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Surveillance of *Batrachochytrium salamandrivorans* in Europe and in the Czech Republic

DISSERTATION

Author: Mgr. Ing. David Lastra González
Supervisor: doc. Ing. Jiří Vojar, Ph.D.
Advisor: Mgr. Vojtech Baláž, Ph.D.

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Ph.D. Thesis: Surveillance of *Batrachochytrium salamandrivorans* in Europe and in the Czech Republic

Thesis supervisor: Assoc. Prof. Dr. Jiří Vojar (Czech University of Life Sciences Prague)

Thesis reviewers: Prof. Dr. Matthew J. Gray (The University of Tennessee, USA)

Prof. Dr. Stefan Lötters (Trier University, Germany)

RNDr. Ivan Rehák, CSc. (Prague Zoo, Czech Republic)

Czech University of Life Sciences Prague

Faculty of Environmental Sciences

Department of Ecology

David Lastra González

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To my grandparents, I wish that you could read it.

Statement of originality

I certify and declare that this doctoral thesis has not been submitted or published elsewhere. My involvement in the manuscript and the research presented in this thesis is specified in the authors’ contributions and implied by the authorship order of the published manuscripts. All relevant literature sources used while writing the following text in this thesis have been properly cited in accordance with the standard practice associated with publications in the biological sciences. Any thoughts by others or literal quotations are clearly referenced.

David Lastra González

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Summary

Nowadays, we are seeing the effects of a novel emerging infectious disease (EID). The general public is starting to become aware as to the consequences of biodiversity loss and how closely this relates also to the rising concern over EIDs. For that reason, species conservation and control of EIDs should be essential tasks upon which researchers as well as policy makers focus their joint efforts. With more than 40% of their species endangered, amphibians have suffered massive declines and become the most threatened group of vertebrates. The EID chytridiomycosis has been demonstrated to be the major cause of this amphibian diversity loss. This fungal disease is caused by two chytrid fungi: *Batrachochytrium dendrobatidis* (*Bd*) and *B. salamandrivorans* (*Bsal*).

The aims of this dissertation thesis are all focused on *Bsal*, especially upon mapping its distribution in Europe, and particularly within the Czech Republic and Spain, and improving the diagnostic methods available in order to make them more accessible while reducing their costs.

Chapter 1 deals with the first monitoring of *Bsal* within the Czech Republic in both wild populations and captive collections. In total, 324 swabs were collected, including from 18 individuals of the largest amphibian species, the Chinese giant salamander (*Andrias davidianus*), reared in Prague's zoological garden. The result of this pioneer *Bsal* test in the Czech Republic ended with non-detection of this pathogen. Nonetheless, its sister species *Bd* was detected in 3 individuals of wild smooth newt (*Lissotriton vulgaris*) and in 1 reared ribbed newt (*Pleurodeles waltl*) in a captive collection.

Chapter 2 focuses on Spanish captive collections. After it was determined that *Bsal* was present in the Spanish amphibian trade, extensive research looking for the salamander killer fungus was a logical and urgent step. This work concluded with 287 samples taken from 7 Spanish captive collections, all of which were found to be free of the pathogen. In addition, two agreements were signed among the Czech University of Life Sciences Prague, BIOPARC Valencia, and Fundación Oceanogràfic, which also provided animals for checking.

In the work encompassed within **Chapter 3**, I had the privilege of leading an international project to conduct extensive monitoring, analyzing 1,135 samples belonging to 10 amphibian species at 47 sites in 6 European countries. Samples from

5 Spanish *Lissotriton helveticus* tested positive for *Bsal*. These were the first such positive findings in the wild for this country and pointed up the importance of better understanding long-distance *Bsal* transmission. We also detected *Bd* in 11 samples from 3 newt species (*L. helveticus*, *L. vulgaris*, and *Triturus cristatus*) from Spain and Montenegro and 1 captive *Cynops ensicauda* newt from the Czech Republic.

Finally, in **Chapter 4**, with the experience and knowledge of how difficult it is to collect samples from remote areas of these particularly elusive species, research was done to improve the existing methodologies. With this upgrade, it became possible for the first time to detect either *Bsal* or *Bd* by examining environmental DNA and the costs were reduced by more than two-thirds relative to similar approaches. The area affected in Spain by *Bsal* was enlarged and it is hoped that through cooperation with regional authorities and NGOs it will be possible to increase awareness about the risk created by *Bsal's* presence.

In summary, **more than 2,300 samples** were collected by skin swabs from amphibians and **47 by water filtration** of aquatic habitats. Amphibian monitoring has been carried out on amphibians in the wild as well as in captive collections (e.g., zoos, hobbyists, private breeders, and university collections). Thus, **more than 60 localities** have been visited in nature and approximately **20 captive collections**. These samplings have resulted in **detecting 17 *Bsal* samples and 27 for *Bd***. Now, local authorities of the affected areas have the knowledge and information to implement mitigation measures and conservation plans to prevent *Bsal* incidence in their amphibian populations.

Abstrakt

Mnoho druhů čelí v současné době nově se vyskytujícím infekčním onemocněním (zkráceně EIDs z anglického, *emerging infectious diseases*). Široká veřejnost si začíná uvědomovat důsledky ztrát biologické rozmanitosti díky vlivu těchto nemocí. Z tohoto důvodu jsou ochrana druhů a kontrola EIDs základními úkoly, na které by vědci, společně se zákonodárci, měli soustředit svou pozornost. Obojživelníci jsou nejohroženější skupinou obratlovců na světě, mezi ohrožené patří více než 40 % druhů. Mezi nejvýznamnější nemoci obojživelníků patří chytridiomykóza, způsobená dvěma druhy chytridiomycet: *Batrachochytrium dendrobatidis* (*Bd*) a *B. salamandrivorans* (*Bsal*).

Tato disertační práce se zaměřuje na druhý jmenovaný druh, především pak na mapování jeho výskytu a šíření v Evropě, zejména v České republice a ve Španělsku. Dalším cílem této práce je optimalizace stávajících metod pro detekci těchto patogenů, aby se tyto metody staly efektivnějšími a dostupnějšími pro jejich širší využití.

Kapitola 1 se zabývá vůbec prvním monitorováním patogenu *Bsal* v České republice, a to jak u volně žijících populací, tak u obojživelníků chovaných v zajetí. Celkem bylo odebráno 324 vzorků včetně 18 stěrů z největšího druhu obojživelníka, velemloka čínského (*Andrias davidianus*), chovaného v pražské zoologické zahradě. Na základě tohoto monitoringu nebyl patogen *Bsal* v České republice sice zjištěn, nicméně jeho výskyt samozřejmě vyloučený není. Příbuzný druh *Bd* byl detekován u tří jedinců čolka obecného (*Lissotriton vulgaris*) z volné přírody a dále u jednoho žebrovníka Waltlova (*Pleurodeles waltl*) chovaného v zajetí.

Kapitola 2 se zaměřuje na monitoring patogenu Bsal u obojživelníků chovaných v zajetí. Poté, co bylo zjištěno, že *Bsal* je přítomen na španělském trhu s obojživelníky, byl zahájen monitoring tohoto patogenu. Bylo odebráno celkem 287 vzorků odebraných ze sedmi španělských chovů. V žádném z těchto chovů nebyl

patogen potvrzen. Kromě toho byly podepsány dvě dohody mezi Českou zemědělskou univerzitou v Praze a BIOPARC Valencia a Fundación Oceanogràfic, v rámci kterých byla testována zvířata chovaná těmito institucemi. Opět s negativními výsledky.

Při práci popsané v **kapitole 3** jsem měl tu čest vést mezinárodní projekt zaměřený na provádění rozsáhlého monitorování, kdy bylo analyzováno celkem 1135 vzorků patřících k 10 druhům obojživelníků na 47 lokalitách v 6 evropských zemích. Z toho pět čolků hranatých (*Lissotriton helveticus*) ze severu Španělska bylo pozitivně testováno na *Bsal*. Jednalo se vůbec o první průkaz patogenu ve volné přírodě v této zemi, a poukázalo se tak na důležitost lepšího porozumění přenosu *Bsal* na dlouhou vzdálenost. Kromě toho byl prokázán také patogen *Bd*, a to v 11 vzorcích od 3 druhů mloků (*L. helveticus*, *L. vulgaris* a *Triturus cristatus*) ze Španělska a Černé Hory a 1 čolka *Cynops ensicauda* chovaného v České republice.

Nakonec, v **kapitole 4**, se zkušenostmi a znalostmi o tom, jak obtížné je někdy získat vzorky pro analýzy pomocí stěrů z pokožky odchycených jedinců, byl proveden výzkum s cílem zefektivnit stávající metodiky detekce obou patogenů. Konkrétně šlo o využití environmentální DNA (eDNA), kdy namísto stěrů z pokožky je detekována DNA patogenů z prostředí, kde žijí, typicky z vody. Jako první jsme byli schopni detekovat pomocí eDNA oba patogeny současně. Současně byla rozšířena dosud známá oblast s výskytem *Bsal* o nové lokality. Bylo prokázáno, eDNA je efektivní metodou snižující náklady na monitoring ve srovnání s klasickým přístupem na jednu třetinu. Doufáme, že prostřednictvím spolupráce s regionálními úřady a nevládními organizacemi bude možné zvýšit povědomí o riziku, které přítomnost *Bsal* v této oblasti přináší.

V rámci výzkumu prezentovaného v této disertační práci bylo odebráno celkem více než 2 300 standardních vzorků (stěrů z pokožky) a 47 vzorků vody pro testování eDNA. Monitorování obojživelníků bylo prováděno na obojživelnících ve volné přírodě i obojživelnících chovaných v zajetí (např. zoologické zahrady, soukromí

chovatelé a univerzitní sbírky). Bylo testováno více než 60 lokalit v přírodě a přibližně 20 chovů, v rámci kterých bylo dokumentováno celkem 17 pozitivních vzorků na *Bsal* a 27 pozitivních vzorků na *Bd.* Znalosti o výskytu patogenů i dostupnost optimalizovaných metod by měly pomoci při ochraně obojživelníků v souvislosti s těmito patogeny.

Resumen

Actualmente, estamos experimentando los efectos de una nueva enfermedad infecciosa (por sus siglas en inglés, *emerging infectious disease,* EID). La gente de a pie está dándose cuenta de los efectos de la pérdida de biodiversidad, de cómo nos afecta y lo íntimamente relacionado que está con la creciente preocupación sobre estas nuevas enfermedades emergentes. Por lo tanto, la conservación de especies y el control de enfermedades infecciosas debería ser una tarea esencial, para la cual tanto científicos como políticos tendrían que aunar esfuerzos. Con más del 40% de las especies amenazadas, los anfibios han sufrido un gran declive y ya son el grupo de vertebrados más amenazados del planeta. Una de estas nuevas enfermedades infecciosas, la quitridiomicosis, se ha demostrado que es la mayor causante de este declive que afecta a los anfibios. Esta enfermedad está provocada por dos hongos, llamados quítridos, *Batrachochytrium dendrobatidis* (*Bd*) y *B. salamandrivorans* (*Bsal*).

Los objetivos de esta tesis doctoral están centrados en este último, *Bsal.* En concreto, en delimitar su distribución en Europa y en la República Checa y España en particular. Además, se pretende mejorar los actuales métodos de diagnóstico para hacerlos más accesible y a su vez, reducir su coste.

El **Capítulo 1** aborda el primer monitoreo de *Bsal* dentro de la República Checa, tanto de poblaciones en estado salvaje como de poblaciones en cautividad. Un total de 324 hisopos fueron recogidos, incluyendo 18 muestras de la especie más grande de anfibios que existe, la salamandra gigante de China (*Andrias davidianus*), provenientes del zoo de Praga. El resultado de este estudio pionero sobre *Bsal* en la República Checa, resultó en la ausencia de detección del hongo patógeno. Sin embargo, el otro hongo quítrido causante de quitridiomicosis, *Bd* fue detectado en 3 ejemplares salvajes de tritón común (*Lissotriton vulgaris*) y de un gallipato (*Pleurodeles waltl*) proveniente de un criador particular.

El **Capítulo 2** se centra en los anfibios provenientes de colecciones particulares o en cautividad. Una vez que *Bsal* se había detectado en el mercado español entre particulares, una búsqueda exhaustiva del hongo asesino de salamandras era un paso a seguir lógico y necesario. Esta investigación concluyó con la recogida de 287 muestras, provenientes de 7 criadores o coleccionistas españoles, en las cuáles no se detectó el hongo patógeno. Además, se firmó dos acuerdos de colaboración entre

la Czech University of Life Sciences Prague, BIOPARC Valencia y la Fundación Oceanogràfic, que también proporcionaron animales para ser testados.

En el **Capítulo 3**, tuve el privilegio de liderar un proyecto internacional para realizar un extenso monitoreo en Europa, donde se recogieron 1135 muestras, de más de 10 especies de anfibios, en más de 47 lugares a lo largo de 6 países europeos. En este caso, 5 individuos españoles de tritón palmeado (*Lissotriton helveticus)* fueron positivos para *Bsal,* dando lugar a la primera notificación sobre la presencia de este hongo en territorio español, y poniendo énfasis en la necesidad de estudiar su posible transmisión a larga distancia. Además, también detectamos el otro hongo patógeno, *Bd,* en 11 animales de 3 especies distintas tritón palmeado (*L. helveticus),* tritón común (*L. vulgaris)* y tritón crestado (*Triturus cristatus*) de España y Montenegro y 1 individuo en cautividad de *Cynops ensicauda* proveniente de la República Checa.

Por último, en el **Capítulo 4**, con la experiencia y el conocimiento de cómo de complicado es llegar a conseguir muestras de lugares remotos de poblaciones aisladas de anfibios, se llevó a cabo una investigación para mejorar las metodologías existentes. Con estas mejoras, se ha conseguido detectar por primera vez, los dos patógenos a la vez, *Bd* y *Bsal,* mediante técnicas de ADN ambiental y a su vez consiguiendo que sea tres veces más barato que protocolos similares. Además, se amplió el área de presencia de *Bsal* en España, por lo cual esperamos que la cooperación entre autoridades regionales, organizaciones conservacionistas y asociaciones haga posible una mayor concienciación sobre el peligro de la presencia del hongo asesino de salamandras en nuestro territorio.

En resumen, más de 2300 muestras en anfibios y 47 ecosistemas acuáticos han sido analizados en busca de la enfermedad quitridiomicosis. Los muestreos en anfibios han sido llevados a cabo tanto en poblaciones salvajes como en cautividad (zoos, coleccionistas, criadores, colecciones científicas). Así, más de 60 puntos en la naturaleza y 20 colecciones privadas han sido testadas. Estos muestreos han dado lugar a 17 muestras positivas para *Bsal* y 27 para *Bd.* Ahora, una vez que las autoridades locales de las áreas afectadas tienen el conocimiento y la información necesaria es el momento de implementar medidas contra esta enfermedad y realizar planes de conservación para impedir que *Bsal* afecte a nuestras poblaciones de anfibios.

Author's Contributions

This dissertation has gathered the work of my Ph.D. It includes four scientific contributions (three published papers and one paper submitted). All of them are focused upon looking for the salamander killer fungus, *Batrachochytrium salamandrivorans* in Czech amphibian captive collections (Chapter 1), in Spanish amphibian captive collections (Chapter 2), in the wild around Europe in six countries (Chapter 3), and by detection using an upgraded environmental DNA design (Chapter 4). In three manuscripts (Chapters 2, 3, 4), I am the first author and in my first paper (Chapter 1), I am a middle author. Details regarding my contributions to the work in the manuscripts are described in the following table.

Table 1. Details of the dissertation author's contributions to the manuscripts presented.

Manuscript	Journal	Concept	Funding	Fieldwork	Labwork	Writing
Baláž et al., 2018	**Salamandra**		√		√	√
Lastra González et al., 2019	**Emerging Infectious Diseases**	√	√	√	√	√
Lastra González et al., 2020	**Diseases of Aquatic Organisms**	√	√	√	√	√
Lastra González et al., 2021 (submitted)	**Methods in Ecology and Evolution**	√	√	√	√	√

The different chapters are:

Chapter 1: Baláž, V., Solský, M., **Lastra González, D.,** Havlíková, B., Gallego Zamorano, J., González Sevilleja, C., Torrent, L. and 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.

Chapter 2: Lastra González, D., Baláž, V., Chajma, P. and Vojar, J. (2020). Surveying for Batrachochytrium salamandrivorans presence in Spanish captive collections of amphibians. *Diseases of Aquatic Organisms* 142, 99-103.

Chapter 3: Lastra González, D., Baláž, V., Solský, M., Thumsová, B., Kolenda, K., Najbar, A., Najbar, B., Kautman, M., Chajma, P., Balogová, M. and Vojar, J. (2019). Recent findings of potentially lethal salamander fungus

Batrachochytrium salamandrivorans. *Emerging Infectious Diseases* 25(7), 1416–1418.

Chapter 4: Lastra González, D., Baláž, V., Vojar, J., Chajma, P. (2021). Dual detection of the chytrid fungi *Batrachochytrium* spp. with an enhanced environmental DNA approach. (submitted)

General Introduction

Nowadays, we are experiencing the first world pandemic of the 21st century. It has been widely discussed that one of the factors leading to this grave situation is connected with the loss of biodiversity (Corlett et al., 2020). Several decades ago, many researchers started to warn about a massive extinction and the decline of many species all across the Earth (Wake and Vredenburg, 2008). These extinction rates are similar to, or even more severe than were, those of the previous five mass extinctions (McCallum, 2015), which means we are currently passing through the sixth mass extinction (Wake and Vredenburg, 2008). Many reasons have been put forward, but there is no doubt that extinction rates have reached levels thousands of times greater than the natural rate (Ceballos et al., 2015).

According to IUCN, the most endangered group of vertebrates consists of the amphibians. Of the more than 7,500 known amphibian species, about 41% have been classified as threatened (IUCN 2020) and roughly 18% as endangered or critically endangered (AmphibiaWeb 2020).

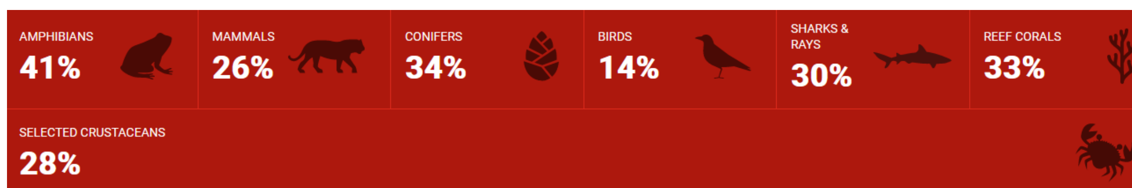

Figure 1. Percentages of endangered animal groups. Source: iucnredlist.org.

Amphibians have existed on Earth for over 300 million years, but in just recent decades there has been an alarming number of extinctions (Houlahan et al., 2000; Stuart et al., 2004). The most up-to-date report about the massive extinction affecting amphibians calculated that over the past half-century at least 501 species of amphibians suffered severe decline and 90 are presumed to have become extinct (Scheele et al., 2019).

Among the main reasons for amphibian decline are:

1- Habitat alteration and overexploitation
2- Global climate change
3- Pollution
4- Invasive species
5- Emerging infectious diseases

(Collins and Storfer, 2003; Stuart et al., 2004; Murray et al., 2011).

At the same time, the amphibian trade and emerging infectious diseases are adding urgency to this conservation concern (Fisher and Garner, 2007).

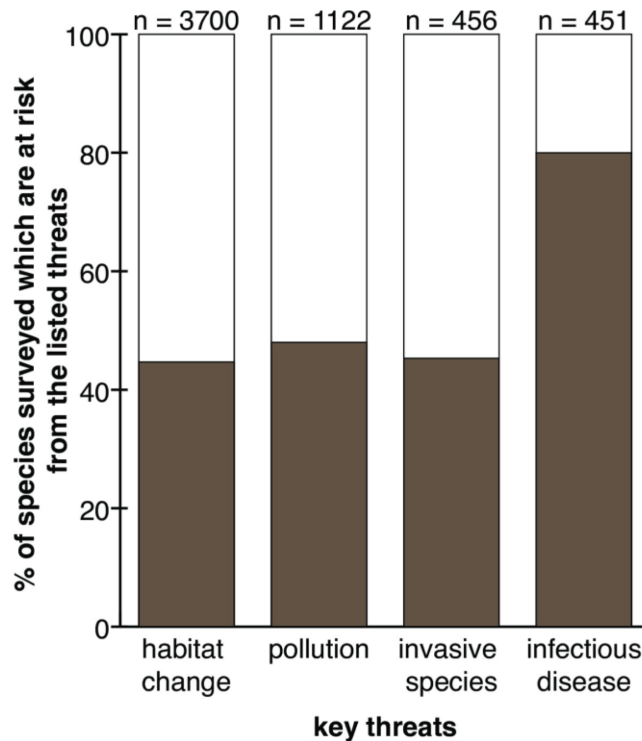

Figure 2. Threats to amphibians. Adapted from Chanson et al. (2008) and Chambouvet et al. (2020).

1- Changes in their habitats dramatically affect the survival of species for obvious reasons, provoke extinctions (e.g., *Atelopus guajano*, IUCN, 2020), prevent dispersal and impede completion of the life cycle (e.g., Wind, 1999), and/or increase the risk of suffering body abnormalities (Soto-Rojas et al., 2017).

2- Amphibians are greatly susceptible to the global climate change because of their ecology. Changes in precipitation, UV radiation, and temperature patterns cause problems of reproduction and physiology while altering the water areas linked to amphibians (Blaustein et al., 2003; Araújo et al., 2006; Hayes et al., 2010). In fact, salamanders are considered the group most vulnerable to climate change (Catenazzi, 2016).

3- Another factor is pollution. It should be highlighted that in comparison with other vertebrates, the skin of amphibians is vital for their

homeostasis, and any pollutant's presence in the habitat will have special relevance. Herbicides like atrazine (causing feminization of male individuals and inhibiting the development of breeding behavior (Hayes et al., 2010) and especially glyphosate, which leads to population declines (Relyea 2005), polychlorinated biphenyls (PCBs) or pesticides (malathion) have been proven to affect the amphibians' populations (Relyea 2011).

4- Additionally, and with increasingly stronger influence, invasive species comprise a factor changing amphibian populations. Invasive species have arrived in various ways, but directly or indirectly these invasions are intermediated by human actions (Stuart et al., 2004; Rowley et al., 2016). Invasive species are able to adjust themselves to new conditions better than are native species (Ficetola et al., 2007). The most common examples are the cane toad (*Rhinella marina*) and the American bullfrog (*Lithobates catesbeianus*), which directly predate native frog species and have cause decline in numerous frog populations (Kats and Ferrer 2003). Non-amphibian species, especially fish, also negatively affect amphibians. Well studied is the case of *Rana muscosa* in the USA, whose populations have declined due to an invasive trout that feeds on amphibian eggs and larvae (Knapp and Matthews, 2000). Less straightforward consequences of invasive species are seen in genetic pollution (e.g., alien species *Triturus carnifex* hybridizing with native *Triturus cristatus*; Beebee and Griffiths, 2005). Lastly, and so as to focus upon the decline factor more related to the scope of this dissertation, invasive species can bring a new pathogen to a healthy population in addition to affecting the local environment (Hayes et al., 2010).

5- As mentioned above, emerging infectious disease is today one of the most important factors influencing amphibian decline (Blaustein et al., 2018). Undoubtedly, diseases always have been a part of ecosystems, but the current rates are abnormally high (Picco and Collins, 2008). Multiple infectious diseases with severe impacts on biodiversity have emerged and have been recognized on a global scale. Worldwide recognized examples among different vertebrate groups are the spring viraemia of carp (Ahne et al., 2002), the *Suttonella ornithocola* bacterial infections in parid birds (Foster et al., 2005), the bat fungal disease called white-nose syndrome (Gargas et al., 2009), snake fungal disease (Allender et al., 2015), and the

ranaviruses (Rafferty, 1965). Amphibians have become the canaries in the coal mine within this pathogen-mediated crisis (Scheele et al., 2019), with chytridiomycosis being the main culprit in their decline (Berger et al., 1998). This disease is caused by two chytrid fungi species: *Batrachochytrium dendrobatidis* (*Bd*) (Longcore et al., 1999) and *B. salamandrivorans* (*Bsal*) (Martel et al., 2013).

Diseases in amphibians

Amphibians certainly suffer from a wide range of infectious diseases, and it definitely is not the scope of this dissertation to make a thorough review of them all. Nonetheless, some of them are related to or provoke coinfections with the chytrid fungi, the pathogen targets of this thesis. Thus, I would like to provide to the tribunal and the reviewers of this text (or to whomever is brave enough to go through research in such an important but positively not entertaining topic) a brief presentation of major diseases affecting amphibians.

Several agents can act as pathogens to amphibians. Luckily, not all of them are able to cause symptomatology in the animals or have clinical significance, that is to say that animals carry on a common behavior. The different groups presented here will be presented in order of their importance, from those less affecting amphibians to the most important causative agents of amphibian diseases.

1. Metazoa

Typically, many parasites belonging to this kingdom affect amphibians, but just a few become infectious (Koprivnikar et al., 2012). An example would be the trematode lungworms belonging to the genus *Rhabdias* that are able to infect wild amphibians. Nonetheless, the amphibians are able to host them without developing disease (Pessier et al., 2018). Sometimes, it could occur that, due to a high number of the metacercariae (i.e., the encysted larva of trematode parasites) in certain parts of the body (e.g., head), skin nodules may form and interfere in the animal's basic functions, such as feeding (e.g., *Clinostomum marginatum* in *Notophthalmus viridescens)* (Perpinan et al., 2010). Encysted trematode larvae of *Ribeiroia ondatrae* are a frequent cause of polymelia (malformation) in free-ranging anurans from the United States of America (USA), and the prevalence of infection is enhanced by environmental eutrophication (Rohr et al., 2008). Malformations are attributed to disruption of the embryonic limb bud by the encysting metacercaria.

Other metazoan parasites are the myxozoans, which target aquatic invertebrates as final host but need amphibians (or fish) as an intermediate host. Organisms in the genera *Chloromyxum*, *Sphaerospora,* and *Hoferellus* inhabit renal tubules and glomeruli of anurans and caudates. In particular, the genus *Sphaerospora* has been found in Brno and surroundings (Jirků et al., 2007). The best-known related infection is Frog kidney enlargement disease in wild-caught African hyperoliid frogs caused by *Hoferellus anurae* (Mutschmann, 2004).

All previous parasites mentioned above are endoparasites. In addition, there are some metazoan ectoparasites that rarely are going to provoke mortalities but are nevertheless noteworthy. For example, myiasis caused by larvae of several species of dipteran flies from such genera as *Notochaeta*, *Batrachomyia*, and *Bufolucillia* occurs in the subcutaneous lymph sacs or nasal cavities of anurans worldwide (Pessier et al., 2018).

2. Bacteria

2.1 Bacteria causing Red leg syndrome

In the past, this disease was thought to be exclusively triggered by *Aeromonas hydrophila*. Subsequent research found that many other bacteria were present in affected animals (e.g., *Pseudomonas aeruginosa, Streptococcus iniae*, and others) (Schadich and Cole, 2010). Symptoms are similar to those of ranavirosis (see below), and it has been discussed that bacteria could be a secondary infection after animals are affected by *Ranavirus* infection (Green et al., 2002).

2.2 Genus *Chlamydia*

These Gram-negative bacteria have been found in anurans as well as in caudates. Two species have been recognized as causative agents of infection in amphibians: *C. psittaci* and *C. pneumoniae* (Berger et al., 1999). These undoubtedly are more relevant due to their effects on humans than for their relevance as pathogenic in amphibians. In fact, a survey carried out in Switzerland by Blumer et al. (2007) found high prevalence of these bacteria in amphibians and they could serve as reservoirs of these pathogens. Nonspecific lesions have been described, and if any symptom is most common it is abnormal shedding of the skin.

3. Protozoa

As true of other groups mentioned in this report, protozoa are not clinically significant unless the organisms are invasive and present in very high numbers or a disease cannot be directly associated with a specific pathogen. Recent work has consistently demonstrated that a wide range of protists of the superphylum Alveolata infects the tissues of larval amphibians (Davis et al., 2007; Jirků et al., 2009; Chambouvet et al., 2016). The alveolates include many organisms like Apicomplexa, chrompodellids, Perkinsozoa, dinoflagellates, and Ciliophora (i.e., ciliates). In some cases, a link with disease has been identified, although formal confirmation equivalent to fulfillment of Koch's postulates is lacking (Chambouvet et al., 2020). Koch's postulates are traditionally the requirements needed to prove that a pathogen is the causative agent of a disease.

3.1 Apicomplexa

3.1.1 Coccidia. Approximately 50 species of the apicomplexan genus *Goussia* have been described infecting a range of hosts, including marine fish and amphibian species (e.g., *Pelophylax* spp., *Rana dalmatina*, *R. temporaria*, *Bufo bufo*, and *Hyperolius viridiflavus*) (Jirků et al., 2009).

3.1.2 Gregarines. These apicomplexan parasites infect tadpoles, and they are represented by *Nematopsis temporariae* (Chambouvet et al., 2016). Gregarines are known to inhabit the intestine and other extracellular spaces of nearly every major group of invertebrates but were thought to be absent from vertebrates. In 2016, this pathogen was detected in Brno and surroundings in the macrophages of tadpoles of *R. dalmatina*, *R. temporaria*, and *Hyla arborea*.

3.2 Perkinsozoans

Perkinsozoa were traditionally thought of as comprising a marine group that infects mollusks or dinoflagellate microalgae (Chambouvet et al., 2015). Marine members of this group have been classified as "emerging disease parasites" and the World Organisation for Animal Health has included the bivalve parasites *Perkinsus marinus* and *P. olseni* in its list of notifiable diseases (OIE, 2019).

The agent of severe Perkinsozoa infection has been primarily identified in tadpoles, although there are some reports of infection in adults (Landsberg et al., 2013). Infection by Perkinsozoa is now considered an emerging disease and has been implied as responsible for die-offs of tadpoles throughout the USA, including

populations of endangered species (Davis et al., 2007), and of tropical anuran tadpoles (Isidoro-Ayza et al., 2017).

3.3 Organisms in the protistan class Mesomycetozoea

Previously classified either as protozoa or fungi, these organisms can be found in both marine and freshwater environments as commensals or parasites of invertebrates, fish, amphibians, as well as birds and mammals (e.g., *Rhinosporidium seeberi*) (Pessier et al., 2018).

The lifecycle of amphibian mesomycetozoans, which have been reported in wild anurans and caudates from North and South America, Europe, and Africa, is quite unknown. Recently, new amphibian mesomycetozoan species from the order Dermocystida have been described and revised. Previously, these parasites were placed within the genera *Dermosporidium*, *Dermocystidium,* or *Dermomycoides*. Now they are placed within *Amphibiocystidium* spp., *Amphibiothecum* spp. (Pascolini et al., 2003; Pereira et al., 2005; Borteiro et al., 2018), and a new species of *Rhinosporidium* spp. (Scheid et al., 2015).

Unlike other pathogens associated with amphibians, these organisms are widely dispersed within Europe (González-Hernández et al., 2010; Courtois et al., 2013; Fiegna et al., 2016; Martínez-Silvestre et al., 2017; among others). Interestingly, one of the first reports in the world about mesomycetozoans was in the Czech Republic, at Hradec Kralové (Broz and Privora, 1952), and to date no more studies have been reported here. Another noteworthy fact about these pathogens is that they have been found in co-infection with chytrids (Borteiro et al., 2014; Ayres et al., 2020). Considering their potential combined effects, it is imperative that this be a subject of future research. During fieldwork, our research team found individuals with lesions compatible with these pathogens. Further research is scheduled to confirm these findings (Lastra González et al., unpublished results).

4. Viruses

As stated above, ranaviruses are, together with chytrid fungi, the main characters responsible for the world amphibian decline. Additional viruses, however, are playing at least secondary roles. Examples include rhabdovirus that causes spring viremia in carp (mentioned above and which recently was isolated in *Hypselotriton orientalis;* Ip et al., 2016) or the ranid herpesviruses, the latest described of which is

the ranid herpesvirus-3 identified by Origgi et al. (2017). This agent affects *Rana dalmatina* and *R. temporaria*, and it provokes various kinds of skin lesions, the most common being an abnormal growth of the skin surface (epidermal hyperplasia).

Increasingly of concern are the ranaviruses. This genus within the family of iridoviruses is considered notifiable to the World Organisation for Animal Health (OIE). The disease is commonly detected in early metamorphosed frogs and tadpoles (Blaustein et al., 2018) and a number of population declines have been associated with different ranaviruses. These include:

- Common midwife toad virus (CMTV) affecting anurans
- Frog virus 3 (FV3) affecting mainly anurans
- *Ambystoma tigrinum* virus (ATV) affecting mainly salamanders
- Bohle iridovirus (BIV) affecting anurans

A notable and worrisome feature is that some of these viruses can also affect other vertebrates, including fish (FV3 and BIV) and reptiles (FV3). Roughly, the typical lesion associated with ranavirus is skin flushing of certain body parts as result of erythemas and internal hemorrhages (Pessier et al., 2018). Nonetheless, it is possible mistakenly to diagnose the infection as being the bacteria *Aeromonas hydrophila* (Cunningham et al., 1996). This Ph.D. candidate was part of the research group presenting in the "Mitigating single pathogen and co-infections that threaten amphibian biodiversity" symposium at the Zoological Society of London in 2019 extensive research on the presence of ranavirus in Central and East Europe. We have observed coinfection of *B. dendrobatidis* chytrid fungus with ranavirus in a decreasing population of water frogs from the genus *Pelophylax* in Poland. We collected live frogs in the field with lesions indicative of ranavirus infection and confirmed *Bd*. Both pathogens were detected also in Belarus from tissue samples of *Pelophylax* spp. Quite surprisingly, we were unable to detect ranavirus in amphibians from the Czech Republic.

5. Fungi

As stated above, the amphibian's skin is the key factor for the homeostasis of these animals. Therefore, the most significant group of pathogens affecting populations of amphibians worldwide are the fungi. This group of organisms finds perfect living conditions in the amphibian's skin due to the permanently moist and moderate temperatures that amphibians need to survive. It is widely recognized, and summarized in Scheele et al. (2019), that chytridiomycosis (a fungal disease) has

been the cause of decline in populations of more than 500 species. Later on, I will focus on this disease more closely. Nevethelees other fungi, too, are relevant to amphibian health.

Probably the most known fungal disease that is not chytrid related is saprolegniasis. This is caused by infections with Oomycete water molds in the genera *Saprolegnia*, *Aphanomyces,* and *Achlya*. It is observed in both captive and wild larvae and aquatic post-metamorphic amphibians. Mortality of egg masses due to infections with water molds is a concern for amphibian conservation in association with environmental factors, such as ultraviolet radiation (Pessier et al., 2018). In larvae, lesions are often located on the keratinized mouthparts, nostrils, or gills (Berger et al., 2001).

Chytridiomycosis

With amphibian biodiversity already under threat, chytridiomycosis has become the major cause of global amphibian decline (Daszak et al., 2003; Scheele et al., 2019). It has been recognized by the World Organisation for Animal Health (OIE) as an invasive pathogen necessitating surveillance and control (Schloegel et al., 2010).

Chytridiomycosis is caused by chytrid fungi (Berger et al., 1998) of the genus *Batrachochytrium*, described as *B. dendrobatidis* (*Bd*) by Longcore et al. (1999) and the recently discovered *B. salamandrivorans* (*Bsal*) (Martel et al., 2013). The two together are the microorganisms responsible for the general and widely distributed decline of amphibians. Both fungi are being spread in many cases by human activities involving infected amphibians, mainly by the pet trade in such amphibians (Spitzen-van der Sluijs and Zollinger 2010; Cunningham et al., 2015). The emergence of chytridiomycosis has contributed significantly to amphibian population declines in the last 30 years (Berger et al., 1998; Bosch and Martínez-Solano 2006; Fisher et al., 2009; Kilpatrick et al., 2009).

Bd infection has been documented in numerous frog species, some salamander species, and 1 caecilian species (*Typhlonectes* sp.) in captivity (Raphael and Pramuk, 2007) as well as 6 from the wild in Cameroon and Tanzania (Gower et al., 2013).

Bd has been found on all continents where amphibians occur (Fisher et al., 2009), and the fungus constitutes one of the greatest threats to vertebrate biodiversity worldwide (Skerratt et al., 2007; Zippel and Mendelson, 2008). Its origin was uncertain for many years, but recently O'Hanlon et al. (2018) showed that *Bd* is native from the Korean Peninsula. The first recorded death of individuals due to this chytrid fungus, after its description, occurred in Spain near Madrid and at least three species of amphibians – spiny toad (*Bufo spinosus*), fire salamander (*Salamandra salamandra*), and common midwife toad (*Alytes obstetricans*) – have currently suffered mass mortality (Bosch et al., 2001). Previously, mortalities in Australia and Panama had already been linked to chytridiomycosis (Berger et al., 1998).

The presence of *Bd* in the Czech Republic was detected in 2008 (Civiš et al., 2012). In a study by Baláž et al. (2014), prevalence of *Bd* was found to be increasing based on a large dataset. Currently, the incidence of the chytrid fungus *Bd* is recorded at many localities in the Czech Republic. Due, however, to a lack of attention to the presence of these chytrid fungi, its incidence is still undetected in most localities (Civiš et al., 2010). Chytridiomycosis caused by the pathogen *Bd* has been responsible for the extinction of nearly 90 amphibian species, mainly frogs and toads, and the decline of more than 500 species (Scheele et al., 2019).

In contrast, *B. salamandrivorans* was originally detected just in caudate amphibians. Today, it has been detected in anurans, which apparently could be carriers of the disease (Nguyen et al., 2017). In 2010, the Netherlands' fire salamander population experienced a sudden 96% decline. *Bsal* was responsible for collapse of the entire natural population of *Salamandra salamandra* in the Netherlands (Spitzen-van der Sluijs et al., 2013). After the first outbreak in the Netherlands, *Bsal* spread to Belgium, causing outbreaks in 2013 and 2014 and adding another target species in the wild, the alpine newt (*Ichthyosaura alpestris*). Global surveys and infection experiments combined with genetic work have shown that it most likely originated in East Asia (Thailand, Vietnam, Japan, and China), where it does not cause obvious disease (Martel et al., 2014; Laking et al., 2017, Yuan et al., 2018). Similar projects have been carried out at other locations around the world and, to date, only in Europe has caused mass mortalities.

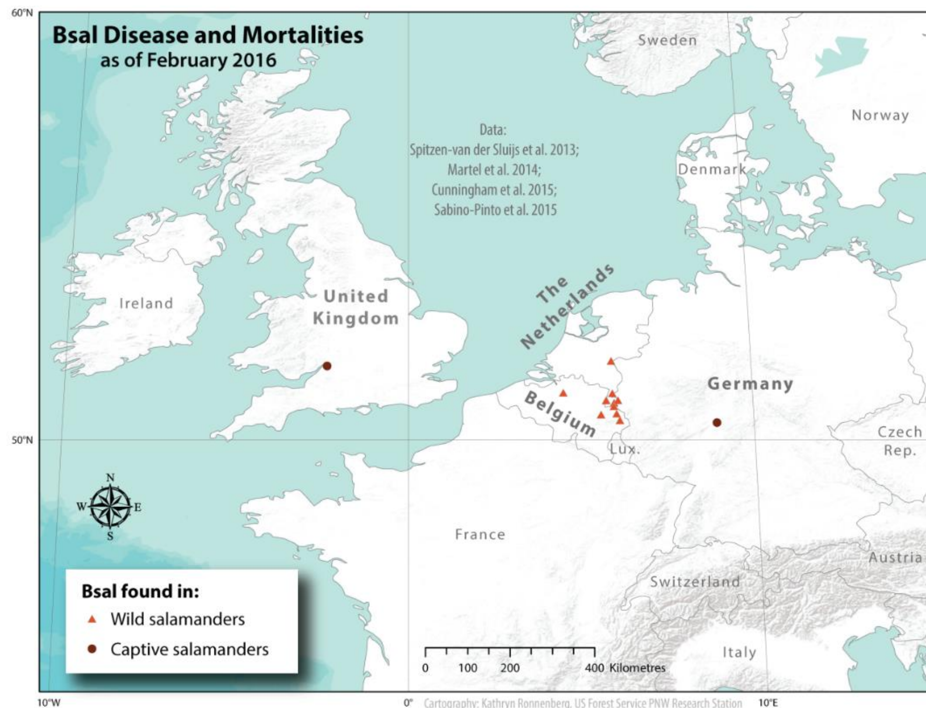

Figure 3. Original *Batrachochytrium salamandrivorans* core area. Source: salamanderfungus.org.

Although it can appear as though the outbreaks are centralized at the border areas of Belgium, the Netherlands, and Germany, (Lötters et al., 2020), there are already more than 50 locations. In addition, findings of infected amphibians in captive collections point to what appears to be the most important way of spreading the fungus (Fitzpatrick et al., 2018). To date, fortunately, preliminary surveys conducted in cooperation with Czech breeders have not found signs of *Bsal* in the Czech Republic (Baláž et al., 2018).

On the other hand, other research of this team conducted in cooperation with institutions from six countries found the first positive in Europe outside of the outbreak area (see Figure 3), in northern Spain, but with no associated mortalities (Lastra González et al., 2019). Thus, dispersal of the pathogen, which was already a complex ecological problem (Stegen et al., 2017), possibly involves birds as a way of spreading or other anthropogenic sources.

The behavior of the disease in caudate amphibians is very complex. Although they are the target of the disease caused by *Bsal*, there are big differences between the different species, with some being more susceptible than others. On the other hand, it is true that, to date, the massive mortalities caused by *Bsal* have been recorded first in fire salamanders (Spitzen-van der Sluijs et al., 2013). The possible existence of resistant species and the mechanisms which might enable resistance comprise a topic in need of deeper research.

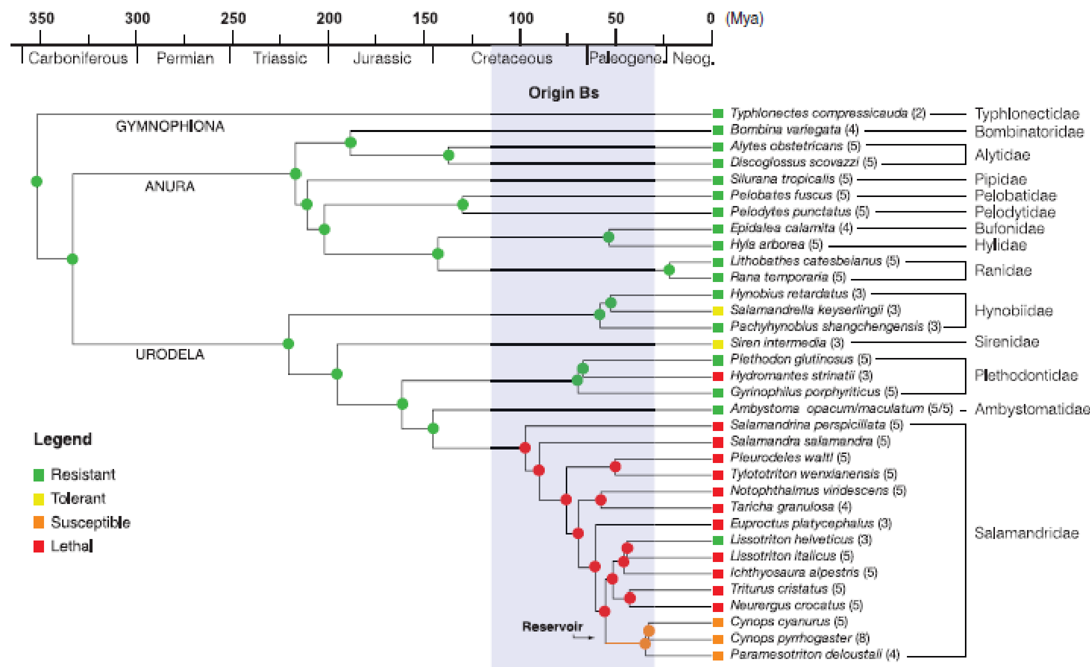

Figure 4. Amphibians ordered by resistance and susceptibility to *Bsal* infection (Martel et al., 2014).

It has been proven, for example, that animals thought to be resistant to the pathogen, like *Lissotriton helveticus* (see Figure 4), in fact are not (Dalbeck et al., 2018; Lastra González et al., 2019). For a better understanding of the figure above, it is necessary to clarify some concepts of susceptibility, tolerance, and resistance to chytridiomycosis. Not all amphibians respond equally to a chytrid infection, and host responses can be roughly categorized as susceptible (infection resulting in disease, either followed by clinical recovery or by mortality), tolerant (persistent infection in absence of disease), or resistant (inhibition or rapid clearance of infection). In the chytrid literature the term resistant (pathogen-inhibiting or pathogen-limiting) is often used for describing species that are actually tolerant (damage-limiting), and definitions may vary according to individual author (Martel et al., 2014).

Especially non-Asian *Salamandridae* seem highly susceptible to *Bsal* (see Figure 4). Although some Asian representatives of the *Salamandridae* family (i.e., *Cynops pyrrhogaster, Cynops cyanurus, Paramesotriton deloustali*) are classified as "susceptible," they are capable of limiting clinical disease (Martel et al., 2014). Tolerant species are able to limit the fitness consequences of infection. Species belonging to this host response category do not succumb to *Bsal* infection either in the wild or under laboratory conditions, although they may be persistently infected. Therefore, they may act as carriers (Martel et al., 2014). Species truly resistant to *Bd*

infection are nevertheless quite few in number. Species resistant to *Bsal* included originally all surveyed anurans and caecilians, as well as several caudate species belonging to the Asian hynobiid, ambystomatid, and North American plethodontid families (Martel et al., 2014; Van Rooij et al., 2015). Some anurans, however, already have been detected to carry *Bsal* (Nguyen et al., 2017; Towe et al., 2020).

Ecology of *Batrachochytrium* genus

To date, the genus *Batrachochytrium* lacks a family name (Incertae sedis). Its members are the unique chytrid fungi known to parasitize vertebrates. Although another parasite of cyprinid fish (*Ichthyochytrium vulgare*) has been identified, *Batrachochytrium spp.* is scientifically recognized as the only known genus that parasitizes vertebrates (Plehn 1916; Červinka et al., 1974; Schäperclaus et al., 1991).

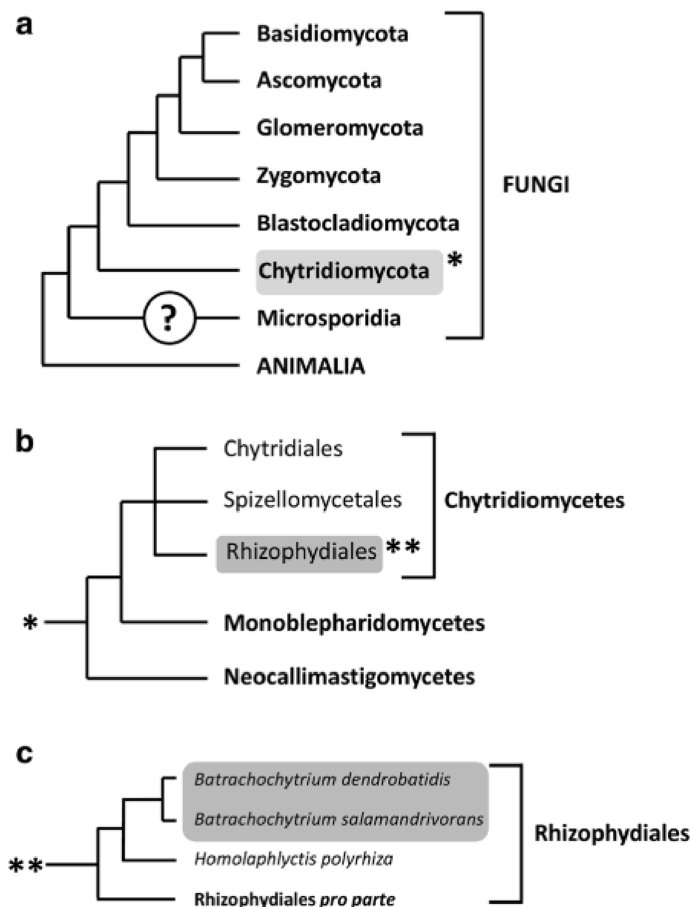

Figure 5. Cladogram tree from Van Rooij et al. (2015) showing topology of the genus *Batrachochytrium* and the taxonomic position of *Bd* and *Bsal*. That position is in (a) the fungal kingdom, (b) the phylum Chytridiomycota, and (c) the order *Rhizophydiales*.

Chytridiomycota have a non-mycelial morphology and a motile-flagellated spore, termed a zoospore. In culture, the zoospores are able to swim only distances up to 2 cm in 24 hours, but they can disseminate more rapidly in swiftly moving water. 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). An aquatic, actively swimming zoospore finds the skin of a host using chemical clues (Moss et al., 2008), encapsulates inside of a living cell of the superficial skin layer, and then grows into a zoosporangium that produces new zoospores (Rosenblum et al., 2010). Zoosporangium size differs between strains, and the most virulent strain has faster reinfection rates (Fisher et al., 2009; Farrer et al., 2011; Voyles 2011). It is important to discriminate between infection by *B. dendrobatidis* and the disease chytridiomycosis. Infection is the state when the fungus is detectable on the host, invades the cells, and eventually proliferates (Voyles et al., 2007). Infection can lead to acute disease accompanied by several clinical signs (Duffus and Cunningham 2010), but it also can remain at subclinical intensity or even disappear (Woodhams et al., 2012).

The complementary part of this chytrid's life cycle, including sexual reproduction and thus genetic hybridization of different lineages, has been evidenced by molecular studies (James et al., 2009; Farrer et al., 2011; McKenzie and Peterson 2012) but has not been observed and seems uncommon. In its life cycle, *Bsal* varies from *Bd* in that there exists an extra stage. It is characterized by the emergence of germ tubes from encysted zoospores, which results in the appearing of novel sporangia and more abundant colonial thalli (Spitzen-Van Der Sluijs 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 an ability to survive in filtered water for up to 30 days (Stegen et al., 2017). As they float, they also can easily adhere to new hosts or, for example, to the feet of waterfowl, which will mediate their distribution into new, non-polluted habitats (Stegen et al., 2017). The preferred temperature for *Bsal* is between 10°C and 15°C, but it grows also at around 5°C and dies at 25°C. Nevertheless, Laking et al. (2017) found *Bsal* in Vietnam at over 26°C. Ulcerations of skin and hemorrhages are typical clinical disease signs in *Bsal* (Mutschmann, 2015; Van Rooij et al., 2015). In the case of *Bd*,

the temperature gradient for growth in culture is from 10°C to 25°C and the optimum temperature is from 17°C to 25°C, with pH 6–7 (Voyles et al., 2012).

A notable feature of *Bd* is the presence of various lineages around the world. The most recent research about this was carried out by Byrne et al. (2019), resulting in a taxonomic revision and the description of new lineages (Figure 6).

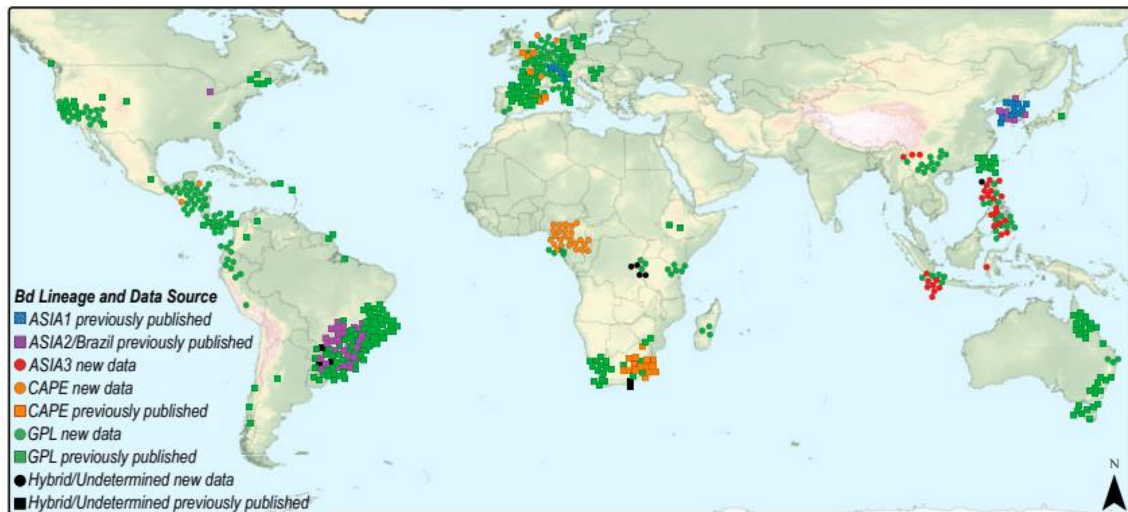

Figure 6. Distribution of *Batrachochytrium dendrobatidis* lineages (Byrne et al., 2019).

Although genomic difference between the *Bsal* isolates have not yet been described, it has been proven that for *Bd* the different virulence of these lineages can make the difference between lethal infections causing mortalities and subclinical infections. For example, it is known that *Bd* Global Panzootic Lineage (GPL) is the most virulent lineage and, even worse, its hybrids (F1) with *Bd* Asia2/Brazil are more virulent still (Byrne et al., 2019). On the other hand, another report from October 2020 relates a contrary effect and points to a possible option for fighting against the chytridiomycosis. In Europe, 90% of *Bd* presence is of *Bd* GPL (Byrne et al., 2019), but some of the isolates of this lineage have shown low virulence and, more importantly, protection against disease after an exposure to either hypervirulent lineage of *Bd* GPL as well as an effect on the infection dynamics of *Bsal* (Greener et al., 2020). Sadly, other studies have tested coinfections with both fungi in laboratory conditions and found a worsening of the disease (Longo et al., 2019). These coinfections already have been detected in the wild (Lötters et al., 2018), and horizontal gene transfer is a frightening scenario that seems likely based upon *Bd's* ability for gene acquisition from oomycete or bacteria (Sun et al., 2011).

The divergence between *Bd* and *Bsal* is dated around 67.3 million years ago. The oldest evidence of *Bsal's* presence was found in a museum specimen of *Cynops ensicauda* from 1861 (Van Rooij et al., 2015). Another difference is that the ploidy of *Bsal* is still unknown. *Bsal* grows well in tryptone-gelatin hydrolysate-lactose predominantly or broth containing peptonized milk, tryptone, and glucose, and it has a lower thermal preference than does *Bd* (Van Rooij et al., 2015). *Bsal* can complete its lifecycle within culture in 5 days at 15°C. Zoosporangia are monocentric (1 thallus = 1 sporangium). Just 8 hours is enough for infection to pass from a sick animal to a clean one. Colonization of the skin can occur within 24 hours and mortality within 2 weeks. Moreover, *Bsal* invades deeper layers of epidermis. (Berger et al., 2015; Van Rooij et al., 2015). One of the latest determined differences between the two fungi regards the sizes of their genomes. While the *Bd* genome occupies 23 Mb, *Bsal* goes to 32 Mb and, even more important, it encodes more than 100 metalloprotease M36 genes (that is three times more than *Bd*) that are thought to degrade host tissue. That is consistent with the typical ulcer symptoms caused by *Bsal* (Farrer, 2019).

Figure 7. *Batrachochytrium salamandrivorans* signs (small black circles) on fire salamander (*Salamandra salamandra*). Photo: Frank Pasmans. Wikimedia Commons (CC0 1.0).

Pathogenesis, signs, and diagnosis

Chytridiomycosis is not known to be associated with internal gross lesions (Van Rooij et al., 2015). Again it is important to discriminate between infection by the fungi and the disease chytridiomycosis. Presence of infection means that one of the fungi is detectable on the host, invades the cells, and eventually proliferates (Voyles et al., 2007). The infection can lead to acute disease with several clinical signs (Duffus and Cunningham 2010), but it also can remain at subclinical intensity or

even disappear (Woodhams et al., 2012). Histopathological evidence most suggestive of *Bsal* infection in the epidermis of salamanders is multifocal epidermal necrosis with loss of distinction between layers of keratinocytes associated with myriad intracellular and extracellular fungal talli (Van Rooij et al., 2015). Many fungal and bacterial pathogens are known to alter both structure and function of the host epidermis and induce changes in water and electrolyte transport by activating or inhibiting ion channels and transporters. Electrolyte transport across the amphibian epidermis is partially accomplished by epithelial sodium channels and sodium/potassium pumps. A study by Campbell et al. (2012) shows that chytrid infection is likely to inhibit epithelial sodium channels, thereby leading to severely reduced sodium absorption through the skin. Low plasma potassium concentrations are linked to abnormal cardiac electrical activity, and cardiac arrest is thought to be the proximate cause of death in diseased amphibians. It is not possible, however, to distinguish *Bd* from *Bsal* histopathologically (Van Rooij et al., 2015). Both fungi are localized to grow on keratinized skin (Longcore et al., 1999; Berger et al., 2006). Infection causes excessive shedding and thus diversely influences exchange of metabolites, minerals, nutrients, and electrolytes (Mutschmann, 2015). Clinical signs are anorexia, ataxia, abnormal body posture, and death (Pessier, 2007). The difference between *Bd* and *Bsal* is that in *Bd* hyperkeratosis and hyperplasia are common side effects on amphibian epidermis, while in *Bsal* amphibian skin is primarily suffering from lesions and focal necrosis (Berger et al., 2005; Martel et al., 2013). *Bd* is present in all forms of amphibians, but it causes greater mortalities on metamorphosed individuals. In adults, it can be found on the outer keratinizing layer of the skin; in tadpoles, it occurs on the external parts around the mouth and eyes and causes depigmentation as a result of the infection (O'Hanlon et al., 2015). Unlike adults, larvae of *S. salamandra* are not affected by *Bsal*. These differences (adult vs larvae) could occur because during metamorphosis the larval epidermis begins to stratify and keratinize. (Berger et al., 2015). Just at metamorphosis the larval epidermis begins to stratify and keratinize, a process that is controlled by the thyroid hormone triiodothyronine (T3). Increased hormone levels during metamorphosis (e.g., T3, corticosteroid hormones) may trigger immune suppression and increased susceptibility to chytrid infection (Rollins-Smith et al., 2011; Thekkiniath et al., 2013).

According to Schmeller et al. (2014), prevalence of *Bd* correlates with the abundance and diversity of aquatic microfauna in mountain lakes. In this particular

case, ciliates and rotifers were found to predate on the aquatic infectious zoospores, thereby reducing the environmental abundance of *Bd*. Besides, zooplankton like water fleas (*Daphnia*) graze on the spores of this chytrid fungus and are known to reduce the risk of infection in aquatic environments (Searle et al., 2013). According to Strauss et al. (2013), variation in the occurrence of *Bd* might also coincide with variation in other biotic factors, including the macroinvertebrate community structure (e.g., midge larvae, dragonflies, water bugs, and snails) and the presence of green algae that interfere with *Bd*, either physically or by allelopathy (the release of secondary metabolites that are detrimental for *Bd*) (Searle et al., 2013). Additional deeper research in this field is necessary to understand the potential against chytridiomycosis of these biotic factors.

In the wild, amphibians are of course exposed to various pathogens as it was briefly exposed above, that include viruses, bacteria, non-chytrid fungi, or helminths that may also cause severe pathology and mortality. In captive amphibians, chytridiomycosis due to *Bd* has been found together with such other pathogens as *Chlamydia pneumoniae*, *Aeromonas hydrophila*, and *Mycobacterium* spp. infection or *Ranavirus* (Reed et al., 2000; Hill et al., 2010; Kik et al., 2012). In the wild, moreover, coinfection with *Bd* and *Ranavirus* has been observed (e.g., Whitfield et al., 2013; Warne et al., 2016). Lately, coinfection with both pathogens, *Bd* and *Bsal*, has been detected (Lötters et al., 2018). To entangle the pathogen interactions even more, a recent case of triple coinfection in a palmate newt (*Lissotriton helveticus*) among *Amphibiocystidium, Ranavirus,* and *Bd* has been detected in northwestern Spain (Ayres et al., 2020). In these cases, it is not clear which pathogen contributes more to the infection or the mortality.

Veterinary treatment

Treatment trials have been conducted with the aim of mitigating the disease in animals. Unfortunately, these procedures are suitable only for individuals in captivity and their application in the wild is limited.

Batrachochytrium dendrobatidis

Bd has been found to decrease under temperature, salt, and antifungal treatments (White, 2006; Garner et al., 2009; Tobler and Schmidt, 2010; Chatfield and Richards-Zawacki, 2011; Geiger et al., 2011). The most effective treatment has been application of the antifungal itraconazole, which removed 100% of the pathogen

from tested individuals (Garner et al., 2009; Tobler and Schmidt, 2010). However, there are possible side effects, such as depigmentation or even death in some species (Pessier and Mendelson, 2011). Another option is temperature treatment (Chatfield and Richards-Zawacki, 2011; Geiger et al., 2011). Tested on both tadpoles and adult individuals, more than 90% of treated individuals were no longer infected with *Bd*. Furthermore, salt treatment in an artificial pond was conducted by White (2006) and it resulted in absence of *Bd* for at least 6 months. Additionally, in the case of this treatment, not all the species can survive the salty environment (White, 2006). According to Bosch et al. (2015), a unique permanent eradication of *Bd* in nature was successfully carried out on Mallorca Island, Spain, and a massive application combining antifungal treatment for tadpoles and environmental chemical disinfection was needed.

Batrachochytrium salamandrivorans

Regarding *Bsal*, exposure to fungus at high temperatures along with fungicidal treatments has proven to be one of the most effective ways of treating amphibians against the disease. Exposure of *S. salamandra* captured from the wild to a temperature of 25°C over a 10–day period resulted in complete elimination of the fungus in 26 specimens (see Figure 8) (Blooi et al., 2015a; Blooi et al., 2015b). In another experiment, the treatment of *S. salamandra* was tried at lower temperature in an effort to achieve the same effective results while reducing the temperature pressure on the animal. For *S. salamandra*, treatment at 20°C for 10 days, together with polymyxin E and voriconazole fungicide application, resulted in complete elimination of *Bsal* infection under laboratory conditions (Blooi et al., 2015b). Drawbacks of this approach include that such high treatment temperatures (20–25°C) may be fatal for some species.

It is important to note that all experiments for *Bsal* treatment were conducted on only one host species (*S. salamandra*). Undoubtedly, more studies are needed to determine species-specific infection dynamics, (Yap et al., 2017). Different infection dynamics of the same pathogen presence already have been seen in a comparison study between *Lissotriton helveticus* and *Triturus cristatus* (Bates et al., 2019). Another possible and less explored treatment would be to manipulate hosts' microbiomes which compete with pathogens for space and resources and, according to Muletz-Wolz et al. (2019), can lead to variation in disease outcome. In relation to host microbial communities, secondary metabolites of some bacteria,

like violacein or prodigiosin, have shown *Bd* and *Bsal* growth inhibition (Woodhams et al., 2017). Unfortunately, it would be challenging to pursue any of these treatments in natural conditions over a vast territory.

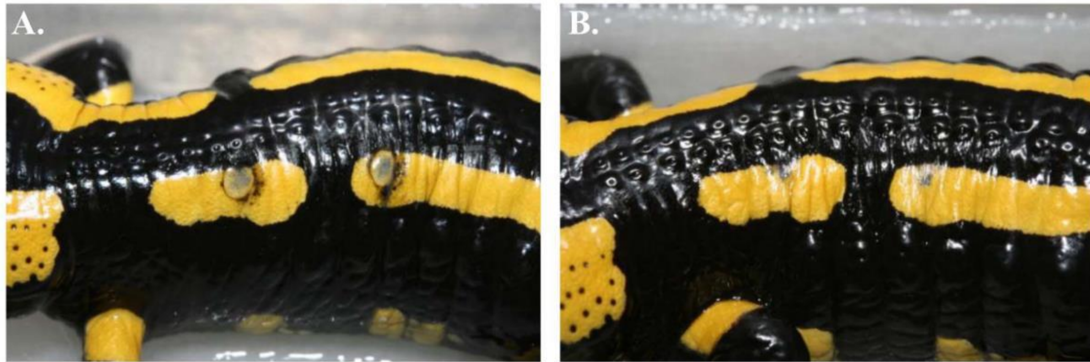

Figure 8. Treatment of *S. salamandra* at 25°C over a 10–day period. (A) infected individual before the treatment with obvious signs of ulceration on skin; (B) recovered individual after the treatment. Figure adopted from Blooi et al. (2015a).

Current status of chytridiomycosis and habitat suitability

The origins of *Batrachochytrium* species have been the subject of long discussion giving rise to two different explanations about how chytridiomycosis emerged. Typically, an emerging infectious disease could be either completely new to an area (novel or spreading pathogen hypothesis, NPH) or it could be endemic and a change in conditions leads to the disease's emergence (endemic pathogen hypothesis, EPH). Alternatively, through time, both theories were accepted and at that time *Bd* geographic origin was still unknown (reviewed in Fisher and Garner 2020). Different studies pointed to possible origin of *Bd* as African (Weldon et al., 2004), Japanese (Goka et al., 2009), East Asian (Bataille et al., 2013), South American (Rodríguez et al., 2014), or North American (Talley et al., 2015). Recently, O'Hanlon et al. (2018) solved the mystery and identified the origin of *Bd* on the Korean Peninsula, based in analysis of 177 isolates. Moreover, this study clarified that the pathogen behavior's matched up with the NPH hypothesis.

As stated above, *Bd* is all over around the world, present on any continent where amphibians dwell, affects more than 1,000 species, and occupies 39% of those field sites analyzed. Nowadays, *Bsal* is the most worrying with regard to its distribution even though it does not yet affect animals in such large numbers as does *Bd*.

Currently, outside of its natural distribution and detection in Vietnam (Laking et al., 2017) and China (Yuan et al., 2018), *Bsal* has been detected in the wild just in the Netherlands, Belgium, Germany, and Spain (Lastra González et al., 2019).

Bsal infections in captive urodelans have been reported in the United Kingdom, Germany, the Netherlands, and Spain (BsalEurope, 2020).

The latest information about contemporary *Bsal*-positive sites confirms a presence in Bavaria, Germany, causing mortality and disease in fire salamanders and alpine newts (Thein et al., 2020; Schmeller et al., 2020). Thus, the distance between *Bsal's* presence and the Czech Lands has been reduced to less than 300 km.

Pathogen suitability

Having discussed the ecology of chytrids and their current distribution, it is useful to mention the suitable areas to which the pathogen could spread. An area suitable for the establishment of *Bsal* will have a combination of the pathogens' requirements and a presence of suitable hosts, together with appropriate environmental conditions.

According to Beukema et al. (2018), Northwestern Europe is highly suited to colonization by the pathogen (see Figure 9).

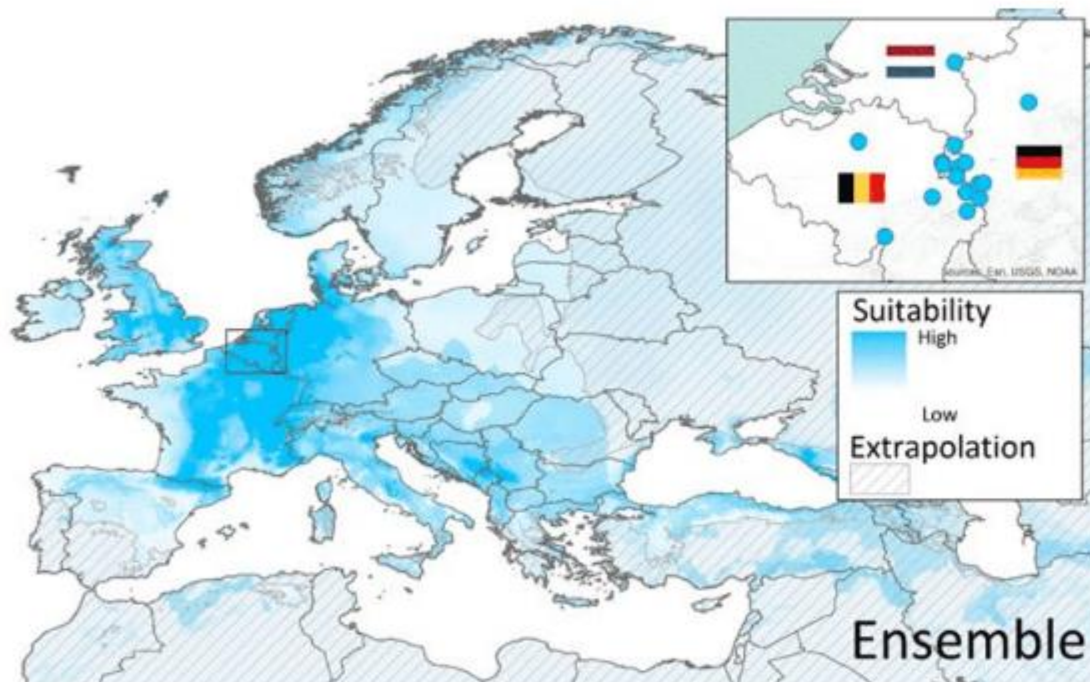

Figure 9. *Batrachochytrium salamandrivorans* suitability according to Beukema et al. (2018).

Nonetheless, it should be pointed out that this map was created prior to the *Bsal* findings in northern Spain and southern Germany. It is likely, therefore, that the areas surrounding these new *Bsal*-positive sites would now have greater chances of being colonized by the fungus.

In different circumstances, other areas not yet colonized by *Bsal* are preparing action plans and assessing the risk of invasion. North America is the world's salamander biodiversity hotspot, with 48% of 676 recognized salamander species representing 9 of the 10 known families within the order Caudata (190 species in the USA, 137 in Mexico, and 21 in Canada (AmphibiaWeb, 2020). For that reason, the two countries with the greatest diversity have developed models to show the possible Bsal colonization. According to Richgels et al. (2016), and as is shown in Figure 10, the most vulnerable areas of the USA against *Bsal* are the Pacific Coast and the Appalachian Mountains.

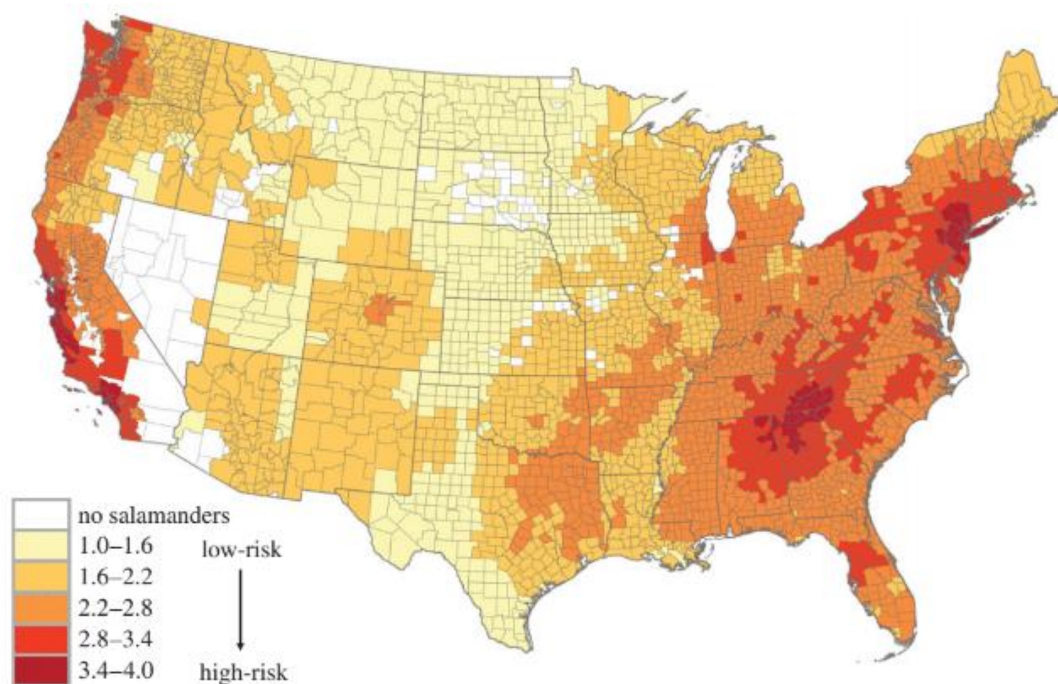

Figure 10. Risk areas of the USA regarding *Bsal* invasion. Adopted from Richgels et al. (2016).

Correspondingly, the other country that is a North American salamander hotspot, Mexico, also demarcated its areas most endangered by *Bsal* invasion. Basanta et al. (2019) found that areas from the Sierra Madre Oriental, Trans-Mexican Volcanic Belt, Sierra Madre del Sur, Mexican Gulf, and Yucatan Peninsula were the most suitable for *Bsal* (see Figure 11).

Figure 11. *Bsal* suitability areas in Mexico. Adopted from Basanta et al. (2019).

Pathogen dispersal, trade, and regulation

Strictly speaking, natural pathogen dispersal is poor and zoospore activity is limited to 2 cm in 24 hours (Mutschmann, 2015). When zoospores are encysted, moreover, they do not swim but remain just floating on the water's surface (Stegen et al., 2017). Thus, the most worrying chytrid fungi spread is the pathogen-mediated dispersal. Several scenarios have been proposed or even tested to explain long-distance pathogen dispersal. Stegen et al. (2017) observed in laboratory experiments how zoospores can attach themselves to bird scales. On the other hand, this trial was not carried out in wild conditions. Another means of spread could be hiking activities or other outdoor activities involving movement of footwear (where zoospores could be present) through *Bsal* confirmed-presence areas (Sachs et al., 2020). In fact, this way of dispersal has been confirmed to play an important role in transmission of Kauri dieback in Kauri trees (*Agathis australis*) in New Zealand, which is originated by the fungus *Phytophthora agathidicida* (Kauri Dieback, 2020). Water sports could also contribute to the translocation of chytrid fungi, as Casais et al. (2019) confirmed for *Ranavirus* in Spain. Moreover, researchers and herpetologists are a dispersal factor. Any stakeholders having direct contact with pathogens must be especially careful with disinfection of their gear and clothes. Implementation of hygiene protocols is a must for any person interacting with the animals (see Gray et al., 2017).

Identically, as stated above, *Bsal* suitability maps would quickly become outdated if a spillover of *Bsal* from a captive collection were to occur. This is

not a recent problem, and it has been suggested as the primary means of introducing *Bsal* to Europe (Martel et al., 2014). Well-known cases of these pathogens' spillover include the spread of ranaviruses through traded bullfrogs (*Lithobates catesbeianus)* (Schloegel et al., 2009) and the movement of ranaviruses and *Bd* in the trade of tiger salamanders (*Ambystoma tigrinum*) as bait (Picco and Collins, 2008). Another confirmed case, and one that is science related, concerns the massive exportation of *Xenopus laevis* during the 20th century, especially for pregnancy tests, which allowed the international spread of *Bd* (Vredenburg et al., 2013). Recently, linked to a reintroduction plan of critically endangered species like *Alytes muletensis,* conservationists from Jersey Zoo unintentionally spread *Bd*CAPE into Mallorca Island in Spain (Doherty-Bone et al., 2019).

Based on the aforementioned cases, it is clear that the enormous global trade in amphibians for the pet trade, consumption, or laboratory experiments affects wild populations by either decreasing host populations or pathogen pollution. Due to globalization, organisms with similar environmental requirements but that have been geographically separated are now being brought together. The lack of effective biosecurity measures allows for pathogens to be transported as stowaways around the world (Franklin et al., 2008), thereby reaching new areas and infecting naïve host populations (Cunningham et al., 2003; Martel et al., 2014; Auliya et al., 2016). Despite an absence of reliable trade data for most species, the volume of animals collected from the wild is large enough potentially to extirpate populations and species (Schlaepfer et al., 2005; Cox et al., 2008; Auliya et al., 2016). Only 3.4% of amphibian species are currently listed in the CITES Appendices or EU Wildlife Trade Regulations Annexes (EFSA, 2017). Non-CITES-listed amphibians are untraceable due to the absence of amphibian specific unique identifier codes in TRACES (the European Union Trade Control and Export System) (Spitzen-van der Sluijs, 2018). That means the majority of trade in amphibians is not regulated (Auliya et al., 2016).

Many species of Caudata from Southeast Asia are threatened due to the worldwide pet trade (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 world's major importer of wild-caught amphibians is the USA. During 2002–2004, legal trade involving more than 26 million individuals was recorded in the USA and included 127 non-native species, probably intended to be used in the pet

trade (Schlaepfer et al., 2005; Jenkins et al., 2007). During 2010–2014, nearly 780,000 live salamanders entered the USA. Of these, about 99% originated from Asia, 98% were species native to Asia, and 91% consisted of species in the genera *Cynops* and *Paramesotriton* (EFSA, 2017). Gray et al. (2015) also reported that in the USA the anuran trade clearly dominates, counting for approximately 94% of the international amphibian trade. As previously mentioned, however, this fact does not diminish the spread of *Bsal*, as these animals can act as carriers (Nguyen et al., 2017; Stegen et al., 2017).

In many European countries (e.g., Austria, Germany, Italy, the Netherlands, Poland, Spain, or the United Kingdom), moreover, it is possible to find online newts from Southeast Asia (Rowley et al., 2016). These are considered to constitute a potential *Bsal* reservoir (Martel et al., 2014). In total, 17 species were found for sale on European Union (EU) websites. Of these, 9 are not included in CITES Appendices and/or EU Wildlife Trade Regulations Annexes and 2 of them (i.e., *Neurergus crocatus* and *Cynops pyrrhogaster*) are susceptible to *Bsal* (UNEP-WCMC, 2016). During 2005–2014, 3,895 salamanders were imported to the EU, 61% for commercial purposes and 24% for scientific purposes. Asian salamanders came from China 65%, Hong Kong 22%, and Japan 9%. Main importers were Germany 79%, the Czech Republic 18%, and Spain 3%, although the data for indirect importations put those proportions at Germany 71%, Spain 23%, and the Czech Republic 5% (UNEP-WCMC, 2016). It is estimated that between 2005 and 2015 around 620,000 individual caudates were imported into the EU (EFSA, 2017).

Full trade data is nevertheless lacking (Yap et al., 2015; Rowley et al., 2016; EFSA, 2017). The CITES database shows that the majority of individuals imported to the EU-28 were traded from unknown sources (EFSA, 2017).

Black market and illegal activities exist as well, such as animal collection within nature reserves from the wild and which subsequently are sold as captive-bred individuals (Auliya et al., 2016; Rowley et al., 2016; EFSA, 2017).

After many expert recommendations calling for a temporary prohibition of amphibian trade (Yap et al., 2015; Rowley et al., 2016; EFSA, 2017; Nguyen et al., 2017) some legal measures were implemented.

In summer 2015, Switzerland temporarily banned the importation of salamanders and newts to protect that country's amphibian native biodiversity (Schmidt, 2016).

The USA, through its U.S Fish and Wildlife Service, implemented in 2016 a ban on trade of 201 salamander species (US Fish and Wildlife Service, 2016). In May 2017, Canada joined the USA and restricted all salamander imports under the law number SOR/2017-86 (Yap et al., 2017). In 2017, *Bsal* was listed in Aquatic Animal Health Code by The World Organisation for Animal Health (commonly referred to as OIE) and a prohibition of trade was issued also in Hungary (Korm. rendelet 199/2017). Later, the European Commission, which already had been under pressure by the Standing Committee of the Bern Convention to implement measures for preventing the introduction and further spread of *Bsal* (Bern Convention 2015; 2017), announced the Decision (EU) 2018/320 in 2018. This decision defined quarantine rules for consignment of salamanders introduced into the EU, the minimum conditions for appropriate establishments of destination, and the examination, sampling, testing, and treatment procedures for *Bsal*.

From the presented background, it should be clear that there are several questions within this topic that remain uninvestigated. In the subsequent chapters, I have endeavored, with the help of many others, to go through some of them.

Chapter 1

In the preceding pages, it was mentioned that the Czech Republic has an important role as importer of Asian salamanders, which are known carriers of the *Bsal* pathogen. According to the United Nations Environment Programme World Conservation Monitoring Centre (UNEP-WCMC), the Czech Republic is the second major importer in Europe. Nothing was known about the pathogen's presence or if *Bsal* was already present in Czech captive amphibian collections. Therefore, here is the pioneer study intended to begin unraveling this situation.

First survey of the pathogenic fungus *Batrachochytrium salamandrivorans* in wild and captive amphibians in the Czech Republic

Vojtech Baláž[1,2], Milič Solský[3], David Lastra González[3], Barbora Havlíková[3], Juan Gallego Zamorano[3], Cristina González Sevilleja[3], Laura Torrent[3] & Jiří Vojar[3]

[1)] Department of Ecology and Diseases of Game, Fish and Bees, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno, Palackého tř. 1/3, 612 42 Brno, Czech Republic

[2)] Department of Biology and Wildlife Diseases, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno, Palackého tř. 1/3, 612 42 Brno, Czech Republic

[3)] Department of Ecology, Faculty of Environmental Sciences, Czech University of Life Sciences Prague, Kamýcká 129, 165 00 Prague-Suchdol, Czech Republic

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Correspondence

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[1] Department of Ecology and Diseases of Game, Fish and Bees, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno, Palackého tř. 1/3, 612 42 Brno, Czech Republic

[2] Department of Biology and Wildlife Diseases, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno, Palackého tř. 1/3, 612 42 Brno, Czech Republic

[3] Department of Ecology, Faculty of Environmental Sciences, Czech University of Life Sciences Prague, Kamýcká 129, 165 21 Prague 6, Czech Republic

Corresponding author: Jiří Vojar, e-mail: vojar@fzp.czu.cz

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The recently discovered fungal pathogen *Batrachochytrium salamandrivorans* (Martel et al. 2013) (hereinafter referred to as *Bsal*) has already received significant scientific and public attention (e.g., Martel et al. 2014, Van Rooij et al. 2015, Yap et al. 2015, Stegen et al. 2017). The *Bsal* epidemic has so far been limited to European newts and salamanders found in the wild (Belgium, Germany and the Netherlands: Martel et al. 2013, Spitzen-van der Sluijs et al. 2016) and in captive populations (Germany: Sabino-Pinto et al. 2015; United Kingdom: Cunningham et al. 2015). In the Netherlands, *Bsal* is responsible for the near extinction of wild fire salamander (*Salamandra salamandra*) populations (Spitzen-van der Sluijs et al. 2013).

The Bern Convention Standing Committee has therefore announced Recommendation No. 176 on the prevention and control of the *Bsal* chytrid fungus. According to this recommendation, European countries should adopt measures that include establishment of monitoring programmes to control the possible further spread of the disease, especially in areas of high risk (e.g., areas near disease outbreaks), and develop emergency action plans that will allow prompt responses in case of *Bsal* occurrence (Council of Europe 2015).

The Czech Republic is a country with relatively high caudate species diversity (Sillero et al. 2014) and shares a western border with Germany, a country with previously proven *Bsal* occurrence (Sabino-Pinto et al. 2015, Spitzen-van der Sluijs et al. 2016). The country, and especially the capital city of Prague, has an active and sizeable community of exotic pet keepers and pet shops, and large exotic pet fairs take place on a regular basis (Havlíková et al. 2015). Furthermore, Prague and its surroundings are known to harbour wild populations of at least four native caudate species: fire salamander, smooth newt (*Lissotriton vulgaris*), alpine newt (*Ichthyosaura alpestris*) and great crested newt (*Triturus cristatus*) (Šťastný et al. 2015). All four are susceptible to *Bsal*-induced mortality (Martel et al. 2013, 2014, Cunningham et al. 2015, Spitzen-van der Sluijs et al. 2016, Stegen et al. 2017). The surrounding areas of such large cities are likely to constitute areas of high risk for wild populations of native amphibians. For this reason, we selected Prague and its surroundings as the first focal area for *Bsal* surveillance efforts in wild populations of Czech caudate amphibians. Considering that *Bsal* is spread through the pet trade in caudates originating in Asia (Martel et al. 2014), we also focused on captive collections of caudate amphibians.

In total, 324 swab samples were tested for both *Batrachochytrium dendrobatidis* (*Bd*) and *Bsal* presence and prevalence. In wild populations, 126 samples of three caudate species (fire salamanders, smooth newts and alpine newts) were collected at nine sites within Prague's urban area and its surroundings during autumn 2015 and spring 2016 (Table 1). Furtheremore, we analysed samples from five captive collections, including those of four private breeders and Prague's zoological garden during the period 2015–2016. Within each collection, only subset of about two to four individuals were sampled from an aquarium. This *Bsal*-targeted sampling in captivity was extended by re-analysing samples of caudates from previous surveillance projects

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Table 1. Numbers (No.) of individuals sampled within nine wild caudate populations in Prague and its surroundings. Date = date of sampling.

Locality name	Coordinates		Species	No.	Date
Podhořský potok, small stream, tributary of Vltava River	50.129947°N	14.404111°E	*Salamandra salamandra*	31	07.10.2015
Únětice, unnamed tributary of Únětický potok	50.144853°N	14.384502°E	*Salamandra salamandra*	25	15.10.2015
Levý Hradec, unnamed tributary of Vltava River	50.169883°N	14.377429°E	*Salamandra salamandra*	12	20.10.2015
Úholičky, unnamed tributary of Podmoráňský potok	50.170698°N	14.344784°E	*Salamandra salamandra*	8	09.11.2015
Lhotecký potok, tributary of Vltava River	49.956059°N	14.411423°E	*Salamandra salamandra*	7	15.10.2015
Chalupecká strouha, near confluence with Zvolský potok	49.930541°N	14.390361°E	*Salamandra salamandra*	1	17.11.2015
Baně, unnamed tributary of Vltava River	49.961229°N	14.392828°E	*Salamandra salamandra*	2	17.11.2015
Ohrobecké údolí, unnamed tributary of Vltava River	49.943775°N	14.413338°E	*Salamandra salamandra*	10	21.10.2015
Botanická zahrada, artificial pond in botanic garden	50.070429°N	14.421077°E	*Lissotriton vulgaris*	28	01.07.2016
Botanická zahrada, artificial pond in botanic garden			*Ichthyosaura alpestris*	2	01.07.2016

searching for *Bd* presence in captive amphibians (Havlíková et al. 2015), including 18 individuals of the largest amphibian species, the Chinese giant salamander (*Andrias davidianus*), reared in Prague's zoological garden. In total, 198 samples of 60 caudate (sub)species were analysed in captive collections (Table 2).

Sampling and DNA extraction were performed according to procedures used in amphibian chytridiomycosis research (Boyle et al. 2004). The first sample subset, consisting of 98 wild and 56 captive samples, was checked for *Bsal* presence by SYBRGreen quantitative polymerase chain reaction (qPCR) following the method described in Blooi et al. (2013) as one possible detection option. Bovine serum albumin (BSA) was added to reduce PCR inhibition (Garland et al. 2010).The identity of amplified DNA was checked by melt curve analysis and compared to results for genomic standards of *Bsal* provided by An Martel (Ghent University). We later adopted the duplex *Bd*+*Bsal* qPCR (Blooi et al. 2013) and used it for additional samples. In this assay, we used genomic standards of *Bd* equivalent to 0.1, 1, 10 and 100 zoospores per 5 µl (strain IA042, Ibon Acherito, Pyrenees, 2004) obtained from the Institute of Zoology, Zoological Society of London. A single quantity sample of *Bsal* genomic DNA was used as a positive control. If any sample showed fluorescence growth in the wavelength of the *Bsal* probe, it would be re-analysed with the full set of *Bsal* standards. In this way, we slightly reduced the cost of analysis. In both detection assays, we used duplicates of all analysed samples, standards, as well as positive and negative controls.

All tested samples yielded negative results for the presence of *Bsal*. *Bd* was detected in three individuals of wild smooth newts and in one reared ribbed newt (*Pleurodeles waltl*) in a captive collection, albeit with no visible signs of the chytridiomycosis. Low *Bd* prevalence in caudates corresponds well with our previous findings in Czech captive collections (Havlíková et al. 2015), and wild populations of caudates in Central and east Europe (Baláž et al. 2014a,b, Vojar et al. 2017).

The 0% *Bsal* prevalence in wild caudates has Sterne-Wald 99% confidence limits of 0.0–4.2%, while in the case of samples from captivity the 99% confidence limits are 0.0–2.6% (Rósza et al. 2000). This does not directly mean that *Bsal* is not present in the Czech Republic. Because the disease outbreaks can occur at very low host densities in wild populations (Schmidt et al. 2017), all host populations of susceptible European caudate species (Martel et al. 2014) are at risk from *Bsal* (Schmidt et al. 2017). In the case of asymptomatic Asian caudates in captive collections, infection may be present in such small prevalence (Martel et al. 2014, Laking et al. 2017) that our sampling was not sufficient. On the other hand, because the intensive sampling of wild fire salamanders covered nearly all localities within Prague where the species presently is known to occur (Šťastný et al. 2015) and no sampled individual exhibited visible disease symptoms, we conclude that *Bsal* probably has not invaded Prague's fire salamander population, at least for now. Similar results of pathogen absence have been found in studies focused on fire salamanders in Austria (Gimeno et al. 2015), eastern hellbenders (*Cryptobranchus alleganiensis*) in the U.S. (Bales et al. 2015), Japanese giant salamanders (*Andrias japonicus*) in Japan (Bletz et al. 2017a), Chinese amphibians (Zhu et al. 2014), five species of newts and fire salamanders in most of tested localities in Belgium, Germany and the Netherlands (Spitzen-van der Sluijs et al. 2016), alpine newts, smooth newts and great crested newts in Germany (Bletz et al. 2017b), and in a study by Parrot et al. (2016) on 17 caudate species across three continents.

We used two available detection assays in our study, both based on DNA amplification with the same pair of *Bsal* primers (Blooi et al. 2013) and differing only in the detection format of the amplicon. The SYBR Green qPCR assay often produced detectable fluorescence growth of nonspecific products, thus complicating interpretation of the results. In several cases, we ran standard PCR followed by gel electrophoresis with samples of equivocal results to confirm the identity of PCR products. Our results indicated a mean melting temperature (Tm) for *Bsal* standards of 77.21°C (SD = 0.29), which differs slightly from the published value of 75.5°C (Blooi et al. 2013). For monitoring *Bsal* presence in wild and captive amphibians, we later adopted and recommend the use of duplex *Bd*+*Bsal* qPCR,

Table 2. List of surveyed species and numbers (No.) of individuals sampled in captivity.

Species	No.	Species	No.
Ambystoma mexicanum	3	*Neurergus derjugina derjugina*	2
Ambystoma tigrinum	2	*Neurergus strauchii barani*	3
Andrias davidianus	18	*Neurergus strauchii strauchii*	3
Calotriton asper	3	*Ommatotriton ophryticus nesterovi*	3
Cynops ensicauda ensicauda	3	*Pachyhynobius shangchengensis*	1
Cynops ensicauda popei	4	*Pachytriton* sp.	2
Cynops orientalis	1	*Paramesotriton caudopunctatus*	7
Cynops pyrrhogaster	3	*Paramesotriton deloustali*	6
Cynops pyrrhogaster "Kanagawa"	6	*Paramesotriton guangxiensis*	4
Cynops pyrrhogaster "Yubana"	2	*Paramesotriton hongkongensis*	3
Euproctus platycephalus	2	*Paramesotriton chinensis*	12
Hynobius dunni	1	*Paramesotriton* sp. "helm"	1
Hynobius leechii	2	*Paramesotriton* sp. "red"	6
Hynobius lichenatus	1	*Paramesotriton yunwensis*	2
Hynobius quelpartensis	2	*Pleurodeles nebulosus*	2
Hynobius retardatus	2	*Pleurodeles waltl*	4
Hypselotriton cyanurus	2	*Salamandra algira tingitana*	2
Hypselotriton cyanurus cyanurus	2	*Siren intermedia*	1
Hypselotriton chenggongensis	3	*Triturus anatolicus*	2
Hypselotriton orientalis	1	*Triturus blasii*	3
Ichthyosaura alpestris	3	*Triturus carnifex*	9
Laotriton laoensis	3	*Triturus cristatus*	2
Lissotriton boscai	3	*Triturus dobrogicus dobrogicus*	2
Lissotriton graecus	3	*Triturus dobrogicus macrosoma*	3
Lissotriton helveticus	3	*Triturus ivanbureschi*	1
Lissotriton italicus	3	*Triturus karelinii*	2
Lissotriton malcani	3	*Triturus macedonicus*	10
Lissotriton meridionalis	3	*Triturus marmoratus*	2
Lissotriton montandoni	3	*Triturus pygmaeus*	3
Neurergus crocatus complex	3	*Tylototriton shanjing*	2

which is designed specifically to detect either of the pathogen species (Blooi et al. 2013). The higher cost of analyses versus using fluorescent probes is counterbalanced by clearer and more specific results.

As not only newts, but also infected anurans and even waterfowl via scales on their feet, may promote fungal spread over large spatial distances (Stegen et al. 2017), the spread of this emerging pathogen is difficult to predict, and we can expect the distribution of *Bsal* to change considerably over time. The risk that new points of entry for *Bsal* into Europe will occur via the pet trade is a constant threat that can be alleviated by collaboration among pet owners, the pet trade, veterinary authorities, and conservationists (Sabino-Pinto et al. 2015). It is essential to prevent this pathogen entering the wild amphibian populations (Cunningham et al. 2015), because there is no effective method to reduce the impact of chytridiomycosis in the field (Garner et al. 2016). Therefore, our next planned steps in the Czech Republic include establishment and issue of biosecurity guidelines for owners of caudates, providing *Bsal* detection in captive collections of amphibians, forming a network of continuously monitored localities in proximity to larger cities, and preparing an action plan in case of *Bsal* occurrence in collaboration with the Nature Conservation Agency of the Czech Republic, the State Veterinary Authority, and the Czech Ministry of Environment.

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Chapter 2

In the section where trade statistics were presented and discussed, it was mentioned that Spain has an important role as importer of Asian salamanders, which are known carriers of the *Bsal* pathogen. According to the United Nations Environment Programme World Conservation Monitoring Centre (UNEP-WCMC), Spain is the third major importer in Europe. Little was known about the pathogen's presence even though *Bsal* already had been found in one Spanish amphibian captive collection linked with the presence of *Bsal* in the UK. Based on the aforementioned, here is the result of a study directed to shedding light on that issue.

Surveying for *Batrachochytrium salamandrivorans* presence in Spanish captive collections of amphibians

David Lastra González[1] Vojtech Baláž[2] Petr Chajma[1] Jiří Vojar[1]

[1)] Department of Ecology, Faculty of Environmental Sciences, Czech University of Life Sciences Prague, Kamýcká 129, 165 00 Prague-Suchdol, Czech Republic

[2)] Department of Ecology and Diseases of Game, Fish and Bees, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno, Palackého tř. 1946/1, 612 42 Brno, Czech Republic

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NOTE

Surveying for *Batrachochytrium salamandrivorans* presence in Spanish captive collections of amphibians

D. Lastra González[1], V. Baláž[2], P. Chajma[1], J. Vojar[1,*]

[1]Department of Ecology, Faculty of Environmental Sciences, Czech University of Life Sciences Prague, Kamýcká 129, 165 00 Prague – Suchdol, Czech Republic

[2]Department of Ecology and Diseases of Game, Fish and Bees, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno, Palackého tř. 1946/1, 612 42 Brno, Czech Republic

ABSTRACT: *Batrachochytrium salamandrivorans* (*Bsal*), a pathogenic fungus causing the fatal disease chytridiomycosis in amphibians, was likely introduced to Europe through the trade in pet salamanders from Asia and then escaped into wild populations. Among European countries, Spain has a large number of private breeders and keepers of pet salamanders, and cases of *Bsal* in wild and captive populations already have been confirmed there. However, surveillance for the pathogen in Spanish collections of amphibians is sparse. Therefore, assisted by private owners and breeders, we surveyed 10 amphibian collections and analysed a total of 317 samples for presence of *Bsal*. All of our analyses yielded negative results. However, this apparent lack of *Bsal* cases in captivity should not encourage relaxation of vigilance, quarantine efforts or good practices. Because amphibian collections represent highly dynamic environments (animals are coming in and out), the pathogen could easily be introduced into a collection by new individuals. Any case of *Bsal* infection in captive animals could lead to its further spread to wild populations of susceptible species, potentially decimating them, and thus should be prevented.

KEY WORDS: Salamander · *Bsal* · Chytridiomycosis · Emerging infectious diseases · Pet keepers

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1. INTRODUCTION

The recently discovered chytrid fungus *Batrachochytrium salamandrivorans* (*Bsal*) has severely impacted many European newts and salamanders (Martel et al. 2013, 2014, Stegen et al. 2017). The first pathogen-related mass mortalities were recorded in wild populations of fire salamanders *Salamandra salamandra* in the Netherlands (Martel et al. 2013, Spitzen-van der Sluijs et al. 2013). Further surveillance in wild populations of caudates has extended our knowledge of *Bsal* presence to Belgium and Germany, mostly in relatively adjacent areas (up to tens of kilometers) from the first disease outbreak (Spitzen-van der Sluijs et al. 2016, Stegen et al. 2017, Lötters et al. 2018). Recently, the fungus was detected in northern Spain, more than 1000 km from the area where *Bsal* was initially detected (Lastra González et al. 2019, Martel et al. 2020). Most recently, the pathogen was found in southern Germany (Bavaria) (Schmeller et al. 2020, Thein et al. 2020).

Thought to be native to Asia, *Bsal* was probably introduced to Europe through the commercial pet trade in salamanders (Martel et al. 2014, Laking et al. 2017). To date, *Bsal* has been identified within private amphibian collections in 5 European countries: Belgium, Germany, the Netherlands, Spain and the UK (Cunningham et al. 2015, Sabino-Pinto et al.

*Corresponding author: vojar@fzp.czu.cz

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2018, EFSA Panel on Animal Health and Welfare et al. 2018). Based on contact-tracking, 16 private amphibian collections epidemiologically linked with the presence of *Bsal* in the UK (Cunningham et al. 2015) have been identified in 4 European countries. Positives for *Bsal* were found in 7 of 11 tested collections, including the first identification of the fungus in 1 of 2 tested collections in Spain (Fitzpatrick et al. 2018).

The 5 European countries with confirmed *Bsal* occurrence are of priority for active surveillance (EFSA Panel on Animal Health and Welfare et al. 2018, Thomas et al. 2019). This applies mainly to countries with widespread trade in amphibians and sizeable communities of exotic pet keepers, such as Germany and Spain. Together with the Czech Republic, these 3 countries are among the main EU importers of live Asiatic Salamandridae (UNEP-WCMC 2016, Baláž et al. 2018). Captive collections can be a dangerous pathway for the spread of *Bsal* into the wild, as has been verified in the Montnegre i el Corredor Natural Park in Catalonia (northeast Spain) (Martel et al. 2020). Surveillance for the pathogen in captive amphibians is nevertheless sparse, and especially in Spain, where, notwithstanding the major role of this country in the trade of amphibians, there has been a lack of specific research focussed upon captive collections. For this reason, we targeted Spanish collections of caudates in conducting the first relatively extensive *Bsal* surveillance there.

2. MATERIALS AND METHODS

2.1. Sampling sites and procedure

Samples were taken from 10 amphibian collections distributed throughout Spain, including 1 private facility where *Bsal*-positives had been found previously (Fitzpatrick et al. 2018) and another 2 with previously known amphibian mortalities compatible with the symptoms of the pathogen. In addition to 8 private collections, 2 institutions dealing with amphibian conservation (BIOPARC® Valencia and Fundación Oceanogràfic de la Comunitat Valenciana) were surveyed. To maintain anonymity of the private owners, the exact locations and information on species composition in their collections are not provided here. A total of 317 animals belonging to 64 amphibian species were sampled between November 2017 and November 2018. These included 16 individuals of 10 anuran species (Table 1), because such animals can serve as reservoirs of *Bsal* (Nguyen et al. 2017, Stegen et al. 2017).

Within each collection, a subset of animals (usually 1 animal per species and aquarium) was swabbed while following the standard procedure for sampling of amphibian chytrid fungi (Hyatt et al. 2007). Each individual was swabbed using 1 sterile dryswab™ (MW113, Medical Wire Equipment). We visited and sampled some of the collections personally. In other

Table 1. Amphibian species swabbed and analysed for detecting *Batrachochytrium salamandrivorans* (*Bsal*). To avoid misunderstandings and maintain anonymity of the breeders, information about subspecies is not included, even though this was available in most cases. N indicates the number of sampled individuals. No sampled individual was positive for *Bsal*

Species	N	Species	N
Anura		**Caudata (continued)**	
Alytes muletensis	3	*Neurergus crocatus*	2
Barbarophyine brongersmai	2	*Neurergus derjugini*	1
Bufotes latastii	1	*Neurergus kaiseri*	7
Sclerophrys mauritanicus	1	*Neurergus strauchii*	3
Bufo spinosus	1	*Ommatotriton ophryticus*	2
Bufotes boulengeri	1	*Pachytriton granulosus*	2
Dendrobates azureus	1	*Paramesotriton caudopunctatus*	2
Hymenochirus sp.	2	*Paramesotriton deloustali*	2
Hyperolius sp.	3	*Pleurodeles nebulosus*	6
Mantella aurantiaca	1	*Pleurodeles waltl*	52
Caudata		*Pseudotriton ruber*	4
Ambystoma mavortium	1	*Salamandra algira*	28
Ambystoma tigrinum	1	*Salamandra atra*	1
Aneides lugubris	2	*Salamandra infraimmaculata*	3
Aneides vagrans	2	*Salamandra salamandra*	31
Cynops cyanurus	4	*Salamandrella keyserlingii*	4
Cynops ensicauda	14	*Siren intermedia*	2
Cynops pyrrhogaster	10	*Taricha granulosa*	2
Desmognathus fuscus	2	*Taricha torosa*	1
Echinotriton andersoni	2	*Triturus anatolicus*	1
Euproctus platycephalus	1	*Triturus carnifex*	16
Euricea guttolineata	1	*Triturus cristatus*	5
Hynobius dunni	3	*Triturus dobrogicus*	19
Hynobius tokyoensis	1	*Triturus ivanbureschi*	5
Hypselotriton orientalis	5	*Triturus karelinii*	3
Ichthyosaura alpestris	12	*Triturus macedonicus*	2
Laotriton laoensis	7	*Triturus marmoratus*	6
Lissotriton boscai	5	*Triturus pygmaeus*	2
Lissotriton helveticus	1	*Tylototriton kweichowensi*	1
Lissotriton italicus	3	*Tylototriton shanjing*	2
Lissotriton vulgaris	1	*Tylototriton verrucosus*	2
Necturus maculosus	1	*Tylototriton yangi*	2
Neurergus cristatus	1		

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cases, the breeders collected the samples and sent them to us by post while following a protocol based on Klocke et al. (2017). Swabs were stored in 1.5 ml Eppendorf tubes with silica gel at −18°C until DNA extraction.

2.2. Laboratory analyses

Genomic DNA was extracted following the protocol of Blooi et al. (2013, 2016). Testing for the presence of *Bsal* was carried out by 2 methods at 2 laboratories with different availability of equipment. All samples were analysed initially at the Czech University of Life Sciences Prague by standard polymerase chain reaction (PCR) with the *Bsal*-specific primers STerF and STerR as used by Martel et al. (2013). Subsequently, electrophoresis was carried out on the amplified target. Two samples that produced equivocal results in standard PCR were then re-analysed by duplex qPCR for *Bsal* and the related chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) at the University of Veterinary and Pharmaceutical Sciences Brno. All analyses were run in duplicate with negative and positive controls (PCR) or with quantification standards (100, 10, 1, 0.1 genome equivalents) in each run (qPCR).

3. RESULTS

Bsal was not detected in any of the samples taken from all 10 collections, including a collection in which the pathogen was present during 2015−2016 (Fitzpatrick et al. 2018). No signs of the disease were observed in any collection at the time of sampling. In the cases of 2 samples with equivocal results from PCR, several repetitions of analyses by qPCR showed no indication of *Bsal* positives.

4. DISCUSSION

4.1. Statistical analyses

Because of the lack of *Bsal* presence in our set of samples, no statistics were used for estimating *Bsal* prevalence. While it certainly is possible to calculate Bayesian credible intervals even for populations with no infection (for example, see Lastra González et al. 2019), the probability of us missing an infected individual is low. In amphibian collections, it is reasonable to assume that, due to the specific conditions within which individuals are kept (e.g. aquaria or small containers, frequent handling), infection would occur in more than 1 individual and thus be detected by our sampling method.

4.2. Interpretation of negative results

Despite the finding of no *Bsal* in the surveyed Spanish collections, the results do not confirm absence with certainty that the pathogen is not or has not been there. There are several possible reasons why the fungus could go undetected despite its past or even current presence in a collection. First, such collections are highly dynamic, as the keepers change the number of individuals kept and the species composition over time. The pathogen could easily be introduced into a collection by new individuals when preventive measures are not applied, or it could be quickly eradicated by proper treatments (EFSA Panel on Animal Health and Welfare et al. 2018). Second, we cannot be sure that all animals were made available for sampling, as some keepers might intentionally have avoided sampling individuals showing symptoms to avoid being labelled as '*Bsal*-positive' breeders and suffer from loss of reputation. Given these circumstances, new approaches should be implemented. Regular mandatory check-ups (as is common in some domestic animals) may provide better results in preventing spread of the disease. Another solution would be to improve accessibility of facilities and services for pathogen detection and disease treatment so that a proactive attitude against *Bsal* is not economically demotivating to non-commercial and hobby breeders.

4.3. Captive collections as reservoirs of *Bsal*

Although our sampling was quite extensive, only a part of all Spanish amphibian collections was sampled. Spain has a sizeable community of amphibian keepers (UNEP-WCMC 2016) and is one where the presence of *Bsal* has already been reported in at least 2 amphibian collections (Fitzpatrick et al. 2018, Martel et al. 2020). Apparently, occurrence of the pathogen within European private collections of amphibians is not rare (see Section 1), and the movement of amphibians, including both legal and illegal interchanges and trade, can easily spread *Bsal* over long distances into new areas (Lötters et al. 2020).

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Current regulations of the European Commission (Decision 2018/320, European Commission 2018) do not completely mitigate the risk of a *Bsal* spillover from captive collections. The regulations do not allow access to private collections if needed for epidemiological tracing and control of biosecurity measures applied there (Martel et al. 2020). The implementation of precautionary measures within private collections is strongly affected by the attitudes of individual breeders. Perhaps certification or classification of collections and breeders could be useful in order to increase 'safe trade' or 'quality trade', where the prestige of having a seal of quality would allow breeders to benefit if they fulfil safety measures or avoid the importation of susceptible species. We would like to highlight the important role of pet keepers in the management of emerging infectious diseases. Only close cooperation between amphibian enthusiasts, breeders, traders, researchers and policy makers can mitigate the risks that *Bsal* poses to captive and wild populations of susceptible salamanders and newts.

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Chapter 3

At the beginning, *Bsal*-affected areas were restricted only to the Netherlands, Belgium, and Germany (see above, Figure 3). On the other hand, according to the pathogen's ecology and preferences as described in that section and the suitability maps (see Figure 9), other amphibian areas could be at risk. Reported in this chapter are the first *Bsal*-positive finding in the wild in Spain as part of a huge monitoring project across six European countries.

Recent findings of potentially lethal salamander fungus *Batrachochytrium salamandrivorans*

David Lastra González, Vojtech Baláž, Milič Solský, Barbora Thumsová, Krzysztof Kolenda, Anna Najbar, Bartłomiej Najbar, Matej Kautman, Petr Chajma, Monika Balogová, Jiří Vojar.

Czech University of Life Sciences Prague, Prague, Czech Republic (D. Lastra González, M. Solský, B. Thumsová, P. Chajma, J. Vojar)

University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic (V. Baláž, M. Kautman)

University of Wrocławski, Wroclaw, Poland (K. Kolenda, A. Najbar)

University of Zielona Góra, Lubuskie, Poland (B. Najbar)

Slovak Academy of Sciences, Košice, Slovakia (M. Kautman)

Pavol Jozef Šafárik University in Košice, Slovakia (M. Balogová)

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Address for correspondence: Fang Huang, Beijing Center for Disease Prevention and Control, Institute for Communicable Disease Control and Prevention, No. 16, Hepingli Middle Av, Dongcheng District, Beijing 100013, China; email: hhffxdd@126.com

Recent Findings of Potentially Lethal Salamander Fungus *Batrachochytrium salamandrivorans*

David Lastra González, Vojtech Baláž, Milič Solský, Barbora Thumsová, Krzysztof Kolenda, Anna Najbar, Bartłomiej Najbar, Matej Kautman, Petr Chajma, Monika Balogová, Jiří Vojar

Author affiliations: Czech University of Life Sciences, Prague, Czech Republic (D. Lastra González, M. Solský, B. Thumsová, P. Chajma, J. Vojar); University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic (V. Baláž, M. Kautman); University of Wrocławski, Wroclaw, Poland (K. Kolenda, A. Najbar); University of Zielona Góra, Lubuskie, Poland (B. Najbar); Slovak Academy of Sciences, Košice, Slovakia (M. Kautman); Pavol Jozef Šafárik University in Košice, Košice (M. Balogová)

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The distribution of the chytrid fungus *Batrachochytrium salamandrivorans* continues to expand in Europe. During 2014–2018, we collected 1,135 samples from salamanders and newts in 6 countries in Europe. We identified 5 cases of *B. salamandrivorans* in a wild population in Spain but none in central Europe or the Balkan Peninsula.

Chytridiomycosis, an amphibian disease caused by the chytrid fungi *Batrachochytrium dendrobatidis* and *B. salamandrivorans*, is responsible for declines of amphibian populations worldwide (*1*). The recently discovered *B. salamandrivorans* (*2*) is severely impacting salamanders and newts in Europe (*3,4*). This emerging fungal pathogen infects the skin of caudates and causes lethal lesions (*2*). It most likely was introduced to Europe by the pet salamander trade from Southeast Asia (*3*). In Europe, the Netherlands, Belgium, and Germany have confirmed *B. salamandrivorans* in wild caudates; the United Kingdom, Germany, and Spain have confirmed the fungus in captive animals (*5,6*). Several countries have established trade regulations (*5*) and a recent European Union decision, no. 2018/320, implements measures to protect against the spread of *B. salamandrivorans* via traded salamanders (*7*). The World Organisation for Animal Health listed infection with *B. salamandrivorans* as a notifiable disease in 2017. In addition to controlling the amphibian pet trade, surveillance of the pathogen is urgently needed to establish disease intervention strategies in affected areas and prevention in *B. salamandrivorans*–free regions.

During 2014–2018, we collected 1,135 samples directly for the detection of *B. salamandrivorans* or as a part of unrelated studies. Samples came from 10 amphibian species at 47 sites in 6 countries in Europe. Most samples came from the fire salamander, *Salamandra salamandra*, which is a known suitable host for *B. salamandrivorans* (*3*), and the palmate newt, *Lissotriton helveticus*, which is known to be resistant to *B. salamandrivorans* (Appendix Table 1, http://wwwnc.cdc.gov/EID/article/25/7/18-1001-App1.pdf).

Most samples were skin swabs collected by following the standard procedure for sampling of amphibian chytrid fungi (*8*). A smaller portion of samples was toe clippings (Appendix Table 2). We extracted genomic DNA following the protocol of Blooi et al. (*9*), and 2 laboratories with different equipment tested for *B. salamandrivorans*. Samples from Spain and the Czech Republic initially were analyzed at the Czech University of Life Sciences (Prague, Czech

Republic) by standard PCR with *B. salamandrivorans*–specific primers STerF and STerR, as described by Martel et al. (*2*), with subsequent electrophoresis on the amplified target. We reanalyzed samples that produced positive or equivocal results by using duplex quantitative PCR (qPCR) for *B. dendrobatidis* and *B. salamandrivorans* (*9*) at the University of Veterinary and Pharmaceutical Sciences (Brno, Czech Republic). Trenton Garner of the Institute of Zoology, Zoological Society of London (London, England), provided DNA for quantification standards of the *B. dendrobatidis* GPL lineage, strain IA042, and An Martel of Ghent University (Ghent, Belgium) provided quantification standards of *B. salamandrivorans*.

We directly analyzed samples from other countries by qPCR. We used negative and positive controls for standard PCR analyses and quantification standards for qPCR analyses. For *B. dendrobatidis*– or *B. salamandrivorans*–positive sites, we estimated prevalence and Bayesian 95% CIs using 3 parallel Markov chains with 2,000 iterations each, a burn-in of 1,000 iterations, and no thinning (Appendix Table 1). We performed all statistical analyses in R 3.3.1 using the R2WinBUGS package and WinBUGS 1.4.3 (*10*).

Samples from 5 *L. helveticus* newts tested positive for *B. salamandrivorans*, implying that this species is not resistant to this fungus as previously indicated by experimental exposures (*3*). The positive cases were found in populations from an isolated area encompassing 2 different regions in northern Spain, Cantabria and Asturias, with remote human populations. Four cases were found in livestock drinking troughs located 150–1,000 m above sea level, and 1 case was found in a pond in a private garden, 30 km from the nearest recorded case. We did not find *B. salamandrivorans*–positive cases in consecutive locations during our monitoring.

Although *B. salamandrivorans* cases have been reported in captive salamanders (*6*), our reported cases were >1,000 km from any area of known *B. salamandrivorans* occurrence (*7*). We also detected *B. dendrobatidis* by duplex qPCR in 11 samples from 3 newt species (*L. helveticus*, *L. vulgaris*, and *Triturus cristatus*) from Spain and Montenegro and 1 captive *Cynops ensicauda* newt from the Czech Republic. The *B. dendrobatidis*–positive cases did not involve co-infection with *B. salamandrivorans*.

We confirmed that the known distribution of *B. salamandrivorans* continues to expand in Europe, indicating that this fungus might be capable of dispersing over long distances (*4*), might be introduced by humans, or might even have been circulating in this geographic range with no detected deaths. Our results should alert the research and conservation community and motivate urgent action to identify regions with early emergence of the disease and implement mitigation measures to prevent further spread of this deadly pathogen.

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About the Author

Mr. Lastra González is a PhD candidate at Czech University of Life Sciences, Prague. His research focuses on amphibian conservation and emerging infectious diseases that affect them.

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Address for correspondence: David Lastra González, Faculty of Environmental Sciences, Czech University of Life Sciences, Kamýcká 129, 165 21 Prague–Suchdol, Czech Republic; email: lastra_gonzalez@fzp.czu.cz

Crimean-Congo Hemorrhagic Fever Virus Genome in Tick from Migratory Bird, Italy

Elisa Mancuso, Luciano Toma, Andrea Polci, Silvio G. d'Alessio, Marco Di Luca, Massimiliano Orsini, Marco Di Domenico, Maurilia Marcacci, Giuseppe Mancini, Fernando Spina, Maria Goffredo, Federica Monaco

Author affiliations: Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale," Teramo, Italy (E. Mancuso, A. Polci, S.G. d'Alessio, M. Orsini, M. Di Domenico, M. Marcacci, G. Mancini, M. Goffredo, F. Monaco); Istituto Superiore di Sanità, Rome, Italy (L. Toma, M. Di Luca); Istituto Superiore per la Protezione e la Ricerca Ambientale, Bologna, Italy (F. Spina)

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We detected Crimean-Congo hemorrhagic fever virus in a *Hyalomma rufipes* nymph collected from a whinchat (*Saxicola rubetra)* on the island of Ventotene in April 2017. Partial genome sequences suggest the virus originated in Africa. Detection of the genome of this virus in Italy confirms its potential dispersion through migratory birds.

Crimean-Congo hemorrhagic fever virus (CCHFV) is a vectorborne virus responsible for severe illness in humans, whereas other mammals usually act as asymptomatic reservoirs. The virus is transmitted through tick bites or by direct contact with blood or body fluids of infected vertebrate hosts. CCHFV, an *Orthonairovirus* within the Nairoviridae family, has a negative-sense tripartite RNA genome characterized by high genetic diversity. The sequences of the circulating strains cluster in 6 genotypes (I–VI) reflecting their geographic origin; worldwide distribution is the result of efficient dispersion through migratory birds, human travelers, and the trade and movement of livestock and wildlife (*1*,*2*). In Europe, CCHFV distribution was limited to the Balkan region until 2010, when the virus was identified in ticks collected from a red deer (*Cervus elaphus*) and, 6 years later, in 2 autochthonous human cases in the same region of Spain (*3*). Sequences from the Iberia strains clustered in the Africa genotype III (*4*), supporting the hypothesis of CCHFV dispersion through ticks hosted by migrating birds.

The role of birds in the potential spread of the virus was confirmed by CCHFV detection in ticks collected from migratory birds in Greece in 2009 (*5*) and Morocco in 2011 (*6*). Because Italy hosts an intense passage of birds migrating along major routes connecting winter quarters in Africa and breeding areas in Europe, the country is potentially exposed to the risk for virus introduction. We report the detection of CCHFV RNA in a tick collected in Italy from a migratory bird.

We conducted tick sampling during March–May 2017 on the island of Ventotene, where a ringing station has been operating since 1988 as part of the Small Islands Project, a large-scale and long-term effort to monitor spring migrations of birds across the central and western Mediterranean. We ringed 5,095 birds and checked ≈80% for ectoparasites. We collected 14 adults, 330 nymphs, and 276 larvae from 268 passerines belonging to 28 species; 18 species were trans-Saharan migrants. We stored ticks in 70% ethanol until morphologic identification and assignment to a genus or, whenever possible, a species (*7*). We then individually

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Recent Findings of Potentially Lethal Salamander Fungus *Batrachochytrium salamandrivorans*

Appendix

Appendix Table 1. Summary of locations, sample size, findings from *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* testing, and prevalence and infection intensities for each*

Location	Species	No.	*Bd*+	*Bsal*+	Bd_{prev} (95% CI)†	$Bsal_{prev}$ (95% CI)†	*Bd* min–max GE	*Bsal* min–max GE
Czech Republic								
Prague	*Cynops ensicauda*	5	1		0.29	0.14	1.91	
					(0.05–0.65)	(0–0.47)		
Montenegro								
Moromish	*Lissotriton vulgaris*	35	4		0.10	0.02	0.28–22.25	
	Triturus cristatuts	22	1		(0.04–0.19)	(0–0.07)	1.05	
Liverovići lake	*L. vulgaris*	31	2		0.09	0.03	1.73–1.83	
					(0.02–0.20)	(0–0.11)		
Spain								
Suances	*L. helveticus*	22		1	‡	0.06		0.42
	T. marmoratus	10				(0.01–0.16)		
Ampuero	*Salamandra salamandra*	9			‡	0.10		
						(0.01-0.26)		
	L. helveticus	10		1				2.73
Teverga	*L. helveticus*	62		2	‡	0.04		0.89–4.36
	T. marmoratus	11				(0.01–0.09)		
Carracedelo	*L. helveticus*	5	1		‡	0.06	0.54	
	T. marmoratus	11				(0–0.20)		
Ruente	*L. helveticus*	50	2	1	‡	0.04	0.24	0.16
						(0–0.10)		

**Bd*, *Batrachochytrium dendrobatidis*; Bd_{prev}, prevalence for Bd; *Bsal*, *B. salamandrivorans*; $Bsal_{prev}$, prevalence for *Bsal*; CI, confidence interval; GE, genomic equivalent; +, positive.
†Calculated using Bayesian probability.
‡Cannot be included because only a subset of the samples were analysed by duplex qPCR.

Appendix Table 2. Locations, species, year, origin, type, and number of samples collected during surveillance for *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans**

Location	Species	Year	Origin	Sample Type	No.	*Bd*+	*Bsal*+
Croatia							
lokva Majkovi	*Lissotriton vulgaris*	2016	W	S	30		
Crna Mlaka	*L. vulgaris*	2016	W	S	1		
	Salamandra salamandra	2016	W	S	1		
Czech Republic							
Kokořínsko	*L. vulgaris*	2017	W	S	44		
Ústí nad labem	*S. salamandra*	2016	W	S	17		
Prague	*Cynops ensicauda*	2017	C	S	5	1	
Montenegro							
Moromish	*L. vulgaris*	2016	W	S	35	4	
	Triturus cristatuts	2016	W	S	22	1	
Liverovići lake	*L. vulgaris*	2016	W	S	31	2	
Lovcén	*L. vulgaris*	2016	W	S	40		
Traktir-Sutorina	*L. vulgaris*	2016	W	S	33		
		2016	W	S	10		
Poland							

Location	Species	Year	Origin	Sample Type	No.	*Bd*+	*Bsal*+
Wąwóz Lipa-Chełmy Landscape Park	*S. salamandra*	2014	W	S	30		
Sady-Ślęża Massif	*S. salamandra*	2014	W	S	9		
		2015	W	S	2		
Złoty Stok-Śnieżnik Landscape Park	*S. salamandra*	2014	W	S	15		
		2015	W	S	3		
Jarnołtówek	*S. salamandra*	2015	W	TC	21		
Bielsko-Biała	*S. salamandra*	2016	W	S	32		
Pleśna	*S. salamandra*	2014	W	TC	4		
		2016	W	TC	30		
Góra Kamińska	*S. salamandra*	2015	W	TC	17		
		2016	W	TC	30		
Rakówka	*S. salamandra*	2015	W	TC	7		
Czarnorzeki	*S. salamandra*	2015	W	TC	24		
Trzciana	*S. salamandra*	2014	W	TC	2		
		2016	W	TC	30		
Sękowiec	*S. salamandra*	2016	W	TC	30		
Southern Otryt	*S. salamandra*	2016	W	TC	18		
Jagiellonian University	*L. vulgaris*	2016	C	S	5		
Slovakia							
Remetské Hámre	*S. salamandra*	2017	W	S	15		
Ruská Bystrá	*S. salamandra*	2017	W	S	10		
Tichá Voda	*S. salamandra*	2017	W	S	18		
Ružín	*S. salamandra*	2017	W	S	5		
Modra	*S. salamandra*	2017	W	S	5		
Pezinok	*S. salamandra*	2018	W	S	12		
		2017	W	S	13		
Bratislava	*S. salamandra*	2018	W	S	13		
Spain							
Boo de Guarnizo	*Lissotriton helveticus*	2017	W	S	28		
Santillana del Mar	*Ichthyosaura alpestris*	2017	W	S	10		
	L. helveticus	2017	W	S	1		
	Ambystoma mexicanum	2017	C	S	1		
Suances	*L. helveticus*	2017	W	S	22		1
	Triturus marmoratus	2017	W	S	10		
Valdáliga	*L. helveticus*	2017	W	S	17		
	I. alpestris	2017	W	S	4		
Voto	*S. salamandra*	2017	W	S	19		
Ampuero	*S. salamandra*	2017	W	S	9		
	L. helveticus	2017	W	S	10		1
Teverga	*L. helveticus*	2017	W	S	62		2
	T. marmoratus	2017	W	S	11		
Villafranca del Bierzo	*Lissotriton boscai*	2017	W	S	20		
Carucedo	*L. boscai*	2017	W	S	4		
	L. helveticus	2017	W	S	20		
Carracedelo	*L. helveticus*	2017	W	S	5	1	
	T. marmoratus	2017	W	S	11		
Chozas de Abajo	*Pleurodeles waltl*	2017	W	S	17		
	T. marmoratus	2017	W	S	1		
Ruente	*L. helveticus*	2017	W	S	50	2	1
Cabuérniga	*S. salamandra*	2017	W	S	19		
Campoo-Cabuérniga	*L. helveticus*	2017	W	S	11		
	I. alpestris	2017	W	S	2		
Los Tojos	*T. marmoratus*	2017	W	S	14		
	L. helveticus	2017	W	S	15		
Comillas	*L. helveticus*	2017	W	S	28		
Campoo de Suso	*L. helveticus*	2017	W	S	7		
	I. alpestris	2017	W	S	16		

**Bd*, *Batrachochytrium dendrobatidis*; *Bsal* = *B.salamandrivorans*; C, captive; S, swab; TC, toe clipping; W, wild; +, positive.

Chapter 4

Monitoring of amphibians in search of chytridiomycosis has some difficulties. It is complicated to find the animals, and it is harder still in areas already affected by chytrids. Environmental DNA is an essential technique that can help to solve that issue. However, current protocols are expensive, dependent on access to reliable power sources, or are valid just for *Bd* or *Bsal*. In addition, DNA from an environment degrades quickly and so a low-cost and universal storage capability is needed even in remote areas. The following study strives to deal with these drawbacks and elucidate a practical solution.

Dual detection of the chytrid fungi *Batrachochytrium* spp. with an enhanced environmental DNA approach

David Lastra González[1] Vojtech Baláž[2] Jiří Vojar[1]Petr Chajma[1]

[1)] Department of Ecology, Faculty of Environmental Sciences, Czech University of Life Sciences Prague, Kamýcká 129, 165 00 Prague-Suchdol, Czech Republic

[2)] Department of Ecology and Diseases of Game, Fish and Bees, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno, Palackého tř. 1946/1, 612 42 Brno, Czech Republic

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Title Page

Dual detection of the chytrid fungi *Batrachochytrium* spp. with an enhanced environmental DNA approach.

David Lastra González, Department of Ecology, Faculty of Environmental Sciences, Czech University of Life Sciences Prague, Kamýcká 129, Prague – Suchdol, 165 00, Czech Republic.

Vojtech Baláž, Department of Ecology and Diseases of Zoo Animals, Game, Fish and Bees, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno, Palackého tř. 1946/1, 612 42 Brno, Czech Republic.

Jiří Vojar, Department of Ecology, Faculty of Environmental Sciences, Czech University of Life Sciences Prague, Kamýcká 129, Prague – Suchdol, 165 00, Czech Republic.

Petr Chajma, Department of Ecology, Faculty of Environmental Sciences, Czech University of Life Sciences Prague, Kamýcká 129, Prague – Suchdol, 165 00, Czech Republic.

Correspondence author: David Lastra González, Department of Ecology, Faculty of Environmental Sciences, Czech University of Life Sciences Prague, Kamýcká 129, Prague – Suchdol, 165 00 Czech Republic. lastra_gonzalez@fzp.czu.cz

Running headline: Simultaneous *Bd* and *Bsal* detection with eDNA.

Abstract

Point 1: Environmental DNA (eDNA) is becoming an indispensable tool in biodiversity monitoring, including the monitoring of invasive species and pathogens. Aquatic chytrid fungi *Batrachochytrium dendrobatidis* (*Bd*) and *B. salamandrivorans* (*Bsal*) are major

threats to amphibians. However, the use of eDNA for detecting these pathogens has not yet become widespread, due to technological and economic obstacles.

Point 2: The new approach was successfully tested in laboratory conditions using manufactured gene fragments (gBlocks) of the targeted DNA sequence. A comparison of storage methods showed that samples kept in ethanol had the best DNA yield. Our results showed that the number of DNA copies in the Internal Transcribed Spacer region was 120 copies per *Bsal* cell. Eradication of emerging diseases requires quick and cost-effective solutions. We therefore performed cost-efficiency analyses of standard animal swabbing, a previous eDNA approach, and our own approach.

Point 3: Using the enhanced eDNA approach (a simple and cheap sampling protocol) and the universally accepted qPCR assay, we confirmed the presence of *Bsal* and *Bd* in previously identified sites in Spain, including four sites that were new for *Bsal*.

Point 4: The procedure presented here was evaluated as the most cost-efficient and the first one being able to detect both pathogens. Our findings will help to disseminate accessible methods to detect the pathogen and prevent the spread of *Bsal*.

Keywords

Batrachochytrium dendrobatidis, *Batrachochytrium salamandrivorans*, *Bd*, *Bsal*, chytridiomycosis, water samples.

Introduction

Species detection is an integral - but often laborious - task in any biological field research. Environmental conditions and species-specific ecology (e.g., hidden way of life, scattered distribution, or small size) can substantially decrease the probability of certain taxa being detected (Mackenzie and Kendall, 2002). Technological progress in the last 15 years has enabled the collection and analysis of DNA traces left by organisms in the environment (eDNA) and has led to a revolution in biodiversity studies. Species can be

detected from soil, sediment, or water without any direct signs of their presence (Thomsen and Willerslev, 2015). The method is broadly used for multiple purposes, for detecting elusive (Goldberg et al., 2011), endemic (Laramie et al., 2015) or invasive species (Ficetola et al., 2008). At the same time, multiple infectious diseases with a detrimental impact on biodiversity have emerged and have been recognized on a global scale (Allain and Duffus, 2019). Amphibians have become the canaries in the coal mine in this pathogen-mediated crisis (Scheele et al., 2019), with chytridiomycosis being the main culprit in their decline (Berger et al., 1998). This disease is caused by two chytrid fungi species: *Batrachochytrium dendrobatidis* (*Bd*) (Longcore et al., 1999) and *B. salamandrivorans* (*Bsal*) (Martel et al., 2013).

Amphibian chytrid fungi are potentially good targets for the application of eDNA detection, owing to their microscopic size, the large quantities of actively dispersing zoospores that are usually produced, occupation of an aquatic environment, and the ready availability of established detection assays. Attempts to use eDNA detection in the case of *Bd* (Walker et al., 2007; Kirshtein et al., 2007; Kamoroff and Goldberg, 2017; Mosher et al., 2017) or *Bsal* (Spitzen-van der Sluijs et al., 2020) are still surprisingly sparse, although qPCR (Boyle et al., 2004; Blooi et al., 2013), nested PCR (Goka et al., 2009) and standard PCR (Martel et al., 2013) assays are sensitive enough to detect the DNA equivalents of individual cells. The reasons limiting the broader use of eDNA in the detection of both chytrid fungi include the perceived difficulty of the procedure, inconsistent results in comparison with standard individual sampling protocols (Walker et al., 2007), and the potentially high cost of sample collection and processing (Spitzen-van der Sluijs et al., 2020).

The crucial factor in eDNA detection of target pathogens is the establishment of an applicable procedure for eDNA collection, sample storage and DNA isolation. Critical considerations stated in Goldberg et al. (2016) should be followed, and the current lack of unified criteria for procedures makes comparison of results challenging. As an

77 example, various types of filters are used – polycarbonate track-etched (Kamoroff and
78 Goldberg, 2017), polyethersulfone (Mosher et al., 2017), nitrate cellulose (Walker et al.,
79 2007) or VigiDNA®(Spygen, Le Bourget du Lac Cedex, France), unspecified material
80 (Spitzen-van der Sluijs et al., 2020). At the same time, the current rapid spread of the
81 pathogens requires simple, fast, robust, and cost-effective surveillance methods. In order
82 to meet these requirements, particular aspects of sample collection and subsequent DNA
83 extraction should be considered: the pore size of the filter, the type of pumping system,
84 the extraction kit and complementary kits (e.g. an anti-inhibitor kit or a purification kit), or
85 the storage method. An extensive review of the range of the above-mentioned
86 parameters within 36 articles can be checked in Figure S1 in Supporting Information.

87 The choice of the storage method requires special attention. The stability of the eDNA in
88 the filter after collection has a strong effect on species detection (Renshaw et al., 2015;
89 Spens et al., 2016), and multiple methods of sample preservation were tested. The ideal
90 sample conservation method should be technically simple and applicable in field
91 conditions, should provide stability of the sample in ambient temperatures, should be
92 safe to use and transport, and should be cost-effective. The most widely used storage
93 methods are freezing and 96% ethanol (see Figure S1 in Supporting Information).
94 However, freezing the samples reduces the yield (Spens et al., 2016), and ethanol
95 evaporates easily even when the enclosed filters are sealed with a cap. Moreover, it is
96 necessary to use a pipette and tips in field conditions, or to transport a portable freezer.
97 Some articles have reported on studies of this crucial topic (Renshaw et al., 2016; Spens
98 et al., 2016), but they were not linked to the detection of *Batrachochytrium* spp. and did
99 not include any other affordable storage method, such as silica gel.

100 Lastly, the economic aspect of any method greatly affects its application potential and
101 how widely it is used. Unfortunately, only a small number of publications have included
102 precise costs or estimates of the costs of their working procedure that would enable non-
103 affordable materials to be rejected, either due to their unavailability or because their high

104 cost makes them unusable when large amounts are needed (but see Goldberg et al.,
105 2011; Hyman and Collins, 2012; Goldberg et al., 2016).

106 The purpose of this research is therefore to find an accessible protocol for detecting *Bsal*
107 with an eDNA approach. Subsequently, the aim is to make a protocol that is compatible
108 for detecting *Bd* at the same time, thus creating the first protocol for detecting both chytrid
109 fungi by eDNA. A further goal was to identify the best storage method for the filters that
110 will be used. Finally, we have calculated a strict comparison of existing protocols to
111 optimize the resources and thus to strengthen efforts to detect these pathogens.

112

113 **2. Materials and Methods**

114 **2.1 Study area and collection of field samples**

115 Since 2017, we have been monitoring by swabbing several caudate populations in the
116 northern part of Spain, which have been positive for *Bsal* (Lastra González et al., 2019).
117 To collect samples for the purposes of this research, we swabbed amphibians and
118 filtered water from the habitat where the animals were collected. We tested the filters in
119 the *Bsal*-positive localities mentioned in (Lastra González et al., 2019), and also in four
120 other localities. We also sampled several Czech localities, near Staré Město, Zlín Region
121 and Sokolov, Karlovy Vary region, where we collected eDNA samples for an evaluation
122 of the presence of *Bd.* In total, 47 filters and 148 swabs were collected in 11 localities
123 (see details in Table 1 Results).

124 Skin swabs were collected following the standard procedure for sampling amphibian
125 chytrid fungi, as described in (Hyatt et al., 2007). The eDNA was collected using
126 SVHVL10RC filters, 0.45 μm pore size, PVDF membrane, with a Luer outlet (Millipore
127 Sigma, Burlington, Massachusetts, USA). These filters were attached to a 50 mL Omnifix
128 Luer Lock syringe (B. Braun, Melsungen, Germany). Each syringe was first rinsed with
129 water from the tested waterbody, was then filled completely with water pre-filtered by

130 nylon fabric (to avoid macroscopic organic material), was attached to the filter and was
131 manually forced to pass through. Pre-filtration was done just if organic material (e.g.
132 leaves, algae, sticks) could block the inlet or the outlet of the filter device (not affecting
133 the capture of zoospores). Typical situation was when a layer of *Lemna minor* covered
134 the water surface. Multiple repetitions of 50 mL with water from all around the perimeter
135 of the water body were carried out, until the filter was clogged. Then, the remaining water
136 in the filter was removed entirely by pushing air with the syringe. The maximum number
137 of repetitions was 20, giving 1000 mL of water filtered per water body. The collected filter
138 samples were preserved with Longmire's buffer and silica gel (see extended information
139 in Table S5 in Supporting Information). A hygienic protocol was used, including a
140 different set of gear (e.g. gloves, boots, or nets) for each locality. The set of gear was
141 disinfected when the procedure had been completed.

142 **2.2 DNA Extraction and qPCR**

143 The filter membrane was removed from the filter case and was cut into smaller fragments
144 to fit in a 2 mL tube. We extracted the DNA with the ISOLATE II Genomic DNA Kit
145 (Bioline, Meridian Bioscience Inc, Cincinnati, Ohio, USA), with the following modifications
146 of the protocol: during the pre-lysis step, the lysis step and the step for adjusting the DNA
147 binding conditions, we triplicated the volume of each reagent to cover the filter
148 completely. The DNA binding step with 96% ethanol was performed in a clean 2 mL tube
149 with all the liquid collected from the previous lysis step. The elution step was performed
150 in two consecutive rounds of 50 µL to increase the yield. The isolated DNA was stored
151 in 1,5 mL tubes at -20°C.

152 The detection of *Bd* and *Bsal* DNA followed the protocol of (Blooi et al., 2013). To avoid
153 false negative results due to PCR inhibition, we ran each eDNA sample with TaqMan™
154 Exogenous Internal Positive Control (IPC) Reagents (Thermo Fisher Scientific, Waltham,
155 Massachusetts, USA). This could not be performed in the same reaction as the detection
156 itself, as the IPC and *Bsal* probe share the same fluorophore parameters. All analyses

157 were performed in duplicate in Roche LightCycler 480 II (Roche Diagnostics, Prague,
158 Czech Republic), using the Roche Probes Master mix (Roche Diagnostics, Prague,
159 Czech Republic). Only in the case of the Czech samples, we used a simplified qPCR
160 assay targeting only *Bd* (using the cycling program after (Blooi et al., 2013), but the mix
161 contained only *Bd* primers and a probe). As the quantification standards for *Bd*, we used
162 100, 10, 1, 0.1 GE per 5 µl (standard volume added in the PCR reaction) dilutions of *B.*
163 *dendrobatidis* genomic DNA, from the GPL lineage, strain IA042, provided to us by
164 Trenton Garner, from the Institute of Zoology, Zoological Society of London (United
165 Kingdom). Quantification standards of *B. salamandrivorans* of 100, 10, 1, 0.1 GE per 5
166 µl were made from DNA provided by An Martel, of Ghent University (Belgium). Later, we
167 adopted gBlocks (see 2.3) as *Bsal* standards. In addition, some samples were sent to
168 Trier University in order to confirm our results. A subset of samples from field and
169 laboratory experiments were retested with a recently-developed assay for *Bsal* eDNA
170 testing (Spitzen-van der Sluijs et al., 2020).

171 In all qPCR tests, the sample was considered positive only if both wells amplified, the
172 increase in fluorescence showed a standard sigmoidal curve, and the Ct value was below
173 40. Samples with single well amplification were retested.

174 **2.3 gBlock *in vitro* testing**

175 We used Genbank sequences of *Bsal* (KC762295.1 and NR_111867.1) in designing
176 gBlock (Integrated DNA Technologies, Inc., Iowa, USA) fragments to be used as a
177 substitute for free eDNA in spiked water tests. We used a *Bsal* gBlock 213 bp in length
178 that overlaps with the *Bsal* primers (Blooi et al., 2013) target sequence by 26 bases on
179 both sides. The same gBlock is also applicable with recently published eDNA qPCR
180 (Spitzen-van der Sluijs et al., 2020). Positions 96 and 97 contain two adenine nucleotides
181 in the position of thymine in the reference sequences, in order to identify possible
182 contamination by the gBlock in the event of equivocal results suspected to have been
183 caused by laboratory cross-contamination.

To test the filtering and the subsequent qPCR sensitivity in detecting *BsaI* eDNA, we made five 500 ml samples of natural pond water spiked with 10^2, 10^3, 10^4, 10^5, 10^6 gBlock copies. The water samples were filtered and the eDNA was isolated on the day of spiking. *BsaI* gBlock sequence:
5´CAGAACTCAGTGAATCATCGAATCTTTGAACGCACATTGCACTCTACTTTGTAGA
GTATGCCTGTTTGAGAATCAATAGTATTTTCTTGTTCTATaaTCTTTTTTTAATTCATT
TCCTTGTCTTTTTATATCATCTAAAAAGTGATATAAAAATAGGGTTAGGGATGAAGA
GGGGGAGATGGAGCAGATAATGAGTGATTAGTTGAGGTTCT 3´

The genomic equivalents (GE) were calculated with the use of gBlocks with 10- fold serial dilutions from 100 to 108 copies per 1 µl. We ran these standards in quadruple repetitions in separate qPCR (Blooi et al., 2013), with 5 µl of the sample and 20 µl of the mastermix in reaction. The produced standard curve was saved and was used to quantify the *BsaI* DNA load of the sample and a single standard concentration was used as the calibrator.

In the case of the assay of Spitzen-van der Sluijs et al. (2020), we used a new set of gBlock standards run in triplicate with the samples and used the in-run standard curve for quantification. In all analyses, we used the calculation of the 2nd derivative maximum, which is available in LightCycler 480 software.

To make our quantification results easier to compare with widely used genomic DNA standards, we compared the gBlock standard curves with the *BsaI* genomic DNA standards, and we calculated how the gBlock copy numbers translate to *BsaI* GE values. For this purpose, we used data available from the *BsaI* detection laboratory ring test performed in 2017 in collaboration with *BsaI* reference laboratory at Ghent University. The equation for the standard curve generated on the basis of gBlocks ($5*10^0$, $5*10^1$, $5*10^3$, $5*10^4$, $5*10^5$, $5*10^6$, $5*10^7$, $5*10^8$) was used to calculate the copy number of ITS based on the Ct value of each genomic *BsaI* standard (10^{-1}, 10^0, 10^1, 10^2 GE per reaction). For each genomic standard, the resulting Ct value was translated into copy numbers, corrected to the given dilution and averaged.

211 The calculation of the efficiency of the eDNA capture was based on the detected copy
212 number, multiplied by 20 to account for the dilution factor during the DNA extraction (total
213 elution into 100 ul, 5µl of undiluted sample used in reaction), and then divided by the
214 original number of copies in the water.

215 **2.4 Storage experiment for eDNA samples**

216 We compared widely-used filter storage methods, i.e. Longmire's buffer (LB), silica gel
217 (S), ethanol (EtOH), together with no storage method as a control (kept at room
218 temperature). A preliminary test of the filter storage method was performed using five
219 litres of water from a pond with amphibian presence but no chytrid fungi, collected in
220 November 2019. The water was pre-filtered to remove the abundant plankton (e.g.
221 *Daphnia* sp., Ephemeroptera larvae) and larger debris. We spiked four bottles containing
222 0,5 L of pond water with 10^6 copies of *Bsal* gBlock each. Filtration was performed as
223 explained above (see 2.1). One filter was preserved by each of the compared storage
224 methods (LB × S × EtOH × control). To ensure repeatability of the methodology, note the
225 following specifications: LB and EtOH were added in an amount of 2 mL pipetted inside
226 the filter; S was 2–5 mm with an orange indicator (P-lab, Prague, Czech Republic); EtOH
227 was 96% and control. LB and EtOH were stored with Luer-Lock caps, and all of them
228 were placed inside a Falcon tube. The filters were stored at room temperature for 9
229 weeks. DNA extraction and qPCR analyses were carried out as stated above (see 2.2).

230 The second test of storage methods used more repetitions and was slightly modified.
231 We used 400 mL of water from the same pond collected in April 2020, and we again
232 spiked it with 10^6 copies of *Bsal* gBlocks. Each storage method was used on three filters.
233 In filters stored in S, we cracked and removed the external layer of the filter and we left
234 the filter in direct contact with the silica gel. The LB and EtOH filters were sealed with
235 Luer Lock caps and all of them were placed inside a Falcon tube. The filters were stored
236 at room temperature for 6 weeks. DNA extraction and qPCR analyses were carried out
237 as stated above (see 2.2).

238 ***2.5 Cost assessment***

239 In order to assess the cost-efficiency of traditional sampling (swabbing), together with
240 our approach and a previously used approach for detecting *Bsal* with eDNA (Spitzen-
241 van der Sluijs et al., 2020), we performed a cost comparison among these sampling
242 protocols. All prices (in euros) and all costs were consulted in July 2020, with the
243 exception of the cost of the services provided by the SPYGEN laboratories in April 2020.
244 If the price was in another currency, the exchange rate valid on July 13, 2020 was used.
245 The budget was envisaged for a single locality, using one filter or swabbing 20 animals,
246 and for two people. The laboratory extraction costs are for one replicate. This calculation
247 covered perishable materials and reagents, but labour costs, transport, usage of
248 university facilities and long-lasting materials (e.g. traps, buckets, disinfection
249 equipment) were not included. We considered these conditions to be the minimum
250 required for developing a complete analysis with total guarantees.

251 **3. Results**

252 ***3.1 Field sampling***

253 Out of 13 filters collected in Spain, 12 were positive for *Bsal.* These filters were from nine
254 different localities, in four of which this was the first case of *Bsal* detection (see Table 1).
255 Moreover, in eight localities we were able to test both sampling options: filters and swabs.
256 Just in three of the localities, both eDNA and swabs tested positive for *Bsal*. This means
257 that eDNA was able to prove the presence of *Bsal* in five localities where no positive
258 trace had been found with the swab methodology.

259 The analysis of the eDNA detected the presence of both amphibian chytrid fungi in three
260 filters in Spain, two from the same locality (see Table 1). This is to our knowledge the
261 first time that both *Batrachochytrium* species have been detected by eDNA within a
262 single sampled site. In addition, one filter in the Czech Republic tested positive for *Bd*

263 (see Table 1). All IPC tests showed no difference among the tested samples and
264 produced typical amplification curves.

265 Table 1. List of *Batrachochytrium salamandrivorans* (*Bsal*) and *B.dendrobatidis* (*Bd*) positive
266 localities in 2019. Number (N), (number of positive samples/number of collected samples).
267 Distances between particular water bodies within the Teverga and Ruente localities are at least
268 5 km.

Country	Locality	First *Bsal* positive	N Filters	N Swabs	*Bsal* DNA copies	*Bd* (DNA copies)
Spain	Ampuero	*2017	1/1	5/5	10.81[a]/8.05-23[b]	
Spain	Teverga 1	*2017	1/1	1/19	96.55[a]/15.05[b]	
Spain	Teverga 2	*2017	1/1	0	15.70[a]	
Spain	Teverga 3[#]	2019	2/2	2/2	12.2-12.45[a]/ 3.27-17[b]	
Spain	Ruente 1	*2017	1/2	0/20	12[a]	
Spain	Ruente 2	2019	1/1	0/11	6.16[a]	28.8[a]
Spain	Suances	*2017	2/2	0/25	22.35-96.05[a]	31.2; 764.4[a]
Spain	Ponga[#]	2019	2/2	0/15	1.51-24.2[a]	
Spain	Cieza	2019	1/1	0/11	33.3[a]	
Czech R.	Stare Mesto	N/A	2/2	11/30	N/A	0.1-7.3[a]/1.91-44.55[b]
Czech R.	Sokolov	N/A	0/32	N/A	N/A	

269 [#]Two different water habitats, but close to each other.
270 [a] are DNA copies - eDNA approach; [b] are DNA copies - swabs approach.
271 **Bsal* positive localities from Lastra González et al. (2019).
272

273 ### ***3.2* gblock *in vitro* testing**

274 The comparison between the gBlock standards (standard curve $y = -1.478\ln(x) +$
275 $40.221 R^2 = 0.998$) and genomic DNA resulted in a mean value of 121.3 (SD = 33.1). The

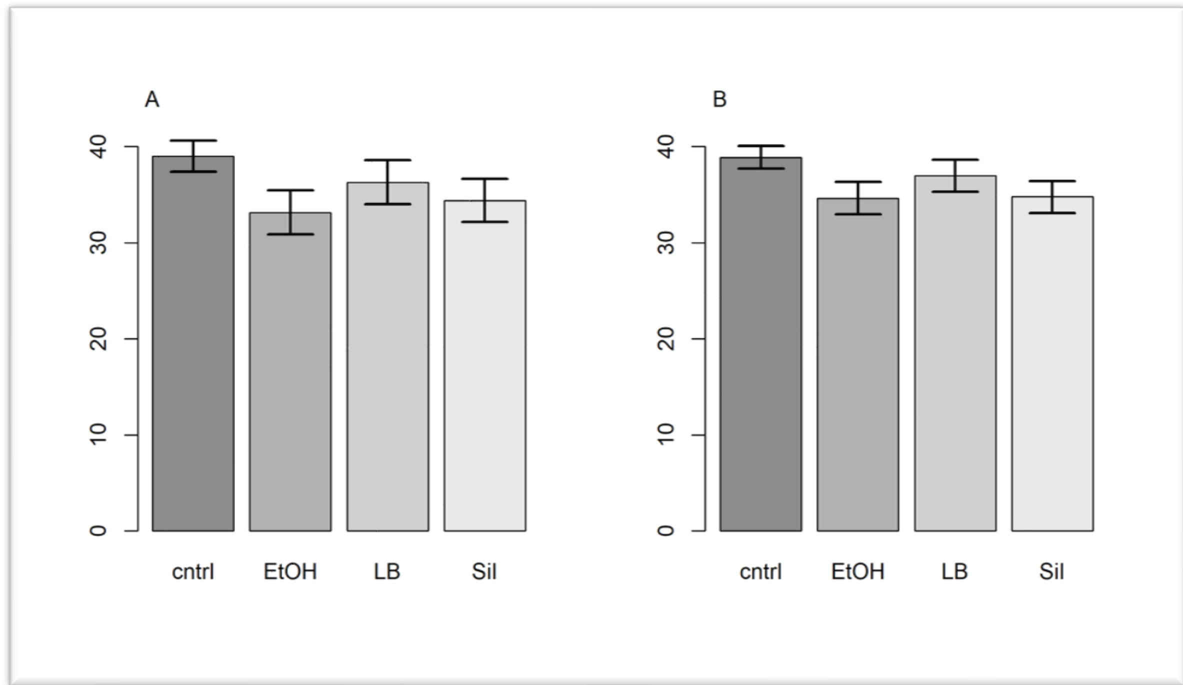
276 targeted ITS region therefore seems to be present in the *BsaI* lineage used for producing
277 genomic standards in approximately 120 copies.

278 The efficiency of eDNA collection evaluated by qPCR testing of pond water filters spiked
279 with 10× serial dilutions of *BsaI* gBlocks (10^2–10^6 copies per 500 ml filtered water)
280 showed that amplification occurred consistently only in 10^4 and higher numbers of gBlock
281 copies. One well of 10^3 copies was positive with Ct 39, while the 100 copies per filter
282 were not detectable. The overall gBlock capture from spiked pond water using freshly
283 isolated filters that tested positive was 20%. However, the lowest concentration
284 equivalent of approximately a single cell did not amplify at all.

285 **3.3 Storage experiment for eDNA samples**

286 In the preliminary test, we detected *BsaI* gBlocks after 9 weeks in all filters. The filter that
287 was preserved in ethanol got the earliest response. In the subsequent test with three
288 replicates, the ethanol filters were again the first to amplify, followed by the filters
289 preserved in silica gel, then LB, and finally the control filters (see Table S3 in Supporting
290 Information). The same results appeared with the primers (Spitzen-van der Sluijs et al.,
291 2020). The filter storage methods had a significant effect on the retention of DNA
292 expressed by the Ct values (primers from [21]: $F = 7.59$, $p = 0.01$; primers from [19]: $F =$
293 8.40; $p = 0.007$). While point estimates from both models showed that the filters stored
294 in ethanol were first to amplify, followed by the filters preserved in silica gel, Longmire's
295 buffer and the control (Fig. 1), Bayesian 95% credible intervals showed that only ethanol
296 and silica gel were substantially better storage methods than the control (Figure 1).

297 Figure 1. Ct values (Y axis) and storage methods (X axis). Control (cntrl), ethanol (EtOH),
298 Longmire's buffer (LB), and silica gel (Sil). The A plots (left side) are the primers from Blooi et al.
299 (2013), and the B plots (right side) are from Spitzen-van der Sluijs et al. (2020).



300

301 Table 2. Comparison of the storage effect on eDNA capture (percentage of eDNA recovered
302 from the original amount added) after six weeks of filters with different storage types (each type
303 three filters) based on two quantification PCR assays from Blooi et al. (2013), and from Spitzen-
304 van der Sluijs et al. (2020).

Storage type	Primers from Blooi et al. (2013)	Primers from Spitzen-van der Sluijs et al. (2020)
Control, room temperature	0.01%*	0.1%*
Longmire's buffer	0.04%	0.2%*
Silica gel	0.19%	1.1%
96% EtOH	0.34%	1.1%

305 *Not all wells amplified

306

307 **3.4 Cost assessment**

308 A cost comparison of the sampling protocols between traditional sampling (swabbing), a
309 previously used approach for detecting *Bsal* with eDNA (Spitzen-van der Sluijs et al.,

310 2020), and our approach presented here revealed our methodology as the most
311 economical option (see Table S2 in Supporting Information), with an approximate cost
312 of 22 euros per locality. In comparison, the costs for the protocol developed in (Spitzen-
313 van der Sluijs et al., 2020) are approximately 72 euros per sample (locality). However,
314 the analysis performed by a private laboratory had a dramatic influence on these costs
315 (see the footnote in Table S2 in Supporting Information). Note that the VigiDNA^{TM} filter
316 cost five times more than our equivalent gear. On the other hand, we added the costs of
317 traditional swabbing monitoring, either the protocol with the Qiagen Blood and Tissue kit
318 (Qiagen, Hilden, Germany) or PrepMan^{TM} (Thermo Fisher Scientific, Carlsbad, USA). In
319 these cases, in order to make a fair comparison, we calculated the costs for 20 samples
320 and gear for two people. With these conditions, the cost per locality for the Qiagen Blood
321 and Tissue kit is around 81 euros, and when PrepMan^{TM} is used the cost is approximately
322 27 euros per locality (see Table S2 in Supporting Information).

323 ### ***3.5 Statistical analyses***

324 Filter storage methods (fixed effect) and their effect on the retention of DNA expressed
325 by the Ct values (response) were compared using linear mixed effects models with a
326 Gaussian error structure and a filter as a random intercept (Bates et al., 2015). Model-
327 based Bayesian 95% credible intervals of Ct were obtained using the 2.5 and 97.5
328 percentiles from the posterior distribution of 5000 simulated values (Gelman and Su,
329 2018). For the purposes of the analyses, Ct values of 0 and 40 or higher were treated as
330 negative and were all set to 40. All statistical analyses were performed in R, version 3.5.3
331 (R Core Team, 2019).

332 ## **4. Discussion**

333 We again found the five *Bsal* positive sites from Lastra González et al. (2019), and we
334 have detected four new ones. We observed inconsistency in the detection through the
335 years, as was also found in Lötters et al. (2020). In Germany, due to the current reduced

336 numbers of adult fire salamanders *Salamandra salamandra* is becoming harder to obtain
337 samples to test the pathogen (Lötters et al., 2020). In addition, almost entire populations
338 can be wiped out from an area after a severe *Bsal* outbreak (Spitzen-van der Sluijs et
339 al., 2018), and swabbing adult individuals is therefore becoming quite a challenging task.
340 It was imperative to develop a protocol that is not dependent on locating amphibians and
341 that unifies the latest advances in laboratory techniques for detecting *Bsal*. However, it
342 is important to note that eDNA detection is not able to discriminate between the presence
343 of viable cells/organisms and the presence of residual DNA fragments (Hyman and
344 Collins, 2012). It is not advisable to collect DNA after heavy rains or after any event that
345 disturbs streams or water bodies (Takahashi et al., 2017). Sediments could re-suspend
346 eDNA up to 6 months later, and this could give a false positive for the presence of species
347 (Goldberg et al., 2015). A comparison between confirming the presence of organisms
348 by eDNA and by traditional monitoring showed that, for example, four visits for amphibian
349 monitoring in Mediterranean ponds were needed to obtain similar detectability as eDNA
350 (Valentini et al., 2016). Nonetheless, in order to obtain 95% probability of detecting *Bd*
351 by eDNA in a site with confirmed positive amphibians, it was necessary to get four
352 samples of 600 ml or five samples of 60 ml (Chestnut et al., 2014).

353 Since the first chytridiomycosis outbreaks were reported, changes have been made in
354 the methodology for swab sample processing before detecting amphibian chytrid fungi.
355 *Bsal* DNA was originally isolated from swabs with the use of PrepMan™ (Thermo Fisher
356 Scientific, Carlsbad, USA) (recommended by Blooi et al. (2013) and Thomas et al.
357 (2018). However, spin-column based DNA extraction kits seem to outperform the simple
358 and affordable option of PrepMan™ (Bletz et al., 2015; Brannelly et al., 2020), because
359 sites with amphibians hosting *Bd* or *Bsal* in low intensities may be falsely identified as
360 negative if the less efficient DNA extraction method is used (Sabino-Pinto et al., 2019;
361 Brannelly et al., 2020].

362 Despite several recommendations and methodological publications on *Bd* and *Bsal*
363 detection (Hyatt et al., 2007; Thomas et al., 2018), there are ongoing inconsistencies
364 among research teams in the definition of a positive result. For example, there is
365 inconsistency in the Ct value set as the threshold to distinguish positives from late
366 amplification false positives (e.g. Klymus et al. (2019) uses 40 cycles, while Bedwell et
367 al. (2020) uses 45 cycles). Our data based on the known number of gBlock fragments in
368 reaction show that a single DNA molecule with the target sequence should amplify at
369 40.2 cycles. We therefore considered samples as positive only if the Ct values were less
370 than 40, and if both wells amplified. Consistency of amplification in all wells where a
371 sample was added is not universally used (see the discussion in Goldberg et al. (2016)),
372 with many studies considering single well amplification sufficient (Chestnut et al., 2014;
373 Mosher et al., 2017; Brannelly et al., 2020; Bedwell et al., 2020). Multiple repetitions of
374 qPCR tests of a single sample as used in (Spitzen-van der Sluijs et al., 2020), not only
375 waste sample material, but increase the risk of in-lab contamination and false positive
376 results. If we had adopted the strategy of one single PCR well to claim a sample as
377 positive, we could have reported the first *Bsal* positive in the Czech Republic. However,
378 we believe that a single amplification should not be considered sufficient for such a
379 serious claim. We advise that this crucial issue should be solved by using sequence
380 identifiable gBlocks or other synthetic DNA standards, not genomic DNA of the tested
381 pathogen. DNA identity in positive results from unexpected scenarios should be
382 confirmed subsequently by sequencing of the amplicon. To reduce the risk of false
383 negative results, Exogenous Internal Positive Controls should be used whenever
384 possible, as already recommended in the past (Hyatt et al., 2007; Thomas et al., 2018).

385 A qPCR assay alone provides less robust support for the presence of disease in tested
386 animals than is provided by histopathology. However, qPCR is usually a much more
387 sensitive method than histopathology, it can be designed to be target-specific, and
388 together with eDNA it is applicable without directly handling the amphibians.

389 Histopathology is not compatible with eDNA filters for confirming samples, and it cannot
390 be used in the absence of animals. If qPCR results are consistent and repeatable, there
391 is no apparent reason for omitting some data completely, as in Martel et al. (2020) or in
392 the reference website BsalEurope, or for marking them "doubtful" or "unconfirmed", as
393 was the case of Lötters et al. (2020) in regard to some studies (e.g. Lastra González et
394 al., 2019). Nevertheless, in order to show the consistency of our results a subset of
395 samples also tested positive in the Trier University (see Table S4 in Supporting
396 Information).

397 Moreover, other features were considered to strengthen this approach. The use of
398 enclosed capsule filters reduces the risk of contamination during transport and storage
399 (Harper et al., 2018). Single-use syringes are easier to carry than various pump systems,
400 and they avoid the risk of cross-contamination with the tubing. We recommend 50 mL
401 syringes with a Luer-Lock tip, as they are easy to use in difficult field conditions. The
402 overall amount of equipment needing disinfection, and the amount of waste that is
403 generated from single-use plastics are considerably smaller than when traditional
404 swabbing sampling schemes are used.

405 The collection efficiency of eDNA observed in our lab experiment may be due to
406 limitations of the selected gBlock approach (see Table 3). The short length of the
407 fragments designed in our case to act as a standard in qPCR likely causes faster
408 degradation in pond water than in water spiked with viable zoospores. Moreover, we
409 followed the DNA isolation protocol with standard lysis times, providing excess time for
410 degradation to take place. It is also important to note that, based in Kirshtein et al. (2007),
411 the recovery of zoospores varied according to where they were diluted. The yield was
412 higher when deionized water was used, instead of the pond water that we used. The
413 biological activity of natural water changes in time (e.g. presence of bacterial enzymes),
414 and this could be the reason why our two different storage experiments produced slightly
415 different Ct values, even with the same storage options. However, one cell contains

416 approximately 120 ITS copies, and this provides a good chance of detection even if a
417 large amount of DNA is degraded. We believe that the collection efficiency would be
418 higher if cells were used, but the gBlock experiment was still effective in testing the whole
419 process. We observed slightly better results when using the assay of Spitzen-van der
420 Sluijs et al. (2020) than when using the assay of Blooi et al. (2013) in the storage tests.
421 The explanation could again be DNA degradation, considering that the first assay targets
422 a shorter fragment within the target sequence. As DNA is degraded predominantly from
423 the ends, it is more likely to not get degraded over the position targeted by the primers
424 and by the probe. The primers used in (Spitzen-van der Sluijs et al., 2020) were in
425 general more sensitive, and we therefore recommend them. Issues with the specificity
426 of the primer should be more critically evaluated if these new primers become as widely
427 used as the primers in Boyle et al. (2004) in the case of *Bd*.

428 Regarding storage methods, we found that filters preserved in ethanol were the first to
429 amplify, followed by silica gel, Longmire's buffer and control samples stored at room
430 temperature. However, the difference in performance between ethanol and silica gel was
431 not statistically significant. In addition, silica gel is simpler to use, because no extra gear
432 is needed. This makes the silica gel our recommended storage method. We advise
433 putting the filter and the silica gel in direct contact with each other. We excluded from our
434 comparison keeping filters at -20ºC until extraction, because the freeze-thaw cycle
435 affects DNA detection (Hinlo et al., 2017), and the process is impractical in most fieldwork
436 scenarios. Despite its good performance, Longmire's buffer has only rarely been used in
437 previous studies (Renshaw et al., 2015; Wegleitner et al., 2015; Spens et al., 2016).
438 Perhaps, the use of certain chemicals, e.g. sodium dodecyl sulfate or sodium azide, and
439 the precautions that need to be taken when working with them, is a constraint. However,
440 according to our results, Longmire's buffer was a low-performance storage method (see
441 Figure 1).

442 In relation to extraction kits, we used the ISOLATE II Genomic DNA Kit (Bioline, Meridian
443 Bioscience Inc, Cincinnati, Ohio, USA). The most widely-used extraction kit is the Qiagen
444 Blood and Tissue Kit (Qiagen, Hilden, Germany) (see Table S1 in Supporting
445 Information), but we found the protocol of the ISOLATE II Genomic DNA Kit (Bioline,
446 Meridian Bioscience Inc, Cincinnati, Ohio, USA) slightly easier. There is a difference in
447 price with the extraction kit that we used (US$ 199 for ISOLATE II Genomic DNA Kit
448 (Bioline, Meridian Bioscience Inc, Cincinnati, Ohio, USA) versus 168 US$ for the Qiagen
449 Blood and Tissue Kit (Qiagen, Hilden, Germany), 50 preparation kits). Other more
450 affordable options for silica spin-column DNA extraction kits are available, e.g. the Gen-
451 aid Genomic DNA mini-kit, US$ 168; the EZNA tissue DNA kit, US$ 87. Traditional
452 phenol-chloroform-isoamyl extraction is much cheaper. According to Renshaw et al.
453 (2015), the cost is around US$ 0.2 per sample, but additional lab equipment is required
454 to make the process safe. In addition, Brannelly et al. (2020b) researched about
455 extraction kits and detected different performance, showing that it is another key
456 parameter for the detection of chytrids.

457 There are other steps that could improve eDNA detection of amphibian chytrids. Although
458 a detailed discussion is beyond the scope of this article, we want to mention other steps
459 here, in order to promote further research. First, it is advisable to collect two filters per
460 locality, as in the case of swabs (Sabino-Pinto et al., 2018). Second, the TaqMan
461 Environmental Master mix performs better than other options (Hinlo et al., 2017), without
462 costing more than the approaches mentioned here (see Table S2 in Supporting
463 Information). Last, other PCR detection methods, such as digital-drop PCR, could be
464 used as in Harper et al. (2018).

465 **Authors' contributions**

466 DLG and VB conceived the ideas and designed the methodology; DLG and JV collected
467 the data; DLG, VB did the laboratory analyses, and PC performed the statistical

468 analyses. All authors contributed critically to the drafts and gave final approval for
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Supp. Information: Dual detection of the chytrid fungi *Batrachochytrium* spp. with an enhanced environmental DNA approach.

Year	Article	Target	Environment	V(L)	FilterType (µm)	Pumping system	Storage	IPC	Kit Extraction	Purification/Antiinhibitor kit
2018	Hundermark & Takahashi	Amphibians	River	1	0.45 NC	Pump	Freeze		Blood and Tissue Kit Dneasy Qiagen (BTK)	
2018	Preissler & Watzal et al.	Amphibians	River	1	0.45NC	Pump	Freeze		Comparison	Comparison Qiagen vs Zymo
2018	Harper et al.	Review						Yes		
2018	Li et al.	Fish	Pond	0.3	Comparison	Pump/Syringe	Freeze		PowerWater DNA Mo Bio	SequalPrep (Invitrogen)
2018	Eiler et al.	Amphibians	Pond	0.5	0.45 Sx	Pump	Freeze		PowerSoil DNA	
2018	Wittwer et al.	Crayfish	Stream	up10	2.0 GF		Freeze			
2018	Fernandez et al.	Fish	Stream	1.5	0.2GF	Pump	Freeze		EZNA Tissue DNA Kit	
2017	Kamoroff & Goldberg	Bd	Lake	0.25	1.2PCTE	Pump	Ethanol		Dneasy BTK Qiagen	
2017	Mosher et al.	Bd	Laboratory	0.2	0.22 Sx	Syringe	Room	Yes	Gentra Puregene Tissue Kits	Zymo inhibitor removal kit
2017	Takahashi et al.	Amphibians	Stream	1	0.45 NC		Freeze	Yes	Dneasy BTK Qiagen	
2017	Trebitz et al.	Recommendations								
2017	Hinlo et al.	Comparison							PCI and CTAB	
2017	Agersnap et al.	Crayfish	Ponds	0.5-1.5	0.22 Sx	60mL Syringe	Freeze		CTAB	Qiagen QIAquick PCR purification
2017	Buxton et al.	Amphibians	Ponds	1	0.7 Glass	100 mL Syringe			Dneasy BTK Qiagen	
2017	Walker et al.	Amphibians							Dneasy BTK Qiagen	
2016	Spens et al.	Fish	Ponds	1	Comparison		Various			
2016	Goldberg et al.	Comparison								Zymo and Bovine Serum Albumine
2016	Civade et al.	Fish	Various	45		Pump	Buffer		Dneasy BTK Qiagen	MinElute PCR purification kit
2016	Lacoursiere-Roussel et al.	Fish	Aquaria	1	Comparison	Pump	Freeze			
2015	Valentini et al.	Review								
2015	Laramie et al.	Protocol		0.25		Various	Ethanol			
2017	DNeasy PowerWater Kit	Handbook	Various		0.22-0.45	Pump	Freeze		Dneasy PowerWater	
2015	Hall et al.	RV		0.25	0.2 NC	Pump	Ethanol	Yes	Qiashredder/Dneasy BTK	
2015	Eichmiller et al.	Fish	Tank	1	Comparison				FastDNA Spin Kit	Dilution 1/5
2015	Kolby et al.	Bd	Rainwater		0.22 Sx	Pump	Freeze		Qiagen ATL tissue lysis buffer/PrepMan	Dilution1/10
2014	Thomsen & Willerslev	Theoretical								
2014	Chestnut et al.	Bd	Ponds	0.35	0.22 Sx	60mL Syringe			Gentra Puregene Tissue Kit	Phosphate Buffered Saline
2014	Johnson & Brunner	RV		1	0.45 PVDF	Pump/Syringe	Freeze	Yes	Gentra Puregene Tissue Kit	
2014	Wimsatt et al.	Bd	Stream	0.32	0.8 Cellulose	Pump	Freeze		Fast DNA spin Kit	
2014	Rees et al.	Review							Several kits	
2013	Schmidt et al.	Bd	Ponds	0.6	0.2 Sx				Gentra Puregene Tissue Kit	Bovine Serum Albumine
2012	Hyman & Collins	Bd	Ponds	0.6	0.22 Sx	60mL Syringe	PBS/Freezer		Gentra Puregene Tissue Kit	Bovine Serum Albumine
2011	Strand et al.	Crayfish			3 PCTE		Freeze		CTAB	Bovine Serum Albumine
2011	Goldberg et al.	Amphibians	Stream	5.0-10	0.45NC	Pump	Ethanol		Dneasy BTK Qiagen+Qiagen Multiplex PCR	
2007	Walker et al.	Bd	Pond/Sediment	<1	0.45NC	50mL syringe	Freeze		MoBio Power Soil DNA	
2007	Kirshtein et al.	Bd	Pond/Sediment	<2.3	0.2 Sx	Pump	Freeze		Gentra Puregene Tissue/Ultraclean Soil DNA	MoBiocleanup/Generereleaser

Fig. S1. Review of manuscripts used to gather different methodologies and technics relevant for this research. *Batrachochytrium dendrobatidis* (Bd), Ranavirus (RV), Nitrate cellulose (NC), Sterivex (SX), Glass fibre (GF), Polycarbonate track-etched (PCTE), Polyvinylidene Fluoride (PVDF) and Internal Positive Control (IPC).

Table S2. Equipment needed per locality (1 filter or 20 swabs) for two people. Note that the following costs are not included: Gear disinfection, personal wages or salaries, perishable materials e.g. ladle, buckets, or any traps. It has to be highlighted that eDNA methodologies are more environmentally friendly as they use less plastic.

Gear Item (Prices in euros)	Lastra González et al. 2020 (eDNA)	Spitzen-van der Sluijs et al. 2020 (eDNA)	Swabs with QIAGEN	Swabs with PrepMan
Syringe	0.23	0.29	0	0
Filter	11.6	63	0	0
Silica	0.88	0	0.15	0.15
Falcon tube	1.08	0	0	0
Gloves (7.5cent/ud)	0.3	0.3	6	6
Bioline Meridian Bioscience Kit	3.5	0	0	0
Qiagen Blood and Tissue Kit/PrepMan	0	0	62.2	8.1
NucleoSpinSoil Macherey-Nagel Kit	0	4.3	0	0
Swabs (0,19 cents/ud) MWE	0	0	3.8	3.8
Internal Positive Controls (ThermoFisher)	1.5	0	0	0
Eppendorf for sampling (0,03/ud)	0	0	0.6	0.6
Ethanol 96%	0.06	0.97	0	0
Disposable plastic bags (individually placed amphibians)	0	0	1.3	1.3
Whirl-Pak bags	0	0.3	0	0
ATL Buffer Qiagen	0	0.32	0	0
Roche Master Mix	2.6	0	0	0
Environmental Master Mix	0	2.56	0	0
Standard Master Mix (0.36 eur/sample)	0	0	7.2	7.2
Total	21.75	72.04*	81.25	27.15

*According SPYGEN laboratories, it should be added a mandatory fieldwork training (80 euros/ person) and the costs of processing the samples (350 euros/ filter with two replicates). For that reason, other costs (e.g. conservation buffer) related to Spitzen-van der Sluijs et al. 2020 eDNA approach are impossible to calculate precisely. In any case, it is a conservative estimate.

Table S3. Ct values corresponding to the storage methods experiment where *Batrachochytrium salamandrivorans* (*Bsal*) primers were tested from two different articles. Internal Positive Control (IPC) just included in the first analyses to discard PCR inhibition. Control filter (CF), Silica gel filter (Sil), Longmire's buffer (LB), Ethanol (EtOH). Blank space means not qPCR positive detection.

Storage method	Primer's Ct values from Blooi et al. 2013	Storage method	Primer's Ct values from Spitzen-van der Sluijs et al. 2020
CF1	39.4	CF1	39.93
CF1		CF1	
CF1	IPC	CF1	39.31
CF2		CF2	
CF2		CF2	
CF2	IPC	CF2	
CF3	36.68	CF3	38.44
CF3	37.93	CF3	36.1
CF3	IPC	CF3	35.97
LB1	39.5	LB1	36.47
LB1	35.67	LB1	34.8
LB1	IPC	LB1	
LB2	35.52	LB2	36.07
LB2	35.82	LB2	36.69
LB2	IPC	LB2	36.58
LB3	35.62	LB3	36.83
LB3	35.54	LB3	38.3
LB3	IPC	LB3	36.93
Sil1	33.07	Sil1	34.71
Sil1	33.98	Sil1	34.62
Sil1	IPC	Sil1	34,56
Sil2	36.58	Sil2	36.64
Sil2	37.17	Sil2	36.83
Sil2	IPC	Sil2	33.47
Sil3	32.77	Sil3	33.94
Sil3	32.66	Sil3	33.97
Sil3	IPC	Sil3	34.32
EtOH1	34.77	EtOH1	36.69
EtOH1	34.63	EtOH1	35.64
EtOH1	IPC	EtOH1	36.11
EtOH2	32.53	EtOH2	34.03
EtOH2	32.95	EtOH2	34.31
EtOH2	IPC	EtOH2	33.81
EtOH3	32.23	EtOH3	33.74
EtOH3	31.66	EtOH3	33.75
EtOH3	IPC	EtOH3	33.22

Table S4. Comparison between our results and an independent university as control from eDNA filters.

Locality	University of Veterinary and Pharmaceutical Sciences Brno*	Trier University*
Ampuero	10.81	130
Teverga 1	96.55	978
Teverga 2	22.35	650
Ruente 1	Negative sample	84
Suances	96.05	528
Ponga	24.2	227
Cieza	33.3	1149

*Numbers are DNA copies

Table S5. Volumes, pore size filter, storage method and Ct value of each filter. Number of filters (N). Distances within Ruente and Teverga localities are at least 5 km.

Locality (N)	Volume filtered (mL)	Pore size (µm)	eDNA Storage Method	Ct value (Mean of wells)
Ampuero	400	0.22	Longmire's Buffer	36.92
Teverga 1	1000	0.45	Silica gel	33.62
Teverga 2	650	0.45	Silica gel	33.63
Teverga 3* (2)	74/88	0.45/0.45	Longmire's Buffer	36.75/36.70
Ruente 1 (2)	1000/1000	0.22/0.45	Silica gel	36.76/Negative
Ruente 2	325	0.22	Longmire's Buffer	37.76
Suances (2)	123/158	0.22/0.22	Longmire's Buffer /Silica gel	35.85 /36.38
Ponga* (2)	1000/409	0.45/0.22	Longmire's Buffer	39.88/35.71
Cieza	138	0.45	Longmire's Buffer	35.24

*Two different water habitats but close to each other. Numbers in brackets are number of filters.

Discussion

Emerging infectious diseases are today more than just a scientific trend drawing fleeting attention. They affect our daily habits and even our freedom of movement. There has been much discussion about the various factors that could increase the troublesome events related to those novel diseases. Frequently, a loss of biodiversity increases disease transmission (Keesing et al., 2006) and therefore may impact on host population sizes, which again might alter trophic interactions and food webs (Preston and Johnson, 2010). These different role interactions are present also in the ecology of chytrids. Greener et al. (2020) have shown just recently how diversity of parasites can contribute positively to amphibian diversity. Their study explains that a diversity of *Bd* isolates affects the subsequent infection dynamic. Thus, amphibians that have been in contact with some isolates that are less virulent than previously known, are less susceptible to infection by more virulent *Bd* isolates or even to *Bsal*. A similar case that has been studied deeply is that of Chestnut blight, a parasitic fungus, *Cryphonectria parasitica*, that infects chestnut trees. In Europe, considering the nature of hypovirulent strains, natural and artificial dissemination has resulted in the restoration of economically valuable chestnuts (Anagnostakis et al., 1998). On the other hand, another example illustrates how parasites have modified competition and may influence and change species composition. The spiny toad (*Bufo spinosus*) could spread in Guadarrama National Park (Spain) after a strong decline of the common midwife toad (*Alytes obstetricans*) due to chytridiomycosis (Bosch and Rincón, 2008). Lastly, the most known negative impact of parasites on biodiversity is seen in the declines and even extinctions of amphibian populations caused by *Bd* (Kilpatrick et al., 2010; Scheele et al., 2019).

This dissertation has explored the reach of another parasite to the amphibian populations in the Czech Republic and other parts of Europe, *Batrachochytrium salamandrivorans* (*Bsal*). In less than a decade since its discovery in 2013 (Martel et al., 2013), *Bsal* has been able to provoke the near extinction of the Dutch fire salamanders (Spitzen-van der Sluijs et al., 2013). Due to that capacity, early detection and global mapping of *Bsal* is fundamentally important. Our mapping efforts and monitoring of *Bsal's* host species have come from different regions and sources: the Czech Republic in Central-East Europe and Spain in Southern Europe,

and from both wild and captive amphibian populations. Although these areas have different biological features and species composition, the collected data can be seen as milestones to check the health status of unstudied populations and for increasing the knowledge about this emerging pathogen. Many new questions continue to be raised.

The persistence and transmission of *Bsal* need further research. As was stated for *Bd*, *Bsal* can be attached to bird scales by passive adherence of floating zoospores and this enables persistence of *Bsal* in amphibian habitats (Van Rooij et al., 2015; Stegen et al., 2017). Other non-amphibian hosts could exist. As has been seen for *Bd*, it is likely that arthropods could act as reservoir (McMahon et al., 2013). Furthermore, less susceptible amphibian species remain infected for longer periods than do most of the vulnerable species, such as *S. salamandra*, which succumb in 7 days. Species like *Ichthyosaura alpestris* or *Alytes obstetricans* could act as reservoir hosts (Stegen et al., 2017).

Issues concerning the role of anuran vectors and spread of *Bsal* should be addressed, and not just for wild European species but in the amphibian trade, too. A high risk of pathogen spillover from anurans carrying *Bsal* has been tested in *Bombina microdeladigitora* (Nguyen et al., 2017) and in *Osteopilus septentrionalis* (Towe et al., 2020). This previous example also encompasses the most likely way of long-distance *Bsal* dispersal: the global amphibian trade. This issue was previously discussed, and this text includes a summary of the European regulations and restrictions applied (see above). Just one infected individual could trigger an outbreak and introduce the disease in a *Bsal*-free country (Kriger and Hero, 2009). *Bsal* is present in the amphibian trade (Martel et al., 2014; Nguyen et al., 2017) and has been found in captive collections in the UK, Germany, and Spain (Cunningham et al., 2015; Sabino-Pinto et al., 2015; Fitzpatrick et al., 2018).

Import prohibition and restriction of the trade in all salamanders have been suggested as comprising the sole most effective mitigation action against *Bsal* (Grant et al., 2017). A transport ban removes altogether the threat of introducing known and novel diseases (Kriger and Hero, 2009; Garner et al., 2016), although a ban on the trade in caudates has been suggested to stimulate illegal trade (Garner et al., 2016). At the same time, release of non-native animals into a garden pond or directly into the wild involves a high risk of pathogen spillover to native

amphibians, as well as to other vertebrate classes, and this already has provoked outbreaks in wild populations (Martel et al., 2020).

Last but not least, I do not want to forget another recent problem that has been detected once *Bsal* arrives to a wild amphibian population. As mentioned above, the ability of *Bsal* to hit and collapse a population within a short time period is alarming. Subsequently, to find amphibian *Bsal* hosts is made difficult (Lötters et al., 2020) as they succumb to the pathogen. Therefore, tracking the status and the infection dynamic is challenging. Solutions and strategies for monitoring the pathogen independently of its host are going to be of essential importance for proper management and timely implementation of conservation measures (see Chapter 4). Undoubtedly, environmental DNA-based techniques, *in situ* pathogen detection, or portable, real-time devices for DNA sequencing may serve as the fundamental diagnostic technologies for pathogen detection, and this will force researchers and conservation authorities to revise those standards and protocols already established. Laboratory techniques, like histopathology, must be redefined as they will of course be useful diagnostic methods, albeit not imperative to confirming a *Bsal*-positive site (Thomas et al. 2018; Lötters et al., 2020) given that it is host dependent. If any stakeholders are not able to adapt themselves to the incessant new scenario provoked by *Bsal,* then a common response and mitigation measures may arrive too late for the amphibians.

Conclusions and further research

This dissertation thesis provides new insights into the current situation of the pathogen *Batrachochytrium salamandrivorans* in Europe while focusing upon its status in the Czech Republic and Spain.

Toward those ends, the author of this thesis has been involved in collecting more than 2,350 samples by skin swabs from amphibians and 47 by water filtration of aquatic habitats. Amphibian monitoring has been carried on in both wild amphibians and captive collections (e.g., zoos, hobbyists, private breeders, and university collections). Thus, more than 60 localities have been visited in nature and approximately 20 captive collections. As a result of these samplings, 17 *Bsal* samples have been detected and 27 for *Bd.*

Therefore, this thesis has several major outcomes that should be highlighted:

(i) Expanded knowledge on chytrid fungi distribution. Several countries included in this research had not been visited to check for presence of *Bsal.* **(Chapters 1-3)**

(ii) First *Bsal* detection in wild amphibians in Spain which, to date, has not been related to a spillover from captive populations and therefore has important implications due to its long distance from the *Bsal* outbreak core in Western Europe (i.e., the Netherlands, Belgium, and Germany). **(Chapter 3)**

(iii) First dual detection of both chytrid fungi by eDNA methods. **(Chapter 4)**

(iv) Detection of *Bsal* in *Lissotriton helveticus*, thus disproving the previously known resistance to *Bsal.* **(Chapter 3)**

This collection of samples has been effectuated in six European countries, in cooperation with many NGOs and political institutions, and through active collaboration with amphibian breeders, conservationists, and other amphibian researchers.

In detail, these include the following:

- ✓ University of Veterinary and Pharmaceutical Sciences in Brno
- ✓ State Veterinary Association of the Czech Republic
- ✓ Natura servis s.r.o
- ✓ Nature Conservation Agency of the Czech Republic

- ✓ Environmental Ministry of the Czech Republic
- ✓ Zoos: Prague Zoo (CZ), Zoo Santillana del Mar, BIOPARC Valencia and Fundación Oceanogràfic Valencia (Spain).
- ✓ NGOs: Red Cambera, FAPAS, SEO-Birdlife Cantabria; Naturea Cantabria. (Spain)
- ✓ Regional Government of Asturias (Spain)
- ✓ Regional Government of Cantabria (Spain)
- ✓ Environmental Ministry of Spain (currently MITECO)
- ✓ Environmental Hydraulics Institute Cantabria (Spain)
- ✓ University of Trier (Germany)
- ✓ University of Tennessee (USA)

Due to that close cooperation with some of these institutions, the Czech University of Life Sciences has been involved in cutting-edge projects related to the topic of this dissertation. For example:

The University of Veterinary and Pharmaceutical Sciences in Brno, the State Veterinary Association of the Czech Republic, and the Czech University of Life Sciences Prague have been funded by the Technological Agency of the Czech Republic (TACR) to carry on the project "Protection of amphibian biodiversity in connection with invasions of new infectious diseases."

Also, the Spanish NGO Red Cambera and the Czech University of Life Sciences Prague have achieved a partnership agreement within the framework of the project "*BsALert*, interregional assessment of the effects by the salamander killer fungus *Batrachochytrium salamandrivorans* in protected / vulnerable amphibians of the mountains of the Cordillera Cantábrica (Northern Spain)," supported by Fundación Biodiversidad, of the Ministry for Ecological Transition and the Demographic Challenge of the Spanish Government.

Nevertheless, many questions on the ecology and infection dynamics of *Batrachochytrium salamandrivorans* remain open and further research is essential.

From this perspective, during the four-year period of work on my Ph.D. dissertation, I was involved in other still ongoing projects related to amphibian diseases. These projects are not yet finalized (and some were even cancelled due to the coronavirus outbreak) and therefore were not included into this thesis, but I would like to include some brief comments on these proposed ongoing and future plans.

In order continuously to expand the knowledge about *Bsal* distribution, this year we are going to enlarge our study area in Spain, and, as part of the Ph.D. work of another teammate, samples from Italy have been collected and were analyzed just prior to the writing of this thesis.

As described above (**see Discussion**), *in situ* detection of chytrids could be a preventive measure allowing for prompt action to reduce the impact of the pathogen in amphibian populations. Currently, the author of this thesis is involved in the refinement and testing of a loop-mediated isothermal amplification (LAMP) system. In combination with eDNA (**see Chapter 4**), these technologies could result in faster and more economical detection that could greatly facilitate the detection of *Bsal* (and also *Bd*) and be linked to citizen-science projects.

In addition, the author is part of the ongoing project SWAMP - Responsible water management in built-up areas in relation to the surrounding landscape.

A current participation not yet complete is the author's involvement (and therefore that of the Czech University of Life Sciences Prague) in an international round robin test to assess the most relevant laboratories in amphibian diseases detection and which is coordinated by the Long Island University (USA).

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Curriculum Vitae

David
Lastra González

DATE OF BIRTH:
08/04/1989

CONTACT

Nationality: Spanish

Kamýcká 129, Praha-Suchdol
16500 Prague, Czechia

lastra_gonzalez@fzp.czu.cz

(+420) 777206189

Skype: davidlg3d

WORK EXPERIENCE

10/2016 – CURRENT – Czechia

PhD student

Czech University of Life Sciences Prague

Related papers published

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

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Lastra González D., Baláž V., Chajma P. & Vojar J. (2020): Surveying for *B atrachochytrium salamandrivorans* presece in Spanish captive collections of amphibians. Diseases of Aquatic Organisms, 1422:99-103

10/2020 – CURRENT – Czechia

Technician

Czech University of Life Sciences Prague

Team member of the project SWAMP - Responsible water management in built-up areas in relation to the surrounding landscape funded by the Ministry of Education, Youth and Sports.

05/2020 – CURRENT – Czechia

Scientific laboratory technician

Czech University of Life Sciences Prague

Laboratory coordinator within the partnership agreement with Red Cambera for the project *BsALert*, interregional assessment of the effects by the salamander killer fungus *Batrachochytrium salamandrivorans* in protected / vulnerable amphibians of the mountains of the Cordillera Cantábrica (Northern Spain)" supported by Fundación Biodiversidad, of the Ministry for Ecological Transition and the Demographic Challenge of the Spanish Government

04/2020 – CURRENT – Czechia

Technician

Czech University of Life Sciences

Team meamber of the project "Protection of amphibian biodiversity in connection with invasions of new infectious diseases" funded by the Technological Agency of the Czech Republic (TACR)

2016 – 2019 – Spain

Forestry Engineer

TRAGSATEC

Forest disease detection and checking status of forests in Cantabria Region, partner of International Co-operative Programme on Assessment and Monitoring of Air Pollution Effects on Forests operating under the UNECE Convention on Long-range Transboundary Air Pollution.

EDUCATION AND TRAINING

2007 – 2011 – Spain

Degree in Environmental Sciences

University of Salamanca

2011 – 2014 – Spain

Forestry Engineering and Environmental

University of León

07/2010 – 09/2012 – Spain

Internship in CETYMA (Environmental Consulting)

CETYMA

08/2010 – Spain

Internship in Zoo Santillana del Mar

Foundation Zoo Santillana del Mar

2013 – 2014 – Czechia

Erasmus in Forestry Engineering

Czech University of Life Sciences Prague

07/2014 – 10/2014 – Czechia

Erasmus for Placements in Ecology Department

Czech University of Life Sciences Prague

CONFERENCES AND SEMINARS

Related conferences

2020 World Congress of Herpetology (Dunedin, New Zealand) Oral presentation

2019 Kostelecké na Inspirování (Kostelec nad Cernymi Lesy, Czechia) Oral presentation

2019 European Herpetology Congress (Milano, Italy) Oral presentation

2019 Czech Herpetological Society Conference (Prague, Czechia) Oral presentation

2018 Zoological days (Prague, Czechia) Organizing comitee member

2018 Emerging amphibian diseases symposium (Zoological Society of London, UK) Poster contribution

2018 Iberian Herpetological Conference (Salamanca, Spain) Oral presentation

2017 European Herpetology Congress (Salzburg, Austria) Poster contribution

HONOURS AND AWARDS

Awards and grants

Winner of Diploma Thesis Extraordinary Awards 2013/2014 (University of León, Spain)

Winner (2017, 2018, 2019) Internal Grant Agency Faculty of Environmental Sciences (Czech University of Life Sciences Prague, Czechia)

6th best research of the year 2019 (Czech University of Life Sciences Prague, Czechia)

TEACHING AND SUPERVISION ACTIVITIES

Teaching and supervision activities

2018 Supervision of MSc student, Barbora Thumsová, Faculty of Environmental Sciences, Czech University of Life Sciences Prague

2020 Supervision of MSc student, Martina Ugrinovic, Faculty of Environmental Sciences, Czech University of Life Sciences Prague

LANGUAGE SKILLS

MOTHER TONGUE(S): Spanish

OTHER LANGUAGE(S):

English

Listening	Reading	Spoken production	Spoken interaction	Writing
C1	C1	C1	C1	C1

Czech

Listening	Reading	Spoken production	Spoken interaction	Writing
A2	A2	B1	B1	A2

French

Listening	Reading	Spoken production	Spoken interaction	Writing
A1	A2	A2	A2	A1

NETWORKS AND MEMBERSHIPS

Former member of AEGEE (European Students' Forum)

Part of organizing team of the massive event AgorAsturias 2015