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DISEASE DYNAMICS OF THE EMERGING FUNGAL PATHOGEN *BATRACHOCHYTRIUM SALAMANDRIVORANS* IN FIRE SALAMANDERS (*SALAMANDRA SALAMANDRA*)

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List of Abbreviations

5.8S rRNA	5.8S subunit ribosomal ribonucleic acid
ATV	*Ambystoma tigrinum* virus
Bd-GPL	*Batrachochytrium dendrobatidis* global pandemic lineage
BIV(-like)	Bohle iridovirus
BSA	Bovine serum albumin
CI	Confidence Interval
CMR	Capture-Mark-Recapture
CMTV	Common midwife toad virus
CRI	Credibility interval
Ct	Cycle treshold
DNA	Deoxyribonucleic acid
EID	Emerging infectious disease
EPH	Endemic pathogen hypothesis
F_{IS}	Inbreeding coefficient
F_{ST}	Fixation index
FV3	Frog virus 3
GE	Genomic equivalents
GIS	Geographic Information System
Ho	Observed heterozygosity
He	Expected heterozygosity
HWE	Hardy-Weinberg equilibrium
ITS	Internal Transcribed Spacers
IUCN	International Union for the Conservation of Nature and Natural Resources
JAGS	Just Another Gibbs Sampler
K	Number of clusters in an analysis on population genetics
N_A	Observed number of alleles
N_E	Expected number of alleles
NPH	Novel Pathogen Hypothesis
OIE	Office International des Epizooties
(q)PCR	(quantitative) Polymerase Chain Reaction
Phi	Survival probability
Psi	Transition probability
p	Recapture rate

Glossary

Basic reproductive ratio (R_0)

The expected number of individuals that become infected as a result of the introduction of a single infective individual in a totally susceptible population. Can be calculated by using e.g.

$$R_0 = \frac{\beta * X}{a + b + \sigma} \qquad eq. 1$$

Co-infection

A co-infection is the simultaneous presence of more than one agent where each agent leads to a specific disease.

Disease

A disease is the deviation of the normal homeostasis of an individual which can manifest in response to environmental alterations (e.g. related to nutrition, toxins or climate), to inherent or congenital defects and to infectious agents. When a population is diseased, it is assumed that some individuals within the population are diseased, while others may only be infected and others remain uninfected (See *infection*).

Ecosystem health

A term related to the capability of an ecosystem to sustain growth and reproduction, support all life and interactions between lifeforms and deal with perturbations made to the ecosystem (Rapport el al. 1998).

Emerging infectious disease

An (re-)emerging infectious disease (EID) is a term related to infectious disease agents which pose a threat to wildlife, or human, health. Emerging infectious diseases either have the ability to cause epidemic outbreaks, have increased their incidence over the last decades or occur at an endemic level in certain populations or areas while being known to have the ability to cause epidemics in other areas. It can also be an infectious agent with important health hazards which is spreading to new areas.

Endemic occurrence

An infection or a disease that is said to occur at an endemic level, implies that the pathogen and its host are in stable coexistence at a population level within a specific area or region (Anderson and May 1979) or that the occurrence rate within the population or area is predictable, regular and expected (Wobeser 2006).

Epidemic occurrence

An epidemic occurrence of an infectious disease is the rapid increase in the incidence of the disease over a short time-span in a population and is mostly unexpected (Anderson and May 1979). Epidemics can occur due to the recent arrival of a disease in a new population where it had not been present before, by increased virulence or changed transmission mode of the disease agent or due to an increased susceptibility of the host (Wobeser 2006).

Epidemiological triangle

The epidemiological triangle is a concept in the epidemiological study of diseases that illustrates the interplay between three factors: a pathogen, its host(s) and the environment. Each factor is situated at a corner of the triangle emphasizing that each factor directly affects the next

Incidence

Incidence is related to the occurrence of an infection or a disease and it is the ratio of the number of new cases of a disease/infection and the number of individuals at risk of developing the disease/infection during a fixed period of time (Wobeser 2006). See *Prevalence*, with which this term is often confused.

Index site

The index site is a term used to refer to the location where something was seen, observed or detected for the first time. In this thesis, the *B. salamandrivorans* index site is a forest in the Southern part of the Netherlands, where a population collapse from fire salamanders was observed, followed by the identification of the pathogen causing this collapse.

Infection

An infection is the acquisition of a pathogen within/on a host or host population, i.e. the penetration or colonization of the host by a pathogen (Scott 1988). An

infection can lead to disease, depending on the environment in which the infection occurs, the pathogen's characteristics (e.g. virulence or infection dose) and the susceptibility of the host which in turn can be an innate trait or a function of the state of the individual in terms of health, stress, nutrition or previous contact. When a population is infected, it is assumed that a pathogen is present in the population and that some individuals may be infected while others have developed the disease associated with the pathogen while yet other individuals are uninfected.

Mixed infection

A mixed infection is the simultaneous infection of more than one agent leading to one disease (Thrusfield 2008).

Prevalence

Prevalence is related to the occurrence of a disease/infection and is the ratio of the number of infected or diseased individuals and the total number of animals in a population at a specific time (Wobeser 2006). See *Incidence* with which this term is often confused.

Chapter 1

General Introduction

1.1. SETTING THE SCENE

The era we live in is more than ever associated with heavy pressure on wildlife and their natural environment due to the needs and consequences of the expanding global human population (Davis 2003; Meyerson and Moony 2007). The most important factor in battling the loss in biodiversity is recognizing the decline and acting against it in order to combat current and future declines and hence conserving populations and/or species. Sadly, textbook examples exist where this was not the case and where species went extinct: as early as the 18th century, the ill-fated dodo (*Raphus cucullatus*) suffered from the European global expansion, going extinct due to the combination of hunting and introduced predators/competitors and some intrinsic traits of the birds (e.g. gigantism and flightlessness) which made them easy to be spotted (as reviewed in Turvey and Cheke 2008).

At present, the total number of extant species that are considered threatened, i.e. classified as either vulnerable, endangered or critically endangered by the International Union for the Conservation of Nature and Natural Resources (IUCN), is estimated to be 24.431 species (protists, fungi, plants and animals) (IUCN, 2017). Although this is an estimation (not every species is known or has been evaluated), it shows that a lot of species are struggling. This estimate includes roughly one third of all known amphibian species, putting these animals at the forefront of the so-called 6th mass extinction (Ceballos et al. 2015).

1.2. A GENTLE INTRODUCTION TO URODELA

Although amphibians reside somewhere at the bottom of the "petability scale" for the general public, they play their role in regulating food webs, converting biomass and transferring energy from aquatic to terrestrial systems (Davic and Welsh 2004). Members of the amphibian taxon are considered as the first vertebrates to successfully explore and exploit the terrestrial environment (Sahney et al. 2010): *amphibia* literally means "living on both sides", referring to their close relationship with water and land. Although a big part of the amphibian species do not fit the following rule, generally amphibians are said to lay their eggs in water, followed by an aquatic larval stage, transitioning into terrestrial development of juveniles and returning to water as adult either permanently or seasonally for reproduction (Duellman and Trueb 1986).

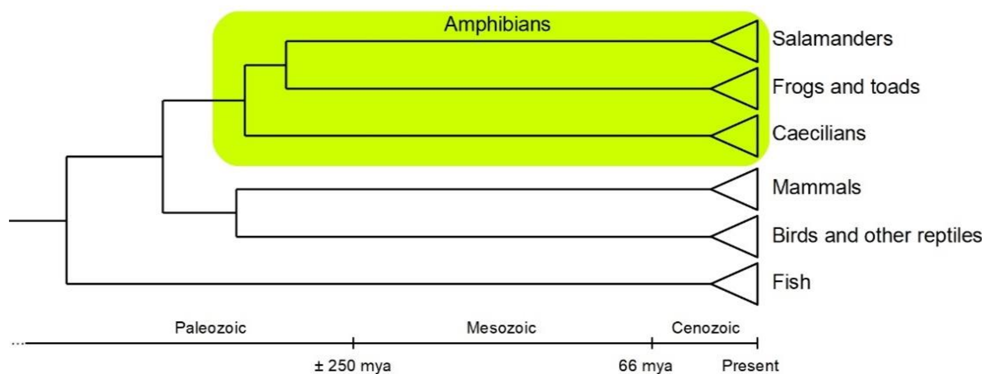

Figure 1.1. Phylogenetic relationships among vertebrates. The three main amphibian groups are highlighted in green. Phylogenetic tree adjusted from San Mauro (2010).

There are three orders of amphibians: Gymnophiona (caecilians), Anura (frogs and toads) and Caudata (salamanders) (Fig. 1.1), which in total contain approximately 7,700 species (Frost 2017). Caudata or salamanders, consisting of approximately 700

species, occur only in the Northern temperate regions and the tropical regions of Central and Northern South America (Raffaëlli 2013).

The skin of salamanders, similar to other amphibians, functions not just as a first protective barrier against diseases and injury to internal organs, it also acts as an organ that regulates osmoregulation, electrolyte homeostasis, and gas exchange (the majority of the salamanders, have even completely "lost" their lungs and depend entirely on cutaneous respiration) (Whitford and Hutchison 1965; Jorgensen 1997; Costa et al. 2013)). Evidently, impaired skin functioning often will lead to life-threatening situations since these vital functions are disturbed.

1.3. The amphibian crisis

The inherent trait of amphibians to depend on aquatic (or moist) and terrestrial environments makes them susceptible to changes and perturbations in both systems. Therefore, this trait makes amphibians ideal barometers to monitor *ecosystem health*: a hard to define term that describes the capability of an ecosystem to sustain growth and reproduction, support all life and interactions between lifeforms and deal with perturbations made to the ecosystems (Rapport et al. 1998). This implies that amphibians are impacted more compared to other groups which depend on either the aquatic or the terrestrial environment. Currently, the IUCN identified five major threats for amphibian biodiversity: habitat loss, pollution, fires, invasive species and diseases (Fig. 1.2). While some of these factors have a clear anthropogenic link (such as habitat loss or pollution), others seem like a natural process but are often facilitated

or induced by human activities (fires, invasive species, diseases). Below, I describe 2 threats which play a major role in the research preformed within this thesis and therefore merit some elaboration.

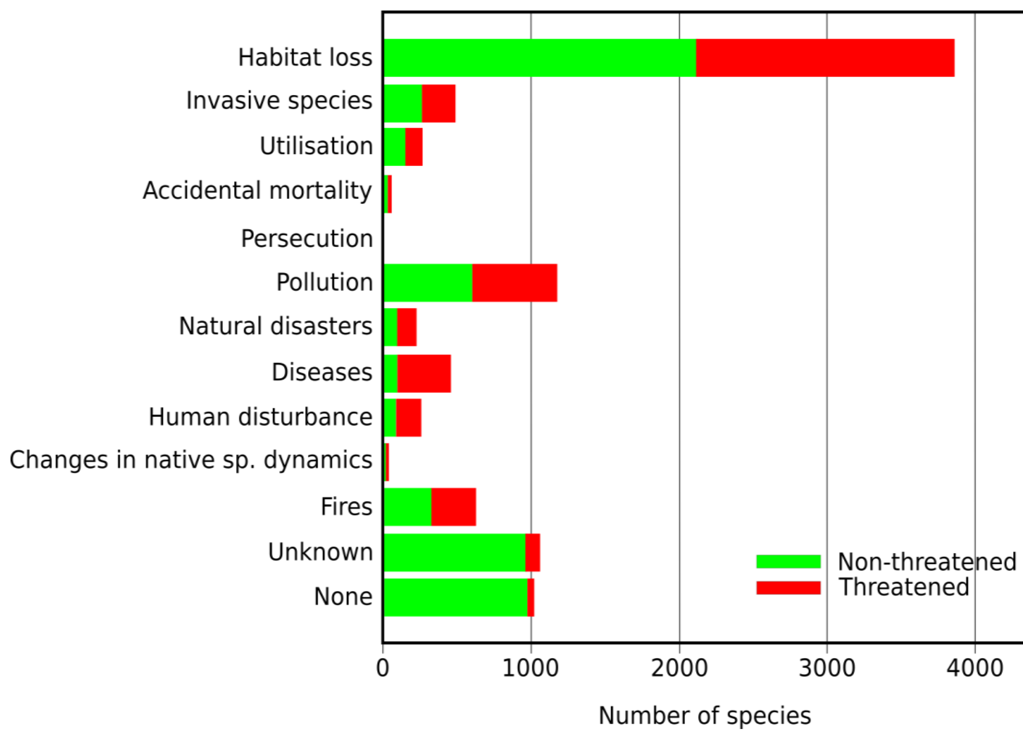

Figure 1.2. **Major threats to amphibian species**. All known threats are listed together with the number of species are impacted by this threat. Species are classified as threatened (listed as vulnerable, endangered or critically endangered by the IUCN) or non-threatened (listed as near threatened or least concern). Only the species that were assessed are shown (6260 species in 2008). (adapted from http://www.iucnredlist.org/initiatives/amphibians/analysis/major-threats 08 September 2017).

1.3.1 Habitat loss

In terms of rapidly declining species, habitat loss and fragmentation have been identified by the IUCN as the largest threat for amphibians more than a decade ago (Stuart et al. 2004). Habitat fragmentation and destruction generally results in altered population and species dynamics and leads to a decrease in ecosystem health (Kraus

et al. 2010). Fragmentation in itself should be considered as a term that describes alterations to a specific ecosystem: it does not have a direct impact on populations or individuals but it is a general concept that combines underlying factors which are at play locally. Such factors are e.g. the edge effect, patch size, patch shape (complexity), matrix permeability or isolation (Boulinier et al. 2001). When an area is fragmented, it is divided into suitable and unsuitable patches. The suitable patches, which have an increased contact zone with the matrix in between the different patches, are in fact smaller than the actual patch size because the outside zone of the patch has different properties (e.g. microclimate or vegetation structure (Didham and Lawton 1999)) compared the core zone of the patch. Therefore, certain species (among which a lot of amphibians), tend to avoid the contact zones between suitable and unsuitable patches (Pfeifer et al. 2017).

In terms of amphibian persistence in a fragmented landscape, between patch matrix permeability and connectivity between patches have been identified as the main driver behind the regional viability of amphibian populations (Cushman 2006). One of the hazards of a reduced connectivity between adjacent populations is the promotion of inbreeding (as reviewed by Fahrig 2003), which likely results in a depauperate genetic diversity of the affected populations (Westmeier et al. 1998). Laboratory experiments and results from captive collections as well as studies on natural populations in a variety of species showed that inbreeding increases the risk of population extirpation (Brook et al. 2002) (e.g. birds (Boulinier et al. 2001), mammals

(Jimenez et al. 1994), fish (Hugueny et al. 2011), butterflies (Saccheri et al. 1998), snails (Chen 1993), Drosphila flies (Bijlsma et al. 2000), plants (Lennartsson 2002) or amphibians as reviewed by Cushman (2006)).Therefore, maintaining connectivity within a fragmented landscape is likely to be beneficial for population persistence. By facilitating gene transfer between neighbouring populations, artificially increasing landscape permeability is often applied as a conservation technique in an attempt to counteract the negative effects of fragmented populations.

However, other than the effect of fragmentation on species distribution and dynamics, increased landscape permeability also affects the rate and pattern of disease spread (McCallum 2008; Rudnick et al. 2012; Gottdenker et al. 2014). Combined with the previous, it is clear that there is some debate on the actions that should be taken in a fragmented landscape when disease spread is also taken into account. On one hand, increasing landscape permeability is beneficial for pathogen spread and thus detrimental for host persistence suggesting that reconnecting infected and uninfected areas should be avoided. On the other hand, the benefits of (re)connecting fragmented areas, cf. rescue effect (Brown and Kodric-Brown 1997), are suggested to outcompete the risk of increased pathogen transmission between such areas (McCallum and Dobson 2002). Likely, there is not one optimal solution and the actions should be placed in perspective of the desired outcome.

1.3.2. Diseases

As mentioned above, diseases are one of the main threats to amphibians. While a variety of diseases have been identified in wild amphibians, their impact on a population-level is not (always) fully understood. While some diseases are the primary cause of clinical symptoms, often, unknown underlying causes affect an individual's immune system which then results in secondary diseases. Some of the disease known to occur in amphibians can be divided based on the type of pathogenic agent: bacterial infections (flavo- and mycobacteriosis, chlamydiosis), viral infections (ranaviruses and herpesvirus), fungal (-like) infections (chytridiomycosis, zygomycosis and chromomycosis, saprolegniasis), protozoan infections (amoeba, ciliates, flagellates, sporozoa), mesomycetozoa infections (amphibiocystidium, amphibiothecum and ichthyophonus) and lastly metozoan infections (nematoda, trematode, myxozoa and arthropods) (Daszak et al. 2000; Pessier 2002; Densmore and Green 2007; Klaphake 2009; Latney and Klaphake 2013; Pasmans et al. 2014). Several of these infectious agents are part of the surrounding environment and harmless to the individual under normal circumstances and only cause diseases in weakened and/or immunosuppressed individuals. *Saprolegnia* species for instance, are oomycetes found in practically all waterbodies as saprophytic organisms, but some species have evolved the ability to parasitize aquatic plants and animals by using specialized strategies such as repeated zoospore emergence (Cerenius and Söderhall 1985). Effects of Saprolegniasis have been observed in toads (Blaustein et al. 1994; Kiesecker et al. 2001) where the fungus-like organism was found on egg masses leading to embryonic deaths

and hatching failures (Kiesecker and Blaustein 1995). Saprolegniosis in adult amphibians can also occur but is usually related to secondary infections on for instance open wounds or damaged gills of neotenic individuals (Fanelli and Goldstein 1964). *Ribeiroia* is a trematode parasite that infects birds and mammals but has snails, fish and larval amphibians as intermediate hosts, where in the latter it has been found in close association with limb malformations (Johnson et al. 1999 & 2004a) although it is unknown whether this parasite has an effect on population level (e.g. stability of population size) (Johnson et al. 2004b).

From the above list of diseases, two diseases in particular have been in the centre of attention lately: ranaviruses and chytridiomycosis. Both diseases have been listed by the World Organisation of Animal Health (Office International des Epizooties, OIE), and following the European guidelines, these diseases have to be reported and efforts should be made to stop the disease from spreading to uninfected areas using sanitary measurements in the trade of amphibians and their product (Schoegel et al. 2010). *Ranavirus* is a genus within the *Iridoviridae* family of viruses (Gray et al. 2015). Until now, there are six species of *Ranavirus* that have been isolated which are known to cause massive die-offs in amphibians: *Frog Virus 3* [FV3] (Granloff et al. 1965) and FV3-like viruses (e.g. Cunningham et al. 1996), Common midwife toad virus [CMTV] (Basleiro 2009), *Ambystoma tigrinum* virus [ATV] (Jancovich et al. 1997), *Bohle iridovirus* [BIV] (Speare and Smith 1992) and BIV-like viruses (e.g. Cheng et al. 2014). Recently, data showed that the virulence of these closely-related viruses is strain

dependent and that the more virulent strains can greatly affect amphibian populations across multiple species even though less virulent strains have been present in that area (Price et al. 2014). Furthermore, additionally to the ranaviruses pathogenic to amphibians, several more ranaviruses exist that cause disease in fish and reptiles (See Duffus et al. (2015) for more detailed information).

1.4. CHYTRIDIOMYCOSIS

Chytridiomycosis is a fungal skin disease caused by chytrid fungi. Chytrid fungi are very abundant in both terrestrial and aquatic habitats where they can act as decomposers of organic matter (Gleason et al. 2008; Grossart et al. 2016) or where they perform in an unknown ecological niche (Vandenkoornhuyse et al. 2002). A number of these species however evolved to become (obligate) parasites in algae, plants or animals (Longcore et al. 1999; Ibelings et al. 2004; Powel 1993). Most of the animal hosts are invertebrates but two genera have become a vertebrate parasite. The first is a monotypic genus, described in the 1920's as a skin and gill parasite of fish, but further scientific details other than the description of *Ichthyochytrium vulgare* are lacking (Plehn 1920). The second genus, *Batrachochytrium*, contains two species: *Batrachochytrium dendrobatidis* and *B. salamandrivorans*, both of which are parasites of amphibians, while their closest relative (*Homolaphlyctis polyrhiza*) is a saprophytic organism found only once in a fishless oligotrophic lake (Davis et al. 1994; Rhodes and Davis 1995; Longcore et al. 2011). Although amphibians are the prime host for *Batrachochytrium* species, they

have also been found to infect invertebrate hosts (e.g. crayfish (McMahon et al. 2013)) and fish (Liew et al. 2017).

The *Batrachochytrium* chytrid fungi are very simple fungi, similar to the other member of the taxon. Their life cycle starts with a flagellated wall-less zoospore that is thought to remain motile for up to 24 hours during which it either attaches itself to a suitable substrate such as amphibian skin/its mucus layer, substrates such as waterfowl feet or environmental fomites or the zoospore dies when no suitable habitat is found (Fig. 1.3A) (Longcore et al. 1999; Piotrowsi et al. 2004; Johnson and Speare 2005; Garmyn et al. 2011). Next, the encysted spore develops into a sporangium where new spores form by mitotic division of the cytoplasm (Fig. 1.3B). As the spores mature in the sporangium (Fig. 1.3C), a pore is formed on the surface of the sporangium through which the motile zoospores will exit the sporangium and the life cycle restarts (Fig. 1.3D). Until now, no evidence has been found that would support a sexual reproductive cycle in this fungal genus, e.g. population genetics studies showed an excess of heterozygosity combined with a low allele diversity (James et al. 2009). One study however also showed that, although the overall allele diversity was low, some local strains had a high genetic diversity and did not share genotypes with other populations suggesting local recombination between different *B. dendrobatidis* lineages (Morgan et al. 2007).

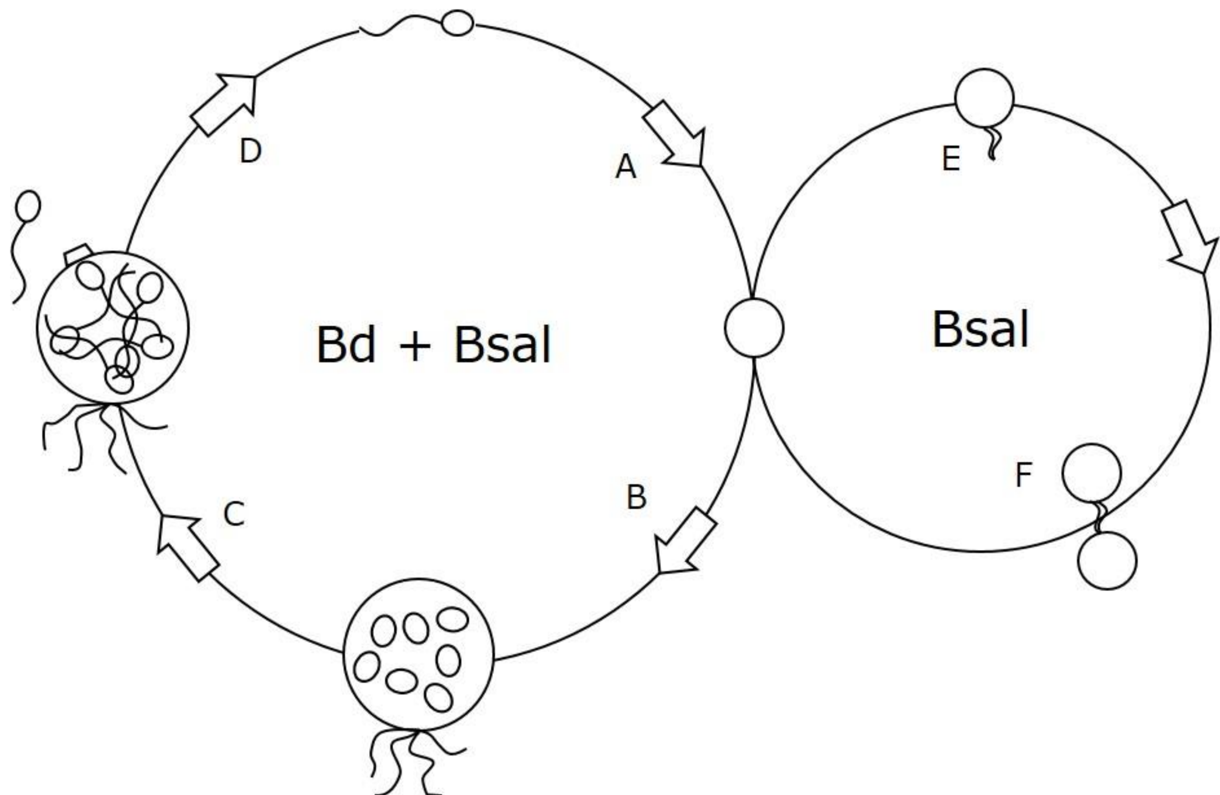

Figure 1.3. Life cycle of *Batrachochytrium* species. The first stage is a flagellated zoospore that will attach itself to suitable substrate and encyst (A). At encystment, the flagellum is absorbed and a young sporangium is formed that develops into an immature sporangium in which the spores are being formed by mitotic division of the cytoplasm (B). This sporangium then matures into a zoosporangium (C) which will release zoospores through a discharge tube (D). *B. salamandrivorans* forms a germtube *in vitro* as well compared to only *in vivo* in case of *B. dendrobatidis* (E) into which it transports its cytoplasm (F). From here, the new thallus develops from where development continues as for *B. dendrobatidis*. Adapted from Van Rooij et al. (2015).

1.4.1. *BATRACHOCHYTRIUM DENDROBATIDIS*

1.4.1.1 THE DISCOVERY

Following marked decreases in amphibian populations in North, Central and South America as well as Europe, Asia, Australia and Africa, the amphibian community was uncertain to what caused these global declines (Blaustein and Wake 1990; Laurance et al. 1996; Houlahan 2000), until at the end of the 20th century the amphibian chytrid disease was discovered (Berger et al. 1998; Longcore et al. 1999).

The fungus responsible for this disease was isolated from a blue poison dart frog (*Dendrobates tincturus*), hence the species name *B. dendrobatidis* (Longcore et al. 1999). Initially, the fungus was thought to be implicated in species declines of Central American and Australian frogs and toads (Berger et al. 1998), but soon after, the fungus was found in other geographical regions and salamanders and caecilians (for instance Daszak 1999; Bosch 2001; Davidson et al. 2003; Gower et al. 2013).

1.4.1.2. The origin

Where this fungus originated from geographically remains under debate: two hypotheses have been brought forward regarding its origin: the endemic pathogen hypothesis or the novel pathogen hypothesis (Rachowicz et al. 2015). The endemic pathogen hypothesis (EPH), suggests that the pathogen has been around for a long time as an endemic disease in specific parts in the world, but due to a change in the environment, the host or the pathogen itself, the host-pathogen relationship has experienced a shift resulting in higher disease prevalence. The novel/invasive pathogen hypothesis (NPH), suggests that the pathogen has spread to naive areas where the local amphibian fauna has not developed any resistance against the pathogen leading to massive outbreaks. Multiple studies have delivered evidence for both scenarios. The NPH for instance was corroborated by studies that linked the trade and translocation of infected bullfrogs (*Lithobates catesbeianus*) and African clawed frogs (*Xenopus laevis*) with *B. dendrobatidis* introductions in new areas (Weldon et al. 2004; Garner et al. 2006; Soto-Azat et al. 2010). The EPH is backed up by the lineages

of *B. dendrobatidis* that have been identified and where different isolates can be clustered in distinct lineages. One lineage specifically, termed the global panzootic lineage or Bd-GPL, is distributed globally and is hyper virulent, while other lineages are endemic lacking signs of amphibian population declines (Farrer et al. 2011). However, it has become clear that there is not one *B. dendrobatidis* strain, but that almost each continent seems to have its own endemic strain(s) and that there is one specific lineage among these strains, the aforementioned Bd-GPL, that is hypervirulent and caused the massive die-offs in amphibian populations (Berger et al. 2005; James et al. 2009; Fisher et al. 2009; Bai et al. 2012; Becker et al. 2017).

Additionally, studies showed the importance of environmental factors such as temperature, acidity, altitude, micropredator presence or macroinvertebrate presence and that these factors influence the infection dynamics in host populations suggesting that altered environmental conditions might also play a role in sudden outbreak events (Johnson et al. 2003; Piotrowski et al. 2004; Puschendorf et al. 2009, Murray et al. 2011 & 2013; Strauss et al. 2013; Schmeller et al. 2014; Spitzen-van der Sluijs et al. 2017).

1.4.1.3. The disease

B. dendrobatidis infects the keratinized parts of amphibians, therefore in anuran larvae infection is restricted to the teeth rows (the only keratinized parts of larval anurans) while in adults, the entire keratinized skin layer (stratum corneum and stratum granulosum) is prone to infection. Although there seems to be a link between the presence of keratinized structures and *B. dendrobatidis* presence, it is unknown

whether the fungus actually feeds on the keratins in these structures (Marantelli et al. 2004; Piotrowski et al. 2004). Clinical signs of a *B. dendrobatidis* infection are very variable and nonspecific. As mentioned above, *B. dendrobatidis* infections in wild larval amphibians have only been identified at the teeth row of anuran tadpoles. Data on larval urodela and caecilians are lacking, but a *B. dendrobatidis* infection has been observed in the epidermis of a captive specimen of the neotenic urodelan Axolotl (*Ambystoma mexicanum*) (Mutschmann 2015), suggesting other amphibian species might also be affected by *B. dendrobatidis*. Although larval infections are not lethal, the infection could affect their development and lead to malnutrition resulting in for instance malformations (Berger et al. 1999). In adult amphibians, infected individuals may be anorectic, lethargic, ataxic, or they might die spontaneously without clinical signs. They might also stay in water for long periods of time and show seizures, convulsions, loss of the righting reflex, and abnormal posture (Mutschmann 2015). Because the infection is localized at the keratinized skin layers, the alterations of the skin can be observed as discoloration, roughening, hyperkeratosis, accumulation of sloughed skin over the body or limbs, and excessive sloughing of the skin (Fig. 1.4) although these signs can also remain absent.

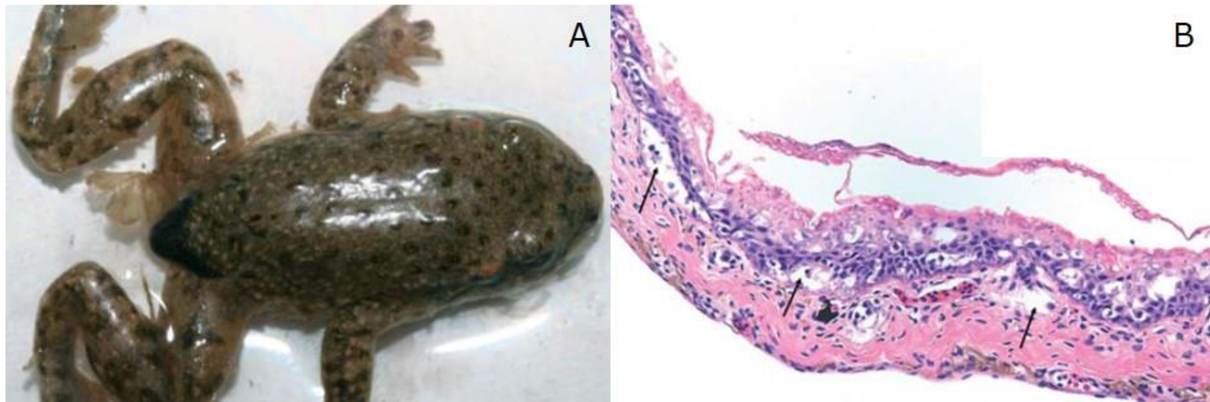

Figure 1.4. Deceased midwife toad (*Alytes obstetricans*) that recently underwent metamorphosis (evident from the black tail remnant). Notice the presence of bits of loose skin shedding, typical as a result of chytridiomycosis (A). Haematoxylin and eosin staining of the skin of a midwife toad (B). Presence of epidermal hyperplasia and hyperkeratosis are indicative of *B. dendrobatidis* infection. Arrows point to intra-epidermal vesicle formation which can result in abnormal skin shedding. Adapted from Pasmans et al. (2010)

The pathogenesis of the disease was long unknown, although it was suggested early on that a disrupted skin function may lay at the basis of the pathology since this is the location where infections start: a zoospore (the infectious stage) arrives at the environment-host barrier (here the skin), attaches itself to the host, germinates and by forming a germ tube, starts penetrating into the hosts cells and eventually invading the cells of the entire epidermal layer where the physical damage occurs (Brutyn et al. 2012, Van Rooij et al. 2012). It is worth noting that the initial phase of a *B. dendrobatidis* infection appeared to be independent of the host's susceptibility to a *B. dendrobatidis* infection: germtube invasion is observed but is only limited to the superficial layers of the skin in unsusceptible species while the invasion proceeds into the deeper layers of the skin accompanied by intracellular growth of the pathogen in susceptible species, the latter being absent in the unsusceptible host (Van Rooij et al. 2012). Eventually, ten years after the discovery of *B. dendrobatidis*, it was shown that an osmotic imbalance

disrupted the normal skin functioning, leading to an electrolyte depletion (as shown by the reduction of plasma osmolality, sodium, potassium, magnesium and chloride concentrations while maintaining normal plasma albumin, hematocrit and urea levels which indicates that the hydration status of the individual was unaffected), and eventually followed by cardiac arrest (Voyles et al. 2007 & 2009).

1.4.1.4. Impact on Belgian amphibian communities

In Belgium, *B. dendrobatidis* has been identified but until now the impact of the disease appears to be non-significant, only one death has been reported as a result of a *B. dendrobatidis* infection and no signs of population declines have been observed that are attributed to chytridiomycosis (Pasmans et al. 2010; Spitzen-van der Sluijs et al. 2010, 2014 & 2017; Martel et al. 2012). Spitzen-van der Sluijs et al. (2017) stated that although the presence of *B. dendrobatidis* comes at a cost for survival probability of individual juvenile yellow-bellied toads (*Bombina variegata*), adults appeared unaffected, resulting in a stable population trend. It should be noted that Spitzen-van der Sluijs et al. (2010), showed that the majority of *B. dendrobatidis* infected individuals in Belgium were invasive American bull frog (*Lithobates catesbeianus*), with a prevalence of approximately 20%, suggesting that these species could potentially increase *B. dendrobatidis* occurrence as they spread from their current distribution. Fortunately, this species is actively eradicated. At present, it is unknown to which lineage the *B. dendrobatidis* strain, or strains, that occur in Belgium belongs to, but the introduction of more virulent strains may lead to problematic situations in our native

amphibian populations. In Europe, *B. dendrobatidis* is widely distributed, generally without observed population declines, although Central Spain did suffer from population decline and large amphibian die-offs associated with *B. dendrobatidis* presence (Bosch et al. 2001 & 2006).

1.4.2. *BATRACHOCHYTRIUM SALAMANDRIVORANS*

1.4.2.1. THE "NEW" CHYTRID

In the Netherlands, the fire salamander (*Salamandra salamandra terrestris*) is at the North-Western edge of its distribution. Only three populations are known in the Netherlands, two of which are native (Bunderbos (N 50°54''51'', E 05°44'59'') and Vijlenerbos (N 50°54'51'', E 05°55'09'')) and one is introduced (Putberg (N 50°51'17'', E 05°57'59'')). From these populations, one population was monitored since the 70's and reached densities up to 1000 individuals/hectare (Bunderbos, with 144 ha suitable habitat), while the other two populations yielded very low numbers during transect counts: Vijlenerbos: 1-5 individuals in 58 ha suitable habitat, last individuals seen in 2010, Putberg: 1-15 individuals in 3 ha suitable habitat, last individuals seen in 2012, compared to 70-240 individuals yearly in Bunderbos (Spitzen-van der Sluijs et al. 2013). From 2008 onwards, dead fire salamanders were found on multiple occasions in Bunderbos and from 2010 onwards, the population was found to be decreasing at an alarming rate (Spitzen-van der Sluijs et al. 2013). Initial attempts to determine the cause of this decline failed and five years after the first dead individuals were found, the cause was elucidated with the description of a new chytrid fungus:

Batrachochytrium salamandrivorans (Martel et al. 2013). As mentioned earlier, this fungus clusters together phylogenetically with *B. dendrobatidis*, forming a monophyletic clade of amphibian-parasitizing chytrid fungi. The life cycle is similar to *B. dendrobatidis*, with the only exception that *B. salamandrivorans* germlings (the recently encysted zoospores), form a germtube both *in vitro* and *in vivo* (Fig. 1.3E) and transfer their cell content into the newly formed thallus and continue their development from this new thallus (Fig. 1.3F) (Martel et al. 2013).

1.4.2.2. THE DISEASE

Given that the pathogen is a recent discovery, few studies had been published regarding this new fungus prior to the work performed for this dissertation. The outbreak of the disease was observed in the Netherlands (Spitzen-van der Sluijs et al. 2013) along with the identification of the etiological agent as well as the first outbreak site in Belgium (Eupen) (Martel et al. 2013 & 2014). Next, the detection of *B. salamandrivorans*, and differentiation from *B. dendrobatidis*, in genetic samples was established (Blooi et al. 2013), followed by the determination of the origin, the presumptive host spectrum and the most likely route of introduction from the pathogen (Martel et al. 2014). The clinical signs of the disease are very similar to the signs observed in *B. dendrobatidis* infected individuals although additionally extensive skin ulcerations (Fig. 1.5B) and haemorrhages can be seen all over the epidermis as well as excessive shedding leading up to death as fast as 7 days after infection (Martel et al. 2013). At the microscopic level, intracellular colonial thalli are distributed in all

epidermal cell layers where *B. salamandrivorans* causes necrosis in the keratinocytes juxtaposed to these sites (Fig. 1.5A) (Martel et al. 2013), rather than causing generalized hyperkeratosis as seen with *B. dendrobatidis* (Longcore et al. 1999) (Fig. 1.4).

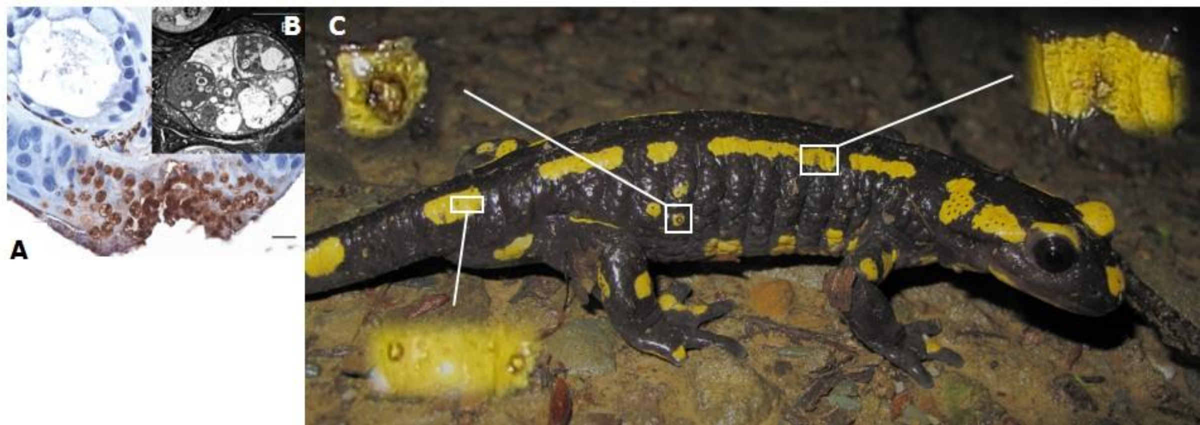

Figure 1.5. Infected fire salamander (*Salamandra salamandra terrestris*). (A) Immunohistochemical skin section of a macroscopic lesion typically seen in *B. salamandrivorans* infected individuals with (B) and transmission electron microscopic image of an intracellular colonial thallus. (C) Infected fire salamander with several macroscopical lesions, ulcers, enlarged in the insets. (A and B adapted from Martel et al. 2013).

The infection process and host invasion have not been studied in detail compared to its sister species, but is most likely germ tube mediated due to the presence of the germ tubes even *in vitro* (Van Rooij et al. 2015). Although the two fungal species, *B. dendrobatidis* and *B. salamandrivorans*, are closely related and the only chytrids pathogenic to amphibians, there are some distinct differences (e.g. host range, environmental conditions tolerated, disease symptoms), the reason for which remain unknown and merit further study to enlarge our understanding of the two pathogens.

1.4.2.3. Distribution of *B. salamandrivorans*

When *B. salamandrivorans* was first described in 2013, the fungus had only been identified in the Netherlands, soon followed by Belgium (Spitzen-van der Sluijs et al. 2013; Martel et al. 2013 & 2014). Samples from traded amphibians showed that positive samples originate from Vietnam, Thailand and Japan while one Japanese museum specimen archived in the mid-19th century also tested positive for the fungus (Martel et al. 2014). Shortly after, the fungus was found to be present at an endemic level in Vietnam, with a prevalence of approximately 3% (Laking et al. 2016), and China, where the prevalence was on average 2-4%, although one population of a heavily traded species showed a prevalence of 50% (Yuan et al. 2018). Therefore, the most likely place of origin is Eastern Asia and the fungus was probably introduced into Western Europe by trade in wild caught salamanders from these areas that were infected at the time of collection (Martel et al. 2014). In the USA, Canada (Wild animal and plant trade regulations SOR/96-263), Switzerland and Hungary (modification to the decree 41/2010), trade bans were promptly installed (Muletz et al. 2014; Bales et al. 2015; Grant et al. 2015; Gray et al. 2015; Yap et al. 2015; Schmidt 2015, Parrot et al. 2016). The discovery of the fungus was most likely “accidental” because the fire salamander population in the Netherlands was closely monitored since the 1970's (Spitzen-van der Sluijs et al. 2013). When and where *B. salamandrivorans* exactly entered Europe is unknown and would need a much wider screening than currently available, combined with a genomic analysis of isolates collected from the different outbreak sites and compared to isolates from the fungus' primary home range.

1.4.2.4. THE HOST(S)

Given that *B. dendrobatidis* was initially only found in frogs and toads (Berger et al. 1998 & 1999), and has later also been shown to infect salamanders (Davidson et al. 2003) and caecilians (Gower et al. 2013), an assessment was carried out to estimate the risk that *B. salamandrivorans* poses to amphibian biodiversity. Therefore, Martel et al. (2014) tested anurans, caecilians and caudata to determine their susceptibility to the fungus. Results of these experiments showed a pattern that allowed animals to be classified in 4 different groups: 1) resistant, 2) tolerant, 3) susceptible and 4) lethal (Fig. 1.6). In species that were *resistant*, no individuals tested positive 7 days after infection and onwards. In species that were termed *tolerant*, infection was detected at 7 days or more after infection, but none of the individuals developed disease and all animals cleared the infection by the end of the experiment. *Susceptible* species were species that got infected and showed clinical signs of the disease but that were also able to recover from the disease and, in some cases, to clear the infection by the end of the experiment. Lastly, the *lethal* species were all successfully infected, developed disease and died as a result of the disease. Species classified within the last category, were all Western Palearctic salamandrid species (except for one species, the palmate newt (*Lissotriton helveticus*)), and one European plethodontid species.

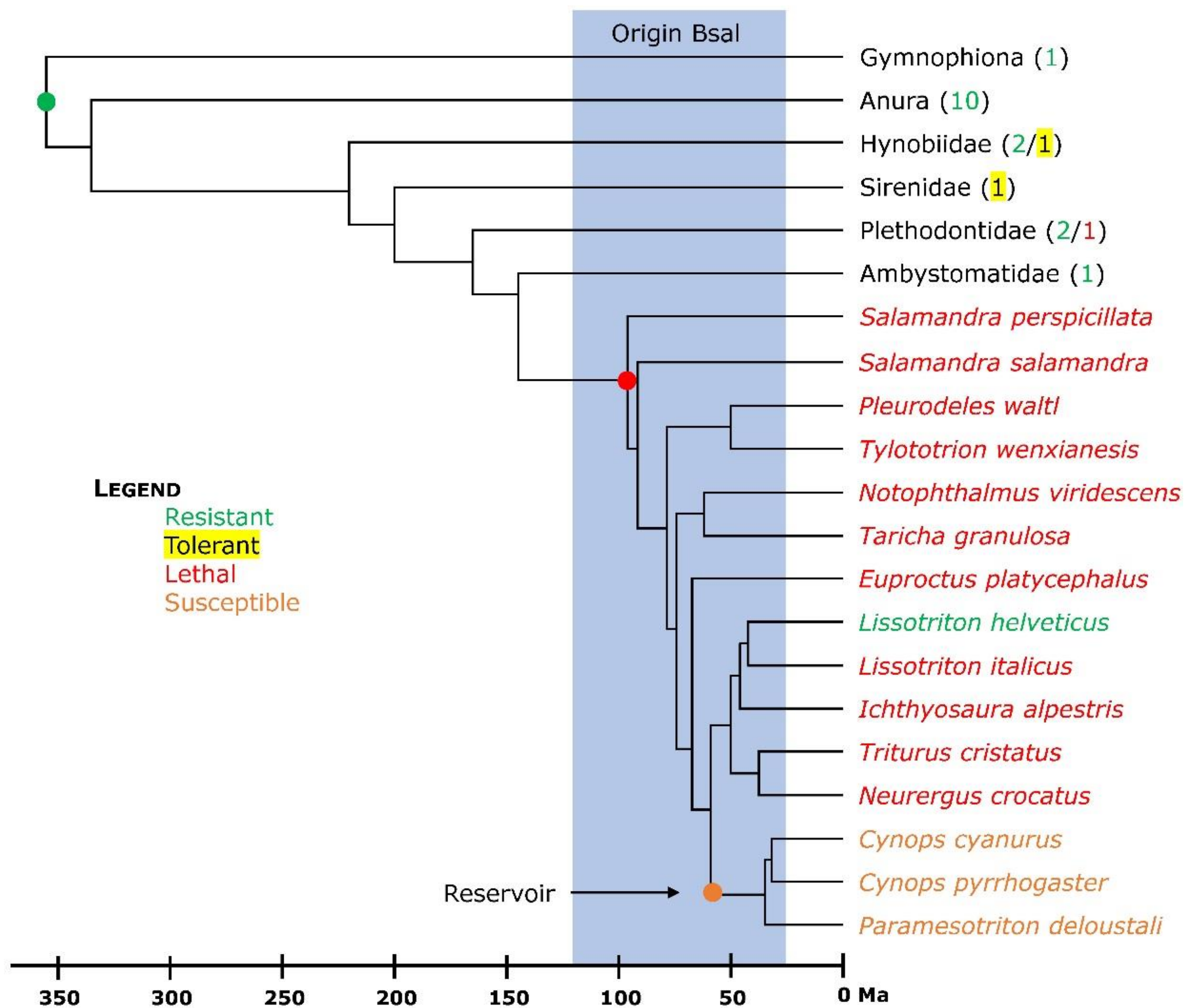

Figure 1.6. Amphibian susceptibility to *Batrachochytrium salamandrivorans* through time. Molecular time scale (Ma) for 34 species. Numbers in brackets are the species tested from that family. Colours indicate the species susceptibility based on experimental infections. Resistant (green): no infection, no disease; tolerant (Black on yellow background): infection in the absence of disease; susceptible (orange): infection resulting in clinical disease with possibility of subsequent recovery; lethal (red): infection resulting in lethal disease in all infected animals. Coloured dots on nodes indicate the results of the maximum likelihood ancestral reconstructions for the respective susceptibility character. The 95% highest posterior density for time of divergence between *B. salamandrivorans* and *B. dendrobatidis* is indicated in light blue. (Adapted from Martel et al. 2014)

1.4.3. Detection of chytridiomycosis in samples

Detection of the fungi is usually done by histology and/or (quantitative) polymerase chain reaction ((q)PCR). Histological sections have the advantage that the infection can be visualized, associated with lesions and that the presence of a chytrid

fungus is unquestionable, especially when using an immunohistochemical staining assay (Berger et al. 2002). The disadvantages of this method are the low sensitivity (the infection itself might be missed during the early stages of the infection), it can take up to several days to process the entire sample, the requirement of a tissue sample such as toe clips in case of adult individuals or whole individuals when working with larval amphibians and lastly the method has a low specificity lacking an etiological diagnosis when using for instance a haematoxylin and eosin staining.

When working with alive and wild animals, a non-invasive or at least non-lethal method is desired. Therefore, methods that can analyse samples quickly and accurately without killing or harming the individual are preferred. A (q)PCR analysis can be performed either for *B. dendrobatidis* or *B. salamandrivorans* alone (simplex) or for both species in one reaction (duplex) on a non-invasively collected skin swab (Boyle et al. 2003; Retallick et al. 2006; Blooi et al. 2013). Primers designed for *B. dendrobatidis* target the regions in the internally transcribed spacers 1 (ITS-1) and the adjacent 5.8S region and aim at amplifying the entire ITS-1 rRNA sequence, the *B. dendrobatidis* probe attaches to this ITS-1 sequence (Boyle et al. 2004). The *B. salamandrivorans* primers and probe target the 5.8S rRNA sequence by using primers that bind at the ITS-1 and the ITS-2 region and a probe which attaches to the 5.8S RNA sequences itself (Martel et al. 2013; Blooi et al. 2013). Some attention is warranted when interpreting the results of a (q)PCR because false negative and false positive results can be present (Hyatt et al. 2007; Thomas et al. 2018). The presence of false negative results occurs due

to inhibitors in samples, such as humic substances which are highly abundant in the humus layer and aquatic environments and can interact with DNA polymerase or anneal to primers blocking the (q)PCR reaction (Tsai and Olso 1992a & b). These inhibitors can be supressed by adding bovine serum albumin (BSA) to the qPCR reaction mixture (Kreader 1996; Garland et al. 2010) or by diluting the DNA tenfold. False positive samples are usually the result of contamination or non-specific amplification. This can be solved by running duplicates of each sample and closely inspecting the outcome of the analysis. Besides the general rules for determining a successful qPCR run (values for efficiency, R^2 and slope, all specific for the setup of the qPCR protocol), the "in-house" rules for defining positive samples are based on the recommendations defined in Thomas et al. (2018): (1) a Ct-value (cycle treshold) is present for all repeated measures from 1 samples, (2) the amplification curve is normal (i.e. logarithmic) for all repeated measures, (3) the variability between the repeated measures is low ($Ct < 0.3$) and (4) Ct values are equal to or larger than, 1 genomic equivalent (GE).

1.5. The Host

1.5.1. Distribution

As mentioned above, a variety of European caudate species are susceptible to infection with *B. salamandrivorans* (Martel et al. 2014), but since the disease was discovered in fire salamanders (*Salamandra salamandra terrestris*), this species has become the model species for research involving chytridiomycosis both in the lab and

in the wild. In contrast to our other native newts (Crested newt (*Triturus cristatus*), Alpine newt (*Ichthyosaura alpestris*), smooth newt (*Lissotriton vulgaris*), palmate newt (*Lissotriton helveticus*)), salamanders are relatively large (up to 30 cm depending on the subspecies (Raffaëlli 2013)), terrestrial and sedentary which makes them more easy to observe than newts which are small, secretive and reside in the water for several months per years. The genus *Salamandra* contains 6 species and is present in Europe, North-Africa and the Middle-East (Fig. 1.7). Within the genus, the species *S. salamandra* occurs from south-western Europe (Portugal) to eastern Europe (Bulgaria, Romania and Western Ukraine) and to Poland and northern Germany (Kuzmin et al. 2009). Within this species, a number of subspecies are known, some of them are recognized while others remain doubtful or have been revoked (e.g. the colour morph *S. s. alfredschmidti* (Fig. 1.8) genetically undifferentiated and sympatric with *S.s. bernadezi* (Beukema et al. 2016)): *S. s. salamandra, S. s. werneri, S. s. beschkovi* and *S. s. carpathica* (Eastern Europe), *S. s. terrestris* (Western Central Europe), *S. s. gigliolii* (Italy), *S. s. fastuosa* (Northern Spain), *S. s. bejarae* and *S. s. almanzoris* (Central Spain), *S. s. bernardezi* (and the colour morph formerly known as *S. s. alfredschmidti*) (Cantabria), *S. s. morenica* and *S. s. longirostris* (Southern Spain) and *S. s. crespoi* and *S. s. gallaica* (Portugal) (Raffaëlli 2013, Vences et al. 2014). This distribution and relatively high number of subspecies is likely due to geographical isolation of populations during glacial periods and interglacial repopulation and -colonization enhancing speciation (Steinfartz et al. 2000, Rodriguez et al. 2017).

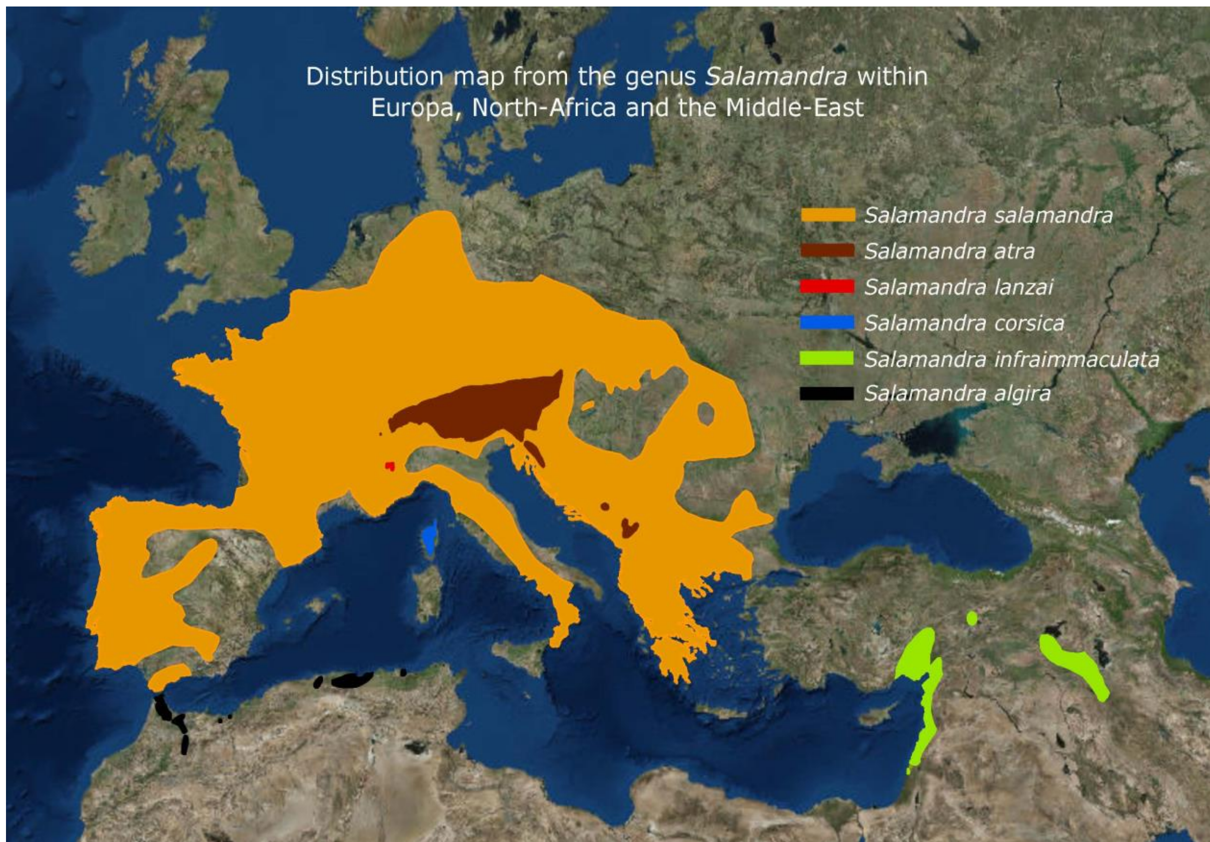

Figure 1.7. Distribution of the genus *Salamandra* in Europe, North Africa and the Middle East. Map constructed using GIS and data from the IUCN assessments for each species (Andreone et al. 2009, Donaire-Barosso et al. 2009; Kuzmin et al. 2009;Miaud et al. 2009; Pappenfuss et al. 2009; Sindaco et al. 2009).

1.5.2. NATURAL HISTORY

A salamander will remain absolutely motionless for an hour on or under some dead leaf, in the trickling waters that wend their way riverward from a mossy spring. To sit or stand for an hour, and watch this immovable creature, is both painful and monotonous, and when, at last, you disturb it, perhaps accidentally, away it goes to some similar spot nearby, and resumes its motionless attitude. Studies of salamander life soon become a bore.

Charles C. Abbott, A Naturalist's Rambles About Home (1884)

Fieldwork is a much desired aspect that biological/ecological researchers aspire to do. Unfortunately, fire salamanders are active after sunset, at high humidity (especially rain), and at temperatures above freezing temperatures (Table 1.1), preferably also at mild wind speeds although they can be quite robust and settle for less optimal conditions when they have been inactive for longer periods as long as

humidity is high enough (personal observation). In contrast to the above citation, multiple researchers have performed one year or long-term studies on the natural history of fire salamanders within different parts of their native range (e.g. France (Joly 1963 & 1968), Germany (Klewen 1985; Seifert 1991; Schmidt et al. 2005 & 2007), Belgium (Denoel 1996), Switzerland (Schmidt et al. 2014 & 2015).

Table 1.1. Data showing some of the variability in climatic conditions required for fire salamanders to be active. (Adapted from Bellenoue et al. 2006).

Location	Climatic conditions for activity	
	Temperature (°C)	Humidity (%)
Germany	>2	85
Germany	>1	67
Belgium	>3	N/A
Spain	6-12	>90
Ukraine	9-26	65-96

Although these studies show that there is a large variability in temporal processes such as mating, deposition of larvae, hibernation, aestivation or the life cycle, the natural history of these study populations is more or less the same. Fire salamanders are mostly found in deciduous forests, often dominated by beech and oak trees, that have a water source (small ponds, brooks, small rivers, …), but they can also be found in mixed forests, woodlands, rocky slopes or humid grasslands (Joly 1963; Klewen 1985; Parent 1985; Seifert 1991; Kuzmin et al. 2009). A typical fire salamander has yellow (sometimes orange to red) spots, blotches and stripes on a black (sometimes dark brown) background (Fig 1.8).

Figure 1.8. Variability in *Salamandra* species and subspecies. Clockwise starting at the top: *Salamandra salamandra fastuosa* (San Sebastian, Spain), *S. s. bernardezi* colour morph (formerly known as *S. s. alfredschmidti* (Tendi Valley, Spain), *S. atra* (Vicinity of Salzburg, Austria), *S. s. terrestris* (Zottegem, Belgium) three dorsal pictures showing the variability in dorsal patterning.

These patterns are unique for each individual and can be used to identify individuals in Capture-Mark-Recapture (CMR) studies (Feldmann 1971), either visually or by using an automated program (e.g. Van Tienhoven et al. 2007; Bolger et

al. 2012; Drechsler et al. 2015; Speybroeck and Steenhoudt 2017). Fire salamanders typically have two periods during which they are more active. Depending on the climatological conditions, these periods are late winter to spring and late summer to autumn, although for instance in Belgium and the Netherlands activity can proceed during summer and winter if temperatures are not too high or too low respectively (Denoël 1996; Goverse et al. 2011). As soon as temperatures allow it, salamanders emerge from their retreats in which they hibernate and start foraging (Table 1.1).

During this first activity period, gravid females deposit their larvae in either streams, rivulets, small ponds or puddles. One study has shown that females preferred either ponds or streams to deposit their larvae, hinting that this behavioural trait, linked to habitat preference, could be a step towards speciation or at least diversification (Caspers et al. 2015). The second main activity period is the mating period and can be observed as early as late spring to autumn. During mating, males have to persuade females to pick up a spermatophore which he deposits on the substrate. This is done by holding the females in a ventral amplexus (an embracement where males positioned themselves ventrally compared to the female), grabbing her front limbs after he has positioned himself under her (Fig. 1.9) (an ideal moment for the transmission of infectious diseases). In several caudate genera, chemical cues have been shown to play a role in this process (Rollman et al. 1999; Houck et al. 2007 & 2008; Van Bocxlaer et al. 2015), but they remain undescribed in the *Salamandra* genus,

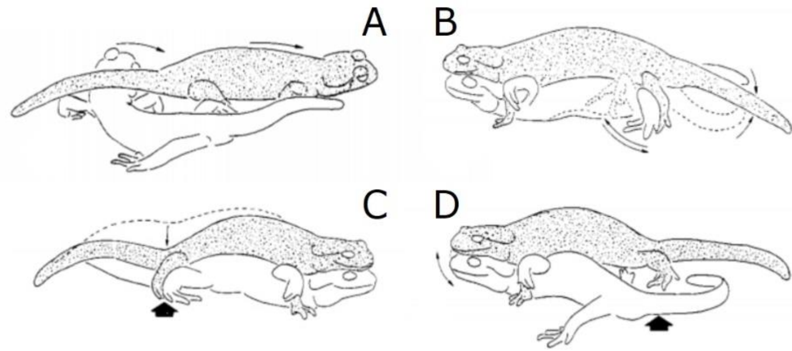

Figure 1.9. Courtship in *Salamandra salamandra*. When male (white) and female (grey) encounters occur, males will try to get underneath females (A) and try to engage in a dorsal amplexus (B). Males will stimulate the female cloaca by moving laterally. Successful mating results in spermatophore deposition by males (C) and subsequent pick up by females (D). Figure adjusted from Arnolds (1986).

although evidence has been given for the presence of olfactory chemical communication in behavioural experiments with fire salamanders (Caspers et al. 2011). When successfully mated and following spermatophore uptake, gestation takes place and up to 50 larvae are deposited into the water since most *Salamandra* (sub)species are ovoviviparous, although some populations give rise to up to 15 fully terrestrial juveniles (e.g. Buckley et al. 2007). Once deposited in the water, larvae remain there for 3-4 months before undergoing metamorphosis to terrestrial juveniles. At this stage, young animals, compared to adult individuals, do not show site fidelity and can migrate to new areas or nearby populations, although it has been suggested that migration rates in adults might be high in one population while low in another (Schmidt et al. 2005, 2007 & 2014). After 4-6 years, fire salamanders are sexually mature and become increasingly sedentary (Seifert 1991). At this stage, general activity is located close to the retreat site where adult fire salamanders, mainly males, have been suggested to show territorial behaviour in the form of physical aggression (mainly

wrestling, again an excellent moment for the transmission of infectious diseases) while biting (observed in e.g. *Plethodon* and *Pachytriton* (Raffaëlli 2013; Bortosky and Mathis 2016)) is absent. In choosing retreat sites, chemical cues have been shown to play a role in a congeneric species (Gautier et al. 2003) and in a more distantly related species (Gautier et al. 2006), which suggests this might also be the case in fire salamanders. In captivity, fire salamanders can live up to 20-30 years (Raffaëlli 2013), confirmed by mark-recapture studies in the wild (e.g. Feldman 1987). Although there is no general average of a decent population densities, population estimates go from 11 individuals per hectare (Bellenoue et al. 2006) to hundreds or more than 1000 individuals per hectare (e.g. Denoël 1996 or Spitzen-van der Sluijs et al. 2013). The persistence of fire salamanders in low densities is likely due to the fact that females are polyandric (Caspers et al. 2014), which likely also allows them to persist in fragmented areas. In a highly urbanized area, different subpopulations were highly differentiated with estimated effective population sizes below the recommended number for preventing inbreeding depression, while those populations only showed a minor loss in genetic diversity (Lourenço et al. 2017). In contrast, subpopulations in continuous areas did not show such a differentiation suggesting that migration and therefore genetic exchange is present (Steinfartz et al. 2007). The fact that fire salamanders, or other salamander species, can persist in low numbers in terms of population size is a promising feature and provides some hope for collapsed populations since *B. salamandrivorans* has been shown to nearly extirpate its host in the index outbreak site (Spitzen-van der Sluijs et al. 2013).

1.6. THE DISEASE TRIANGLE

After a new wildlife disease has been identified, the first scientific reference is usually the description of the disease and its effect on the host, i.e. the disease on an individual level. This is a very crucial first step, because we should understand the pathology/pathogenesis of the disease before we can look at ways to cure diseased individuals. Afterwards, the focus should shift towards understanding the disease in the wild. At this point, there are three variables that as a whole make up the disease dynamics: the pathogen, the host and the environment. This is referred to as the epidemiological triangle and is used to illustrate that the interactions between these three variables determine the outcome of the disease, i.e. whether it is in favour of the host or the infection (Wobeser 2006). A triangle is used as a graphical representation because each corner is connected to the next, emphasizing the interplay between the different variables and that they all affects each other (Fig. 1.10). Understanding the disease triangle is key in terms of looking for mitigation measures because ultimately, the aim is to manipulate the dynamics that are described by the triangle so that it is in favour of the host.

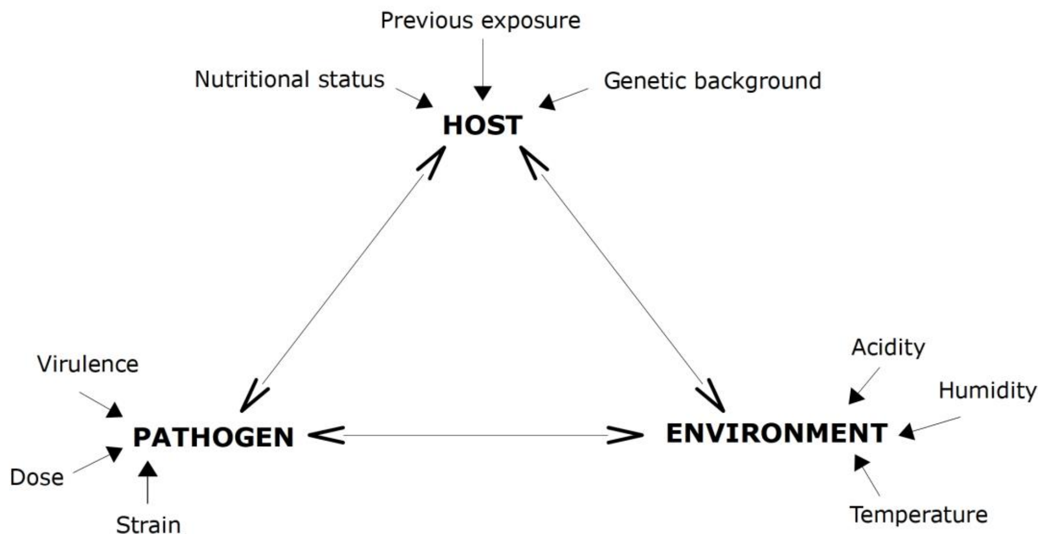

Figure 1.10. Representation of the concept of the epidemiological triangle where all three major variables that have an effect on the disease dynamics (host-pathogen-environment) are linked with each other, while each of them is also a function of parameters that determine their contribution to the epidemiological triangle.

The concept of the epidemiological triangle is the framework of this thesis. It is the concept that links the different studies: ultimately, we try to unravel how *B. salamandrivorans* interacts with the environment and the host and aim at identifying measurements which can alter the disease dynamics to increase the survival chance of the host.

1.7. Thesis outline

In total, this thesis is divided into ten chapters. Chapter one, which can be found on the previous pages, describes the *General Introduction* to this thesis. The next chapter defines the *Scientific Aims* of the work performed in this thesis. Chapters three and four comprise the scientific work that has been performed. Next, a *General Discussion* places the results of chapters three and four in perspective of our current knowledge. Finally,

Chapters six to ten are *Summary* and Dutch *Samenvatting*, followed by my *Curriculum Vitae* and *Bibliography* of the scientific papers I have co-authored and the presentations which have been given through the years. At the end the *Dankwoord* is found.

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Chapter II

Scientific Aims

Batrachochytrium salamandrivorans is the causative agent of lethal chytridiomycosis in fire salamanders and has caused havoc in the largest population of fire salamanders in the Netherlands. Since *B. salamandrivorans* poses an imminent threat to almost all European caudate species, the urgency for disease mitigation was high, but at the same time the knowledge was falling short owing to the recent discovery of the disease. Such valuable information includes e.g. the role of sympatric amphibian species and the environment in sustaining the fungus outside its host, the development of host immunity against the disease or whether infected host populations are likely to go extinct after pathogen introduction. This type of information is crucial when efficient mitigation measures are to be developed.

Therefore, the general scientific aim of this thesis is focused on unravelling the epidemiological triangle, more specifically to understand how *B. salamandrivorans* is capable of causing population crashes in fire salamander populations. In this thesis, I:

- Determine the strategies adopted by the pathogen that result in marked population crashes (**Study 1**)

- Determine the dispersal capability of *B. salamandrivorans* between an infected and non-infected subpopulation (**Study 2**)

Chapter III

Study I

Drivers of salamander extirpation mediated by *Batrachochytrium salamandrivorans*

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3.1. Published manuscript

The recent arrival of *Batrachochytrium salamandrivorans* in Europe was followed by rapid expansion of its geographical distribution and host range, confirming the unprecedented threat that this chytrid fungus poses to western Palaearctic amphibians (Martel et al. 2014; Spitzen-van der Sluijs et al. 2016). Mitigating this hazard requires a thorough understanding of the pathogen's disease ecology that is driving the extinction process. Here, we monitored infection, disease and host population dynamics in a Belgian fire salamander (*Salamandra salamandra*) population for two years immediately after the first signs of infection. We show that arrival of this chytrid is associated with rapid population collapse without any sign of recovery, largely due to lack of increased resistance in the surviving salamanders and a demographic shift that prevents compensation for mortality. The pathogen adopts a dual transmission strategy, with environmentally resistant non-motile spores in addition to the motile spores identified in its sister species *B. dendrobatidis*. The fungus retains its virulence not only in water and soil, but also in anurans and less susceptible urodelan species that function as infection reservoirs. The combined characteristics of the disease ecology suggest that further expansion of this fungus will behave as a 'perfect storm' that is able to rapidly extirpate highly susceptible salamander populations across Europe.

The past two decades have seen the emergence of novel fungal diseases that globally affect biodiversity, leading to the potential extinction of animal and plant

species (Fisher et al. 2012; Martel et al. 2013; Cheng et al, 2011; Kim and Harvell 2004; Frick et al. 2010; Gross et al. 2014). When fungal pathogens are vectored into naive ecosystems, firm pathogen establishment and extensive host population decline typically precede elucidation of the disease ecology, which is required for the development of threat abatement plans (Fisher et al. 2012; Garner et al. 2016). The chytrid fungus *Batrachochytrium salamandrivorans* is a prime example of an emerging infectious disease that has recently become a threat in Europe, where it causes massive decline of salamander populations and poses an unprecedented threat to western Palaearctic amphibian diversity (Martel et al. 2013 & 2014; Spitzen-van der Sluijs et al. 2013). Here we unravel the fundamental mechanisms of amphibian extirpation mediated by the recent arrival of *B. salamandrivorans*. Immediately after the discovery of the first signs of disease (April, 2014) in a population of fire salamanders in Robertville, Belgium, 57 km from the *B. salamandrivorans* index site in the Netherlands (Martel et al. 2013), we began to continuously monitor infection, disease and host population dynamics for two years. Our study demonstrates how the combined characteristics of host susceptibility, pathogen virulence and environmental persistence create a 'perfect storm' with high probability of extirpation after pathogen arrival in a susceptible host population.

Our monitoring revealed that introduction of *B. salamandrivorans* leads to a fast host population collapse, without any sign of recovery, owing to sustained and disproportionate mortality of adults, which leads to a demographic shift in the

population (Fig. 3.1a, b). Across ten- day intervals, we found a probability of infection of 0.33 (95% credible interval (CRI) = 0.169–0.512). Infection resulted in a six-fold difference in survival rate (mean survival in infected versus non-infected animals (0.13 ± 0.11 s.d., 95% CRI = 0.004–0.403) versus (0.84 ± 0.10, 95% CRI = 0.63–0.99)).

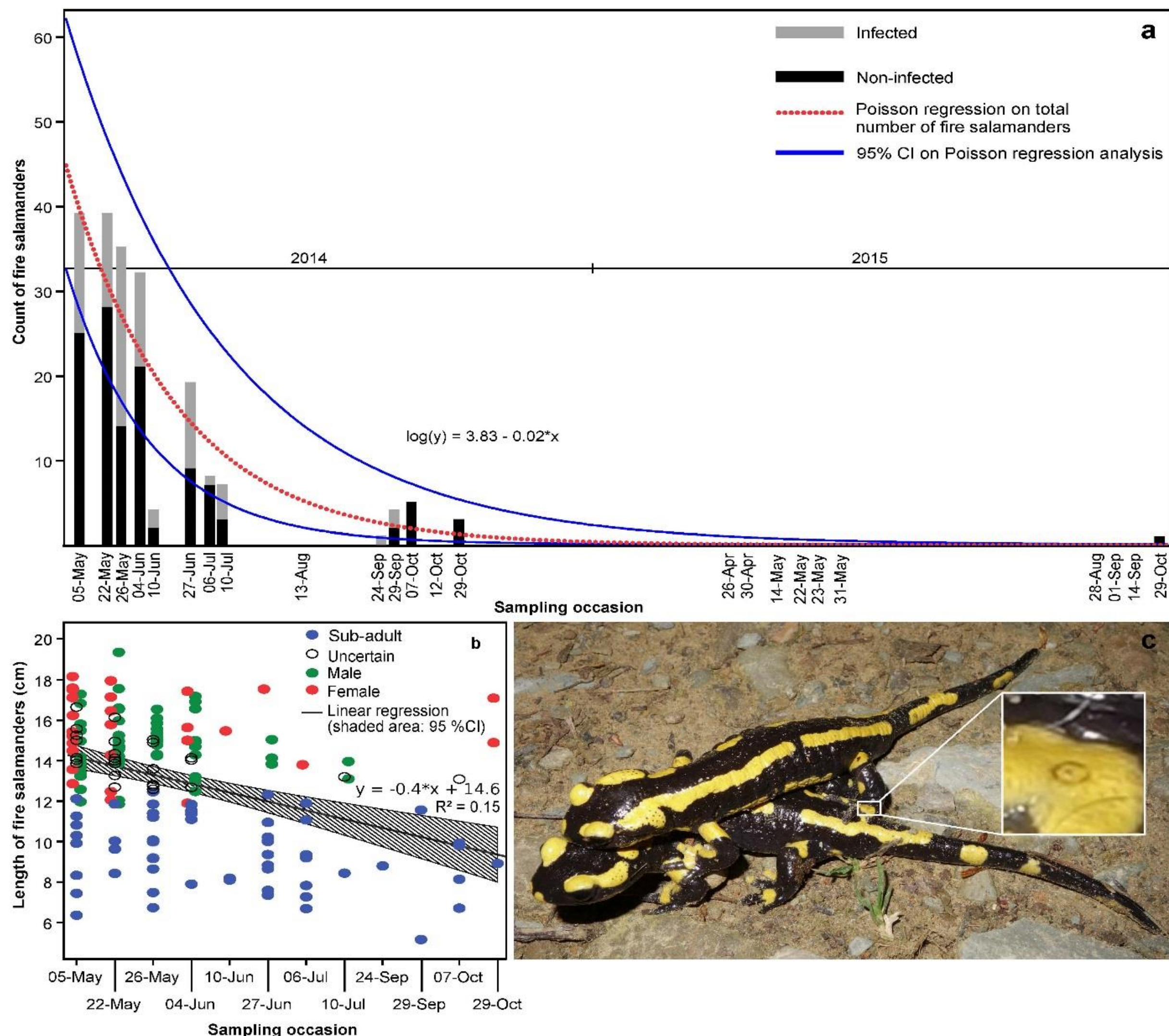

Figure 3.1. *B. salamandrivorans* leads to fire salamander population extirpation a, b, An estimated 90% decline within 6 months (**a**) coincides with a decrease of the average length and a demographic shift (**b**). CI, Confidence interval. **c**, Contact between an infected salamander (on top, skin lesions shown on insert) with an uninfected one during courtship.

In a series of infection trials, we studied the host–pathogen interaction underpinning the susceptibility of fire salamanders to infection with *B. salamandrivorans*. We demonstrate that the outcome of the disease in this species is dose- and temperature-independent and that infected animals do not mount any protective immune response. Experimental inoculation of fire salamanders with a high or low dose of four different *B. salamandrivorans* isolates resulted in lethal disease in all animals, despite a slower build-up of infection load at the low dose (Fig. 3.2a, b). When comparing infection dynamics of *B. salamandrivorans* at the fungus' optimal temperature (15 °C) (Martel et al. 2014) with those at 4 °C, all animals again developed lethal infection, with slower build-up of infection at 4 °C (Fig. 3.2c, d), indicating that the pathogen is able to infect and kill amphibians over a broad temperature range. In a final experiment, we assessed build-up of protection in salamanders after five cycles of exposure treatment. Contrary to the theory that non-lethal exposure to the pathogen could provide opportunities to mount a protective immune response (McMahon et al. 2014), this experiment confirmed that resistance against infection did not increase (Fig. 3.2e, f).

The inability of salamanders to mount resistance against *B. salamandrivorans* infection largely excludes vaccination as a mitigation measure for susceptible salamander species and could preclude build-up of population immunity. Indeed, the few salamanders that were still present at the outbreak site after two years were still highly susceptible to infection with the local *B. salamandrivorans* isolate, showing a

100% mortality rate after exposure.

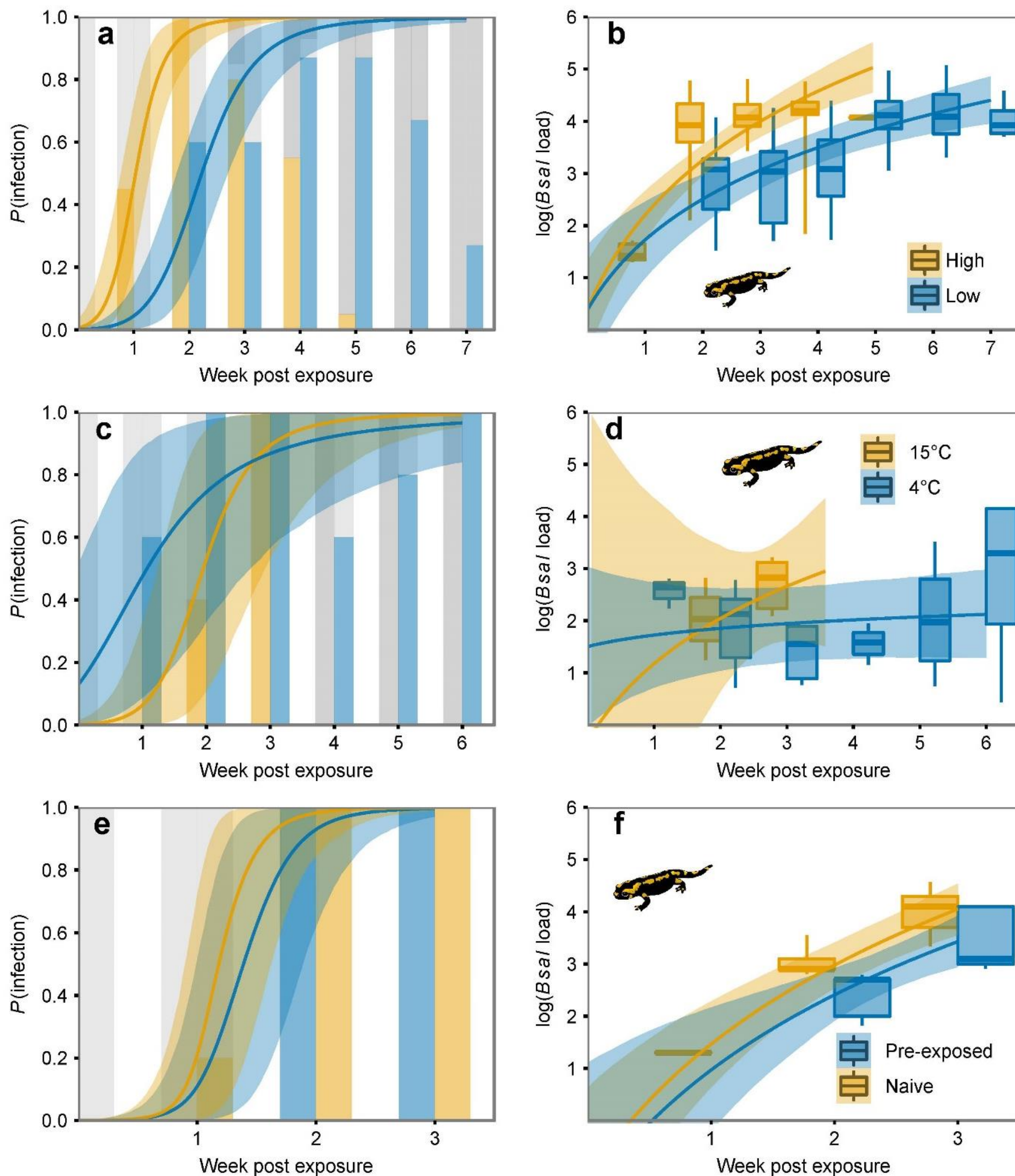

Figure 3.2. Effect of different variables on infection dynamics of *B. salamandrivorans* in fire salamanders. **a**, **b**, A lower dose results in slower build-up of infection and delayed mortality. **c**, **d**, At lower temperatures, similar infection intensities are reached more slowly. **e**, **f**, Previous exposure to *B. salamandrivorans* does not protect against re-infection. Bars (**a**, **c**, **e**) indicate the proportion of alive and infected (coloured), alive and uninfected (light grey) and dead (dark grey) individuals; curves indicate the estimated treatment-specific probability of infection (shading: 95% CRI). Box plots (**b**, **d**, **f**) indicate infection loads in genomic equivalents per swab (bars indicate 2.5th and 97.5th percentiles); curves indicate the estimated treatment-specific load (shading: 95% CRI).

Given the continuous high mortality and high transmission rate, persistence of any susceptible European host population after infection would only be likely when compensated by elevated recruitment (Muths et al. 2011). However, we found that *B. salamandrivorans* disproportionately infects and kills sexually mature animals (Fig. 3.1b), quickly resulting in a demographic shift that massively decreases the recruitment potential of the population that would be necessary to compensate for adult mortality (Schmidt et al. 2005). Increased infection probability and subsequent mortality of adult fire salamanders compared to sub-adults can be explained by intimate contact with infected adult conspecifics during territorial displays and reproduction (Fig. 3.1c), and back-and-forth migration of females to the streams where they give birth to aquatic larvae. Sub-adults show less surface activity and interactions with conspecifics (Seifert 1991), with reduced probability of becoming infected. Our analyses indicate that the rapid population decline can be largely associated with two fungal pathogen determinants: sustained fungal virulence and the presence of an environmentally resistant encysted spore. Sustained virulence of the fungus in its novel susceptible host species was demonstrated by the persistence of highly virulent *B. salamandrivorans* two years after the outbreak in our study site, despite almost complete depletion of the host population. Indeed, isolates cultured from infected animals at the end of our two-year monitoring period were equally capable of killing 100% of the experimentally inoculated salamanders as an initial isolate. The sustained virulence is assisted by the fact that, in addition to motile spores as in its closest sister species *B. dendrobatidis*, *B. salamandrivorans* produces a second type of infectious

encysted spores both *in vitro* and *in vivo* in salamander skin, with a distinct fungal infection, dissemination and persistence strategy (Fig. 3.3a). Whereas zoospores actively swim to their host (Rosenblum et al. 2010), encysted spores float at the water–

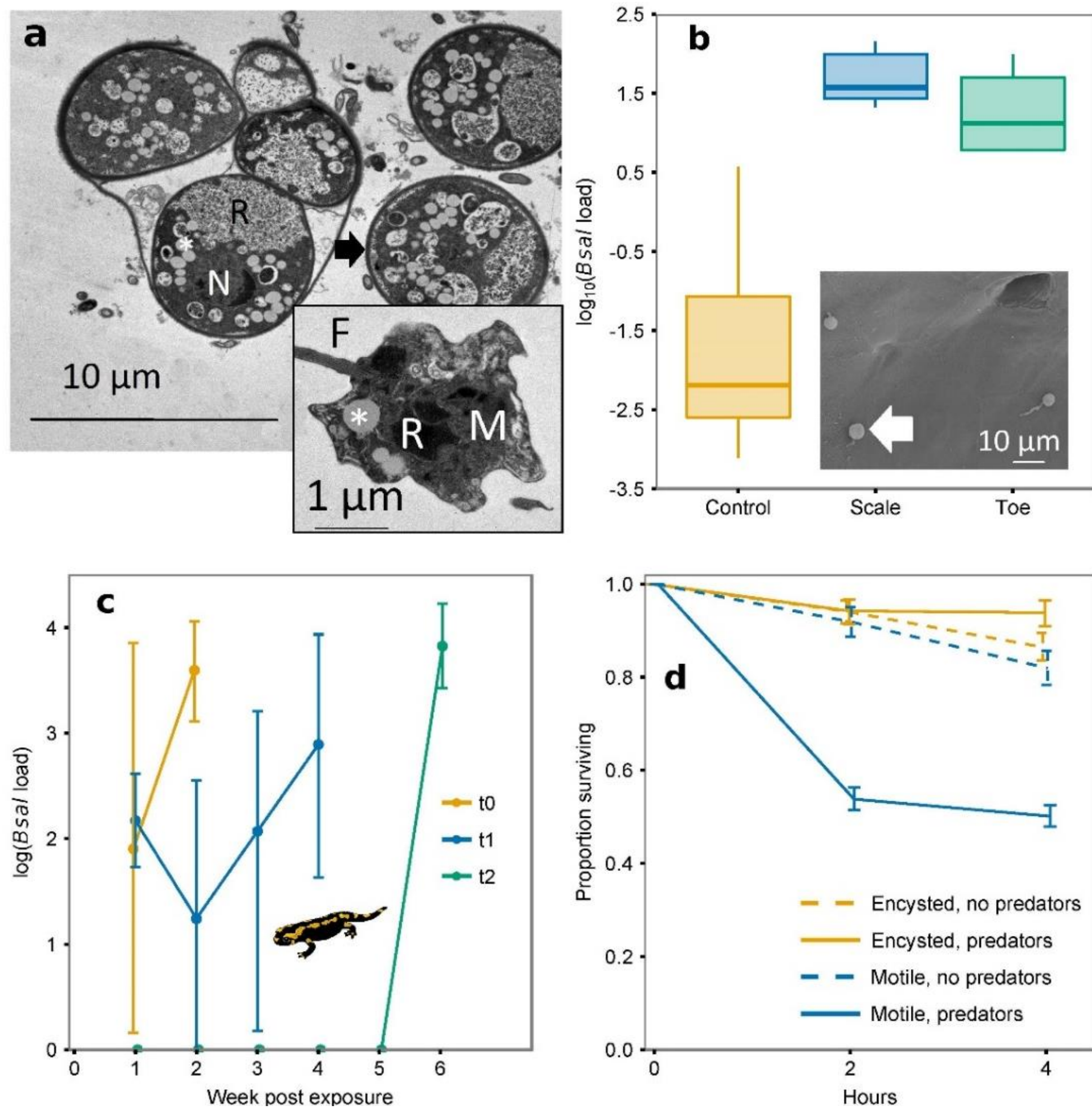

Figure 3.3. *B. salamandrivorans* encysted spores avoid predation and infect fire salamanders. (a) Transmission electron microscopic picture of a sporangium containing encysted spores and free encysted spores in the superficial epidermis and a zoospore (insert). (*) lipid globule; (arrow) cell wall; (F) flagellum; (M) mitochondrion; (N) nucleus; (R) ribosomal mass. (**b**) Adhesion of encysted spores to salamander skin and scales from goose feet (control: skin dipped in culture supernatant; insert: scanning electron microscopic image of attachment to salamander skin). (**c**) *B. salamandrivorans* infection loads in salamanders exposed to encysted spores that were either freshly collected (*t*0, with *n = 6*) or were incubated in pond water for 14 or 31 days (*t*1 and *t*2, with *n = 4* and *n = 2*). (**d**) Estimated survival of motile and encysted spores after exposure to aquatic micropredators. Bars indicate the 2.5th and 97.5th percentiles in (**b**, **c**) and 95% CRI in (**d**). *B. salamandrivorans* loads (**b**, **c**) are expressed in genomic equivalents per swab

air interface (online material; Supplementary Video 1) and are capable of quickly adhering to salamander skin and to scales of the feet of waterfowl (Fig. 3.3b). This passive adherence to inert matrices may promote fungal spread over large spatial distances. Encysted spores survived and remained infective for fire salamanders for at least 31 days in filtered pond water (Fig. 3.3c) and were more resistant than zoospores to predation by zooplankton (Fig. 3.3d), highlighting their potential to persist in an aquatic environment.

Long-term persistence of *B. salamandrivorans* is further promoted by its presence on less-susceptible amphibian pathogen reservoirs, as we demonstrated by experimental infection of anuran and urodelan hosts. A proportion of the four used *B. salamandrivorans* isolates was capable of infecting anuran hosts (midwife toads, *Alytes obstetricans*) at low intensities for several weeks after experimental inoculation. Whereas the toads showed no sign of disease, their colonization with the pathogen was sufficient to transmit *B. salamandrivorans* to susceptible salamanders (Fig. 3.4a, b, Extended Data Table 3.1).

In urodelans, experimental infection of Alpine newts (*Ichthyosaura alpestris*), a species that co-occurs syntopically with fire salamanders, showed a dose-dependent disease course. Whereas infection with a high dose resulted in disease and death after an average of three weeks, exposure to a low dose resulted in significant *B. salamandrivorans* shedding for several months with eventual fungal clearing and clinical cure (Fig. 3.4c, d). However, previous infection was shown to provide the

newts with no protection against re-infection and mortality (Fig. 3.4e). These newts thus meet the criteria for a pathogen reservoir. Indirect transmission would also favour pathogen maintenance in host populations; therefore, in a final infection experiment we demonstrated the potential for pathogen transmission via contaminated forest soil. Infected salamanders were shown to contaminate the forest soil, in which the fungal

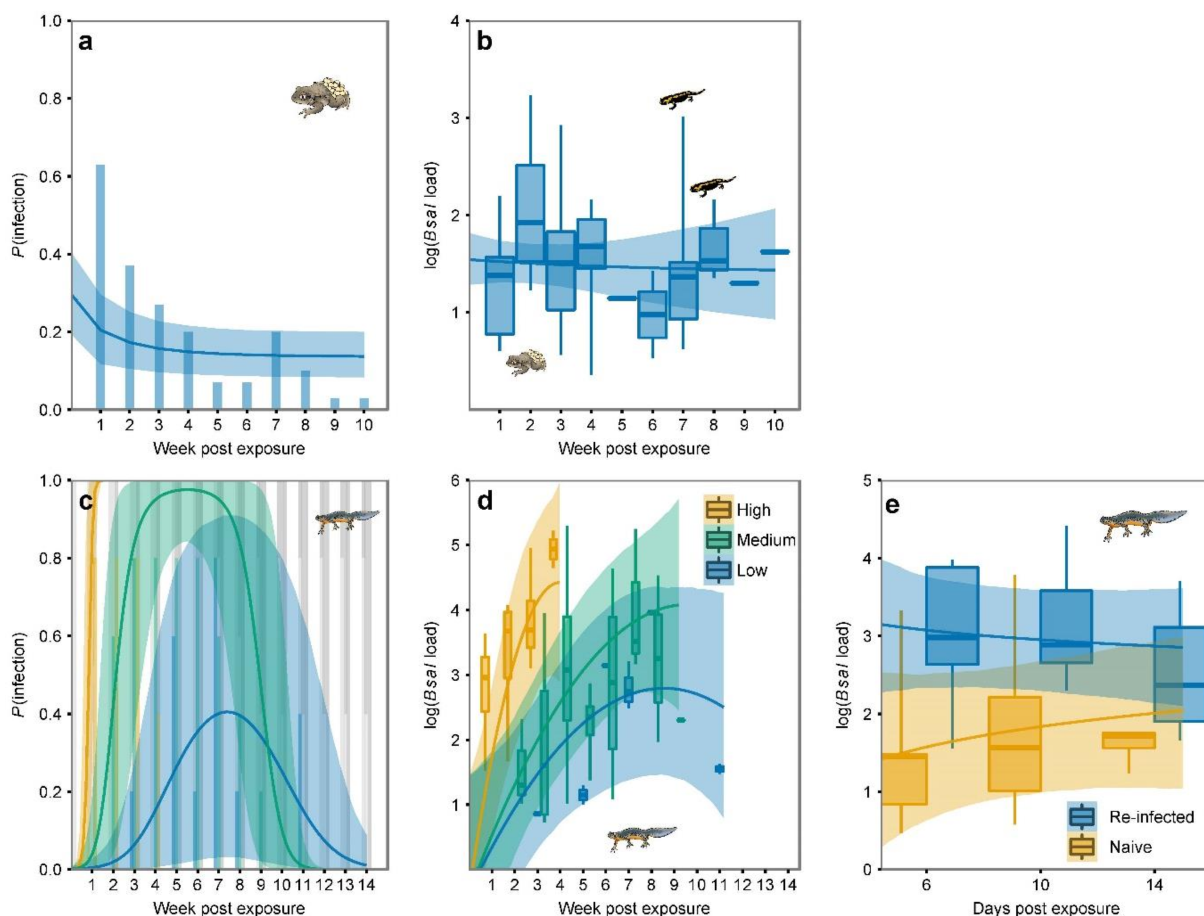

Figure 3.4: Anuran and urodelan reservoirs promote *B. salamandrivorans* sustenance. **a**, **b**, Probability of infection and infection load in midwife toads. **a**, Bars indicate the proportion of infected individuals (no mortality observed) and the curve indicates the estimated probability of infection. **b**, Box plots and curve indicate, respectively, observed and estimated infection load of *B. salamandrivorans*-positive individuals; salamander icons indicate infection in fire salamanders that had been co-housed with the toads from the second week post exposure onwards. **c**, **d**, Probability of infection and infection load in Alpine newts after exposure to 10,000 (orange), 1,000 (green) or 100 (blue) spores. **c**, Bars indicate the proportion of alive and infected (coloured), alive and uninfected (light grey) and dead individuals (dark grey); curves indicate the probability of infection. **d**, Box plots and curves indicate, respectively, observed and estimated infection load of *B. salamandrivorans*-positive individuals. **e**, Infection loads for naive or previously infected Alpine newts; box plots and curves indicate, respectively, observed and estimated infection load of *B. salamandrivorans*-positive individuals. *B. salamandrivorans* loads (**b**, **d**, **e**) are expressed in genomic equivalents per swab. In all plots, shading around curves indicates 95% CRI; box plot bars indicate 2.5th and 97.5th percentiles

DNA could be detected even after 200 days. Actual transmission through contaminated forest soil was demonstrated up to 48 h after the soil had been in contact with an infected animal (Extended Data Fig 3.1, 3.2, Extended Data Tables 3.2, 3.3). Altogether, the presence of a resistant spore with the ability to persist environmentally and to transmit through contaminated water and soil, combined with the occurrence of long-term-infected and pathogen-shedding amphibian hosts, creates the potential for extensive environmental reservoirs and hampers any effort to eradicate *B. salamandrivorans* from an infected ecosystem.

Our study reveals that the multifaceted ecology of this expanding fungal disease is likely to result in fast extirpation of highly susceptible salamanders, with no available options to halt the spread or to mitigate the disease *in situ*. Although several potential measures to counteract the effect of chytrid fungi on amphibian communities have been proposed (Garner et al. 2016), *ex situ* conservation programmes are currently the only intervention available that will effectively avert loss of susceptible urodelan populations upon *B. salamandrivorans* arrival. Given the continuous range expansion of the disease and the speed of its effects, the development of a pan-European early warning system to monitor the fungal invasive front and the enforcement of emergency action plans that allow fast implementation of *ex situ* conservation in acutely threatened urodelan species are urgently needed. A thorough understanding of the host, pathogen and environment determinants underpinning susceptibility to *B. salamandrivorans* may yield the tools required for risk analysis of the actual threat of

the fungal disease to western Palaearctic urodelans, which will guide prioritization of conservation efforts. Indeed, although most western Palaearctic urodelan taxa were shown to be susceptible to *B. salamandrivorans* in laboratory trials (Martel et al. 2014), we demonstrate marked interspecific differences. Fire salamanders represent a hyper-susceptible species in which *B. salamandrivorans* causes acute, dose-independent mortality and population extirpation. The rapid and consistent mortality at least in species of the genera *Salamandra*, *Euproctus*, *Neurergus*, *Pleurodeles* and in *Lissotriton italicus* after exposure to *B. salamandrivorans* (Martel et al. 2014; Sabino-pinto et al. 2015) suggests a similar risk of disease driven extirpation for many other species of western Palaearctic urodelans. As eradication of *B. salamandrivorans* from the European continent is highly unlikely, long-term sustainable mitigation should aim at host–pathogen co-existence, which implies the development of intervention strategies that permanently increase resistance of susceptible species against *B. salamandrivorans* (Garner et al. 2016). For regions that are currently considered free of *B. salamandrivorans*, such as the Americas, prevention of introduction in naive environments should be considered the sole effective control measure available. This will require knowledge of all introduction pathways that, besides amphibians in trade (Martel et al. 2014, Yap et al. 2015; Price et al. 2016), may also include non-amphibian sources carrying resistant spores.

3.2. METHODS

3.2.1. *BATRACHOCHYTRIUM SALAMANDRIVORANS* ISOLATES AND CULTURE CONDITIONS.

Four *B. salamandrivorans* isolates were isolated from wild fire salamander populations that are declining owing to a *B. salamandrivorans* outbreak in the Netherlands (AMFP13/1) and Belgium (AMFP14/1 (Robertville, 2014); AMFP 15/3 (Robertville, 2015) and AMFP14/2 (Luik) (Martel et al. 2013; Spitzen-van der Sluijs et al. 2016). One isolate originated from a captive population suffering from a *B. salamandrivorans* disease outbreak in Germany (AMFP15/1) (Sabino-Pinto et al. 2015). All isolates were grown in a 1.6% tryptone, 0.4% gelatin-hydrolysate and 0.2% lactose monohydrate liquid medium at 15 °C. Motile or encysted spores were collected in distilled water after 5–7 days of growth. Zoospores were obtained by washing the culture flasks with filtered pond water (0.2-µm filter). Floating encysted spores were collected from the water surface using a 10 µl inoculation loop. Purity of the spore suspension was assessed using inverted microscopy. Encysted spore suspensions were only used in experiments when motile spores were absent. No statistical methods were used to predetermine sample size.

3.2.2. DNA EXTRACTION AND *B. SALAMANDRIVORANS* QPCR.

DNA extraction of skin swabs and *B. salamandrivorans* qPCR were performed as described in Blooi et al. (2013 & 2016). Animals were considered positive for *B. salamandrivorans* infection when the following conditions were fulfilled: (1) the qPCR sample quantity was above the detection limit of 0.1 genomic equivalent (GE)/qPCR

reaction for both replicates, (2) the mean starting quantity value of each sample was higher than the standard deviation of its starting quantity, and (3) the amplification curve from each replicate was logarithmic.

3.2.3. Infection trials

For all experimental replicates, all fungal cultures were grown independently. All animals were housed individually in terraria at the fungus thermal preference of 15 °C (unless otherwise stated) on moist tissue with access to a hiding place. All animals (males/females) were captive bred, clinically healthy and free of *B. dendrobatidis*, *B. salamandrivorans* and Ranavirus as assessed by sampling the skin using cotton-tipped swabs and subsequently performing qPCR (Blooi et al. 2013 & 2016) or PCR (Mao et al. 1997). Infection experiments were carried out as described in Martel et al. (2014). Individuals were randomly assigned to treatments. All animals were clinically inspected daily. Skin sampling was done weekly and the swabs were analysed for the presence of *B. salamandrivorans* using qPCR described in Blooi et al. (2013 & 2016). Investigators were blinded to allocation during experiments and outcome assessment.

3.2.4. Hygiene and biosafety protocols

The animal experiments were performed under strict BSL2 conditions. During the fieldwork, each individual was handled with a new pair of nitrile gloves. At the end of each field visit, boots and other equipment which came into contact with the environment were disinfected with a 1% Virkon solution for at least 5 min.

3.2.5. Ethics statement

The animal experiments were performed with the approval of the ethical committee of the Faculty of Veterinary Medicine (Ghent University EC2013/10; EC2014/170; EC2015/29; EC2015/42; EC2016/87). The capture, handling, sampling and transport of wild salamanders and access to the sampling site were permitted by the Wallonian Department of Nature and Forests (Département de la Nature et des Forêsts) (reference 2014/RS/n°23).

3.2.6. Infection and disease dynamics during a *B. salamandrivorans* outbreak in fire salamanders: a 2-year follow-up study

In May 2014, a *B. salamandrivorans* outbreak with mass mortality was identified in a population of fire salamanders (*Salamandra salamandra terrestris*) in the forest of Robertville (50° 27′ 10″ N, 06° 06′ 10″ E), Belgium. Over the course of 2 years, the population was monitored during the activity periods of the salamanders: (1) May to October 2014 and (2) April to September 2015. Over a fixed 475 m-long transect, salamanders were detected using a visual encounter survey and sampled by collecting skin swabs. To investigate whether *B. salamandrivorans* can be detected in terrestrial environments in the wild, soil samples were taken in the close vicinity of animals with chytridiomycosis (Extended Data Fig. 3.1). The animals that were found during the second year, one on the study transect and 11 outside the transect, were brought to our quarantine facilities to test their susceptibility to chytridiomycosis. None of these tested positive for *B. salamandrivorans* when sampled at location. However, the animals

developed severe to lethal signs of chytridiomycosis within 3 weeks after exposure to the fungus. In total, 24 visits resulted in 197 captures of fire salamanders. Individual salamanders were identified by their conspicuous yellow marks; from each captured individual, dorsal and lateral photographs were taken. On the basis of the capture-mark-recapture data from the first two visits between which there were confirmed recaptures, the population of fire salamanders on the study-transect was estimated to consist, at the beginning of the study, of 239 (95% confidence interval, 112–459) individuals, using the Lincoln–Petersen index (Williams, Nichols and Conroy, 2002). Host population dynamics analysis was performed on the basis of a multistate capture-mark-recapture model (Lebreton et al. 2009). On the basis of the qPCR results from the skin swabs, each individual was classified as either infected or non-infected. We constructed a Bayesian multistate capture–recapture model (Kéry and Schaub 2011) to estimate survival probabilities of non-infected (PhiA) and infected individuals (PhiB) as well as transition probabilities between these two states. The transition probability (Psi) from a non-infected to an infected state is the infection probability or rate (PsiAB), the transition probability from an infected to a non-infected state is the recovery probability or rate (PsiBA). Because the population was declining rapidly and no recovered individuals were observed, all parameters were set constant through time, reencounter probability (pA) of infected individuals was assumed to be equal to reencounter probability of uninfected salamanders. The interval between two sampling periods was highly influenced by weather conditions and activity periods of salamanders. To adjust for the unequal time intervals, dummy sampling occasions

were added to the dataset to equalize the intervals between two sampling periods to approximately 10-days. The reencounter probability was set to 0 at the dummy occasions. The estimated survival and transition probabilities refer therefore to 10 day intervals. Analyses were performed for the data collected during the first period only (sampling occasions 1–14). The multistate model was fitted in JAGS (Plummer et al. 2003) through package jagsUI in R (Kéry and Schaub 2011). Vague priors were chosen for all the parameters (uniform between 0 and 1) and convergence was inferred by R-hat values <1.1 (Extended Data Table 3.4).

3.2.7. DRIVERS OF *B. SALAMANDRIVORANS* DYNAMICS IN SUSCEPTIBLE FIRE SALAMANDERS

3.2.7.1. DOSE DEPENDENCY OF B. SALAMANDRIVORANS *INFECTION AND DISEASE DYNAMICS*

Four *B. salamandrivorans* isolates (AMFP13/1, AMFP14/1, AMFP14/2, and AMFP15/1) were used to expose 40 juvenile fire salamanders. Animals were infected with one isolate. For each isolate two doses were used: either 10^4 spores (high dose) or 100 spores (low dose). For each dose-by-isolate combination, 5 animals were inoculated per isolate and each animal was housed individually. The course of disease was followed up by daily clinical inspection and weekly sampling of the animals for 8 weeks (Fig. 3.2a, b).

3.2.7.2. TEMPERATURE DEPENDENCY OF B. SALAMANDRIVORANS *INFECTION DYNAMICS*

In this experiment, the infectivity of *B. salamandrivorans* after incubation in water for 4 weeks was evaluated. A suspension of 1.3 x 10^4 GE *B. salamandrivorans* per

ml environmental water was incubated for 4 weeks at both 4 °C and 15 °C. After 4 weeks, the concentration of *B. salamandrivorans* was 2.6 x 10^4 GE per ml water (4 °C) and 1.3 x 10^4 GE per ml water (15 °C). This water was used to inoculate 10 salamanders as described in (Martel et al. 2014). Water containing *B. salamandrivorans* incubated at 4 °C was used to infect salamanders which were later housed individually at 4 °C. Water containing *B. salamandrivorans* incubated at 15 °C was used to infect salamanders which were later housed individually at 15 °C. The course of disease was followed up by daily clinical inspection and weekly sampling of the animals for 10 weeks (Fig. 3.2c, d).

3.2.7.3. Effect of previous infection on infection and disease dynamics of B. salamandrivorans

Five fire salamanders were infected with 10^3 spores of AMFP13/1. After increase of the GE load in two subsequent swabs (weekly sampling), animals were treated at 25 °C for 10 days as described in Blooi et al. (2015). One month after finishing the treatment, the animals were re-infected. This exposure–treatment cycle was repeated five times. For the subsequent challenge experiment, infection dynamics of *B. salamandrivorans* in these five pre-exposed salamanders were compared their initial infection dynamics (Fig. 3.2e, f).

3.2.8. *B.* SALAMANDRIVORANS PRODUCES INFECTIOUS, ENCYSTED AND ENVIRONMENTALLY PROTECTED SPORES

3.2.8.1. Production of encysted spores by B. salamandrivorans

Using microscopy and transmission electron microscopy, we identified two types of spores in *B. salamandrivorans*: a motile (zoo)spore and a non-motile, encysted spore with a cell wall. The ultrastructure of the different spores was investigated in *in vitro* cultures of *B. salamandrivorans* and in skin samples of infected fire salamanders. Transmission and scanning electron microscopy was performed as described in Martel et al. (2013) (Fig. 3.3a, insert Fig. 3.3b).

3.2.8.2. Brief contact with floating, encysted spores results in adherence of B. salamandrivorans *to salamander skin and scales from goose feet*

Encysted spores were collected and inoculated in filtered (0.2-µm filter) environmental water at a concentration of 10^6 spores per ml water. A salamander toe and scale from goose feet were dipped in the suspension for 1 s. Controls to quantify *B. salamandrivorans* DNA contamination consisted of toes, dipped in filtered (0.5 µm filter) culture supernatant. *B. salamandrivorans* load in all samples was determined using qPCR (Blooi et al. 2013 & 2016). Two independent experiments were performed in triplicate. Results are expressed as mean number of genomic equivalents per mm^2 ± standard deviation of the respective tissue (Fig. 3.3b).

3.2.8.3. Survival in the aquatic environment and infectivity of encysted spores

Encysted spores were collected and inoculated in filtered environmental water

at a concentration of 10^8 spores per ml water. At time points 0 (immediately after collection), 1 (15 days after collection) and 2 (31 days after collection) fire salamanders (n = 6 at t0, n = 4 at t1 and n = 2 at t2) were inoculated by dropping 100 µl of this suspension on the salamanders' dorsum. The course of disease was followed up by daily clinical inspection and weekly sampling of the animals for 10 weeks. Infection loads were determined by quantifying *B. salamandrivorans* DNA in skin swabs using qPCR (Blooi et al. 2013 & 2016). Results are presented as average infection loads ± standard deviation (Fig. 3.3c).

3.2.8.4. PREDATION OF MOTILE AND ENCYSTED SPORES

Predation of motile and encysted spores by micropredators present in pond water was tested as described in Schmeller et al. (2014). Briefly, 10^6 motile or encysted spores were incubated with 1 ml of pond water containing 456 zooplanktonic organisms per ml, for four hours in 24-well plates at 15 °C. The water contained copepods (30 per ml), ciliates (paramecium (338 per ml) and peritrich ciliates (18 per ml)), rotifers (16 per ml), ostracods (35 per ml), heliozoans (1 per ml) and water fleas (18 per ml) as determined by counting the total content in 1 ml of pond water using light microscopy. For comparison, zoospores and encysted spores were incubated in pond water that was filtered using a 5 µm filter. After 4 h incubation the number of remaining spores was counted using a Bürker counting chamber. Removal of spores from the aquatic environment was quantified as proxy for spore ingestion. Ingestion was calculated as the proportion of remaining encysted or zoospores at a given time

point compared to the number of spores recovered in the wells with filtered pond water at that time point. Three independent experiments were carried out in triplicate. Results shown are experimental means with standard error of the mean (Fig. 3.3d).

3.2.9. VECTORS AND POTENTIAL RESERVOIRS OF *B. SALAMANDRIVORANS*

3.2.9.1. ANURAN RESERVOIRS OF B. SALAMANDRIVORANS

Four *B. salamandrivorans* isolates (AMFP13/1, AMFP14/1, AMFP14/2, and AMFP15/1) were used to expose 32 juvenile midwife toads (*Alytes obstetricans*) to 10^5 spores. Eight animals were inoculated per isolate. To assess whether infected midwife toads are capable of transmitting *B. salamandrivorans* to susceptible fire salamanders, from 14 days after inoculation, five randomly selected midwife toads per isolate were selected and each toad was housed together with a juvenile fire salamander in a new terrarium. The course of disease was followed up by daily clinical inspection and weekly sampling of the animals for 10 weeks (Fig. 3.4a, b). Two fire salamanders developed infection and clinical disease. They were taken out of the experiment and treated as described in Blooi et al. (2015).

3.2.9.2. URODELAN RESERVOIRS OF B. SALAMANDRIVORANS

The holotype isolate AMFP13/1 was used to inoculate 20 Alpine newts (*Ichthyosaura alpestris*). Four different infection doses (5 animals per infection dose) were used: 10^4, 10^3, 10^2 and 10 spores. The course of disease was followed up by daily clinical inspection and weekly sampling of the animals for 14 weeks (Fig. 3.4c, d). As exposure to 10 spores resulted in only 2 out of 5 animals becoming infected, results

were omitted from further analyses.

3.2.9.3. *PREVIOUS INFECTION DOES NOT PROTECT ALPINE NEWTS AGAINST* B. SALAMANDRIVORANS *REINFECTION*

In this study, we assessed whether Alpine newts that had been chronically infected by *B. salamandrivorans* are protected against re-infection with *B. salamandrivorans*. From previous experiments in which Alpine newts were exposed to a low (10^3 zoospores) dose of *B. salamandrivorans* (see above in "*urodelan reservoirs of* B. salamandrivorans"), nine animals developed chronic infection without subsequent mortality. Skin swabs from these animals were positive for *B. salamandrivorans* between 28 and 175 days after exposure to *B. salamandrivorans* (average ± s.d. = 95 ± 45 days), with average *B. salamandrivorans* counts per sample of log10(2.02 ± 0.54). All except one animal cleared *B. salamandrivorans* infection before experimental re-infection. Of the animals that cleared infection, the time between the last positive skin sample and the experimental re-infection ranged between 54 and 567 days (average ± s.d. = 278 ± 218 days). Re-infection with 10^6 zoospores of *B. salamandrivorans* was performed as described before and animals were followed up by determining *B. salamandrivorans* infection loads in skin swabs using qPCR (Fig. 3.4e). For comparison, five negative control animals were included that had never been exposed to *B. salamandrivorans*.

3.2.9.4. *FOREST SOIL AS A VECTOR FOR* B. SALAMANDRIVORANS *TRANSMISSION*

Fifteen gram of forest soil (moisture content: 47.24 ± 0.07%) was moistened with

10 ml of distilled water and inoculated with 1 ml of *B. salamandrivorans* suspension containing 6.0 x 10^6 GE. A fire salamander was exposed to 1 g of this soil for 24 h either immediately after inoculating the soil, after 8 h incubation, after 24 h incubation, after 2 days incubation, after 4 days incubation or after one week incubation. At the different time points, the amount of *B. salamandrivorans* in soil was determined with qPCR after DNA extraction using the Powerlyzer Powersoil DNA Isolation Kit (MO BIO Laboratories Inc.). This experiment was done in triplicate at 15 °C and 4 °C. (Extended Data Fig. 3.2, Extended Data Table 3.2). Fourteen infected salamanders (mean infection load 3.2 x 10^3 ± 4.4) were housed individually on forest soil for 24 h. Afterwards the animals were removed and were replaced by a non-infected individual. In one group (7 animals), the replacement was done immediately after removal of the infected animal. In the other group, animals were replaced with a non-infected individual 24 h after removal of the infected animal. The course of disease was followed up by daily clinical inspection and weekly sampling of the animals for 4 weeks (Extended Data Table 3.3). An animal was considered to be infected after two positive skin swabs in two subsequent weeks.

3.2.10. Data analysis

To assess the response of animals to *B. salamandrivorans* infection under different conditions, we carried out a two-step analysis. First, we modelled the presence or absence of infection across all individuals using logistic regression; second, we modelled the average load of positive individuals only using linear regression. For

estimation of the infection load, we always used the natural logarithm of the GE as the response variable. For all analyses where repeated measures of the same individual or sample were taken, we used a random effect to account for pseudoreplication. We modelled probability of infection and average load as a linear function of treatment and as an asymptotic (*S. salamandra*), quadratic (*I. alpestris*) or exponential (*A. obstetricans*) function of time, and included the appropriate treatment - time interactions. For the zoospore predation experiment, we used an open-population N-mixture model with a robust design (Dail and Madsen 2011), accounting for sampling variability in the repeated measures for each sample and mortality between sampling occasions. We fitted all models in JAGS (Plummer 2003) through package jagsUI in R (Kéry and Schaub 2011), using uninformative priors for all coefficients including intercepts, sampling 10,000 posterior values from three Markov chains after a burn-in of 10,000 iterations. Convergence was inferred by R-hat values <1.1.

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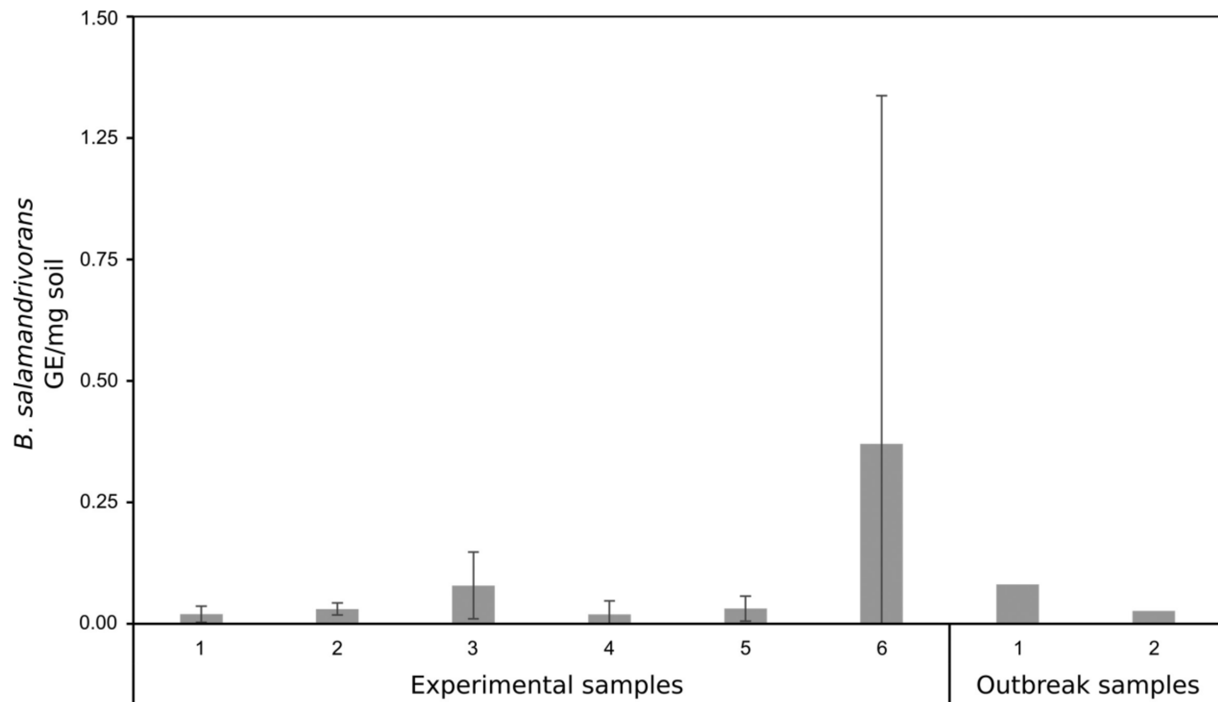
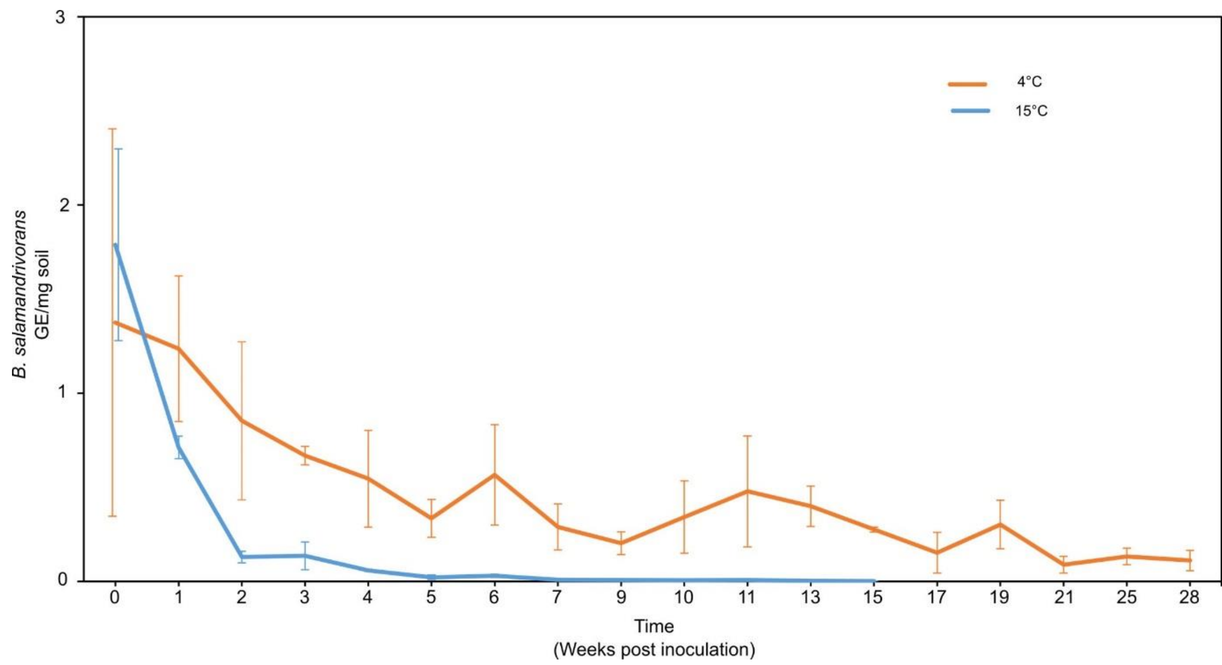
3.4. EXTENDED DATA



Extended Data Figure 3.1. *B. salamandrivorans* GE loads in soil. To investigate whether *B. salamandrivorans* can be detected in terrestrial environments, soil samples were taken in the close vicinity of experimentally infected animals (experimental samples) and naturally infected salamanders in the Robertville outbreak area (outbreak samples). Error bars depict s.d.



Extended Data Figure 3.2. *B. salamandrivorans* GE loads detection in experimentally infected soil, incubated at 4 °C and 15 °C. Error bars depict s.d.

Extended Data Table 3.1. Infection loads (expressed in GE/qPCR reaction) for midwife toads (green) and fire salamanders (orange). Four *B. salamandrivorans* isolates (AMFP13/1, AMFP14/1, AMFP14/2, and AMFP15/1) were used to expose 32 juvenile midwife toads to 10^5 spores. Eight animals were inoculated per isolate. To assess whether infected midwife toads are capable of transmitting *B. salamandrivorans* to susceptible fire salamanders, from 14 days after inoculation, five randomly selected midwife toads per isolate were selected and each toad was housed together with a juvenile fire salamander in a new terrarium (* indicates the time point of co-housing of midwife toads with fire salamanders in a new terrarium). The course of disease was followed up by daily clinical inspection and weekly sampling of the animals. Increased infection load and the development of ulcerations was used as an endpoint to stop the experiment for the fire salamanders: two animals were taken out of the experiment (blue).

Strain	Weeks after inoculation of midwife toads																			
	Week 1		Week 2		Week 3		Week 4		Week 5		Week 6		Week 7		Week 8		Week 9		Week 10	
	Al		Al	*	Al	Sal	Al	Sal	Al	Sal	Al	Sal	Al	Sal	Al	Sal	Al	Sal	Al	Sal
AMFP13/1																				
1	-		-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	6		-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	6		-		-	-	-	-	-	-	-	-	-	-	-	-	20	-	-	-
4	10		-		-	-	-	-	-	-	-	-	-	-	22	-	-	-	-	-
5	-		-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	35		-		-		-		-		-		-		-		-		-	
7	-		-		-		26		14		-		-		-		-		-	
8	-		-																	
AMFP14/1																				
1	6		46		-	-	-	-	-	-	-	-	-		-		-		-	
2	10		250		-	-	-	-	-	-	-	-	36	600	156	8609	-		42	
3	-		134		-	-	-	-	-	-	-	-	-		-		-		-	
4	26		-		-	-	-	-	-	-	-	-	-		-		-		-	
5	24		84		-	-	-	-	-	-	-	-	-		-		-		-	
6	-		56		-		-		-		-		25		-		-		-	
7	-		24		-		65		-		-		-		-		-		-	
8	-		15		12		-		-		-		-		-		-		-	
AMFP14/2																				
1	46		-		20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	30		-		1210	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	32		-		-	-	-	-	-	-	-	-	22	-	-	-	-	-	-	-
4	4		-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	4		-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	66		-		52		35		-		28		4		-		-		-	
7	-		-		-		-		-		-		-		-		-		-	
8	-		-																	
AMFP15/1																				
1	4		-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-		-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-		-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-		1140		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	24		1940		52	-	152	-	14	-	3	36	1660	9564	340		-		-	
6	89		-		3		-		-		-		-		-		-		-	
7	252		422		7		2		-		-		6		-		-		-	
8	39		23		150		100		-		-		-		-		-		-	

Extended Data Table 3.2. Infectivity of experimentally infected *B. salamandrivorans* soil at 4°C and 15°C.

Time points (hours)	Incubation temperature (°C)	*B. salamandrivorans* positive animals (% of individuals)
0h	-	83%
8h	4°C	67%
	15°C	0%
24h	4°C	67%
	15°C	33%
48h	4°C	67%
	15°C	33%
96h	4°C	0%
168	4°C	0%

Extended Data Table 3.3. Infectivity of *B. salamandrivorans* in soil. Infected animals (source animals) were housed in a terrarium at 15 °C with a forest soil substrate and replaced with a healthy individual after 24 h (immediate replacement) or removed after 24 h and replaced by another 24 h later (replacement after 24 h). GE, genomic equivalent

	log_{10}(GE)	*B. salamandrivorans* transmitted to sentinel animal
Immediate replacement	168	NO
	8560	YES
	10	NO
	204	NO
	12240	YES
	3	NO
Replacement after 24 hours	3714	YES
	9421	YES
	335	NO
	3329	YES
	625	NO
	245	NO

Extended Data Table 3.4. Host population dynamics for an infected population of fire salamanders. Phi: survival probability of uninfected (A) or infected (B) salamanders. Psi: Transition probability from uninfected to infected (AB) or infected to uninfected (BA). pA: recapture probability. Parameter estimates based on three chains of 100.000 iterations, with a burn-in of 20.000 iterations and a thin rate of 250. Successful convergence based on Rhat values (successful convergence if Rhat<1.1).

	Mean	**s.d.**	**2.5 %**	**97.5 %**	**Rhat**
PhiA	0.84	0.101	0.631	0.99	1.004
PhiB	0.131	0.109	0.004	0.403	1.008
PsiAB	0.33	0.089	0.169	0.512	1.009
PsiBA	0.377	0.271	0.017	0.928	0.999
pA	0.106	0.037	0.052	0.192	1.001
deviance	111.994	8.475	94.413	128.159	1

JAGS code for estimating host population dynamics. Model is based on Lebreton et al. (2009) and Kéry and Schaub (2011). Parameters were estimated by performing three replicate runs, each with 100.000 iterations, a burn-in of 20.000 iterations and a thin rate of 250 iterations.

```
model {
 # -----------------------------------------------------
 # Parameters:
 # phiA: survival probability of uninfected individuals
 # phiB: survival probability of infected individuals
 # psiAB: Infection rate
 # psiBA: Recovery rate
 # pA: recapture probability of uninfected individuals
 # pB: recapture probability of infected individuals
 # -----------------------------------------------------
 # States (S):
 # 1 alive and uninfected
 # 2 alive and infected
 # 3 dead
 # Observations (O):
 # 1 seen, uninfected
 # 2 seen, infected
 # 3 not seen
 # -----------------------------------------------------
 # Priors and constraints

  for (t in 1:(n.occasions-1)) {
  phiA[t] <- mean.phiA
  phiB[t] <- mean.phiB
  psiAB[t] <- mean.psiAB
  psiBA[t] <- mean.psiBA
#  pA[t] <- mean.pA
#  pB[t] <- mean.pA
    }

pA[1] <- 0
for (t in 2:5){   pA[t] <- mean.pA }
pA[6] <- 0
for (t in 7:9){   pA[t] <- mean.pA }
for (t in 10:13) { pA[t] <- 0 }
pA[14] <- mean.pA
for (t in 15:19) { pA[t] <- 0 }
for (t in 20:22 ) {   pA[t] <- mean.pA  }
pA[23] <- 0
for (t in 24:(n.occasions-1)){   pA[t] <- mean.pA  }

# for (i in 1:1) {
 mean.phiA ~ dunif(0, 1) # Priors for mean state-spec. survival
 mean.phiB ~ dunif(0, 1) # Priors for mean state-spec. survival
 mean.psiAB ~dunif(0,1)
 mean.psiBA ~dunif(0,1)
# mean.psiBA ~dbeta(1,5)
 mean.pA ~ dunif(0, 1) # Priors for mean state-spec. recapture
# mean.pB[i] ~ dunif(0.01, 0.95) # Priors for mean state-spec.
recapture
```

```
#   }

  # Define state-transition and observation matrices
  for (i in 1:nind){
  # Define probabilities of state S(t+1) given S(t)
  for (t in f[i]:(n.occasions-1)){
  ps[1,i,t,1] <- phiA[t] * (1-psiAB[t])
  ps[1,i,t,2] <- phiA[t] * psiAB[t]
  ps[1,i,t,3] <- 1-phiA[t]
  ps[2,i,t,1] <- phiB[t] * psiBA[t]
  ps[2,i,t,2] <- phiB[t] * (1-psiBA[t])
  ps[2,i,t,3] <- 1-phiB[t]
  ps[3,i,t,1] <- 0
  ps[3,i,t,2] <- 0
  ps[3,i,t,3] <- 1

  # Define probabilities of O(t) given S(t)
  po[1,i,t,1] <- pA[t]
  po[1,i,t,2] <- 0
  po[1,i,t,3] <- 1-pA[t]
  po[2,i,t,1] <- 0
  po[2,i,t,2] <- pA[t]
  po[2,i,t,3] <- 1-pA[t]
  po[3,i,t,1] <- 0
  po[3,i,t,2] <- 0
  po[3,i,t,3] <- 1
  } #t
  } #i

  # Likelihood
  for (i in 1:nind){
  # Define latent state at first capture
  z[i,f[i]] <- Y[i,f[i]]
  for (t in (f[i]+1):n.occasions){
  # State process: draw S(t) given S(t-1)
  z[i,t] ~ dcat(ps[z[i,t-1], i, t-1,])
  # Observation process: draw O(t) given S(t)
  Y[i,t] ~ dcat(po[z[i,t], i, t-1,])
  } #t
  } #i
  }
```

Chapter IV

Study II

Post-epidemic salamander persistence in a disease-free refugium suggests poor dispersal ability of *Batrachochytrium salamandrivorans*

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4.1. ABSTRACT

Lack of disease spill-over between adjacent populations has been associated with habitat fragmentation and the absence of population connectivity. We here present a case which describes the absence of the spill-over of the chytrid fungus *Batrachochytrium salamandrivorans* between two connected subpopulations of fire salamanders (*Salamandra salamandra*). Based on neutrally evolving microsatellite loci, both subpopulations were shown to form a single genetic cluster, suggesting a shared origin and/or recent gene flow. Alpine newts (*Ichthyosaura alpestris*) and fire salamanders were found in the landscape matrix between the two sites, which are also connected by a stream and separated by no obvious physical barriers. Performing a laboratory trial using alpine newts, we confirmed that *B. salamandrivorans* is unable to disperse autonomously. Vector-mediated dispersal may have been impeded by a combination of sub-optimal connectivity, limited dispersal ability of infected hosts and a lack of suitable dispersers following the rapid, *B. salamandrivorans* driven collapse of susceptible hosts at the source site. Although the exact cause remains unclear, the aggregate evidence suggests that *B. salamandrivorans* may be a poorer disperser than previously hypothesized. The lack of *B. salamandrivorans* dispersal between neighbouring salamander populations opens perspectives for disease management and stresses the necessity of implementing biosecurity measures preventing human-mediated spread.

4.2. Introduction

Emerging infectious disease of wildlife are a leading cause of biodiversity loss worldwide (Daszak et al. 2000). Because successful mitigation of epidemics remains extremely challenging (Garner et al. 2016), most recommended strategies for controlling disease impacts focus on creative local, context-specific solutions that minimize the spatial diffusion of pathogens, mostly through generally applicable biosafety measures and restrictions to trade and other human-mediated movements of wildlife (Woodhams et al. 2011; Grant et al. 2016). Devising effective actions aimed at minimizing disease spread, that go beyond those general biosafety precautions, would require a better understanding of the dynamics of such spread and its preferential pathways. This type of information is especially vital at the early stages of an emerging disease invasion (Langwig et al. 2015). Dispersal abilities of pathogens, hosts and vectors (biotic and abiotic), the presence and role of barriers to dispersal, as well as stochastic processes that determine whether spread occurs or not, all need to be investigated (Woodhams et al. 2011; Grant et al. 2016).

In North-western Europe, the recently detected chytrid fungus *Batrachochytrium salamandrivorans* (Martel et al. 2013) has brought several populations of fire salamanders (*Salamandra salamandra*) to the brink of extinction within a short time frame of five years or less (Martel et al. 2013; Spitzen-van der Sluijs et al. 2013 & 2016; Goverse et al. 2016; Stegen et al. 2017). The fungus persists in natural systems and currently no viable solution is at hand to eliminate *B. salamandrivorans* from infected

wild populations or to reduce its impact (Canessa et al. 2018). Previous studies have hypothesized that *B. salamandrivorans* should be able to spread rapidly, similarly to *Batrachochytrium dendrobatidis* (Lips et al. 2008), thus posing a concrete risk of a novel amphibian pandemic (Schmidt et al. 2017; Yap et al. 2017). On the other hand, Canessa et al. (2018) suggested that high mortality rates mean that *B. salamandrivorans* infected fire salamanders are generally unlikely to move long distances, although resistant spores and other hosts and vectors may still facilitate dispersion (Stegen et al. 2017).

In October 2013, a population of fire salamanders was discovered in the Netherlands in a marginal habitat site (hereafter referred to as Broek) located 800 m from the index-case of *B. salamandrivorans* in Europe (Bunderbos) (Martel et al. 2013; Spitzen-van der Sluijs et al. 2013). The landscape between the two sites consists of built-up areas alternating with agricultural land, lined with hedgerows and small forest patches and a partially underground stream that connects the two subpopulations (Broek and Bunderbos). In the absence of obvious physical barriers such as highways, invasion by *B. salamandrivorans* in the newly discovered subpopulation and its ensuing total collapse were considered imminent. However, to date these events have not occurred and the Broek subpopulation remains apparently free from *B. salamandrivorans*.

To clarify the potential factors determining this failure of *B. salamandrivorans* spread between two neighbouring sites, we carried out field surveys at both the Broek and Bunderbos subpopulations to estimate fire salamander abundance, to quantify *B.*

salamandrivorans prevalence and infection loads, and to estimate the genetic differentiation and gene flow between the subpopulations. We also conducted a laboratory experiment with alpine newts (*Ichthyosaura alpestris*, a demonstrated *B. salamandrivorans* vector sympatric with fire salamanders (Stegen et al. 2017)), in which we determined the ability of *B. salamandrivorans* to spread autonomously when host contact is physically impeded. We present the results of these analyses and discuss their implications for *B. salamandrivorans* dispersal and potential mitigation strategies.

4.3. Results

4.3.1. Persistence of a stable *B. salamandrivorans*-free salamander subpopulation in the vicinity of a *B. salamandrivorans* outbreak site

To quantify the *B. salamandrivorans* prevalence and infection load we collected skin swabs from the ventral side of salamanders. In the Broek subpopulation, 176 unique fire salamanders were caught over 64 site visits for a total of 510 sightings. This included 139 adults, 33 sub-adults and 4 juveniles (considering the oldest age class of capture for each individual). Sex ratio was slightly biased towards males (78 M:69 F) although 29 animals could not be sexed with certainty. Individual fire salamanders were recaptured between 1 and 16 times (mean = 2.9 times; median = 2 times); the majority of individuals (n = 71) were sighted once, and 35 animals were sighted twice or three times (n = 24). Eight animals, all adult males, were sighted ten times or more. Fitting a Jolly-Seber model (Kéry and Schaub 2011) to individual mark-recapture data (See JAGS code in Extended Data), we estimated the Broek subpopulation size to have

fluctuated between 75 and 115 individuals over the study period (Fig. 4.1), showing a seasonal pattern consistent with the breeding season of fire salamanders (juveniles emerging between August and October).

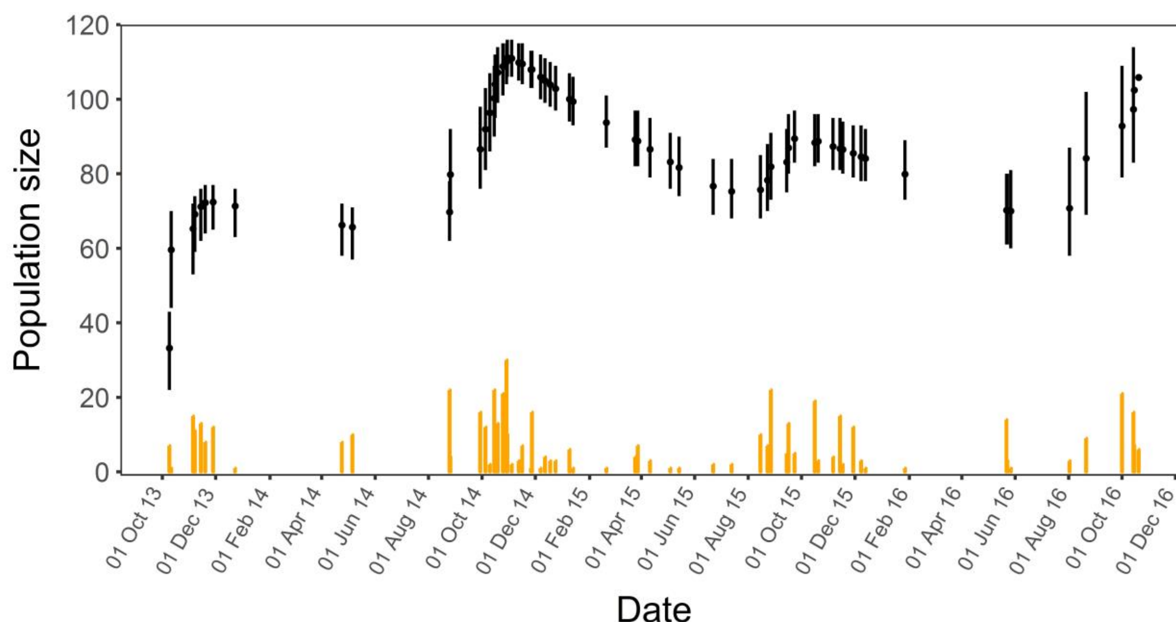

Figure 4.1. Population size of fire salamanders at the new site, estimated from open-population Jolly-Seber model. Bars indicate 95% credible intervals (CRI). Orange bars indicate the total count of captures on a given survey.

The mean estimated weekly survival was 0.991 (95% CRI: 0.989-0.993, corresponding to a mean yearly survival of 0.625) and recapture probability was relatively low (mean probability throughout the year 0.12, 95% CRI: 0.11-0.13). In the Broek subpopulation, we collected a total of 207 skin swabs, all from fire salamanders (2013: 57 swabs; 2014: 43 swabs; 2015: 29 swabs and 2016: 78 swabs), none of which tested positive for *B. salamandrivorans*. One alpine newt was sighted in 2015 at the Broek site, but not sampled.

In the Bunderbos subpopulation, we sighted 15 and 7 adult fire salamanders in 2015 and in 2016 respectively (47 and 24 site visits, both diurnal and nocturnal), as well as one dead salamander in 2015. At this site we also observed 66 adult/subadult alpine newts in 2015 and 74 in 2016. All sighted post-metamorphic newts and salamanders were sampled for *B. salamandrivorans*. For all data prior to 2015, we refer to Spitzen-van der Sluijs et al. (2016). In 2015, three fire salamanders out of 16 and two alpine newts out of 66 tested positive for *B. salamandrivorans*. Two living salamanders showed loads of 23 and 90 GE (Genomic Equivalent) per swab, one dead salamander showed histopathological lesions and had a GE load of 5.3 10^3 per swab. The two alpine newts showed loads of 440 and 322 GE per swab respectively. In 2016, no fire salamanders or alpine newts tested positive for *B. salamandrivorans* (0/7 and 0/74 respectively).

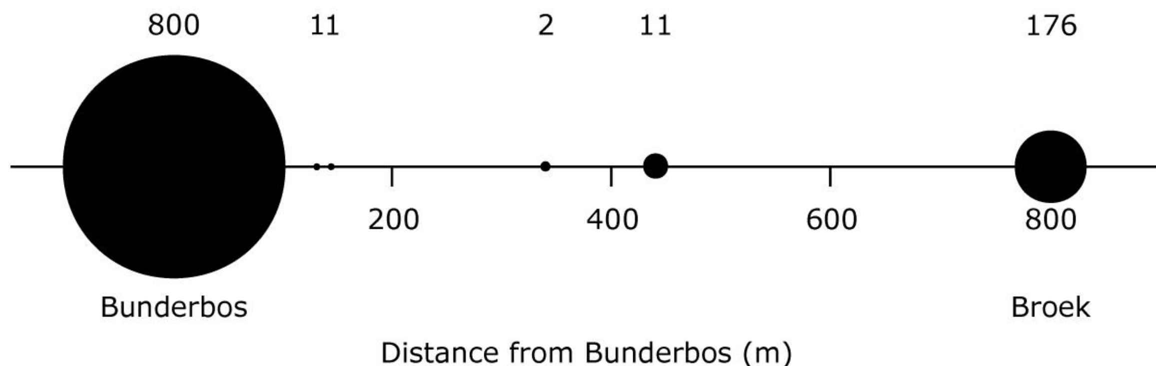

Figure 4.2. Schematic representation of the distance (in meters) between the Bunderbos and Broek subpopulations and in the matrix in between the two subpopulations. The size of circles corresponds to the number of fire salamanders observed from 2007-2017 (indicated above each circle).

In the intermediate matrix between the two sites, ad hoc sightings of fire salamanders are not exceptional. Between 2010 and 2017, a total of five sightings of

larvae, juvenile and adult fire salamanders have been reported from four points which are located between the Bunderbos and Broek (Fig. 4.2).

4.3.2. Salamanders from Bunderbos and Broek form a genetic cluster

A total of 76 individuals were genotyped for 18 microsatellites: buccal swabs were collected from 63 salamanders originating from the Bunderbos (now in an *ex situ* conservation program), and from 11 individuals of the Broek subpopulation along with two tail clips from traffic victims from a road immediately next to the Broek subpopulation. The analysis with STRUCTURE showed that the two subpopulations from the Netherlands cluster together genetically as one population when compared to 50 individuals of the reference population from the Kottenforst (Germany; K=2, mean Ln P(K)=-4745, Delta K=9469) (Fig. 4.3a, Table 4.1, Extended Data Fig. 4.1a, b) while an analysis with the Bunderbos and Broek subpopulations alone did not show a clear differentiation between the two subpopulations (K=3, Delta K=37; K=4, mean Ln P(K)=-2472) (Fig. 4.3b, Table 4.1, Extended Data Fig. 4.2a, b). The self-assignment test confirmed the initial result, assigning 100% of the German Kottenforst fire salamanders to that population while individuals from the two Dutch populations were assigned to either one of them (Bunderbos: 63% correctly assigned, 37% assigned to Broek; Broek: 23% correctly assigned, 77% assigned to Bunderbos).

Table 4.1. Evanno table identifying the most probable hypothetical K within the dataset. #K: number of hypothetical populations in run, Reps: number of replications performed per K, Mean LnP(K): Mean of the log likelihood of the data, Stdev Ln P(K): standard deviation log likelihood of the data, Ln'(K): first order rate of change in the likelihood of the data, \|L''(K)\|: absolute value for the second order rate of change in the likelihood of the data, Delta K: *ad hoc* quantity related to the second order rate of change of the log probability of the data between successive K values.

	# K	Reps	Mean LnP(K)	Stdev Ln(P(K))	Ln'(K)	|Ln''(K)|	Delta K
Broek + Bunderbos + Kottenforst samples	1	20	-5710	0.3	NA	NA	NA
	2	20	-4745	0.1	965	863	9469
	3	20	-4644	64	101	19	0,3
	4	20	-4562	12	82	39	3
	5	20	-4519	12	43	NA	NA
Broek + Bunderbos samples	1	20	-2679	0.3	NA	NA	NA
	2	20	-2612	25	67	34	13
	3	20	-2512	16	100	60	37
	4	20	-2472	49	40	156	32
	5	20	-2588	187	-116	NA	NA

4.3.3. Physical barriers prevent *B. salamandrivorans* transmission

We conducted a laboratory experiment with alpine newts (*Ichthyosaura alpestris*) to test if *B. salamandrivorans* can spread autonomously over short distances. Fourteen pairs, each co-housing one experimentally infected and one non-infected newt, were divided in two treatment groups of seven pairs each: in the first group, infected and non-infected newts were physically separated from each other by a double-sided mesh (mesh size: 1.3 x 1.6 mm.) while in the second group the newts in each pair were free to come into contact with one another. The average infection load of the 14 infected newts before they were co-housed with uninfected animals was 3.5 ± 1.02 (log10

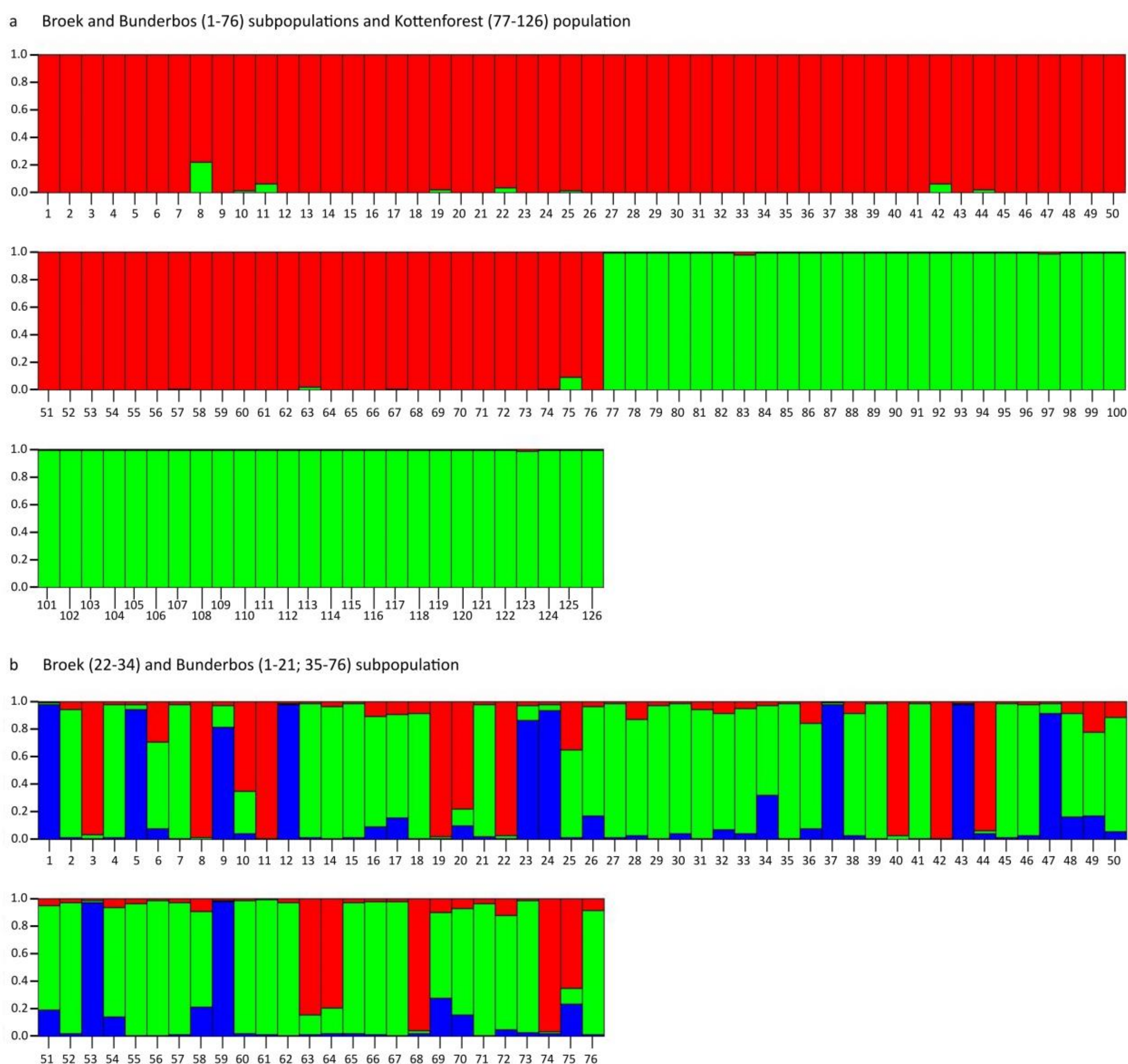

Figure 4.3. Output from Structure where the most likely number of K is plotted with the data. When K=2 (red and green), the samples analysed originated from the Broek subpopulation, the Bunderbos subpopulation (1-76) and the Kottenforst population (77-126) **(a)**. When K=3 (red, green and blue), the samples analysed originated from the Broek (22-34) and the Bunderbos subpopulation (1-21, 35-76) only **(b)**.

GE/swab; 3.6 ± 0.92 in the contact group and 3.3 ± 1.15 in the no-contact group). One week after co-housing, the infection load was not statistically different compared to the beginning (Mann-Whitney U-test: p = 0.344). Animals were selected randomly for each pairing, as confirmed by the lack of statistically significant differences in loads between the two groups (Mann-Whitney U test; p = 0.535; Fig. 4.4). At the end of the

experiment, after four weeks, the difference in the proportion of individuals infected per group was evident (one-sided Fisher's exact test, p = 0.01). In the contact group, 5/7 of the 'non-infected' individuals had become infected; in the no-contact group, none of the non-infected individuals (0/7) tested positive for *B. salamandrivorans*.

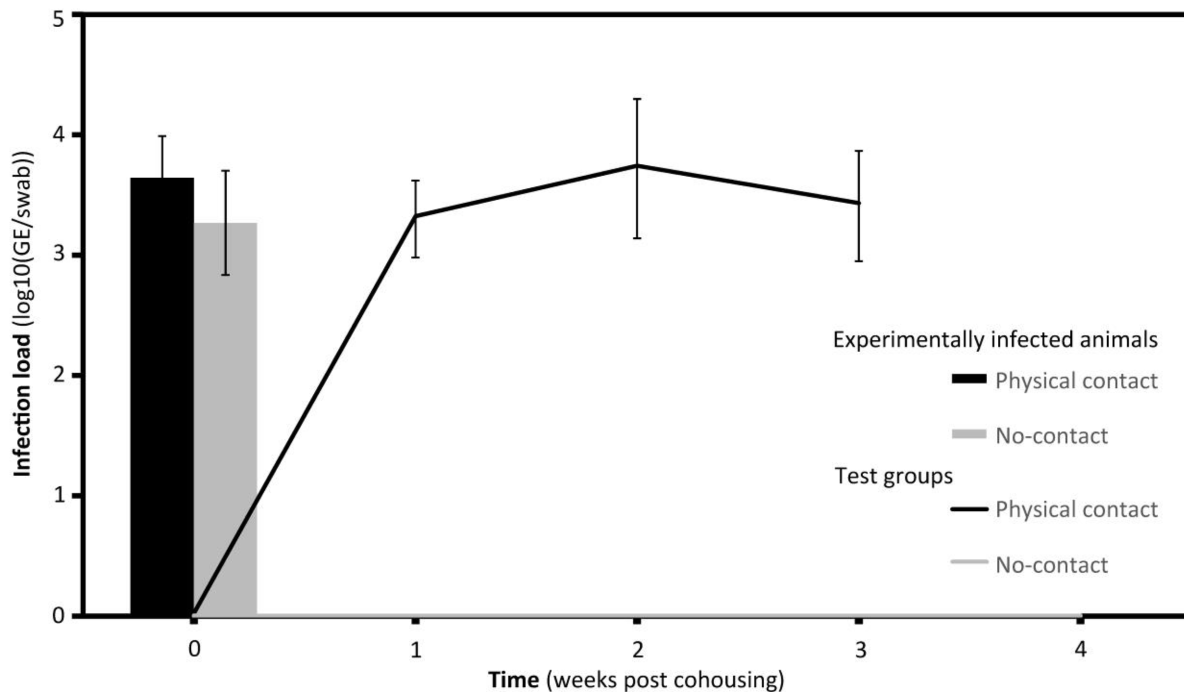

Figure 4.4. In vivo infection experiment with alpine newts (*Ichthyosaura alpestris*). Average infection load for infected newts in each group (Contact vs no-contact group). In the group where physical contact was possible (black), 5 out of 7 newts developed chytridiomycosis while none of the newts in the group where contact was prevented (grey) tested positive for *B. salamandrivorans* nor developed chytridiomycosis. Bars: experimentally infected newts in the physical contact group (black) and in the no-contact group (grey). Lines: average infection load of newts that developed clinical signs of chytridiomycosis from the physical contact group (black) and the no-contact group (grey). Error bars represent standard error of the mean.

4.4. Discussion

We found no evidence of *B. salamandrivorans* spread between two neighbouring fire salamander subpopulations, despite several possible pathways of dispersion via infected hosts or (a)biotic vectors. The size and trend of the Broek subpopulation

suggest that the fungus has so far been unable to bridge the 800-m distance between the two sites. This situation sharply contrasts with available knowledge about *B. dendrobatidis* (Lips et al. 2008) and previous hypotheses about the imminent threat of a rapid *B. salamandrivorans* spread (Schmidt et al. 2017; Yap et al. 2017). The Broek subpopulation has so far persisted in a *B. salamandrivorans* -refuge in the vicinity of the *B. salamandrivorans* index site, although the exact reason remains unclear. In the remainder of this discussion, we consider the possible scenarios and the implications for future *B. salamandrivorans* mitigation.

Autonomous dispersal by *B. salamandrivorans* can be ruled out with some certainty. In our experiment, the fungus was unable to cross even the small distance (< 1 cm) between the two sides of a permeable (to the pathogen although not to the host) physical barrier in a terrarium. Of non-autonomous dispersal pathways, direct spread of *B. salamandrivorans* by infected hosts is arguably the most intuitive. In the surroundings of a known *B. salamandrivorans* outbreak site near Liège in Belgium (Spitzen-van der Sluijs et al. 2016), *B. salamandrivorans* -free fire salamanders can still be found as close as 3 km from where the outbreak was originally identified (Stegen, unpublished results). Here, several highways intersecting the continuous forest habitat may represent physical barriers for migrating fire salamanders and other amphibian and non-amphibian vectors. The situation in our study is less clear. Although the matrix between the Broek and Bunderbos sites largely consists of human-modified landscape and small, fragmented habitat patches, we found evidence

of at least some connection between the two salamander subpopulations. The matrix allows for overland migration of fire salamanders and alpine newts, and individuals have occasionally been sighted in this connecting landscape (Fig. 4.2). We found that the Bunderbos and Broek subpopulations cluster together genetically on the basis of microsatellite loci differentiation when compared to the fire salamanders from the Kottenforst (Germany), suggesting they have a shared population history, which is also underpinned by the analysis of mitochondrial D-loop haplotypes (Extended Data Table 4.2a, b) as well as historical maps dating back to 1868 which show natural connectivity between the two sites. Additionally, there is an indication of recent or ongoing gene flow between the two subpopulations (but see Extended Data for a more detailed discussion of the genetic analysis). More in general, both healthy fire salamanders and alpine newts have sufficient dispersal capabilities to cover the distance between the two sites (Schmidt et al. 2007; Hendrix et al. 2017). However, the dispersal capability of *B. salamandrivorans* infected individuals is a more relevant parameter here. Canessa et al. (2018) predict that infected fire salamanders would move on average less than 100 m before succumbing to infection. Schmidt et al. (2017), while predicting a rapid spatial spread of *B. salamandrivorans*, also recognise the possibility that infected individuals may not move far enough to transmit the disease to neighbouring forest patches.

Ultimately, spread is a stochastic process, and a larger source host population will produce a greater number of dispersers, particularly rare long-range ones. In this

sense, dispersal of *B. salamandrivorans* from the original site in the Bunderbos to the Broek subpopulation may have been impeded by the rapid collapse of the fire salamander hosts at the former site. The virulence of the pathogen may have hindered its spread by functionally removing most potential dispersers, and thus further reducing the stochastic chances. On the other hand, alpine newts persist in the Bunderbos at higher densities than fire salamanders. Newts may survive *B. salamandrivorans* infection and even carry it asymptomatically (Stegen et al. 2017) and are known to have higher dispersal abilities than fire salamanders (Jehle and Sinsch 2007; Bülow 2011; Kovar et al. 2009), making them potentially more important dispersers of the pathogen. However, no infected newts were found in the Bunderbos in 2016, and only one newt was sighted in the newly discovered fire salamander subpopulation, suggesting this host also provides low chances for *B. salamandrivorans* dispersal, at least in this area.

If dispersal by infected hosts is restricted, whether by dispersal abilities of the hosts themselves, by sub-optimal matrix permeability, or by the small number of available hosts (possibly as a result of *B. salamandrivorans* epidemic dynamics at the source), vectors may represent the next most likely pathways. Dispersal by biotic (non-susceptible) vectors is possible: Stegen et al. (2017) demonstrated *B. salamandrivorans* spores can attach themselves to scales of goose feet. Bird vectors are also unlikely to be significantly affected by sub-optimal permeability of the matrix between the two sites.

As for abiotic vectors, waterways are considered highly suitable for fungal survival and spread (Stegen et al. 2017): a stream directly connects the two subpopulations in our study. More than half of this stream is subterranean, and the aboveground part contains fish, possibly making it unsuitable habitat for vector species such as alpine newts. Fire salamanders in the Bunderbos have been demonstrated to deposit their larvae upstream: zoospores and fire salamander larvae can be expected to flush to the downstream naive subpopulation. However, the current absence of *B. salamandrivorans* from the Broek subpopulation suggests to date spread by water or by such passive vectors as flushed amphibian larvae has also been unsuccessful, whether as a result of a deterministic (e.g. due to barriers preventing vector movements) or stochastic process (e.g. due to low numbers of potential vectors and consequent low chances of successful dispersal).

Our results provide important information about the potential of *B. salamandrivorans* to disperse rapidly through the landscape, suggesting such potential might not be as high as previously thought (Schmidt et al. 2017; Yap et al. 2017) or as its congeneric species *B. dendrobatidis* (Lips et al. 2008). In turn, this information has important implications for *B. salamandrivorans* mitigation. Although mitigation is likely to prove highly challenging during the epidemic event (Canessa et al. 2018), if the risk of spread remains low the disease might effectively eradicate itself by extirpating its hosts; mitigation actions could be implemented during or after the outbreak to further reduce spread (for example by actively removing individuals

(Canessa et al. 2018)). Population reinforcement and reintroductions might be implemented after the disease has faded out, or to buffer remaining populations against stochastic extinctions.

Moreover, the possibility that *B. salamandrivorans* is indeed a weaker disperser than originally hypothesized further reinforces the need to prevent its human-mediated dispersal. The currently known distribution of *B. salamandrivorans* in Europe is discontinuous, with apparent jumps (Spitzen-van der Sluijs et al. 2016) for which human-mediated dispersal cannot be ruled out under current evidence. Quarantine and biosafety protocols should be rigorously implemented, and more radical actions considered (such as restriction of access by quarantine fences). The case we have described may provide directions for disease management in highly threatened, range-restricted, isolated or locally endemic salamander species, such as *Salamandra atra pasubiensis*, *S. atra aurorae*, *S. lanzai* or *Calotriton arnoldi*, which might face fast extinction in the event of *B. salamandrivorans* arrival within their ranges.

4.5. Methods

4.5.1. Site

We do not disclose the exact location of the novel site (Broek subpopulation) to prevent pathogen pollution or otherwise harmful activities (Phillott et al. 2010; Lindenmayer and Scheele 2017). The new site is small (0.57 ha.), is located within a one km radius of the Bunderbos (Spitzen-van der Sluijs et al. 2013) and consists of an artificial habitat: a fast-flowing stream with a steep, concreted slope passes through

the area, which is void of a water body suitable for fire salamander reproduction. Both the terrestrial and aquatic habitat are marginal. Multiple creeks merge underground into this stream, including water that originates from the Bunderbos area, which was the first location at which *B. salamandrivorans* was detected (Spitzen-van der Sluijs et al. 2013). Old maps of the area, dating back to 1868, show a natural connection of the current stream, through meadow and brook land forest with the Bunderbos. The landscape between the two subpopulations is characterized by an urbanized and agricultural zone. We checked the national databank flora and fauna for sightings of fire salamanders in this matrix in the period 2007 - 2017[1]. Elevation of the Broek subpopulation ranges between 40 m and 56 m above sea level[2], and the vegetation consists of poplar trees, shrubs, bushes and grassland.

4.5.2. Inferring demographics of the new fire salamander population (Broek subpopulation)

Standardized monitoring of the fire salamanders started immediately upon discovery of the Broek subpopulation in October 2013. Transect counts were continuously done after sunset, either in the late evening or at night, under humid or wet conditions with temperatures ≥ 5°C, according to the national standard to monitor fire salamanders (Goverse et al. 2015). The transect covers the entire area and measures 665 m in total added length. Over the period October 2013 - October 2016, the site was

[1] www.ndff-ecogrid.nl; accessed 16 Nov. 2017
[2] www.ahn.nl; accessed 7 Sept. 2017

visited 64 times: 8 times in 2013 (October – December); 22 times in 2014 (April, May, August - December); 24 times in 2015 (January - December); and 10 times in 2016 (January - October). The mean interval between site visits was 17.6 days (range: 1-122; median: 61.5).

During all 64 visits between October 9th 2013 and October 20th 2016, the dorsal pattern of each individual fire salamander was recorded by photography. These patterns, unique for each individual (Feldman and Bösperde 1971), allowed us to identify recaptures on the basis of dorsal spot patterns using the program AMPHIDENT (Matthé et al. 2017). We used these mark-recapture data to estimate the survival, recapture probability and population size using the Jolly-Seber open-population model (Kéry and Schaub 2011). We assumed constant apparent survival, and modelled the probability of entry and that of recapture using a cosine function to reflect seasonal variation in salamander migration (entry) and activity patterns (detection). We rescaled survival and entry on a weekly period, to account for the variable intervals between surveys. We fitted the model in JAGS (Kéry and Schaub 2011; Plummer 2003) using uninformative priors for all parameters (model code in Extended Data). We drew 50,000 samples from the posterior distributions of all parameters, from three Markov chains with overdispersed initial values, after discarding the first 25,000 as a burn-in and applying a thinning rate of 10. We assessed convergence by visual inspection of the chain histories, and through the R-hat statistic.

4.5.3. Detection of *B. salamandrivorans*

Ventral skin swabs were taken from post-metamorphic salamanders and newts, using aluminium sterile cotton-tipped dryswabs (rayon-dacron, COPAN, UNSPSC CODE 41104116) following the procedure and biosecurity measures described in Hyatt et al. (2007) and Van Rooij et al. (2011). All samples were kept frozen at -20°C until further analysis for the presence of *B. salamandrivorans* DNA through real-time PCR, as described by Blooi et al. (2013). Skin histopathology as described in Martel et al. (2013) was performed to detect *B. salamandrivorans* infection on dead salamanders.

4.5.4. Genetic analyses

We collected genetic samples - buccal swabs - from fire salamanders at the Broek and the Bunderbos subpopulation to test the origin of the Broek subpopulation and to draw conclusions on the overall genetic constitution of both subpopulations. We hypothesize that the Broek subpopulation has a relict origin, although an anthropogenic introduction has been suggested and is also deemed possible. The samples were used to assess the population structure between the Broek and the Bunderbos subpopulations on the basis of neutrally evolving microsatellite loci. Therefore, samples from the Broek and the Bunderbos subpopulation were genotyped for 18 microsatellite loci as described in Steinfartz et al. (2004) and Hendrix et al. (2010) and compared to the well-studied population of fire salamanders in the Kottenforst, near Bonn (north-Rhine Westphalia in Germany, approximately 100 km from the Bunderbos as the crow flies) (Steinfartz et al. 2007; Caspers et al. 2014; Hendrix et al.

2017). We assessed recent gene flow between the Broek and the Bunderbos subpopulation and the Kottenforst (Germany) using the program STRUCTURE, followed by Structure Harvester to identify the most probable number of populations (K) (Pritchard et al. 2000; Falush et al. 2003 & 2007; Hubisz et al. 2009; Earl and vonHoldt 2012). Structure was run using 20 iterations for each K and K was *a priori* assumed to be between 1 and 20 clusters, each iteration had a burn-in of 100.000 runs followed by 2.000.000 runs after burn-in. No *a priori* information on sample origin (LOCPRIOR) was fed into the program. The most probably K was defined using the delta K method as described by Evanno et al. (2005) as well as the posterior likelihood of the data as described by Pritchard et al. (2000), only when both methods identified the same K, we inferred this K as the most likely. Lastly, we used the program GENECLASS2 (Piry et al. 2004) in order to assign individuals to their respective subpopulation (Bunderbos and Broek, e.g. Valbuena-Ureña et al. 2017).

4.5.5. PHYSICAL BARRIERS ACT TO RESTRAIN *B. SALAMANDRIVORANS* TRANSMISSION

Although soil can act as a vector for *B. salamandrivorans* (Stegen et al. 2017), it is unknown whether the fungus can spread actively over short distances. We studied the role of physical barriers in *B. salamandrivorans* transmission between infected and non-infected alpine newts (*Ichthyosaura alpestris*). Alpine newts were chosen because they act as vector but can survive infection and co-occur with *Salamandra* populations frequently. During the experiment, direct physical contact between infected and non-infected individuals was either allowed (contact group) or prevented (no-contact

group) by placing a physical barrier (double sided mesh; mesh size: 1.3 mm x 1.6 mm, the sides placed 0.5 cm apart) in the middle of the containers from the no-contact group. At the onset of the experiment, 14 newts were infected with 10^5 zoospores of the *B. salamandrivorans* type strain (AMFP13/1) suspended in 1 ml distilled water following Martel et al. (2014) to ensure 100% *B. salamandrivorans* prevalence. During the experiment, all animals were clinically examined every day. Seven days after the initial inoculation we collected skin swabs to determine the infection status and load for each animal. Each infected individual was randomly assigned to either the contact or no-contact group and co-housed with a non-infected individual. Each group (contact versus no-contact) therefore contained seven pairs. After seven days of co-housing, the 14 experimentally infected individuals were swabbed, removed from the experiment and heat treated to cure the animal from the *B. salamandrivorans* infection as described by Blooi et al. (2015). Hereafter, skin swabs were collected every seven days for three consecutive weeks from the non-infected animals to determine the *B. salamandrivorans* infection loads. An animal was considered infected when two consecutive swabs were positive, or if the genomic load was higher than 3 log10 (GE; genomic equivalents). As soon as a newt tested positive for *B. salamandrivorans*, the animal was removed from the experiment and treated as described by Blooi et al. (2015). Each container (19 x 12 x 7 cm) was filled with a layer of unsterilized and moisturized forest soil (from a *B. salamandrivorans* -free forest), and kept constantly at 15°C. Crickets, which were also unable to cross the barrier, were provided as food items *ad libitum* twice a week. To avoid cross contamination, each individual was

handled with a new pair of nitrile gloves. Prior to taking part in the experiment all animals were tested for, and proved free of *B. salamandrivorans*, *B. dendrobatidis* and ranavirus – two other infections causing major amphibian diseases.

4.5.6. Ethics statement

All methods involving animals were approved by and carried out in accordance with the guidelines and regulations of permit EC2015/29 issued by the ethical committee of Ghent University and permit FF/75A/2016/015 issued by the Netherlands Enterprise Agency.

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AUTHOR CONTRIBUTIONS A.S., A.M., F.P. and G.S. conceived and designed the study. A.S., S.B., N.J. coordinated and assembled the field sampling. S.C. conducted statistical analyses. G.S., A.M. and F.P. performed the lab study. S.S. and G.S. conducted the genetic analyses. All authors wrote the article, and all authors agreed to submission of the manuscript and accept the responsibility for the accuracy and integrity of the manuscript.

4.7. EXTENDED DATA

4.7.1. METHODS

The samples were used to determine the origin of the Dutch fire salamanders by analysing the mitochondrial D-loop and to assess the population structure between the Broek and the Bunderbos subpopulations on the basis of neutrally evolving microsatellite loci. Samples from two focal populations were sequenced for the mitochondrial D-loop using primers as described in Steinfartz *et al*. (2000). Obtained sequences were categorized according to D-loop haplotypes found across Europe following the study of Weitere *et al*. (2004) to determine whether the populations consist of native fire salamanders or whether they might have been introduced in the past from other regions in Europe. In addition, samples from the Broek and the Bunderbos subpopulation were genotyped for 18 microsatellite loci as described in Steinfartz *et al*. (2004) and Hendrix *et al*. (2010) and compared to the well-studied population of fire salamanders in the Kottenforst, near Bonn (north-Rhine Westphalia in Germany, approximately 100 km from the Bunderbos as the crow flies) (Hendrix et al. 2017, Steinfartz et al. 2007, Caspers et al. 2014). This population serves here as a reference population in terms of genetic composition. Population genetic analyses were performed with programs GENALEX 6.5b2 (Peakell & Smouse 2012) and ARLEQUIN ver3.5.1.2. (Excoffier & Lischer 2010) to determine deviations from Hardy–Weinberg equilibrium in the Bunderbos subpopulation for each locus, which provides an exact probability value (Guo & Thompson, 1992). We then estimated the populations' inbreeding coefficients (F_{IS} and F_{ST}).

4.7.2. RESULTS

4.7.2.1. HAPLOTYPE

For the mitochondrial D-loop analysis, 31 individual samples were analysed (20 from Bunderbos and 11 from Broek). Eight of these were identified as Type Ia (western evolutionary lineage) and one as a Type II (eastern evolutionary lineage) on the basis of a major haplotype network which spans Europe (Weitere et al. 2004) (Table Extended Data Table 4.1a), which have also recolonized major parts of western Germany while the remaining 22 samples represent a new D-loop haplotype, that differed by a single mutational step from Type I (Extended Data Table 4.1b).

Extended Data Table 4.1a. Mitochondrial D-loop analysis. Classification of haplotypes found in the Bunderbos and the Broek fire salamander subpopulations compared to the Kottenforst data (Weitere et al. (2004)).

Locality	Individuals analysed for the D-loop	Haplotypes assignment (frequencies)
Bunderbos	20	Type Ia (35%), Type I-like (65%)
Broek	11	Type Ia (9%), Type I-like (82%), Type II (9%)
Kottenforst	37	Type Ia (78%), Type Ib (22%)

Extended Data Table 4.1b. Comparison of the nucleotide sequences in the mitochondrial D-loop haplotypes used in this study. All sequences where previously known (Weitere et al. 2004), except for the type I-like sequence.

<table>
<tr><th>D-loop</th><th colspan="11">Nucleotide site</th></tr>
<tr><th>haplotype</th><th>1-26</th><th>27</th><th>28-274</th><th>275</th><th>276-336</th><th>337</th><th>338-428</th><th>429</th><th>430-589</th><th>590</th><th>591-724</th></tr>
<tr><td>Type I</td><td rowspan="6">IDENTICAL</td><td>C</td><td rowspan="6">IDENTICAL</td><td>A</td><td rowspan="6">IDENTICAL</td><td>-</td><td rowspan="6">IDENTICAL</td><td>A</td><td rowspan="6">IDENTICAL</td><td>C</td><td rowspan="6">IDENTICAL</td></tr>
<tr><td>Type Ia</td><td>C</td><td>A</td><td>-</td><td>A</td><td>-</td></tr>
<tr><td>Type Ib</td><td>C</td><td>A</td><td>T</td><td>A</td><td>C</td></tr>
<tr><td>Type I-like</td><td>C</td><td>T</td><td>-</td><td>A</td><td>C</td></tr>
<tr><td>Type II</td><td>T</td><td>A</td><td>-</td><td>A</td><td>C</td></tr>
<tr><td>Type IIa</td><td>T</td><td>A</td><td>-</td><td>G</td><td>C</td></tr>
</table>

4.7.2.2. Genetic diversity

The genetic diversity of the Dutch fire salamanders, which was assessed using the samples from Bunderbos, indicated that 5 loci deviated from Hardy-Weinberg equilibrium (Extended Data Table 4.2), while only two loci did so for the reference population from the Kottenforst. Across the 18 genotyped loci of individuals from Bunderbos, we detected a total of 87 alleles with a mean number of 4.83 alleles per locus (Na). The mean number of effective alleles (Ne) was 2.40 (range: 1.16-3.88). Mean observed heterozygosity (Ho = 0.48) was lower than expected (He = 0.53) and the mean inbreeding coefficient (F_{IS}) was 0.10. Compared to the Kottenforst, 88 alleles with a mean number of 4.89 alleles per locus were detected. The mean number of alleles was 2.65 (range: 1.25-4.57). Mean observed heterozygosity (Ho = 0.55) was also lower than expected (He = 0.57). Mean inbreeding coefficient (F_{IS}) was 0.05. The genetic distance (F_{ST}) between the two populations (Kottenforst and Bunderbos) was estimated to 0.20.

Extended Data Table 4.2. Genetic diversity indices for Bunderbos based on 18 microsatellite loci. Significance testing was corrected for multiple comparisons by the Bonferroni correction (adjusted p-value = 0.0028). N: number of samples, Na: number of alleles, Ne: effective number of alleles, I: Shannon's information index, Ho: observed heterozygosity, He: expected heterozygosity, $\chi 2$: Chi-square test for Hardy-Weinberg equilibrium, Signif: p-value for $\chi 2$ test, Post Bonferroni corr: significance of p-value after Bonferroni correction (ns: not significant; SIGNIF: significant), F_{IS}: inbreeding coefficient.

	Locus	N	Na	Ne	I	Ho	He	χ^2	Significance	Post Bonferroni corr*	F_{IS}
BUNDERBOS	**SalE8**	60	5	3,36	1,32	0,75	0,7	11,25	0,338	ns	-0,06
	IIA6	59	6	3,88	1,49	0,76	0,74	43,81	0	**SIGNIF**	-0,02
	E11	61	7	2,76	1,24	0,61	0,64	42,46	0,004	ns	0,06
	IA6	60	5	2,15	1,06	0,45	0,53	41,49	0	**SIGNIF**	0,17
	B11	62	8	3,02	1,38	0,73	0,67	13,23	0,992	ns	-0,08
	SalE6	62	3	1,35	0,51	0,29	0,26	1,79	0,618	ns	-0,12
	C3	62	4	1,16	0,32	0,15	0,14	0,38	0,999	ns	-0,05
	C2	53	3	1,4	0,49	0,17	0,28	13,31	0,004	ns	0,41
	Sal3	61	5	1,62	0,77	0,39	0,38	24,5	0,006	ns	-0,02
	SalE7	62	5	2,64	1,16	0,6	0,62	13,53	0,196	ns	0,05
	SalE11	61	4	3,34	1,28	0,69	0,7	32,9	0	**SIGNIF**	0,03
	SalE14	61	3	1,93	0,73	0,39	0,48	3,68	0,299	ns	0,19
	F10	52	5	2,13	1	0,15	0,53	78,34	0	**SIGNIF**	0,71
	SalE2	61	7	3,18	1,38	0,23	0,69	212,21	0	**SIGNIF**	0,67
	SalE12	61	6	3,24	1,33	0,69	0,69	13,9	0,533	ns	0,01
	G9	63	4	2,17	0,99	0,57	0,54	9,43	0,151	ns	-0,05
	SalE5	63	3	2,1	0,87	0,57	0,52	1	0,801	ns	-0,08
	Sal29	62	4	1,74	0,74	0,47	0,43	11,33	0,079	ns	-0,09
	MEAN	60,33	4,83	2,4	1	0,48	0,53	-	-	-	0,10
	SE	0,71	0,35	0,19	0,08	0,05	0,04	-	-	-	0,06

* adjusted p-value: 0,0028

4.7.3. DISCUSSION

4.7.3.1. HAPLOTYPE

We found that the Bunderbos and Broek subpopulations cluster together genetically on the basis of microsatellite loci differentiation when compared to the fire salamanders from the Kottenforst (Germany), suggesting they have a shared population history, which is also underpinned by the analysis of mitochondrial D-loop

haplotypes. They most likely have a shared origin with the German Western evolutionary lineage, which has recolonized this part of Central Europe following the last glaciation approximately 9000 years ago (Weitere et al. 2004, Steinfartz et al., 2007). The presence of the Type Ia lineage, in combination with the occurrence of a new D-loop haplotype only a single mutation step apart, makes it very unlikely that the Bunderbos population has been founded on introduced salamanders from other areas in Europe, which debunks a pertinacious rumour in the Netherlands.

4.7.3.2. SIMILAR GENETIC CONSTITUTION OF THE SUBPOPULATIONS

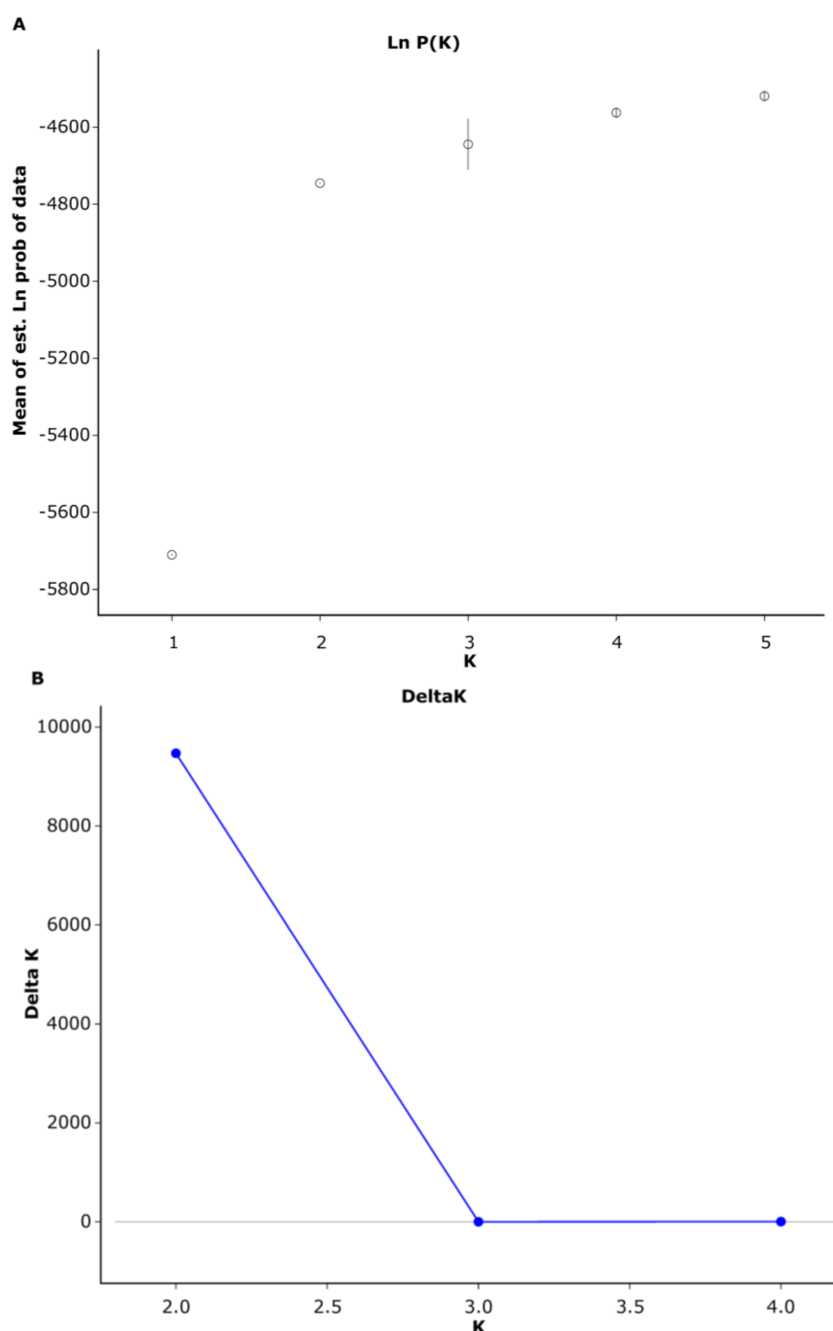
The outcome of the STRUCTURE analysis indicating the presence of three genetic clusters (K = 3; see Extended Data Fig. 4.2b), has no impact on the observed genetic population structure and differentiation between the two subpopulations. Here, only the individual genotypes of the Bunderbos and Broek subpopulations were analysed and the clustering of individuals does not follow a structure of underlying subpopulations. It is difficult and speculative to say why we observe a K = 3 at this level. Possibly, this might be because the samples from Bunderbos already represent non-random samples as they were collected after the collapse of the subpopulation due to *B. salamandrivorans*. Such as biased sampling could violate the assumptions of Hardy-Weinberg equilibrium. Most importantly, however, we do not see that the estimation of K = 3 has an impact on the population structure and genetic differentiation of both subpopulations, stating that the overall genetic constitution of

both subpopulations should be rather similar and is not influenced by genetic structure based on microsatellite loci differentiation population structure.

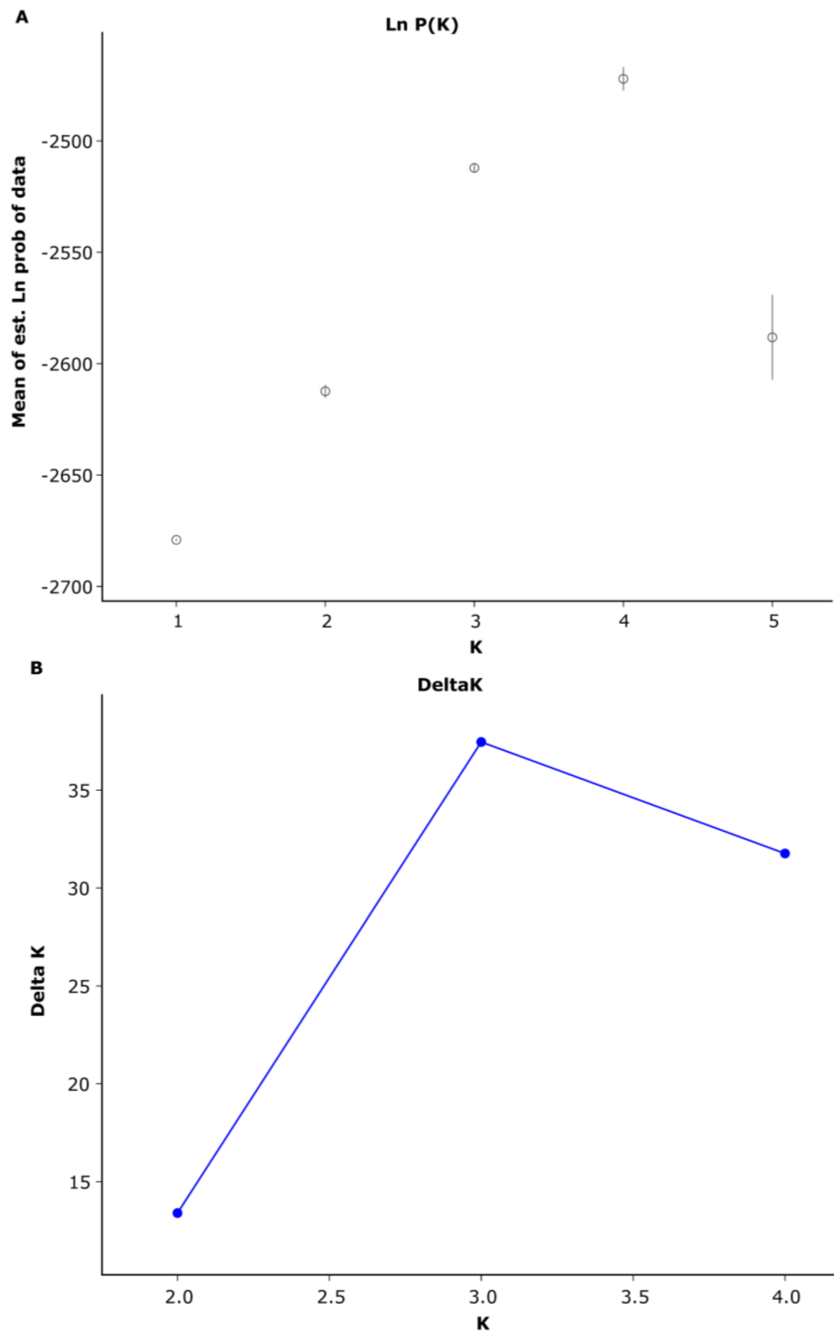
4.7.3.3. Genetic diversity

Although the microsatellite loci analysis revealed significant deviations from HWE for 5 microsatellite loci, two of these also deviated from HWE in the German reference population. The mean inbreeding coefficient (F_{IS}) was low, which allows us to conclude that there is no significant excess or deficiency of heterozygotes that indicates a recent population bottleneck followed by inbreeding (non-random mating) or genetic drift. Alternative explanations for the deviation from HWE might be the violation of the assumptions of the theorem (e.g. finite population, non-random mating, mutation, immigration) or the presence of null alleles within the sample. Immigration and non-random mating can likely be discarded as causative agents of the deviation as there is no substantial other fire salamander population from which fire salamanders might have immigrated to the Bunderbos population, and fire salamanders are polygynandrous (polyandrous females in Caspers et al. (2014); polygynous males in Helfer et al. (2012)). This assumption is corroborated by recent empirical studies on the critically endangered Montseny brook newt (*Calotriton arnoldi* (Valbuena-Urena et al. (2017)) and urban fire salamanders in Spain (Lourenço et al. 2017). These populations show no signs of inbreeding or loss of heterozygosity despite facing strong habitat fragmentation and display low census population sizes. Possibly, the loci C2 and C3 act as null alleles because the allele frequencies are reduced, but did

not show a significant statistical bias or a shifted genetic variation when compared with the reference population. A third reason for the deviation from HWE might be the timing of collection of the samples from Bunderbos, i.e. after the population collapse. Therefore, our sampling cannot be considered random and the deviations from HWE might be the result of an unknown underlying process. Still, it seems that there is sufficient genetic variability left among the surviving individuals, which should suffice in maintaining an *ex situ* breeding program for a possible re-introduction program in the future.



Extended Data Figure 4.1. Selection of the most likely number of K in the microsatellite data from the Bunderbos, Broek and Kottenforst fire salamanders. Plots illustrate the selection of the most likely number of clusters by the two most common two methods: The Ln P(K) method (**A**) and the delta K method (**B**). The first method identifies the most likely K when the likelihood of the data at a specific K-value is maximal or, if there is no clear maximum, when the likelihood reaches a plateau. The second method identifies the most likely K when the second order rate of change in the likelihood of the data between successive values for K is largest. In both cases, the most likely K equal 2.



Extended Data Figure 4.2. Selection of the most likely number of K in the microsatellite data from the Bunderbos and Broek fire salamander subpopulations. Plots illustrate the selection of the most likely number of clusters by the two most common two methods: The Ln P(K) method (**A**) and the delta K method (**B**). The first method identifies the most likely K when the likelihood of the data at a specific K-value is maximal or, if there is no clear maximum, when the likelihood reaches a plateau. The second method identifies the most likely K when the second order rate of change in the likelihood of the data between successive values for K is largest. Using the Ln P(K) method, the most likely K is 4, while the delta K method identifies K=3 as the most likely K.

JAGS code for the Jolly-Seber model for estimation of *S. salamandra* population size.

Model adapted from Ch. 10 in Kery & Schaub (2011)

```
MODEL{
#
===================================================================
===
# LIKELIHOOD
for(i in 1:M){
# The latent state must be 1 in the first occasion
z[i,1] <- 1for(t in 2:n.surv){
# State
z[i,t] ~ dcat(ps[z[i,t-1],t-1,])
# Observation
y[i,t] ~ dcat(po[z[i,t],t-1,])
}
}
#
===================================================================
===
# PRIORS AND CONSTRAINTS
# Phi: survival rate (constant)
for(t in 1:(n.surv-1)){
phi[t] <- pow(mean.phi,days[t]/7) # Weekly survival
# Gamma: entry probability - varies seasonally
gamma[t] <- pow(logit_gamma[t],days[t]/7)
#  Weekly  entry  (to  reflect  variable  length  of  intervals  between
surveys)
logit(logit_gamma[t]) <- a.g+b.g*cos(date[t]*2*3.1416/365-offset.g)
# P: detection probability - varies seasonally
logit(p[t]) <- a.p+b.p*cos(date[t]*2*3.1416/365-offset.p)
}
# Transition matrices
# Define probabilities of state S(t+1) given S(t)
for(t in 1:(n.surv-1)){
ps[1,t,1] <- 1-gamma[t]
ps[1,t,2] <- gamma[t]
ps[1,t,3] <- 0
ps[2,t,1] <- 0
ps[2,t,2] <- phi[t]
ps[2,t,3] <- 1-phi[t]
ps[3,t,1] <- 0
ps[3,t,2] <- 0
ps[3,t,3] <- 1
# Define probabilities of O(t) given S(t)
po[1,t,1] <- 0
po[1,t,2] <- 1
po[2,t,1] <- p[t]
po[2,t,2] <- 1-p[t]
po[3,t,1] <- 0
po[3,t,2] <- 1
}
# Priors
```

```
mean.phi ~ dunif(0.5,1)
mean.gamma ~ dunif(0,0.2)
a.p ~ dnorm(0,0.001)
b.p ~ dnorm(0,0.001)
offset.p ~ dunif(0, 6.283185)
a.g ~ dnorm(0,0.001)
b.g ~ dnorm(0,0.001)
offset.g ~ dunif(0, 6.283185)
#
====================================================================
===
# POPULATION SIZE
for(t in 1:(n.surv-1)){
qgamma[t] <- 1-gamma[t]
}
cprob[1] <- gamma[1]
for(t in 2:(n.surv-1)){
cprob[t] <- gamma[t]*prod(qgamma[1:(t-1)])
}
psi <- sum(cprob[])
for(t in 1:(n.surv-1)){
b[t] <- cprob[t]/psi
}
# Living individuals at time t-1
for(i in 1:M){
for(t in 2:n.surv){
al[i,t-1] <- equals(z[i,t],2)
}
for(t in 1:(n.surv-1)){
d[i,t] <- equals(z[i,t]-al[i,t],0)
}
alive[i] <- sum(al[i,])
}
for( t in 1:(n.surv-1)){
N[t] <- sum(al[,t])
B[t] <- sum(d[,t])
} # t
for(i in 1:M){
w[i] <- 1-equals(alive[i],0)
}
# Total size of each population
Nsup <- sum(w[])
}
```

4.7.4. References

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Chapter V

General Discussion

As mentioned in the beginning of Chapter I, we find ourselves in the midst of the sixth mass extinction event (e.g. Barnosky et al. 2011; Ceballos et al., 2015; Hallmann et al. 2017; Wake and Vredenburg, 2008). This mass extinction event has multiple anthropogenic causes, such as the focal pathogenic organism of this thesis and its non-deliberate introduction into wild-living salamander populations through trade (Martel et al. 2014). In the discussion below, four aspects on *B. salamandrivorans* are discussed in light of the findings from the previous two chapters as well as some future perspectives which I find merit further attention.

5.1. CAN *B. SALAMANDRIVORANS* AND FIRE SALAMANDERS CO-OCCUR

As shown in study I, host population dynamics are altered dramatically when *B. salamandrivorans* enters a fire salamander (*Salamandra salamandra*) population. Here I discuss whether it might be possible for the host and the pathogen to co-occur, i.e. both occurring at a specific site (in contrast to co-exist). First, the relatively low ten-days survival probability of uninfected adults (Study 1, *phiA*= 0.84, compared to monthly survival probability in uninfected populations *phi*= 0.98-0.99 (Schmidt et al. 2014)), is likely due to a combination of the general low recapture rates of fire salamanders (study I, *pA*= 0.11 compared to recapture rate in uninfected population *p*= 0.04-0.08, depending on the season (Schmidt et al., 2014)), and the difficulty of detecting the disease in the early stages of infection (Thomas et al. 2018). The relatively low survival rate of uninfected individuals can be explained by the fact that upon sampling, individuals might have already been infected but tested negative and given

that 10 days would suffice to succumb to the disease (Martel et al. 2013), they might have deceased prior to the subsequent sampling rendering recapture of (assumed) healthy individuals unlikely. This artificially lowers the recapture probability of healthy individuals, although it is not possible to observe this in data. Second, the decrease in survival probability of infected individuals (Study I, *phiB*=0.13) is to be expected given the fast and lethal disease progression (Martel et al. 2013). However, the six-fold reduction in survival probability is much greater compared to estimated survival probability in several *B. dendrobatidis* infected populations (Table 5.1), suggesting that fire salamanders and *B. salamandrivorans* co-existence is excluded. One important work in this respect is the work done by Spitzen-van der Sluijs et al. (2017) who show that there is a stable co-existence between *B. dendrobatidis* and two yellow-bellied toad populations (*Bombina variegata*) in the Netherlands but that this co-existence is driven by the current environmental conditions highlighting the fragility of this relationship. These researchers show that there is a link between temperature and infection incidence, where infection incidence increases with increasing temperature. If therefore the average temperature would increase, the number of individuals that are infected would also increase shifting the stable co-existence in favour of the pathogen leading to consistent population declines.

Table 5.1 Estimated survival probabilities of some *B. dendrobatidis* infected populations

	Survival probability
Pilliod et al (2010)	Reduced in infected individuals by 0.31-0.42
Mutsch et al. (2011)	Uninfected: 0.74-0.78 compared to infected: 0.51-0.54
Sapsford et al. (2015)	No effect on survival probability, although populations have collapsed due to *B. dendrobatidis*
Scheele et al. (2015)	Infected individuals: 0.04

Regardless of disease presence or absence, a decrease in the number of adults is obviously detrimental to the stability of the population. As shown by Schmidt et al. (2005), population trends are determined more by the stability in number of adult individuals, compared to a constant recruitment. Populations infected with *B. salamandrivorans* first of all collapse within a very short time span, as shown in Study I, due to the rapid disease progression and high mortality rate in infected individuals. In addition, the demographic properties of a fire salamander population also preclude future population recovery. In *B. dendrobatidis* infected populations, it has been shown that the persistence of populations can be ensured by recruitment (Scheele et al., 2015): although larval anurans can be infected with *B. dendrobatidis*, they rarely die from infection and can therefore make it through metamorphosis and the terrestrial juvenile/sub-adult stage. Once sexually mature, these individuals return to the infected breeding sites where they mate and get infected with *B. dendrobatidis*. However, they are able to produce offspring before succumbing to the disease (Scheele et al. 2015). In fire salamanders, females store the male sperm and after the eggs are

fertilized, gestation requires multiple months (e.g. Klewen 1985). Therefore recruitment in fire salamanders is a function of adult female survival over the gestation period which is much longer than the time from infection with *B. salamandrivorans* to death. Hence, the infection of adult females during mating, will lead to death of the individual long before larvae are deposited. Additionally, uninfected females could deposit larvae in *B. salamandrivorans* contaminated water. In this scenario, recruitment is theoretically possible and larvae will develop in this water since they are not susceptible to infection. This however cannot be considered as a trait that will ensure population persistence because although we know that fire salamander larvae are not susceptible to *B. salamandrivorans* infection (Van Rooij et al. 2015), we don`t know at what moment in the continuous process of metamorphosis from larvae to juvenile, fire salamanders become susceptible to infection. Therefore, they might still carry the fungus until they become susceptible after which they will soon die as a result of the disease. In this case, the recruitment is compromised at the moment these individuals become susceptible to the disease.

Although recruitment is hampered by the disease, the existing sub-adult age class present prior to the disease outbreak event might still ensure population persistence. As discussed briefly in the introduction of this thesis, juveniles are not restricted to specific sites or do not show homing or territorial behaviour in contrast to adults individuals. Sub-adults more often reside outside the core area of the fire salamander population (usually made up of the most suitable fire salamander habitat),

therefore they have less chance to come into contact with infected adult individuals or they reside in suboptimal environment where the fungus is possibly still absent. From these areas, either new populations can be formed in disease free refugia, or these individuals could spread to the initial population area where either the fungus might have disappeared or is still present. The latter could then lead to a re-emergence of the disease. Forming a population in a disease free-refugium is possibly what happened in the disease free fire salamander subpopulation (Broek) of study II.

To conclude, given the data we have now, a continued host-pathogen co-occurrence appears unlikely. However, given that fire salamanders are still present in the index outbreak site (as mentioned in study II) and that *B. salamandrivorans* is able to remain present in the environment (as shown in study I), host-pathogen co-occurrence might be possible for some amount of time. After the initial outbreak has decimated the host population, the outbreak-event will fade out. At this stage it is a matter of stochasticity whether the population is able to recover (e.g. the spatial distribution of remaining individuals, their sex or their age). If there are some individuals left that are able to reproduce, it is likely a matter of time before the population density is sufficiently high. Then, followed by the environmental infection of one individual, the population might collapse again. Lastly, it has only been five years since the description of the pathogen and therefore, hypotheses on population trends in post-epidemic events remain just that: hypotheses. Trends are by default

long-term studies (Blaustein et al. 1994) which is what we need to determine the true possibility of host-pathogen co-occurrence or co-existence.

5.2. HOW DOES *B. SALAMANDRIVORANS* SPREAD?

Since Belgium is one of the few countries were *B. salamandrivorans* occurs in the wild as an emerging fungal pathogen, the prevention of fungal spread into nearby uninfected areas or neighbouring countries should be of paramount importance. When investigating possibilities for mitigation and risk assessment of diseases, knowledge on the spread of the disease and possibilities for outside host survival are indispensable. In study I, outside host survival has been discussed while study II showed that fungal spread did not occur over a distance of less than 1 km in a period close to ten years (the first signs of the outbreak where observed in 2008 (Spitzen et al. 2013)). This shows that although *B. salamandrivorans* has the potential to survive outside its host (e.g. vectors and the environment), it appears to be unable to cross a relatively small distance. One of the reasons that could explain this observation, is the intrinsic property of the pathogen to be so virulent that it wipes out its host at such a high pace, that the host population dynamics are altered profoundly, leading to the inability of the fungus to spread over any distances. We now know that the fungus is able to survive outside its host in soil and in water. Vectors which have been shown to play a role in the spread of *B. dendrobatidis*, likely also fulfil this role in spreading *B. salamandrivorans* (study I). However, we currently lack more detailed spatial data on the distribution and spread of the pathogen. Spitzen-van der Sluijs et al. (2016)

provided data for the known outbreak sites up to 2016 which clearly showed a disjunct distribution of the fungus. The patchy distribution of the fungus is confirmed by the absence of new outbreak sites during a one year screening of fire salamander populations in Wallonia (Fig. 5.1).

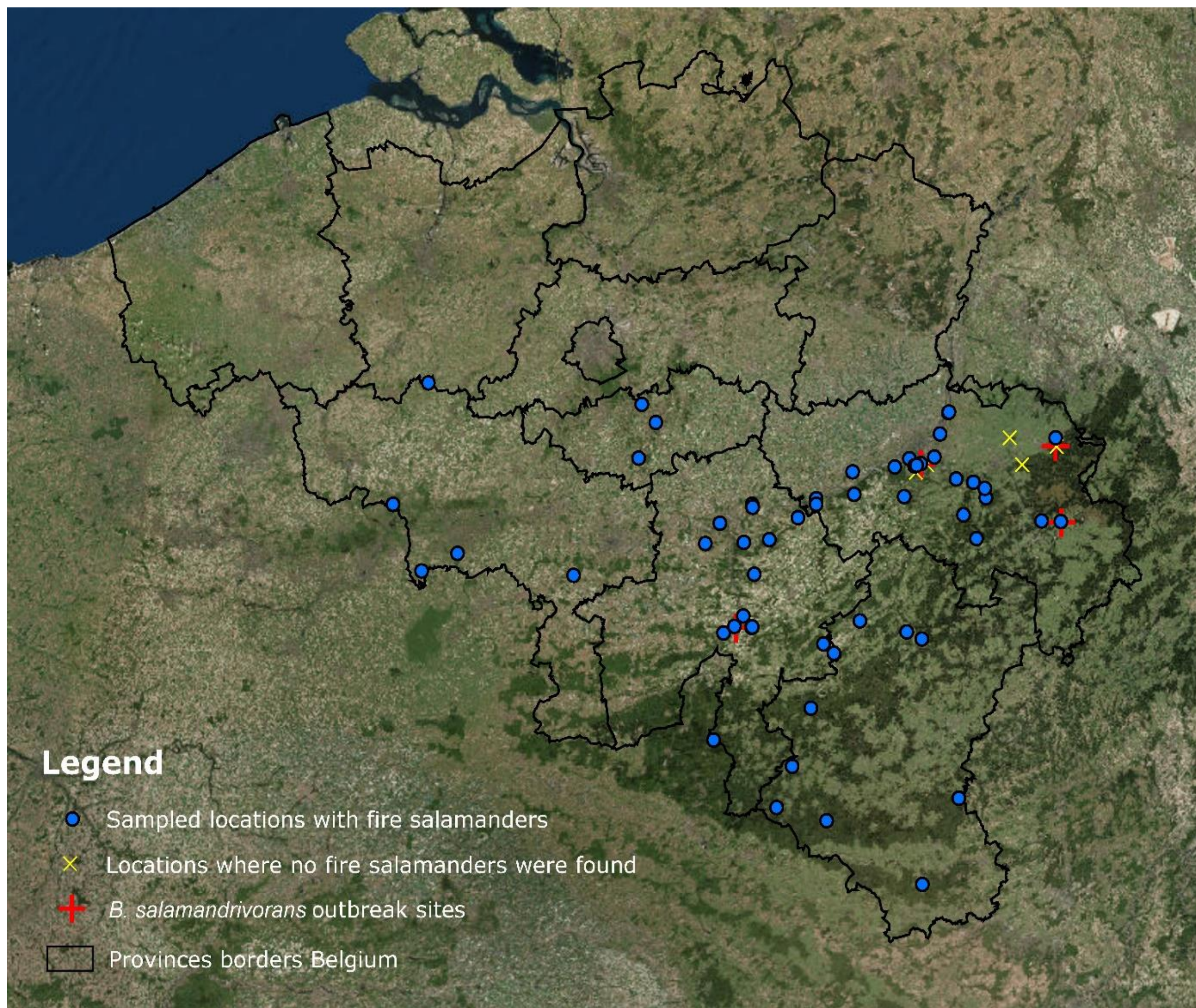

Figure 5.1. Geographical localization of the sampled fire salamander (*Salamandra salamandra terrestris*) populations for the presence of *B. salamandrivorans* in Wallonia over the course of one year. The known outbreak sites (red plus-signs) have been re-visited, but no population recovery was apparent. In five areas, no individuals were found (yellow crosses) although the habitat was suitable and historical observations have been made at these sites. In the other 56 sites (blue circles), salamanders were found (between 2 and 39 individuals per location) (Stegen et al. unpublished data).

During this screening, fire salamanders were found at 56 sites located throughout Wallonia without the identification of new outbreak sites. This confirms that the fungus indeed has a disjunct distribution. Based on the findings of study II and the findings from the screening of fire salamander populations, it seems likely that host-mediated (i.e. fire salamanders) fungal spread is close to absent. Otherwise the distribution of the fungus would be more continuous across and in between the outbreak sites. Therefore it would be safe to assume that external factors drive the spread of the fungus. These are likely biotic and/or abiotic vectors that aid the fungus in reaching new areas. Evidence for this is provided in study I were the production of encysted spores was shown. These encysted spores are capable of surviving much longer in environmental water compared to for instance motile spores in *B. dendrobatidis* (less than 5% still active after 24 hours, Piotrowski et al. 2004) and are also predated less than motile zoospores. Similar to motile spores, they can attach themselves to and survive on the scales of goose feet (Garmyn et al. 2011).

The results from the screening for new *B. salamandrivorans* outbreak sites, other than the fact that we did not find new outbreak sites, suggests that *B. salamandrivorans* is a very slow spreader, much slower than the previous estimates of 11 km/year (Schmidt et al. 2017). This estimate was comparable to the estimate for *B. dendrobatidis* (25-252 km/year, Lips et al. 2008). However, as the authors suggest, increased fungal detection might be due to increased sampling efforts during the years following *B. salamandrivorans* discovery rather than actual fungal spread. Given the contradiction

between the estimates of fungal spread and the empirical data from the wild, this is likely to be true since the last known outbreak in Wallonia dates back to spring 2016, approximately two years ago.

5.3. CONSEQUENCE OF A *B. SALAMANDRIVORANS* INTRODUCTION INTO AN AMPHIBIAN COMMUNITY WITH AN ENDEMIC *B. DENDROBATIDIS* INFECTION

In the disease ecology of the fungal genus *Batrachochytrium*, there is one unexplored aspect: their interaction. This information is especially important when estimating diseases risks to specific species. The *B. dendrobatidis* strain(s?) present in Belgium, do not have a significant effect on host population dynamics (Spitzen-van der Sluijs 2010 & 2017). Although it is debatable whether chytridiomycosis caused by *B. salamandrivorans* and *B. dendrobatidis* actually is the same disease, this is not the point of this discussion and I will use the term mixed infection (as defined in the glossary, in contrast to co-infection) to describe the simultaneous presence of the two fungal species. In the genus *Batrachochytrium*, only two species are known. In Belgium, one is endemic (*B. dendrobatidis*) while the other is emerging (*B. salamandrivorans*), as described in chapter I. Estimating the threat that the emergent fungus poses, includes determining what happens when these two fungal species are present simultaneously and whether this increases host susceptibility to the emerging fungus. Previously, studies have determined the effect of combined exposure of *B. dendrobatidis* and pesticides, herbicides or probiotics and have shown that sub-lethal exposure of *B. dendrobatidis* infected larvae to pesticides increased survival through metamorphosis (Hanlon and Parris 2013), while others did not find a correlation between prior

exposure to pesticide or herbicides and susceptibility to *B. dendrobatidis* (Peatow et al. 2012; Jones et al. 2017). Interactions with bacteria isolated from the amphibian skin microbiome have been shown to inhibit *B. dendrobatidis* and have served as a basis for probiotic treatments (Harris and James 2006).

Considering *B. dendrobatidis* is widely distributed while *B. salamandrivorans* is more localized, the latter is likely to spread to areas where *B. dendrobatidis* is present at an endemic level. When this occurs, three outcomes are considered plausible: a neutral effect (no/additional effect), antagonistic effect (decreased effect) and synergistic effect (increased effect).

- The first outcome is the result of both fungal species not interacting with each other. The effect is simply the sum of the two. What this means in terms of infection development is that species are either susceptible to infection or not and whether individuals develop an infection remains a stochastic process unaffected by the presence of the other fungal species. The same is then true for the disease dynamics.
- An antagonistic interaction is an interaction where the effects are less than the sum of the individual parts. This can be compared to competitive exclusion where the presence of one species precludes successful colonization of a second species. Given that *B. dendrobatidis* is present at an endemic level in the local amphibian communities, such an effect would be desirable.

- When a synergistic interaction would occur, the impact of the combined presence of two pathogens is more than that of the individual parts. This means that the presence of one fungal species increases the probability of developing an infection with the other fungal species or that either or both of the pathogens show an increase in virulence.

In a first attempt to estimate the risk that further spread of *B. salamandrivorans* poses, experimental infections were done to determine whether prior exposure to *B. dendrobatidis* increases the susceptibility to *B. salamandrivorans*. We exposed Alpine newts (*Ichthyosaura alpestris*) to either *B. salamandrivorans* alone or to *B. dendrobatidis* first, followed by exposure to *B. salamandrivorans*. Results showed that previous exposure to an endemic *B. dendrobatidis* isolate does not increase susceptibility to *B. salamandrivorans* (Fig. 5.2). Multiple exposures occurred to ensure successful infection of the individuals but only individuals that were not infected were re-exposed (white vertical dashed or dotted lines, Fig. 5.2). In both groups, 50% of individuals developed an infection with *B. salamandrivorans*. A penalized maximum likelihood generalized linear mixed effects model was used to determine whether any of the explanatory variables (fixed variables: group and exposures; random variable: individual) had a significant impact on the binary response variable (i.e. infection or no infection). No variables (i.e. group, number of exposures or individual) had a significant effect on determining the infection outcome. As a result, preliminary data suggests that it is

unlikely that previous exposure to *B. dendrobatidis* increases susceptibility to *B. salamandrivorans*.

Following from this preliminary result, it seems that there is no increase in susceptibility when *B. salamandrivorans* enters a *B. dendrobatidis* infected population (i.e. a neutral effect). This implies there are no extra mitigation measures or precautions that need to be taken on a population level to protect *B. dendrobatidis* infected populations. To conclude, it is worth noting that the preliminary conclusion might not apply to the individual case when the individual has developed chytridiomycosis following a *B. dendrobatidis* infection and subsequently exposed to *B. salamandrivorans*: as mentioned before, previous studies have shown that there are no detrimental effects of *B. dendrobatidis* on a population level, while individuals can develop the disease (Pasmans et al. 2010; Spitzen- van der Sluijs et al. 2017). In the experiment described above, individuals were exposed to *B. dendrobatidis* but none of these individuals became diseased. From an individual level, it would be interesting to see how *B. dendrobatidis* infected or diseased individuals respond to *B. salamandrivorans* exposure.

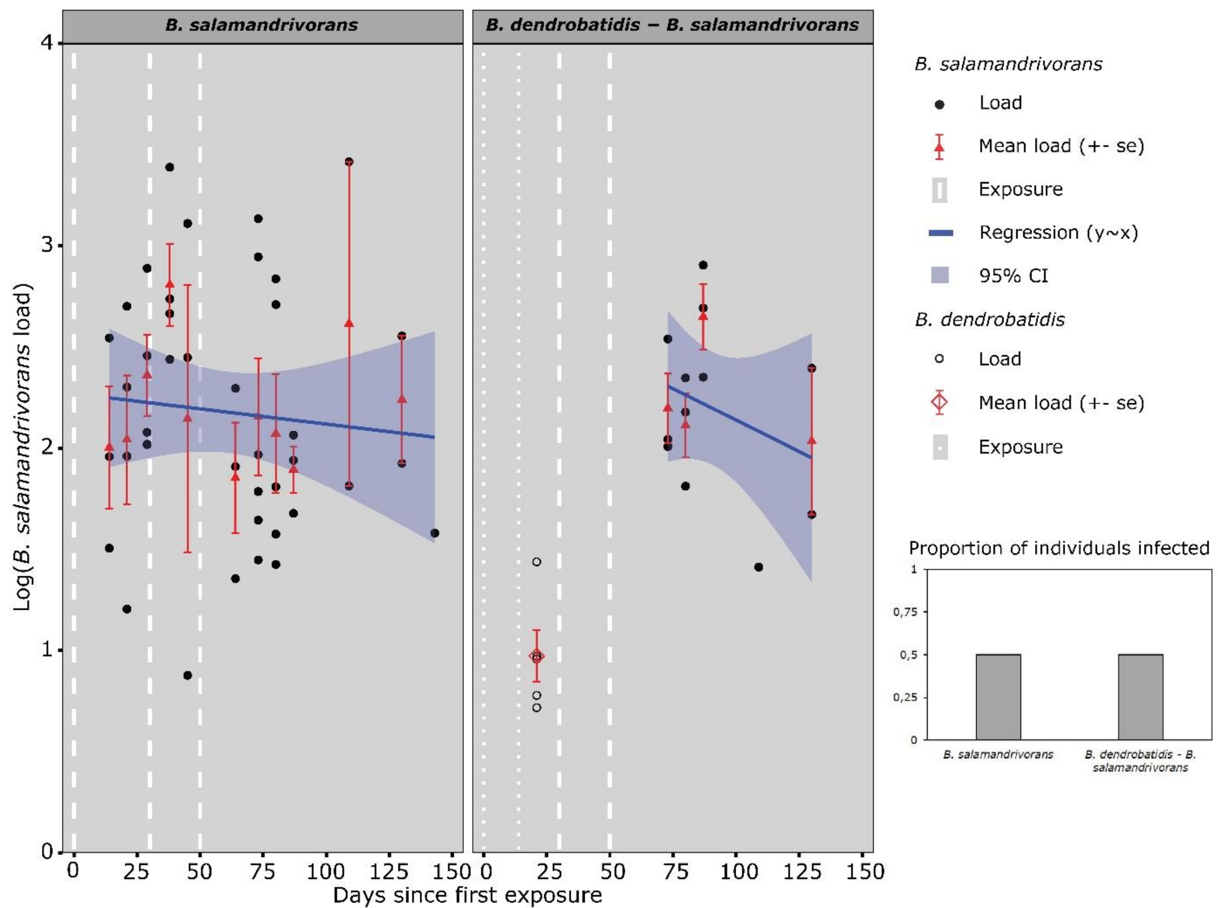

Figure 5.2. Previous exposure to *B. dendrobatidis* does not increase susceptibility to *B. salamandrivor*ans. Two groups are compared for the susceptibility to *B. salamandrivorans*. The first group (*B. salamandrivorans*) was exposed only to *B. salamandrivorans*. The second group was first exposed to B. dendrobatidis and then to *B. salamandrivorans*. Comparison between the two groups was done by determining the probability of developing an infection (binary response, i.e. infected or not infected) as a function of the group and the number of exposures). Multiple exposures occurred to increase the number of positive individuals (white lines; dashed: *B. salamandrivorans* exposure, dotted: *B. dendrobatidis*).

5.4. Mitigating the fungal threat

One of the main lines of research involving *B. salamandrivorans,* is looking for successful mitigation. Results form study I (e.g. fungal persistence outside the host and multiple vectoring species) show that mitigating chytridiomycosis is a difficult task, which has been discussed more in detail by Grant et al. (2017) and Canessa et al. (2018). In the case of *B. salamandrivorans*, based on the available information, disease

mitigation comes down to preventing disease spread and/or removing the disease from the environment. Below, I approach the mitigation issue from two sides: mitigating disease in an infected population and mitigating disease spread.

Disease mitigation *in situ*, is likely to be unsuccessful. For *B. dendrobatidis*, one study has shown to be successful in removing the disease from the environment (Bosch et al. 2015). Here, a very potent and aspecific chemical disinfectant, Virkon S, was administered to the environment of an endangered toad (*Alytes muletensis*). This lead to the successful elimination of the fungus, but can hardly be called a sustainable way of acting against a disease. The authors themselves argue to have opted for this treatment given the imminent threat of extirpation and necessity to act against this process. Although this might be an interesting experiment in a complex system such as a forest, such actions would greatly impact the forest's ecosystem, where a multitude of species depend on clean and potable water and where a disruption of the delicate balance of the aquatic and terrestrial system may lead to a much greater loss in biodiversity than the pathogen itself. As shown in study I, the currently available options for disease mitigation were deemed inadequate (e.g. a lack of immunity build up precludes vaccination) and it has been shown recently that probiotics or antifungal treatment would need to interrupt disease transmission with more than 90% to be successful (Canessa et al., 2018). Therefore, currently the only option to safeguard a vulnerable population from possible extirpation is *ex situ* conservation. As Canessa et al. (2018) highlight, the challenge lies in the interruption of the disease transmission

and artificially lowering the reproductive rate so that is decreases from an estimated 9.6 to less than 1. When R_0 would be smaller than one, the epidemic is stopped and the infection will die out. However, in order to reach this critical value ($R_0<1$), the *in situ* mitigation measures at hand (e.g. probiotic treatment, antifungal treatment, culling (or removal of individuals)) would have to be so unrealistically effective (high success rate of antifungal treatment and near complete removal of individuals) that it is unachievable from a practical point of view. This is also not considering outside host survival and given the results from study I (environmental survival, presence of vectors, thermal effects on disease dynamics), we are far from having an applicable method for *in situ* mitigation.

The results from study II, where a population of fire salamanders was able to persist uninfected in the close vicinity of the index outbreak site for close to ten years now, are very interesting findings in light of the second mitigation goal: stopping the disease from spreading. This suggests that a fragmented landscape, in terms of migration and connectivity, is detrimental to fungal spread. It has been suggested that infected individuals do not move very far (Schmidt et al. 2017; Canessa et al. 2018), so their dispersal will be very low. An often applied conservation strategy consists of (re)connecting isolated or fragmented areas, which should be given priority over the increased chance of pathogen introduction according to epidemiological models (McCallum and Dobson 2002). The currently available data suggest that *B. salamandrivorans* is a poor natural spreader and thus determining the effect of

decreased landscape permeability might be a part of the puzzle when considering *B. salamandrivorans* mitigation measures. This however should be seen as a quarantine measure that isolates the infected patch from nearby uninfected patches rather than fragmenting the entire landscape and isolating numerous populations in order to prevent pathogen spread. While artificially increasing landscape resistance for migrating individuals is rather controversial, it could fit in a broader approach when trying to stopping or at least slowing the disease from spreading to nearby areas by preventing individuals from an infected population to migrate towards nearby areas. Unsuitable areas, such as unforested areas, negatively affect salamander migration (Todd et al. 2009). Therefore, considering that infected fire salamanders are unlikely to move very far, especially over unsuitable habitat, also infected vectoring species such as alpine newts or midwife toads should be contained within the infected area by decreasing their likelihood or potential to migrate. This is a controversial proposal more detailed information is required on vectoring and maintenance hosts as well as the long term effects of isolating amphibian communities. When the forests are far apart, successful migration of amphibians, or other vectors, between these forests is unlikely, or at least decreases, hereby reducing the risk of fungal spread by migrating individuals. In case *B. salamandrivorans* would be introduced into one of these areas, natural spread of the disease to other naive areas (by physical movement of the pathogen or by using vectoring species) would be unlikely.

Additional to increasing landscape resistance, which indirectly influences the movement of amphibians between different sites, amphibian movement can also be influenced directly. During seasonal migration events for instance, newts, frogs and toads are prevented from crossing roads by using fences alongside roads to prevent migrating amphibians to end up as road kill (e.g. in 2017, more than 170.000 amphibians were caught using those fences in Flanders[3]), suggesting that fences might be successful in stopping amphibian migration (Aresco 2005). Additionally, increasing awareness of the fact that the general public might play a (big) role in spreading the disease and informing them how to prevent introducing *B. salamandrivorans* into new areas should also be done (e.g. keeping the public away from infected areas or inform them on successful hygienic measures that eliminate the disease from e.g. walking boots). Anthropogenic spread might explain why the current distribution of the fungus is so disjunct. If measurements are taken to keep the public out of infected areas or if uninfected areas are shielded of from the public, the chance of fungal spread are also likely to decrease.

As a final note for this general discussion, it should be noted that we currently lack specific knowledge which would help us determine successful mitigation measures. One of these is fungal spread. What has been discussed in the paragraphs above, should not be considered as the sole and effective possible measures but should

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[3] https://www.hylawerkgroep.be/po/Po_RptPerGemeente.php?jaar=2017 (visited on 01 March 2018)

serve as a starting point for future research projects that aim at filling our knowledge gaps.

5.5. Future perspectives

The information gathered within this thesis is a step forward in understanding why *B. salamandrivorans* has impacted fire salamander populations so dramatically. Detailed information has become available that may contribute to future disease mitigation or is a step towards more specific research on the topic of *B. salamandrivorans*. While there still is a lot to be discovered, below I describe some of the topics that would be interesting to investigate further.

First of all, for the moment, we don't know how the fungus spreads. Therefore, as a logic first step in investigating the diseases epidemiology further, the pathogen's spatial epidemiology merits attention. Spatial epidemiology, or landscape epidemiology, is a research field dedicated to the spatial structuring of the landscape and its influences on the disease occurrence (Ostfeld et al., 2005). This concept was formed based on three observations:

1) The spread of a disease is geographically delimited

2) Biotic and abiotic factors of the landscape explain the geographic delimitation of the disease

3) Knowing why the disease is delimited geographically, it is possible to determine the present and future risk of disease introduction or spread in currently disease free areas.

By determining the spatial epidemiology of the disease, a more accurate understanding of how it spreads (e.g. anthropogenic spread, environmental spread or spread by migrating (vector) individuals/species) should allow us to define more specific measures that stop the disease from spreading further.

Second, results from e.g. Spitzen-van der Sluijs et al. (2016) or Fig. 5.1 show that the fungus has a disjunct distribution. When isolation of the fungus occurs at each outbreak site, these different isolates can be used to perform a study on the genetic variation between the different isolates. Although this likely requires whole genome sequencing, results from such a study would provide us with information on fungal evolution. Additionally, the genetic variations can be time calibrated since each isolate can be linked with an outbreak event thus providing information on fungal evolution in general. Using the same whole genome sequences, a phylogenetic analysis would provide information on how the fungus has spread. This will allow us to determine in retrospect whether *B. salamandrivorans* has been introduced multiple times (if there would be no link between geographic distribution and phylogenetic relatedness) or whether the isolates from nearby outbreak sites cluster together phylogenetically (which would imply a single introduction, followed by dispersal from this outbreak sites).

Third, as shown in study I, temperature has been shown to play a significant role in the infection dynamics: when the environmental temperatures decreases to 4°C, the disease dynamics change and individuals remain infectious longer but also survive

longer. *In vitro*, the fungus does not do well at temperatures above 20°C. It has been shown that keeping infected individuals for ten days at either 25°C or at 20°C combined with an polymyxin E and voriconazole treatment cures the disease (Blooi et al. 2015a & b). This shows that salamanders can withstand these temperatures while the fungus cannot. In areas where *B. salamandrivorans* occurs at the moment, 20-25°C is unlikely to be reached during the night. However, following fungal spread to e.g. the Mediterranean region, such temperatures might be more common. Therefore, it would be interesting to see how the thermal biology of fire salamanders relates to the environment and *B. salamandrivorans* disease ecology at temperatures above the fungus' preferred temperature (i.e. > 20°C).

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Chapter VI

Summary

The focal organisms of this thesis are fire salamanders (*Salamandra salamandra*) and the fungal pathogen *Batrachochytrium salamandrivorans*. The fungus was described for the first time in 2013 when a population of fire salamanders was brought to the edge of extinction. In this thesis, the first steps were taken towards understanding the impact and consequences of *Batrachochytrium salamandrivorans* presence on fire salamander populations.

In **study I**, the mechanisms underpinning population and disease dynamics of an infected fire salamander (*Salamandra salamandra terrestris*) population are described. Results show that based on the capture-mark-recapture data, populations infected with *Batrachochytrium salamandrivorans* are extirpated within six months. Population recovery was shown to be unlikely due to the prolonged presence of the fungus in the wild and the lack of immunity build up by the host. Prolonged persistence of the fungus in soil and water was attributed to the production of encysted spores, which are released directly from sporangia and may constitute an environmental reservoir. In contrast to motile spores, which are also found in the fungus' sister species *B. dendrobatidis*, the encysted spores are hitherto unique to *B. salamandrivorans* and aggregate at the air-water interface in aquatic environments hereby escaping predation by micropredators. Adherence of these encysted spores to salamander skin and goose feet after brief contact showed that these spores do not require active movement towards a suitable substrate for attachment.

Comparison of the disease dynamics between fire salamanders and alpine newts (*Ichthyosaura alpestris*), showed that the disease is dose independent in fire salamanders while the course of the disease is dose dependent in alpine newts who can clear the disease when infected with a low dose of *B. salamandrivorans* and become fungal carriers and reservoirs at intermediate doses, yet succumb to infection at high doses. Disease dynamics were shown to be also affected by the environmental temperature, with prolongation of the disease process at lower temperatures.

Finally, midwife toads (*Alytes obstetricans*) were shown to act as an anuran reservoir, exhibiting low infection intensities which nevertheless allowed transmission of the disease to susceptible fire salamanders.

In **study II**, a healthy population of fire salamanders was discovered at less than 1 km from the *B. salamandrivorans* index outbreak site in the Netherlands (Bunderbos). This population is genetically similar to the population residing in the Bunderbos suggesting animal movement between the two sites. Since the two sites are environmentally connected and in close proximity and *B. salamandrivorans* has been causing an ongoing epidemic in the Bunderbos for ten years, the fungus is likely a poor natural disperser. This was corroborated by a lab trial where cohoused individuals only developed an infection if physical contact was possible. Persistence of a healthy host population at close proximity of a large outbreak and in the presence of incomplete barriers opens perspectives for future *in situ* mitigation of the effects of *B. salamandrivorans*.

Chapter VII

Samenvatting

In 2013 werd een uiterst pathogene schimmel beschreven die op verschillende locaties in Nederland en België heeft gezorgd voor het verdwijnen van vuursalamander populaties. De resultaten die in deze thesis zijn beschreven, dienen als basis voor het opstellen van maatregelen en richtlijnen waarmee getracht word om de ziekte te stoppen of op zijn minst de verspreiding ervan tegen te gaan/vertragen en zo ontelbare amfibie populaties te redden.

In **studie I** werden de karakteristieken beschreven die verklaren waarom de schimmel *Batrachochytrium salamandrivorans* zo'n verwoestend effect heeft op vuursalamanders (*Salamandra salamandra terrestris*). De resultaten tonen aan dat een geïnfecteerde populatie binnen enkele maanden wordt uitgeroeid en het onwaarschijnlijk is dat dergelijke populaties zich herstellen doordat de schimmel lang in de omgeving aanwezig blijft en de gastheer geen immuniteit opbouwt tegen de ziekte. Langdurige aanwezigheid van de schimmel in de omgeving werd experimenteel aangetoond door proeven met grond en water waaruit bleek dat de schimmel infectieus bleef in deze milieus. Ook werd een nieuw type spore beschreven die resistenter is tegen omgevingsfactoren dan motiele sporen. Deze geëncysteerde sporen zijn niet motiel en drijven op het water oppervlak waardoor ze onder andere ontsnappen aan predatie. Ook hechten ze zich makkelijk aan salamanderhuid of schubben van ganzepoten. Vervolgens vergeleken we de ziekte dynamieken tussen vuursalamanders en alpenwatersalamanders (*Ichthyosaura alpestris*) waaruit bleek dat vuursalamanders een dosis onafhankelijk ziekteverloop kennen.

Alpenwatersalamanders daarentegen kennen een dosis afhankelijk verloop waarbij infecties met lage dosissen, die hoogst waarschijnlijk meer relevant zijn voor infecties in het wild, door het individu kunnen worden verwijderd. De ziektedynamiek werd ook beïnvloed door de omgevingstemperatuur, het ziekteverloop langer duurde bij lage temperaturen in vergelijking met de optimale groei temperatuur van de schimmel. Ook werd er aangetoond dat vroedmeesterpadden (*Alytes obstetricans*) kunnen dienen als reservoir, waarbij de infectie zo goed als afwezig is bij padden, maar toch voldoende aanwezig is om vuursalamanders te infecteren.

In **studie II** werd een recent ontdekte vuursalamanderpopulatie beschreven op vlak van genetica, populatiedichtheid en aanwezigheid van de schimmel *B. salamandrivorans*. Uit de resultaten bleek dat de schimmel afwezig was en dat de vuursalamanders van de nieuwe populatie genetisch niet te onderscheiden is van de salamanders uit het Bunderbos. Het Bunderbos is de locatie waar de schimmel voor het eerst werd vastgesteld en ligt op minder dan 1 km van de recent ontdekte vuursalamanderpopulatie. Dat de salamanders van de nieuwe locatie genetisch niet te onderscheiden zijn van salamanders uit het Bunderbos, wijst op huidige of historische migratie van individuen. Dit doet vermoeden dat de schimmel een slechte verspreider is aangezien het bijna 10 jaar geleden is sinds de eerste tekenen van populatie dalingen werden waargenomen in het Bunderbos. De slechte verspreidingseigenschap van de schimmel werd bevestigd door een experiment dat aantoonde dat salamanders die samen gehuisvest werden, niet geïnfecteerd werden als ze fysisch gescheiden werden.

Chapter VIII

Curriculum Vitae

Gwij Stegen werd geboren op 07 mei 1987 te Ukkel. Na het succesvol afronden van de secundaire graad, richting Moderne Talen-Wetenschappen, in het Sint-Niklaas Instituut te Anderlecht in 2006, begon hij met de studie Biologie aan de Vrije Universiteit Brussel (VUB). In 2010 behaalde hij het diploma "Bachelor in Biologie, richting Ecologie" en in 2013 gevolgd door het diploma "Master of Science in Biology: Environment: Biodiversity and Ecosystems: Profile Herpetology" met als thesis onderwerp: "Identification and characterization of courtship pheromones in newts (Salamandridae: *Lissotriton*)".

In November 2013 begon hij aan de doctoraatsopleiding bij de vakgroep Pathologie, Bacteriologie en Pluimveeziekten aan de faculteit Diergeneeskunde van de Universiteit Gent. Binnen de onderzoeksgroep "Wildlife Health Ghent" geleid door Prof. Martel en Prof. Pasmans, heeft hij onderzoek gedaan naar de impact van een pathogene schimmel, *Batrachochytrium salamandrivorans*, en het effect ervan op wild-levende amfibie-populaties en meer specifiek vuursalamanders. Dit onderzoek werd gefinancierd door het Bijzonder Onderzoeksfonds (BOF) van de Universiteit Gent.

Gwij nam deel aan verschillende internationale congressen en zijn wetenschappelijk onderzoek leidde tot verschillende wetenschappelijke publicaties in internationale tijdschriften.

Chapter IX

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9.1. Scientific publications

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9.2. ORAL PRESENTATIONS

Conference: 8th World Congress for Herpetology, Hangzhou, China. August 2016
Title: "The highly infectious, lethal and persistent fungal pathogen *Batrachochytrium salamandrivorans*: recipe for rapid disease driven extinction."

Conference: 19th European Congress of Herpetology, Salzburg, Austria, September 2017
Title: "Conservation by fragmentation: a case study using a recently discovered fire salamander (*Salamandra salamandra*) population near the index outbreak site, free of *B. salamandrivorans*."

Conference: EAZA: Amphibian Tag Meeting, Emmen, Nederland. September 2017
Title: "*Batrachochytrium salamandrivorans*: emerging threat to European urodelan diversity"

Chapter X

Dankwoord

Boy, time sure goes fast. After four years, this is the last chapter of my PhD (in a literal and figurative sense). I remember the first day I arrived at the faculty as if it was yesterday. Although I hope this is not the end, there are a number of people that have played a crucial role during these years. As you will see, I have not listed names explicitly because I fear I might forget some. But since you are reading this, consider your name to be here.

Eerst en vooral zou ik Prof. Martel en Prof. Pasmans willen bedanken. Het is als gisteren dat ik een mailtje kreeg met de boodschap dat ik een PhD had "gewonnen". Sinds dan kreeg ik de vrijheid om de chytridiomycose hyperspace te ontdekken. Het is vanzelfsprekend dat er zonder jullie geen *Wildlife Health Ghent* zou zijn, maar jullie werkethiek is er eentje voor in de boekskes. Jullie deur staat altijd open en jullie zijn bijna altijd bereikbaar en dat wordt ten zeerste geapprecieerd en bijna ongeloofelijk. Tijdens de laatste 4 jaren (ongeveer toch) ben ik door onze samenwerking niet alleen gegroeid als onderzoeker, maar ook als mens. Heel erg bedankt daarvoor.

Professor Haesebrouck, als vakgroep voorzitter en hoofd van de *Helicobacter* groep hebben we weinig officiële contacten gehad, maar de officieuze contacten verliepen altijd met een lach. Bedankt dat ik mocht starten aan uw departement en dat u deze zo feilloos lijkt te leiden. U was steeds paraat voor een aangename babbel bij bv. de fietsenstalling na de tocht Brussel/Melle/Zottegem-Merelbeke of tijdens de vakgroep uitstappen.

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Next, I would like to thank all colleagues present and past. Maybe to begin with Pascale, Mark and Connie, I remember well that when I started, the chytrid group was relatively small compared to what it is now. But thanks to your knowledge and tips and tricks on how to do all things chytrid related, everything turned out well in the end. Then, everyone from the present "Wildlife Health Ghent". It has been a joy getting to know all of you. I find it very enriching to work together with people who have such a diverse background. Don't forget to let me know when it is your time to defend because I'll be cheering you on. But who knows, I might still be around in which case I'll see you soon. Lieze and I have promised a bike tour/walk in de Vlaamse adrenne and a bbq so start preparing your legs because it is coming. Next, I would like to thank everyone from the department of pathology, bacteriology and poultry diseases (although I must say that I have lost track of who is who since the stay at the VRB). Small chats during labwork, lunchbreaks, … were always accompanied by laughter and that is what made me enjoy what I was doing. So thank you for making the work environment a pleasant place to be.

I would also like to thank everyone who has played a part in the scientific output of this thesis as a co-author but four people in particular: Annemarieke (and

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