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Monitoring of the pathogen *Batrachochytrium salamandrivorans* in captive populations in Spain

Master thesis

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DIPLOMA THESIS ASSIGNMENT

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Nature Conservation

Thesis title

Monitoring of the pathogen Batrachochytrium salamandrivorans in captive populations in Spain

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Objectives of thesis

The recently discovered fungal pathogen Batrachochytrium salamandrivorans (hereinafter referred to as Bsal) has already received significant scientific and public attention. The Bsal epidemic has so far been limited to European newts and salamanders found in the wild (in Belgium, Germany and the Netherlands) and in captive populations (in United Kingdom and recently in Spain). 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. Therefore, the thesis will be focused on monitoring of the pathogen in Spain, mainly in urodelan amphibians in captivity. In the case of confirmation of Bsal presence, the appropriate measures to mitigate spread of the fungus will be proposed.

Methodology

The thesis is focused on monitoring of the pathogen in Spanish amphibian collections. Within each collection, only subset of about two to four individuals will sampled from an aquarium. The goal is to collect about 150 samples of at least five collections. This sampling will be realized from November 2017 to final of January 2018 and will be carried out non-destructive skin swab from individuals (followed by ”Hygiene Protocol for Bsal Fieldwork and Amphibian Husbandy”). The samples will be transported to Czech University of Life Science and there analyzed by PCR for Bsal absence/presence. Sampling and DNA extraction will be performed according to procedures used in amphibian chytridiomycosis research.

The proposed extent of the thesis

ca 40 pages

Keywords

Chytridiomycosis, amphibian conservation, amphibian diseases, captive amphibians, PCR

Recommended information sources

BLOOI, M., F. PASMANS, J. E. LONGCORE, A. SPITZEN-VAN DER SLUIJS, F. VERCAMMEN & A. MARTEL (2013): Duplex Real-Time PCR for rapid simultaneous detection of Batrachochytrium dendrobatidis and Batrachochytrium salamandrivorans in amphibian samples. – Journal of Clinical Microbiology, 51: 4173–4177.

BOYLE, D. G., D. B. BOYLE, V. OLSEN, J. A. MORGAN & A. D. HYATT (2004): Rapid quantitative detection of chytridiomycosis (Batrachochytrium dendrobatidis) in amphibian samples using real-time Taqman PCR assay. – Diseases of Aquatic Organisms, 60: 141–148.

COUNCIL OF EUROPE. Convention on the Conservation of European Wildlife and Natural Habitats, 35th meeting; 2015 Dec 1–4; Strasbourg, France. Recommendation No. 176 (2015) on the prevention and control of the Batrachochytrium salamandrivorans chytrid fungus [cited 2017 Mar 23].

LAKING, A. E., H. N. NGO, F. PASMANS, A. MARTEL & T. T. NGUYEN (2017): Batrachochytrium salamandrivorans is the predominant chytrid fungus in Vietnamese salamanders. – Scientific Reports, 7: 44443.

MARTEL, A. et al. (2013): Batrachochytrium salamandrivorans sp. nov. causes lethal chytridiomycosis in amphibians. – Proceedings of the National Academic of Sciences USA, 110: 15325–15329.

MARTEL, A. et al. (2014): Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. – Science, 346: 630–631.

SPITZEN-VAN DER SLUIJS, A. et al. (2016): Expanding distribution of lethal amphibian fungus Batrachochytrium salamandrivorans in Europe. – Emerging Infectious Diseases, 22: 1286–1288.

STEGEN, G., et al. (2017): Drivers of salamander extirpation mediated by Batrachochytrium salamandrivorans. – Nature, 544: 353–356.

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DECLARATION

I hereby declare that I wrote this thesis entitled „Monitoring of the pathogen *Batrachochytrium salamandrivorans* in captive populations in Spain“ independently, under the direction of doc. Ing. Jiří Vojar, Ph.D. and under the consultant specialist of David Lastra González. I have listed all literature and publications from which I have acquired information.

In Prague, 10. 12. 2018 ----------

Barbora Thumsová

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My sincere thanks belong in the first place to my supervisor of doc. Ing. Jiří Vojar, Ph.D., especially for his approach, patience, trust, motivation and that he was always there for me when I needed. Furthermore, I would like to give my thanks to my consultant David Lastra González, who was on the line and prepared to help me with all of the doubts I had almost 24 hours a day. Also I am sending many thanks to the Internal Grant Agency of the Faculty of Environmental Sciences, which supported the whole project under the no. 20184247 („Monitoring of the pathogen *Batrachochytrium salamandrivorans* in Czech and Spanish captive collections and threatened wild populations of caudate amphibians“). During the sampling in Spain I met a lot of people, which, without knowing me, gave me full confidence and lent me a helping hand. Thus, I am sending special thanks to Spain as well mainly to David, Vicente, Sergio, Cati, Jose Mari and to all of the collaborating parties and keepers who gave me access to theirs collections. Many people helped me during the data collection. I can not imagine that I could make everything alone, so thanks to Martin, Estefania, Fenia and Andrea, we were really good team. For the language support I am very grateful to my friends Ing. Eva Karbanová and her husband Ing. Václav Karban, because without them it never would have been possible to finish the writing part of this thesis. I am also thankful to my new family I found in Spain, so thank you Carmen and Luis for giving me a new home. Sincerely I can say, that the whole work was a very good experience for me, one which brought me a lot of new friends and which made me much stronger.

I dedicate this diploma thesis to my mother Radka and to my father Petr: you were my greatest psychical as well as financial support. Without your love and approach I never would have been, where I am now.

ABSTRACT AND KEY WORDS

Chytridiomycosis is emerging and fatal amphibian skin disease and it has been identified as a major driver of amphibian declines and extinctions worldwide. It is caused by two similar fungi – *Batrachochytrium dendrobatidis* (*Bd*) and recently discovered *Batrachochytrium salamandrivorans* (*Bsal*). *Bsal* is serious threat for European caudate species, causing significant mortality and morbidity within living specimens of salamanders. Until now it was identified in wild populations of salamanders in the Netherlands, Belgium, Germany, Spain and in kept salamander populations in Germany, the United Kingdom and Spain. Spain, together with Germany and the Czech Republic, has a sizable community of exotic pet keepers and the country is important in the amphibian pet trade which is considered as the main cause of the pathogens‘ transmission. In Spanish captivity, *Bsal* was first time detected as part of passive surveillence in 2015 but the active monitoring of this pathogenic chytrid fungus was still missing. Therefore, this diploma thesis is aimed on monitoring of *Bsal* in Spanish captive collections. As part of this work 287 samples from 7 Spanish captive collections of amphibians (mostly composed of urodeles species) were taken and 249 samples were analysed in the laboratory of Czech University of Life Sciences (CULS) by using the standard PCR. *Bsal* absence has been confirmed in all of the analysed samples (the results will serve as a basis for a manuscript for a scientific journal). Furthermore, two agreements were concluded about the collaboration in *Bsal* research between CULS, BIOPARC Valencia and Fundación Oceanogràfic de la Comunitat Valenciana. Outside the scope of this diploma thesis, I was participating in the monitoring of the pathogen *Bsal* in Spanish wild populations, where our team confirmed the further spreading of *Bsal*. From those results a manuscript was prepared which has been submitted to Emerging Infectious Diseases journal (IF – 7,422). The text of the manuscript can be found in Appendix 3.

Keywords: chytridiomycosis, amphibian conservation, amphibian diseases, captive amphibians, PCR.

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1. INTRODUCTION

Amphibians live on our planet more than 360 millions years (Baruš & Oliva 1992). During the 20th century, the first amphibian declines have been observed at former field sites (Collins & Storfer 2003), and amphibians are recently the most endangered species from the group of vertebrates (Stuart et al. 2004). Among the IUCN Red List of Threatened Species, 41% of the more than 7,500 known amphibian species are considered as a threatened (IUCN 2015), and around 80% of the monitored populations demonstrate a decreasing trend (Houlahan et al. 2000; Baillie et al. 2010). Furthermore, it was established that amphibians are declining more rapidly than either birds or mammals (Stuart et al. 2004).

The habitat loss, its fragmentation and degradation is considered to be the main cause of amphibians‘ decreases (Hamer & McDonnell 2008). In addition, climate change, increase of UV-B radiation, introduction of invasive species and diseases are recorded as others threats contributing to the wild populations‘ exctinctions as well (Collins & Storfer 2003). Therefore, one of the biggest threats to amphibians is emerging and fatal amphibian skin disease of chytridiomycosis which has been identified as a major driver of amphibian declines and extinctions worldwide (Lips et al. 2006). It is associated with disruptions of amphibian skin, following with dysfunction in the exchange of the respiratory gases, water and electrolytes. Consequent osmotic imbalance leads to cardiac arrest and the death of the animal (Voyles et al. 2009).

Chytridiomycosis is caused by two similar fungi – *Batrachochytrium dendrobatidis* (*Bd*) (Berger et al. 1998; Longcore et al. 1999) and *Batrachochytrium salamandrivorans* (*Bsal*) (Martel et al. 2013). Well known *Bd* has been first described in 1999 (Berger et al. 1998; Longcore et al. 1999), and up to now, it has caused massive amphibian population declines globally (Mutschmann 2015; Grant et al. 2016). Moreover, it is able to infect various species of this class, including the individuals of anurans, urodeles, and caecilians orders (Mutschmann 2015). The pathogenic fungus of *Bsal* has been discovered recently (Martel et al. 2013) and actually is causing several die-offs in European salamander populations (Spitzen-van der Sluijs et al. 2016). *Bsal* unlike *Bd* has slightly different morphology and infects different amphibian hosts (Berger et al. 2016). It has been documented, that this

occurs mainly in post-metamorphic urodeles (salamanders and newts), but recently was shown *Bsal*‘s ability to infect anurans as well (Stegen et al. 2017).

The fungus of *Bsal* was most likely introduced to European wild populations from East Asia via pet trade (Spitzen-van der Sluijs et al. 2016). Firstly, it was detected in wild population of *Salamandra salamandra* in the Netherlands and after that in Belgium (Martel et al. 2013; Spitzen-van der Sluijs et al. 2013). In addition, it has since been found at others places across Germany (Spitzen-van der Sluijs et al. 2016) and Spain (Lastra González et al. *in prep.*, see Appendix 3). Moreover, it has been identified in captive urodeles collections in Germany (Sabino-Pinto et al. 2015), the United Kingdom (Cunningham et al. 2015) and in Spain (Fitzpatrick et al. 2018). It has been noted, that in Europe, one of greatest amphibian richness, as well as one of the highest concentration of the threatened and endemic salamanders‘ species, is in countries of Southern Europe (including Spain or Italy) (Temple & Cox 2009). Therefore, the *Bsal* expansion to those localities would have the fatal consequences for its wild populations (Richgels et al. 2016; Yap et al. 2017).

The risk of pathogen spillover to native fauna can be reduced through sanitary measures in the live amphibian (Nguyen et al. 2017). For that reason, The Bern Convention Standing Committee has announced Recommendation No. 176 (2015) in order to prevent and control *Bsal* chytrid fungus. It ensures to accept, by European countries, the preventive measures like the monitoring programmes of the pathogen in high risk areas. Furthermore, on February 2018, the EU laid down, by Comission Implementing Decision 2018/320, certain animal health protection measures for import and intra-Union trade in salamanders. It is providing for the appropriate quarantine, diagnostic testing and treatment of living urodeles individuals. In addition it is establishing the obligation to always have the certification of health status of the imported/traded salamanders.

As stated above, recent studies showed the positive results in the wild (Lastra González et al. *in prep.*, see Appendix 3), as well as in one captive collection of urodeles (Fitzpatrick et al. 2018) in Spain. In view of the fact, that Spain, together with the Czech Republic and Germany, has a sizable community of exotic pet keepers, and the country is very important in the amphibian pet trade (UNEP-WCMC 2016), it is necessary to focus on this country and to start with intensive control of

the currently state of *Bsal* distribution. Therefore, the active monitoring of this pathogenic chytrid fungus in captive collections of Spain was established as the main goal of this thesis.

1.1. AIMS OF THE THESIS

Considering that the current state of knowledge about the *Bsal* presence is limited (Fitzpatrick et al. 2018; EFSA AHAW Panel 2018), the effective method to reduce the impact of this pathogen in wild populations is still missing (Garner et al. 2016). That being said, it is essential to prevent its spreading (Cuningham et al. 2015) and start to focus on its detection across the European wild populations as well as captive collections (EFSA AHAW Panel 2018). Therefore, the main objectives of the diploma thesis are:

(i) within the literature review describing the main characteristics of chytridiomycosis (focus especially on *Bsal*);

(ii) summarising the existing law regulations and proposing measures for the salamanders‘ protection from the *Bsal* introduction/spreading;

(iii) monitoring of the pathogen in Spanish captive amphibian collections (sampling carried out non-destructive skin swab from individuals, during November 2017 until January 2018; the goal is collecting of about 150 samples of at least 5 collections; further participation on PCR analysis to *Bsal* absence/presence, performed at the laboratory in the Czech University of Life Sciences;

(iv) suggestion of proposal mitigation measures (in the case of *Bsal* presence confirmation).

Outside the scope of my diploma thesis, I am going to participate on the monitoring of the pathogen *Bsal* in Spanish wild populations of newts and salamanders and on preparation of a manuscript, as the output of the project (target scientific journal Emerging Infectious Diseases, IF – 7,422 (see Appendix 3).

2. LITERATURE REVIEW

Amphibian populations are in decline worldwide and the devasting fungal infection of chytridiomycosis belongs to major drivers of their extinctions (Martel et al. 2013). This disease was first described in 1999 as responsible for the disappearance of many amphibian individuals in Central America and Australia (Berger et al. 1998; Longcore et al. 1999). Nowadays, it is distributed globally (Fisher et al. 2012) and records extinctions in more than 200 species of amphibians from all over the world (Martel et al. 2013).

Chytridiomycosis affects the vital function of amphibian skin and it is caused by the fungal pathogens of *Bd* and *Bsal*. It has been described as the worst infectious disease ever recorded among vertebrates in terms of the number of species impacted (Skerratt et al. 2007; Berger et al. 2016). Moreover, it represents a real threat to amphibian species diversity on our planet (Gascon et al. 2007). For the better understanding of this issue, in the first part of the following text, the main characteristics of *Bsal* (its transmission, origin, distribution, host range, symptoms, diagnosis, treatment) will be specified. After that, the second part will focus on regulations applied to reduce negative impacts of *Bsal* to salamander populations.

2.1. MAIN CHARACTERISTICS OF *BSAL*

Basic characteristics

Bsal, as well as *Bd*, belongs to the phylum of Chytridiomycote. Currently, they are placed in the class of Chytridiomycetes and in the order of Rhizophydiales (Longcore et al. 1999; Hibbett et al. 2007; Martel et al. 2013; Van Rooij et al. 2015). Generally, the chytrid fungi are dependent on the water and their desiccation is fatal for both of them (Van Rooij et al. 2015). It is possible to find them in aquatic environments and soils as a free-living or commensal organisms. Mostly, they are the parasites of algae, invertebrates, fungi and plants (Fisher et al. 2009). Moreover, *Bd* and *Bsal* are known like almost the unique two Chytridiomycota with the ability to infect vertebrate hosts. The next one, which can parasites on vertebrates, is called *Ichthyochytrium vulgare*, and it affects freshwater fishes (Martel et al. 2013).

Both pathogens are characterised by two main life stages. They are an infectious aquatic zoospore stage and a sedentary zoosporangium stage. The first stage is an infective stage, where the free-living zoospores use their flagella for moving (between hosts or within a host). After the contact with the skin of an amphibian, they encyst and enter keratinized skin cells (Martel et al. 2013; Yap et al. 2017). The second life stage is a growth stage wherein asexual zoospores develop into a thallus and produce termed zoosporangium which reaches a size of 15,7–50,3 μm (in the case of *Bsal*). The thallus can be monocentric (forming one zoosporangium) or colonial (forming multiple zoosporangia along internal septa). *Bsal* thalli are mainly monocentric and the colonial thalli are more abundant, than those of *Bd* (Martel et al. 2013; Van Rooij 2015). In addition to that, the ability of *Bsal* to produce the other type of spores has recently been discovered. These spores are non-motile and just float on water's surface (Stegen et al. 2017).

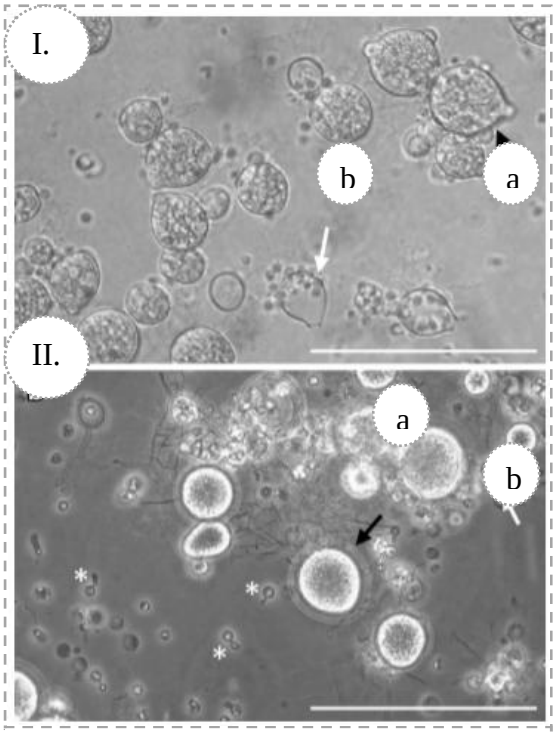

Fig. 1: *Batrachochytrium's morphology in culture.*

I. *Bd*, a- abundant mature zoosporangia with zoospores, b- empty, discharged zoosporangia.

II. *Bsal*, a- predominant monocentric thalli, b- few colonial thalli, *- zoospore cysts with germ tubes.

Scale bars 100 μm. Taken from Van Rooij et al. 2015.

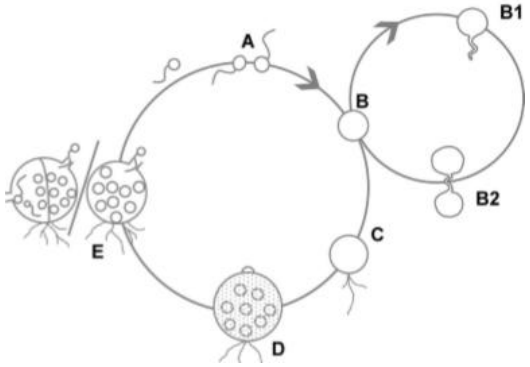

Fig. 2*: The lifecycle of Batrachochytrium species in culture.*

A- flagellated motile zoospores;

B- encysted zoospore;

B1- germling with germtube;

B2- transfer of the cell contents into a newly formed thallus;

C- zoospore cyst with rhizoids;

D- immature sporangium;

E- mature monocentric zoosporangium with discharge tube (at the right), colonial thallus containing several sporangia, each with their own discharge tube (at the left).

Bd A–E (without B1 and B2), *Bsal* A–E (including B1 and B2).

Taken from Van Rooij et al. 2015.

Transmission of the pathogen

Bsal pathogen can be transmitted either directly, or indirectly via healthy carrier animals by encysted spores, motile zoospores or contaminated soil (EFSA AHAW Panel 2017). The direct transmission can occur intraspecies (between individuals of the same salamanders‘ species), interspecies (different salamanders‘ species) or between salamanders‘ and anurans‘ species (Stegen et al. 2017; Nguyen et al. 2017; EFSA AHAW Panel 2017). Specifically wild birds, frogs, toads or wild mammals are considered to be *Bsal* carriers and they play an important role in its spreading (EFSA AHAW Panel 2018). It is mainly the human beings that significantly affect the pathogen transmission, for instance during pet trade (Schloegel et al. 2009; Auliya et al. 2016) or even during research and conservation purposes (because of non-complying with the health conditions) (Civiš et al. 2010; EFSA AHAW Panel 2018).

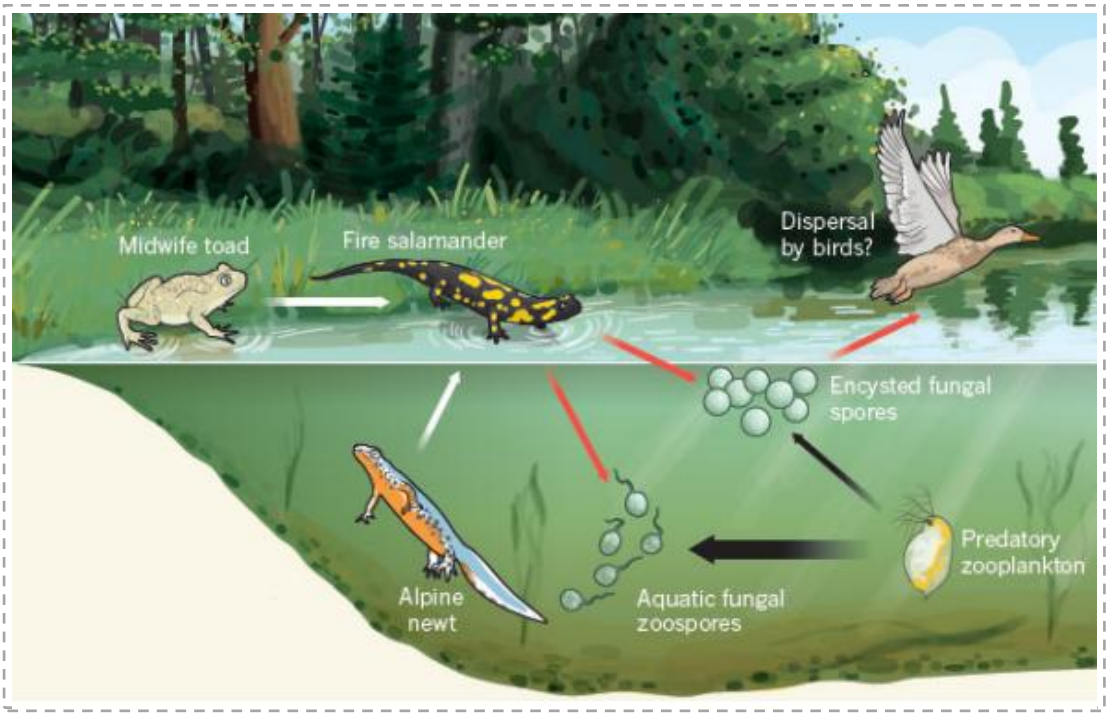

Fig. 3: *The possible means for Bsal spread and persistence in wild (taken from EFSA AHAW Panel 2018).*

The origin of the pathogen and its distribution

Phylogenetic analysis showed, that the pathogen of *Bsal* diverged from *Bd* almost 67,3 million years ago in the late Cretaceous or early Paleogene. Given its detection

in a more than 15–year–old museum sample of the Asiatic newt *Cynops ensicauda*, the origin of this fungus is hypothesized to be in Asia (Martel et al. 2014). It was confirmed by finding, that *Bsal* is probably endemic in Vietnam, because its geographically widespread in local salamanders‘ populations at low prevalence (2,9%) was recorded there. Moreover, these Vietnamese species have the ability to resist the infection, so they may serve as a reservoir hosts of *Bsal* (Laking et al. 2017; Yap et al. 2017).

Bsal was likely imported to Europe via pet trade. Until now there has been no detection outside either Asia or Europe (Martel et al. 2014; Spitzen-van der Sluijs 2016). *Bsal* was first isolated from the skin of infected European fire salamanders (*Salamandra salamandra*), following a mass die-off event in the locality of Bunderbos in the Netherlands (Spitzen-van der Sluijs et al. 2013). Unfortunately, there is still a lack of knowledge about the possible source of *Bsal* contamination during the Netherland's outbreak (EFSA AHAW Panel 2018). Nevertheless, it is predicted, that this country could be the initial point of *Bsal* entry into wild populations of European salamanders (Martel et al. 2014). Probably from there, the pathogen expanded to Belgium and afterwards to Germany. Until 2016, the range of *Bsal* distribution in wild may have been up to 10 000 km^2 across Western Europe (Spitzen-van der Sluijs 2016). Nevertheless, the recent studies confirmed further spreading of the *Bsal* fungus. It has now been also detected in another locality of Germany, following the massive mortality event of fire salamander in Essen (Dalbeck et al. 2018). Furthermore, the first positive findings were recorded in population of *Lissotriton helveticus* in Spain as well (Lastra González et al. *in prep.*, see Appendix 3). Additional monitoring programmes were realised, but without confirmation of *Bsal* presence (Spitzen-van der Sluijs 2016). Its presence in the wild was not also proved in some localities of Austria (Gimeno et al. 2015), in the Czech Republic (Baláž et al. 2018), on the Balkan Peninsula (Lastra González et al. *in prep.*, see Appendix 3), or in USA (Parrott et al. 2016).

Beyond, there have been announced positives results of *Bsal* in captive urodeles in the UK (Cunningham et al. 2015), Germany (Sabino-Pinto et al. 2015) and the Netherlands (Sabino-Pinto et al. 2018). Further, the pathogen was detected in one Spanish captive collection after the import of infected animals from the UK (Fitzpatrick et al. 2016; Fitzpatrick et al. 2018).

Fig. 4: *Actual distribution of Bsal in wild populations (taken from EFSA AHAW Panel 2018).*

Fig. 5: *Positive detection of Bsal in captivity (taken from EFSA AHAW Panel 2018).*

According to niche modelling (based on *Bsal* native records), regions susceptible to colonisation by this chytrid fungus were identified. Its results showed, that the distribution range of European salamanders overlaps with climate conditions, considered as a suitable for *Bsal* (Beukema et al. 2018). Furthermore, niche climatic model demonstrated its predicted spread and expansion. It was estimated across Europe, some parts of North African Mediterranean shore and Anatolia. That means, that the Western Palaearctic salamanders‘ wild populations are in an increasing risk of *Bsal* invasion (Beukema et al. 2018; EFSA AHAW Panel et al. 2018).

Host range and symptoms of the disease

As previously stated, the chytridiomycosis has been recorded in all three orders of amphibians (anurans, urodeles and caecilians). While pathogen of *Bd* can cause mortality both in anurans and salamanders (Olson et al. 2013; Yap et al. 2017), *Bsal* is probably pathogenic for most Palaearctic salamander and newt taxa (Martel et al. 2013; Yap et al. 2017). Furthermore, the capability of *Bsal* pathogen to infect anurans as well has recently been discovered. Although they are not susceptible to disease, they pose a threat to salamanders‘ species as infectious carriers (Stegen et al. 2017).

Response of the animal to the infection by pathogen of *Bsal* can vary. The clinical outcome of the infection depends on the amphibian host, the fungal virulence and the environmental conditions (Van Rooij et al. 2015). According to host response, amphibians are classified into following categories: resistant (no infection, no disease), tolerant (infection, any symptoms of the disease), susceptible (infection, symptoms of the disease with possibility of recovery) and lethal (infection following into a lethal disease) (see the Table 1) (Martel et al. 2014). The animals of the categories „tolerant“, „susceptible“ and „lethal“ are considered, from the epidemiological point of view, as potentional carriers of the *Bsal* pathogen. The example of the amphibians, that can serve as a potentional reservoirs of this fungus, are some Asiatic species (*Cynops cyanurus*, *Cynops pyrrhogaster* and *Paramesotriton deloustali*). They are able to coexist with *Bsal* without developing any clinical signs of the disease, therefore they may be a threat for the other species (UNEP-WCMC 2016). Beyond, the most threatened are European and American species belonging to the family of Salamandridae (European and American species),

because they were documented as mostly lethally susceptible to this pathogenic chytrid fungus (Martel et al. 2014). The species of the family Ambystomatidae were assigned as a resistant (Martel et al. 2014), but it should be interprated with caution, because the study to *Bsal* susceptibility is based on results from a small number of study animals (EFSA 2017). The overview of tested species, divided according to their response to the pathogen presence, showed in detail in Table 1.

Category	Family	Species
Resistant	Ambystomatidae	*Ambystoma maculatum*
		Ambystoma opacum
	Hynobiidae	*Hynobius retardatus*
		Pachyhynobius shangchengensis
	Plethodontidae	*Gyrinophilus porphyriticus*
		Plethodon glutinosus
	Salamandridae	*Lissotriton helveticus*
Tolerant	Hynobiidae	*Salamandrella keyserlingii*
	Sirenidae	*Siren intermedia*
Susceptible	Salamandridae	*Cynops cyanurus*
		Cynops pyrrhogaster
		Paramesotriton deloustali
Lethally susceptible	Plethodontidae	*Euproctus platycephalus*
	Salamandridae	*Ichthyosaura alpestris*
		Lissotriton italicus
		Neurergus crocatus
		Notophthalmus viridescens
		Pleurodeles waltl
		Salamandra salamandra
		Salamandrina perspicillata
		Taricha granulosa
		Triturus cristatus
		Tylototriton wenxianensis

Table 1: *Amphibian susceptibility to Bsal according to Martel et al. 2014 (taken from EFSA 2017).*

As previously explained, the effects of the chytridiomycosis to the amphibian host can be different and the signs of *Bsal* infection can be missing until the final stage of the disease. Metamorphosed urodelans invaded by *Bsal* mostly display the disease by multifocal superficial erosions and extensive epidermal ulcerations all over the body

(see Fig. 6). Further erosive vs hyperplastic/hyperkeratotic skin lesions and cutaneous haemorrhages can develop (Martel et al. 2013; EFSA 2017).

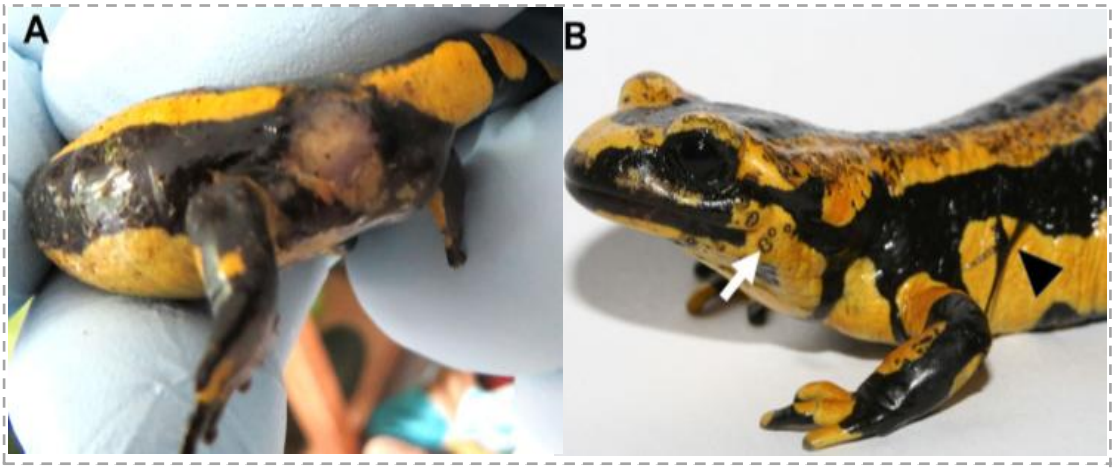

Fig. 6: *Typical skin lesions caused by Bsal; A– Salamandra salamandra bernadezi, B– Salamandra salamandra fastuosa (taken from Sabino-Pinto et al. 2015).*

The clinical sings of the infection may be anorexia, apathy or lethargy, but frequently the animal can die spontaneously without any signs of disease (Martel et al. 2013; Van Rooij et al. 2015). The duration of the infectious period, the mortality rate and the time, at which the death can occur, can differ across species. The duration of infectious period varies and is ranging from 2 weeks to more than 4 months, at a constant temperature of 15°C and in controlled experiments (in nature it is not known) (Stegen et al. 2017; EFSA AHAW Panel 2017). While the mortality of healthy susceptible individual can come in 2 weeks, the infected lethaly susceptible specie can die within 7 days (Martel et al. 2013; EFSA 2017).

Diagnosis

Chytridiomycosis caused by *Bsal* is diagnosed on the presence of numerous intracellular colonial thalli, that spread all over the epidermis. Methods that are useful to detect the pathogen include histological examination, microscopy, polymerase chain reaction (PCR), isolation and culture (Blooi et al. 2013; Martel et al. 2013; White et al. 2016; Yap et al. 2017; EFSA 2017). While microscopy, histological and culture method require biopsy (e.g. toe clipping), the PCR provides non-invasive testing from swab samples. That is why the PCR is considered as the best method used for screaning in living specimens (Blooi et al. 2013; Martel et al. 2013). A duplex real-time quantitative PCR (qPCR) method allows to estimate the

number of infective zoospores (Blooi et al. 2013; Yap et al. 2017). In fact, the PCR and culturing methods can only confirm the occurrence of *Bsal*, but not automatically the presence of the infection or disease. Therefore, the definitive diagnosis must include positive results from PCR (or culture) and histopathology as well, that can provide support for the chytridiomycosis (White et al. 2016; Yap et al. 2017).

For the analysis, it is possible to use skin swabs, toe and skin clipping, or whole juvenile individuals (Civiš et al. 2010). But the preferred method to survey for *Bsal* infections is non-invasive sampling using skin swabs. This can be provided by amphibian‘s skin rubbing by a disposable cotton swab. After the swabbing, the cotton swab has to be reinserted into the protective case and placed into a refrigerator set to the temperature of 4°C. Bodies of dead animals should be conserved frozen, or in 70% EtOH. During the fieldwork and laboratory activities it is always necessary to follow the hygiene protocol created as a prevention of pathogen spreading (Hyatt et al. 2007; Civiš et al. 2010).

Treatment

As the *Bsal* was discovered recently, the information about the infection treatments are limited. There is only a record of inexpensive procedures to eliminate *Bsal* in captive animals. It has been demonstrated, that the temperature is a determining factor for pathogens growing (Blooi et al. 2015a; Yap et al. 2017). *Bd* can grow at 10–25°C with optimal growth at 17–25°C and it is starting to die from 30°C (Davidson et al. 2003; Piotrowski et al. 2004). *Bsal* has lower thermal characteristics than *Bd* and is capable to optimal growth at 15–20°C (Blooi et al. 2015a), but recently has been shown, that it is able to persist in ponds with water temperatures between 20–25°C (Laking et al. 2017). The study of Blooi et al. (2015) demonstrated, that the heat treatment is a viable option for *Bsal* disposal, therefore the infected animals being exposed to temperatures of 25°C for a 10-day period is enough for disease and infection elimination (Martel et al. 2013; Blooi et al. 2015a). Furthermore, the study of Blooi et al. (2015b) specifies another method to remove the infection. It may be the application of the fungicides polymyxin E and voriconazole for 10 days at an ambient temperature of 20°C. This suggests that the animals with lower heat tolerance can benefit from this kind of combined treatment (Yap et al. 2017). In any case, the election of suitable treatment has to be taking with

consideration the clinical stage of the disease and the animals thermal tolerance (Martel et al. 2013; Blooi et al. 2015a). It is important to realise that it should be interpreted with caution, because all experiments for *Bsal* treatment were only conducted on one host specie (*Salamandra salamandra*). Therefore more studies, to determine species-specific infection dynamics, are needed (Yap et al. 2017).

2.2. PROTECTION FROM *BSAL* SPREADING

This chapter is focused on presenting of protection activities, including the law regulation (at EU and national level) and risk-mitigating measures proposed as a prevention from *Bsal*.

2.2.1. LAW REGULATION

The comercial trade is considered to be likely the main cause of *Bsal* entrance to new geographical regions (Gray et al. 2015; Yap et al. 2015; Auliya et al. 2016). The problem is, that only 3.4 % of amphibian‘s species are currently listed in CITES Appendices or EU wildlife Trade Regulations-Annexes (EFSA 2017). That means, the majority of trade in amphibians is not regulated (Auliya et al. 2016). Even in many European countries, such as Austria, Germany, Italy, the Netherlands, Poland, Spain and the UK is possible to find online pet trade with southeast Asian newts (Rowley et al. 2016), which are considered as a potentional reservoir of this pathogen and could be a serious threat for the other amphibians (Martel et al. 2014; UNEP-WCMC 2016). China, the United States, Hong Kong-SAR and Japan are estimated to be the main providers to EU-28 of caudata species listed in CITES Appendices and/or EU wildlife Trade Regulations-Annexes between 2005–2015 (EFSA 2017). Furthermore, a lack of trade data is reported (Yap et al. 2015; Rowley et al. 2016; EFSA 2017) which means, that in CITES database the majority of individuals imported to EU-28 in 2005–2015 for „commercial purpose“, were traded from unknown sources (EFSA 2017). Because the threat of *Bsal* spreading via international pet trade of Asiatic amphibians is high, some authors have pointed out trade-bans as a necessary tool for risk management (Yap et al. 2015; Rowley et al. 2016; EFSA 2017; Nguyen et al. 2017).

It is important to be aware that illegal activities with the animals exist as well, for example collecting of animals within nature reserves, laundering of wild-caught animals as captive-bred (Auliya et al. 2016; EFSA 2017) and ilegal trade (Rowley et al. 2016; EFSA 2017). In accordance with the fact that more than 95% of the world's amphibian species commercial trade is not regulated and the trade data is lacking. Therefore, the import restrictions to limit pathogen invasion should be the priority. Enacting legislation, that requires control measures, hygienic procedures, restricting salamander movements, can ensure safe pet trade of amphibians (EFSA 2017). For that reason some countries, including the whole EU, adopted some prevention and management activities to stop the *Bsal* spreading (UNEP-WCMC 2016).

Law regulations in European Union

For the prevention and control of the *Bsal* chytrid fungus, the Bern Convention Standing Committee announced Recommendation No. 176 (2015). According to this Convention, the signatories accepted a number of precaution to prevent the pathogen spreading. It includes applying biosafety rules during the working with wild or captive animals, establishing monitoring programmes to control the possible spread of disease and developing emergency action plans (in case of the *Bsal* occurence).

Furthermore, European Food Safety Authority (hereinafter referred to as EFSA) has prepared for request of European Comission three scientific opinions and technical assistances about *Bsal*, to summarize the contemporary knowledge and to demonstrate the risk of pathogen spreading without adopting more stringent measures for its prevention and control. These documents are based on the critical analysis of the available data and include concretes proposals of mitigation measures. They propose for example restricting salamander movements, the requirment to test the animals to demonstrate freedom from *Bsal* or hygienic biosecurity measures before and during movements etc. (EFSA AHAW Panel 2017; EFSA 2017; EFSA AHAW Panel 2018).

In 2017, the infection of *Bsal* was listed in Aquatic Animal Health Code by The World Organisation for Animal Health (hereinafter referred to as OIE), as a disease affecting amphibian individuals (http://www.oie.int/fileadmin/Home/eng/About_us/docs/pdf/Session/2017/A_FR_2017_public.pdf, cit. 20. 9. 2018). But no further international standards are available yet; thus, there is still a lack of

information. Therefore, according to proposals of the EFSA, the European Comission announced the decision (EU) 2018/320 of 28 February 2018, to lay down salamander health protection measures for intra-Union trade, in order to ensure that the pathogen is not spreading anymore and to get more available data about this chytrid fungus. It requires diagnostic testing of salamanders, the certification of their health status for the trade in or introduction into the Union and the annual reporting. The decision defines 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*. We expect that more information will be available, so this decision is aplicable only for transitional period, which is until 31th of December 2019 (EU 2018/320).

Before the EU laid down this Implementing Decision, for example Belgium arranged the legal basis for prevention of *Bsal* spreading, including prohibition of Asian salamander species import (UNEP-WCMC 2016). Besides realising its own Action Plan, including preventive measures and recommendations of best practice (SPF Public Health 2017).

National law regulations – examples

USA

North America is the world hot spot of species diversity of salamanders. Therefore, the introduction of *Bsal* to USA could likely be devasting for these wild populations (Richgels et al. 2016; Yap et al. 2017). Although the pathogen has not yet been detected in the USA (http://www.salamanderfungus.org/wp-content/uploads/2018/06/News-Release-Bsal-Rapid-Response-Plan-Now-Available.pdf, cit. 22. 9. 2018), for the safety of local salamanders‘ richness, the U. S. Fish and Wildlife Service (hereinafter referred to as USFWS) declared 201 species of salamanders as „injurious wildlife“ under the Lacey Act in 2016 (UNEP-WCMC 2016). It is an interim rule, which lays down pet trade bans for species listed as injurious. They can not be imported to USA, or transported between the continental U.S., the District of Columbia, Hawaii, the Commonwealth of Puerto Rico or any territory or possession of the USA, by any means without the authorized permission (permits) granted by USFWS (18 USC 42-USFWS 2016). Following the *Bsal* spreading through the EU continent, Association of Fish and Wildlife Agencies (hereinafter referred to as

AFWA) prepared The *Bsal* Rapid Response Plan template. It provides tools to facilitate preparation for the potentional *Bsal* outbreak, and the actions to minimaze impacts of the disease. This plan has been started as a product of colaboration between the AFWA Amphibian and Reptile Conservation Committee and the *Bsal* Task Force, which includes participation from Canada, Mexico and USA (http://www.salamanderfungus.org/wp-content/uploads/2018/06/News-Release-Bsal-Rapid-Response-Plan-Now-Available.pdf, cit. 22. 9. 2018).

Switzerland

Switzerland drew on example from the USA and in summer of 2015, the country temporarily banned the importation of salamanders and newts to protect their amphibian native biodiversity (Schmidt 2016).

Canada

The Canadian Wildlife Authorities prepared the assessment of the threat posed by *Bsal* fungus, which identified two species (*Taricha granulosa* and *Notophthalmus viridescens*) as the most vulnerable to the infection (Stephen et al. 2015). In May 2017, Canada joined USA, and restricted all salamander imports under the law number SOR/2017-86 (Yap et al. 2017).

2.2.2. PROPOSED RISK-MITIGATING MEASURES

Following measures were proposed as part of scientific opinion published by EFSA. The working group is presenting control efforts that should be established as a feasible and effective protection from further *Bsal* introducing/spreading (EFSA AHAW Panel 2018).

To ensure safe international and intra-EU pet trade EFSA AHAW Panel 2018 recommended:

- preventative heat treatment based on temperature increase in a terrarium/aquarium, where the animals are kept at 25°C for 10 days;
- premovement health certification as an acknowledgement of the *Bsal* pathogen absence;

- quarantine, which implies keeping of the traded salamanders at entrance point in isolation conditions for 6 weeks (incubation time of *Bsal* in *Salamandra salamandra* specie);
- testingfor *Bsal* before importation at the entry point, including keeping traded animals for the necessary time of one week to carry out the *Bsal* testing;
- ban and restriction of salamanders‘ import, based on progress in the knowledge about this pathogen;
- identifying amphibians‘ shipments by a unique code to gather complete movement data and trade records;
- hygiene procedures (as cleaning and desinfection of equipment);
- guidelines with a good practice code for traders.

For the protection of captive individuals was proposed (EFSA AHAW Panel 2018):

- the screening of kept collections and newly obtained individuals for *Bsal* presence/absence;
- identification and treatment of the infected individuals according to the reported protocol (see chapter of 2.1);
- increasing of the keepers‘ awareness by providing disease information for breeders and stores via Internet, presentations etc.;
- developing guidelines and hygiene protocols as a manual for the right management and used equipment in salamanders‘ keeping/selling;
- registration of the keepers and pet-shops that kept/traded the salamanders (information about location, kept species, number of animals kept);
- training courses for the breeders, based on correct management of amphibians.

For wild salamanders‘ protection EFSA Panel AHAW 2018 designed:

- site definition and visitation, including identification of potential risks for the spread, habitat and population characteristics;
- hygiene procedures as cleaning and desinfication of field worker body parts and equipments, using of disposable materials when animals are handled;

- avoiding/minimising the capture, handling and housing of wild salamanders;
- translocation of potentionally infected amphibians;
- prevention of the return of captive individuals into the wild, which means that salamanders‘ keeper should not release animals kept to the wild;
- public awareness and participation increasing (education campaign, information sign installation, informational flyers);
- active and passive surveillance, where the active surveillance is based on the populations screening and testing for *Bsal* presence/absence, whereas the passive one involves the collection and analysis of dead individuals (could be complied with by setting of emergence teams);
- wild population monitoring for actual data completion and acquiring.

Moreover, it is recomended to introduce a harmonised protocol for *Bsal* detection throughout the EU; forbid any movement of the animals with *Bsal* known health status; increase the data based on actual information about abundance and distribution of salamanders and enhance the public awareness. According to the analysis and evaluation of each risk-mitigation measures, the ban and restriction on salamanders importation; hygiene procedures; manuals of best practice; identification; treatment of positive collection; translocation of wild individuals; increasing of salamanders keepers‘ awareness to do not release kept salamanders into wild and support of passive surveillance through setting emergency teams, were considered as the most feasible and effective (EFSA AHAW Panel 2018).

Several authors are adding the urgency for the instalment of measures, as quarantine and entry controls, not only for urodeles, but also including anurans. As it was described before, because anurans are considered to be a vector of *Bsal*, they could be potentional threat for the susceptible and lethally susceptible species, thus their testing for *Bsal* presence/absence is important as well (Nguyen et al. 2017; Stegen et al. 2017).

3. METHODOLOGY

3.1. PROJECT PLAN

Project summary

The objective of this diploma thesis was the active monitoring of the pathogen *Bsal* in captive collections of Spain. The thesis represents results of 249 analysed samples of 5 private keepers and 2 public institutions (Fundación Oceanogràfic de la Comunitat Valenciana and BIOPARC Valencia) from three autonomous communities of Spain, namely Andalusia, Catalonia and Valencia. The particular contacts were mainly found on social networks or they were recomended after the first samplings. Before data collection, all of the breeders were informed about the method to be used. Sampling has always taken place in the home of the keeper, or in the place, where each collection was placed (garage, cellar). The whole process was conducted in a non-invasive manner (non-invasive skin scrapings) and it was followed by the hygiene protocol for disease control proposed by Hyatt et al. (2007). After that, the samples were sent to the laboratory at the Czech University of Life Sciences (CULS), where they were analysed for the presence of *Bsal*'s DNA by standard PCR. When the analyses were done, the results of each collection were gathered to aggregated tables (Table 4; Appendix 1) and then they were presented to respective owners. The results are published under conditions that ensure the anonymity of each of the collaborating parties. In following sub-chapters, the whole process is specified in more details.

Acquiring of contacts

Because of the different conditions, language and generally an unknown environment, in which I appeared, the initial stages provided some difficulties. In the first place, it was important to perform research of urodeles' keepers. Within public sector they were found and later contacted two ZOOs (BIOPARC Valencia, ZOO in Barcelona) and one aquarium (Fundación Oceanogràfic de la Comunitat Valenciana) specialized in urodeles keeping. Two of them were interested in colaboration. The institutions like Fundación Oceanogràfic and BIOPARC Valencia, among other things, are focused on *Pleurodeles waltl* breeding (https://www.oceanografic.org/especie/gallipato/;https://www.bioparcvalencia.es/gallipato-anfibio-bioparc-

valencia/, cit. 3. 10. 2018), which is considered as a specie lethally susceptible to *Bsal* chytrid fungus (Martel et al. 2014). Therefore, its sampling was important for *Bsal* absence confirmation. Besides, Fundación Oceanogràfic is very active in conservation *in-situ* of this species. Hence, they provided for the analysis of their samples from wild populations as well (the results of this experiment are, however, out of scope of this thesis).

Afterwards, in term of the Spanish urodeles‘ pet trade analysis, petshops offering the sale of live urodeles on the Internet were contacted (see Table 2), with the intention to test the health of their animals. Unfortunately, no interest has been recorded.

Website	Type of site
http://www.animalcenter.es/	Amphibian importer/wholesaler
http://www.bichosfera.com/	
http://tierraexotica.es/	
http://reptilesyanfibiosonline.blogspot.co.uk/	
http://exofauna.com/	

Table 2: *Caudate species offered for sale on website within the Kingdom of Spain (taken from UNEP-WCMC 2016).*

During the search for collaborating parties, the reptile and amphibian market in Madrid has been visited. This event, called Expoterraria, is the most important within amphibian and reptile pet trade across southern Europe (https://expoterraria.es, cit. 4. 10. 2018). It is taking place approximately five times per year, alternatively in Barcelona and in Madrid. Flyers with the information about *Bsal* and the possibility of free testing for its presence (see the Appendix 2) were handed out during the event. Not many people were interested in the issue. The rest of the contacted cooperating breeders were found through social media in closed community, which is bringing together the people keeping/selling/changing salamanders and newts in the area of Spain. In addition, some of the collectors were recommended by currently colaborating keepers, some even contacted the research team from their own initiative. All of the selected collectors were owners of extensive collections of not only urodeles species breeded for various reasons (mostly for hobby, sale or exchange between each others). Access to private collections was always realised

with the permission of the owner of each collection, which was obtained under the condition of anonymity.

3.2. DATA COLLECTION

Target species

The samples were collected in autumn and winter of 2017–2018, more precisely between 11. 11. 2017 and 13. 3. 2018. The tested species were chosen according to their response to *Bsal* pathogen. In particular, the sampling was focused on caudata individuals tolerant to infection as determined by Martel et al. (2014, i.e. *Salamandrella keyserlingii*, *Siren intermedia*) and on the asiatic species (*Cynops cyanurus*, *Cynops pyrrhogaster* and *Paramesotriton deloustali*, see in Appendix 1). These species are considered as potentional reservoirs of the *Bsal* fungus, therefore they could be a serious threat to other individuals (EFSA 2017) and their sampling should be mandatory.

Afterwards salamanders species susceptible and lethally susceptible to disease were sampled, as for example *Ichthyosaura alpestris*, *Lissotriton italicus*, *Pleurodeles waltl* – see more in Appendix 1 (Martel et al. 2014), to confirm the absence of the pathogen, or failing that, so that the treatment could be started in time. Withs regard to recently discovered capability of *Bsal* pathogen to invade anurans too (Stegen et al. 2017), the samples from some of the anura species were taken as well (for example *Bufotes latastii*, *Alytes muletensis*, *Bufo spinosus* etc., see Appendix 1). Because there is still a lack of information about the anurans and their role in this fungus epidemiology (Nguyen et al. 2017), it is necessary to not underestimate any possibility. The species outlined by Martel et al. (2014) as resistant (for example *Ambystoma maculatum*, *Hynobius retardatus* etc., see the Table 1) to the infection were not tested. The one exception was *Lissotriton helveticus*. Although it was noted by Martel et al. (2014) as a specie resistant to *Bsal*, recently the presence of this pathogen was detected in wild populations of both Germany and Spain (Dalbeck et al. 2018; Lastra González et al. *in prep.*, see Appendix 3). Therefore, when this specie was presented in a collection, it was sampled as well. The majority of the sampled species (for example *Triturus dobrogicus*, *Triturus carnifex*, *Triturus*

marmoratus etc., see Appendix 1) were chosen for the unknown category of their susceptibility to this pathogen, to ascertain whether they are infected or not.

Sampling

The sampling was realised in various locations of Spain, including Catalonia, Valencia and Andalusia. Within each collection only a subset of about 2–3 individuals from an aquarium was taken. The sampling was conducted in a non-invasive manner (non-invasive skin scrapings) and it was processed following the hygiene protocol for disease control (Hyatt et al. 2007). Those principles were published in order to be used for safe fieldwork and amphibian husbandry to ensure that the potentional infection will not be transmitted. For this reason, the handling of each animal was done with disposable materials (powder-free vinyl gloves and dry cotton swab tip MW113). Alternatively, between samplings in different aquariums, all equipments used were subjected to proper desinfection procedures. The skin of each living individual was rubbed firmly, 10 times on the abdomen, 10 times on the ventral tail and 10 times on the underside of a foot. Living animals were captured individually, swabbed within a few minutes, and immediatly returned back to their reservoir. Each amphibian was thoroughly inspected for visual signs, potentionally indicating chytridiomycosis, as well. Dead individuals were sampled by removing a piece of their body part.

Afterwards, the cotton swab/part of the dead animal was inserted into sterile protective case with grains of silica gel (used as a sample cabinet). All of the protective cases were identified by a specific mark and kept in an impervious container. The information about the individual‘s life stage (adult/subadult), sex and number of animals sharing its aquatic environment were also recorded in order to obtain a complete picture. The information about the specific origin of the animal was considered as important as well, but given the size of the collections it was difficult to determine (the keepers mostly did not know).

For the samples‘ transport, the impervious container was used with sufficient amount of ice. After sampling, the samples were stored frozen at –20°C until the DNA-extraction. The collected data with the results are summarised below in the Table 4 and in Appendix 1.

3.3. GENETIC ANALYSIS

The samples were sent from Spain to the Czech University of Life Sciences (CULS), where they were checked for the presence of *Bsal*'s DNA by standard PCR. This method allows to detect the presence of DNA of the pathogen, but it does not provide the amount of the fungus in samples (genomic equivalents, hereinafter referred to as GE). The amount of the GE could be estimated only by quantitative PCR (qPCR) (Blooi et al. 2013). Because the CULS laboratory does not have the equipment necessary for conducting qPCR, the positive samples would subsequently be sent to the University of Veterinary and Pharmaceutical Sciences Brno (UVPS Brno). In order to save money, prior standard PCR analysis allows to reduce the total amount of samples, which should be quantified by qPCR analysis at UVPS Brno.

The laboratory work was conducted following the detailed protocol by the Department of Biology and Wildlife Diseases from UVPS Brno. This protocol is used for detection of *Bd,* as well as *Bsal,* and it is based on the protocol used at the Zoological Society of London (Boyle et al. 2004).

Firstly, for the extraction of DNA, the sterile corresponding centrifuge tubes (2 ml) were marked and into all of them, zirconium/ silica beads (0.04–0.05 g; 0.5 mm) were weighted out. After that, within the sames tubes, the PrepMan® Ultra reagent was pipetted (for skin swab samples 60 µl; for toe clips 50 µl) and the cotton of swabs (sample collected during the fieldwork) were placed by sterile scalpel. In the next step, the samples were homogenized by using the machine MagNA lyser for 45 seconds at speed 6500 and centrifuged for 30 seconds at 14500 rpm. According to the protocol, these two steps were done twice, to ensure a complet extraction from the swab. The centrifugation was followed by putting the samples in the thermoblock (set up on 100°C for 10 minutes). After, they were cooled (around 2 minutes) and the centrifugation was done again (14500 rpm, 3 minutes). In the last step of extraction, as much supernatant as possible was collected and stored into a marked and sterile Eppendorf.

After the extraction, the reaction mix with extracted DNA was prepared. This was done via standard PCR in flow box, by using primers developed according to Martel et al. 2013 (STerF TGCTCCATCTCCCCCTCTTCA and STerR

TGAACGCACATTGCACTCTAC), further by using Taqman Universal PCR Master Mix, bovine serum albumin (BSA) and distilled water (DH_2O). Reagents were added to a screw cap tube in this order:

Reagent	per well [µl]	per 96 well plate [µl]
Taqman Universal PCR Master Mix	12.5	1200
SterF	0.625	60
SterR	0.625	60
BSA	0.2	19.2
DH_2O	1.05	100.8
Total	**15**	**1440**

Table 3: *The amount of the reagents for preparation of the PCR reaction mix.*

Besides, it was necessary to make a dilution with water, this has been done tenfold of the whole reaction mix. Therefore , to each well of full plate PCR strip 9 µl of water, 1 µl of extracted DNA and 15 µl of mixed reagents was added, the total volume of the reaction was, thus, 25 µl. Negative and positive control samples (DH_2O and 1 µl of *Bsal* zoospores respectively) were placed as last two samples on the PCR plate.

In the next step, the samples were amplified using thermocycler. The following PCR conditions were used: 2 min at 50°C, 10 min at 95°C, followed by 15 s at 95°C and 1 min at 60°C for 50 cycles.

From the amplified samples, the results of PCR could be assessed following pulsed-field gel electrophoresis, using a 1% agarose gel. To create it, we used 1.6 g of agarose and 160 ml of buffer (for the big gel of 56 wells). The small gel with 32 wells was created by mixing of 0.8 g of agarose and 80 ml of buffer. After the dissolution of agarose by warming, followed cooling at 50°C and adding 1.5 µl (for the big gel, for the small one 1 µl) of ethidium bromide (EtBr). The liquid was transfered to the Electrophoresis plate. As soon as the gel was solid, it was put 3 µl of Gene Ruler (50 bp) to the first well of each line and 8 µl of the sample to the rest of the wells. The negative and the positive controls were placed at the end of the plate. The program of the electroforesis was set up for 40 min and 135 V (conditions for the big gel, for the small one 40 min and 90 V). Results were interpreted in transilluminator (see Fig. 7).

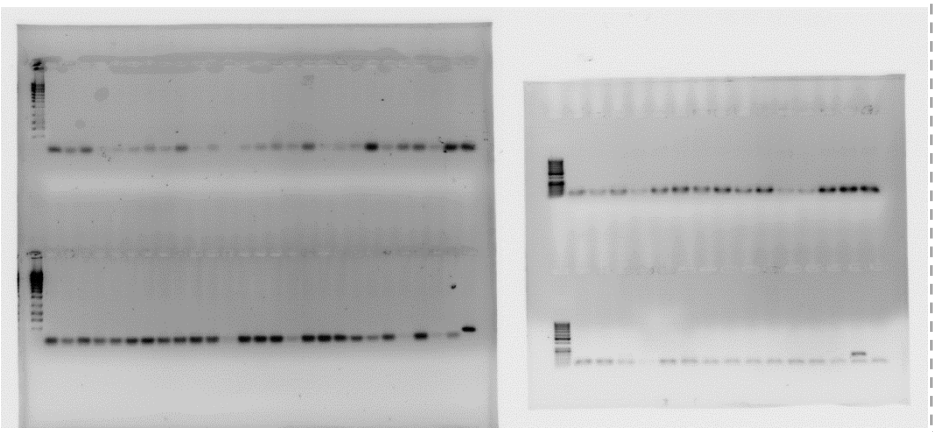

Fig. 7: *Pictures made by transilluminator shows the absence of Bsal pathogen. The positive control, down right, shows the different length comparing with all negative samples.*

4. RESULTS

4.1. PRESENCE OF THE PATHOGEN

During the swab period 287 samples were taken from 7 different sources, including private amphibian collections, the BIOPARC Valencia and the Fundación Oceanogràfic. All of the 7 keepers, as soon as the sampling methodology was explained, granted the team access to sample their animals for *Bsal* presence. Appendix 1 presents the list of species with numbers of sampled individuals. Unfortunately, sometimes, the conditions for sampling were not appropriate, thus excessively dirty individuals could not always be sufficiently rinsed off before swabbing. Therefore, some of the samples (38) contained remainders of soil, which prevented their analysing, because soil and dirt both contain potent PCR inhibitors. That means, they could make a *Bsal* positive result appear to be negative. It follows that together 249 samples were analysed.

After the samples were analysed, PCR showed positive detection only in the positive control samples. **It was thus confirmed, that all the tested samples yielded negative results for the presence of *Bsal* chytrid fungus.**

The Table 4 summarises important data about each collection, including location, the number of sampled aquariums and individuals (divided for urodeles and anurans species), number of analysed samples and results of the analysis. Unfortunately, the published data has to be limited. The sampling in private captive collection was realised on the grounds of anonymity and specific details could help to identify individual keepers. Although both of the mentioned public instituions agreed with their name appearing, they are kept anonymous in summary results as well (for uniform structure mantaining).

Collection	Autonomous community	No. of sampled aquariums	No. individuals sampled		No. samples analysed	No. *Bsal*-positive samples
			of which urodeles	of which anurans		
A	Andalusia	32	63	6	58	0
B	Andalusia	34	61	2	58	0
C	Catalonia	18	32	0	32	0
D	Catalonia	26	45	0	38	0
E	Catalonia	14	27	0	25	0
F	Valencia	8	9	8	17	0
G	Valencia	8	34	0	29	0
Total						
8	**3**	**140**	**271**	**16**	**249**	**0**
			287			

Table 4: *Numbers (No.) of individuals sampled and after analysed within 7 captive collections from three autonomous communities of Spain.*

4.2. PROPOSED PREVENTIVE ACTIONS

Absence of the *Bsal* in tested collections is a positive finding, but it does not mean that this pathogen is absent in all the captive collections in Spain, even in tested collections. There is still an urgent need to stop the further *Bsal* spreading. It should be realised via setting of and strictly complying with the preventative measures, which are mentioned above in chapter „2.2.2. Proposed risk-mitigating measures“. Moreover, to prevent the intra- and international spread of this pathogen within Spanish captive collections, it could be more specifically recommended:

- establishment of active (systematic checking and testing of all wild populations) as well as passive (opportunistic detection and testing of dead animals) *Bsal* surveillance and to bring its importance to the keepers/pet store attention;
- during the animals‘ screening the anurans species can not be underestimated either;
- providing the manuals of the best practice, sharing new knowledge about the pathogen, in short to make all the information more accessible and comprehensible (for example via the flyers, movies, or to do one

summarizing multiple language website, where could be possible to find new research results and all useful information of good practice);

- registration of all subjects keeping salamanders (keepers, pet stores, public institutions such as ZOOs, aquariums, rescue centers etc.) with specification of their location, contacts and information about the breeding of animals;
- education and training of stakeholders through courses, where the right keeping and handling will be shown;
- cooperative effort across non-governmental organisations, government agencies, scientests, ZOOs, pet shops and concerned citizens to organise joint meetings and conferences, where the topical issues can be shared and the following efforts could be proposed with the participation of all said parties;
- to ensure more cooperation between scientists and organisations, orientated on amphibian breeding, for improvement of their activities in conservation *in-situ* as well as *ex-situ* and to increase public awareness (could be provided through creation of the international campaign for amphibian protection from the spreading pathogens, such as *Bsal*, *Bd*, Ranavirus);
- more preventive measures for safe pet trade and protection of the captive as well as wild animals are mentioned in the chapter above.

5. DISCUSSION

The main purpose of this thesis was the monitoring of the *Bsal* pathogen in Spanish captive urodeles collections. When the topic of the work was suggested (the year of 2016), there were no records about positive finding of this fungus in Spain. An article about the first positive finding of *Bsal* in Spanish captivity was first published by Fitzpatrick et al. in (2018) (before that there was only an abstract from 2016, but without any specific information, Fitzpatrick et al. 2016). This study was based on analysis of received dead animals (Fitzpatrick et al. 2018).

Thus, the project presented within this diploma thesis is to our knowledge the first survey in active monitoring of this chytrid fungus in Spanish captive collections. Concerning this topic, the testimony about its absence is a positive result, and furthermore such an information is equally scientifically important as a confirmed presence would have been (Vojar et al. 2017).

When the method of obtaining the samples was explained, and anonymity was promissed, the majority of the contacted persons and organisations were willing to cooperate in this research. All of them had at least basic information about this pathogen and its negative impacts to urodeles. Nevertheless, in terms of this thesis, the keepers were familiarised with the newest research in the field, prevention measures to avoid the *Bsal* introduction to theirs collections, how to recognize and what to do, when their animals are infected. In addition, one of the private keepers had direct experience with this chytrid fungus, as its presence was detected around two/three years ago in his collection. He lost 4 individuals of *Triturus marmoratus*, when his friend brought them for him from abroad. Both of them sent the dead animals and swab samples from their whole collections for analysis to the United Kingdom. There the infection by *Bsal* has been confirmed. These results were probably published as part of the above-mentioned article published by Fitzpatrick et al. (2018). This statement can not, however, be fully verified as some facts appear to be at odds. The results of this diploma thesis has, nevertheless, shown that the particular keeper‘s collection seem to be clear from this chytrid fungus.

As part of this research, the Department of Ecology of the Faculty of Environmental Sciences of the CULS concluded two cooperation agreements. One of them was with

the BIOPARC Valencia and the second one with Fundación Oceanogràfic de la Comunitat Valenciana. Subject of these agreements is specification of the mutual co-operation in *Bsal* rescue, where CULS will be responsible for the detection of this pathogenic fungus via PCR. Moreover the BIOPARC Valencia/Fundación Oceanogràfic will act as facilitators of samples of amphibian, with the aid and supervision of the CULS. Besides, all of the organisations are involved in amphibians‘ *ex-situ* conservation, the Fundación Oceanogràfic has hitherto been actively working on amphibian *in-situ* conservation in the area of Valencian Community (https://www.oceanografic.org/especie/gallipato/; https://www.bioparc valencia.es/gallipato-anfibio-bioparc-valencia/, cit. 3. 10. 2018). Therefore the developing collaboration between CULS and aforementioned organisations is very beneficial and can bring new knowledge about the *Bsal* fungus spreading and ensure effective conservation of amphibians.

Outside the scope of this diploma thesis, I was participating in the monitoring of the pathogen *Bsal* in Spanish wild populations. In term of this research, further spreading of *Bsal* was confirmed and the infection was detected in wild population of *Lissotriton heleveticus* in Spain for the first time. I have also participated subsequently on preparation of a manuscript which has been submitted to Emerging Infectious Diseases journal (IF – 7,422). The text of the manuscript can be found in Appendix 3.

Due to lack of information (EFSA AHAW Panel 2018; Fitzpatrick et al. 2018), there is still urgent need to continue in the *Bsal* monitoring, investigating and co-operating on European emergancy action plan. Only through mutual co-operation between countries, as well as between research organisations, governments and the general public it is possible to prevent the next *Bsal* spreading and thus avoid a world-wide decline of amphibians.

6. CONCLUSIONS

(i) Amphibians are recently the most endangered species from the group of vertebrates. One of the biggest emerging threats is the fatal amphibian skin disease of chytridiomycosis (identified as a major driver of amphibian declines and extinctions worldwide), which is caused by two similar fungi – *Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrivorans* (*Bsal*).

(ii) Recently discovered pathogen fungus of *Bsal* is a serious threat for European caudate species and it is causing significant mortality and morbidity within living specimens of salamanders.

(iii) The main purpose of this thesis was the monitoring of the *Bsal* pathogen in Spanish captive urodeles collections.

(iv) Although *Bsal* was first detected in Spanish captivity as a part of passive surveillence in 2015 (Fitzpatrick et al. 2018), the active monitoring of this pathogenic chytrid fungus, is the first survey of this type to focus on Spanish captive urodeles.

(v) Focus on Spanish captive collections was necessery, because Spain together with Germany and Czech Republic has a sizable community of exotic pet keepers, and it is important in the amphibian pet trade which is considered as the main cause of the pathogens‘ transmission.

(vi) In terms of this work were:

- described the main characteristics of the *Bsal* pathogen, summarised the existing law regulations and presented risk-mitigating measures proposed as a protection from *Bsal* spreading;
- taken 287 samples from 7 Spanish captive collections of amphibians (mostly composed of urodeles species), where in each aquarium/terrarium 2–4 individuals were sampled, none from the sampled animals showed any signs of disease;
- analysed 249 samples in laboratory of CULS by using standard PCR, in all of them *Bsal* absence has been confirmed;

- proposed actions as a part of preventive measures from *Bsal* spreading/introduction;
- concluded two agreements about the collaboration in *Bsal* research (between CULS, BIOPARC Valencia and Fundación Oceanogràfic)

(vii) The results will serve as a basis for a manuscript for a scientific journal.

(viii) Furthermore, outside the scope of this diploma thesis, I was participating in the monitoring of the pathogen *Bsal* in Spanish wild populations, where further spreading of *Bsal* was confirmed and the infection was detected in wild population of *Lissotriton heleveticus* in Spain, after from these results the manuscript has been prepared and submitted to Emerging Infectious Diseases journal (IF – 7,422) (see Appendix 3).

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Appendix 1: The list of sampled animals.

Order	Family	Species	No.	Susceptibility (if it is known)[1]
Anura	Alytidae	*Alytes muletensis*	3	(no data)
	Bufonidae	*Barbarophryne brongersmai*	2	(no data)
		Bufo spinosus	1	(no data)
		Bufotes boulengeri	1	(no data)
		Bufotes latastii	1	(no data)
		Sclerophrys mauritanica	1	(no data)
	Dendrobatidae	*Dendrobates azureus*	1	(no data)
	Hyperoliidae	*Hyperolius* sp.	3	(no data)
	Mantellidae	*Mantella aurantiaca*	1	(no data)
	Pipidae	*Hymenochirus* sp.	2	(no data)
Caudata	Cryptobranchidae	*Andrias davidianus*	1	(no data)
	Hynobiidae	*Hynobius dunni*	1	(no data)
		Hynobius tokyoensis	1	(no data)
		Salamandrella keyserlingii	4	Tolerant
	Plethodontidae	*Aneides lugubris*	2	(no data)
		Aneides vagrans	2	(no data)
		Desmognathus fuscus	2	(no data)
		Pseudotriton ruber	2	(no data)
	Salamandridae	*Calotriton arnoldi*	2	(no data)
		Calotriton asper	3	(no data)
		Cynops cyanurus	4	Susceptible
		Cynops ensicauda ssp.	12	(no data)
		Cynops orientalis	4	(no data)
		Cynops pyrrhogaster ssp.	8	Susceptible[2]
		Ichthyosaura alpestris	2	Lethally susceptible
		Ichthyosaura alpestris ssp.	8	Lethally susceptible[2]
		Laotriton laoensis	4	(no data)
		Lissotriton boscai	5	(no data)
		Lissotriton helveticus	1	Resistant
		Lissotriton italicus	2	Lethally susceptible
		Lissotriton maltzani	2	(no data)
		Neurergus crocatus	3	Lethally susceptible
		Neurergus kaiseri	6	(no data)
		Neurergus strauchii	2	(no data)

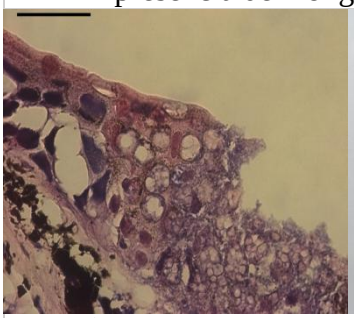
		Ommatotriton ophryticus	2	(no data)
		Pachytriton granulosus	2	(no data)
		Paramesotriton caudopunctatus	2	(no data)
		Paramesotriton deloustali	2	Susceptible
		Pleurodeles nebulosus	5	(no data)
		Pleurodeles waltl	53	Lethally susceptible
		Pleurodeles waltl x albinum	3	(no data)
		Salamandra algira	5	Linked with observed mortality
		Salamandra algira ssp.	23	Linked with observed mortality[2]
		Salamandra salamandra ssp.	24	Lethally susceptible[2]
		Salamandra atra	1	(no data)
		Salamandra infraimmaculata	2	(no data)
		Taricha torosa	1	(no data)
		Triturus anatolicus	1	(no data)
		Triturus carnifex	11	(no data)
		Triturus carnifex x cristatus	2	(no data)
		Triturus carnifex x pygmaeus	2	(no data)
		Triturus carnifex x dobrogicus	1	(no data)
		Triturus carnifex x marmoratus	1	(no data)
		Triturus cristatus	5	Lethally susceptible
		Triturus dobrogicus	13	(no data)
		Triturus dobrogicus ssp.	6	(no data)
		Triturus ivanbureschi	2	(no data)
		Triturus karelinii	3	(no data)
		Triturus macedonicus	2	(no data)
		Triturus marmoratus	6	(no data)
		Triturus pygmaeus	2	(no data)
		Tylotriton shanjing	2	(no data)
		Tylototriton verrucosus	1	(no data)
		Tylotriton yangi	2	(no data)
	Sirenidae	*Siren intermedia*	1	Tolerant
Total: 287 individuals				

[1] *Sources: Martel et al. 2014; Sabino-Pinto et al. 2015*
[2] *It was followed the fact, that subspecies susceptibility is stated as the same as their species*

Appendix 2: Flyer with the information about *Bsal* and the possibility of free testing for its presence in Spanish captive collections (*presented in Spanish*).

GRATIS **Pruebas de *Bsal*** **GRATIS**

Que es?	**Sintomas**
➔ Un patógeno llamado *Batrachochytrium salamandrivorans* (*Bsal*).	➔ Apatia, anorexia, ataxia, letargia, ulceras de la piel.
➔ Una grave amenaza para salamandras y tritones.	
➔ Causa de la mortalidad de los urodelos en Europa.	
Diagnosis	**Por que es importante?**
➔ Metodo no cruento.	➔ Disminuye riesgo de dispersion de la enfermedad.
➔ Mediante un frotis con bastoncillo de algodon.	➔ Deteccion puede salvar sus animales!
➔ Analisis moleculares de ADN (qPCR) para deteccion de la presencia del hongo *Bsal*.	➔ Vas a ayudar detectar el nivel de expansión del patogeno.


© Martel A., Pasmans F.


© Pasmans F.


© McCreary B.

Quiénes somos?	**Contactos**
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➔ Trabajamos en un trabajo fin de master (el cual trata sobre salamandras y tritones criados en cautividad en la Península Ibérica).	David Lastra Gonzalez lastra_gonzalez@fzp.czu.cz.

Más informaciones sobre *Bsal*
http://bsaleurope.com/
https://www.savethesalamanders.com/killer-fungus-disease/
http://www.salamanderfungus.org/november-2016/

Appendix 3: Scientific report about the results of our *Bsal* detection in Spanish wild populations (manuscript submitted to Emerging Infectious Diseases journal in november 2018).

Running Title: New findings of *Batrachochytrium salamandrivorans*

Keywords: chytridiomycosis, amphibians, newts, caudates, chytrid fungus, Lissotriton helveticus

Title: Recent Findings of Salamander Killer Fungus *Batrachochytrium salamandrivorans,* the Invasion Continues

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Abstract–49

Distribution of the chytrid fungus *Batrachochytrium salamandrivorans* (*Bsal*) in Europe continues to spread. We collected 1135 samples of salamanders and newts

from 6 European countries during 2014–2018. We identified 5 positives of *Bsal* within a wild population in Spain but not in Central Europe or the Balkan Peninsula.

Text–791

Chytridiomycosis, an amphibian disease caused by the chytrid fungi *Batrachochytrium dendrobatidis* (*Bd*) and *B. salamandrivorans* (*Bsal*), is responsible for declines of amphibian populations worldwide (*1*). The recently discovered *Bsal* (*2*) has severe impact on European salamanders and newts (*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 salamander pet trade from Southeast Asia (*3*). The occurrence of *Bsal* in Europe has been confirmed in the Netherlands, Belgium, and Germany in the wild and in the UK, Germany, and Spain in captive animals (*5*,*6*). Therefore, regulation of trade has been established in several countries (*5*) and the recent decision of the European Union no. 2018/320 implements measures to protect against the spread of *Bsal* by traded salamanders (*7*). Furthermore, infection with *Bsal* was listed by the World Organisation for Animal Health (OIE) in 2017. Together with the necessity to control the amphibian pet trade, there is an urgent need for surveillance of the pathogen to establishing disease intervention strategies in affected areas and prevention in *Bsal*-free regions.

Samples were collected during 2014–2018, either directly for the detection of *Bsal* or as a part of unrelated studies. Altogether, 1135 samples of 10 species at 47 sites of 6 European countries were accumulated and used to test for *Bsal*. The amphibians most surveyed were, the known suitable host, the fire salamander (*Salamandra salamandra*) and, the classified as resistant (*3*), the palmate newt (*Lissotriton helveticus,* Appendix).

Most of the samples were collected as skin swabs following the standard procedure for sampling of amphibian chytrid fungi (*8*). A smaller portion of samples was collected as toe clips (Supplementary Appendix). Genomic DNA was extracted following the protocol of Blooi et al. (*9*). Testing for the presence of *Bsal* was carried out at two laboratories with different equipment availability. All samples from Spain and the Czech Republic were initially analyzed at Czech University of Life Sciences Prague by standard polymerase chain reaction (PCR) with *Bsal*-specific primers STerF and STerR as used by Martel et al. (*2*). Subsequently, electrophoresis was

carried out on the amplified target. Any samples that produced positive or equivocal results in standard PCR were then reanalyzed by duplex qPCR for *Bd* and *Bsal* (*9*) at University of Veterinary and Pharmaceutical Sciences Brno. Trenton Garner, Institute of Zoology, Zoological Society of London, provided us with the DNA for quantification standards of the *Bd* GPL lineage, strain IA042, and An Martel, Ghent University, provided us the quantification standards of *Bsal*. The samples from other countries were directly analyzed by the qPCR. All analyses were run with negative and positive control (in PCR) or with quantification standards in each run (in qPCR). For *Bd* or *Bsal* positive sites its prevalence and its Bayesian 95% credible interval were estimated using three parallel Markov chains with 2000 iterations each, a burn-in of 1000 iterations and no thinning (Appendix). All statistical analyses were performed in R 3.3.1 using the R2WinBUGS package and WinBUGS 1.4.3 (*10*).

A total of 5 *Lissotriton helveticus* individuals tested positive for *Bsal*, which implies that this species is not resistant to *Bsal* as it had been indicated by experimental exposures (*3*). The positive cases were from Spanish populations, in the northern part of Spain, situated in isolated areas with remote human populations. Four of them are located in drinking troughs from 150 to 1000 meters above sea level in two different regions, Cantabria and Asturias. Furthermore, the other *Bsal* positive was found in a pond within a private garden, 30 km distant from the nearest other recorded *Bsal* positive. In addition, the *Bsal* positives were not found in consecutive locations during our monitoring.

To our knowledge, this is the first detection of *Bsal* in the wild in Spain, however positive cases in captive salamanders were already observed (*6*) and at the same time it is far (more than 1000 km) from any known area of the fungus's occurrence (*7*). In the samples that were analyzed by duplex qPCR, we also detected a presence of *Bd* in 11 individuals (with no co-infection in *Bsal* positive individuals) of three newts species *(Lissotriton helveticus, L. vulgaris, Triturus cristatus*) from Spain and Montenegro, and one captive *Cynops ensicauda* from the Czech Republic.

We confirm that *Bsal* continues to expand within Europe. This may confirm *Bsal'*s capability for long distance dispersal (*4*), indicate a human mediated introduction, or even point to a longer presence of the fungus within this geographical range with no detected mortalities. Our results should alert the research

and conservation community and motivate urgent action to identify all regions where there is early emergence of the disease. It is imperative at this point to implement mitigation measures to prevent further spread of *Bsal*.

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Appendix

Table. Summary of locations with additional information on sample size with the numbers of *Bd*- and *Bsal*-positive findings, prevalence for *Bd* and *Bsal* and infection intensities for *Bd* and *Bsal**.

Location	Species	N*	*Bd* +*	*Bsal* +*	Bd_{prev} (95% CI)*	$Bsal_{prev}$ (95% CI)*	*Bd(min-max GE values)**	*Bsal (min-max GE values)**
Czech Republic								
Prague	*C. ensicauda*	5	1		0.29 (0.05-0.65)	0.14 (0-0.47)	1.91	
Montenegro								
Moromish	*L. vulgaris*	35	4		0.10 (0.04-0.19)	0.02 (0-0.07)	0.28-22.25	
	T. cristatuts	22	1				1.05	
Liverovići lake	*L. vulgaris*	31	2		0.09 (0.02-0.20)	0.03 (0-0.11)	1.73-1.83	
Spain								
Suances	*L. helveticus*	22		1	†	0.06 (0.01-0.16)		0.42
	T. marmoratus	10						
Ampuero	*S. salamandra*	9			†	0.10 (0.01-0.26)		
	L. helveticus	10		1				2.73
Teverga	*L. helveticus*	62		2	†	0.04 (0.01-0.09)		0.89-4.36
	T. marmoratus	11						
Carracedelo	*L. helveticus*	5	1		†	0.06 (0-0.20)	0.54	
	T. marmoratus	11						
Ruente	*L. helveticus*	50	2	1	†	0.04 (0-0.10)	0.24	0.16

**Bd = Batrachochytrium dendrobatidis*, *Bsal = B. salamandrivorans*, N = number of samples, *Bd*+ / *Bsal*+ = number of positive samples for *Bd*/*Bsal*, Bd_{prev} / $Bsal_{prev}$ (95% CI) = prevalence for *Bd* or *Bsal* with Bayesian 95% credible intervals, *Bd*/*Bsal* (min-max GE values) = minimum and maximum genomic equivalent values for *Bd*/*Bsal* .

† Bd_{prev} (95% CI) cannot be included because just a subset of the samples were analysed by duplex qPCR

Supplementary Appendix

Supplementary Table. Locations where samples were collected along with additional information on year of sampling, origin of sampled population, sample type, sample size with the numbers of *Bd* and *Bsal* positive findings*.

Location	Country	Species	Year	Origin	SampleType	N*	*Bd* +*	*Bsal* +*
lokva Majkovi	Croatia	*L. vulgaris*	2016	W*	S*	30		
Crna Mlaka	Croatia	*L. vulgaris*	2016	W*	S*	1		
		S. salamandra	2016	W*	S*	1		
Kokořínsko	Czech Republic	*L. vulgaris*	2017	W*	S*	44		
Ústí nad labem	Czech Republic	*S. salamandra*	2016	W*	S*	17		
Prague	Czech Republic	*C. ensicauda*	2017	C*	S*	5	1	
Moromish	Montenegro	*L. vulgaris*	2016	W*	S*	35	4	
		T. cristatuts	2016	W*	S*	22	1	
Liverovići lake	Montenegro	*L. vulgaris*	2016	W*	S*	31	2	
Lovcén	Montenegro	*L. vulgaris*	2016	W*	S*	40		
Traktir-Sutorina	Montenegro	*L. vulgaris*	2016	W*	S*	33		
			2016	W*	S*	10		
Wąwóz Lipa-Chełmy Landscape Park	Poland	*S. salamandra*	2014	W*	S*	30		
Sady-Ślęża Massif	Poland	*S. salamandra*	2014	W*	S*	9		
			2015	W*	S*	2		
Złoty Stok-Śnieżnik Landscape Park	Poland	*S. salamandra*	2014	W*	S*	15		
			2015	W*	S*	3		
Jarnołtówek	Poland	*S. salamandra*	2015	W*	TC*	21		
Bielsko-Biała	Poland	*S. salamandra*	2016	W*	S*	32		
Pleśna	Poland	*S. salamandra*	2014	W*	TC*	4		
			2016	W*	TC*	30		
Góra Kamińska	Poland	*S. salamandra*	2015	W*	TC*	17		
			2016	W*	TC*	30		
Rakówka	Poland	*S. salamandra*	2015	W*	TC*	7		
Czarnorzeki	Poland	*S. salamandra*	2015	W*	TC*	24		
Trzciana	Poland	*S. salamandra*	2014	W*	TC*	2		
			2016	W*	TC*	30		
Sękowiec	Poland	*S. salamandra*	2016	W*	TC*	30		
Southern Otryt	Poland	*S. salamandra*	2016	W*	TC*	18		
Jagiellonian University	Poland	*L. vulgaris*	2016	C*	S*	5		
Remetské Hámre	Slovakia	*S. salamandra*	2017	W*	S*	15		
Ruská Bystrá	Slovakia	*S. salamandra*	2017	W*	S*	10		
Tichá Voda	Slovakia	*S. salamandra*	2017	W*	S*	18		
Ružín	Slovakia	*S. salamandra*	2017	W*	S*	5		
Modra	Slovakia	*S. salamandra*	2017	W*	S*	5		
Pezinok	Slovakia	*S. salamandra*	2018	W*	S*	12		
Bratislava	Slovakia	*S. salamandra*	2017	W*	S*	13		
			2018	W*	S*	13		
Boo de Guarnizo	Spain	*L. helveticus*	2017	W*	S*	28		
Santillana del Mar	Spain	*I. alpestris*	2017	W*	S*	10		
		L. helveticus	2017	W*	S*	1		
		A. mexicanum	2017	C*	S*	1		
Suances	Spain	*L. helveticus*	2017	W*	S*	22		1
		T. marmoratus	2017	W*	S*	10		
Valdáliga	Spain	*L. helveticus*	2017	W*	S*	17		
		I. alpestris	2017	W*	S*	4		
Voto		*S. salamandra*	2017	W*	S*	19		
Ampuero	Spain	*S. salamandra*	2017	W*	S*	9		
		L. helveticus	2017	W*	S*	10		1
Teverga	Spain	*L. helveticus*	2017	W*	S*	62		2
		T. marmoratus	2017	W*	S*	11		
Villafranca del Bierzo	Spain	*L. boscai*	2017	W*	S*	20		

Carucedo	Spain	*L. boscai*	2017	W*	S*	4		
		L. helveticus	2017	W*	S*	20		
Carracedelo	Spain	*L. helveticus*	2017	W*	S*	5	1	
		T. marmoratus	2017	W*	S*	11		
Chozas de Abajo	Spain	*P. waltl*	2017	W*	S*	17		
		T. marmoratus	2017	W*	S*	1		
Ruente	Spain	*L. helveticus*	2017	W*	S*	50	2	1
Cabuérniga	Spain	*S. salamandra*	2017	W*	S*	19		
Campoo-Cabuérniga	Spain	*L. helveticus*	2017	W*	S*	11		
		I. alpestris	2017	W*	S*	2		
Los Tojos	Spain	*T. marmoratus*	2017	W*	S*	14		
		L. helveticus	2017	W*	S*	15		
Comillas	Spain	*L. helveticus*	2017	W*	S*	28		
Campoo de Suso	Spain	*L. helveticus*	2017	W*	S*	7		
		I. alpestris	2017	W*	S*	16		

**Bd* = *Batrachochytrium dendrobatidis*, *Bsal* = *B.salamandrivorans*, N = number of samples, *Bd*+ / *Bsal*+ = number of positive samples for *Bd*/*Bsal*, W = wild populations, C = captive populations, S = swab, TC = toe clip.