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Monitoring of the patogen *Batrachochytrium salamandrivorans* within populations of fire salamander *Salamandra salamandra* in Posázaví region

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Declaration

"I hereby declare that this thesis entitled Monitoring of the patogen Batrachochytrium salamandrivorans *within populations of fire salamander* Salamandra salamandra *in Posázaví region, is my own work and all the sources have been quoted and acknowledged by means of complete references."*

In Prague, 27. 4. 2017

…….

Tomáš Caska

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Abstract

Chytridiomycosis is the emerging infectious disease and is one of the major factors causing global amphibian declines. This diploma thesis is aimed to the monitoring of recently discovered pathogenic chytrid fungus the *Batrachochytrium salamandrivorans* (*Bsal*), likely originated in South Asia, at fire salamanders (*Salamanadra salamandra*) in two localities in Czech Republic. It is a serious disease which infects newts and salamanders and led to massive population decline of fire salamanders in Netherlands and Belgium. The infection is lethal for European caudate species. This chytrid fungus caused erosive skin diseases and fast mortality of infected animals. Research was conducted at two locations closed to Sázava city. Skin swabs samples were collected from 45 fire salamanders in total. Samples were analysed for pathogen presence by duplex- real time PCR method. In tested samples, the presence of *Batrachochytrium salamandrivorans* and *Batrachochytrium dendrobatidis* was not detected. The results of this research contribute to mapping of possible amplification of this pathogen in Czech Republic. Another part of this research, was to estimate the population size of fire salamanders on possibly threatened studied locality Radvanice, using capture- mark-recapture method. Locality is threatened mainly by clear cutting of forest and possibly by contamination of the brook. The threats and prospects for population of fire salamanders were evaluated and the future steps and care plan was suggested.

Keywords: chytridiomycosis, amphibian protection, amphibian diseases, *Batrachochytrium dendrobatidis*

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AOPK- Agentura ochrany přírody a krajiny

Bsal- *Batrachochytrium salamandrivorans*

Bd- *Batrachochytrium dendrobatidis*

1. Introduction and literature review

Amphibians are the most endangered group of vertebrates (Gray et al., 2015). One of the main threats for them is the fungal disease chytridiomycosis, which infected amphibian skin and devastated amphibian populations in last few years worldwide. Recently discovered species *Batrachochytrium salamandrivorans* (*Bsal*) is responsible for 99% population decline in fire salamanders in Netherlands (Martel et al., 2013; Sabino-Pinto et al., 2015). Main goal of this thesis is analysis of *Bsal* presence in fire salamanders, the threatened species in Czech Republic. Due to this fact, following chapters of introduction are about threats to amphibians, biology of studied species, amphibian skin functions and chytridiomycosis itself.

1.1. Current state of threats to amphibians

Amphibians are considered as environmental indicator thanks to their permeable skin and two life stages- the aquatic and terrestrial (Kolby and Daszak, 2016). Amphibian decline is complex problem with no simple solution. Decline of populations around the world is influenced by habitat destruction, agrochemical a chemical pollution, UV- B irradiation, diseases, introduced species (Baruš and Oliva, 1992; Beebee and Griffiths, 2005; Kolby and Daszak, 2016) and interaction among several factors. All these factors lead back directly or indirectly to human (McCallum, 2007).

Habitat destruction is the most obvious factors contributing to amphibian decline. Clearcutting forest and draining wetlands lead to amphibian population decline (Blaustein and Kiesecker, 2002). Environment fragmentation is connected with infrastructure development and direct habitat destruction and reduction. The main parameter which influences mortality of amphibians is road width, density and traffic intensity (Vojar, 2007).

Connected to habitat destruction, the environment contamination represents another serious factor which influences amphibian population decline. Semipermeable skin of amphibians is easy way for contaminants as heavy metals and pesticides (Vojar, 2007). Due to use of pesticides amphibians lose their food source. Chemical compounds

in pesticides can also negatively affect amphibian larvae metamorphosis (Mikátová and Vlašín, 2002).

Another the big problem is over- exploitation. According to Drinkwater (2016), over 38 million of amphibians, reptiles, birds and mammals are imported to the USA every year. Trade with amphibians is one of the most important ways, how the diseases can be spread all around the world (Martel et al. 2014; Agarwal, 2015). Salamanders represented 5.5% of the amphibians imported to US from 2004 to 2014 and 95% of those belong to four genera: *Cynops, Paramesotriton, Salamandra, Tylotriton*. These genera contain at least one or more species which are susceptible to *Bsal* infection. The Asian genera *Cynops* and *Paramesotriton* may be the greatest threat. The genera *Tylotriton* susceptibility has not been tested yet (Gray et al., 2015). The genera *Salamandra* is representative of European amphibians and the mortality on *Bsal* caused approximately extinction of this species in Netherlands (Spitzen van-der Sluijs et al., 2016). The U.S. Fish and Wildlife Service accept in 2016 the regulations which banned import of 201 injurious species of salamanders except by permit for zoological, educational, medical or scientific purposes (US Fish and Wildlife Service, 2016).

There are several types of infectious diseases or pathogen fungi of amphibians, which play a big role in global amphibian declines. For example, we can mention ranavirus disease, saprolegniosis, diseased caused by *Ribeiroia* sp. and chytridiomycosis (Daszak et al., 2003). Chytridiomycosis is caused by two pathogens *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans*. *Bsal* seems to be lethal for European newts and salamanders (Martel et al., 2013). As a prevention, adult amphibians and larvae should not be transferred from one to another population or released from captive conditions into wildlife. Reintroduction programs must have clear rules to eliminate risks of the disease spread (Mutschmann, 2015). Also the trade with amphibians can possibly spread the amphibian diseases as mentioned before and should be controlled.

Situation in the Czech Republic

The Czech Republic has very rich assemblage of amphibians compare to other European countries (13 anura and 8 caudata). All amphibian species in Czech Republic are protected by law except *Rana dalmatina* and *Triturus dobrogicus* which are not involved to notice (Vyhláška č. 395/1992 Sb.). *Triturus dobrogicus* is not involved probably by a mistake. However all 21 species are involved to national Red List of amphibians and reptiles of Czech Republic (Zavadil and Moravec, 2003). Majority of amphibian species are threatened by habitat destruction which started after Second World War. Main factors of habitat destruction in Czech Republic are drainage of wetlands, creation of uniform environment (large monoculture fields) and fishery intensification (Vojar, 2007).

1.2. Biology of studied species

The main subject of this diploma thesis research is the presence of *Bsal* in fire salamander (*Salamandra salamandra*). The fire salamander belongs to the family Salamandridae (phylum; Chordata; class, Amphibia; order, Caudata). Fire salamanders are found throughout the Europe, reaching the southern borders distribution in Israel and Northern Africa. Moreover, the current state of protection of this species is critically endangered in Czech Republic (Vyhláška č.395/1992 Sb.). According to Kuzmin et al. (2009), fire salamander is classified as least concern, with declining population trend. Adult fire salamanders are relatively demanding to habitat, in terrestrial period of life. In Czech Republic, they mostly live in deciduous or mixed forests with streams, bourns and springs (Baruš and Oliva, 1992). They are most abundant on overgrown talus slopes with oak or beech forest with fallen logs (Mikátová and Vlašín, 2002). Talus slopes and fallen logs provide a lot of hiding places and serve as place for hibernation which is necessary for salamanders (Zavadil et al., 2011). The salamanders use a variety of hibernation sites including natural cavities, spaces under large stones, logs and drain pipes, cellars and mines (Wells, 2010). Elevation range of salamanders is between 200 – 1000m above sea level. (Baruš and Oliva, 1992). According to biolib.cz (2017), fire salamanders occur on 51% of the area of Czech Republic (Fig. 1).

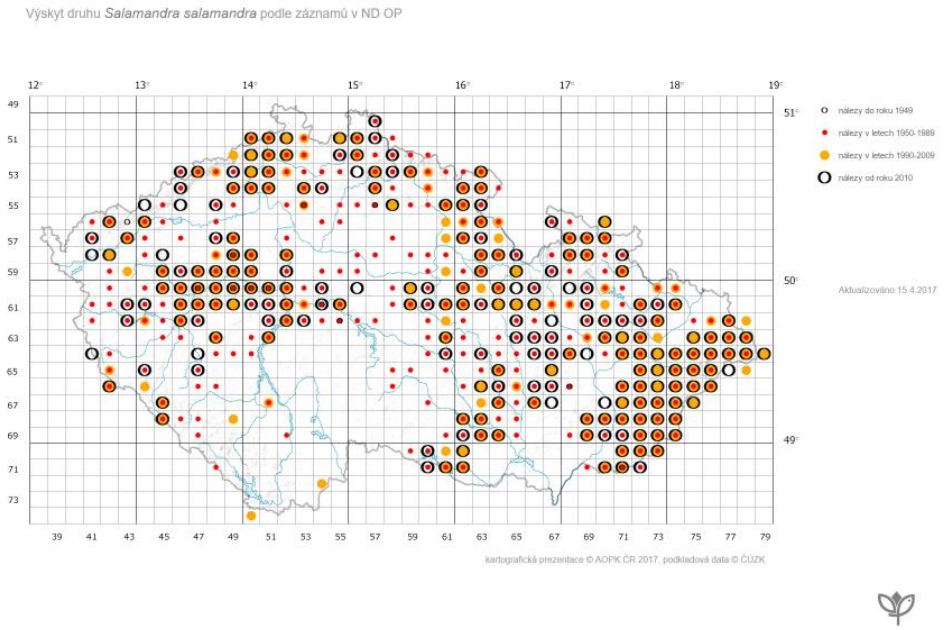

Figure 1 Distribution map of fire salamanders (Salamandra salamandra) in Czech Republic. (AOPK, 2017).

Description of fire salamander

Fire salamander grows up to 20cm, while the males are smaller, about 10 – 16cm and the females are bigger, 12 – 20cm, occasionally 22cm. The body is covered by very soft black skin with relatively large yellow spots, which are unique for each individual (Baruš and Oliva, 1992). Yellow spots on back have aposematic function. Aposematic coloration is often characterized by blocks or colour spots with apparent and sharp borders that are easily recognized. Aposematism is connected with unpalatability and have warning function (Stevens and Merilaita, 2011). Yellow colour is always present on upper eyelid and parotids (Baruš and Oliva, 1992). The conspicuous parotids behind the eyes are yellow with a prominent outlets of poison glands (Speybroeck et. al., 2016). Parotids are imbedded in trunk mussels that can be contracted to spray whitish, viscous, acidic fluid, which contain steroid alkaloid samandrin, used against predators (Wells, 2010). Male cloaca is bigger and swollen than female cloaca, especially during mating season (Speybroeck et al., 2016). The highest age is only known from captive breeding. Generally salamanders can live from 18 to 24 years (Baruš and Oliva, 1992).

Behaviour and mating

Fire salamanders are mostly nocturnal they leave their hiding places in daytime only during or after heavy rain and more often during mating season (Baruš and Oliva 1992). According to Wells (2010), the fire salamanders can be found at same home ranges for seven years and similar fidelity was observed also in hibernation sites, where some individuals hibernate in the same cave for 20 years. They are active when temperature is above 5°C (Spitzen- van der Sluijs et al., 2013). Mating season begins in summer and autumn, usually during the months September- October but it can differ between populations (Speybroeck et al., 2016). Mating takes place on land. When the male find possible mate, he will block her path. Male produces spermatophore, which is taken up by female. Fertilization is internal and gestation period is quite long and take place during hibernation. Fire salamanders are ovoviviparous, female moved from their summer home ranges to deposit larvae in the water (Wells, 2010). In this time they can migrate at the distance up to 100 m (Baruš and Oliva, 1992). Females usually lay the larvae into water during first decade of March (Zavadil et al., 2011). Fire salamanders exploit for breeding small streams without fish, pools closed to streams, bourns and human made oligotrophic and mesotrophic ponds. Larvae feed on insect larvae like a Mayflies (Ephemeroptera) or Caddisfly (Trichoptera) and crustaceans like (*Gammarus sp*.). Larvae of salamanders are sensitive to water temperature and amount of oxygen in water. They do not need sunlight for successful development, against other larvae of amphibians which live in Czech Republic. Larvae live in water for about 3 – 5 months (Baruš and Oliva, 1992).

Subspecies

Fire salamander creates several subspecies according to Speybroeck et al., (2016):

Salamandra salamandra salamandra: this subspecies live in east Europe up to central Germany, Alps and from north to central Italy. It is large subspecies which can grows up to 20 cm. This variation has irregular yellow blotches on body.

Salamandra salamandra almazoris: Lives mainly in high altitudes in Sierra de Gredos and Guadarrama in Spain but also in some lowland locations. Coloration of high- altitude salamanders can show restricted pattern of yellow spots.

Salamandra salamandra bejarae: Lives in Sistema Central in Spain in low altitudes and in other southern mountain areas. This subspecies is characterized by very short tail and toes and short pointed muzzle. Color pattern is variable, usually with restricted irregular yellow and red spots.

Salamandra salamandra bernardezi: Common salamander through west Cantabria and Asturias in Spain. It is medium sized salamander which grows about 15 cm. It has round muzzle. This subspecies has variable pattern, with yellow dorsolateral stripes of variable range. In some cases black or completely yellow individuals can exist. Some individuals can have dirty yellow coloration and are attributed to *Salamandra salamandra alfredschmidti.* But they live at the same place with *S. s. bernardezi* and they are genetically indistinguishable with them.

Salamandra salamandra crespoi: This subspecies lives in south Portugal. Remarkable long toes and tails. It has tiny whitish specks on belly.

Salamandra salamandra fastuosa: Lives in west and central Pyrenees and in North Spain. Yellow pattern is very considerable mostly with dorsolateral stripes.

Salamandra salamandra galaica: Lives in Northwestern Spain and North and Central Portugal. Muzzle is short and pointed. Toes and legs are short. Color pattern is highly variable, mostly characterized by yellow and red dots.

Salamandra salamandra gigliolli: It is sole representative of Italia. Head of this subspecies is flattened. The body is considerable of yellow spots. Completely yellow individuals are common.

Salamandra salamandra longirostris: Hills in provinces of cities Cádiz and Málaga are their habitat. Sharp nose with overbite is characteristic for this subspecies. Color pattern is represented from bright whitish to yellow spots.

Salamandra salamandra morenica: Occurs in Sierra Morena, North Andalucia and Valencia in general in South and Southeast Spain. Snout is short and pointed equally toes and legs. Pattern is highly variable, but mostly formed of very small yellow and red dots.

Salamandra salamandra terrestris: Lives in east Pyrenees and northeast Spain throughout France, Belgium, Netherlands, Switzerland and west part of Germany. It has typically two dorsolateral rows of yellow spots.

Salamandra salamandra werneri: Occurs in Greece on Peloponnese together with *S. s. salamandra.* But it differs with additional red spots. But the validity of this subspecies is not supported by any study.

1.3. Amphibian skin function

Infection by chytridiomycosis leads to total failure of the amphibian skin function. Amphibian skin is unique among vertebrate species (Voyles et al., 2009, Clarke, 1997). Skin plays very important role in amphibian survival. Amphibians usually pass through two life stages- from aquatic form to a terrestrial form (Kardong, 2012). Skin is a morphological, biochemical and physiological complex organ with wide range of functions necessary for amphibian survival. Amphibian skin is not only used for respiration, water regulation, exchange electrolytes (Voyles et al., 2009; Wells, 2010), anti- predator, anti- microbial and anti- fungal defence, but it has much more functions including excretion, temperature control, sexual dimorphism, protection against irradiation and camouflage function (Baruš and Oliva, 1992) Amphibian skin glands basically consists of two types- (Fig. 2) mucus glands and granular glands (Kardong, 2012). Mucus glands helps to keep the amphibian body moist, skin surface slippery and prevents against mechanical (abrasive) damage to the soft skin. Amphibians keep the skin moist via direct movement of water across the skin surface that is facilitated by extensive epidermal sculpturing (Wells, 2010). Mucus glands also protect amphibian body against harmful effects of prolonged contact with water. Furthermore, they also retard water evaporation, have bacteriostatic effect and equally have mechanical function against potential microbial and fungal pathogens. But this is not the only protections against pathogens. The granular glands (poison glands) produce toxic proteinaceous substances. Glands synthesize wide range of chemical compounds, which provide protection against bacterial and fungal infection, as well as against predators (Baruš and Oliva, 1992; Clarke, 1997; Wells, 2010; Kardong, 2012). According to Woodhams et al. (2006), the skin synthetized peptides can inhibit growth of *Bd.* In some cases symbiotic bacteria is partially involved in protection against *Bd*, giving amphibian

possibility to produce skin antimicrobials peptides (Kolby and Daszak, 2016). For example *Janthinobacterium lividum* is the common bacterium living on amphibian skin and producing antifungal metabolite violacein which is important antagonist against *Bd* (Mutschmann, 2015). Granular glands can be spread over the whole body or can create enlarged clusters like parotids at salamanders or frogs family *Bufonidae* (Baruš and Oliva, 1992). Secretions contain alkaloids which can be used against predators and acts on heartbeat, weakens breathing, paralyze mussels, induces vomiting and in some tropical species can cause death. Salamander poison contains alkaloid samandarin which induces cramps after oral ingestion (Baruš and Oliva, 1992).

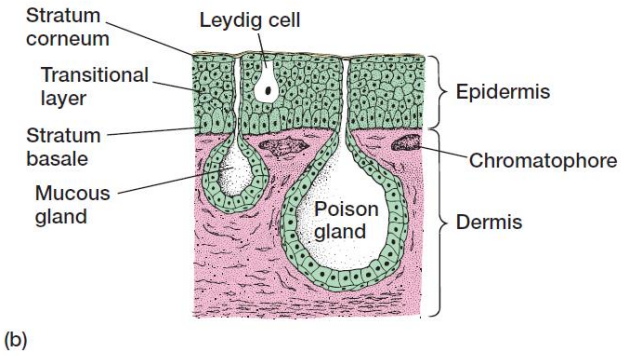

Figure 2 Diagram view of amphibian skin showing mucous and granular glands and their secretion (Kardong, 2012).

1.4. Chytridiomycosis

Basic characteristic and transmission

Chytridiomycosis is a serious amphibian disease which causes damage and loss of skin function (Berger et al., 2005). The amphibian chytrid was placed in a new genus *Batrachochytrium* (Phylum Chytridiomycota, Class Chytridiomycetes, order Rhizophydiales) (Berger et al., 2016). It thought that chytridiomycosis is caused by one species *Bd,* until 2013. But there were some cases with symptoms of chytridiomycosis, but *Bd* was not found there (Drinkwater, 2016). This die-offs was explained by describing the new species- the *Bsal* (Martel et al., 2013). These two chytrids *Bd* and *Bsal* are only members of this phylum, to cause disease in vertebrates (Berger et al., 1998; Daszak et

al., 1999; Berger et al., 2005; Bales et al., 2015; Martel et al., 2015; Mutschmann, 2015), and subsequently *Ichtyochytrium vulgare* mold, which attacks skin and gills of fishes (Červinka et al., 1974). Both species which parasites at amphibians are commonly called chytrids and cause disease chytridiomycosis (Mutschmann, 2015). They are found in aquatic habitats and moist soil where they degrade cellulose, chitin and keratin (Berger et al. 1998; Daszak et al. 1999). Parasitic chytrids mainly infect plants, algae, protists and invertebrates. As Martel (2015) says, chytridiomycosis has resulted in the serious decline and extinction of more than 200 species of amphibians worldwide and poses the greatest threat to biodiversity of any known disease. Infection with pathophysiological changes leads to mortality (Voyles et al., 2009). This disease is restricted to the skin in which sporangia were found in the *stratum coleum* and *stratum granulosum* and also to the keratinized mouth parts of tadpoles (Berger et al., 1998; Mutschmann, 2015).

Batrachochytrium dendrobatidis is ancient organism existing for thousands of years without any mass outbreaks (Kolby and Daszak, 2016). Chytridiomycosis has been recorded from Australia, New Zeeland, Europe, Africa, Asia and South, Central and North America from a wide range of habitats (Berger et al., 2005) *Bd* was recognized as pathogenic fungus in 1998 from moribund and dead adult amphibians collected at sites of mass deaths in Australia and Panama from 1993 to 1998 (Daszak et al., 1999). As a new chytrid species, it was described in 1999 (Longcore et al., 1999). The origin of the fungus is not clear. It may have been spread by international trade with *Xenopus sp.* In past frogs *Xenopus laevis* were used for pregnancy tests in humans and the animals was caught in wild and transported around the world in high numbers (Karesh et al., 2005; Mutschmann, 2015; Kolby and Daszak, 2016). Chytridiomycosis was signed in Wildlife disease list in 2001 (Civiš et al., 2010).

Batrachochytrium salamandrivorans is recently discovered salamander-specific species of chytrid fungus which is threat to distribution and abundance of salamanders and newts within Europe and United States (Martel et al., 2013; Sabino- Pinto et al., 2015; Grant et al., 2016; Laking et al., 2017). Frogs and toads seem to be resistant to infection. Fungus was introduced from East Asia into Europe via pet trade with caudate amphibians (Martel et al., 2014; Cunningham et al., 2015; Sabino- Pinto et al., 2015;

Drinkwater, 2016; Laking et al., 2017). According to Lips (2016), museum samples shows that *Bsal* was presented in Asia for over 150 years without any negative impacts on local species.

Both pathogens *Bd* and *Bsal* are spreading in the same manner. People who work with amphibians should follow basics hygienic measures as a disinfection of used fomites and hands, especially when travelling from site to site. It is strictly recommended to use latex gloves when manipulating with amphibians, new for each individual (DECC, 2008). For *Bsal* it is necessary to prevent entering this pathogen to the wild nature in Europe. It can be spread by releasing of infected animals or discarding of contaminated water and fomites in amphibian habitats (Cunninghan et al., 2015). The Bern Convention Standing Committee has announced strong recommendations for *Bsal* disease screening and trade restrictions. According to this issue European countries should accept actions for establish monitoring programs to control spreading of *Bsal* pathogen (Council of Europe, 2015). In *Bsal* case it is clear, that the pet trade is the main spreading route. Responsible behaviour of hobby pet keepers with a strict adherence to basic biosafety rules is at most importance (Sabino- Pinto et al., 2015).

Life cycle

Chytrids are primitive fungi that develop without hyphae and produce flagellated moveable zoospores (Berger et al., 1998; Mutschmann, 2015). *Bd* occurs in two forms- as embers spherical zoosporangium with a diameter 10 – 40 µm or mobile zoospores of size approximately 2µm (Civiš et al., 2010). The *Bd* life cycle is consists of two distinct life stages. It is quite simple development from zoospore to the growing organism, which is called thallus and which produces zoosporangium cleaves to new zoospores exit through papillae (Fig. 3). According Berger et al. (2005), at 22°C the life cycle takes 4 – 5 days to complete. *B. dendrobatidis* start life as aquatic uniflagellated zoospore released from mature zoosporangia, which are embedded to amphibian skin. When the zoospore encysts in the amphibian skin it develops to the second life cycle stage, the thallus and start to produce zoosporangium (Longcore et al., 1999). New zoospores are released from discharge tube and can survive in water for 7 weeks (Johnson and Speare, 2003).

Both amphibian chytrids *Bd* and *Bsal* are characterized by an asexual reproduction but the phenotypic diversity in *Bd* is uncommonly high (Mutschmann, 2015).

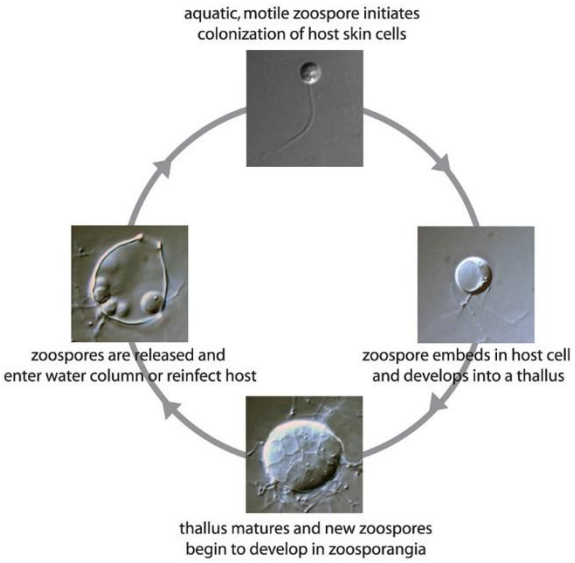

Figure 3 Life cycle of Batrachochytrium dendrobatidis(Rosenblum et al., 2013).

Temperature plays very important role in ecology of chytridiomycosis and can explain seasonality of infections in some regions (Berger et al. 2015). As Berger et al. (2004) wrote in his research, the optimal temperature for grow of *Bd* fungus in vitro is 23°C, and also grow well at 16°C, survive at 4°C and dies above 29°C. At this high temperature sporangia are killed by drying out. While the Mutschmann (2015) wrote that the *Bd* is temperature intolerant fungus and grow optimal in cultures from 17°C to 25°C. Development is very slow or can stop while the temperatures is below 10°C or above 28°C (Berger et al., 2015; Mutschmann, 2015). The highest mortality caused by chytrids in Australia was recorded constantly in winter months. Berger et al. (2004) confirm that the *Bd* does not like high temperatures. Frogs which were exposed to lower temperature 17°C and 23°C all died, but only 50% of exposed frogs died at 27°C.

Bsal has a lower optimal growth temperature in vitro than *Bd* (10 – 15°C versus 17 – 25°C, respectively) (Martel et al., 2013; Bales et al., 2014; Berger et al., 2015). According to Laking et al. (2017), majority of infected animals in Vietnam live in ponds and streams

with temperature between 20 – 25°C even reaching 26.43 °C in one positive location. Under laboratory conditions the *Bsal* infection is lethal for many urodeles not originated from the Asia and the mortality led to 100% in few days (Martel et al., 2014; Feldmeier et al., 2016).

Both chytrids are adapted to water and they are susceptible to desiccation. Desiccation for 3 hours on direct sunlight kills reliably 100% of infective stages (Berger et al., 2015).

Distribution of pathogen

Bd can lead to mortality in all amphibian orders (Anura, Caudata, Gymnophiona) (Baláž et al., 2014, Sabino- Pinto et al., 2015). Nowadays *Bd* is widespread and has broad host range detected in over 520 of amphibian species including frogs, toads, caecilians, salamanders and newts from 56 countries (Fig. 4) (Sabino-Pinto et al., 2015; Berger et al., 2015). It is responsible for greatest disease-caused extinction and declines of 200 anuras species in history (Skerratt et al., 2007). Some species can be resistant. This species include some major invasive amphibians like a North American bullfrog (*Lithobates catesbeanius*) and African clawed frogs. Presence of *Bd* DNA was found also in non- amphibian samples such as crayfish (*Procambarus* sp., *Orconectes virilis*), lizards and waterfowl (Kolby and Daszak, 2016). Berger et al. (2015) argues that retrospective diagnosis (more than 80 years) shown that *Bd* was occurring in Asia, Africa and Brazil and frogs have not undergone population declines associated with mass extinctions. *Bd* caused decline or extinction within orders Anura, Urodela and Gymnophiona worldwide (Laking et al., 2017). It was also discovered in many amphibians populations in central Europe, but it have not led to mass mortality or declines (Sabino- Pinto et al., 2015). During the years 2001-2003 the fire salamanders were found death in Peñalara Natural Park in Spain. According later examination of epidermis the presence of *Bd* was confirmed (Bosch and Martínez- Solano, 2006). In Czech Republic *Bd* was found in 2008 a year later the occurrence was confirmed. It was detected at 4 of 10 sampled species (Civiš et al., 2012). Pathogen presence was confirmed at common toad (*Bufo bufo*) and water frogs (*Pelophylax* sp.), European fire- belied toad (*Bombina bombina*) and yellow-belied toad (*Bombina variegata*) (Baláž et al., 2014).

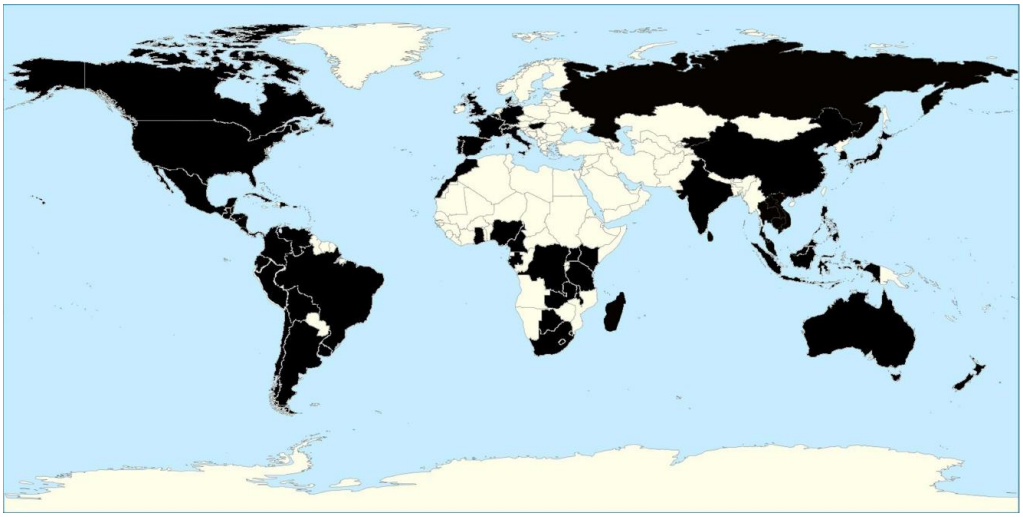

Figure 4 Detection of Batrachochytrium dendrobatidis. Black shading represent positive findings. (Kolby and Daszak, 2016).

Bsal origin is in East Asia (Thailand, Vietnam and Japan) (Martel et al., 2014) as was mention before. *Bsal* causes dramatic declines of native fire salamanders in Netherland and Belgium (Lips, 2016; Sabino-Pinto et al., 2016). First record of this pathogen was in September 2013 in Netherlands in fire salamander (Fig. 5) (Martel et al., 2013; Sabino-Pinto et al., 2015). *Bsal* is responsible for 99.9% natural population decline of fire salamanders the Netherlands between years 2010 and 2013 (Spitzen- van der Sluijs et al., 2016; Drinkwater, 2016). In 2015 *Bsal* was confirmed in private captive collection of salamanders (*S. salamandra, S. algira, S. corsica and S. infraimmaculata*) in Germany (Sabino-Pinto et al., 2015). Positive findings of *Bsal* are also known from captive collection in United Kingdon (Cunnighan et al., 2015; Sabino-Pinto et al., 2015). According to Spitzen-van der Sluijs et al. (2016), *Bsal* also attack Alpine newt (*Ichtyosaura alpestris*) and Smooth newt (*Lissotriton vulgaris*). According to Laking et al. (2017), *Bsal* in Vietnamese species of salamanders is more widespread than sister species *Bd*. Researched animals do not shown any symptoms or signs of *Bsal* so it was assumed that South East Asian salamander species could be a reservoir of this pathogen.

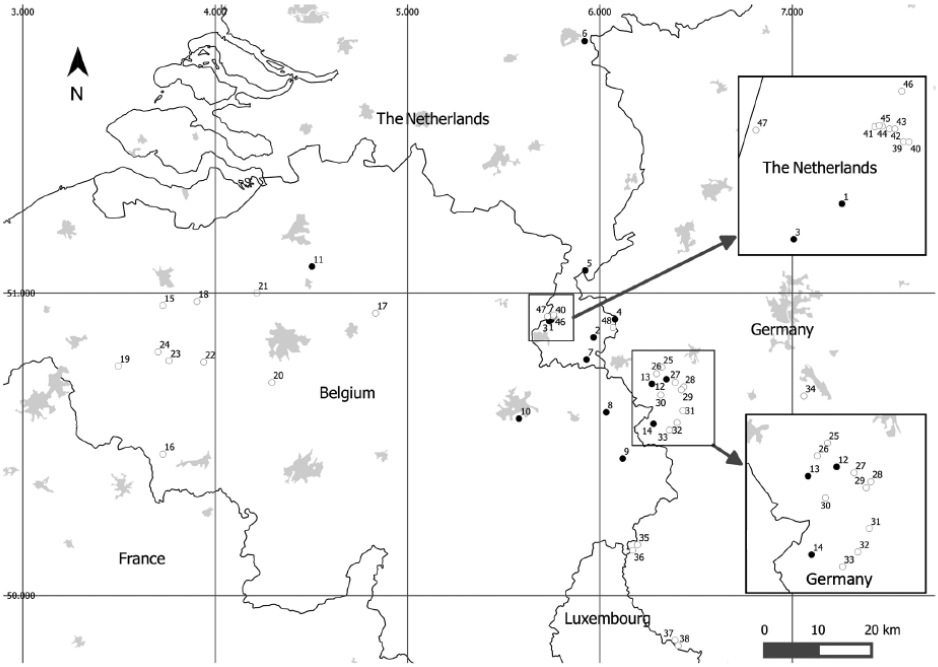

Figure 5 Distribution map of *Bsal* in Europe. *Bsal* detected; open circles, *Bsal* not detected. Larger cities are indicated in light gray. Note that there are additional sites where the fungus remained undetected (not shown). (Spitzen- van der Sluijs et al., 2016).

Symptoms and detection of the pathogen

Clinical signs of amphibian chytridiomycosis include abnormal posture, lethargy and loss of righting reflex (Daszak et al. 1999). As mentioned previously, amphibian skin is morphological, biochemical and physiological complex organ with wide range of functions necessary for amphibian survival. Infection in anuran larvae is limited to the keratinized cells of the oral region and can result in deformed, eroded, or missing mouthparts, which can be used for diagnosis (Berger et al., 1998).

Infected salamanders show symptoms of this disease like anorexia, apathy and ataxia and dying in relatively short period (Martel et al, 2013). As similar to *Bd* it also infects epidermal cells but invades into deeper layers. Result of this infection is haemorrhages and more erosive and ulcerative skin (Berger et al., 2015; Mutschmann, 2015). The pathology contains multifocal superficial erosion and ulcerations in skin over the body (Martel et al., 2013). Ulcerations with prominent degradation of the epidermis, can be sometimes visible macroscopically and very apparent histologically (Fig. 6 and

Fig. 7). Secondary bacterial infection or infections of other fungi is very common (Mutschmann, 2015). Disruption of epidermal integrity influence vital skin functions (electrolyte homeostasis, fluid balance, gas exchange and barrier against opportunistic pathogens) leads to death in two or three weeks after exposure (Martel et al., 2013; Gray et al., 2015). Infection and disease dynamics of *Bsal* in urodelas is given by environmental temperature (Blooi et al., 2015).

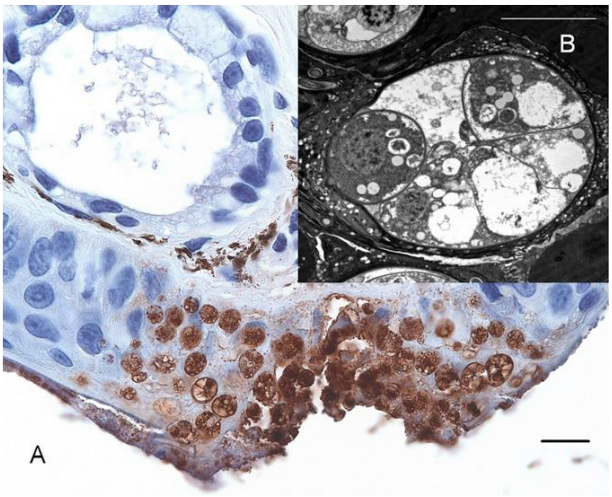

Figure 6 Microscopy of the skin of death fire salamander. (**A**) Cell layers are associated with erosive lesions. (**B**) Electron microscopy picture of an intracellular colonial thallus of pathogen (Martel et al. 2013).

Both species of chytrid fungus can be detected in two ways. Current PCR screening test for *Bd* described by Annis (2004) or *Bd* real-time PCR described by Boyle (2004) are not able to accurate detects the *Bsal* (Blooi et al., 2013; Zhu et al., 2014). For diagnosis of both chytrids duplex real- time PCR was developed, which allows detection of *Bd* and *Bsal* in amphibian samples with high sensitivity and specificity (Blooi et al., 2013). This method was already used in several projects on *Bsal* detection (Baláž et al., 2017).

Microscopic observations of the pathogen in the skin of infected amphibians is possible. Sample types are skin scrapings or sections from live animals. The histological examination is possible in dead animals. The fungus is found most often in stratum corneum of the digits, then the ventral surface. The epithelium can be scanned at x200 power. The suspicious areas should be examined at x400 power to confirm the diagnosis (Berger et. al., 1999). The swab- PCR examination is more significant than toe- clip

histology (Kriger et al., 2006). Histological examinations cannot be used as a diagnostic test to differentiation between two fungi (Baláž et al., 2017). The confirmation of chytridiomycosis structures relies on the level of expertise of the examiner. Diagnosis is easier if mature zoosporangia are seen (Berger et al., 1999).

Figure 7 Fire salamander infected by *Bsal*. Skin lesions are evident. (Gray et al., 2015).

Treatment of chytridiomycosis

Chytridiomycosis can be cured. For antimycotic treatment of *Bd* using voriconazole at a concentration of 1.25 µg/ ml for one week is highly successful and safe. The antimycotic protocols using itraconazole or voriconazole alone or in combination with polymyxin E, which were developed for related fungus *Bd* failed. Blooi et al., 2015A in his research successfully cure the *Bsal* with heat treatment at 25°C during 10 days. However this temperature may be critical for several species of urodelas. Later the new treatment system was invented. According to previously synergy in vitro cultures between voriconazole and polymyxin E, which inhibit *Bsal* growth and previously determined temperature dependent infection dynamics of the disease. Voriconazole and polymyxin E were used in same concentrations, but the temperature was raised to

20°C which successfully eliminate the *Bsal* and is not critical for most species (Blooi et al., 2015B).

Vulnerability to Czech Urodela species

Czech Republic has eight species of urodelas (Vojar, 2007): fire salamander (*Salamandra salamandra*), smooth newt (*Lissotriton vulgaris*), palmate newt (*Lissotriton helveticus*), Carpathian newt (*Lissotriton montandoni*), alpine newt (*Ichtyosaura alpestris*), north crested newt (*Triturus cristatus*), Italian crested newt (*Triturus carnifex*) and Danube crested newt (*Triturus dobrogicus*). According to Martel et al. (2014) and Spitzen- van der Sluijs et al. (2016), all of this species are vulnerable to *Bsal* except palmate newt which is resistant to this disease and showing no mortality on infection.

2. Aims of the thesis

Main aim of my research was to test the presence of fungal pathogen *Batrachochytrium salamandrivorans* in *Salamandra salamandra* species in two selected localities in Central Bohemia in Posázaví region. Another aim was to estimate the population size of fire salamanders at Radvanice locality, using capture- mark- recapture method and to evaluate sources of danger and prospects of these stocks, including concrete proposals for protective measures.

3. Materials and methods

3.1. Study area

Research was conducted in two selected locations in Central Bohemia in Posázaví region (Fig. 10). Studied areas are situated in Benešovská pahorkatina. This areas were chosen because of high human population density and possible pathogen introduction. Locations are situated on shores of Sázava river. Localities were visited after rain in the morning or at night, when the humidity was higher. Temperature range varied between 5°C to 20°C.

First location was chosen closed to village Radvanice (49°52'35.6"N 14°55'32.2"E). It is situated on right shore of Sázava river. The location is surrounded by fields on the northern border. It is deep ravine with west oriented talus slope and dominant rocks (Fig. 8). Flora is mainly represented by spruce, black locust and pine on the top of western slope. This slope is inhabited by salamanders, offering a lot of hiding places which also served as hibernaculum.

Figure 8 Talus slope and spruce forest in locality Radvanice. (photo by T. Caska).

Another representatives of amphibians were also find on this locality like a common European toad and European common frog (*Rana dalmatina*). Small brook with some

pools flow through the ravine. The minimum depth is 8 cm and the maximum depth is 30 cm. Bottom of the stream is covered by flattened stones. Aquatic vegetation is present in few pools. No fish were recorded in the stream. Aquatic invertebrates are represented by *Gammarus* sp.*,* Caddisflies (*Trichoptera sp.)* and Mayflies (*Emphemeroptera sp.*) Shore is mainly gradual with roots and moss, being very steep. Shores are covered by garlic mustard (*Alliaria petiolate*), Alternate-leaved Golden-saxifrage (*Chrysosplenium alternifolium*), Forget me not (*Myosotis nemorosa*). Forest mainly consists of spruce (*Picea abies*) and some solitaire black locust (*Robinia pseudoacacia*).

Second selected locality was protected nature area Ve Studeném (49°52'36.7"N 14°51'23.7"E). It is located in between villages Samechov and Dojetřice, on the left shore of Sázava river and on the north hillside of Spálený vrch, 459m above sea level. Locality is surrounded by pastures and grasslands on south. The slope orientation is predominantly northern and northeast. The altitude ranges is between 300 – 460m above sea level. As national nature area, it was declared in 1935. Reason for protecting are natural forests with primeval structure formed by natural communities of beech (*Fagus sylvatica*) and talus forests. Majority of area is created by herb-rich beech forest.

Figure 9 Original beech forest in national nature area Ve Studeném (photo by T. Caska).

Most vegetation has primeval character with sufficient dynamics of natural recovery and high species diversity (Fig. 9). The oldest trees are approximately 170 –200 years old. Bedrock consists of granodiorites in the western part interfere migmatised metabasists and in eastern and south slopes emerge biotite gneisses (Pokorný et al., 2010). It is a non- intervention area with a lot of old fallen beech tree trunks, which contributes to extraordinary amount of biodiversity. Slope is around 50 –60%. The canopy is represented by typical species mixture: European beech, sycamore (*Acer pseudoplatanus*) and European hornbeam (*Carpinus betulus*). Significant outcrops are mainly concentrated in the ravine from the part of railway and in the eastern part of the steepest section of hill (www.nature.cz, 2006). Part with occurrence of fire salamanders is situated in western part of the natural reserve in deep ravine with small brook.

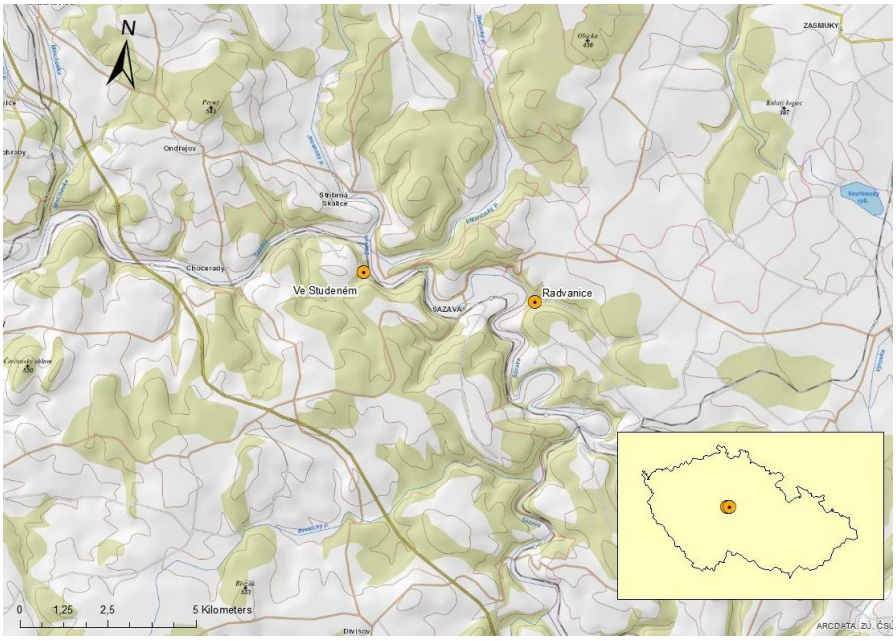

Figure 10 Map of studied localities in Posázaví region. Map was created in ArcGis 10.2.2.

3.2. Data collection

During 16th April 2016 to 31th October 2016, skin swab samples were collected from free- living populations of salamanders. Permission for manipulation with fire salamanders was necessary, gained from National Conservation Agency of Czech Republic. Salamanders were swabbed by dry cotton swabs (Medical Wire and

Equipment Co. Ltd., UK) and new latex gloves were used for each individual. 1.5 mm screw cap tubes were used for transporting the sample, labelled with permanent ethanol marker. Skin swabs were collected by standardized method according to Boyle et al. (2004), in total from 45 salamanders. Salamanders were sampled opportunistically, which means that every visible salamander was captured. Every sampled salamander was preliminary visually examined for signs of chytridiomycosis such a skin ulcerations, lethargy and poor body condition. Every sampled animal was swabbed 20 times in total from belly, legs, back, thighs and skin folds. Amphibians are fragile animals, so it must be on mind when they are handled. After collecting, the swab was broke and dropped into empty screw cap tube with PrepMan Ultra (Thermo Fisher Scientific Company, USA). Every cap tube with sample was labelled by permanent marking pen. Samples were kept in fridge. During research additional information was also collected such as: temperature, time, weather, sex and photos.

Extraction of DNA

Laboratory sample processing took place at the University of Veterinary and Pharmaceutical sciences in Brno. We extracted the DNA from 45 field skin swabs. For DNA extraction protocol was used, based on protocol used at Zoological Society of London (Hyatt et al., 2007). First step is to weigh out 0.04-0.05g of 0.5mm zirconium/silica beads into 2ml screw top centrifuge tube, after that 60 µl of Prepman Ultra was pipetted into centrifuge tube. All tubes were labelled by permanent marker. Tip of swabs were sliced off by sterile scalpel in sterile Petri dish and place into corresponded centrifuge tube. New blade was used for each sample. Next step was to homogenize samples using MagNa lyser (Roche Company, Switzerland) for 45 seconds at speed 6500 and centrifuge for 30 seconds at 14500 rpm. After centrifuge, these two steps were repeated again. Samples were put into thermoblock which was set on 100°C and kept there for 10 minutes. After boiling, samples were cooled for 2 minutes and centrifuged at 14500 rpm for 3 minutes. Next step was to collect as much supernatant as possible and store in a sterile 0.5 ml Eppendorf. Pipette 36µl of sterile PCR water into a sterile 0.2ml Eppendorf and add 4µl of supernatant and mix well.

Duplex Real- Time PCR

Real- time PCR is the method, which continuously collects the fluorescent signal from one or more polymerase chain reaction over a range of cycles. This method is the conversion of fluorescent signals from each sample to numerical value for each sample (Dorak, 2006). The samples were tested for presence of both pathogens *Bd* and *Bsal.* Analysis was based on methodology described by Boyle et al. (2004). For sample analysing duplex real- time PCR was used as described by Blooi et al. (2013). It is method, which allows analysis of *Bd* and *Bsal* at the same time. The infectious status was detected using quantitative polymerase chain reaction (qPCR) with *Bd* and *Bsal* primers and TaqMan probes. Bovine serum albumin (BSA) was added to reduce PCR inhibition. Into 5ml screw cap tube solution of Taqman Universal PCR Master Mix 12.5µl, Fwd. 5.8sChytr 0.625µl, Rev. its1-3Chytr 0.625µl, SterFwd 0.625µl, SterRev 0.625µl, Taqman B.d probe 6FAM 0.03125, Taqmann B.s probe (VIC) 0.3125µl, DH_2O 4.7375µl, BSA 0.2µl, DNA (1/10 dilution) 5µl should be added, 25µl in total. Pipetted 20µl of reagent mix was added in each well. Next step was to mark the position of *Bd* standards, negative control and *Bsal* positive control on 96- well plate (Fig. 11). In this test genomic standards of *Bd* equivalent 0.1, 1, 10 were used and 100 zoospores per 5µl, which were obtained from Institute of zoology, Zoology society of London. Single sample of *Bsal* genomic DNA was used as positive control. Than we add 5µl of the 1/10 diluted sample to the first well, while the tip was changed after each sample. This was repeated with all samples/standards/controls. In both detection tests, duplicates off all analysed samples and standards were used as well as positive and negative control. Into negative well 5 µl of sterile PCR water was added.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D	0.1Bd	0.1Bd	1Bd	1Bd	10Bd	10Bd	100Bd	100Bd	NC	NC	Bsal+	Bsal+
E												
F												
G												
H												

Figure 11 Schema of 96 well plate with standards for both chytridiomycosis and negative control.

A position of each sample was written down on the plate. When the plate is completed it was covered by Clear PCR seal which must fit around each well. The plate is vortexted and centrifuged. The plate was placed in the PCR machine LightCycler 480 Instrument II (Roche Company, Switzerland) in right orientation as soon as possible. Last step of preparation of qPCR was setting the machine. New experiment was created using program template. Reaction volume 25 µl, detection filters FAM 465-510 and VIC 533-580. Melting 50°C for 2 minutes and 95°C for 10 minutes. The replication 95°C for 15 seconds, 60°C for 1 minute. Number of replication cycles was set to 60. Sample names were filled in program table. If any sample showed florescence growth in wavelength of *Bsal* probe, it would be re-analysed with full set of *Bsal* standards.

3.3. Estimation of population size

Data from captures were used for estimation of population size of fire salamanders in location Radvanice. This location has high potential risk of clear cutting of forest and this situation can strongly affect existence of population of fire salamanders (Fig. 12). All salamanders were photographed and they were determined by gender. Every individual has a specific pattern of yellow spots. Individual identification by colour pattern in amphibian was used for different species such as: fire salamander, alpine newt, palmate newt and fire-belied toad (Carafa and Biondi, 2004).

Field visits were conducted to locate already photographed animals or to take photo of new individuals. Photos were sorted according date of visit. After every visit the taken photos of individuals were compared with other photos from previously visits. The dorsal coloration pattern is generally stable (Carafa and Biondi, 2004). Analysing of

mark- recapture data was done by Cormack- Jolly- Seber model in MARK 8.1. software. The basic unit of analysis is the capture history with a sequence of 0 and 1 which means when the animal was seen in experiment.

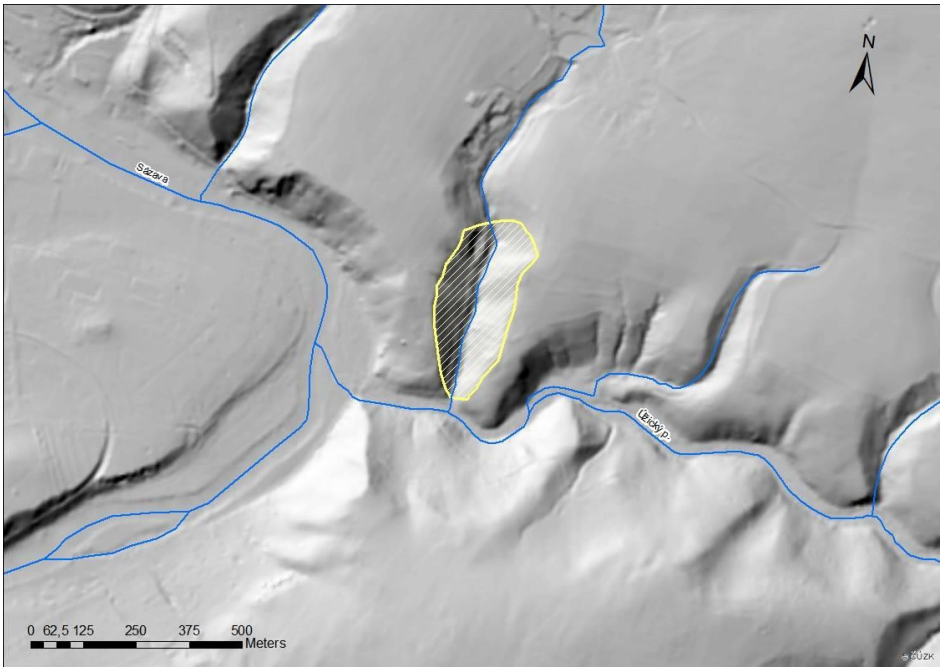

Figure 12 Study area Radvanice relief map for estimation of population size. Map was created in ArcGis 10.2.2.

4. Results

4.1. Detection of chytridiomycosis in selected locations

The PCR shows the positive detection in the positive controls of *Bsal.* None of 45 skin swabs samples from both locations we positive to *Bd* and *Bsal* pathogen. Absence of the *Bsal* pathogen is positive finding. Especially in case of studied locations, which are in densely populated area with high risk of pathogen introduction.

4.2. Population estimation on Radvanice

Altogether were caught 77 individuals (27 males and 50 females) with only six recaptures. As for the estimation of population size, the data had low informative value, because of small number of recaptured individuals. For this reason Jolly- Seber model for estimation of population size could not been applied. Data could have been analysed only with Cormack- Jolly- Seber model which estimates survival and probability of capture. Survival or the likelihood of catching the salamanders selected population did not differ between the sexes or in time. The males and females had same chance of catching them- 4.3% and the same chance of surviving in the next capture action 72.1%. (Tab. 1).

Real Function Parameters of { ϕ(.),p(.) }

95% Confidence Interval

Parameter	Estimate	Standard Error	Lower	Upper
1:Phi	0.7210298	0.1907833	0.2871124	0.9431389
2:p	0.0430994	0.0325451	0.0095004	0.1745815

Table 1 Estimates of the **phi** (The probability of survival of individual, between each capture action, in the studied population) and **p** (The probability of capture and recapture of the individual in the individual capture action, in the studied population) values for the fire salamander population in Sázava from the best Cormack Jolly Seber model

4.3. Evaluation sources of danger for fire salamander population

Fire salamanders are very sensitive to any changes in their habitat (Zavadil et al., 2011). The locality closed to Radvanice village is threaten by depositing composts or by creating a smaller black dumps individually. The inhabitants of the village and vacationers from nearby cottages throw away unnatural varied thrash, especially plastic bottles. However the biggest threat for fire salamanders on this locality is clear cutting of the forest. This locality was already been affected by clear cutting, but the most valuable part is still in its original form. Logging caused temperature rise and loss of shadow and damage of talus slope, which provide sheltering for fire salamanders. There is also potential risk of stream chemical contamination from upper village and fields which are treated by pesticides. Absence of fish in shallow stream is very important for reproduction of fire salamanders (Manenti et al., 2009. Compare to that the locality Ve Studeném already has high protection level as national nature area. There is also some human impact like a depositing compost and some waste, but nothing what can really threat the fire salamander population. This locality is not used for tourism for its steep slopes.

5. Discussion

Batrachochytrium salamandrivorans

The main purpose of this study was to examine the fire salamanders for the presence of fungal pathogen *Bsal.* According to our knowledge, the pathogen *Bsal* in Czech Republic is not presented yet. In case of *Bsal* pathogen, the negative results are scientifically equally important as well as finding of presence of pathogen. There were also published research with negative samples in China and Austria (Zhu et al., 2014; Gimeno et al., 2015). The pathogen free areas, which can serve as refugium (Teacher et al., 2009, Heard et al., 2015), in case that this pathogen appears in Czech Republic will be known. It is only a matter of time and responsibility of amphibian breeders and importers, when and if ever disease appear in Czech environment. Breeders of salamanders and newts should follow bio-safety rules for prevention pathogen introduction. I suggest greater public awareness about the disease and design manual in cooperation with government institutions, in case that the pathogen will be present in wildlife (Matthew et al., 2015). All imported caudate should pass through preventive screening for presence of *Bsal.* If the presence of *Bsal* pathogen will be confirmed in Czech wildlife I recommend to ban the trade with newts and salamanders for better protection of indigenous species (Baláž et al., 2017). This diploma thesis research was a part of first monitoring of *Bsal* in Czech Republic, it is necessary to continue in this monitoring and cooperate with other European countries on emergency action plan.

Estimation of population size

Estimation of population size cannot be calculated because of low number of recaptured individuals, which are necessary for the model. Fire salamander activity is highly affected by weather. Locality was visited after rain in morning or at night when the occurrence of fire salamanders should be high. The locality should have been visited several times for higher chance of recaptures and research should take more than one season as in Schmidt et al. (2007). Carafa and Biondi (2004) from 15th July 2000 to 6th November 2001 did a total of 50 sampling days, but only in 18 were individuals found active out of their shelters. Locality Radvanice will be still monitored, because it is necessary to know the population size to ensure protection and also potential impact of

the *Bsal* pathogen on population. Detailed data on distribution and population sizes of fire salamanders in countries with possible pathogen spreading are very important to know for controlling the impact of pathogen. According to Baláž et al. (2017), only few countries in EU has detailed data on national level. The advantages of the method which was used are: a) sampling of the animals with minimum disturbance; b) individual identification that is performed using information science supports and avoiding the stress caused to animals by application of invasive methods; c) easier analysis, management and transferring of the image files (Carafa and Biondi, 2004).

Evaluation of sources of danger

Future steps for protection of Radvanice locality should be consulted with government organization Nature Conservation Agency of the Czech Republic and with the owner of the forest. The protection of locality should be done by declaring protected area or, for example, contractual protection, which is often the protection of localities from undesirable environmental influences. Protection should always be preferential in the form of an agreement with the owners of the forest, which should be such as to suit the amphibians. Appropriate care must be taken of both aquatic and terrestrial habitats. Absence of fish in shallow stream is important for successful reproduction (Manenti et al., 2009). Support more deciduous trees, so that in most area the forest is at least mixed. More environmentally friendly farming and primarily forest management practices should be introduced. Selected natural and nature-friendly forest stands and it would be appropriate to apply nature-friendly management to create richly structured forests with high age, species and spatial diversity. Is better to logging in selective way and do not create the glades. Very important is to do not remove the forest floor. Salamanders are losing shelter and source of food (Zavadil et al., 2011). In Czech forests there is generally a shortage of dead wood, which should be changed. It is necessary to keep small heaps of branches, grass, logs, stones, or even fallen trees in areas with occurrence of threaten species as a fire salamander. These habitats are important places for hiding and hibernation.

6. Conclusions

- The pathogen *Bsal* is the serious threat for European caudate species. Monitoring of this fungal pathogen ongoing in the Czech Republic since 2015 without positive findings. This study was a part of the national monitoring programme of *Bsal*. This research analysed the presence of the fungal pathogen *Bsal* in two selected localities in Posázaví region.
- From live fire salamanders were taken skin swabs which are analysed using duplex real-time PCR method. All 45 collected samples from both localities were evaluated as negative for the presence of the pathogen. The absence of the pathogen in Czech fire salamanders is important finding for creating plan against introduction of *Bsal* pathogen to the Czech Republic.
- Unfortunately, the study failed to meet the goal of population estimation size of fire salamander in locality Radvanice due to lack of data. Monitoring of the fire salamander population on locality Radvanice will continue.
- Fire salamanders on locality Radvanice are mainly threaten by clearcutting of the forest. In this study were suggested solutions for locality protection like a: supporting more deciduous trees, nature-friendly management to create richly structured forests with high age, species and spatial diversity. The next steps for locality protection will be contacting the owner and possible agreement on locality conservation.
- Public can help with reporting suspicious death of salamanders and newts. As a prevention against *Bsal* introduction is necessary to examine the imported caudate for *Bsal* presence or to banned the amphibian trade. If the fungus was discovered in a salamander or newt populations, the most important thing to do would be to prevent the dispersal of the pathogen to other, healthy populations.

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8. The Appendices

List of appendices

Appendix 1 Nearby road caused death of few individuals of fire salamanders in Radvanice locality.

Appendix 2 Clearcutting of forest on Radvanice locality.

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Appendix 5 Real-time PCR: an example assay of *Bsal* results from the VFU Brno. Only positive controls of *Bsal* were detected.

Appendix 6 Real-time PCR: an example assay of *Bd* results from the VFU Brno. Only *Bd* standards were detected, all samples were negative.

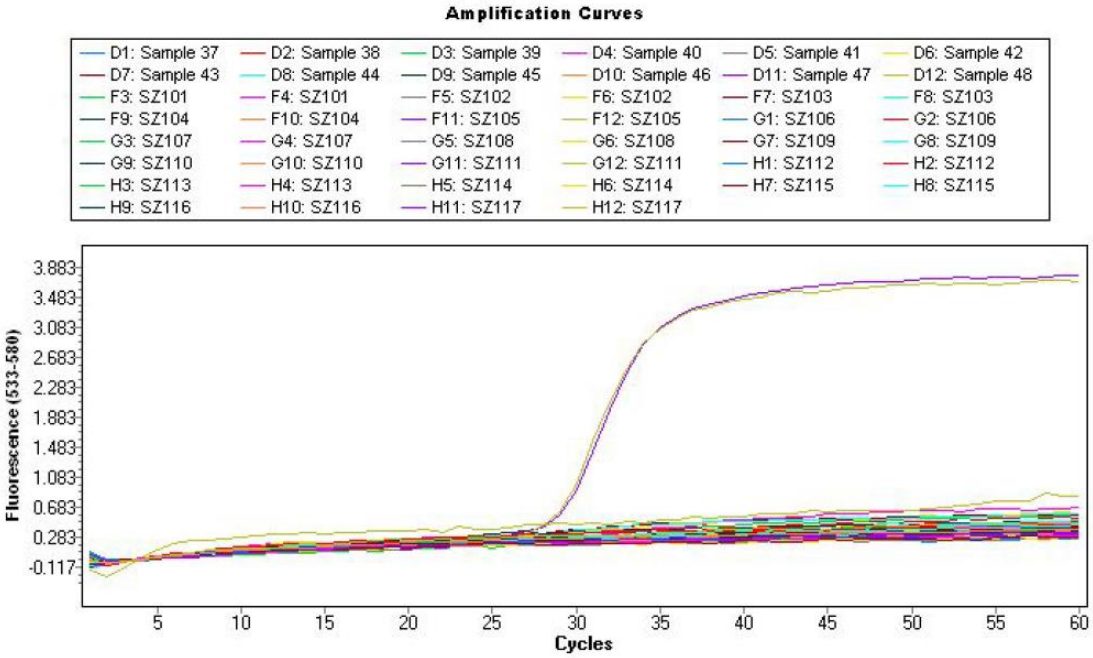
Appendix 7 Capture history of fire salamanders in Radvanice locality

Appendix 1 Nearby road caused death of few individuals of fire salamanders in Radvanice locality (photo by T. Caska).

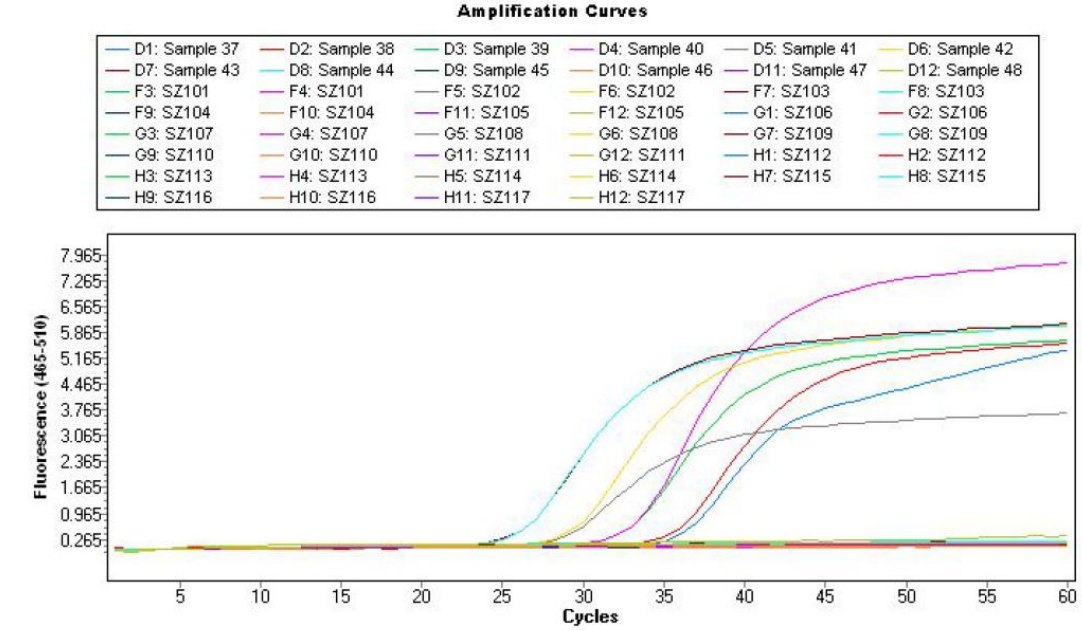
Appendix 2 Clearcutting of forest on Radvanice locality (photo by T. Caska).

Appendix 3 Field sample collecting (photo by T. Caska).

Appendix 4 Recaptured individual. **A)** was captured on 4.5. 2016, **B)** was captured on 23.2. 2017. No pattern changes are visible.



Appendix 5 Real-time PCR: an example assay of *Bsal* results from the VFU Brno. Only positive controls of *Bsal* were detected.



Appendix 6 Real-time PCR: an example assay of *Bd* results from the VFU Brno. Only *Bd* standards were detected, all samples were negative.

Appendix 7 Capture history of fire salamanders in Radvanice locality

Sample ID	Capture	Sex		Sample ID	Capture	Sex
S1	11000000	0 1;		S41	00100000	1,0
S2	00110000	1,0		S42	00100000	0,1
S3	01001000	0,1		S43	00100000	1,0
S4	00001001	1,0		S44	00100000	1,0
S5	00100001	0,1		S45	00100000	1,0
S6	01100000	0,1		S46	00100000	0,1
S7	10000000	0,1		S47	00100000	1,0
S8	10000000	0,1		S48	00100000	1,0
S9	10000000	0,1		S49	00100000	1,0
S10	10000000	0,1		S50	00100000	1,0
S11	10000000	0,1		S51	00100000	0,1
S12	10000000	0,1		S52	00100000	1,0
S13	10000000	1,0		S53	00100000	0,1
S14	10000000	0,1		S54	00100000	1,0
S15	10000000	0,1		S55	00100000	0,1
S16	10000000	0,1		S56	00100000	1,0
S17	10000000	0,1		S57	00100000	1,0
S18	10000000	1,0		S58	00100000	0,1
S19	01000000	0,1		S59	00100000	0,1
S20	01000000	1,0		S60	00100000	0,1
S21	01000000	0,1		S61	00100000	0,1
S22	01000000	0,1		S62	00010000	1,0
S23	01000000	0,1		S63	00010000	1,0
S24	01000000	1,0		S64	00001000	0,1
S25	01000000	0,1		S65	00001000	1,0
S26	01000000	0,1		S66	00000100	1,0
S27	01000000	1,0		S67	00000010	0,1
S28	00100000	1,0		S68	00000010	0,1
S29	00100000	0,1		S69	00000001	1,0
S30	00100000	0,1		S70	00000001	0,1
S31	00100000	0,1		S71	00000001	0,1
S32	00100000	0,1		S72	00000001	0,1
S33	00100000	0,1		S73	00000001	0,1
S34	00100000	0,1		S74	00000001	0,1
S35	00100000	1,0		S75	00000001	0,1
S36	00100000	1,0		S76	00000001	0,1
S37	00100000	0,1		S77	00000001	0,1
S38	00100000	0,1				
S39	00100000	0,1				
S40	00100000	1,0				