra.com

Blaustein, A. R., Romansic, J. M., Kiesecker, J. M., & Hatch, A. C. (2003). Ultraviolet radiation, toxic chemicals and amphibian population declines. *Diversity and Distributions*, *9*(2), 123–140. https://doi.org/10.1046/j.1472-4642.2003.00015.x

Blaustein, A. R., Urbina, J., Snyder, P. W., Reynolds, E., Dang, T., Hoverman, J. T., … Hambalek, N. M. (2018). Effects of emerging infectious diseases on amphibians: A review of experimental studies. *Diversity*, *10*(3). https://doi.org/10.3390/d10030081

Blaustein, A. R., Walls, S. C., Bancroft, B. A., Lawler, J. J., Searle, C. L., & Gervasi, S. S. (2010). Direct and indirect effects of climate change on amphibian populations. *Diversity*, *2*(2), 281–313. https://doi.org/10.3390/d2020281

Bletz, M. C., Perl, R. G. B., Bobowski, B. T. C., Japke, L. M., Tebbe, C. C., Dohrmann, A. B., … Vences, M. (2017). Amphibian skin microbiota exhibits temporal variation in community structure but stability of predicted Bd-inhibitory function. *ISME Journal*, *11*(7), 1521–1534. https://doi.org/10.1038/ismej.2017.41

Bletz, M. C., Vences, M., Sabino-Pinto, J., Taguchi, Y., Shimizu, N., Nishikawa, K., & Kurabayashi, A. (2017). Cutaneous microbiota of the Japanese giant salamander (Andrias japonicus), a representative of an ancient amphibian clade. *Hydrobiologia*, *795*(1), 153–167. https://doi.org/10.1007/s10750-017-3126-2

Blooi, M., Martel, A., Haesebrouck, F., Vercammen, F., Bonte, D., & Pasmans, F. (2015). Treatment of urodelans based on temperature dependent infection dynamics of Batrachochytrium salamandrivorans. *Scientific Reports*, *5*(January 2015), 8037. https://doi.org/10.1038/srep08037

Blooi, M., Pasmans, F., Longcore, J. E., Spitzen-Van Der Sluijs, A., Vercammen, F., & Martel, A.

(2013). Duplex real-Time PCR for rapid simultaneous detection of Batrachochytrium dendrobatidis and Batrachochytrium salamandrivorans in amphibian samples. *Journal of Clinical Microbiology*, *51*(12), 4173–4177. https://doi.org/10.1128/JCM.02313-13

Blooi, M., Pasmans, F., Rouffaer, L., Haesebrouck, F., Vercammen, F., & Martel, A. (2015a). Successful treatment of Batrachochytrium salamandrivorans infections in salamanders requires synergy between voriconazole, polymyxin e and temperature. *Scientific Reports*, *5*(June 2015), 1–8. https://doi.org/10.1038/srep11788

Blooi, M., Pasmans, F., Rouffaer, L., Haesebrouck, F., Vercammen, F., & Martel, A. (2015b). Successful treatment of Batrachochytrium salamandrivorans infections in salamanders requires synergy between voriconazole, polymyxin e and temperature. *Scientific Reports*, *5*(March 2015), 1–8. https://doi.org/10.1038/srep11788

Bonacci, O. (2003). *Ekohidrologija vodenih resursa i otvorenih vodotoka (Ecohydrology of water resources and free-surface watercourses)*. Građevinsko-arhitektonski fakultet sveučilišta u Splitu.

Boyle, D. G., Boyle, D. B., Olsen, V., Morgan, J. A. T., & Hyatt, A. D. (2004). Rapid quantitative detection of chytridiomycosis (Batrachochytrium dendrobatidis) in amphibian samples using real-time Taqman PCR assay. *Diseases of Aquatic Organisms*, *60*(2), 141–148. https://doi.org/10.3354/dao060141

Brenes, R., Gray, M. J., Waltzek, T. B., Wilkes, R. P., & Miller, D. L. (2014). Transmission of ranavirus between ectothermic vertebrate hosts. *PLoS ONE*, *9*(3), 1–6. https://doi.org/10.1371/journal.pone.0092476

Briggs, J. C. (2017). Emergence of a sixth mass extinction? *Biological Journal of the Linnean Society*, *122*(2), 243–248. https://doi.org/10.1093/biolinnean/blx063

Brucker, R. M., Harris, R. N., Schwantes, C. R., Gallaher, T. N., Flaherty, D. C., Lam, B. A., & Minbiole, K. P. C. (2008). Amphibian chemical defense: Antifungal metabolites of the microsymbiont Janthinobacterium lividum on the salamander Plethodon cinereus. *Journal of Chemical Ecology*, *34*(11), 1422–1429. https://doi.org/10.1007/s10886-008-9555-7

Brunner, J. L., Storfer, A., Gray, M. J., & Hoverman, J. T. (2015). Ranavirus Ecology and Evolution: From Epidemiology to Extinction. In M. Gray & V. Chinchar (Eds.), *Ranaviruses*. https://doi.org/https://doi.org/10.1007/978-3-319-13755-1_4

Cardinale, B. J., Duffy, J. E., Gonzalez, A., Hooper, D. U., Perrings, C., Venail, P., … Naeem, S. (2012). Biodiversity loss and its impact on humanity. *Nature*, *486*(7401), 59–67. https://doi.org/10.1038/nature11148

Ceballos, G., Ehrlich, P. R., Barnosky, A. D., García, A., Pringle, R. M., & Palmer, T. M. (2015). Accelerated modern human-induced species losses: Entering the sixth mass extinction. *Science Advances*, *1*(5), 1–6. https://doi.org/10.1126/sciadv.1400253

Chatfield, M. W. H., & Richards-Zawacki, C. L. (2011). Elevated temperature as a treatment for Batrachochytrium dendrobatidis infection in captive frogs. *Diseases of Aquatic Organisms*, *94*(3), 235–238. https://doi.org/10.3354/dao02337

Chen, C., Yang, S., Guo, Y., Sun, C., Gu, C., & Xu, B. (2009). Photolytic destruction of endocrine disruptor atrazine in aqueous solution under UV irradiation: Products and pathways.

Journal of Hazardous Materials, *172*(2–3), 675–684. https://doi.org/10.1016/j.jhazmat.2009.07.050

Ćirović, R., Vukov, T. D., Radović, D., Džukić, G., & Kalezić, M. L. (2008). Distribution patterns and environmental determinants of European newts in the Montenegrin karst area. *Biologia*, *63*(5), 745–752. https://doi.org/10.2478/s11756-008-0136-6

Cockell, C., & Blaustein, A. R. (2001). *Ecosystems, Evolution, and Ultraviolet Radiation*. https://doi.org/10.1007/978-1-4757-3486-7

Converse, K., & Green, D. (2005). Diseases of salamanders. In S. Majumdar, J. Huffman, F. Brenner, & A. Panah (Eds.), *Wildlife diseases: landscape epidemiology, spatial distrib-ution and utilization of remote sensing technology* (pp. 118–130). Easton: Pennsylvania Academy of Science.

Cooper, E. W. T. (2016). Current Trade Patterns into Canada Regarding Introduction of Fungus Batrachochytrium salamandrivorans. *Environment and Climate Change Canada*, 118.

Cunningham, A. A. (1996). Pathological and microbiological findings from incidents of unusual mortality of the common frog (Rana temporaria). *Philosophical Transactions of the Royal Society B: Biological Sciences*, *351*(1347), 1539–1557. https://doi.org/10.1098/rstb.1996.0140

Cunningham, Andrew A., Beckmann, K., Perkins, M., Fitzpatrick, L., Cromie, R., Redbond, J., … Fisher, M. C. (2015). Surveillance: Emerging disease in UK amphibians. *Veterinary Record*, *176*(18), 468. https://doi.org/10.1136/vr.h2264

D'Amore, A., Hemingway, V., & Wasson, K. (2010). Do a threatened native amphibian and its invasive congener differ in response to human alteration of the landscape? *Biological Invasions*, *12*(1), 145–154. https://doi.org/10.1007/s10530-009-9438-z

D'Amore, A., Kirby, E., & Mcnicholas, M. (2009). Invasive species shifts ontogenetic resource partitioning and microhabitat use of a threatened native amphibian. *Aquatic Conservation: Marine and Freshwater Ecosystems*, *19*, 34–541. https://doi.org/DOI: 10.1002/aqc.1026

Densmore, C. L., & Green, D. E. (2007). Diseases of amphibians. *ILAR Journal*, *48*(3), 235–254. https://doi.org/10.1093/ilar.48.3.235

Dodd Jr., C. K. (2010). *Amphibian Ecology and Conservation, A Handbook of Techniques*. Oxford University Press.

Dodd Jr., C. K., & L. Smith, L. (2003). Habitat Destruction and Alteration. Historical Trends and Future Prospects for Amphibians. *Amphibian Conservation*, (January 2018), 94–112.

Džukić, G., & Kalezić, M. (2004). *The biodiversity of amphibians and reptiles in the Balkan Peninsula* (H. I. Griffiths, B. Kryštufek, & J. M. Reed, Eds.). https://doi.org/https://doi.org/10.1007/978-1-4020-2854-0

European Commission. (2020a). European Commission, European Red List. Retrieved from https://ec.europa.eu/environment/nature/conservation/species/redlist/amphibians/status.htm?fbclid=IwAR0wSgK-MmDdaFhiIDl9Sh4Pi2_w65k6mjh35WcvKMOpCU0Y8x-tq0OLVpI

European Commission. (2020b). European Commission, Live animals - Trade & Imports. Retrieved from https://ec.europa.eu/food/animals/live_animals/other_en

Ficetola, G. F., Thuiller, W., & Miaud, C. (2007). Prediction and validation of the potential global distribution of a problematic alien invasive species - The American bullfrog. *Diversity and Distributions*, *13*(4), 476–485. https://doi.org/10.1111/j.1472-4642.2007.00377.x

Fisher, M. C., & Garner, T. W. J. (2007). The relationship between the emergence of Batrachochytrium dendrobatidis, the international trade in amphibians and introduced amphibian species. *Fungal Biology Reviews*. https://doi.org/10.1016/j.fbr.2007.02.002

Fisher, M. C., Henk, D. A., Briggs, C. J., Brownstein, J. S., Madoff, L. C., McCraw, S. L., & Gurr, S. J. (2012). Emerging fungal threats to animal, plant and ecosystem health. *Nature*, *484*(7393), 186–194. https://doi.org/10.1038/nature10947

Fitzpatrick, L. D., Pasmans, F., Martel, A., & Cunningham, A. A. (2018). Epidemiological tracing of Batrachochytrium salamandrivorans identifies widespread infection and associated mortalities in private amphibian collections. *Scientific Reports*, *8*(1), 1–10. https://doi.org/10.1038/s41598-018-31800-z

Garner, T. W.J., Garcia, G., Carroll, B., & Fisher, M. C. (2009). Using itraconazole to clear Batrachochytrium dendrobatidis infection, and subsequent depigmentation of Alytes muletensis tadpoles. *Diseases of Aquatic Organisms*, *83*(3), 257–260. https://doi.org/10.3354/dao02008

Garner, Trenton W.J., Walker, S., Bosch, J., Hyatt, A. D., Cunningham, A. A., & Fisher, M. C. (2005). Chytrid Fungus in Europe. *Emerging Infectious Diseases*, *11*(10), 1639–1641. https://doi.org/10.3201/eid1110.050109

Geiger, C. C., Küpfer, E., Schär, S., Wolf, S., & Schmidt, B. R. (2011). Elevated temperature clears chytrid fungus infections from tadpoles of the midwife toad, Alytes obstetricans. *Amphibia Reptilia*, *32*(2), 276–280. https://doi.org/10.1163/017353711X556970

Gimeno, A., Meikl, M., Mühl, S., Pitt, A., Winkler, M., & Berninger, U. G. (2015). Testing of Fire Salamanders around Salzburg for Batrachochytrium salamandrivorans within a school project. *Eco.Mont*, *7*(1), 72–76. https://doi.org/10.1553/eco.mont-7-1s72

González, D. L., Baláž, V., Solský, M., Thumsová, B., Kolenda, K., Najbar, A., … Vojar, J. (2019). Recent findings of potentially lethal salamander fungus batrachochytrium salamandrivorans. *Emerging Infectious Diseases*, *25*(7), 1416–1418. https://doi.org/10.3201/eid2507.181001

Grant, E. H. C., Muths, E., Katz, R. A., Canessa, S., Adams, M. J., Ballard, J. R., … White, C. L. A. (2017). Using decision analysis to support proactive management of emerging infectious wildlife diseases. *Frontiers in Ecology and the Environment*, *15*(4), 214–221. https://doi.org/10.1002/fee.1481

Grasselli, E., Bianchi, G., Dondero, L., Marchianò, V., Carafa, M., Perrone, M., & Salvidio, S. (2019). First screening for batrachochytrium salamandrivorans (BSAL) in wild and captive salamanders from Italy. *Salamandra*, *55*(2), 124–126.

Gray, M. J., Lewis, J. P., Nanjappa, P., Klocke, B., Pasmans, F., Martel, A., … Olson, D. H. (2015). Batrachochytrium salamandrivorans: The North American Response and a Call for Action.

PLoS Pathogens, *11*(12), 1–9. https://doi.org/10.1371/journal.ppat.1005251

Gray, M. J., Miller, D. L., & Hoverman, J. T. (2009). Ecology and pathology of amphibian ranaviruses. *Diseases of Aquatic Organisms*, *87*(3), 243–266. https://doi.org/10.3354/dao02138

Gutierrez, J. B., & Teem, J. L. (2006). A model describing the effect of sex-reversed YY fish in an established wild population: The use of a Trojan Y chromosome to cause extinction of an introduced exotic species. *Journal of Theoretical Biology*, *241*(2), 333–341. https://doi.org/10.1016/j.jtbi.2005.11.032

Hanlon, S. M., Lynch, K. J., Kerby, J., & Parris, M. J. (2015). Batrachochytrium dendrobatidis exposure effects on foraging efficiencies and body size in anuran tadpoles. *Diseases of Aquatic Organisms*, *112*(3), 237–242. https://doi.org/10.3354/dao02810

Hanski, I. (1999). *Metapopulation Ecology*. New York: Oxford University Press.

Hayes, T. B., Falso, P., Gallipeau, S., & Stice, M. (2010). The cause of global amphibian declines: A developmental endocrinologist's perspective. *Journal of Experimental Biology*, *213*(6), 921–933. https://doi.org/10.1242/jeb.040865

Hayes, Tyrone B., Khoury, V., Narayan, A., Nazir, M., Parka, A., Brown, T., … Gallipeau, S. (2010). Atrazine induces complete feminization and chemical castration in male African clawed frogs (Xenopus laevis). *Proceedings of the National Academy of Sciences of the United States of America*, *107*(10), 4612–4617. https://doi.org/10.1073/pnas.0909519107

HBSD. (2020). Hrvatsko biospeleološko društvo: HBSD. Retrieved from https://www.hbsd.hr/ugrozenost_i_zastita/

Hyatt, A. D., Boyle, D. G., Olsen, V., Boyle, D. B., Berger, L., Obendorf, D., … Colling, A. (2007). Diagnostic assays and sampling protocols for the detection of Batrachochytrium dendrobatidis. *Diseases of Aquatic Organisms*, *73*(3), 175–192. https://doi.org/10.3354/dao073175

Hyla. (2020). Hrvatsko herpetološko društvo, Hyla, Udruga Hyla. Retrieved from http://www.hhdhyla.hr/

IPCC. (2007). *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change* (S. Solomon, D. Qin, M. Manning, Z. Chen, M. Marquis, K. B. Averyt, … H. L. Miller, Eds.). Retrieved from https://www.ipcc.ch/site/assets/uploads/2018/05/ar4_wg1_full_report-1.pdf

IPCC. (2013). *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* (T. F. Stocker, D. Qin, G.-K. Plattner, M. Tignor, S. K. Allen, J. Boschung, … P. M. Midgley, Eds.). Retrieved from https://www.ipcc.ch/site/assets/uploads/2018/02/WG1AR5_all_final.pdf

IUCN. (2020a). IUCN - A brief history. Retrieved from https://www.iucn.org/about/iucn-a-brief-history

IUCN. (2020b). IUCN Red List of Threatened Species. Retrieved from https://www.iucnredlist.org/

Iwanowicz, D. D., Schill, W. B., Olson, D. H., Adams, M. J., Densmore, C., Cornman, R. S., … Chestnut, T. (2017). Potential Concerns with Analytical Methods Used for the Detection of Batrachochytrium salamandrivorans from Archived DNA of Amphibian Swab Samples, Oregon, USA. *Herpetological Review*, *48*(482), 352–355. Retrieved from http://www.salamanderfungus.org/wp-content/uploads/2017/07/Iwanowicz-et-al-2017.pdf

Jelić, D., Kuljerić, M., Koren, T., & Dag, T. (2012). *Crvena knjiga vodozemaca i gmazova Hrvatske*. Ministarstvo zaštite okoliša i prirode, Državni zavod za zaštitu prirode, Hrvatsko herpetološko društvo HYLA.

Jenkins, P. T., Genovese, K., Ruffler, H., Muffett, C., Burgiel, S., Malsch, K., … Chavarria, G. (2007). *The regulation of live Animal imports in the United states*. Retrieved from https://defenders.org/sites/default/files/publications/broken_screens_report.pdf

Johnson, M. L., & Speare, R. (2003). Survival of Batrachochytrium dendrobatidis in water: Quarantine and disease control implications. *Emerging Infectious Diseases*, *9*(8), 922–925. https://doi.org/10.3201/eid0908.030145

Johnson, S. A., & Barichivich, W. J. (2004). A Simple Technique for Trapping Siren lacertina, Amphiuma means , and Other Aquatic Vertebrates. *Journal of Freshwater Ecology*, *19*(2), 263–269. https://doi.org/10.1080/02705060.2004.9664540

Kats, L. B., & Ferrer, R. P. (2003). Alien predators and amphibian declines: Review of two decades of science and the transition to conservation. *Diversity and Distributions*, *9*(2), 99–110. https://doi.org/10.1046/j.1472-4642.2003.00013.x

Knapp, R. A., & Matthews, K. R. (2000). Non-native fish introductions and the decline of the mountain yellow-legged frog from within protected areas. *Conservation Biology*, *14*, 428–438.

Kolby, J. E. (2014). Presence of the amphibian chytrid fungus Batrachochytrium dendrobatidis in native amphibians exported from Madagascar. *PLoS ONE*, *9*(3). https://doi.org/10.1371/journal.pone.0089660

Kolby, J. E., Ramirez, S. D., Berger, L., Griffin, D. W., Jocque, M., & Skerratt, L. F. (2015). Presence of amphibian chytrid fungus (Batrachochytrium dendrobatidis) in rainwater suggests aerial dispersal is possible. *Aerobiologia*, *31*(3), 411–419. https://doi.org/10.1007/s10453-015-9374-6

Kriger, K. M., & Hero, J. M. (2009). Chytridiomycosis, amphibian extinctions, and lessons for the prevention of future panzootics. *EcoHealth*, *6*(1), 6–10. https://doi.org/10.1007/s10393-009-0228-y

Kudeljnjak, M. (2009). *ZNAČAJKE I UGROŽENOST VODOZEMACA CHARACTERISTICS AND VULNERABILITY OF AMPHIBIANS IN PODRAVINA*. Sveučilište u Zagrebu Prirodoslovno-matematički fakultet.

Kupferberg, S. J., Palen, W. J., Lind, A. J., Bobzien, S., Catenazzi, A., Drennan, J., & Power, M. E. (2012). Effects of Flow Regimes Altered by Dams on Survival, Population Declines, and Range-Wide Losses of California River-Breeding Frogs. *Conservation Biology*, *26*(3), 513–524. https://doi.org/10.1111/j.1523-1739.2012.01837.x

Kuzmin, S., Papenfuss, T., Sparreboom, M., Ugurtas, I. H., Anderson, S., Beebee, T., … Ishchenko, V. (2009). Salamandra salamandra. *The IUCN Red List of Threatened Species 2009*. https://doi.org/10.2305/IUCN.UK.2009.RLTS.T59467A11928351.en

Laking, A. E., Ngo, H. N., Pasmans, F., Martel, A., & Nguyen, T. T. (2017). Batrachochytrium salamandrivorans is the predominant chytrid fungus in Vietnamese salamanders. *Scientific Reports*, *7*(October 2016), 1–5. https://doi.org/10.1038/srep44443

Liew, N., Moya, M. J. M., Wierzbicki, C. J., Hollinshead, M., Dillon, M. J., Thornton, C. R., … Mostowy, S. (2017). Chytrid fungus infection in zebrafish demonstrates that the pathogen can parasitize non-amphibian vertebrate hosts. *Nature Communications*, *8*, 1–10. https://doi.org/10.1038/ncomms15048

Lips, K. R. (2016). Overview of chytrid emergence and impacts on amphibians. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *371*(1709). https://doi.org/10.1098/rstb.2015.0465

Lips, K. R., Brem, F., Brenes, R., Reeve, J. D., Alford, R. A., Voyles, J., … Collins, J. P. (2006). Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(9), 3165–3170. https://doi.org/10.1073/pnas.0506889103

Longcore, J. E., Pessier, A. P., Nichols, D. K., Longcore, J. E., Pessier, A. P., Nichols, D. K., … Nichols, D. K. (1999). *chytrid pathogenic to amphibians*. *5514*. https://doi.org/10.1080/00275514.1999.12061011

Martel, A., Blooi, M., Adriaensen, C., Van Rooij, P., Beukema, W., Fisher, M. C., … Pasmans, F. (2014). Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. *Science*, *346*(6209), 630–631. https://doi.org/10.1126/science.1258268

Martel, An, Sluijs, A. S. der, Blooi, M., Bert, W., Ducatelle, R., Fisher, M. C., … Pasmans, F. (2013). Batrachochytrium salamandrivorans sp. nov. causes lethal chytridiomycosis in amphibians. *Proceedings of the National Academy of Sciences*, *110*(38), 15325–15329. https://doi.org/10.1073/PNAS.1307356110

Martel, An, Spitzen-Van Der Sluijs, A., Blooi, M., Bert, W., Ducatelle, R., Fisher, M. C., … Pasmans, F. (2013). Batrachochytrium salamandrivorans sp. nov. causes lethal chytridiomycosis in amphibians. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(38), 15325–15329. https://doi.org/10.1073/pnas.1307356110

Martel, An, Vila-Escale, M., Fernández-Giberteau, D., Martinez-Silvestre, A., Canessa, S., Van Praet, S., … Pasmans, F. (2020). Integral chain management of wildlife diseases. *Conservation Letters*, (January), 1–6. https://doi.org/10.1111/conl.12707

Miller, D., Gray, M., & Storfer, A. (2011). Ecopathology of ranaviruses infecting amphibians. *Viruses*, *3*(11), 2351–2373. https://doi.org/10.3390/v3112351

More, S., Angel Miranda, M., Bicout, D., Bøtner, A., Butterworth, A., Calistri, P., … Gortázar Schmidt, C. (2018). Risk of survival, establishment and spread of Batrachochytrium salamandrivorans (Bsal) in the EU. *EFSA Journal*, *16*(4). https://doi.org/10.2903/j.efsa.2018.5259

More, S., Angel Miranda, M., Bicout, D., Bøtner, A., Butterworth, A., Calistri, P., … Gortázar Schmidt, C. (2018). Risk of survival, establishment and spread of Batrachochytrium salamandrivorans (Bsal) in the EU (M. Lukač, Faculty of Veterinary Medicine, University of Zagreb, Croatia). *EFSA Journal*, *16*(4). https://doi.org/10.2903/j.efsa.2018.5259

Muletz, C., Caruso, N. M., Fleischer, R. C., McDiarmid, R. W., & Lips, K. R. (2014). Unexpected rarity of the pathogen Batrachochytrium dendrobatidis in Appalachian Plethodon salamanders: 1957-2011. *PLoS ONE*, *9*(8). https://doi.org/10.1371/journal.pone.0103728

Murphy, M. B., Hecker, M., Coady, K. K., Tompsett, A. R., Jones, P. D., Du Preez, L. H., … Giesy, J. P. (2006). Atrazine concentrations, gonadal gross morphology and histology in ranid frogs collected in Michigan agricultural areas. *Aquatic Toxicology*, *76*(3–4), 230–245. https://doi.org/10.1016/j.aquatox.2005.09.010

Murray, K. A., Rosauer, D., McCallum, H., & Skerratt, L. F. (2011). Integrating species traits with extrinsic threats: Closing the gap between predicting and preventing species declines. *Proceedings of the Royal Society B: Biological Sciences*, *278*(1711), 1515–1523. https://doi.org/10.1098/rspb.2010.1872

Mutschmann, F. (2015). Chytridiomycosis in Amphibians. *Journal of Exotic Pet Medicine*, *24*(3), 276–282. https://doi.org/10.1053/j.jepm.2015.06.013

Natuurpunt. (2020). Save our salamanders. [Web Application]. Retrieved from https://www.natuurpunt.be/salamanders-and-batrachochytrium-salamandrivorans

Nazir, J., Spengler, M., & Marschang, R. E. (2012). Environmental persistence of amphibian and reptilian ranaviruses. *Diseases of Aquatic Organisms*, *98*(3), 177–184. https://doi.org/10.3354/dao02443

O'Hanlon, S. J., Rieux, A., Farrer, R. A., Rosa, G. M., Waldman, B., Bataille, A., … Fisher, M. C. (2018). Recent Asian origin of chytrid fungi causing global amphibian declines. *Science*, *360*(6389), 621–627. https://doi.org/10.1126/science.aar1965

Olson, D. H., Aanensen, D. M., Ronnenberg, K. L., Powell, C. I., Walker, S. F., Bielby, J., … Fisher, M. C. (2013). Mapping the Global Emergence of Batrachochytrium dendrobatidis, the Amphibian Chytrid Fungus. *PLoS ONE*, *8*(2), e56802. https://doi.org/10.1371/journal.pone.0056802

Oludairo, O. O., Aiyedun, J. O., Olorunshola, I. D., Dibal, M. A., Gungbias, A. A., Ayeni, A. M. J., & Adeyi, A. J. (2017). Transboundary Diseases and Wildlife Management: an Overview. *Bangladesh Journal of Veterinary Medicine*, *14*(2), 123–130. https://doi.org/10.3329/bjvm.v14i2.31380

Parmesan, C., & Yohe, G. (2003). A globally coherent fingerprint of climate change impacts across natural systems. *Nature*, *421*(6918), 37–42. https://doi.org/10.1038/nature01286

Parrott, J. C., Shepack, A., Burkart, D., LaBumbard, B., Scimè, P., Baruch, E., & Catenazzi, A. (2016). Survey of Pathogenic Chytrid Fungi (Batrachochytrium dendrobatidis and B. salamandrivorans) in Salamanders from Three Mountain Ranges in Europe and the Americas. *EcoHealth*, *14*(2), 296–302. https://doi.org/10.1007/s10393-016-1188-7

Pask, J. D., Woodhams, D. C., & Rollins-Smith, L. A. (2012). The ebb and flow of antimicrobial skin peptides defends northern leopard frogs (Rana pipiens) against chytridiomycosis.

Global Change Biology, *18*(4), 1231–1238. https://doi.org/10.1111/j.1365-2486.2011.02622.x

Pessier, A.P., & Mendelson, J. R. (2011). A manual for control of infectious diseases in amphibian survival assurance colonies and reintroduction programs. *Iucn/Ssc Cbsg*, (January 2011), 1–284.

Pessier, Allan P. (2007). Amphibian Chytridiomycosis. In M. Flower & E. Miller (Eds.), *Zoo and Wild Animal Medicine* (pp. 137–143). https://doi.org/10.1016/B978-141604047-7.50020-8

Piotrowski, J. S., Annis, S. L., & Longcore, J. E. (2004). Physiology of Batrachochytrium dendrobatidis, a chytrid pathogen of amphibians. *Mycologia*, *96*(1), 9–15. https://doi.org/10.1080/15572536.2005.11832990

Plumb, J. A., & Zilberg, D. (1999). Survival of largemouth bass iridovirus in frozen fish. *Journal of Aquatic Animal Health*, *11*(1), 94–96. https://doi.org/10.1577/1548-8667(1999)011<0094:SOLBII>2.0.CO;2

Pounds, J. A., Fogden, M. P. L., & Campbell, J. H. (1999). Biological response to climate change on a tropical mountain. *Nature*, *398*(6728), 611–615. https://doi.org/10.1038/19297

Pounds, J. A., Fogden, M. P. L., Savage, J. M., & Gorman, G. C. (1997). Tests of null models for amphibian declines on a tropical mountain. *Conservation Biology*, *11*(6), 1307–1322. https://doi.org/10.1046/j.1523-1739.1997.95485.x

Price, S. J., Wadia, A., Wright, O. N., Leung, W. T. M., Cunningham, A. A., & Lawson, B. (2017). Screening of a long-term sample set reveals two Ranavirus lineages in British herpetofauna. *PloS One*, *12*(9), e0184768. https://doi.org/10.1371/journal.pone.0184768

Priroda Hrvatske. (2020). Priroda Hrvatske, Ugroženost Prirode. Retrieved from http://prirodahrvatske.com/ugrozenost-prirode/

Rachowicz, L. J., Hero, J. M., Alford, R. A., Taylor, J. W., Morgan, J. A. T., Vredenburg, V. T., … Briggs, C. J. (2005). The novel and endemic pathogen hypotheses: Competing explanations for the origin of emerging infectious diseases of wildlife. *Conservation Biology*, *19*(5), 1441–1448. https://doi.org/10.1111/j.1523-1739.2005.00255.x

Richgels, K. L. D., Russell, R. E., Adams, M. J., White, C. L., & Grant, E. H. C. (2016). Spatial variation in risk and consequence of Batrachochytrium salamandrivorans introduction in the USA. *Royal Society Open Science*, *3*(2). https://doi.org/10.1098/rsos.150616

Rollins-Smith, L. A. (2017). Amphibian immunity–stress, disease, and climate change. *Developmental and Comparative Immunology*, *66*, 111–119. https://doi.org/10.1016/j.dci.2016.07.002

Rollins-Smith, L. A., Ramsey, J. P., Pask, J. D., Reinert, L. K., & Woodhams, D. C. (2011). Amphibian immune defenses against chytridiomycosis: Impacts of changing environments. *Integrative and Comparative Biology*, *51*(4), 552–562. https://doi.org/10.1093/icb/icr095

Rosa, G. M., Sabino-Pinto, J., Laurentino, T. G., Martel, A., Pasmans, F., Rebelo, R., … Bosch, J. (2017). Impact of asynchronous emergence of two lethal pathogens on amphibian assemblages. *Scientific Reports*, *7*(February). https://doi.org/10.1038/srep43260

Rowley, J. J. L., Shepherd, C. R., Stuart, B. L., Nguyen, T. Q., Hoang, H. D., Cutajar, T. P., … Phimmachak, S. (2016). Estimating the global trade in Southeast Asian newts. *Biological Conservation*, *199*, 96–100. https://doi.org/10.1016/j.biocon.2016.05.001

Sabino-Pinto, J., Bletz, M., Hendrix, R., Perl, R. G. B., Martel, A., Pasmans, F., … Steinfartz, S. (2015). First detection of the emerging fungal pathogen Batrachochytrium salamandrivorans in Germany. *Amphibia Reptilia*, *36*(4), 411–416. https://doi.org/10.1163/15685381-00003008

Sabino-Pinto, J., Veith, M., Vences, M., & Steinfartz, S. (2018). Asymptomatic infection of the fungal pathogen Batrachochytrium salamandrivorans in captivity. *Scientific Reports*, *8*(1), 1–8. https://doi.org/10.1038/s41598-018-30240-z

Savage, A. E., Grismer, L. L., Anuar, S., Onn, C. K., Grismer, J. L., Quah, E., … Zamudio, K. R. (2011). First record of batrachochytrium dendrobatidis infecting four frog families from peninsular Malaysia. *EcoHealth*, *8*(1), 121–128. https://doi.org/10.1007/s10393-011-0685-y

Schlaepfer, M. A., Hoover, C., & Dodd, C. K. (2005). Challenges in Evaluating the Impact of the Trade in Amphibians and Reptiles on Wild Populations. *BioScience*, *55*(3), 256. https://doi.org/10.1641/0006-3568(2005)055[0256:cietio]2.0.co;2

Schmidt, B. (2016). Import ban for salamanders and newts in Switzerland. Why? (Importverbot für Salamander und Molche in die Schweiz: Warum?.). *Terraria/Elaphe*, *57*, 8–9.

Secretariat of the CBD. (2010). *Pets, aquarium, and terrarium species: best practices for addressing risks to biodiversity*. Retrieved from https://www.cbd.int/doc/publications/cbd-ts-48-en.pdf

Semlitsch, R. D., Todd, B. D., Blomquist, S. M., Calhoun, A. J. K., Gibbons, J. W., Gibbs, J. P., … Rothermel, B. B. (2009). Effects of Timber Harvest on Amphibian Populations: Understanding Mechanisms from Forest Experiments. *BioScience*, *59*(10), 853–862. https://doi.org/10.1525/bio.2009.59.10.7

Sparling, D. W., Fellers, G. M., & McConnell, L. L. (2001). Pesticides and amphibian population declines in California, USA. *Environmental Toxicology and Chemistry*, *20*(7), 1591–1595. https://doi.org/10.1002/etc.5620200725

Spitzen-Van Der Sluijs, A., Bosman, W., Pasmans, F., Goverse, E., Martel, A., De Zeeuw, M., … Kik, M. (2013). Rapid enigmatic decline drives the fire salamander (salamandra salamandra) to the edge of extinction in the netherlands. *Amphibia Reptilia*, *34*(2), 233–239. https://doi.org/10.1163/15685381-00002891

Spitzen-van der Sluijs, A., Martel, A., Asselberghs, J., Bales, E. K., Beukema, W., Bletz, M. C., … Lötters, S. (2016). Expanding distribution of lethal amphibian fungus Batrachochytrium salamandrivorans in Europe. *Emerging Infectious Diseases*, *22*(7), 1286–1288. https://doi.org/10.3201/eid2207.160109

Stark, T., Martel, A., Pasmans, F., Thomas, V., Gilbert, M., & Spitzen, A. (2018). A European early warning system for a deadly salamander pathogen. *AArk Newsletter*, *42*(April), 12–14.

Stegen, G., Pasmans, F., Schmidt, B. R., Rouffaer, L. O., Van Praet, S., Schaub, M., … Martel, A. (2017). Drivers of salamander extirpation mediated by Batrachochytrium salamandrivorans. *Nature*, *544*(7650), 353–356. https://doi.org/10.1038/nature22059

Stephen, C., Forzan, M. J., Redford, T., & Zimmer, M. (2015). Batrachochytrium salamandrivorans, a threat assessment of salamander chytrid disease. *The Canadian Wildlife Health Cooperative*, 30.

Stuart, S. N., Chanson, J. S., Cox, N. A., Young, B. E., Rodrigues, A. S. L., Fischman, D. L., & Waller, R. W. (2004). Status and trends of amphibian declines and extinctions worldwide. *Science*, *306*(5702), 1783–1786. https://doi.org/10.1126/science.1103538

Subba, B., Sen, S., Ravikanth, G., & Nobis, M. P. (2018). Direct modelling of limited migration improves projected distributions of Himalayan amphibians under climate change. *Biological Conservation*, *227*(May), 352–360. https://doi.org/10.1016/j.biocon.2018.09.035

Swei, A., Rowley, J. J. L., Rödder, D., Diesmos, M. L. L., Diesmos, A. C., Briggs, C. J., … Vredenburg, V. T. (2011). Is chytridiomycosis an emerging infectious disease in Asia? *PLoS ONE*, *6*(8). https://doi.org/10.1371/journal.pone.0023179

Tamara, Y., Ohtsuka, M., Kato, K., Manabe, S., Kuroda, K., Sanada, M., & Ueda, M. (2006). Application of the arming system for the expression of the 380R antigen from red sea bream iridovirus (RSIV) on the surface of yeast cells: A first step for the development of an oral vaccine. *Biotechnology Progress*, *22*(4), 949–953. https://doi.org/10.1021/bp060130x

Thekkiniath, J. C., Zabet-Moghaddam, M., San Francisco, S. K., & San Francisco, M. J. (2013). A novel subtilisin-like serine protease of Batrachochytrium dendrobatidis is induced by thyroid hormone and degrades antimicrobial peptides. *Fungal Biology*, *117*(6), 451–461. https://doi.org/10.1016/j.funbio.2013.05.002

Thomas, V., Blooi, M., Van Rooij, P., Van Praet, S., Verbrugghe, E., Grasselli, E., … Martel, A. (2018). Recommendations on diagnostic tools for Batrachochytrium salamandrivorans. *Transboundary and Emerging Diseases*, *65*(2), e478–e488. https://doi.org/10.1111/tbed.12787

Thurman, E. M., & Cromwell, A. E. (2000). Atmospheric Transport, Deposition, and Fate of Triazine Herbicides and Their Metabolites in Pristine Areas at Isle Royale National Park. *Environ. Sci. Technol.*, *34*(15), 3079–3085. Retrieved from https://doi.org/10.1021/es000995l

Tobler, U., & Schmidt, B. R. (2010). Within- and among-population variation in chytridiomycosis-induced mortality in the toad Alytes obstetricans. *PLoS ONE*, *5*(6), 1–8. https://doi.org/10.1371/journal.pone.0010927

UNEP-WCMC. (2016). *Review of the risk posed by importing Asiatic species of Caudata amphibians (salamanders and newts) into the EU.* 35. Retrieved from www.unep-wcmc.org

US Fish and Wildlife Service. (2016). Injurious wildlife species: Listing salamanders due to risk of salamander chytrid fungus. *Federal Register*, *81*(8), 1534–1556. Retrieved from www.regulations.gov.

Van Rooij, P., Martel, A., Haesebrouck, F., & Pasmans, F. (2015). Amphibian chytridiomycosis: A review with focus on fungus-host interactions. *Veterinary Research*, *46*(1), 0–22. https://doi.org/10.1186/s13567-015-0266-0

Velo-Antón, G., Zamudio, K. R., & Cordero-Rivera, A. (2012). Genetic drift and rapid evolution of viviparity in insular fire salamanders (Salamandra salamandra). *Heredity*, *108*(4), 410–418. https://doi.org/10.1038/hdy.2011.91

Vojar, J., Havlíková, B., Solský, M., Jablonski, D., Iković, V., & Baláž, V. (2017). Distribution, prevalence, and amphibian hosts of Batrachochytrium dendrobatidis in the Balkans. *Salamandra*, *53*(1), 44–49.

Vörös, J., Bosch, J., Dán, Á., & Hartel, T. (2013). First record of Batrachochytrium dendrobatidis on amphibians in Romania. *North-Western Journal of Zoology*, *9*(2), 446–449.

Vörös, J., & Jelić, D. (2011). First steps to survey chytrid fungus in Croatia Prvi koraci u istraživanju chytrid gljivica u Hrvatskoj J. *Hyla*, *2011*(1), 31–33.

Voyles, J., Johnson, L. R., Briggs, C. J., Cashins, S. D., Alford, R. A., Berger, L., … Rosenblum, E. B. (2012). Temperature alters reproductive life history patterns in Batrachochytrium dendrobatidis, a lethal pathogen associated with the global loss of amphibians. *Ecology and Evolution*, *2*(9), 2241–2249. https://doi.org/10.1002/ece3.334

Vujica, L. (2018). *Planinski vodenjak, Alpine Newt* (Sveučilište u Zagrebu Prirodoslovno-matematički fakultet). Retrieved from https://urn.nsk.hr/urn:nbn:hr:217:892000

Wang, S., Zhu, W., Fan, L., Li, J., & Li, Y. (2017). Amphibians testing negative for Batrachochytrium dendrobatidis and Batrachochytrium salamandrivorans on the Qinghai-Tibetan plateau, China. *Asian Herpetological Research*, *8*(3), 190–198. https://doi.org/10.16373/j.cnki.ahr.170010

Warren, R., Vanderwal, J., Price, J., Welbergen, J. A., Atkinson, I., Ramirez-Villegas, J., … Lowe, J. (2013). Quantifying the benefit of early climate change mitigation in avoiding biodiversity loss. *Nature Climate Change*, *3*(7), 678–682. https://doi.org/10.1038/nclimate1887

White, A. (2006). A trial using salt to protect green and golden bell frogs from chytrid infection. *Herpetofauna*, *36*, 93–96.

Wind, E. (1999). Effects of Habitat Fragmentation on Amphibians. *Proceedings of a Conference on the Biology and Management of Species and Habitats at Risk, Kamloops, B.C*, *2*, 15–19. Retrieved from http://www.env.gov.bc.ca/wld/documents/st17wind.pdf

Wyler, L. S., & Sheikh, P. A. (2013). International illegal trade in wildlife: Threats and U.S. policy. *Combating Wildlife Trafficking: National Strategy, Implementation Plan and Restrictions on Elephant Ivory Trade*, 79–112.

Yap, T. A., Koo, M. S., Ambrose, R. F., Wake, D. B., & Vredenburg, V. T. (2015). Averting a North American biodiversity crisis: A newly described pathogen poses a major threat to salamanders via trade. *Science*, *349*(6247), 481–482. https://doi.org/10.1126/science.aab1052

Yap, T. A., Nguyen, N. T., Serr, M., Shepack, A., & Vredenburg, V. T. (2017). Batrachochytrium salamandrivorans and the Risk of a Second Amphibian Pandemic. *EcoHealth*, *14*(4), 851–

864. https://doi.org/10.1007/s10393-017-1278-1

Yuan, Z., Martel, A., Wu, J., Van Praet, S., Canessa, S., & Pasmans, F. (2018). Widespread occurrence of an emerging fungal pathogen in heavily traded Chinese urodelan species. *Conservation Letters*, *11*(4), 1–8. https://doi.org/10.1111/conl.12436

Žeželj, B. (2011). *PRIRODOSLOVNO-MATEMATIČKI FAKULTET UTJECAJ REGULACIJE RIJEČNOG TOKA NA RAZNOLIKOST VRSTA I STANIŠTA INFLUENCE OF RIVER MANAGMENT ON THE DIVERSITY OF HABITATS AND SPECIES SEMINARSKI RAD Barbara Žeželj Preddiplomski studij znanosti o okolišu Undergraduate S*. Sveučilište u Zagrebu Prirodoslovno-matematički fakultet.

Zhu, W., Xu, F., Bai, C., Liu, X., Wang, S., Gao, X., … Li, Y. (2014). A survey for Batrachochytrium salamandrivorans in Chinese amphibians. *Current Zoology*, *60*(6), 729–735. https://doi.org/10.1093/czoolo/60.6.729