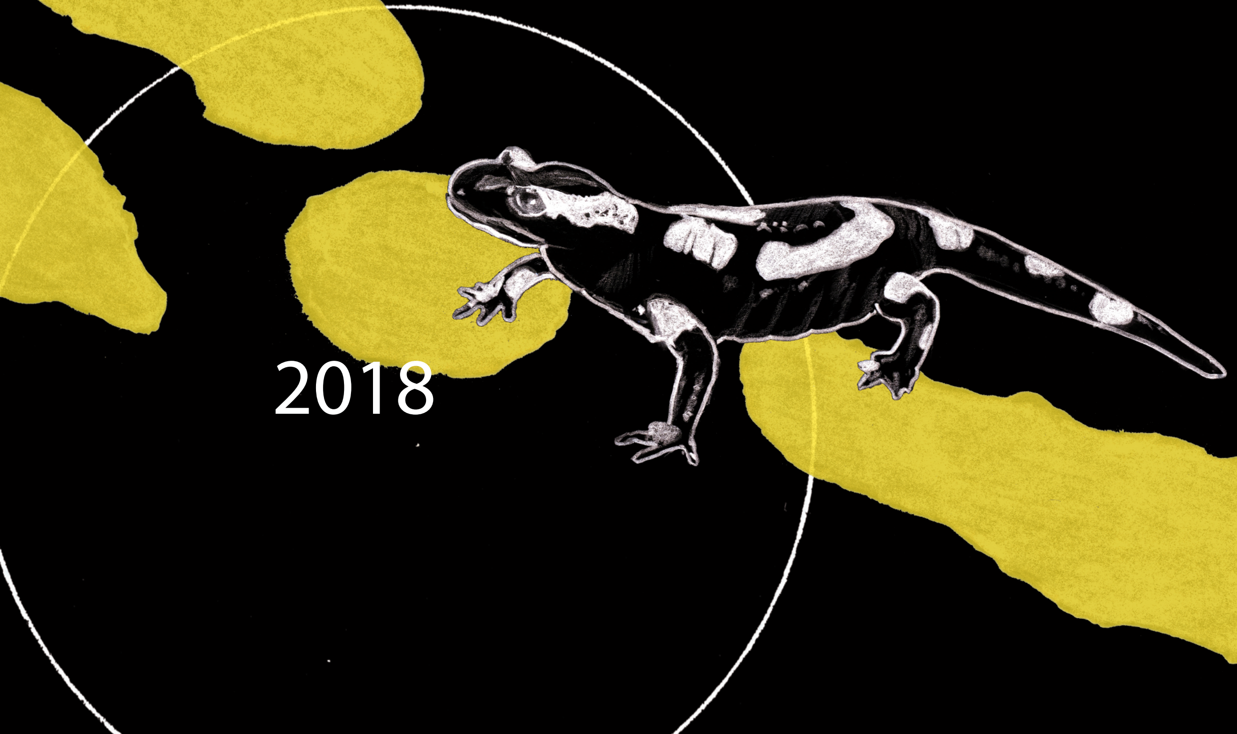

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It Takes Three To Tango

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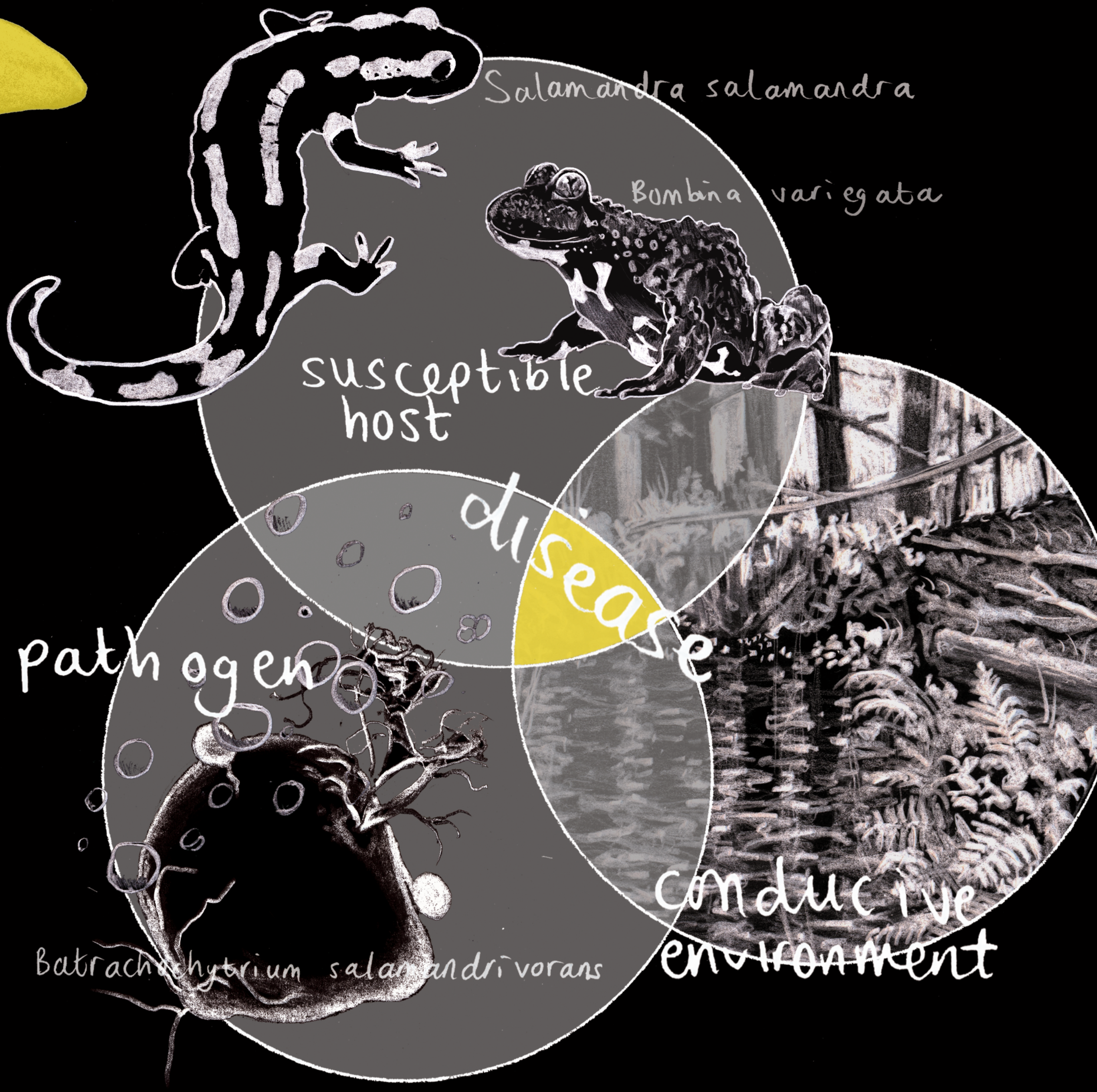

It takes three to tango

The impact of chytridiomycosis on native amphibians in the Netherlands

Annemarieke Spitzen-van der Sluijs

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Glossary

AIS	Alien Invasive Species
ART	Assisted Reproductive Technologies. Technologies like IVF (*in vitro* fertilization) and hormone therapy used to enhance the reproductive outcome of amphibian populations kept in captivity (ex-situ).
Bd	*Batrachochytrium dendrobatidis*
BdASIA-1	Bd lineage. Bd ancestor from East Asia. Genetic features of ancestral population that seeded the panzootic (O'Hanlon et al. 2018).
BdASIA-2/BdBRAZIL	Bd lineage. Second Asian-associated lineage (BdASIA-2), closely related to enzootic lineage from Brazil (BdBRAZIL). Not possible to infer the direction of intercontinental spread between isolates within this lineage, so it was named BdASIA-2/BdBRAZIL (O'Hanlon et al. 2018).
BdCAPE	Bd lineage, first isolated from sites in the South African Cape (Farrer et al. 2011).
BdGPL	Global Panzootic Lineage. Highly virulent Bd lineage, spread early 20th century (O'Hanlon et al. 2018).
Bsal	*Batrachochytrium salamandrivorans*
Chemotaxis	Directed movement of flagellated pathogens towards the host.
Chytridiomycosis	Amphibian disease caused by the fungus *Batrachochytrium*. Animals that test positive for the presence of Bd/Bsal may show no symptoms of the disease.
CMTV	Common Midwife Toad Virus. A ranavirus originally isolated from common midwife toad tadpoles (*Alytes obstetricans*) from the Picos de Europa National Park in Northern Spain in September 2007 (Balseiro et al. 2009). Also occurring in the Netherlands.
e-DNA	environmental DNA. e-DNA refers to DNA that can be extracted from environmental samples (such as soil, water or air), without first isolating any target organisms (Taberlet et al. 2012).
EID	Emerging Infectious Disease; Infectious diseases that have increased recently or are threatening to increase in the near future.
Endemic	Infection is maintained at low or non-detrimental levels
EPH	Endemic Pathogen Hypothesis. Posits that Bd co-existed with its host in equilibrium but changing conditions, such as environmental change, trigger disease emergence.
Enzootic	Host–pathogen dynamics that support coexistence over time, mortality may occur, but prevalence is stable.
Epizootic	Describes pathogens that are increasing in frequency (outbreak of infectious disease in animals), that is, have not reached a stable equilibrium.
EWS	Early Warning System
Exotic species	Introduced non-native species that occurs in an area where it did not evolve, but causes no harm.
Ex-situ	Off-site. Ex-situ conservation refers to the management of a captive population indoors or outside the natural habitat.

In-situ	On-site. In-situ conservation is the conservation of species diversity within normal and natural habitats and ecosystems.
Invasive species	Non-native species that causes major ecological, health or economic problems.
Landscape resistance	Degree of permeability of the landscape. Resistance of environmental variables impacting amphibian dispersal.
Lethal	The host becomes infected, infection results in lethal disease, no recovery from disease.
Matrix permeability	The degree of landscape resistance that determines the permeability for amphibians of the landscape between two suitable habitat patches.
MHC	Major Histocompatibility Complex. The MHC is a group of genes known to be directly involved in the immune response in vertebrates, and variation at the MHC has been linked to disease resistance and susceptibility.
NPH	Novel or Spreading Pathogen Hypothesis. Posits that pathogens emerge by the translocation of a virulent strain into a new geographic location or into a host species that has no evolved resistance.
Panzootic	An epizootic that spreads over a large region, or even globally.
Pathogenicity	The ability of an organism to cause disease.
Pathogen pollution	Human-mediated introduction of a pathogen to a new host or region.
Propagule pressure	The success of invasive species in a new environment is dictated by the interaction of propagule pressure (which includes both the number of release events – propagule number – and the number of individuals released – propagule size), location and species (Lockwood et al. 2005).
Resistant	Host does not become infected, there is no disease.
R0	Basic reproductive ratio: the average number of infections one infected individual generates in a population of susceptible hosts over the course of its infectious period.
Susceptible	The host becomes infected, and infection leads to clinical diseases with the possibility of recovery from disease.
TRACES	The EU trade Control and Export System (https://ec.europa.eu/food/animals/traces_en)
Tolerant	The host becomes infected, but there is no disease or mortality.
VGC-system	Veterinarian Border Control System of the Netherlands (Veterinair Grens Controle Systeem)
Virulence	The degree to which an organism can cause damage to a host.
WND	White-nose disease. An EID caused by *Geomyces destructans*, a fungus in the family Myxotrichaceae, causing severe mortality in some bat species.
Zoospore	Flagellated motile spore. Both Bd and Bsal have motile flagellated (single flagellum) spores or so-called zoospores.
Zoosporangium	Container for zoospores (Berger et al. 2005)

Introduction

Introduction

Preface

Global biodiversity is in decline. Emblematic for this so-called biodiversity crisis is the global amphibian crisis. Amphibians around the world suffer from the emerging infectious disease chytridiomycosis and up to now, there is no satisfactory universal set of measures that can be executed to combat the disease. Also in Europe this is a concrete problem, and there is a need for practical conservation tools that can be applied in the field. The present thesis represents a series of studies discussing the presence and impact of *Batrachochytrium* sp. in and on wild and captive amphibians in the Netherlands.

The introduction is structured in the way that I first sketch the global biodiversity problem, and the role of emerging infectious diseases within this biodiversity crisis, after which I discuss the causes of the global amphibian declines, such as habitat destruction, trade and pollution. Then I zoom in on chytridiomycosis, which is the topic of this thesis. For the definition of conservation actions (which traits can be targeted?) it is important to have as much information available as possible on the specific traits of the pathogen, which I therefore discuss elaborately.

1. Biodiversity crisis

The pressure of the human population size, its unsustainable growth rate and the herewith associated consumptive behaviour is the overarching driver of species extinction (Pimm et al. 2014; Crist et al. 2017). The unsustainable human population size and its associated problems like global climate change, changes in land-use, contaminants and infectious diseases all impact on global biodiversity [biodiversity = the diversity of genes, species and ecosystems]. The magnitude of species extinctions has exploded since 1980 (McCallum, 2015), and current rates are about 1000 times the expected background rate of extinction (Pimm et al. 2014). These current extinction estimates are similar, or even more severe than the previous five mass extinctions (McCallum, 2015), which means we are currently passing through the sixth mass extinction (Wake and Vredenburg, 2008).

The biodiversity crisis can be recognised across all species groups and ecosystems. In 2017, corals are in severe decline, 25% of all mammal species are threatened with extinction, 183 of all bird species have disappeared, 1,375 (13%) bird species are threatened with extinction, and invertebrates are declining at great speed (IUCN Red List, update May 2017; Hallmann et al. 2017). Amphibians are the most threatened species group; an estimated 42% of all known species are currently threatened (IUCN Red List, update May 2017). The extinction of *Taudactylus diurnus* (Mt Glorious day frog) and the two last species of gastric brooding frogs (*Rheobatrachus silus* and *R. vitellinus*) are exemplary of these recent extinctions (Czechura and Ingram, 1990; White, 1995; Hero et al. 2004).

To allow for the consistent monitoring of the status of ecosystems, a global framework is set up to develop an IUCN Red List of Ecosystems (Keith et al. 2015; Bland et al. 2017). A full evaluation of all ecosystems will be realized in 2025. It is already obvious that at larger and regional scales, declining trends are recognized in a.o. the extent of forest (for instance the Tapia Forest in Madagascar), mangroves, sea beds (like the kelp beds in Alaska) and the condition of coral reefs

(Butchart et al. 2010; Keith et al. 2013). The global loss of biodiversity is unsettling. At higher levels of diversity, ecosystems are more stable and more productive (Cardinale et al. 2012), and the loss of biodiversity frequently increases disease transmission (Keesing et al. 2006; 2010).

2. Diseases

Emerging infectious diseases (EIDs)[1] challenge the conservation of native species, as they may cause the extinction of species. Due to the chytrid fungus *Batrachochytrium dendrobatidis* [Bd], the causative agent of chytridiomycosis, several amphibian species have suffered severe declines and even extinction (e.g. Schloegel et al. 2006; Scheele et al. 2017). EIDs may also have indirect effects. For instance, if green frog tadpoles (*Lithobates clamitans*) are exposed to trematode parasites, the tadpoles' avoidance response increases and they become more active, through which they then experience greater predation rates by dragonfly larvae (Marino and Weber, 2013). Additionally, EIDs may also alter habitats that are already threatened by fragmentation or global climate change (Daszak, 2000; Epstein et al. 2003). For example, coral reefs that are already stressed by global warming and eutrophication, additionally suffer from opportunistic pathogens, which has led to significant deterioration of coral integrity, cover and biodiversity (Weil, 2004).

EIDs can be either completely new to an area (novel or spreading pathogen hypothesis, NPH) or they can be endemic, but changing conditions trigger disease emergence (emerging endemic pathogen hypothesis, EPH). White-nose disease (WND) is an example of a NPH. The fungus causing disease presumably originated in Europe and causes population collapses of common bat species in North America (Frick et al. 2010). An example of an EPH is the nonzoonotic *Mycoplasma gallisepticum*, known as a major pathogen of domestic poultry, that has caused mass mortality events in house finches (*Carpodacus mexicanus*) and goldfinches (*Carduelis tristis*) in the eastern United States. Its spread is facilitated by bird feeders and presumably also by the limited genetic pool of the eastern house finch population (Fischer et al. 1997).

2.1 Disease and biodiversity

A high biodiversity could either provide for a large source of pathogens, but may also reduce pathogen transmission of both long-established and novel diseases (Keesing et al. 2010). This means that biodiversity plays a double role in the persistence and emergence of infectious diseases, which pleads for considering the entire ecological context of in-situ host-parasite relationships. Parasites may shape community- and ecosystem structures by influencing host behaviour and host fitness. These influences may impact on host population sizes, which again might alter trophic interactions and food webs (Preston and Johnson, 2010). This is illustrated by three examples. The first example illustrates how parasites can positively contribute to biodiversity. Parasite-modified competition may allow the co-existence of two competing species. The lizard *Anolis gingivinus* is the superior competitor and outcompetes *A. wattsi*, except where the malarial parasite *Plasmodium azurophilum* occurs. This parasite affects *A. gingivinus*, allowing co-occurrence with the inferior competitor *A. wattsi* (Schall, 1992). The second example illustrates how parasite-modified competition may lead to a shift in species composition. The common toad (*Bufo bufo*) could expand in Peñalara NP (Spain) after a strong

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[1] Infectious diseases that have increased recently or are threatening to increase in the near future

decline of the common midwife toad (*Alytes obstetricans*) due to chytridiomycosis (Bosch and Rincón, 2008). The negative impact of parasites on biodiversity is illustrated by the mass declines and even extinctions of amphibian populations caused by Bd (Kilpatrick et al. 2010).

2.2 Disease and climate change

The effects of global climate change on disease dynamics are complex and multifaceted (Lindgren et al. 2012). For instance, the arrival, establishment and spread of new pathogens are predicted to be facilitated by the increase in global trade and travel, in combination with climate change (Suk and Semenza, 2011). The warming of the climate is the overarching factor explaining pathogen emergence in geographically separated populations, and it is hypothesized to be the true common that accounts for pathogen emergence (Collins, 2010). It is expected that the changes in the climate will increase the vulnerability of populations and/or species to already existing threats (Suk and Semenza, 2011). Host susceptibility, transmission and survival rates may be impacted by changes in regional or local climatic conditions (Harvell et al. 2002; Walther et al. 2002).

2.3 Disease and globalization

Organisms with similar climatic requirements, but normally geographically separated by considerable distances, are being brought together by globalization. Due to globalization and the lack of effective biosecurity measures (Franklin et al. 2008), pathogens can easily be transported as passive passengers around the world (pathogen pollution) where they can infect naïve host populations (pathogen spillover[2]). Examples of pathogen movement due to commercial activities are the movement of ranaviruses through traded bullfrogs (*Lithobates catesbeianus*; Schloegel et al. 2009), the movement of ranaviruses and Bd in the trade of tiger salamanders as bait (Picco and Collins, 2008; Picco et al. 2010) and the recent introduction of *Batrachochytrium salamandrivorans* (Bsal) in Europe from East Asia (Martel et al. 2013).

Pathogen movement from wild to domesticated animals has received widespread recognition of being problematical (from a commercial perspective), as for instance the role of badgers (*Meles meles*) in the transmission of tuberculosis to cattle (Godfray et al. 2013), or brucellosis transfer from wild bisons and elk to cattle in the USA (Scurlock and Edwards, 2010). Alternatively, the impact of pathogen transfer from domestic animals to wildlife may be equally significant, which can be illustrated by the spill-over from domestic dogs of rabies to African wild dogs and Ethiopian wolf, and canine distemper virus to lions in the Serengeti, which has decimated populations (Power and Mitchell, 2004).

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[2] Pathogen spillover is defined as the transmission of pathogen propagules from one host population to another sympatric population

3. Amphibians

Amphibians form a very diverse group of ectotherms. The word ‘amphibian’ is derived from the Greek and means ‘two kinds of life’. Currently there are 7,823 described species of amphibians globally. The class Amphibia includes three orders; Anura (6,899 species of frogs and toads), Caudata (717 species of newts and salamanders) and Gymnophiona (207 caecilian species) (from: Frost, 2018; accessed 28 March 2018). Frogs and toads form a diverse group, inhabiting a great diversity of terrestrial and aquatic habitats, from lowlands to mountaintops. They can be found on all continents, apart from Antartica. Salamanders and newts are tailed amphibians and are largely a Northern Hemisphere group. It is only the group of the lungless salamanders (Plethodontids) that occur in South America, but all others are restricted to the temperate and subtropical areas of Eurasia, North America and North Africa. The caecilians form a secretive group of amphibians, with cylindrical, legless bodies. They live in moist soil, although a few species are aquatic, in the tropics, apart from Oceania and Madagascar.

Fillet of a fenny snake,
In the caldron boil and bake;
Eye of newt, and toe of frog,
Wool of bat, and tongue of dog,
Adder's fork, and blind-worm's sting,
Lizard's leg, and howlet's wing,--
For a charm of powerful trouble,
Like a hell-broth boil and bubble.

Figure 1. Old and new art featuring amphibians. Left *Danse Macabre* by Brandon Ballengée (2014) and right Macbeth (IV, i, 14-15) by William Shakespeare (1606).

Amphibians are omnipresent; they may function as a food source, especially in Southeast Asia, they provide potential for new medication and they play prominent roles in mythology, literature and arts (Figure 1). In some communities, amphibians are considered key-stone[3] species (Sparling et al. 2003). Amphibians are important contributors to regional diversity, and they form an integral part of many aquatic and terrestrial foodwebs. Adults and some larvae tend to be intermediate predators, feeding on invertebrate prey (Walton, 2005; Walton and Steckler, 2005; Durant and Hopkins, 2008) whilst simultaneously serving as food for a wide range of larger predators. Large mole salamander larvae (*Ambystoma talpoideum*) can consume up to 900 mosquito larvae in one day (Durant and Hopkins, 2008). Anuran larvae of *Hyla*

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[3] keystone species: one whose importance is so great that drastic declines or extirpation would lead to serious ramifications within the entire community (Sparling et al. 2003).

septentrionalis and *H. cinerea* (now *Osteopilus dominicensis* and *Dryophytes cinereus*) were found to consume 13 - 45 *Culex* sp. larvae/tadpole per day (Spielman and Sullivan, 1974; Ritchie, 1982). Amphibians are exposed to problems in both aquatic and terrestrial environments due to their bi-phasic life, which means that this group of animals may be the first to signal environmental impairment through either a reduction in numbers or in distribution (Sparling et al. 2003).

3.1 Global amphibian declines

Pollution, habitat loss and climate change are often proposed as the causative agents of the current amphibian crisis, however many declines have happened in areas that were not, or at least not heavily, affected by habitat alterations and environmental pollution (Alford, 2011). Collins (2010) lists six major causes of amphibian declines and extinction, of which many work in concert. These leading causes are habitat destruction, pollution, climate change, commercial trade, introduced (non-native) species, and infectious diseases.

3.1.1 Habitat destruction

Habitat loss, fragmentation and degradation threaten amphibian population persistence through a.o. downsizing and the isolation of populations, inbreeding, and edge effects (e.g. Cushman, 2006; Becker et al. 2007; Temple and Cox, 2009). Habitat loss and habitat degradation negatively impact on nearly four times more species than the next most common threat, pollution (IUCN, 2017a; accessed Dec. 20th 2017).

The negative impact of the loss of habitat on the survival of populations and species is evident. For instance, habitat loss is suggested to be responsible for the presumed extinction of the El Empalme worm salamander (*Oedipina paucidentata*) and the Guanujo stubfoot toad (*Atelopus guajano*) (IUCN, 2017b). The fragmentation of habitat reduces the connectivity between the suitable habitat patches, as well as patch size. This immediately affects (seasonal) amphibian migratory movements and may lead to an amplified risk of stochastic, genetic and demographic events, which may increase the risk of local population extinction, or even species extinction (Rudnick et al. 2012). Additionally, Soto-Rojas et al. (2017) found that habitat degradation may also increase the frequency of morphological abnormalities in amphibians, which obviously affects fitness and survival.

3.1.2 Pollution

Environmental pollution is the second most common threat to amphibians, following habitat destruction (IUCN, 2017a; accessed Dec. 20th 2017). The density, health and diversity of amphibians is proven to be, either directly or indirectly, affected by exposure to lethal or sub-lethal biocide concentrations (Whitfield et al. 2016). Many studies provide evidence for the negative effects of pesticides on amphibians:

- The frequently applied glyphosate-based herbicide may cause high rates of mortality in several amphibian species in both a terrestrial and aquatic situation, which could lead to population declines (Relyea, 2005).
- The exposure of leopard frogs (*Rana pipiens*) to low concentrations of the herbicide atrazine in the laboratory and in the field led to hermaphroditism (Hayes et al. 2002; 2003)

- In England and Wales, in a study at sites with varying levels of agricultural intensity, a relationship was found between agricultural intensity and the presence of intersex individuals (Orton and Routledge, 2011).
- Immuno-suppression by pesticides increases the infection intensity with *Ribeiroia sp.* in amphibians, which leads to limb deformities (Kiesecker, 2002).
- Exposing larval common frogs to environmentally relevant concentrations of de-icing salt causes reduced locomotor behaviour in *Rana temporaria* tadpoles, which detrimentally affects their response to predation and competition (Denoël et al. 2010).

Nitrogen pollution, of which the main sources are manure and fertilizers, can seriously affect amphibian health, but often it is difficult to establish a cause-effect relationship because of the presence of other contaminants in the environment (Marco and Ortiz-Santaliestra, 2009). Exposure to deleterious substances can be an important cofactor suppressing the amphibian immune system which would facilitate outbreaks of infectious diseases and result in reduced adult fitness or mortality (Mann et al. 2009). The effects of nitrogen pollution may be lethal or sublethal to amphibians and cause deformities, impact reproduction or alter their behaviour (Marco and Ortiz-Santaliestra, 2009).

3.1.3 Climate change

Future predictions on climate change include smaller temperature diurnal ranges and increased evaporation (IPCC, 2013). The global surface temperature is predicted to result in more frequent hot and fewer cold temperature extremes. Additionally, an increase of the frequency and intensity of heavy precipitation events over land is predicted. The intensity, duration and spatial extent of heat waves and warm spells will be varying spatially and temporally (IPCC, 2013).

Because amphibians are ectothermic, have shell-less eggs and have permeable skins, they are particularly vulnerable for changes in their environment. It is even suggested that salamanders are the group of terrestrial vertebrates most vulnerable to climate change (Catenazzi, 2016). Even climatic changes within their noncritical range may increase mortality or reduce body mass (Rohr and Palmer, 2013). Amphibians have several compensatory mechanisms that they can use in response to changes in climatic conditions. They may respond to climatic shifts behaviourally (behavioural thermoregulation); they might seek refuge during warm and dry spells and are hereby forced to reduce the available time for foraging or mating (Rohr and Madison, 2003; Walls et al. 2013) or they might migrate to other – more climatically suitable – areas (Raxworthy et al. 2008). Alternatively, they may be able to display plasticity in physiological processes (e.g. metabolic compensation or depression; Catenazzi, 2016). Another compensatory mechanism is the evolutionary adjustment of traits that can mitigate the impact of climate change, like for instance morphological changes in the red-backed salamander (*Plethodon cinereus*; Gibbs and Karraker, 2006) and in amphibian breeding phenology (Green, 2017 and references therein). Parmesan (2007) looked at the relation between global warming and the breeding dates of 203 species in the northern hemisphere. She showed that from all taxonomic groups, amphibians had the strongest shifts towards earlier breeding, which was even more than twice as fast as birds, trees and butterflies.

In the UK, climate induced reduction in body size of the common toad (*Bufo bufo*) was associated with reduced survivorship and reduced reproductive success (Reading, 2007). A reduction in body size may induce changes in thermal and energetic requirements, and because of this, result in changes in activity patterns and survival (Blaustein et al. 2010). Most amphibian species operate within relatively narrow optimal temperature ranges, and climate warming demands metabolic compensation (Catenazzi, 2016), which forces a thermally stressed animal to allocate more energy to maintenance and reproduction than to growth (Caruso et al. 2015).

3.1.4 Commercial trade

The vast international trade in amphibians for consumption, as laboratory animals or for pet trade, directly and indirectly affects wild populations; directly by depletion of host populations and/or indirectly by pathogen pollution. The anthropogenic movement of pathogens through trade allows pathogens to move out of their geographical range and infect naïve populations (Cunningham et al. 2003; Fisher and Garner, 2007; Martel et al. 2014; Auliya et al. 2016). Despite the absence of accurate trade data for most species, the volume of animals collected from the wild is large enough to potentially extirpate populations and species (Schlaepfer et al. 2005; Cox et al. 2008; Warkentin et al. 2009; Collins, 2010; Auliya et al. 2016). Currently, non-CITES listed amphibians are untraceable by the absence of an amphibian specific unique identifier code in TRACES (the EU Trade Control and Export System).

3.1.5 Bio-invasion

The intentional or unintentional release of non-native species may heavily impact on native amphibians. The introduction of invasive species often disrupts the ecological balance. The newcomers may not be impacted by the native predators, competitors, endemic pathogens and parasites, which consequently may lead to increased mortality of native populations (Forti et al. 2017). Hence, the invasive alien species can cause various problems, like for instance hybridization between native *Triturus cristatus* and invasive *T. carnifex* (Beebee and Griffiths, 2005; Meilink et al. 2015). The success of invasive species in a new environment is dictated by the interaction of propagule pressure (which includes both the number of release events – propagule number – and the number of individuals released – propagule size), location and species (Lockwood et al. 2005).

3.1.6 Infectious diseases

Diseases are part of the natural system (Dobson and Hudson, 1986), but infectious diseases are emerging at unusually high rates (Epstein et al. 2003). In the current era, many pathogens have been anthropogenically introduced (pathogen pollution) to new areas, especially by the trade in amphibians (Picco and Collins, 2008; Rodgers et al. 2011). These novel infectious diseases can severely impact on naïve wildlife populations (Berger et al. 1998), which in turn can have substantial impacts in ecosystem integrity (Anderson and May, 1979 (part I)).

A condense overview of emerging infectious diseases that currently are considered as conservation concern for wild amphibians is listed in Table 1. Ranaviruses represent a group of pathogens that infect a wide host range, and although species extinction has not been reported yet, local populations may collapse. In a National Park in Spain an outbreak of CMTV-like ranaviruses (Common Midwife Toad Virus) infected a wide range of amphibian hosts and caused multispecies host declines (Price et al. 2014). Three ranid herpesviruses have been described in frogs which may cause tumours, edema, hyperplasia and degeneration of the mucous glands (Origgi et al. 2017). Herpesviruses might be more widespread in Europe than previously considered, and they are known to be lethal to at least the common spadefoot (*Pelobates fuscus*) (Mutschmann and Schneeweiss, 2008; Garner et al. 2013). Trematodes are flatworm parasites and some, such as *Ribeiroia ondatrae*, may cause limb abnormalities in frogs and toads (Blaustein et al. 2012).

A bacterial infection that is currently gaining attention as a common cause of disease in amphibians (Berger et al. 1999; Martel et al. 2012a) is caused by the family *Chlamydiaceae*, which was already known to cause mortality in captive amphibians. Recently novel species of Chlamydiales were discovered in captive salamanders (Martel et al. 2012b) and in exotic invasive bullfrog tadpoles from an introduced population in the Netherlands (Martel et al. 2012a). Dermocystid infections have been reported for over a long time, but much is yet unknown. For instance, the infection cycle has yet to be described (Garner et al. 2013). Dermocystid infections in European amphibians are referred to as *Amphibiocystidium*, which has been accepted as non-pathogenic for a long time. There is however mounting evidence that infections with Amphibiocystidium may result in high mortality rates (Garner et al. 2013), but it remains to be studied to which extent *Amphibiocystidium* impacts amphibian populations. It is under debate if saprolegniasis is to be considered as an EID, but it is obvious that infection with *Saprolegnia ferax* may cause significant and consistent/persistent mortality in eggs, larvae and metamorphosed animals (Kiesecker and Blaustein, 1995; Garner et al. 2013). Spawn of various anuran species, among which the common spadefoot and natterjack toad (*Bufo* (now *Epidalea*) *calamita*) were found to be susceptible to infection with *Saprolegnia* sp. in the Netherlands (Strijbosch, 1979). It should however be studied to which extent saprelegniosis outbreaks impact long-term population dynamics.

3.1.7 Disease triangle

'Everything is everywhere, but the conditions determine the outcome of an infection' (Baas Becking, L.G.M., 1934; DeWit and Bouvier, 2006). The varying responses of host species and populations to an infection with the same parasite are a case in point. In Spain, CMTV-like ranavirus has caused the near collapse of an entire amphibian community, whereas in the Netherlands CMTV-like ranavirus cause mortality in amphibians, but less severe and disruptive (Price et al. 2014; Spitzen- van der Sluijs et al. 2016a,b). In stable systems, pathogens may not cause disease. Introducing new elements usually has a disruptive element (Poelman, 2016). The current and on-going mass mortality events in amphibian populations throughout the world may demonstrate that currently severe alterations in our environment are occurring.

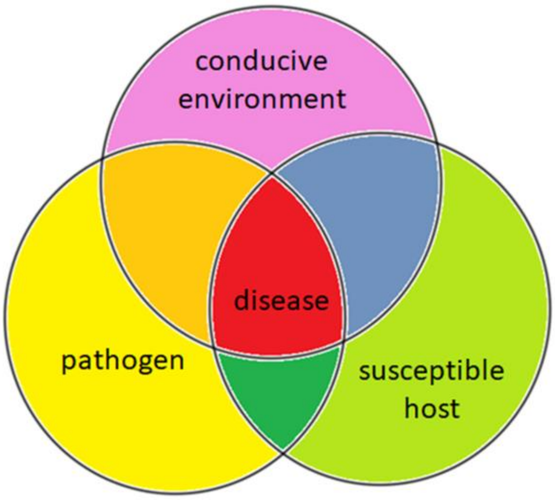

Figure 2. The disease triangle. A susceptible host, conducive environment and a pathogen are required to cause disease emergence.

The disease triangle (Fig. 2) is a theoretical model that illustrates the interactions between the host, environment, and an infectious (or abiotic) agent, and shows how variation in these factors lead to varying disease outcomes (Scholthof, 2007; Gurr et al. 2011). It is a tool that allows to evaluate, beginning at every edge, the triggers that are conducive for disease emergence (McNew, 1960).

4. Chytridiomycosis

Amphibian declines are currently one of the most impactful conservation issues and herein the disease chytridiomycosis plays a major role. This disease is suggested to be: "*the worst infectious disease in wildlife ever recorded among vertebrates. Chytridiomycosis is notorious for the number of host species impacted and its propensity to drive them to extinction*" (Gascon et al. 2007). Although Bd, which may cause chytridiomycosis, is distributed globally in amphibian communities, the individual and population level responses of hosts to Bd infection have shown to be varying from resistant (Márquez et al. 2010; Woodhams et al. 2012; Pasmans et al. 2013), tolerant (Garner et al. 2006; Schloegel et al. 2010; Brannelly et al. 2012) to susceptible (Pounds et al. 2006; Rachowicz et al. 2006; Walker et al. 2008). Biodiversity losses due to Bd are currently restricted to well-known outbreak regions e.g. in Central America, the western USA, Australia and southern Europe (Berger et al. 1998; Bosch et al. 2001; Stuart et al. 2004; Rachowicz et al. 2006). In most other regions (Moreno et al. 2011), Bd appears in a state of endemism, without obvious negative effects on local amphibian communities.

The disease chytridiomycosis, caused by an infection with Bd, kills amphibians by affecting the keratinized skin layers. The skin is an important organ, involved in hydration, gas exchange, and protection from other pathogens (Voyles et al. 2009). The clinical symptoms are variable. In anuran larvae, mouthpart abnormalities can be observed (Berger et al. 1998; Blaustein et al. 2005). In metamorphosed amphibians the symptoms are variable, but clinical chytridiomycosis is most often characterized by lethargy, extensive shedding and death. Epidermal hyperplasia and hyperkeratosis are consistent histological lesions (Berger et al. 2005; Pasmans et al. 2010).

4.1 Life cycle

The life cycle of Bd involves the development from a zoospore to the thallus. The thallus is the reproductive body, which produces a zoosporangium (= container for zoospores; Berger et al. 2005). Bd discharges zoospores and exhibits monocentric or colonial growth (Longcore et al. 1999). Sexual reproduction has not been observed in Bd, but sexual recombination and hybridization are likely to play a role in the evolutionary history of Bd (Rosenblum et al. 2013). Schloegel et al. (2012) found a hybrid between Bd-GPL (Global Panzootic Lineage) and a divergent Bd lineage from Brazil. This finding suggests that genetically distinct isolates can hybridize (isolate CLFT024-02). Recently two new hybrids of BdGPL and BDCAPE were identified (O'Hanlon et al. 2018).

Table 1. Condense overview of emerging infectious diseases that are seen as conservation concern for wild amphibians.

	Geography	**Susceptible hosts**	**Field symptoms**	**Netherlands**	**References**
Viruses					
Ranaviruses (e.g. CMTV, FV3, ATV)	Global	anurans and caudates	Often absent, but may comprise cutaneous erythema, skin erosion and haemorrhages	Mass mortality events in north of NL in water frogs and common spadefoots, now endemic. Additional cases in smoot newt, crested newt, common toad. In south NL a second, hypothesized less virulent strain, present in populations.	Cunningham et al. 2007; Kik et al. 2011; Gray and Chinchar (eds.), 2015; Saucedo et al. 2018
Herpesviruses	Europe, America's	anurans	Often absent, epidermal hyperplasia, tumours	Occasional report of infections from the public. No information on distribution, prevalence and host impact.	Mutschmann and Schneeweiss, 2008; Garner et al. 2013; Antonucci et al. 2014; Origgi et al. 2017
Fungi					
Batrachochytrium dendrobatidis (e.g. BdGPL, JEL423)	Global	anurans and caudates	Often absent, also variable; excessive shedding, erythema, lethargy, abnormal posture	National distribution, endemic, but infection is costly for the individual host.	Berger et al. 2009; Spitzen-van der Sluijs et al. 204; Van Rooij et al. 2015
Batrachochytrium salamandrivorans	Europe	caudates	Erosion of the skin, epidermal ulcerations and ataxia, excessive shedding, anorexia	Currently limited to south of the Netherlands, infecting and killing fire salamanders in vast numbers. Alpine newts asymptomatic carriers.	Martel et al. 2013; 2014; Van Rooij et al. 2015
Trematodes					
Ribeiroia ondatrae	Europe, USA	anurans	Limb abnormalities	No information on distribution, prevalence and host impact.	Blaustein et al. 2012; Garner et al. 2013
Bacteria					
Chlamydiales	Europe, USA	anurans and caudates	Nno clinical signs, anorexia, lethargy, edema (swelling)	Novel species discovered in invasive bullfrogs in south of NL, no further known studies in NL. No information on distribution, prevalence and host impact.	Berger et al. 1999; Martel et al. 2012a,b

Mesomycetozoa					
Amphibiocystidium	Europe, North- and South America	anurans and caudates	ulcerative skin lesions and nodular skin cysts distributed across the entire body of the host	Reported from various sites in the Netherlands (north to south), broad host range, currently large-scale study conducted on the palmate newt which currently has the highest prevalence. No information on distribution, prevalence and host impact.	Berger et al. 2009; Garner et al. 2013; Stark and Guex, 2014; Borteiro et al. 2018
Oomycetes					
Saprolegnia	Europe, USA, Australia	anurans and caudates	Fluffy white hyphae on surface of skin or protrude from wound, also infects eggs. Also ulcerated and red skin	Frequent reports of the public showing photos of presumed infected eggs, newts or anurans. It often seems to be a secondary infection, but there is no information available on distribution, prevalence and host impact.	Strijbosch, 1979; Kiesecker and Blaustein, 1995; Berger et al. 2009; Garner et al. 2013

Reported common frog with possible herpesvirus infection in 2011, province Flevoland in the Netherlands. Photo credits: A. Lodder

Palmate newt with Amphibiocystidium infection. Collected in province Brabant in the Netherlands. Photo credits: J. Herder

Water frog with ranavirus infection. Collected in province Drenthe in the Netherlands. Photo credits: J. Janse

In amphibian skin, the sporangia infect cells in the epidermis. In the epidermis the zoospores encyst; the flagellum is absorbed and a cell wall is formed. Then, the zoospore cyst germinates and develops a germ tube that invades the epidermis of the host. Immature sporangia occur within the deeper, more viable cells while mature zoosporangia and empty sporangia are more prevalent in the outer keratinized layers. Immature sporangia are carried from the deeper skin layers to the skin surface by differentiating epidermal cells. When the sporangia have developed discharge tubes containing mature zoospores, they are back up in the *stratum corneum* and the mature zoosporangia are released in the environment (Longcore et al. 1999; Berger et al. 2005). Bd displays a chemotactic response towards nutritional cues in its nearby environment; zoospores are attracted by the mucus on amphibian skin. That said, the mucus plays a dual role as it may also contain Bd-inhibitory mechanisms, such as skin bacteria that produce antifungal metabolites (Moss et al. 2008; Garmyn et al. 2012; Van Rooij et al. 2015).

The optimal growth temperature of Bd lies between 17 and 25°C and at pH 6–7 (Piotrowski et al. 2004). At 10°C or lower the fungus grows slowly. Above 28°C the growth slows down, and after four hours at 37°C, or five minutes at 60°C, the zoospores are killed off (Piotrowski et al. 2004; Johnson et al. 2003). The lifecycle of Bd, from zoospore to zoosporangium, takes 4 to 5 days at 22°C (Berger et al. 2005).

Keratin, a fibrous structural protein, is not essential for the growth of Bd. The fungus is able to grow on agar (Longcore et al. 1999), and Bd is known to be able to grow on the chitinous exoskeleton of crayfish (McMahon et al. 2013a). The fungus has also been found on other carriers such as birds (Johnson and Speare, 2005; Garmyn et al. 2012), reptiles (Kilburn et al. 2011), nematodes (Shapard et al. 2012), on moist, sterile river sand (Johnson and Speare, 2005) and in water (Johnson and Speare, 2003), but there is no evidence yet that these carriers support the actual growth and transmission of the fungus and hereby add to the long-term persistence of the fungus in the environment (McMahon et al. 2013a). Resistant resting spores have not been found (Berger et al. 2005).

4.2 Origin

There has been a lot of debate about the geographical origin of Bd. It has been hypothesized that it originated from the America's (Rodriguez et al. 2014; Talley et al. 2015), Africa (Weldon et al. 2004) and Asia (Goka et al. 2009; Bataille et al. 2013) and was transported over the world by the trade in amphibians, mainly *Xenopus laevis* and *Lithobates catesbeianus* (Weldon et al. 2004; Schloegel et al. 2012; Auliya et al. 2016). Two not mutually exclusive hypotheses on the emergence of Bd have been proposed, the novel or spreading pathogen hypothesis (NPH; Bd is a novel pathogen and has been spread across the world (Lips et al. 2008)), and the endemic pathogen hypothesis (EPH; the fungus has been around, but recent environmental changes are responsible for the outbreaks (Rachowicz et al. 2005)). Recently the riddle was elucidated, and both hypotheses hold. East Asia (Korean peninsula) is the geographic origin of Bd. Here a novel lineage BdASIA-1 was discovered. This lineage shares more diversity of the population of Bd than any other lineage, and now four main diverged lineages are defined: BdGPL, BdASIA-1 (which includes BdCH isolate), BdCAPE and BdASIA-2/BdBRAZIL. Expansion of BdGPL in the 20th century coincided with the increase in amphibian trade volume. Additionally, hybrid genotypes have been found, which shows that Bd exchanges haplotypes amongst lineages when they contact (O'Hanlon et al. 2018).

4.3 Treatment and mitigation

Various treatments and mitigation measures are available for either hosts or habitats.

- Captive amphibians can be treated successfully for an infection with Bd, for instance by the daily spraying of the animals for 7 days with a voriconazole solution (e.g. Parker et al. 2002; Martel et al. 2010; Young et al. 2012; Brannelly, 2014; Hardy et al. 2015).
- Immunization by vaccination in the field should ideally trigger a strong protective response across all life stages, across many species against a broad spectrum of relevant and virulent chytrids. It should be safe to use and easy to apply (Garner et al. 2016). Despite some successful studies (Ramsey et al. 2010; McMahon et al. 2014), vaccination has not been shown to be effective in others (Rollins-Smith et al. 2009; Stice and Briggs, 2010; Cashins et al. 2013). For instance, immunization against Bd induced an increase in Bd antibodies in the mucus secretion of *Xenopus laevis* (Ramsey et al. 2010), but treating boreal toads (*Bufo* (*Anaxyrys*) *boreas*) and mountain yellow-legged frogs (*Rana muscosa*) with heat-killed Bd zoospores did not protect them from infection (Rollins-Smith et al. 2009; Stice and Briggs, 2010).
- Up to date there is no safe tool to mitigate the disease effectively in the field. In Spain, an ex-situ island population *Alytes muletensis* was treated successfully for Bd by simultaneously releasing treated larvae (with itraconazole) in an environment that was treated with VirkonS. They found that the complete drainage and drying of the ponds was insufficient to eliminate Bd. The application of VirkonS to the rocks, crevices and vegetated areas surrounding the breeding site did eliminate Bd successfully even up to two years post-application (Bosch et al. 2015). However, in this study the effects of the frequent application of VirkonS on the natural system (like vegetation and invertebrates) was not monitored, hence more study is needed before it can be applied at a larger scale. Repeatedly (5-7 times) exposing tadpoles to the veterinary disinfectant F10SC for 15 minutes reduced infection with 86% in the African common toad (*Sclerophrys gutturalis*) and 100% in Phofung river frogs (*Amietia hymenopus*) (De Jong et al. 2018). It has been postulated that garlic can be used as a disinfectant against *Bd* and that planting garlic in amphibian enclosures would cure infected amphibians (Fog et al. 2011). No scientific evidence has been found that supports this idea.
- Habitat management that alters temperature or humidity at micro-scale may impact pathogen growth or host immune responses (James et al. 2015) and are therefore potential manipulative sources to reduce disease impact. In *Bufo bufo* and *Alytes obstetricans* natural UV-B intensities reduced – but not eliminated – Bd prevalence in tadpoles of these species, which could implicate conservation measures such as increased sun exposure in ponds (Walker et al. 2010; Ortiz-Santaliestra et al. 2011). This inverse correlation between UV-B and Bd prevalence is not ubiquitous. Others have not found synergistic interactions between UV-B and Bd (Garcia et al. 2006; Searle et al. 2010). Overall, UV-B radiation is considered as a common stressor with negative effects on amphibians (e.g. Blaustein et al. 1997; Croteau et al. 2008).
- Elevating water temperature has been suggested as a possible tool to suppress disease impact of aquatically transmitted Bd; with increasing pond water temperature, Bd prevalence gradually decreases (Forrest and Schlaepfer, 2011).
- A similar relationship has been suggested between salinity and Bd infection loads (Stockwell et al. 2014; Clulow et al. 2018). Here, the physiological limits of the host species are key.

- A general conservation tool for amphibians is the regular desiccation of reproductive waterbodies to reduce the number of invertebrate predators and the persistence of fish (Van Delft, 2009). Environmental Bd is also not thought to survive/persist long dry periods of drying. In the laboratory, 100% mortality of zoospores was observed after 3 hours of drying (Johnson et al. 2003; 2005). It was shown that Bd occurs primarily in permanent ponds (Kriger and Hero, 2007). Besides drying the environment as a conservation tool, it was shown that if Bd re-exposed boreal toads (*Anaxyrus boreas*) had access to dry microenvironments they'd survive three times longer than animals that could not escape wet environments (Murphy et al. 2011), but in Mallorca and in the Netherlands, pond drying was not a waterproof conservation tool as it did not prevent the return of Bd and the concurrent infections (Lubick, 2010; Spitzen-van der Sluijs pers. obs.).
- Aquatic microfauna can quickly reduce the density and abundance of Bd zoospores, even small densities of *Daphnia* may reduce zoospore populations significantly (Woodhams et al. 2011). Schmeller et al. (2014) found Bd zoospores are effectively eaten by ciliate and rotifer microorganisms. This finding opens perspective to manipulate the infection intensity of Bd by natural augmentation of predatory microorganisms.
- Bioaugmentation is the introduction of beneficial microbes (probiotics) by humans to the environment or an organism to produce a desired effect (Muletz et al. 2012). In temperate zones, it has been shown that bioaugmentation with the antifungal bacterium *Janthinobacterium lividum*, which is a gram-negative bacterium producing an antifungal metabolite violacein (Brucker et al. 2008), can be effective in the laboratory via individual treatment (Becker et al. 2009; Harris et al. 2009), and potentially also in the field, by inoculation of soil (Muletz et al. 2012) or water (Rebollar et al. 2016), however, in *Atelopus zeteki*, treatment with *J. lividum* did not prove to be successful (Becker et al. 2011). Some reservations exist with regard to the application of probiotica. It is for instance unsure if successful application in vitro translates in effective inhibition in vivo, if it is safe to use in situ, and if bioaugmentation can be applied to multiple Bd isolates (Garner et al. 2016).
- Vegetation Plants may indirectly affect Bd growth by providing suitable thermal conditions, but may also directly have an effect on the infectivity of Bd. Certain plant extracts were shown to reduce pathogen load in the tiger salamander (*Ambystoma tigrinum*) (Davidson et al. 2012).
- Some more experimental alternatives that are still in their infancy could be the selective breeding of susceptible hosts for disease resistance or genetic manipulation of the pathogen. The context-dependent outcome of the interaction between host and pathogen however, complicate a solution that would be applicable to multiple populations. However, there is evidence that natural amphibian populations have the evolutionary potential to adapt to chytridiomycosis (Savage and Zamudio, 2016; Voyles et al. 2018).
- Alternatively, the (preventive) culling of reservoir and superspreader hosts has also been suggested (Langwig et al. 2015; Garner et al. 2016) as a feasible control mechanism to stop or slow the spread of disease emergence.
- An important action is to prevent the transport of pathogens via field equipment or terraria. The fungus has low tolerance to chemicals like 70% ethanol and desiccation (Johnson et al. 2003; Webb et al. 2007; Phillott et al. 2010; Gold et al. 2013; Van Rooij et al. 2017), and the disinfection of equipment is urgently warranted. As can be derived

from the above examples there is yet no universal safe and effective treatment to mitigate Bd across amphibian communities and ecosystems.

4.4 Host-pathogen-environment interactions

4.4.1 Pathogen

The susceptibility to Bd varies among and within amphibian species, across regions and according to the infecting Bd strain (Bosch et al. 2001; Farrer et al. 2011; Dang et al. 2017; O'Hanlon et al. 2018). It has been shown that Bd sensu stricto is composed of deep genetic lineages which are emerging through international trade in amphibians (O'Hanlon et al. 2018). Several Bd lineages exist that appear to co-exist with the local amphibian communities, suggesting host-pathogen co-evolution (Farrer et al. 2011; Rodriguez et al. 2014; Voyles et al. 2018; Bates et al. 2018). Simultaneously, BdGPL (Global Panzootic Lineage) is the most virulent strain known, but it also holds for this strain that the the population-level outcomes of infection are context dependent. Other strains than BdGPL may also cause population declines (O'Hanlon et al. 2018).

4.4.2 Host

Not all amphibian hosts are equally susceptible to an infection with Bd. The variation in susceptibility may result from innate and/or learned defence mechanisms/capabilities. Species responses to Bd exposure are variable (Smith et al. 2009; Crawford et al. 2010). Some hosts are highly susceptible such as several *Atelopus* species (La Marca et al. 2005) but others can persist, and carry Bd asymptotically like the American bullfrog (Garner et al. 2006), although also in this species the response to Bd infection is strain dependent and it is suggested that the bullfrog is a less efficient long-term carrier of Bd than has previously been considered (Gervasi et al. 2013). Even within species, there is variation in the interaction between host and pathogen, probably depending on strain and/or environmental conditions (Bosch et al. 2001).

Skin defence mechanism

Multifold studies are being conducted on the natural defence mechanism of the amphibian skin. Infection and colonization of the skin by Bd may be inhibited by the presence of antimicrobial peptides and symbiotic bacteria on the skin. As Bd attaches to the external surface of the skin, the presence of inhibitory bacteria and fungi in the skin mucosome could prevent establishment and growth of Bd (e.g. Brucker et al. 2008; Woodhams et al. 2007; Kueneman et al. 2016; Kearns et al. 2017). The natural defence mechanisms of amphibians and the antimicrobial role of the amphibian mucus is thoroughly reviewed in Rollins-Smith et al. (2011) and VanRooij et al. (2015). Briefly, antimicrobial peptides (AMP's), secreted from granular glands in amphibian skin form an important line of defense in amphibians, and there is a strong correlation between species-specific susceptibility for Bd and the effectiveness of AMP's in inhibiting Bd. A second line of defense against fungal infections are antifungal metabolites that are being secreted by symbiotic bacteria which may be present on amphibian skin (Harris et al. 2009).

Behavioural adaptation

Some amphibian species can adapt behaviourally to Bd. In an experimental set-up, specimens from two species of frogs learned to avoid the fungus after exposure and temperature-induced clearance (McMahon et al. 2014). Additionally, amphibians are capable of inducing behavioural fever to eliminate a Bd infection. Indications of 'behavioural fever' in the field come from

Richards-Zawacki (2010) who observed that Panamanian golden frogs (*Atelopus zeteki*) would raise their body temperature above normal during a Bd epidemic, by modifying their thermoregulatory behaviour. Likewise, Bd-infected metamorphic American toads (*Anaxyrus americanus*) behaviourally increased their body temperatures (Karavlan and Venesky, 2016), although Han et al. (2008) did not observe behavioural fever or altered thermoregulation in *Bufo boreas*, *Rana aurora*, *R. cascadae* and *Pseudacris regilla* Bd-infected larvae versus non-infected conspecifics. An infection with Bd may alter the thermal tolerance of the host up to 4°C. This increased heat sensitivity may discourage amphibians to perform adaptive behaviours, such as 'behavioural fever', even at temperatures in the noncritical range (Greenspan et al. 2017).

Evolution

The major histocompatibility complex (MHC) binds pathogen molecules and presents them to T-cells to activate destruction (Savage and Zamudio, 2016). The response of MHC class I and class II genes to an infection with Bd is variable (Fu and Waldman, 2017). Heritable Bd tolerance is evolving in some populations, but not in others (Savage and Zamudio, 2016; Fu and Waldman, 2017). The activation of an immune response may be costly for the fitness of an individual, so if Bd does not pose an immediate threat, the MHC alleles that confer disease resistance might not be selected (Fu and Waldman, 2017). Amphibians with specific major MHC genotypes seem to have an advantage when infected with Bd in their acquired immune response, and across populations MHC heterozygosity is a significant predictor of survival (Savage and Zamudio, 2011).

The observed variation in resistance between and within species is due to genetic variation and due to environmental factors which influence the interaction between host and pathogen (Ellison et al. 2015; Fu and Waldman, 2017). Recently it was found that Bd gene expression is strongly host specific, which results in host-dependent differences in Bd gene expression. The pathogen responds to a different host with shifts in resource allocation to either growth or reproduction, rather than responding through a short-term evolutionary process (Ellison et al. 2017). For a generalist pathogen as Bd this is advantageous as it allows quick shifts in pathogenicity (Brem et al. 2013). Likewise, there is a clear indication of host-pathogen evolution affecting disease dynamics. In Spain, the bacterial skin diversity of Pyrenean midwife toads (*A. obstetricans*) differed between epizootic and enzootic Bd situations, while there was no difference in Bd isolate (Bates et al. 2018), and in Panama 9 out of 12 species severely affected by Bd managed to recover in a period of 5 – 13 years by a shift in host response (Voyles et al. 2018).

4.4.3 Environment

Besides geographic variation in the distribution of hosts and Bd strains, another important factor influencing chytridiomycosis epidemics are the local environmental conditions (Savage et al. 2011). These conditions may influence growth rate and reproduction of Bd (Stevenson et al. 2013).

Naturally fluctuating environmental conditions might provide advantage for either host or pathogen, which is illustrated by Longo and Zamudio (2017). They found that the interplay between temperature and host-associated bacteria contributed to the infection outcome. Seasonal environmental fluctuations changed the diversity, composition and structure of the bacterial community in amphibian skin. During weather conditions favouring the host, frogs recruited beneficial bacteria, but during conditions favouring the pathogen, frogs shifted their defence strategy to tolerance (Longo and Zamudio, 2017).

It is suggested that cooler temperatures promote Bd outbreaks (e.g. Savage et al. 2011), as the release of Bd zoospores is induced by drops in temperature (Woodhams et al. 2008). In Australia, Retallick et al. (2004) observed a seasonal peak of infection in cooler months, but in Spain Bosch et al. (2007) showed that rising temperatures were linked to the occurrence of chytrid-related disease. Cohen et al. (2017) found that cold- and warm-adapted hosts had the greatest susceptibility for Bd at respectively warm and cool temperatures. This finding provides evidence for the thermal mismatch hypothesis[4], which might help explain the manifold observed variation in host' responses to infection with Bd. Host species may experience thermal stress outside their preferred temperature range, whereas parasites usually have broad thermal breadths. In amphibian disease dynamics, temperature-dependent immunity is important, and hence changes in environmental temperatures steer the immune system of the amphibian host away from an optimal level (Raffel et al. 2006).

Climate: Altitude and climate change

Bd has a pan-global distribution. Within this global distribution, there's a more restricted set of geographic regions where Bd has led to actual epizootic outbreaks, leading to the loss of amphibian biodiversity. The fungus has been found in a great diversity of habitats, but does best in relatively wet and cool habitats in tropical, temperate and in alpine areas (Berger et al. 2016). In Europe Bd is omnipresent as well (Garner et al. 2013), and lethal chytridiomycosis has been reported in both captive and wild amphibian species in Spain, Switzerland, Portugal, Belgium, Italy, Germany and in the United Kingdom (Mutschmann et al. 2000; Bosch et al. 2001; Stagni et al. 2004; Cunningham et al. 2005; Bosch and Martínez-Solano, 2006; Bielby et al. 2009; Pasmans et al. 2010; Tobler and Schmidt et al. 2010; Rosa et al. 2013). However, mass mortality events with strong impact on the host population have only been reported from Spain and Portugal where the common midwife toad suffered severe population declines due to chytridiomycosis (Bosch et al. 2001; Walker et al. 2010; Rosa et al. 2013). It is assumed that the observed strong decline of fire salamander in Peñalara Natural Park, a protected alpine area, several years after the initial collapse of the midwife toad, was also caused by chytridiomycosis (Bosch and Martínez-Solano, 2006).

The lethal occurrences of chytridiomycosis in European wild amphibians were observed at various altitudes (100 – 941 m a.s.l. in Italy and 450 m a.s.l. in Belgium (Stagni et al. 2004; Bielby et al. 2009; Pasmans et al. 2010) and 651 – 1955 m a.s.l. in Portugal (Rosa et al. 2013) and 1800–2430 m a.s.l. in Spain (Bosch and Martínez-Solano, 2006)). The simultaneous measurement of Bd infection rate in the Italian peninsula at sites above and below 800 m a.s.l. in three susceptible species showed an erratic infection pattern. Some species (*Mesotriton alpestris apuanus*) showed a higher prevalence at the higher sites, whereas others showed no relationship with altitude and infection status (*Salamandra salamandra gigliolii*, *Rana italica*, *Bombina pachypus*) (Zampiglia et al. 2013). It has been suggested that overwintering larvae may sustain Bd infection at higher altitudes (Catenazzi et al. 2013). In montane regions, the larvae may have a prolonged aquatic period before completing metamorphosis, or they may even overwinter in the water (Bosch et al. 2001; Bosch and Martínez-Solano, 2006; Briggs et al. 2010). These low temperatures do not

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[4] Thermal mismatch hypothesis posits that hosts should be more susceptible to parasites when environmental conditions shift away from the thermal optima of the host (from: Cohen et al. 2017).

protect the toadlets from an infection with Bd, and as such may provide a continuous reservoir of Bd for other more susceptible life stages (Catenazzi et al. 2013). Kriger and Hero (2008) found no evidence for a difference in either prevalence and infection intensity between high-altitude and low-altitude frog populations in Australia. They suggest that possibly the coinciding of metamorphosis and optimal temperatures for Bd growth, and the longer persistence of Bd at high altitudes may account for the declines of many southeast Queensland's montane frog populations. A recent study in the Peruvian Andes showed that at mid-elevation, where the amphibian declines were steepest, the proportion of anti-Bd isolates on amphibians' skin was lowest (Catenazzi et al. 2018). This association between anti-Bd isolates and elevation does not fully explain the observed variance of amphibian deaths at various altitudes, as the researchers found high proportions of anti-Bd isolates (40 and 45%) in two non-susceptible frog species, but counterintuitively, also a low proportion (13%) in a third non-susceptible host.

Climate change has been reported to be a key factor for the spread of Bd and a catalysator for the devastating impact (Bosch et al. 2007; Collins, 2010). Even small shifts in temperature or humidity within their noncritical range, may cause sharp or gradual added mortality in ectothermic vertebrates, by altering metabolic demands, ecological interactions, disease susceptibility, hydration, or foraging effort (Pounds et al. 1999; Rohr and Palmer, 2013). So, a change in climatic conditions shifts the amphibian host away from its optimal temperature through which the probability of increased host susceptibility to infectious disease might increase (Cohen et al. 2017). Presumably the largest risk from large scale and long-term climatic changes is the increase in climate variability, which makes climate less predictable (Raffel et al. 2013). Because of the increasing maximum temperatures over the years, climate change results in greater drops in temperature occurring on shorter timescales (Raffel et al. 2013). As Bd has a generation time of 4 – 10 days, it is likely to acclimatise faster to shifts in temperature than its amphibian host (the 'lag effect'), for which it takes 3 – 6 weeks for its immune system to adjust (Raffel et al. 2006). This allows the parasite to exploit this period of suboptimal host immunity.

Modelling studies on the geographic range of Bd in relation to the expected climatic changes, suggest that climate change reduces the geographic distribution of Bd (Rödder et al. 2010). In tropical and subtropical lowland regions, temperatures may rise above the thermal limit of the fungus, rendering these regions unsuitable for Bd, because transmission efficiency will be decreased by both a reduction in growth and fecundity of the fungus. In addition, temperate regions are likely to become less suitable as well, although this is contrasted by an expected increase in suitability for Bd in the Northern Hemisphere (Rödder et al. 2010). The presence of Bd does however not necessarily imply clinical disease, which is a sometimes underexposed topic in modelling, and different scenarios for the pathogen responses to climate change are likely (Altizer et al. 2013).

For instance, the harmful effect of UV-B on the amphibian immune response is well known (e.g. Blaustein et al. 1997; Kiesecker et al. 2001). Due to stratospheric ozone depletion, solar irradiance has been increasing at both temperate and polar latitudes (Kerr and McElroy, 1993; Madronich et al. 1998), and therefore one would expect an increasing susceptibility for infectious pathogens such as Bd, with increasing UV-B intensity. Nevertheless, Bd infection probability is either not (Garcia et al. 2006), or inversely correlated with the intensity of UV-B (Ortiz-Santaliestra et al. 2011). Also, the behaviour and natural thermal regime affects the vulnerability of frogs in becoming infected by Bd and this also affects the final outcome of infection (Stevenson et al. 2014). So even though amphibian flexibility is limited due to their small size and their dependence on environmental temperature and humidity (Burrowes, 2009), their behavioural plasticity may buffer climate and may reduce mortality during extreme climate events (Scheffers et al. 2014).

Invasive species

The most important role of exotic invasive species in the epidemiology of Bd is that of an environmental reservoir. Normally micro-parasites, such as Bd, demand high densities of susceptible hosts in order to be able to persist in the environment, but with the presence of non-clinical reservoir species that are able to disperse the pathogen into the environment for extended periods of time this demand is circumvented (Daszak et al. 1999).

Reservoir species of Bd, like the American bullfrog and crayfish (*Procambarus* spp.) are transported for trade around the globe and are able to disperse Bd into novel environments (Mazzoni et al. 2003; Schloegel et al. 2009). Concrete evidence for the role of the bullfrog as vector is lacking, and is even ruled out for Brazil, as it is for *Xenopus* (Rodriguez et al. 2014). Also, the trade in plants, fish or waterfowl can all potentially aid Bd in its spread (Johnson and Speare, 2003, 2005; Garmyn et al. 2012; Wimsatt et al. 2014). Crayfish have been identified as hosts of Bd (McMahon et al. 2013b) capable of transmitting Bd to susceptible amphibians for at least 12 weeks after their initial infection. Next to serving as vectors of Bd, presence of invasive exotics may also lead to changes in the host responses of local amphibian assemblages when Bd is introduced. An increase in the frequency and intensity of stress levels experienced by local amphibian communities as a result of introduction of invasive predatory exotics might not directly be related to prevalence and severity of infectious diseases in amphibians (Haislip et al. 2012; Reeve et al. 2013; Searle et al. 2014). However, it suppresses their immune system, and may synergistically exacerbate the effects of other environmental stressors (Relyea et al. 2005)

Pollution

The exposure of amphibians to chemicals in their habitat may have an adverse impact on their health and growth rate, and may alter predator-prey interactions (Battaglin et al. 2016). Alternatively, pathogens may also be killed, or their growth inhibited by pesticides (McMahon et al. 2013b). Because of the ubiquity of pesticide residuals in the environment, studying this relationship in-situ is important (Relyea et al. 2005), but the results may be contradictory. Jones et al. (2017) found no effects of sublethal pesticide mixtures and Bd on anuran survival and no effects of pesticides on Bd infection load, which is in concord with Reeves et al. (2017) who also observed a negative relationship between Bd zoospore abundance in water and neonicotinoid concentrations in surface water. The exposure of Cuban tree frog tadpoles (*Osteopilus septentrionalis*) to fungicides increased Bd-induced mortality, whereas in culture the tested fungicides killed Bd (Rohr et al. 2017). Exposure to these fungicides during their larval stages increased post-metamorphosis disease susceptibility. Even the use of itraconazole, which is frequently applied as a prophylaxis against Bd (Garner et al. 2009; Brannelly, 2014), increased the susceptibility of booroolong frogs (*Litoria booroolongensis*) for Bd (Rohr et al. 2017). Interestingly, in-situ measurements at several amphibian habitats across 7 states in the USA showed that Bd-positive frogs were associated with higher fungicide concentrations in water and sediment, but with lower insecticide concentrations in relation to Bd-negative frogs (Battaglin et al. 2016).

The spill of agricultural-derived phosphorus results in accelerated eutrophication of surrounding water bodies (Bennett et al. 2001; Ulén et al. 2007), which normally is considered to have a negative effect on local freshwater and marine environments through induction of harmful algal blooms (Paerl et al. 2001; Anderson et al. 2003). However, a limited increase in the trophic state of a water body can also positively influence zooplankton biomass (Johansson, 1983; Blancher, 1984). This in turn can lead to an increase in possible predators of Bd (Schmeller et al. 2014). A shared feature in regions were Bd has had an enormous impact on local amphibian assemblages is the pristine character of these water bodies (Kriger and Hero, 2006; Fisher et al. 2009). Here, the nutrient content is generally low, and –however counterintuitive - this could theoretically explain the dramatic effects of Bd seen in these environments due to low concentrations of Bd predators.

There is currently no conclusive evidence that environmental pollutants unambiguously influence local Bd epidemiology, but the combined effects of impaired host immunity, the exacerbating or abating effect on Bd, and the altered composition of local zooplankton or microbial communities may collectively impact site-specific Bd epidemiology.

Multiple pathogen exposure

Most amphibian disease studies focus on the interaction between a single host and pathogen, which does not reflect the complex reality in which amphibian hosts are exposed to simultaneous infections with various parasites that interact with the host and with each other. An increasing parasite diversity may increase the risk of disease in the host (Huang et al. 2016), or decrease the disease risk because of antagonistic parasites (Johnson et al. 2013). The increase of host and parasite richness reduced transmission success of the trematode *Ribeiroia ondatrae* from snails to frogs with 50% (Johnson et al. 2013). Co-infections are widespread and prevalent (Stutz et al. 2017), but the effects of parasite richness on the individual host is context-dependent (Johnson and Hoverman, 2012). The complexity and the relevance of context is shown by the outcomes of various studies. Infection by the trematode *R. ondatrae* was positively correlated with ranavirus and Bd, but infection by the trematode *Echinostoma* sp. was negatively correlated with ranavirus and Bd (Stutz et al. 2017). Rosa et al. (2017) found little evidence for an interaction between pre-existing infections with Bd and ranavirus emergence in Portugal, yet their cumulative impact threatens amphibian communities in the National Park. The Bd-tolerant *Xenopus laevis* (Weldon et al. 2004) exhibits clinical signs and lesions of chytridiomycosis when the animals are concurrently infected with mycobacteria (Hill et al. 2010). Underlying bacterial infections such as Chlamydiales or Mycobacterium infections (Reed et al. 2000; Hill et al. 2010) may at least in part explain why the related species *Silurana tropicalis* (formerly *Xenopus*) is sometimes considered susceptible to chytridiomycosis (Parker et al. 2002; Rosenblum et al. 2009), despite several failed attempts to reproduce clinical chytridiomycosis in otherwise healthy specimens of this species (Pasmans, unpublished results; James et al. 2009). In short, considering the frequent co-occurrence of multiple pathogens in amphibian communities, there is a need for a better understanding of coinfection dynamics.

5. Management

Emerging infectious diseases pose a complex and relevant threat to amphibian biodiversity globally, and countries respond to this problem differently (Box 1). Problems never come alone and never occur in isolation. It is therefore important to look at the entire suit of context-specific determining factors that make or break a successful infection. Also in Europe, and at a smaller scale in the NL, amphibians are decreasing rapidly, demonstrated by half of the 16 species being red listed as a species of high conservation concern. Half of the native species are either critically endangered (n=1), endangered (n=3), vulnerable (n=3) or near threatened (n=1). Currently the fire salamander can be considered as a functionally extinct species in the Netherlands considering its decline of 99.9% over the last few years (Goverse and de Zeeuw, 2017). The Living Planet Index Netherlands (CBS et al. 2018) indicates that generally species show a slight recovery from the historical decline pre-1990, apart from butterflies, freshwater fish and amphibians. Habitat has been lost because of the human population growth in the Netherlands from 5 to 17 million people between 1900 – 2015, and the expansion of land for agriculture (WNF, 2015). The combination of habitat fragmentation, desiccation, eutrophication and pollution have severely affected the quality of nature since 1900 (CBS et al. 2014), especially since the seventies and eighties of the last century. These problems all remain urgent, although improvements have been made. In the Netherlands the most pressing conservation problems are desiccation, the influx of alien invasive species and climate change in addition to the permanent effects of habitat-loss and fragmentation (WNF, 2015).

Successfully managing the challenges of emerging infectious diseases requires the understanding of the full ecological context of infection and transmission, and we need to look beyond the interaction between the single host and single pathogen (Johnson et al. 2015). The availability of monitoring data and absence/presence data predating a disease outbreak is needed to single out the impact of disease from that of other forces influencing population dynamics (LaDeau et al. 2007). If we are able to quantify the relative contributions of the diverse processes, we can define an ecological foundation for guiding resources into the appropriate management strategy.

Box 1. Overview of international Bd and/or Bsal Action Plans

B. dendrobatidis

Chytridiomycosis-related management is very often post-hoc `crisis management'. For instance, the first conservation plans for Bd were developed in 2005 and 2006 in Australia and the United States (Grant et al. 2017). In the global Amphibian Conservation Action Plan actions relating to the detection and control of chytridiomycosis are listed (Gascon et al. 2007). There are no national management or action plans in Europe and New Zealand with regard to Bd. In New Zealand it is mandatory to follow hygiene protocols in state-owned frog habitat. In Spain, regional species specific action plans are effective, trying to control or eradicate Bd from sites and to protect and conserve *Alytes obstetricans*, *A. muletensis* and *Salamandra salamandra* (J. Bosch, pers. comm.). Australia has updated its Treat Abatement Plan in 2016 (DEE, 2016). The overarching goal is to minimize the adverse impacts of amphibian chytrid fungus on affected native species and ecological communities. Financial support to reach the objectives is not covered completely by the Australian Government.

Both Sweden and Norway do not have action plans for Bd and/or Bsal (S. Kärvemo and A. Taugbol pers. comm.). In the UK syndromic surveillance and an EWS for Bd and Bsal is supported by Garden Wildlife Health, ARG UK and ZSL.

B. salamandrivorans

The response to Bsal in the US is pro-active, as a Bsal Task Force has been initiated (Grant et al. 2016), prior to the detection of Bsal in this continent. This Task Force consists of several working groups that meet regularly. They have developed a Rapid Response Template in order to facilitate all organizations and institutions that will need to respond to the pathogen. This template is intended to be incorporated in a National Bsal Strategic Framework (www.salamanderfungus.org).

An overarching European response to Bsal is assembled in www.BsalEurope.com. This project aims to establish a European EWS, delineate the current range of Bsal, develop an Emergency Action Plan and mitigation measures. In the European countries where Bsal has been detected in wild amphibians, the national response varies. There is no national or regional Bsal action plan in the Netherlands, but there is an Early Warning System (www.sossalamander.nl). In Belgium, a national action plan (2017-2022) is initiated and includes an emergency plan, an information network and a communication plan (FOD Volksgezondheid, 2017). In Germany a federal project is ongoing (Monitoring und Entwicklung von Vorsorgemaßnahmen zum Schutz vor der Ausbreitung des Chytridpilzes Bsal im Freiland) that should form the base for a future national Bsal management plan.

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Scientific Aims

Although native Dutch amphibians seem to be spared from population extinctions due to emerging infectious diseases, the impact of often experienced cryptic development of disease outbreaks might be more severe than previously considered. It is often felt that there is no ‘management perspective’ for in-situ amphibian disease management, and therefore information on the actual impact of emerging infectious diseases on native amphibian populations is required as well as on the interaction between host, pathogen and environment that ultimately dictate disease dynamics.

The general aim of this PhD thesis was to elucidate the presence and impact of chytridiomycosis on amphibians in the Netherlands and define practical conservation tools. To be able to define the urgency and required measures, several studies were conducted that contribute to the understanding of the ecological context of infection and transmission. This knowledge can be translated in conservation tools to mitigate the impact of disease on susceptible hosts.

The specific aims were:

I. To study the presence and impact of *B. dendrobatidis* in captive amphibian collections and wild populations in the Netherlands
II. To elucidate the cause of the observed mass mortality event in fire salamanders in the Netherlands
III. To examine the epidemiology of *B. dendrobatidis* and the novel causative agent for chytridiomycosis

Chapter 1

Clinically healthy amphibians in captive collections and at pet fairs: A reservoir of *Batrachochytrium dendrobatidis*

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Batrachochytrium dendrobatidis is the cause of the fungal disease chytridiomycosis, a potentially lethal skin disease of amphibians. Asymptomatically infected amphibians may pose a risk for environmental pathogen pollution. This study therefore assessed the role of healthy, captive amphibians as a reservoir of *Batrachochytrium dendrobatidis*. Samples were collected from captive amphibians in Belgium, the Netherlands, Germany and France (559 from anurans, 330 from urodelans and 4 from gymnophians) from private owners, zoos, and laboratories. In addition to which, 78 anurans from 19 living collections were sampled during a pet fair in the Netherlands. Nearly 3% of the captive amphibians were infected by *B. dendrobatidis*, and 13.6% of the collections yielded at least one positive result. At the fair, 7 out of 78 anurans, representing 2 collections were positive. None of the animals that tested positive showed any obvious health problems at the time of sampling. Our results demonstrate the potential of the amphibian pet trade as a vehicle for the spread of *B. dendrobatidis.*

Introduction

Chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (Bd), is an emerging infectious disease (Daszak et al. 2000) of amphibians that is causing mass mortality and population declines worldwide (Berger et al. 1998; Rachowicz et al. 2006). Bd is listed on the World Organisation of Animal Health (OIE) wildlife disease list and has been declared an international notifiable disease (Schloegel et al. 2010). A number of case reports describe the occurrence of clinical chytridiomycosis in captive amphibians worldwide. Since infection with Bd is not always accompanied by clinical disease (e.g. Daszak et al. 2004), asymptomatically infected amphibians might represent an important chytrid reservoir. Although supposedly common in amphibians in captivity, asymptomatic chytrid infection has been documented only on a few occasions in large scale studies (Frías-Alvarez et al. 2008; Goka et al. 2009). This study therefore assessed the role of healthy, captive amphibians as a reservoir of Bd.

Methods

Samples were collected from captive amphibians in Belgium, the Netherlands, Germany and France, between February and October 2009. A total of 559 swabs from anurans, 330 swabs from urodelans and 4 swabs from gymnophians were included in this study (Table 1). Two laboratories, 4 zoos and 38 private owners participated. In August 2008, 78 anurans from 19 owners were sampled during a pet fair (40th international frog day, organised by Dendrobatidae Netherlands (DN) in Haarlem, the Netherlands) for private owners (Table 2). All applicants were accepted as no distinction was made either in the size of a collection (= all amphibians from one owner or institution), the species concerned, or the background of the animals. Samples were taken as described by Hyatt et al. (2007) using cotton tipped swabs (MW102 sterile cotton-tipped dryswab™, MWE medical wire, UK). The swabs were stored with one silicagel granule (VWR International, NL) at −20°C until further analysis. All individuals were visually checked for disease symptoms or malformations. The owners were asked to report health problems in their animals. In one collection containing dendrobatid frogs with confirmed chytrid infection, animals were retested 4 months later. From this same collection, 50 ml. water samples were collected from the water basins of 5 terraria containing *B. dendrobatidis* infected frogs and of 5 terraria in which Bd was not detected in the frogs. These water samples were filtered and processed to quantify numbers of Bd as described in Kirshtein et al. (2007). For the quantification of Bd DNA in the samples, real-time Taqman PCR assays were conducted using an Applied Biosystems Prism 7700 Sequence Detection System (2009 samples) or Applied Biosystems Prism 7300 Sequence Detection System as described by Boyle et al. (2004). All samples were examined in duplicate. An animal was considered positive when both qPCR replicates yielded a ct value corresponding to at least 0.1 genomic equivalents.

Results and Discussion

Of the 893 samples from living collections of amphibians, 26 (2.9%) tested positive for Bd. Six of the 44 collections tested (13.6%) yielded at least one positive result. Apart from one collection in which both tested dendrobatid frogs were positive, the prevalence of Bd in six collections with at least one infected amphibian was 1.4% of 70 samples, 3.2% of 31 samples, 7.8% of 45 samples, 10.3% of 39 samples, 14.3% of 7 samples and 17.8% of 45 samples. Results are summarized in Table 1. Twenty one of the 559 (3.8%) anuran samples yielded a positive result. Twenty of these infected frogs were dendrobatid frogs (dendrobatid frogs s.l., including genera formerly included in the genus *Dendrobates*). Infected dendrobatid frogs were detected in 3 of 30 (10%) of the collections examined. All of these were private collections. In one dendrobatid collection with established Bd infection, 8 animals were still infected 4 months after the first sampling. One infected toad was found among recently confiscated and wild caught fire bellied toads (*Bombina variegata*) held in quarantine in a zoological institute. Five of the 330 urodelan samples (1.5%) examined were infected with Bd, including, a wild caught adult Kaiser's spotted newt (*Neurergus kaiseri*) derived from the pet trade, and four ambystomid salamanders. None of the infected urodeles showed any health problems at the time of sampling. Under the assumption that the observed animals and collections are a random sample from all captive amphibians and collections in Belgium, the Netherlands, Germany and France, the 95% confidence interval for Bd infection in captive amphibians is between 1.9%-4.2% and between 5.2%-27.4% for collections.We found at the fair in the Netherlands that, 7 out of 78 anurans, belonging to 2 out of 19 collections were infected with Bd (Table 2). None of the water samples contained Bd.

We show that chytrid infections are present in captive amphibian collections without a clinical history of chytridiomycosis. Moreover, repeated testing of positive frogs in a captive collection showed that 8 frogs remained positive for Bd over a four month period. Although there is no doubt that chytridiomycosis is capable of causing severe disease across a range of amphibian hosts, including dendrobatid frogs (e.g. Nichols et al. 2001) persistence of Bd without the occurrence of clinical signs as shown in the present study supports a context driven course of infection.

The overall incidence of Bd infection (2.9%) was low comparable with the results of a similar study in Japan (Goka et al. 2009), where prevalence was 28% in pet shops and 5.9% in institutions (zoos, aquariums and museums) and also low compared to the 100% infection rate in Catenazzi et al. (2010). This difference might be due to the amphibian populations sampled. Whereas we included animals from private captive collections, institutions and a pet fair, the Japanese study mainly included amphibians from pet shops and Catenazzi et al. (2010) sampled wild caught frogs traded at markets in the tropical Andes. The prevalence of chytrid infections in captive amphibians was lower than the prevalence in wild amphibians in Belgium (5.4%) and the Netherlands (3.7%; Spitzen-van der Sluijs et al. 2010) and lower than the prevalence in the susceptible midwife toad (*Alytes obstetricans*) in the Iberian peninsula (25% of the populations infected with a median prevalence of 0.63% in infected populations; Walker et al. 2010). Captive amphibians may nonetheless function as a reservoir for native amphibian populations, and they may introduce highly virulent strains that do not yet occur in Europe. Hence, amphibian keepers should test for the presence of Bd regularly and implement quarantine and biosafety measures for newly acquired animals, as is also suggested by Kriger and Hero (2009). Testing for Bd presence solely by water samples is not advisable because the sampling of the water basins of terrariums containing infected frogs did not yield any positive results. A sample size of 59 individuals gives a near 95% probability of detection if the prevalence of Bd is around 5% (DiGiacomo and Koepsell, 1986). In our study, the overall prevalence was 2.9% and sample size was only equal to or higher than 59 for 4 out of tested 23 families. It is therefore likely that some families tested falsely negative.

Table 1. Amphibian taxa from captive collections sampled in for the presence of *B. dendrobatidis* infection.

Order	Family	Number of positive animals / number of animals sampled	Number of positive collections / number of collections sampled	Positive tested species
ANURA	Amphignathodontidae	0/1	0/1	
	Alytidae	0/5	0/2	
	Arthroleptidae	0/1	0/1	
	Bombinatoridae	1/25	1/3	*Bombina variegata*
	Bufonidae	0/22	0/7	
	Dendrobatidae	20/398	3/30	*Dendrobates tinctorius;* *Phyllobates terribilis;* *Oophaga pumilio;* *Dendrobates leucomelas;* *Ranitomeya fantastica*
	Eleutherodactylidae	0/2	0/1	
	Hylidae	0/61	0/11	
	Hyperoliidae	0/4	0/1	
	Leptodactylidae	0/5	0/2	
	Mantellidae	0/3	0/2	
	Microhylidae	0/3	0/2	
	Pipidae	0/23	0/5	
	Pyxicephalidae	0/2	0/1	
	Ranidae	0/3	0/2	
	Rhacophoridae	0/1	0/1	
CAUDATA	Ambystomatidae	4/41	1/9	*Ambystoma mavortium;* *Ambystoma ordinarium;* *Ambystoma velasci*
	Cryptobranchidae	0/9	0/2	
	Hynobiidae	0/26	0/7	
	Plethodontidae	0/60	0/8	
	Salamandridae	1/193	1/16	*Neurergus kaiseri*
	Sirenidae	0/1	0/1	
GYMNOPHIONA	Caeciliidae	0/4	0/3	
TOTAL		26/893	6/44	

Most of the positive animals (77%) were dendrobatid frogs. This finding is not that curious, since this group had the largest representation in the tested animals, which is interrelated to the very intense worldwide trade in wild caught and captive bred dendrobatid frogs. The only other positive anuran was a wild caught *Bombina variegata* that was illegally collected in Croatia. This animal was allocated to a zoo that participates in the "Amphibian Ark initiative" of IUCN in order to maintain a captive breeding stock of endangered amphibian species ex situ.

Table 2: Amphibian taxa from a pet fair sampled in for the presence of *B. dendrobatidis* infection

Order	Family	Number of positive animals / number of animals sampled	Number of positive collections / number of collections sampled	Positive tested species
ANURA	Arthroleptidae	1/2	0/1	*Leptopelis vermiculatus*
	Dendrobatidae	4/67	2/18	*Dendrobates tinctorius;* *Epipedobates tricolor;* *Oophaga pumilio*
	Hylidae	1/5	1/4	*Phyllomedusa hypchondrialis*
	Hyperoliidae	1/3	1/1	*Hyperolius mitchelli*
	Pipidae	0/1	0/1	
TOTAL		7/78	2/19	

This finding emphasizes the risk of introducing disease in established amphibian colonies and confirms the need to impose strict quarantine measures for new animals. It appears that trade is indeed contributing to the global spread of Bd (Fisher and Garner, 2007) and may negatively affect both conservation and trade (Schloegel et al. 2010). Results from the pet fair confirm the presence of infected amphibians in pet trade.

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Chapter 2

Environmental determinants of recent endemism of *Batrachochytrium dendrobatidis* infections in amphibian assemblages in the absence of disease outbreaks

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The inconsistent distribution of large-scale infection mediated die-offs and the subsequent population declines of several animal species, urges us to understand how, when, and why species are affected by disease. It is often unclear when or under what conditions a pathogen constitutes a threat to a host. Often, variation of environmental conditions plays a role. Globally *Batrachochytrium dendrobatidis* (Bd) causes amphibian declines; however, host responses are inconsistent and this fungus appears equally capable of reaching a state of endemism and subsequent co-existence with native amphibian assemblages. We sought to identify environmental and temporal factors that facilitate host–pathogen coexistence in northern Europe. To do this, we used molecular diagnostics to examine archived and wild amphibians for infection and general linear mixed models to explore relationships between environmental variables and prevalence of infection in 5 well-sampled amphibian species. We first detected infection in archived animals collected in 1999, and infection was ubiquitous, but rare, throughout the study period (2008–2010). Prevalence of infection exhibited significant annual fluctuations. Despite extremely rare cases of lethal chytridiomycosis in *A. obstetrican*s, Bd prevalence was uncorrelated with this species' population growth. Our results suggest context dependent and species-specific host susceptibility. Thus, we believe recent endemism of Bd coincides with environmentally driven Bd prevalence fluctuations that preclude the build-up of Bd infection beyond the critical threshold for large-scale mortality and host population crashes.

Introduction

Large-scale die-offs of wild animal species resulting in population declines have been caused by a number of emerging or re-emerging infectious diseases that are characterized by efficient pathogen transmission and build-up of lethal pathogen loads in infected hosts (e.g. Foley et al. 2011; Lawson et al. 2012). Such pathogens are recognized as serious conservation threats (e.g. Daszak et al. 1999; Kock, 2006), but even lethal pathogens may not, under some circumstances, cause significant illness and mortality in susceptible hosts (Woolhouse, 2002). The interactions and processes underlying a disease outbreak are complex and vary between geographic regions. Thus, situations where significant mortality attributable to an emergent wild animal pathogen is detected may not constitute a conservation threat to susceptible hosts. Because the occurrence of a potentially threatening pathogen does not equate with threat and conservation efforts to mitigate infectious diseases are costly, labor-intensive, and take extensive time and effort to develop (Cross et al. 2007; Harrison et al. 2010), the risk of disease should be assessed locally to ascertain if a threat exists. This requires putting disease in the context of the local environment because the value of predictive modeling for disease outbreaks increases substantially when parameters affecting local host species-specific infection dynamics are considered at a local scale (e.g., Paaijmans et al. 2009).

Chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (Bd), is a prime example of an environmentally driven disease that has caused global loss of vertebrate biodiversity. The recent and relatively concurrent occurrence of global amphibian declines due to chytridiomycosis and the detection of a globalized, hypervirulent, and newly evolved genetic lineage of Bd indicate that it is a novel, emerging, pandemic pathogen (Vredenburg et al. 2010; Farrer et al. 2011). Some amphibian assemblages persist with potentially lethal Bd, but coexistence may or may not indicate a lack of threat. Australian amphibian species experience persistent mortality from chytridiomycosis that may lead to host declines (Phillott et al. 2013). In Europe, host responses to Bd infection are geographically and taxonomically inconsistent and are influenced by environmental factors (e.g. Walker et al. 2010; Garner et al. 2011; Baláž et al. 2013) or strain dependent variation in virulence (Fisher et al. 2009; Farrer et al. 2011). This last finding suggests some forms of Bd infected amphibians long before the 20th century global amphibian declines occurred (Farrer et al. 2011; Schloegel et al. 2012), a scenario which should result in endemic, rather than threatening, host–pathogen dynamics. Because some ecological conditions result in low virulence, the pathway to nonthreatening, endemic dynamics is likely mediated by environmental factors.

Northern European countries provide an opportunity for determining what conditions lead to endemism because chytridiomycosis-driven mass mortality and species decline has yet to be reported despite the presence of Bd (e.g. Scalera et al. 2008; Tobler et al. 2012) and the occurrence of rare Bd-caused mortality in susceptible hosts such as the midwife toad (*Alytes obstetricans*) (Pasmans et al. 2010). We investigated whether Bd has reached a state of endemism in northern Europe and identified environmental factors steering infection dynamics toward host–pathogen coexistence. Specifically, we estimated the time of Bd arrival in this region, determined spatial and temporal distribution of Bd infections in northern European amphibian communities over 3 years, determined the population dynamics of a sentinel species and how infection might be related, and identified environmental determinants defining Bd infection dynamics in the study region.

Methods

Sampling for Bd

We sampled preserved amphibians from 2 natural history museums in the Netherlands, the Zoological Museum Amsterdam and Naturalis in Leiden. Sampled specimens dated from 1837 to 2007. We collected 116 swabs from 409 individuals (Supporting Information). Because of the potential for cross-contamination among animals stored in the same container, we pooled specimens so stored and treated a positive test for any one individual from a container as a single positive. On average 3.5 individuals were pooled per swab (range 1–29, 13 swabs were pools of 6 or more individuals).

For 3 consecutive years, we sampled breeding sites of amphibians across Belgium and the Netherlands (Figure 1). Our sampling criterion for selecting breeding sites was opportunistic in that we sampled ponds where amphibians were known to occur (Supporting Information). However, each year we focused on specific questions and target hosts and sampled guided by the principles outlined by Hyatt et al. (2007) and Skerratt et al. (2008). In 2008 (August through October) we sampled invasive, nonnative *Lithobates catesbeianus* (American bullfrog) in Belgium. In 2009 (March through September) we sampled both native and nonnative amphibians at breeding sites across the Netherlands and Belgium (in Flanders). From March to October 2010, we focused on 2 species (*Alytes obstetricans* and *Bombina variegata*) that are highly susceptible to infection (Baláž et al. 2013). For this survey, we sampled a smaller geographic area (province of Limburg in the Netherlands) more intensively per unit area. In total, 279 breeding ponds were sampled during the 3 years of field surveillance. We distinguished between 4 life history stages: larvae, juveniles before their first hibernation, subadults (stage between the first hibernation and breeding condition), and adults (sexually mature). To minimize the possibility of false positives, we temporarily held each specimen separately and followed standard biosecurity measures. Animals were handled while wearing a fresh pair of nonpowdered, disposable vinyl gloves. Equipment and field clothing was cleaned and disinfected between visits to sampling locations. To detect infections, we collected samples and swabbed the superficial skin surface of metamorphosed animals, the mouthparts of larval anurans, or the toes of larval caudates (MW102 sterile cotton-tipped dryswabTM; MWE medical wire, U.K.).

Acquisition of Environmental Data

Weather data were obtained from weather stations nearest to each sampling location (range: 0.9– 32 km, mean: 13.0 km; Royal Netherlands Meteorological Institute, www.knmi.nl). Because interpolated weather data were nearly 1:1 correlated with the nearest neighbor values (see Supporting Information), we used the latter in the analyses. We calculated mean, minimum, and maximum daily temperature; sum of daily precipitation; mean, maximum, and minimum humidity (daily relative atmospheric humidity); and mean daily UV radiation (global radiation) estimated over 2 periods: 7 and 30 d prior to each individual swab date (Kriger and Hero, 2007; Rödder et al. 2008; Walker et al. 2010; Ortiz-Santaliestra et al. 2011).

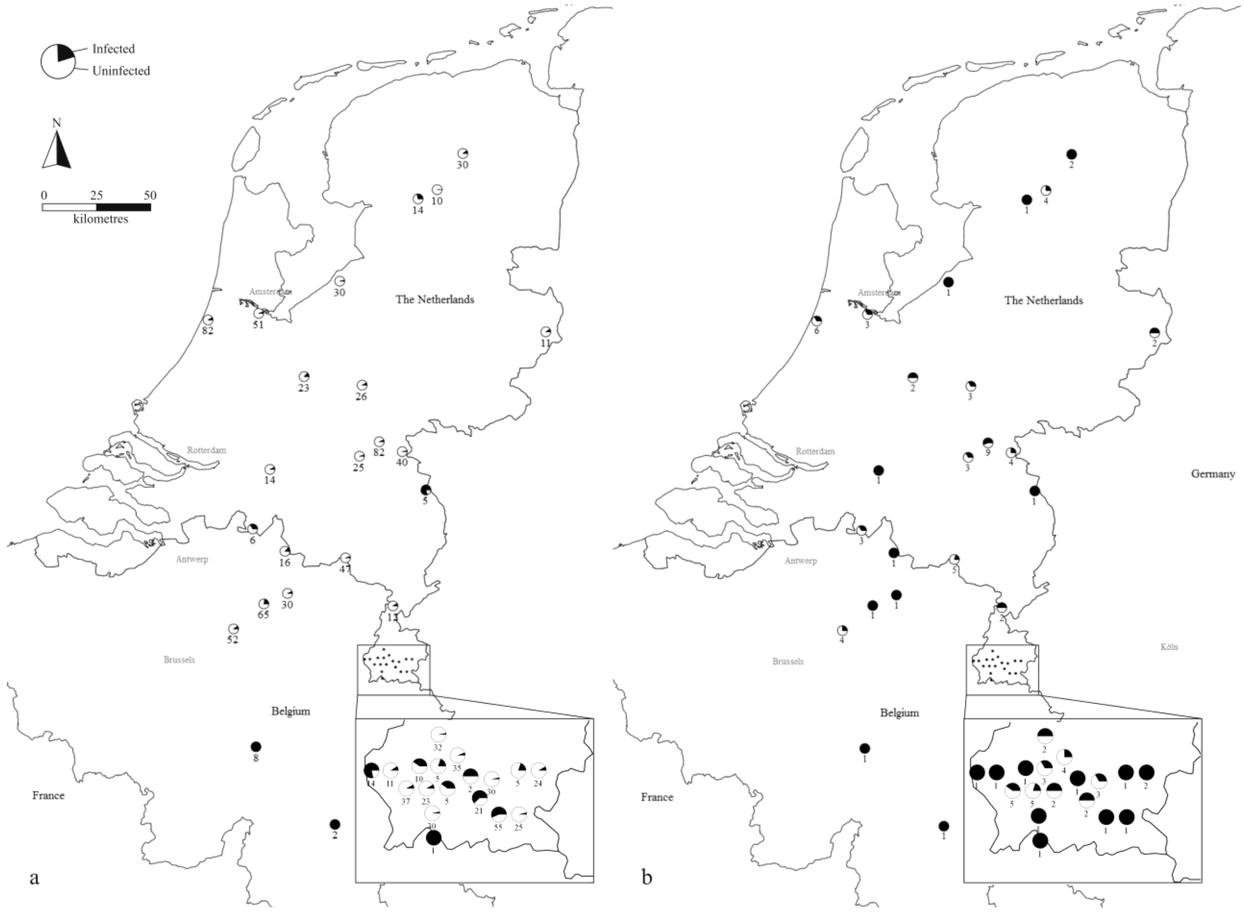

Figure 1. The proportion of (a) infected individuals and (b) infected species per sample location (location indicated by placement of pie charts*)*.

The time spans were selected as representative of the time scale over which infection dynamics and Bd patterns of growth are known to vary significantly (Stockwell et al. 2010; Garner et al. 2011). We classified the water bodies into 5 categories: pool, pond, ditch or watercourse, garden pond, and land. Depth of water bodies was measured at the deepest point, and we categorized the perimeter of all ponds and pools as 0–9 m, 10–19 m, 20–99 m, 100–199 m, or ≥200 m. Based on our field data, we classified the predominant landscape within which the water body was embedded as urban (n=14), village (n=15), agricultural or rural (n=78), or nature reserve (n=171) and assessed the effect proximity to urban areas had on prevalence. Because close proximity to people may increase infection rates (St-Amour et al. 2008), we incorporated the Euclidean distance from sampling location to the nearest urban area (Corine land cover database [European Environment Agency], 100-m resolution).

Laboratory Analysis of Samples

All swabs tested were stored at −20°C until DNA extraction. For the quantification of Bd DNA, we first extracted all swabs following the protocol of Boyle et al. (2004). Quantitative PCR (qPCR) was carried out with a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Extractions were diluted 1/10 and all assays were performed in duplicate. Samples were considered positive for Bd when there was a clear log-linear amplification and both repeats were above the detection limit >0.1 mean genomic equivalents (GE), which for analyses we treated as mean infection intensity.

Statistical Analyses

We defined Bd prevalence as the number of positive amphibians in a category divided by the total number of amphibians sampled in that category (Table 1). We compared variation of prevalence between species and life history stages over all years with χ^2 tests and estimated differences in infection intensity between species by means of univariate analysis of variance (ANOVA). We used generalized linear mixed models (GLMM) with a binomial error structure to assess monthly and yearly variation in Bd prevalence. Due to our surveillance strategy, not all ponds were sampled each year or repeatedly within a year. Because of this, we restricted this analysis to a subset of sites for which we had repeated samples for 2009 and 2010 (n=22 water bodies; all located in the Netherlands). For this analysis we used data for all species and life history stages for the 22 water bodies. We accounted for possible non-independence of swabs collected at the same locations by including a random intercept effect for each unique pond and a nested random intercept and year effect for species. Finally, we added month and year as fixed effects. We did not expect serial autocorrelation to be present in the data because swab samples were predominantly taken at 40-d intervals.

We tested whether weather covariates were correlated with Bd prevalence in the Netherlands in 5 species (*A. obstetricans*; *B. variegata*; *B. bufo*; *Ichthyosaura alpestris* [alpine newt]; and *Pelophylax* spp.), which we selected based on data sufficiency (>200 individuals sampled per species, with a prevalence >1% per species, sampled in both 2009 and 2010; Table 1) and diversity in species traits. We pooled data for all water frog species (*Pelophylax* spp., *P.* kl. *esculentus*, and *P. lessonae*) to increase the sample size for this taxon. For this analysis, infection status (Bd detected or not detected) was used as the unit of analysis, and we constructed an a priori set of competing GLMMs followed by model selection as per Burnham and Anderson (2001). We started by specifying the global model, a model in which we included all weather variables described above. We also specified all possible subsets (simplified models) of the global model. From this model list, we excluded all models that included confounding pairs of covariates (absolute correlation coefficient > 0.6) and ensured that covariates in the models were measured over 30 or 7 d. This resulted in a set of 47 candidate models for each species, ranked according to their Akaike's Information Criterion (AIC) values. We based our conclusions regarding model reporting as suggested by Burnham and Anderson (2001); all models with ΔAICc ≤ 2 (AICc, correction Akaike's Information Criterion). Because an individual's infection status is not independent of the infection status of other amphibians in a pond (Han et al. 2008), we included a random intercept effect for each individual pond to account for possible nonindependence.

To assess how characteristics of water bodies correlated with infection metrics, we pooled swabs (all ponds, species, and stages over all years) and conducted logistic regression (LR) analysis with number of infected and uninfected individuals (binary trials) as the response variable. Because correlation among depth and perimeter was <30%,we treated them as independent. The number of amphibian species detected in a pond was included as an additional, independent covariate. In addition, we evaluated all possible pairwise interactions by adding each interaction term (one at a time) to a model including all main effects and investigated how the deviance changed by means of AIC and LR. None of the interactions proved significant or could be estimated with sufficient accuracy. The model was subsequently simplified until only significant terms (following LR) were retained.

Because *A. obstetricans* is the most susceptible species in this study region, we calculated the average annual population growth rates of all populations we sampled for Bd presence. These populations are annually monitored following a standardized method (Goverse et al. 2006), where all breeding ponds are visited once yearly between May and October and larvae are counted. We estimated the average annual population growth rate over the years 2001–2010 as the average slope of population size over time. We considered larval counts of each population separately. We used GLM models with Poisson error distribution and a log link to relate the counts to the year the sample was taken (as a continuous covariate). We present the exponent of the slope for each population. We calculated the correlation coefficient between Bd prevalence and population growth. When amphibians were found dead in the study region, they were subject to necropsy and histopathology and microbial analyses using standard protocols as described elsewhere (Pasmans et al. 2010). Clinical chytridiomycosis was diagnosed based on evidence of a heavy infection, typical histological lesions, and the absence of other obvious causes of death.

Results

The oldest and only Dutch museum sample where we detected Bd infection came from a natterjack toad (*Epidalea calamita*) collected in 1999 near Amstelveen in the west of the Netherlands (52°18′34.70′′N; 4°53′36.53′′E; GE=14.2). Infection was confirmed through immunohistochemical staining (Berger et al. 2002).

We collected and processed 3050 swabs (Belgium n=273; the Netherlands n=2777), including all native amphibian species, apart from the marsh frog (*Pelophylax ridibundus*) (Table 1). Of all swabs, 4.8% tested positive for Bd DNA. Estimates of GE were not exceptionally strong or weak (mean GE [SE]=16.6 [4.9]). Infection was detected across a broad geographic range (46 of 279 sites were infected; Figure 1) and a broad range of hosts (10 of 18 hosts were infected; Table 1). Prevalence differed significantly among species ($\chi^2=133.9$, $P<0.001$, df=14), among life history stages ($\chi^2=46.8$, $P<0.001$, df=3), and between caudate and anuran amphibia ($\chi^2=47.5$, $P<0.001$, df=2).

Table 1. *Batrachochytrium dendrobatidis* infection prevalence and infection intensity in amphibians collected in Belgium and the Netherlands from 2008 through 2010.

Species	Year	Sample size (l/m)[a]	Percent individuals Bd positive (n)	Mean infection intensity (SE, range)	Fraction infected sites
Native					
Salamandra salamandra	2009	12 (5/7)	0	-	0/5
	2010	-	-	-	-
Ichthyosaura alpestris	2009	250 (51/199)	3.2 (8)	13.3 (8.5, 0.1-54)	3/43
	2010	134 (131/3)	0.7 (1)	1.27	1/10
Triturus cristatus	2009	74 (32/42)	0	-	0/13
	2010	5 (0/5)	0	-	0/1
Lissotriton helveticus	2009	60 (0/60)	0	-	0/4
	2010	-	-	-	-
Lissotriton vulgaris	2009	415 (46/369)	1.0 (4)	0.8 (0.3, 0.3-1.5)	2/59
	2010	19 (7/12)	0	-	0/6
Alytes obstetricans	2009	245 (212/33)	22.9 (56)	13.1 (5.4, 0.1-261)	10/30
	2010	255 (236/19)	1.6 (4)	0.84 (0.31, 0.3-1.67)	5/23
Bombina variegata	2009	200 (0/200)	7.5 (15)	42.8 (39.4, 0.008-594)	2/38
	2010	243 (0/243)	2.1 (5)	68.3 (32.7, 0.8-154)	4/33
Pelobates fuscus	2009	9 (0/9)	0	-	0/4
	2010	1 (0/1)	0	-	0/1
Bufo bufo	2009	202 (6/196)	4.0 (8)	9.1 (5.2, 0.7-43.9)	2/26
	2010	6 (0/6)	0	-	0/1
Epidalea calamita	2009	62 (0/62)	0	-	0/6
	2010	5 (0/5)	80 (4)	6.5 (2.8, 1.6-14)	1/1
Hyla arborea	2009	77 (4/73)	2.6 (2)	26.8 (25.7, 1.2-52.5)	2/11
	2010	-	-	-	-
Rana arvalis	2009	47 (0/47)	0	-	0/4
	2010	-	-	-	-
Rana temporaria	2009	155 (8/147)	0.6 (1)	4.7	1/41
	2010	9 (0/9)	0	-	0/3
Pelophylax spp.	2009	111 (18/93)	5.4 (6)	4.4 (2.8, 0.2-18.2)	3/16
	2010	26 (17/9)	0	-	0/2
Pelophylax klepton *esculentus*	2009	224 (1/223)	4.9 (11)	12.0 (8.2, 0.008-90.6)	7/39
	2010	8 (0/8)	0	-	0/1
Pelophylax lessonae	2009	85 (0/85)	4.7 (4)	26.6 (23.0, 0.2-95.4)	3/15
	2010	1 (0/1)	0	-	0/1
Non-native					
Xenopus laevis	2009	10 (0/10)	0	-	0/1
Triturus marmoratus	2009	1 (0/1)	0	-	0/1
Triturus carnifex	2009	11 (0/11)	0	-	0/3
Lithobates catesbeianus	2008	88 (82/6)	20.5 (18)	10.6 (5.9, 0.01-107.9)	7/16
Total		3050	4.8 (147)	16.6 (4.9, 0.008-594.0)	

[a] (l/m) = number of larvae/number of metamorphosed specimens. Post-metamorphic life stages were pooled for convenience of comparison and because Bd is supposed to have the largest impact during metamorphosis.

[b] *Pelophylax spp.* includes all water frogs that could not be reliably identified to species.

Of all 981 caudates, 13 (1.3%) tested positive, whereas 134 of 2069 (6.5%) anurans tested positive. Prevalence increased as age decreased (2.9% of adult amphibians infected [51 infected out of 1744]; 6.7% of subadults [23 of 342]; 8.0% of juveniles (9 of 103]; 9.3% of larvae [75 of 809). Total number of samples does not add up to 3050 because some swab labels did not indicate life stage. Prevalence in anuran larvae (11.9%) was significantly higher than in metamorphosed anurans (4.7%, sum of juveniles, subadults, and adults; χ^2=32.2, P<0.0001, df=1), but prevalence between caudate larvae (0.4%) and metamorphosed caudates (1.7%; χ^2=3.4, P=0.065, df=1) did not differ. At 5 locations more than one species was infected, but in 26 of 46 sites only one species was sampled (Figure 1). Mean infection intensity did not differ among host species ($F_{1,3}$=103.9, P=0.965). Of the 4 nonnative species, only *L. catesbeianus* tested positive for Bd at half the sites we sampled where the species occurred (3 of 6 sites). Although GE values for this species were again moderate (10.6±5.9), prevalence was relatively high (20.5%).

Prevalence in 2010 was significantly lower than in 2009 (χ^2=17.2, P<0.001, df=1). No such pattern was detected among months within years even when variation among species and years was accounted for (χ^2=3.2, P=0.517, df=4; Supporting Information). Interannual dynamics of 3 well-sampled species further illustrate this. In 2009 overall prevalence in *A. obstetricans* was highest out of all species (n=245, 22.9% infected; mean GE [SE]=13.1 [5.4]) and 4 of 10 *A. obstetricans* populations contained infected individuals. Eighty-seven percent of *A. obstetricans* that we sampled were tadpoles, but there was no significant difference in prevalence among larval and postmetamorphic life history stages (χ^2=2.8, P=0.095, df=1). Postmetamorphic life stages were pooled to obtain sufficient sample size. Prevalence of Bd in *A. obstetricans* in the same 10 populations in 2010 was 1.57% (n=255 specimens of which 92% were larvae, no difference in prevalence among life history stages; χ^2=0.4, P=0.554, df=1; mean GE=0.84 [0.31]). Infection was not detected at 1 of the 4 locations where infection had been detected in 2009. Overall prevalence in *B. variegata* in 2009 was 7.5% (n=200; mean GE=42.8 [39.4]), and we detected infected frogs at 2 out of 5 of the *B. variegata* sites. In 2010 prevalence was 2.1% (total decrease −72%; n=244; mean GE=68.3 [32.7]), but infection was detected at a site where no infection was detected in 2009. In both years and at the same 5 sites only postmetamorphic individuals were sampled (Table 1). Prevalence in *I. alpestris* was 78% lower in 2010 than in 2009. Although here different populations were sampled between years and the proportionate change was substantial, the true change in prevalence was actually small for this species (2009; n=250 of which 20% were larvae, prevalence 3.2%, mean GE=13.3 [8.5] and 2010; n=134, 98% larvae, prevalence 0.7%; mean GE=1.27).

Response of Bd prevalence to weather covariates was inconsistent across species. For *B. variegata* and *I. alpestris*, none of the weather covariates improved model fit over an intercept-only model, suggesting that prevalence of Bd was unaffected by weather for those species. Although several models proved a better fit than the intercept only model for *Pelophylax* spp., there was no obvious pattern in weather covariates included in the top models. Similarly, top *B. bufo* models were inconsistent in terms of environmental parameters. However, all models with good fit (ΔAIC<2) involved weather metrics recorded 7 d prior to swab sampling. A similar pattern emerged for *A. obstetricans*, but for this species weather predictors measured 30 d previous to the date of swabbing were consistently involved in best-fit models (Supporting Information).

Table 2. Results of logistic regression of the main habitat effects and interaction terms of human proximity, amphibian biodiversity, water depth, landscape, water body circumference and water body on *Batrachochytrium dendrobatidis* infection metrics.

	df	Deviance	P (χ^2)	Coefficient estimate	SE
Intercept[a]		352.18			
Distance urban area – sample site	1	388.29	0.000		
No. of species	1	385.72	0.000		
Water depth (cm.)	1	356.77	0.032		
Area type	3	393.66	0.000		
village				-0.532	0.469
nature area				-1.272	0.221
urban				-1.920	0.617
Circumference (m)	4	405.70	0.000		
10-19				0.880	0.959
20-99				1.377	0.930
100-199				-1.937	0.913
200≥				2.282	9.618
Water body type	4	391.05	0.000		
pond				-3.834	0.923
ditch or watercourse				-21.247	788.820
garden pond				-4.917	1.008
pool				-3.372	1.002

[a] The intercept (estimate 0.880 with SE 0.959) for prevalent landscape type is the category agricultural area for perimeter 0–9 m. for the water body category the intercept was "on land."

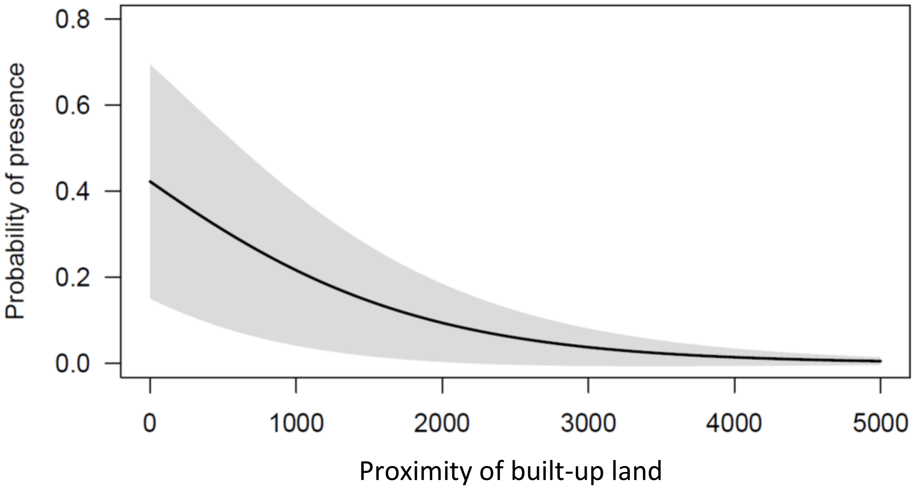

Figure 2. The relationship between the proximity of built-up land to ponds and the probability of *Batrachochytrium dendrobatidis* presence

We detected significant effects of physical characteristics of sample sites, amphibian community, and landscape on prevalence (Table 2). The presence of Bd was negatively correlated with distance to built-up land (Figure 2), and Bd prevalence increased when the perimeter of water bodies was large and as the number of amphibian species increased. Prevalence of Bd also varied significantly between water types: amphibians on land (n=16) were most likely infected, whereas amphibians sampled in ponds (n=223), pools (n=8), and garden ponds (n=24) were similarly likely to be infected. Prevalence of Bd was greater in agricultural areas than in nature areas, which in turn was higher than in urban areas.

Growth rate of A. *obstetricans* populations did not correlate (ρ=0.35) with Bd prevalence (Supporting Information). Chytridiomycosis associated mortality was diagnosed in 2 dead juvenile *A. obstetricans* with high GE loads (3600 GE) (N 50°50′09″, E 5°46′87″, 105-m a.s.l.).

Discussion

We demonstrated that Bd can be broadly present in amphibian communities at low infection loads and relatively low prevalence in most species. Even in the highly susceptible host *A. obstetricans*, Bd prevalence and infection intensity was near zero in tadpoles and was consistently well below prevalence observed in study regions where Bd exerts a marked negative impact on *A. obstetricans* populations (Walker et al. 2010). In our study area, Bd does not follow the pattern of a rapidly spreading pathogen reaching persistent saturation quickly in susceptible hosts (Vredenburg et al. 2010). Rather, the relatively low and fluctuating prevalence coincided with erratic and cryptic mortality (Pasmans et al. 2010; this study) but without population declines.

Bd is one of several important pathogen threats to biodiversity that has become globalized, but despite its widespread presence, infection and population declines occur nonrandomly (Smith et al. 2009; Baláž et al. 2013). This indicates that species-specific traits are affecting pathogen distribution, prevalence, and disease susceptibility, which may ultimately affect the species composition in communities. However, not all variation can be attributed to host specific responses to exposure and infection (Walker et al. 2010; Baláž et al. 2013). In our system, Bd has been present for at least 13 years at low levels without prevalence increasing to potentially threatening levels. These results are in line with the first reported cases of infection and disease in other European countries (Garner et al. 2005). Presence preceding 1999 cannot be excluded because fixation history was not known for all specimens, and some may have been originally fixed in formalin, which reduces the chance of detecting Bd (Soto-Azat et al. 2009). Although mass mortality events in amphibians do occur in our study area, none have been attributed to chytridiomycosis (Kik et al. 2011; Martel et al. 2012; 2013). Hypotheses explaining the observed pattern of infection and lack of severe costs associated with Bd presence include the fungus is endemic and either exhibits reduced virulence or host species exhibit increased tolerance or resistance, or Bd is recently arrived but has yet to firmly establish or reach a critical threshold where more detectable costs to hosts can be detected. Both have been suggested as possible explanations for the distribution and effects of Bd (e.g., Rachowicz et al. 2005; Stockwell et al. 2010). Our results support the first hypothesis, suggesting that arrival of Bd in amphibian communities containing susceptible host species does not necessarily result in wave-like declines (Vredenburg et al. 2010). Instead, Bd may have reached an equilibrium state in our region, and we hypothesize that the current environmental context precludes the build-up of infection loads and prevalence beyond the threshold necessary to induce mortality and decline.

Bd infection may have disappeared in one *A. obstetricans* population, and reduced prevalence over the course of our study was also observed in 2 other host species. A wide geographic distribution is difficult to reconcile with unstable pathogen presence and the possibility of local pathogen extirpation without substantial capacity for repeated pathogen introductions. This could be accomplished through the presence of an alternative host (e.g., Johnson and Speare 2005; Garmyn et al. 2012; McMahon et al. 2013); however, this is far more likely in a system where prevalence is high and where, accordingly, the probability of a migrant being infected is also high. Our results highlight the need to incorporate species-specific (micro)climatic data in predictive models, and climatic differences between regions may at least in part explain why a single species such as *A. obstetricans* suffers marked losses in some regions (Bosch et al. 2001) but not in others (our study). However, for most species, weather conditions failed to explain Bd infection dynamics. For these species, environmental characteristics that could be expected to promote establishment and persistence of Bd infection at the community level predicted Bd prevalence: relatively large ponds that support greater amphibian diversity harbor more susceptible host species, and are located close to or influenced by human activities. The positive relationships between human activities, artificial water bodies, and the detection of infection suggests that repeated introductions, if occurring, are mediated anthropogenically, including the possibility of direct introductions of infected amphibians. Certainly, introductions of suitable hosts in the region are not uncommon, often are undetected, and are associated with amphibian trade (Holsbeek et al. 2010). Nonnative North American bullfrogs sampled in this study exhibit disproportionately high prevalence relative to the study mean. Other studies also underline the positive association between human proximity and Bd presence (St-Amour et al. 2008; Adams et al. 2010), but Pullen et al. (2010) found that urbanization has a minimal impact on the prevalence of Bd.

Our finding of differential, context-dependent host susceptibility to Bd is supported by Doddington et al. (2013) and may be a pattern exhibited by other fungal pathogens that may threaten wildlife hosts (Fisher et al. 2012). The fact that threatening pathogens are not ubiquitously threatening, directly affects the framing and development of conservation efforts to mitigate infectious diseases. Surveillance for threatening disease must involve long-term monitoring that includes an assessment of local environmental conditions because changing environmental conditions could shift the balance from coexistence to significant mortality in some species but not in others. Furthermore, even endemic chytridiomycosis can still result in mortality that can reduce the probability of population persistence (Phillott et al. 2013). The apparent coexistence between Bd and the Dutch and Belgian amphibian communities is in sharp contrast with the recent emergence of the related chytrid fungus *B. salamandrivorans* (Martel et al. 2013) that caused near extinction of fire salamanders (*Salamandra salamandra*) in the region (Spitzen-van der Sluijs et al. 2013). Highly divergent disease dynamics of 2 related and potentially lethal pathogens in the same amphibian populations further emphasize the importance of small scale host, pathogen, and environment interactions in the eventual disease impact at the population level. Studying the patterns of local disease dynamics is crucial to understanding how extinctions affect biodiversity at larger spatial scales; hence, we stress the exigency to identify how local factors may exacerbate or reduce the impact of an infectious disease.

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Supporting Information. Results of the screening of museum specimens (Appendix S1), sampling intensity and prevalence per species per year and month (Appendix S2), output of the model selection with competing GLMMs (Appendix S3), correlation graphs interpolated weather data (Appendix S4), fluctuations in prevalence and sampling intensity (Appendix S5), and correlation between Bd prevalence and *A. obstetricans* population growth rate (Appendix S6) are available online. The authors are solely responsible for the content and functionality of these materials. Queries (other than absence of the material) should be directed to the corresponding author.

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Appendix S1

Results of retrospective screening of museum specimens. The number of positive swabs and the total number of tested swabs (x/y) per multidecadel time period are reported.

Species	1837-1900	1901-1920	1921-1940	1941-1960	1961-1980	1981-2000	2001-2007
Salamandra salamandra	0	0/1	0	0	0	0/1	0
Ichthyosaura alpestris	0/2	0/6	0/1	0	0	0/2	0
Lissotriton vulgaris	0	0	0	0	0	0/10	0/13
Alytes obstetricans	0	0/2	0	0	0	0/3	0
Bombina variegata	0	0	0/5	0	0	0	0
Bufo bufo	0/5	0/1	0/2	0/64	0	0/13	0/30
Epidalea calamita	0	0	0	0	0	1/8	0
Hyla arborea	0	0/2	0/3	0	0	0	0
Rana arvalis	0	0	0	0	0/1	0/1	0
Rana temporaria	0	0	0	0	0	0/5	0/3
Pelophylax synkl. *esculenta*	0	0	0	0	0	0/3	0
Pelophylax lessonae	0	0	0	0	0	0/38	0
Pelophylax kl. *esculentus*	0/1	0	0/7	0	0	0/56	0

Appendix S2 Sampling intensity and prevalence per species, per year (2009 and 2010) and per month (infected/tested)

	Year	March	April	May	June	July	August	September	October	January
Salamandra salamandra	2009	-	-	(0/3)	(0/8)	-	-	-	-	-
	2010	-	-	-	-	-	-	-	-	-
Ichthyosaura alpestris	2009	(0/6)	(2/93)	(0/28)	(3/50)	(3/73)	-	-	-	-
	2010	-	-	(0/3)	-	-	(0/108)	(1/23)	-	-
Triturus cristatus	2009	(0/8)	(0/8)	(0/22)	(0/3)	(0/29)	(0/4)	-	-	-
	2010	(0/3)	(0/2)	-	-	-	-	-	-	-
Lissotriton helveticus	2009	(0/11)	-	(0/6)	(0/43)	-	-	-	-	-
	2010	-	-	-	-	-	-	-	-	-
Lissotriton vulgaris	2009	(0/17)	(1/183)	(3/115)	(0/30)	(0/70)	-	-	-	-
	2010	(0/5)	(0/5)	(0/2)	-	-	-	(0/6)	(0/1)	-
Alytes obstetricans	2009	-	(0/15)	(1/4)	(3/9)	(45/197)	(7/20)	-	-	-
	2010	-	-	(0/1)	-	-	(14/197)	(0/33)	(0/34)	-
Bombina variegata	2009	-	-	(2/44)	(0/79)	(0/51)	(13/26)	-	-	-
	2010	-	-	(2/41)	-	(1/48)	(2/132)	(0/22)	-	-
Pelobates fuscus	2009	-	(0/9)	-	-	-	-	-	-	-
	2010	-	-	(0/1)	-	-	-	-	-	-
Bufo bufo	2009	(0/20)	(6/149)	(0/1)	(0/14)	(2/18)	-	-	-	-
	2010	(0/1)	(0/5)	-	-	-	-	-	-	-
Epidalea calamita	2009	-	(0/41)	(0/20)	(0/1)	-	-	-	-	-
	2010	-	(4/5)	-	-	-	-	-	-	-
Hyla arborea	2009	-	(1/39)	(0/1)	(0/16)	(1/21)	-	-	-	-
	2010	-	-	-	-	-	-	-	-	-
Rana arvalis	2009	-	(0/31)	-	-	(0/16)	-	-	-	-
	2010	-	-	-	-	-	-	-	-	-
Rana temporaria	2009	(0/6)	(0/60)	(0/22)	(0/41)	(1/20)	(0/1)	(0/5)	-	-
	2010	(0/1)	(0/2)	(0/4)	-	-	(0/2)	-	-	-
Pelophylax spp.	2009	-	(1/22)	(5/48)	(0/8)	(0/33)	-	-	-	-
	2010	(0/1)	-	-	-	-	(0/18)	(0/7)	-	-
Pelophylax klepton *esculentus*	2009	-	(2/26)	(9/98)	(0/21)	(0/64)	-	(0/15)	-	-
	2010	(0/1)	(0/7)	-	-	-	-	-	-	-
Pelophylax lessonae	2009	-	(0/2)	(3/55)	(0/8)	(1/20)	-	-	-	-
	2010	-	(0/1)	-	-	-	-	-	-	-
Xenopus laevis	2009	-	-	-	-	-	-	-	-	(0/10)
	2010	-	-	-	-	-	-	-	-	-
Triturus marmoratus	2009	-	-	-	(0/1)	-	-	-	-	-
	2010	-	-	-	-	-	-	-	-	-
Triturus carnifex	2009	-	-	(0/8)	-	-	(0/3)	-	-	-
	2010	-	-	-	-	-	-	-	-	-
Lithobates catesbeianus	2009	-	-	-	-	-	-	-	-	-
	2010	-	-	-	-	-	-	-	-	-

Appendix S3

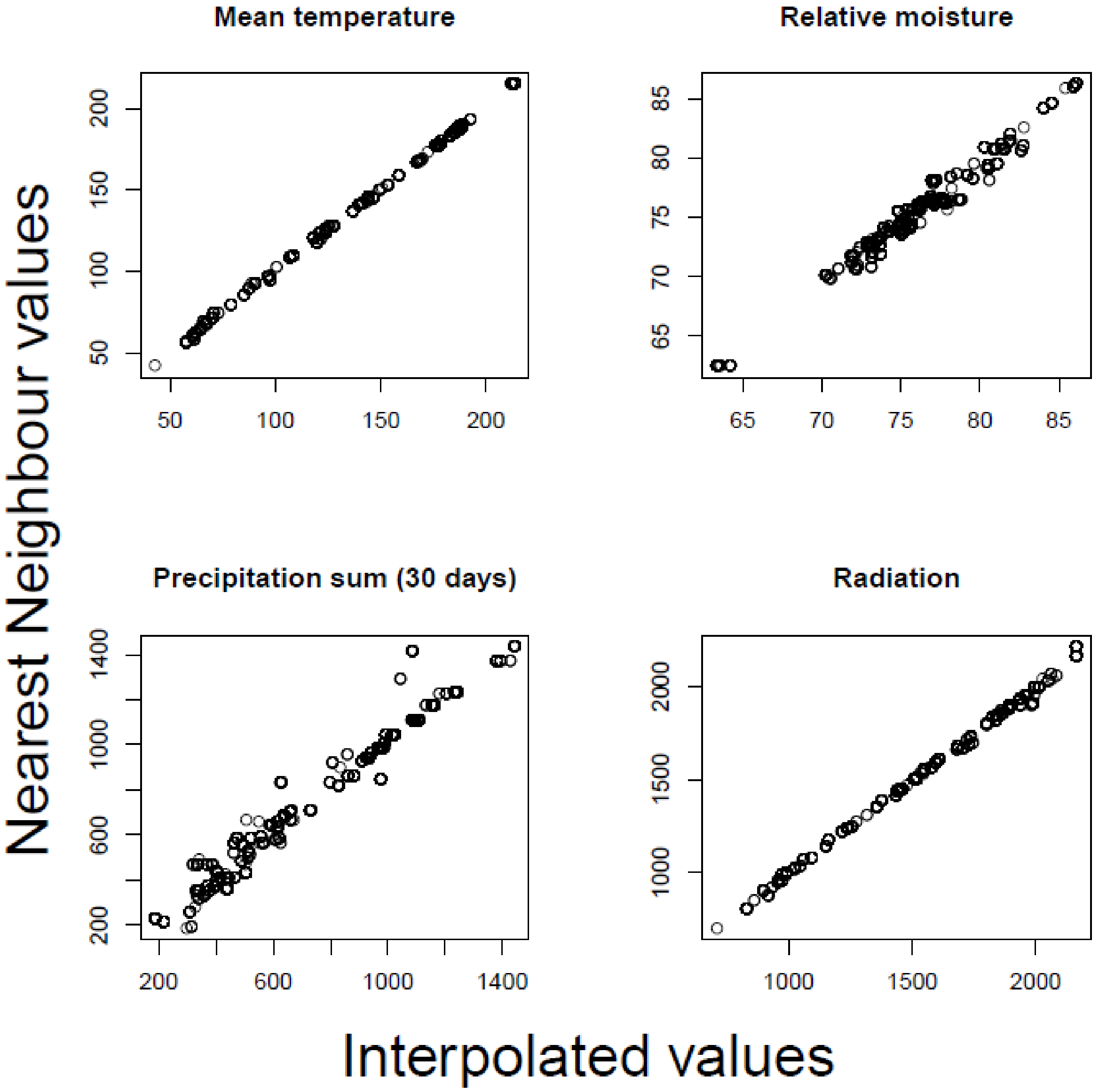
Output of the model selection using competing Generalized linear mixed models (GLMM), having ΔAIC ≤ 2. *Abbreviations*: AIC, Akaike Information Criterion; ΔAIC, difference in AIC between the current and the best model; k, number of covariates in the model. *Model abbreviations*: TG, Daily mean temperature (in 0.1 °C); TN, Minimum temperature (in 0.1 °C); TX, Maximum temperature (in 0.1 °C); Q, Global radiation (in J/cm^2); RH, Daily precipitation amount (in 0.1 mm); UG, Daily mean relative atmospheric humidity (in percents); UX, Maximum relative atmospheric humidity (in percents); UN, Minimum relative atmospheric humidity (in percents). The numbers 7 and 30 following the abbreviations refer to the concerning time frames prior to swab sampling.

	model	AIC	ΔAIC	k
Pelophylax spp				
	TN7+UX7	162.292	0.000	2
	RH30+TX30	162.471	0.179	2
	TN7	162.614	0.323	1
	TN7+UG7	162.758	0.466	2
	RH30	162.852	0.561	1
	RH7+UG7	162.991	0.700	2
	RH30+UG30	163.073	0.782	2
	RH7+TN7+UG7	163.495	1.204	3
	RH7+TX7+UG7	163.545	1.253	3
	RH7+TX7+UX7	163.711	1.419	3
	TN7+UG7+UX7	163.776	1.485	3
	RH7+TX7	163.872	1.580	2
	TX7+UN7+UX7	163.997	1.705	3
	RH7+TN7+UX7	164.131	1.839	3
	TN7+UN7+UX7	164.175	1.883	3
	RH7+TG7+UG7	164.204	1.912	3
	Q7+TN7+UX7	164.212	1.920	3
	Q30+RH30	164.222	1.931	2
	Q7+TN7	164.263	1.971	2
A. obstetricans				
	Q30	233.514	0.000	1
	Q30+RH30	234.762	1.247	2
	UX30	234.809	1.294	1
	Q30+UN30	235.122	1.607	2
	UG30+UN30	235.217	1.703	2
	Q30+UX30	235.245	1.730	2
	UG30+UX30	235.436	1.922	2
	Q30+TN30	235.436	1.922	2
B. bufo				
	UX7	42.292	0.000	1
	TG7+UX7	42.423	0.132	2
	TG7	42.856	0.564	1
	Q7+UX7	43.490	1.198	2
	UG7+UX7	43.681	1.389	2
	RH7+TG7+UX7	43.877	1.585	3
	UN7+UX7	43.960	1.668	2
	RH7+TG7	44.011	1.720	2
	Q7+TG7+UX7	44.240	1.948	3
	TG7+UG7+UX7	44.253	1.961	3
	Q7+UN7+UX7	44.267	1.976	3
I. alpestris				
	Intercept	31.796	0.000	1
	RH7	33.457	1.661	1
	model	**AIC**	**ΔAIC**	**k**
I. alpestris				
	UN30	33.505	1.709	1
	UG30	33.622	1.826	1

	RH30	33.691	1.895	1
	UN7	33.715	1.919	1
	UX7	33.729	1.932	1
	UG7	33.745	1.949	1
	TN7	33.783	1.986	1
	Q7	33.787	1.990	1
	UX30	33.789	1.992	1
	TN30	33.795	1.999	1
	Q30	33.796	1.999	1
	TG30	33.796	2.000	1
B. variegata				
	Intercept	105.842	0.000	1
	RH7+TN7+UG7	106.304	0.462	3
	UG7	107.472	1.631	1
	RH7+TN7+UG7+UX7	107.583	1.741	4
	RH7	107.638	1.796	1
	UN7	107.663	1.821	1
	Q7	107.840	1.998	1

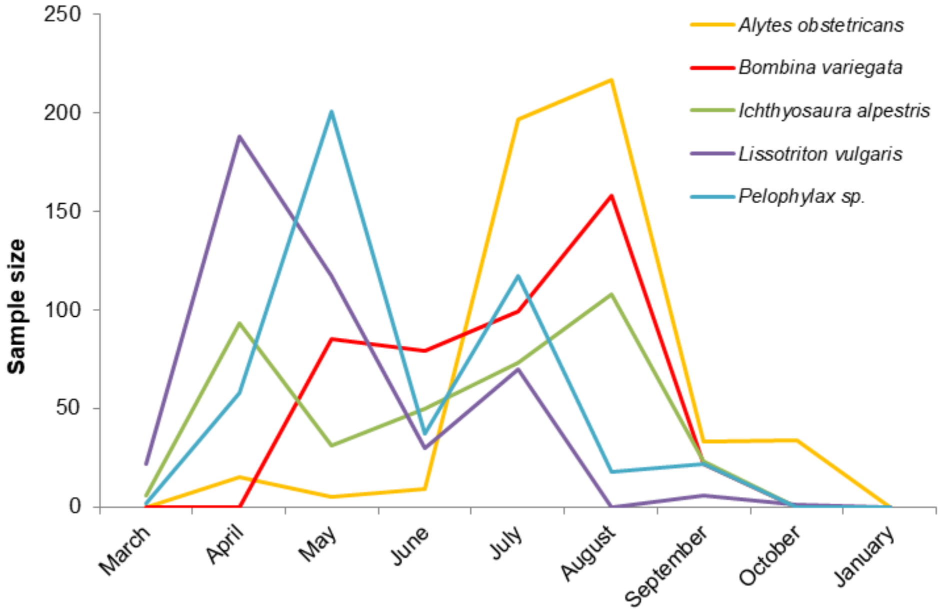
Appendix S4

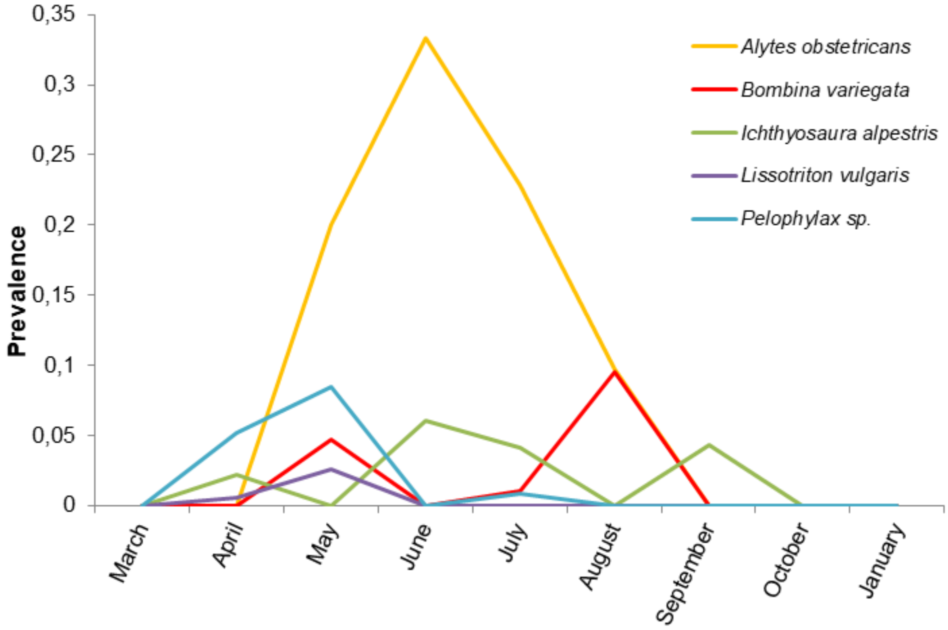
Correlation graphs showing the correlation between the interpolated weather data and the data from the nearest weather stations. Weather data were obtained from weather stations nearest to each sampling location (range: 0.9 – 32 km., mean: 13.0 km; Royal Netherlands Meteorological Institute, www.knmi.nl). Because interpolated weather data was nearly 1:1 correlated with the Nearest Neighbour values, we used the latter in the analyses.



Appendix S5

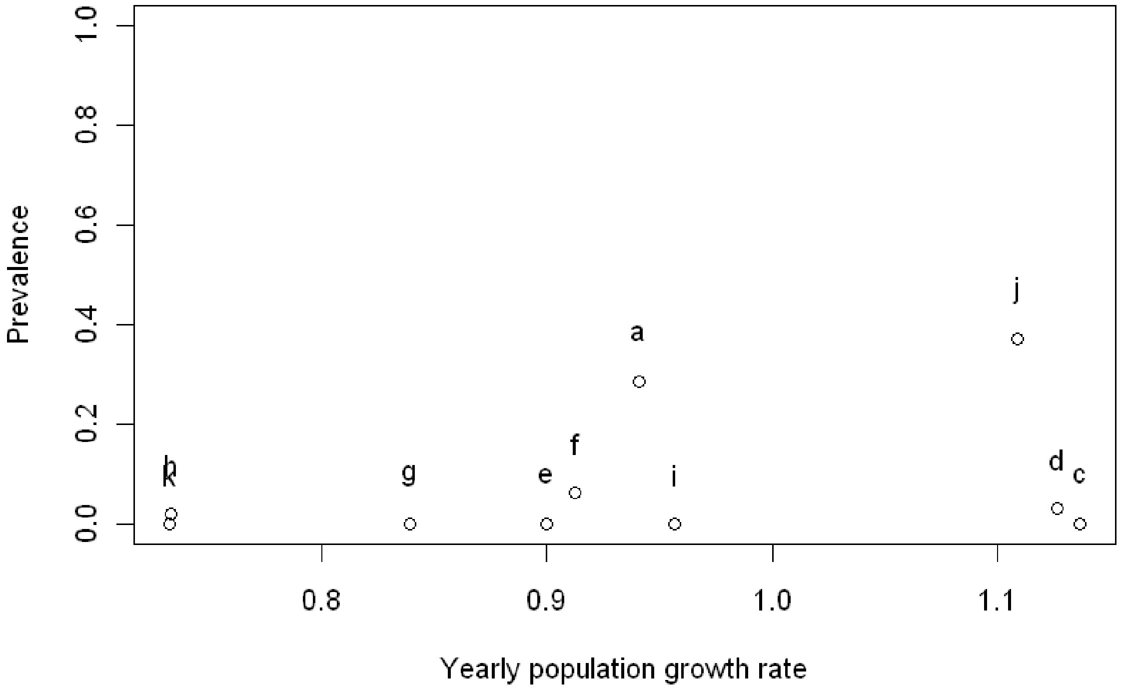
Fluctuations in prevalence and sample size per month over the whole study period presented for species with at least 30 tested individuals per month.





Appendix S6

We calculated the average annual population growth rates of all *Alytes obstetricans* populations that we also sampled for *Bd* presence. The letters a – k represent the ten different populations. The average annual population growth rate over the years 2001-2010 was estimated as the average slope of population size over time. We used GLM models with Poisson error distribution and a log link relating the counts to year of sample (as continuous covariate). The exponent of the slope for each population is presented. The population growth rate of *A. obstetricans* populations showed no correlation (ρ = 0.35) with *Bd* prevalence.



Chapter 3

Fragile coexistence of a global chytrid pathogen with amphibian populations is mediated by environment and demography

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Unravelling the multiple interacting drivers of host–pathogen coexistence is crucial in understanding how an apparently stable state of endemism may shift towards an epidemic and lead to biodiversity loss. Here, we investigate the apparent coexistence of the global amphibian pathogen *Batrachochytrium dendrobatidis* (Bd) with *Bombina variegata* populations in The Netherlands over a 7-year period. We used a multi-season mark–recapture dataset and assessed potential drivers of coexistence (individual condition, environmental mediation and demographic compensation) at the individual and population levels. We show that even in a situation with a clear cost incurred by endemic Bd, population sizes remain largely stable. Current environmental conditions and an over-dispersed pathogen load probably stabilize disease dynamics, but as higher temperatures increase infection probability, changing environmental conditions, for example a climate-change-driven rise in temperature, could unbalance the current fragile host–pathogen equilibrium. Understanding the proximate mechanisms of such environmental mediation and of site-specific differences in infection dynamics can provide vital information for mitigation actions.

Introduction

Emerging wildlife diseases are a key driver of global biodiversity loss (e.g. Daszak et al. 2000). The impact of an infectious disease on a wildlife population is evident when the population crashes during a mass mortality event (Price et al. 2014). However, the impact on population persistence may be equally significant, but less obvious and more difficult to assess, when the pathogen has a long-term presence in the population (Scheele et al. 2017). Overall, the impact of wildlife diseases is highly variable and most often depends on a complex interplay between multiple host, pathogen and environmental factors (Muths et al. 2011).

Chytridiomycosis, caused by the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (Bd) is a particularly relevant globally emerging wildlife disease, detected in more than 500 amphibian species (Olson et al. 2013). Yet within this global context, there is no universal inter- or intraspecific response to Bd infection. In Australia and the Neotropics, chytridiomycosis outbreaks have led to species extinctions (Lips et al. 2006; Skerratt et al. 2016). Conversely, many amphibian species across the globe persist in spite of recurrent or ongoing Bd infections. In Europe, Bd outbreaks have been limited in number and are variable in extent (Bosch et al. 2001; Simoncelli et al. 2005; Rosa et al. 2012). In particular, in northern Europe, Bd was shown to have been present at least since the late 1990s and is widespread, but mass mortalities and consistent negative effects on population trends have not been observed (Spitzen-van der Sluijs et al. 2014). In northern Europe, Bd seems to have reached a state of endemism and the build-up of lethal Bd infection-loads is hypothesized to be precluded by unfavourable environmental conditions (Spitzen-van der Sluijs et al. 2014). However, the true nature of this hypothesized coexistence and its driving mechanisms are not known.

Ecological theory and empirical evidence suggest a range of different mechanisms may facilitate coexistence. Firstly, the pathogen may not impact the hosts' survival or recruitment rate, instead behaving as a commensal (Briggs et al. 2010). Secondly, the pathogen may be parasitic but have compensatory, rather than additive, effects on the vital rates of individual hosts; that is, the individuals that die of disease would also have had higher mortality rates in the absence of the pathogen (Jolles et al. 2006). Alternatively, the parasitic pathogen may inflict additive mortality, but this is compensated at the host population level, for example through increased recruitment and a shift in age structure (Muths et al. 2011; Scheele et al. 2015; McDonald et al. 2016). Understanding whether, and by what mechanisms, host populations truly coexist with a pathogen is crucial for predicting and eventually managing the potential impacts of a disease.

Here, we analyse a multiple-year dataset of Bd infection dynamics and demographic data in two populations (multiple-pond systems) of *B. variegata*, a locally endangered amphibian species hypothesized to coexist with Bd (Wagner et al. 2017). This dataset allowed us a rare possibility to study the drivers of coexistence in the absence of obvious mortality effects at the population level. Rather than focusing on a single potential coexistence mechanism, we simultaneously addressed the role of individual body condition, environmental mediation and demographic compensation in shaping individual- and population-level effects of Bd infection. We show that in our study system, despite potential negative effects at the individual level, coexistence at the population level is most probably maintained by environmental conditions and demographic compensation. Key to long-term population persistence is the maintenance of high-quality habitat to safeguard the compensatory mechanism as climate change and positive feedback between host demography and pathogen prevalence may threaten the stability of this equilibrium.

Material and methods

(a) Study species and area

The yellow-bellied toad (*B. variegata*) is a species of high conservation concern in the European Union (Kuzmin et al. 2009). The species exhibits slow development (toads usually become sexually mature in their third summer), high longevity (a lifespan of over 20 years) and relatively small clutch sizes for an amphibian (up to 130 eggs per female, deposited in several clutches over a prolonged period between April and September (Gollman and Gollmann, 2012 and references therein). In The Netherlands, the natural range of the yellow-bellied toad only covers the southernmost part of the country, where it occurs in seven isolated populations (Bosman et al. 2009).

The magnitude of Bd-induced mortality for this species in the wild seems limited (Wagner et al. 2017). While prevalence varies between 4.5% and 32%, infection intensities are generally low (Statecsny and Glaser, 2011; Civiš et al. 2012; Gál et al. 2012; Ohst et al. 2013; Vörös et al. 2013; Spitzen-van der Sluijs et al. 2014; Scheele et al. 2015; Wagner et al. 2017). Nonetheless, lethal chytridiomycosis has been observed in the Apennine (continental Italy) subspecies of yellow-bellied toad, *B. variegata pachypus* (Stagni et al. 2004). Also, Woodhams et al. (2014) found that post-metamorphic survival of the yellow-bellied toad was substantially reduced after an infection experiment with the Swiss lineage Bd TG739. A confirmed chytridiomycosis disease outbreak in captive juvenile yellow-bellied toads shortly after metamorphosis resulted in a 50% mortality rate (15/30 animals died). Mortality ceased after initiation of antifungal treatment (F. Pasmans 2017, unpublished data).

We monitored two populations of yellow-bellied toads in The Netherlands over a 7-year period (2010–2016). The populations, relatively small but apparently stable, occupy two separate sites, 'Groeve 't Rooth' (hereafter Rooth; 15.8 ha) and 'Wahlwiller' (3.9 ha), 11 km apart in a straight line. In 1982 six yellow-bellied toads were brought from Rooth to Wahlwiller (Bosman et al. 2009). Rooth is a marl quarry that has been actively operated since 1938, whereas Wahlwiller is a southern exposed hill slope with extensive agricultural activity (pasture and vineyard). Within the sites, there are respectively 35 and 19 artificial, irregularly maintained ponds, of which respectively 31 and 17 ponds are suitable for reproduction by yellow-bellied toads.

Between 2010 and 2016 we visited each site on one (2015) or more occasions (all other years except 2014 when no surveys were carried out), totalling up to 22 visits to Rooth and 24 visits to Wahlwiller. We caught and 'marked' each individual by photographing the unique belly pattern. We classified photographs using the program AMPHIDENT, an automatic algorithm to identify individuals on the basis of ventral spot patterns (Matthé et al. 2008; 2017; Drechsler et al. 2014).We classified each individual in one of four life stages: larvae, juveniles (metamorphosed toadlets before their first hibernation), subadults (stage between the first hibernation and breeding condition) and adults (sexually mature) (Spitzen-van der Sluijs et al. 2014). In contrast to other studies (Gollmann and Gollmann, 2012), we found that the belly patterns of juvenile toads often changed considerably until the sub-adult life stage, making recognition possibly subject to error. Therefore, we compiled multi-season capture–recapture data only from the sub-adult life stage onwards. For the juvenile stages we compiled count data and mark–recapture data only for the single season of the first capture. Animals were also weighed to the nearest 0.1 g using a digital spoon scale (Konsait Electronic Spoon weight Scale, Stainless LCD Display 0.1 g/500 g) and snout to vent length was measured to the nearest millimetre using a regular ruler. We calculated body condition using the ‘scaled mass index’ ($\widehat{M}$), a method that takes account of the scaling between body components and body size (Peig and Green, 2009).

We obtained a skin sample from every individual at each capture, using aluminium sterile cotton-tipped dry swabs (rayon-Dacron, COPAN, UNSPSC CODE 41104116) following the procedure and biosecurity measures described in Spitzen-van der Sluijs et al. (2014). All swabs tested were stored at -22°C until DNA extraction. For the quantification of Bd-DNA, we first extracted all swabs following the protocol of Boyle et al. (2004). Quantitative PCR was carried out with a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Extractions were diluted 1/10 and all assays were performed in duplicate. Samples were considered positive for Bd when there was a clear log-linear amplification and both repeats were above the detection limit >0.1 mean genomic equivalents (GE), which for analyses we treated as mean infection intensity. Infection intensity was measured as GE/swab. We obtained weather data from the weather station nearest to the sampling locations (Maastricht; 3 km (Rooth) and 14 km (Wahlwiller); Royal Netherlands Meteorological Institute, www.knmi.nl). For each sampling occasion we calculated daily mean temperature, the minimum temperature and the maximum temperature (all in 0.1 degrees Celsius) estimated over two periods: 7 and 30 days prior to each site visit (Spitzen-van der Sluijs et al. 2014). We measured pond pH using HI 2211 pH/ORP Meter (Hanna® instruments, Temse, Belgium), and water temperature at each visit 1 m. from the shore - or in the centre of the pond if the pond had a diameter smaller than 1 m. -using an HI 98311 DiSTt5 EC/TDS/temperature tester (Hanna Instruments, Nieuwegein, The Netherlands) to the nearest 0.1°C repeatedly at the same position and depth (10 cm.) at each site.

(b) Statistical analyses

To understand the mechanisms of coexistence between *B. variegata* and Bd, we estimated first the survival and transmission dynamics at the individual level and their individual and environmental predictors, and then survival, recruitment and Bd prevalence at the population level. To this end we modelled three datasets: (i) individual mark–recapture data of individuals older than 2 years (grouping together sub-adults and adults), using a Jolly–Seber model; (ii) individual mark–recapture data of juveniles during their first year, using a Cormack–Jolly–Seber model; and (iii) counts of juveniles for each year, using a state-space model.

(i) Adult mark–recapture data

We built an open-population Jolly–Seber model (Lebreton et al. 1992) with four states (‘not entered’, ‘alive, not infected’, ‘alive, infected’, ‘dead’). State transitions are described as survival (φ, allowed to differ between infected and non-infected individuals), probability of infection (ϑ, transition from non-infected to infected), probability of clearing infection (χ, transition from infected to non-infected), and probability of entry (γ, probability that a new individual enters the pool of individuals available for capture).

We began our analysis with a full model, in which the transition probabilities φ, ϑ and χ were estimated as a logistic regression of individual body condition, the mean minimum air temperature in the week before the survey -after preliminary analyses suggested this was the most supported predictor over mean and maximum temperatures, over 7 or 30 days prior to the survey- an interaction between those two covariates, and a site-specific fixed effect. We included temperature to reflect the potential influence of environmental conditions on infection dynamics (Daskin et al. 2014; Raffel et al. 2015), and body condition to assess whether the endemic infection was more likely to affect individuals with a lower body condition, as reflected by the transition probabilities. Since the body condition is unobserved every time an individual is not caught, we imputed missing values randomly from the lognormal distribution of observed values, after preliminary modelling suggested no time-, site-, sex- or individual-specific pattern in body conditions. Similarly, for infected individuals we included the infection load (log genomic equivalent) as a predictor of survival, assessing the possibility of pathogen overdispersion. We followed the same procedure as above for imputation of unobserved infection loads. We used uninformative priors for these three coefficients and centred all covariates.

We modelled the probability of entry (γ) to vary between the first and subsequent occasions every year. This reflects our belief that, given the marked isolation of sites is likely to prevent immigration, γ can be interpreted as recruitment only. Therefore, we expect entries to occur at the beginning of each sampling season: that is, sub-adults captured during a season are most likely to have entered at the beginning of that season (after their first hibernation). We expressed this belief using different prior distributions, respectively an uninformative uniform (0,1) for the first occasion and an informative uniform (0,0.05) for subsequent occasions in every year. We allowed the probability of individual detection p to vary temporally but not by site, since ponds are largely similar and effort identical, and to be independent of the infection state, since we observed no differences in behaviour or external features between infected and non-infected individuals. We fitted the model in JAGS (Plummer, 2003). We ran each model for 50,000 iterations, after discarding the first 25,000 as a burn-in and applying a thinning rate of 10. We ran three Markov chains with overdispersed initial values and assessed convergence by visual inspection of the chain histories, and through the R-hat statistic. We progressively simplified the model by removing covariate predictors of φ, ϑ and χ for which the 95% credible intervals of the coefficient's posterior distribution encompassed zero (Kéry and Schaub, 2011).

(ii) Juvenile mark–recapture data

Although we found that individuals could not be tracked with certainty between their first and second year, we were still able to model individual mark–recaptures during their first year (between metamorphosis and before or just following their first hibernation). We fitted a Cormack–Jolly–Seber in JAGS, following the same procedure and predictors as for the adult data; the main difference being that the Cormack–Jolly–Seber model conditions on first capture and does not allow estimation of new entries and population sizes. We modelled the progressive change of belly patterns and effective loss of marking using an exponential decay function for the probability of individual recapture over time. In other words, the model estimated survival of juveniles within the first year accounting for the fact that as individuals grew after metamorphosis, it would become increasingly unlikely that their photographs could be matched to those of the previous captures.

(iii) Juvenile count data

Finally, we modelled the maximum number of juveniles counted at any occasion in every year as the outcome of a binomial observation process (to account for imperfect detection) and a Poisson fecundity process, where the mean fecundity rate per adult was modelled as a function of population density in the current year, using a log link. We assumed each female to lay between 40 and 70 eggs, with a maximum of 130, and a mean 10% survival from egg to metamorphosis

(Morand, 1997; Barandun et al. 1997); the number of non-breeding females is compensated by the number of females that lay a second clutch, approximately 12% according to (Barandun et al. 1997). We ran the model in JAGS using the same settings and diagnostics described above.

(iv) Derived parameters

After obtaining the final versions of the three models above, we combined them to derive population-level parameters of interest. For the first survey occasion of every year we calculated the number of individuals in each age class. For juveniles, the age class size was estimated directly; for sub-adults and adults, by summing the number of individuals estimated to be alive in the respective latent states. Similarly, we calculated the prevalence of Bd infection as the proportion of the individuals alive at any given occasion that were in the latent state ‘infected’. We calculated adult survival as the proportion of adults alive at year t-1 that were still alive at t; juvenile survival and recruitment as the number of new entries in the adult class on the first occasion of year t, divided respectively by the number of juveniles at year t-1, and the number of adults alive on the first occasion of year t-1; and population growth as the ratio of population sizes on the first occasions of years t and t-1. All ratios were rescaled by the length of the intervals between first occasions (in days). We excluded the first survey of the study from the calculation of recruitment, since γ and p were not identifiable separately on the first occasion (Kéry and Schaub, 2011). Finally, we calculated the density of adults by dividing the size of this class by the total area of each site, and the density of juveniles by dividing their estimated number by the total surface of water bodies at each site (reflecting the respective use of both terrestrial and aquatic habitats, for adults, and of water bodies and immediate surroundings only, for juveniles). We were not able to directly estimate the relationship between Bd patterns (infection, prevalence) and host density, the inclusion of which in the model led to poor fit and wide estimates; therefore, we were forced to limit our analysis to a visual assessment of the correspondence between host density and prevalence patterns.

Results

(a) Survey results

We made 1008 captures of 608 uniquely marked yellow-bellied toads (326 individuals in Rooth, and 282 in Wahlwiller; 400 adults, 97 sub-adults and 111 juveniles) throughout the study period, including multiple captures for 206 individuals. Forty individuals changed infection state at least once (became infected or recovered) and 11 changed state twice, with none recaptured in an infected state after recovery. The strongest infection intensity recorded prior to recovery was 3,500 GE/swab, while the highest overall infection intensity was 29,600 GE/swab in a larva (second highest load: 26,800 in a juvenile toad). The observed prevalence of Bd in yellow-bellied toads (all age classes, both sites) fluctuated between years, ranging from 0.5% (in 2011) to 22.2% in 2012 and 2015. When observed over the entire study period, the younger life stages showed higher prevalence rates than the older life stages (larvae: 18.7%, juveniles: 40%, sub-adults: 5.5%; adults, 6.6%). The observed infection intensity varied by life stage and particularly by site, with maximum loads for adults and juveniles respectively one and two orders of magnitude greater at Rooth (adults: at Rooth median 34.9 GE, range 3.44–10,820; at Wahlwiller median 28.8 GE, range 5.36– 276; juveniles: at Rooth median 1724 GE, range 4.74–26,800; at Wahlwiller median 17.55 GE, range 11.30–6680) (Table 1).

(b) Individual infection patterns

The survival of infected individuals was lower than that of non-infected individuals, both for adults and juveniles (Figure 1a). Weekly survival for non-infected adults was $\varphi_{(A)NI}$=0.993 (95% CRI: 0.990, 0.993) at Rooth and $\varphi_{(A)NI}$=0.994 (0.992, 0.994) at Wahlwiller (Figure 1a). Survival of infected individuals decreased markedly with higher infection loads, particularly for juveniles, although considerable uncertainty surrounded estimates for high infection loads (Figure 1b). For adults with the median infection load of 23.2 GE, $\varphi_{(A)I}$=0.989 (0.949, 0.999) at Rooth and $\varphi_{(A)I}$=0.921 (0.827, 0.976) at Wahlwiller. Weekly survival for non-infected juveniles was $\varphi_{(A)NI}$=0.975 (95% CRI: 0.959, 0.987) at Rooth and $\varphi_{(A)NI}$=0.940 (0.910, 0.964) at Wahlwiller (Figure 1a). For infected juveniles, we found no significant difference between sites; for juveniles with the median observed load of 351 GE, weekly survival was $\varphi_{(A)I}$=0.248 (0.017, 0.674). We found no evidence for an effect of body condition or temperature on survival, with the posterior distribution of regression coefficients for these covariates always encompassing zero.

The probability of an adult becoming infected increased with higher minimum temperatures in the week preceding the survey and was always higher at Rooth than Wahlwiller (Figure 2), and was not correlated to body condition. In average observed conditions (median of the observed temperatures) the probability of infection was 0.69 (0.338, 0.929) at Rooth and 0.37 (0.121, 0.705) at Wahlwiller. We found no evidence of the probability of an adult clearing infection being correlated to either body condition or temperature, with a mean value of $\chi_{(A)}$=0.738 (95%CRI: 0.504, 0.966). For juveniles, the mean probability of becoming infected was $\vartheta_{(J)}$=0.11 (0.037, 0.209) and the mean probability of clearing the infection was $\chi_{(J)}$=0.791 (0.413, 0.992); neither were strongly correlated to body condition or temperature, with the posterior distribution of regression coefficients for these covariates always encompassing zero.

(c) Population-level patterns

The two sites showed a markedly similar pattern in adult survival, recruitment and Bd prevalence, although the latter showed greater magnitude of change at Rooth. The survival of adults decreased between 2010 and 2013 at both sites, then increased in 2014 and 2015 (Figure 3a,b; we refer to survival in a given year as the survival from that year to the next). In the year of minimum survival (2013), both sites showed a marked increase in recruitment (Figure 3a,b). At Rooth, the decrease in adult survival in 2010–2013 matched a constant increase in adult population density; at Wahlwiller, the low adult survival in 2013 occurred after a decline in density had already begun in 2012. The number of juveniles produced per adult remained mostly constant throughout the study period, as highlighted by the correspondence between adult and juvenile densities (Figure 3a–d). The density of adults, calculated over the total site area, was generally higher at Wahlwiller than at Rooth (Figure 3a,b), but the density of juveniles, calculated over the surface of water bodies at each site, was largely comparable (Figure 3c,d).

Table 1 Observed Bd-prevalence in two *Bombina variegata* populations (Rooth and Wahlwiller) between 2010 – 2016

				P/N (SQ mean; SQ range) per age class			
		P/N (SQ mean; SQ range)	Fraction infected ponds	adult	subadult	juvenile	larvae
Wahlwiller	2010	1/65 (15.68; -)	1/8	1/46 (15.68; -)	0/12 (-;-)	0/7 (-;-)	NA
	2011	1/143 (276;-)	1/11	0/107 (-;-)	1/36 (276; -)	NA	NA
	2012	34/287 (233.7; 4.1-6680)	3/13	10/186 (43.1; 19.5-166.2)	0/6 (-;-)	1/26 (6680)	23/69 (36.6;4.1-129.2)
	2013	0/73 (-;-)	0/8	0/69 (-;-)	0/2 (-;-)	0/2 (-;-)	NA
	2015	21/73 (471.2; 3.4-7520)	5/6	1/7 (33.2)	1/2 (20.2)	18/36 (545.4; 3.4-7520)	1/28 (24.8)
	2016	10/67 (56.03; 5.4 - 367)	2/4	1/29 (5.36;-)	1/2 (367;-)	8/33 (23.5; 11.3 - 70)	0/3 (-;-)
Rooth	2010	0/40 (-;-)	0/12	0/33 (-;-)	0/6 (-;-)	0/1 (-;-)	NA
	2011	0/60 (-;-)	0/14	0/53 (-;-)	0/1 (-;-)	NA	0/6 (-;-)
	2012	123/420 (2241.9;4.7-29600)	9/22	23/96(439.0;7.3-4200)	3/22(3654.4;23-10820)	53/92(3332.7;4.7-26800)	44/210(1774.2;23.2-29600)
	2013	15/131 (30.6;3.4-224)	7/19	13/91(33.3;3.4-224)	2/39(13;9.0-17.1)	0/1 (-;-)	NA
	2015	3/35 (73.5; 8-204)	2/6	1/2 (8.02;-)	0/1 (-;-)	NA	2/32 (106.3; 8.6 - 204)
	2016	1/88 (49; -)	1/6	0/41 (-;-)	1/17 (49;-)	0/4 (-;-)	0/26(-;-)

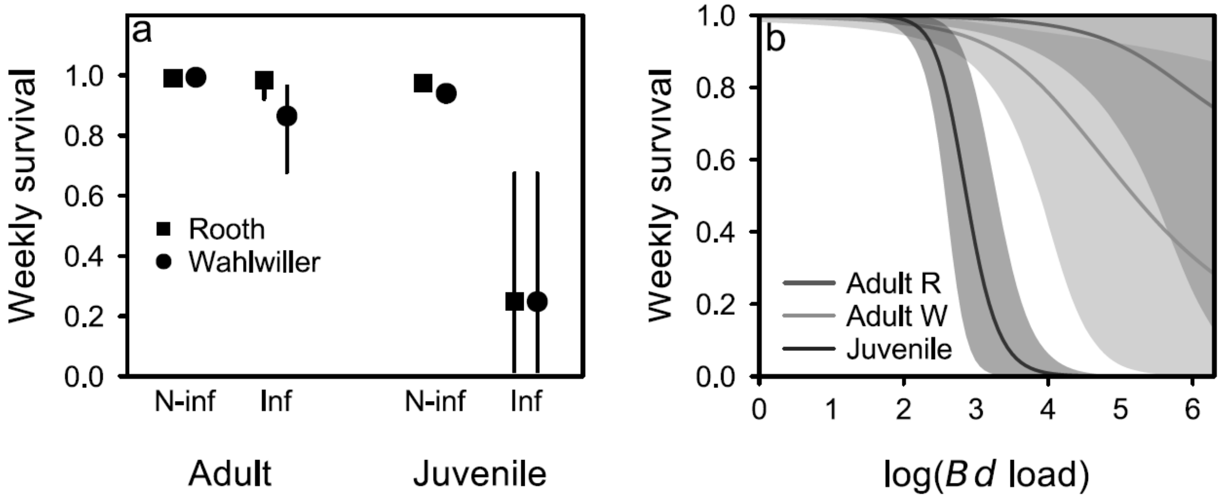

Figure 1. (a) Estimated survival over a 7-day period for adult (more than 2 years old) and juvenile (first-year) *B. variegata*, in different infection states (not infected 'N-inf', and infected 'Inf') at two sites. For infected toads, values indicate survival for the median of the observed infection loads in the respective age classes (adults=23.2 GE; juveniles=351 GE). Bars indicate 95% credible intervals. (b) Estimated survival over a 7-day period as a function of the infection load at t expressed in GE, for adult toads at each site and juvenile toads at both sites. Shaded areas indicate 95% credible intervals. R, Rooth; W, Wahlwiller.

At Rooth, the trend in the prevalence of Bd in the adult population closely matched that of the density of juveniles in ponds (Figure 3c). The growth rate of the population, particularly of the adult age class, also matched the trend in prevalence: in particular, prevalence appeared to follow the population growth in the previous year (Figure 3e). At Wahlwiller, juvenile density showed greater variability, but Bd prevalence remained lower throughout the study period, although the year of highest juvenile density was also the year of highest Bd prevalence (Figure 3d). The trend in population growth suggested a more stable population (Figure 3f); although a growth rate of λ=2 was estimated in the first year, Wahlwiller was only surveyed once in 2010 and the number of new entries in 2011 might therefore have been overestimated.

Discussion

Our results highlight the complexity behind apparent host–pathogen coexistence. In our study region Bd is historically present but has not been associated with declines in amphibian populations (Spitzen-van der Sluijs et al. 2014). In the absence of obvious disease-driven mass mortality for our *B. variegata* populations, we found a potential cost of endemic Bd at the individual host level. The parasitic fungus randomly infects individuals and lowers the survival of infected individuals; however, this additive mortality does not noticeably translate to a negative effect at the level of the population, and the size of both our focal populations appeared relatively stable over the study period.

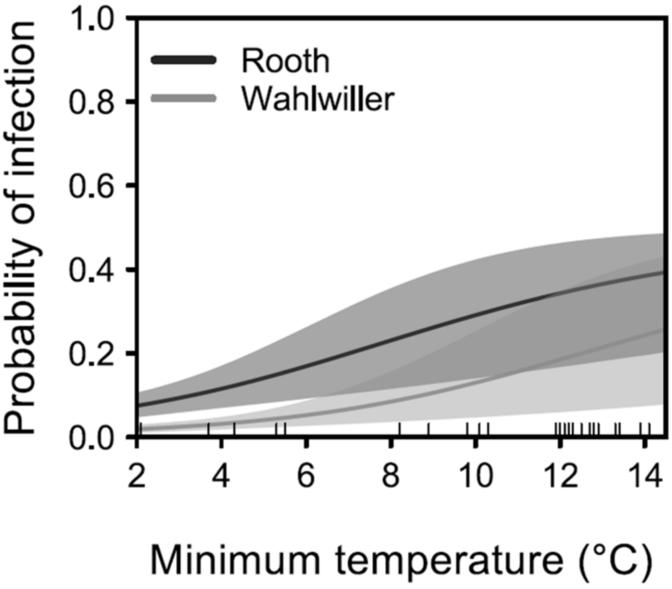

Figure 2. Probability of becoming infected over a 7-day period for adult *B. variegata*, as a function of site and the mean minimum temperature in the week prior to the survey. The shaded area indicates 95% credible intervals.

(a) Individual-level patterns

At the individual level, environmental mediation represented the most likely mechanism of host–pathogen coexistence. The survival of individual toads was significantly reduced by Bd infection, in line with previous observations for this species (Stagni et al. 2004; Woodhams et al. 2014) and with two separate cases of chytridiomycosis induced mortality in captive yellow-bellied toads that were recorded in Belgium (F. Pasmans 2017, unpublished data). We found no evidence that body condition correlated significantly with probability of infection or survival of either healthy or infected individuals. Within the limitations of our analysis, the individual probability of infection appeared random, and survival was clearly reduced for infected individuals, suggesting a potential true cost of Bd infection for *B. variegata*.

Infection intensity may determine transmission probability and pathogenicity; the presence of an over-dispersed pathogen load distribution (where most infections are weak, and only a small number of hosts are heavily infected) might therefore have a stabilizing effect on host–pathogen dynamics (Grogan et al. 2016). Our results appear to support this possibility, suggesting high infection loads, uncommon within the studied populations, are correlated with a marked decrease in survival. Uncertainty surrounding survival at high infection loads reflects their rare occurrence and the need to impute a large proportion of unobserved (latent) covariates.

The probability of an individual toad becoming infected clearly increased with temperature at both sites, suggesting environmental conditions play an important role in infection dynamics. Temperatures vary seasonally, and this seasonality largely coincides with the host activity patterns, potentially confounding the Bd–temperature relationship. Density dependent transmission may become more likely as temperatures increase during the season, first with breeding activity and later with increasing numbers of juveniles in and near the ponds.

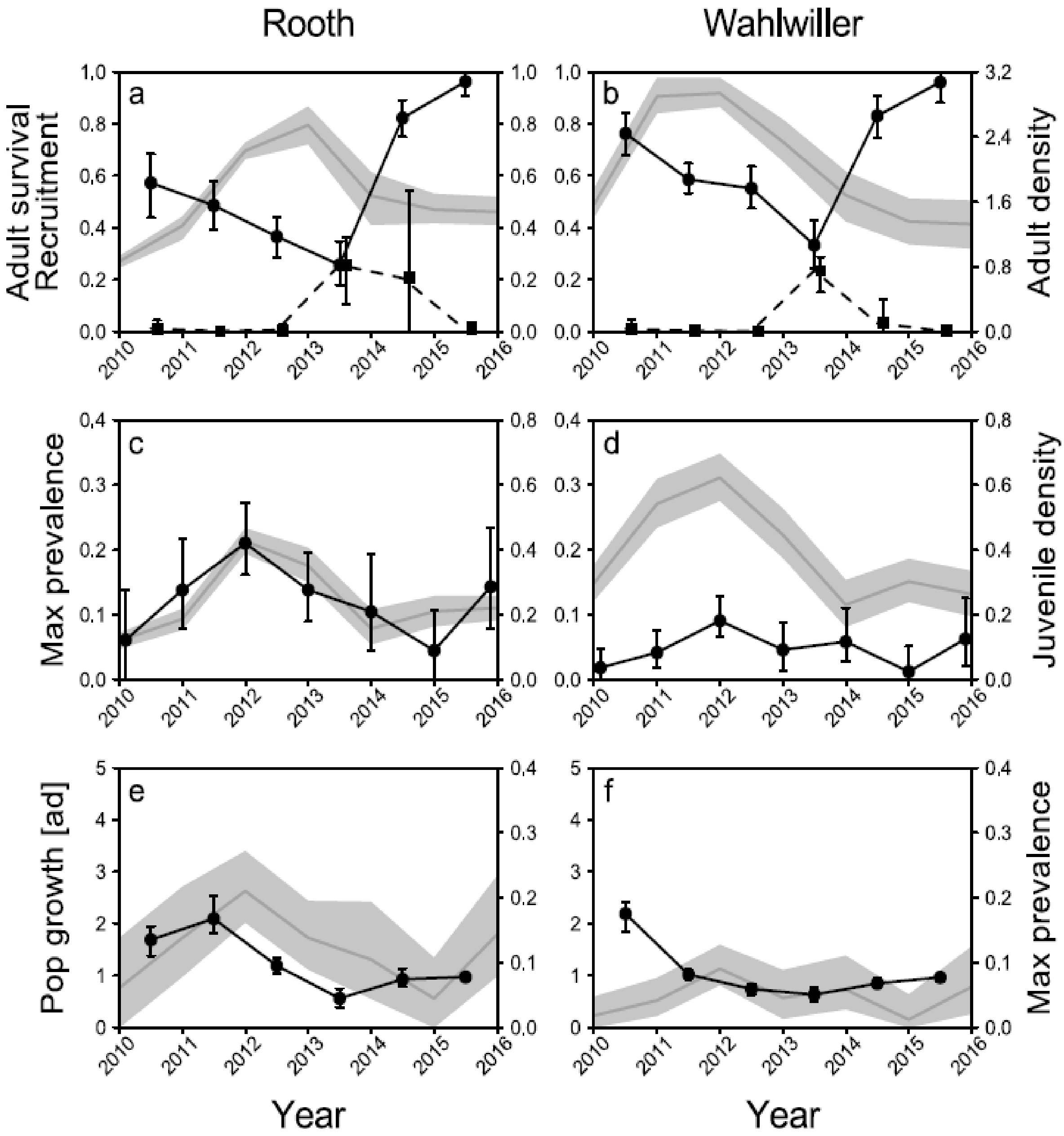

Figure 3. Patterns of host demography and Bd prevalence patterns at two sites during the study period. The left y-axis indicates the line and error bars; the right y-axis indicates the curve surrounded by the shaded area. In (a,b), the solid line indicates adult survival, the dashed line indicates recruitment. Values that fall exactly on the year are estimated during the sampling season (i.e. density in 2014); values that fall between years indicate the value from one year to the other (i.e. survival between 2013 and 2014 indicates the proportion of adults alive at the start of 2013 that were still alive at the start of 2014). Bars and shaded areas indicate 95% credible intervals. Juvenile density is calculated over the total surface of water bodies at each site; adult density is calculated over the total area of sites (15.8 and 3.9 ha respectively), scaled by 10^{-1} for ease of presentation.

However, the multi-season analysis showed Bd prevalence did not vary consistently within each season; rather, higher Bd prevalence occurred consistently in warmer years, while host activity remained largely similar (highlighted, for example, by consistent recapture probabilities and numbers of offspring per female), suggesting the relationship between Bd infection and temperature is not simply an indirect result of modified host behaviour. As longer time series become available, the model can be adapted to separate these effects.

A marked site-specific difference in the probability of infection also supported a role of environmental mediation, although the exact dynamics are more difficult to interpret than temperature patterns. Moreover, high infection loads were more common for Rooth than for Wahlwiller for both age classes, although the model did not suggest a significant site-specific difference in survival for a given load. These results suggest unidentified factors disrupt pathogen transmission and subsequent build up at Wahlwiller more than at Rooth, but once a given load is reached its negative effect on individual survival is similar. The topographic and physical structure of the two sites is largely similar and they host identical amphibian species assemblages. Although water temperature at Wahlwiller was consistently 1-2°C higher than at Rooth, both remain within the suitable thermal conditions for Bd growth for most of the *Bombina* breeding season (Longcore et al. 1999; Piotrowski et al. 2004), although other biotic interactions may be playing a role. For example, Longo and Zamudio (2017) found a strong relationship between seasonality and experimental Bd infection in *Eleutherodactylus coqui*, reflecting seasonal changes in the microbial community of the host species. The populations studied are close genetically, Wahlwiller having been established from a recent translocation from Rooth, although from a small founder population that may have magnified different levels of genetically determined disease tolerance. Identifying the proximate cause of these site differences represents a priority for future research, given the potential to inform mitigation actions.

(b) Population-level patterns

Further mechanisms for coexistence may operate at the population level, such that even observable negative effects of Bd on the individual host do not automatically translate to negative effects at the population level (Muths et al. 2011; Tobler et al. 2012). In our case, both populations appeared relatively stable over the study period. The observed pattern of adult survival and recruitment suggests compensatory recruitment might occur in years of particularly low adult survival. Importantly, this apparent compensation occurred in the same year at both sites, under different Bd prevalence but following a similar trend in adult and juvenile density, suggesting a process independent of Bd prevalence and infection load.

Also, the site with a more stable demographic pattern showed the smaller fluctuations in prevalence, suggesting some level of feedback between the two. At both sites, the maximum prevalence was reached in the year of maximum density of juveniles in ponds. The correspondence between Bd prevalence and juvenile density was particularly close at the site that appeared most favourable for Bd transmission (Rooth). The higher prevalence and intensity of infection in juveniles may reflect less developed immune responses or immunosuppression, following the stress of metamorphosis (Warne et al. 2011), increasing both transmission probabilities and pathogen growth on infected individuals. Late-stage tadpoles and early metamorphs may thus provide an infection reservoir in and around the waterbodies, increasing the probability of infection for adults that move to the water for reproduction (Medina et al.

2015; Valencia-Aguilar et al. 2016). Moreover, since new recruits (sub-adults) do not breed in the first year, their contact rate would be smaller than older, breeding-age adults; this explains how, in spite of compensatory recruitment in 2014, juvenile density and therefore prevalence showed a 1-year lag before increasing again in 2016 (when those replacement recruits would have entered the breeding age class). These observations are consistent with a classic infectious disease pattern, where higher density leads to higher transmission rates and ultimately higher population costs. Given the role of environmental mediation, where favourable conditions occur (either reflecting site suitability or weather/climate patterns), Bd prevalence can amplify the underlying density-dependent dynamics in a positive feedback cycle. The altering global pattern in precipitation and temperature shifts is likely to impact local environmental conditions and through this increase the infection probability of an individual host and overall infection prevalence (Clare et al. 2016). However, feedbacks between environmental mediation, infection and host demography such as we have observed for *B. variegata* increase the difficulty of predicting the ultimate effect on population trends. In the face of such uncertainty, maintaining the capacity of host populations to compensate reduced survival (regardless of its cause) through recruitment is crucial for their long-term persistence. These considerations have been made for amphibian hosts in epizootic situations (Muths et al. 2011; Scheele et al. 2014; Heard et al. 2017), but they also hold for endemic, temporarily stable host–pathogen systems, particularly where environmental factors that currently mediate coexistence might change in the future. To counter this risk, several studies have suggested that long-term population coexistence may be promoted by actively optimizing the environmental conditions, for example through habitat manipulation (Vredenburg et al. 2010; Savage et al. 2011; Heard et al. 2017).

Conclusion

Our results reinforce the findings of previous studies about the role of environmental conditions and demography in regulating Bd infection in amphibian hosts (Muths et al. 2011; Spitzen-van der Sluijs et al. 2014; Scheele et al. 2015; McDonald et al. 2016). Furthermore, by explicitly modelling both individual- and population-level effects, we could use our results to support the initial hypothesis of coexistence between Bd and *B. variegata* at population level, despite a potential cost of Bd infection for individual toads, and to suggest this equilibrium is most probably maintained by environmental mediation. Positive feedbacks between host demography (particularly juvenile density) and pathogen prevalence suggest changes in the mediating environmental factors, for example because of climate change, might lead to a breakdown of the current equilibrium. Future research should focus on the effect of those factors on pathogen and host ecology, as well as on the proximate causes of site-specific differences in infection dynamics, given both have potential for informing disease mitigation actions. Most importantly, the complexity of the system we have described, and the implications for conservation in the face of environmental change, highlight why situations of apparent host–pathogen coexistence still require monitoring and analysis, even in the absence of obvious mass mortalities.

Ethics. No animals were harmed in conducting this study. Surveys and sample collections were conducted in accordance with the landowners. The collection of skin swabs in the field was conducted under permit FF/75A/2016/015. To prevent research-mediated spread of Bd between populations, all field research equipment that contacted water or amphibians was disinfected with 1% Virkon S solution.

Data accessibility. All data have been deposited in the Dryad Digital Repository at http://dx.doi.org/10.5061/dryad.1m207.

Authors' contributions. All authors contributed ideas, A.S.-v.d.S. assembled the field data, S.C. conducted statistical analyses, A.M. conducted the laboratory analyses and all authors wrote the article. All authors agreed to submission of the manuscript and accept the responsibility for the accuracy and integrity of the manuscript.

Competing interests. We have no competing interests.

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Chapter 4

Rapid enigmatic decline drives the fire salamander (*Salamandra salamandra*) to the edge of extinction in the Netherlands

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In the Netherlands, the fire salamander (*Salamandra salamandra*) is at the edge of its geographic range and is restricted to three small populations in the extreme south of the country. Despite the species being listed as 'Endangered' on the national Red List, the situation was considered to be stable. However, from 2008 onwards dead individuals were seen on more than one occasion. A sharp decline in numbers has been observed since 2010 (96%;$P<0.01$), but we were unable to attribute this to any known cause of amphibian decline, such as chytridiomycosis, ranavirus or habitat degradation. The present work describes this enigmatic decline, and we discuss these results in the context of possible causes.

Introduction

Amphibians are experiencing declines globally, exemplified not only by population declines, but also by range reductions and extinctions of some species (Houlahan et al. 2000; Stuart et al. 2004; IUCN, 2012), and they may be part of a sixth major extinction event (Wake and Vredenburg, 2008), for which there is no single cause (Blaustein et al. 2011; Hof et al. 2011). In the Netherlands, despite 50% of the amphibian species being on the Red List (Van Delft et al. 2007), there were, until quite recently, no indications of an acute decline that could result in the extinction of a species from the country. Even when the two major infectious drivers of amphibian declines were present, *Batrachochytrium dendrobatidis* and ranavirus (Daszak et al. 2003; Spitzen-van der Sluijs et al. 2010; Kik et al. 2011) no specific decline has been observed.

However, the sudden, steep decline of the fire salamander (*Salamandra salamandra terrestris*) in the Netherlands is a new phenomenon. In the Netherlands, fire salamanders live at the very edge of their distribution and are confined to the old growth stages of deciduous forests on hillsides. Surface activity is limited to humid periods with night temperatures above 5°C. Mating usually occurs in autumn, the larvae are deposited from late winter until spring. In the Dutch populations, the animals mature at an age of 6 years in both males and females. Specimens can live for 20 years in this region (Gubbels, 2009). Listed as 'Endangered' on the national Red List, the species' range has decreased by 57% since 1950. This is probably due to the drying up of streams, or their canalisation and intensive cleaning. Additional threats are collection for the pet trade and the use of herbicides in surrounding agricultural land (Van Delft et al. 2007; Gubbels, 2009).

Currently, the species is only known from two native populations and another small, introduced, non-native population, all three in the extreme south of the Netherlands. The largest population ('Bunderbos') has been monitored since 1971, and yielded high estimates of population densities in the most suitable area (350-500 individuals in 0.5 ha). It was estimated that the whole population comprised several hundred individuals (Gubbels, 2009). All populations have been monitored yearly since 1997 within the Network Ecological Monitoring framework (Goverse et al. 2006). From 2008 onwards, repeat findings of dead adults were recorded for the first time and from 2010 onwards, there was an extremely sharp decline in the number of sightings of living salamanders (Figure 1). Even though amphibians are known for their large fluctuations in abundance (Green, 2003), and the fire salamander may have a strong dispersal capacity (Schmidt et al. 2007), the rapidity of the decline seemed more to be indicative of a disease or a toxin (Blaustein and Kiesecker, 2002). In this paper we analyse this enigmatic decline and we describe our attempts so far to elucidate its causes.

Materials and methods

The fire salamander currently has a very limited range in the Netherlands, being only present in the extreme south of the province Limburg. The largest native population is not far from the city of Maastricht in 'Bunderbos' (N 50°54'51'', E 5°44' 59''), an 8 km^2 area of brook valley woodland of which 144 ha is suitable habitat. Further to the east in 'Vijlenerbos' (N 50°45'44'', E 5°56'33''; size: 6 km^2, 58 ha suitable habitat) the species is present near six small brooks in the valley of the river Geul. The non-native, introduced population in 'Putberg' (N 50°51'17'', E 5°57'59'', size: 12 ha, 3 ha suitable habitat) occurs south of the city Heerlen. At all three sites, fire salamanders seem to occur in patches, thus not occupying the entire suitable habitat. The three areas are geographically isolated from each other, being 17-21 km apart, straight-line distance. The intervening landscape is unsuitable for the species, rendering migration between populations highly unlikely. Standardized monitoring of amphibian populations and the calculation of population trends started in 1997. Transect counts are always done in the late evening or at night, under humid or wet conditions with temperatures ≥ 5°C, according to the national standard (Groenveld et al. 2011). Transect length ranges between 50-500 m (mean±SD=275 ± 162; median value=300m). The Bunderbos population has received the most attention. Between 1997-2007, volunteers monitored five transects four times yearly. One transect was situated too close to a railroad and for reasons of safety, monitoring was stopped during 2009 and continued the same year on three new transects at a safer distance nearby. For 'Bunderbos' there is no data available for 2006 and 2008. Monitoring effort was intensified by the volunteers due to their concern to the near absence of live animals and the discovery of dead ones. From 2009 onwards, covering all transects (7) were monitored between 4-30 times/year (mean±SD = 18.9±7.8). The transects in 'Vijlenerbos' (4) (mean±SD=4.1±5.2 times/year; median value=3) and 'Putberg' (1) (range: 0-1 times/year) have only been monitored irregularly.

Indices and trends were calculated using TRIM (Trends & Indices for Monitoring data), a statistical program based on log-linear Poisson regression designed for fauna monitoring data with missing values (Pannekoek and Van Strien, 2001; Van der Meij et al. 2009). The overall trend between 1997 and 2012 and the associated slope were calculated. Annual indices represent the yearly numbers as a percentage of the numbers in the first year (1997) of monitoring. If the standard deviation is below 0.02, the trends are considered to be reliable. The indices are based on the estimated abundance of the species, calculated from the sum of the mean number of salamanders seen per transect per year, supplemented by the estimated values for the missing years as calculated by TRIM. The calculation of the population trend is based on the average change of the indices per year, for the three areas. The yearly indices and the trend are calculated for the whole period, taking serial correlation between years into account.

We conducted linear regression analyses to see if the number of fire salamanders sighted was influenced by temperature, humidity, observer-effect, or the time of year. Climatic data (mean, minimum and maximum daily temperature, total daily precipitation, and the mean daily relative atmospheric humidity) were obtained from the weather station of the Royal Netherlands Meteorological Institute (www.knmi.nl) in Maastricht (N 50°55', E 5°47'). None of these predictor variables significantly influenced the number of sighted salamanders, and were therefore excluded from further analysis. We chose to use data from the eight visits with the highest counts to compensate for the effect of more frequent monitoring on the average counts per year for 2010-2012. We could not define a clear rationale to choose four visits in these years comparable to the four in previous years. Using all visits of four randomly chosen visits would probably result in a lower average while using the four visits with highest counts could have the opposite effect. Using the eight visits with higher counts may have resulted in a too conservative population trend, but this was preferred above a Type I error which may have exaggerated the rate of decline.

Post-mortem examination was severely limited by the rapid autolysis of the animals, and post mortem studies were conducted on 5 specimens (Ghent University), including macroscopic examination and (q)PCRs for the detection of *Batrachochytrium dendrobatidis*, ranavirus, Chlamydiales, herpes viruses and circovirus (Mao et al. 1997; Boyle et al. 2004; Halami, 2007;

Martel et al. 2012). Due to severe autolysis, resulting in a lack of recognizable internal organs, routine histology, bacteriology and virology were not performed on these specimens. On a single specimen, found in 2010, histological, bacteriological and cytological tests were performed, in addition to macroscopic examination (Utrecht University).

Results

In the period 1997-2010, the maximum number of fire salamanders sighted annually over all areas fluctuated between 10 and 241 (Table 1). In the largest population 'Bunderbos', annual maximum numbers fluctuated between 71 and 241 for that period, and in 2011 the total number dropped to four, despite intensive monitoring (26 visits that year). Taking the monitoring period as a whole (1997-2012) the species showed a very strong and significant decrease in all populations (Figure 1). Over this time frame, the total population decreased by 96%, illustrated by the trend, which is expressed as a slope of −0.2189 ± 0.018 ($P<0.01$), indicating an annual decrease of 19.7%. In 'Vijlenerbos', only five individuals were seen in 2010; none were sighted in 2012 despite 57 visits to monitor the site that year. The population discovered in 'Putberg' in 1994 (Janssen and Huijgens, 2001) was visited infrequently the last years. Earlier visits saw the maximum number of adults fluctuating between 1 and 15 per year. The fire salamander (2) was last seen there in 2010.

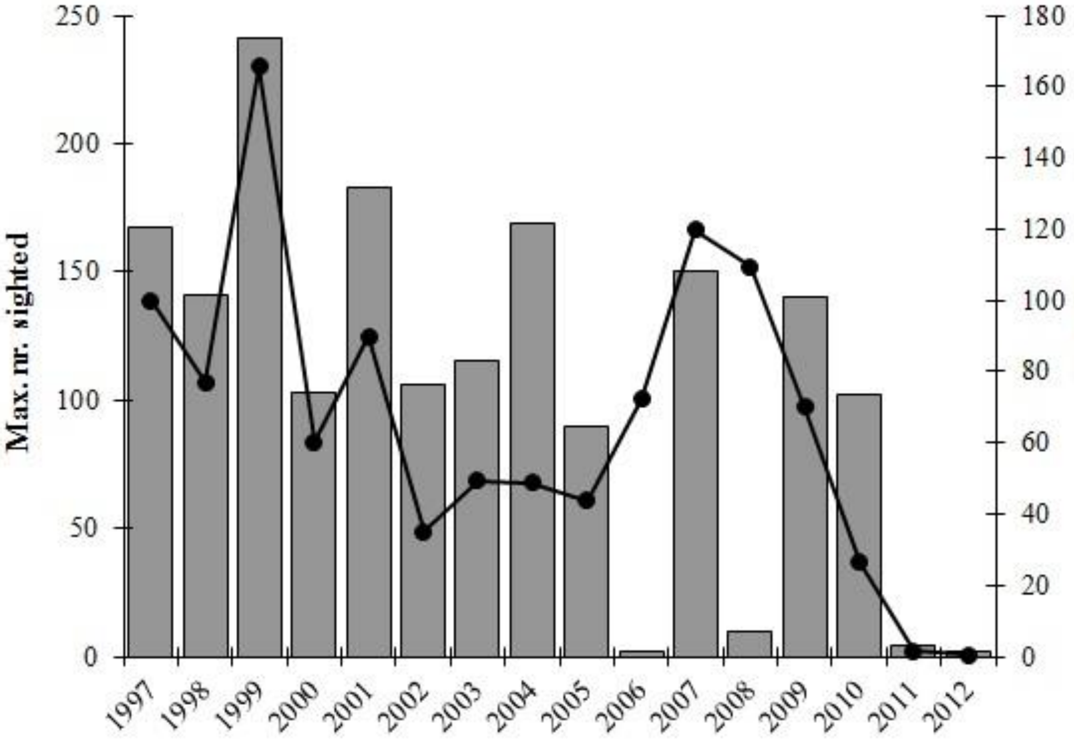

Figure 1. Maximum number of fire salamanders seen/year (bars) and the index (line) in the period 1997-2012. Index (calculated by TRIM) set at 100 at the start of the monitoring programme, shows a dramatic and continuing decline since 2008.

The first reports of dead animals reached us in 2008. They were generally found without external signs of injury on footpaths in broad daylight (Figure 2). To date 25 have been found (2008: 3; 2009: 0; 2010: 16; 2011: 6), twenty in 'Bunderbos', and five in 'Putberg'. During the years 2009 and 2012 no dead or moribund specimens were encountered in the field. A total of six fire salamanders was necropsied. All specimens were too decomposed for extensive pathological examination. However, the tests we carried out yielded no conclusive results about the cause of death; we did not detect *Batrachochytrium dendrobatidis*, ranavirus, Chlamydiales, herpes viruses or circovirus. In 2012, twenty-two visits searching outside the monitoring transects for animals for the ex-situ conservation programme, yielded a total of 39 individuals.

Table 1. Monitoring results per year, indicated as the maximum number of seen salamanders per year over all transects (second column), yearly indices and standard deviation (SD) of the index (calculated by TRIM), as well as the number of transects and the mean number of visits per transect per year.

Year	Sighted	Index	SD (index)	# Transects	Mean nr visits/transect
1997	167	100	0	8	3
1998	141	77.1	0.030	7	4
1999	241	165.5	0.060	6	4
2000	103	60.3	0.025	7	4
2001	183	89.8	0.038	8	3
2002	106	35.2	0.020	9	3
2003	115	49.2	0.025	9	3
2004	169	48.8	0.026	7	4
2005	90	43.6	0.035	9	3
2006	2	72.3*	0.033	1	5
2007	150	119.8	0.047	6	4
2008	10	109.5*	0.036	1	3
2009	140	70.1	0.042	8	7
2010	102	26.4	0.015	11	15
2011	4	1.4	0.001	10	16
2012	2	0.3	0.001	11	13

* Estimated values by TRIM (Trends & Indices for Monitoring data).

Figure 2. Deceased fire salamanders were generally encountered without external lesions (photo: M. van Mullekom). This figure is published in colour in the online version.

Discussion

Although the population of the fire salamander in the Netherlands has always been small, it was not considered to be at risk. However, the extent and speed of decline of the population is worrying and it has now shrunk to a size approaching extinction.

Although the monitoring was not standardized optimally and the trend analyses did not fully account for the possible effect of pseudo-replication, the decline is to such an extent and the level of significance so strong, that it cannot be overlooked. Likewise, any bias due to variation in sighting probability would not lead to a different conclusion about the extent of the decline. To our knowledge, no similar decline in occurrence has been observed in the neighbouring regions of Belgium (pers. comm. R. Jooris, D. Verbelen, A. Laudelout) and western North Rhine-Westphalia (Germany) (pers. comm. M. Aletsee).

Although the cause has not yet been established, considering the sudden and steep nature of the decline and the number of dead individuals found, the most likely cause seems to be either an infectious agent or intoxication, possibly in combination with other causes. Amphibian population declines are caused by various abiotic and biotic factors acting together in a context-dependent fashion (Blaustein and Kiesecker, 2002). The decline we describe in this report strongly resembles the population crashes after entry and subsequent build-up of *B. dendrobatidis* infections in Australia, Central America, the Sierra Nevada (USA) and southern Europe (Berger et al. 1998; Bosch et al. 2001; Lips et al. 2006; Vredenburg et al. 2010). However, we did not find any trace of *B. dendrobatidis* in any of the fire salamanders sampled. Other possible causes such as climate change, habitat degradation, disturbed population demography, genetic erosion and the illegal capture of animals, or a combination of these, cannot be excluded at this moment. Indeed, habitat degradation has caused a strong range reduction of *S. salamandra* in the Netherlands in the past, but it is unlikely that this has caused the present collapse in the species' status. Although it is expected that climate change will cause streams to dry up (Goosen et al. 2010), this has not been seen to date. Habitat degradation can also be ruled out: the remaining habitat consists of a climax vegetation, the management of which has not changed since 1952. Genetic erosion or a demographic problem would result in a more gradual decline, and the occurrence of dead individuals cannot be explained by activities of illegal collectors. Emigration can more or less be ruled out as a possible cause of decline. This is supported by the absence of sightings of the species in any other areas, the lack of suitable habitat between the three source populations and fact that there have never been any records of road casualties in the surroundings.

However, it is known that high nitrate concentrations can seriously affect amphibian health (Rouse et al. 1999; Camargo et al. 2005; Ortiz- Santaliestra et al. 2012). In the 'Bunderbos' the nitrate concentrations are very high in both the spring water and in the top layer of the forest soil. In the area where the fire salamander is found, mean nitrate concentrations of up to 2000 μmol l^{-1} were measured in the spring water in 2001 (Hendrix and Meinardi, 2004), which are lethal values for tadpoles (Baker and Waights, 1993; Camargo et al. 2005). Exposure to deleterious substances can be an important cofactor suppressing the amphibian immune system which would facilitate outbreaks of infectious diseases and result in reduced adult fitness or mortality (Mann et al. 2009). However, a clear association between the high nitrate levels and the observed decline could not be made since the dead animals were in a severe state of postmortal decay, rendering proper histopathological examination impossible.

To date, 11 juvenile and 28 sub-adult and adult fire salamanders have been safely removed from 'Bunderbos'; they were collected from outside the monitoring transects. They are being kept in captivity to safeguard them from possible threats on that site. These individuals will form the basis of a breeding programme for repopulating the sites once the cause or causes of the decline are clear and reversed (Stuart et al. 2004). These captive animals will be intensively examined for the presence of pathogens. Should a mortality occur, it could provide valuable material to help to elucidate the cause of the decline. Currently, a demographic study is being carried out, together with one on the genetic variation of the whole population. There is also an investigation being carried out into the possibility of pesticide residues in the brain of the dead individuals that have been found, and additionally monitoring in the field will be continued in 2013.

The present case illustrates how small populations believed to be stable can suddenly collapse and possibly go extinct. Stochastic variation greatly influences demographic rates in small populations. This was recognized at a relatively early stage due to monitoring, early enough, we hope, to prevent the complete loss of the fire salamander from the Netherlands.

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Chapter 5

Batrachochytrium salamandrivorans sp. nov. causes lethal chytridiomycosis in amphibians

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The current biodiversity crisis encompasses a sixth mass extinction event affecting the entire class of amphibians. The infectious disease chytridiomycosis is considered one of the major drivers of global amphibian population decline and extinction and is thought to be caused by a single species of aquatic fungus, *Batrachochytrium dendrobatidis* (Bd). However, several amphibian population declines remain unexplained, among them a steep decrease in fire salamander populations (*Salamandra salamandra*) that has brought this species to the edge of local extinction. Here we isolated and characterized a unique chytrid fungus, *Batrachochytrium salamandrivorans* sp. nov. (Bsal), from this salamander population. This chytrid causes erosive skin disease and rapid mortality in experimentally infected fire salamanders and was present in skin lesions of salamanders found dead during the decline event. Together with the closely related Bd, this taxon forms a well-supported chytridiomycete clade, adapted to vertebrate hosts and highly pathogenic to amphibians. However, the lower thermal growth preference of Bsal, compared with Bd, and resistance of midwife toads (*Alytes obstetricans*) to experimental infection with Bsal suggest differential niche occupation of the two chytrid fungi.

Introduction

Amphibians have become an icon of the global biodiversity crisis (Mendelson III et al. 2006). Although a variety of factors are involved in amphibian decline worldwide, fungal chytridiomycosis has been identified as one of the major infectious diseases involved, resulting in the extirpation of >40% of amphibian species in areas in Central America and widespread losses across Europe, Australia, and North America (Crawford et al. 2010; Fisher et al. 2012). Chytridiomycosis is currently considered to be caused by a single species of fungus, *Batrachochytrium dendrobatidis* (Bd), which is the only chytridiomycete taxon known to parasitize vertebrate hosts. However, Bd and other factors known to cause amphibian decline fail to explain several recent amphibian population losses (Caruso and Lips, 2013; Spitzen-van der Sluijs et al. 2013).

A dramatic and enigmatic mortality event, which has brought this species to the edge of extinction, was recently reported among fire salamanders (*Salamandra salamandra*) in the Netherlands (Spitzen-van der Sluijs et al. 2013). Since 2010, the species has declined, with only 4% of the population remaining in 2013. This rapid decline coincided with the finding of dead animals in the field (Spitzen-van der Sluijs et al. 2013). The recent startup of an ex-situ conservation program for 39 of the remaining fire salamanders was compromised by the unexplained death of 49% of the captive animals between November and December 2012. Attempts to identify known amphibian infectious agents, including Bd, in these salamanders yielded negative results (Spitzen-van der Sluijs et al. 2013). Instead, we found, isolated, and characterized a second, highly pathogenic chytrid fungus from this decline event that occupies a different niche compared with Bd.

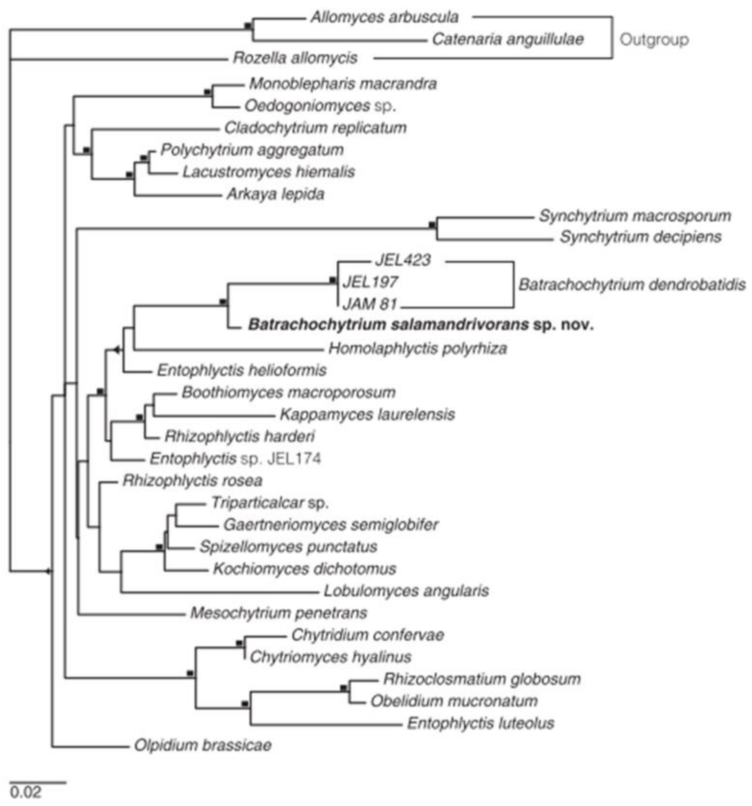

Figure 1. Maximum likelihood tree (−Ln L = 9,562.04266) for the analysis of a 1,513-bp data matrix of partial 18S + 28S rRNA genes. Together with *B. dendrobatidis*, *B. salamandrivorans* sp. nov. forms a well-supported clade [maximum parsimony bootstrap support = 100; maximum likelihood bootstrap support (MLBS) = 100; Bayesian posterior probability (BPP) = 100] of Chytridiomycota that parasitize amphibians with potentially lethal consequences. Squares on branches indicate MLBS > 70 and BPP > 95; triangles indicate MLBS < 70 and BPP > 95.

Significance
Chytridiomycosis has resulted in the serious decline and extinction of >200 species of amphibians worldwide and poses the greatest threat to biodiversity of any known disease. This fungal disease is currently known to be caused by *Batrachochytrium dendrobatidis*, hitherto the only species within the entire phylum of the Chytridiomycota known to parasitize vertebrate hosts. We describe the discovery of a second highly divergent, chytrid pathogen, *Batrachochytrium salamandrivorans* sp. nov., that causes lethal skin infections in salamanders, which has resulted in steep declines in salamander populations in northwestern Europe. Our finding provides another explanation for the phenomenon of amphibian biodiversity loss that is emblematic of the current global biodiversity crisis.

Results and Discussion

The chytrid fungus was isolated from the skin of fire salamanders from the affected population in Bunderbos (N50°54′51″, E5°44′59″), the Netherlands. Phylogenetic analyses including a broad range of representative chytrid species show that this fungus represents a previously undescribed lineage that forms a clade with Bd (Figure 1; Table S1). Its considerable genetic distance from Bd (3.47–4.47% for the 1,513 18S + 28S rRNA base pairs) compared with the shallow divergences between Bd isolates (Rosenblum et al. 2013) warrants the description of a unique species within the chytridiomycote order Rhizophydiales (family incertae sedis): *Batrachochytrium salamandrivorans* spec. nov. (Bsal). The unique chytrid represented by isolate AMFP13/1 (the holotype in liquid nitrogen at Ghent University) is the second chytrid known to parasitize and kill amphibians. In vitro, the unique taxon produces motile zoospores, which emerge from colonial (a single thallus containing multiple, walled sporangia) or monocentric thalli (Figure 2A). The most obvious morphological differences, compared with the Bd type strain, are the formation of germ tubes in vitro (Figure 2B; Figure S1) and the abundant formation of colonial thalli both in vitro and in vivo (Figure 3B). Bsal grew at temperatures as low as 5°C, with optimal growth between 10°C and 15°C and death at ≥ 25°C, a markedly lower thermal preference compared with Bd (Longcore et al. 1999) (Figure 4).

Infected fire salamanders died within 7 d after a short episode of anorexia, apathy, and ataxia. The pathology consistently comprised multifocal superficial erosions and deep ulcerations in the skin all over the body. Keratinocytes with eosinophilic necrosis and marginated nuclei were at the periphery of the erosions. Each of these keratinocytes contained one centrally located thallus, the majority being segmented (colonial thalli). Bacteria superficially colonized the ulcers. Additionally, anywhere in the skin, small foci of keratinocytes immediately below the damaged keratin layer were found. These presented similar eosinophilic necrosis, marginated nuclei, and centrally located colonial thalli. The intraepidermal organisms did stain with immunohistochemistry (Hyatt et al. 2007) (Figure 3A). Transmission electron microscopic examination of the skin lesions confirmed the presence of intracellular structures consistent with the colonial thalli (Figure 3B). All animals were also screened for a wide array of other infectious diseases, but no evidence for any other pathology was found: neither PCR (Annis et al. 2004) nor quantitative PCR (qPCR) (Boyle et al. 2004) suggested the presence of chytrid Bd DNA in the skin samples. Virological examination [including PCR for the detection of herpes viruses (Doszpoly et al. 2008), adenoviruses (Wellehan et al. 2004), and ranaviruses (Mao et al. 1997) and inoculation of IgH2 (iguana heart epithelial cells) and RTG (rainbow trout gill) cell cultures for general virological investigation] was negative. Ziehl Neelsen staining, PCR for

Chlamydiaceae (Martel et al. 2012), and bacterial isolation attempts did not yield any evidence of bacterial infections.

To further demonstrate that salamandrid mortality was caused by Bsal, we performed infection experiments on healthy fire salamanders (n=5) by exposing them to 5,000 zoospores of Bsal for 24 h. All animals died 12–18 d after inoculation after a 1- to 2-d episode of ataxia. Isolation was attempted and succeeded from one deceased salamander. PCR (described below) showed that Bsal DNA was present in all five infected animals, coinciding with histopathological lesions consisting of focal epidermal ulceration with very high numbers of colonial thalli of Bsal, which matched the lesions found in wild animals. Bsal-induced lesions are characterized by marked skin ulceration, opposed to those caused by Bd, which typically induces epidermal hyperplasia and hyperkeratosis (Berger et al. 2005). No clinical signs or histopathological lesions were observed in the uninfected negative control animals (n=5). Additionally, we put two healthy fire salamanders in a terrarium with an infected individual for 2 d. One salamander died 22 d after contact and the other 27 d after being placed with the infected animal. Histology, immunohistochemistry (Hyatt et al. 2007), and PCR demonstrated the presence of high numbers of Bsal in their epidermal layers, with lesions identical to those described above. Cohousing on damp toweling effectively transmitted Bsal and caused death in <1 mo. Experimentally infected midwife toads (*Alytes obstetricans*), the species that is most highly susceptible to infection by *B. dendrobatidis* in Europe (Bosch et al. 2001; Walker et al. 2010), did not show any signs of colonization by Bsal, as determined by immunohistochemistry and PCR, or disease, suggesting a differential amphibian host range for the two chytrids.

Amphibians will clearly benefit from the rapid identification of areas in which Bsal is present. We therefore designed diagnostic species-specific PCR primers to amplify the 5.8S ribosomal RNA gene and its flanking internal transcribed spacer regions: ITS1 and ITS2. Our set of primers STerF and STerR amplified Bsal in all positive tissues examined. Importantly, these primers did not amplify any of the nine tested strains from all three Bd lineages known to infect Europe and therefore provide a rapid noninvasive method for detecting of Bsal infections. Furthermore by using the newly developed PCR primers, we were also able to detect Bsal DNA in remains of the epidermises of six wild fire salamanders (from Bunderbos, the Netherlands) that were found dead in 2010 or 2011 and were stored at −70°C. Bsal was found present in skin swabs from all five experimentally infected and moribund fire salamanders, but in none of the midwife toads and noninfected fire salamanders. Additionally, 13 of 33 swabs collected from live fire salamanders from the declining population in Bunderbos, The Netherlands, in 2010 tested positive with this PCR, in contrast to 0 of 51 swabs from fire salamanders from a stable population in Belgium. Our PCR method thus allows the rapid screening of both extant populations and archived specimens for the presence of Bsal-induced chytridiomycosis.

Chytridiomycosis in amphibians can no longer be attributed to a single species of chytrid, but can be caused by either Bd or Bsal. Our results reveal striking similarities and differences between Bsal and the behavior of the hypervirulent global pandemic lineage of *B. dendrobatidis* (Farrer et al. 2011). Both fungal species share at least the following hallmarks: (i) induction of a lethal skin disease and (ii) association with mortality events and severe population decline. In contrast, it is as yet unclear to what extent Bsal is capable of infecting a broad amphibian host range, as is the case for Bd (Fisher et al. 2012). However, development of erosive vs. hyperplastic/hyperkeratotic skin lesions, failure to experimentally infect midwife toads, and relatively low thermal preferences of Bsal suggest differential host specificity of the two pathogens and possibly a differential effect on amphibian assemblages. Because the majority of recent Bd surveillance worldwide is based on the *B. dendrobatidis*–specific qPCR (Boyle et al. 2004), it is currently impossible to estimate the extent and impact of Bsal on amphibian populations worldwide using the Bd mapping framework (Olson et al. 2013). However, the emergence of the pathogenic Bsal chytrid fungus is worrying and warrants close monitoring, urgent risk analysis, and its inclusion in any monitoring program assessing amphibian population health.

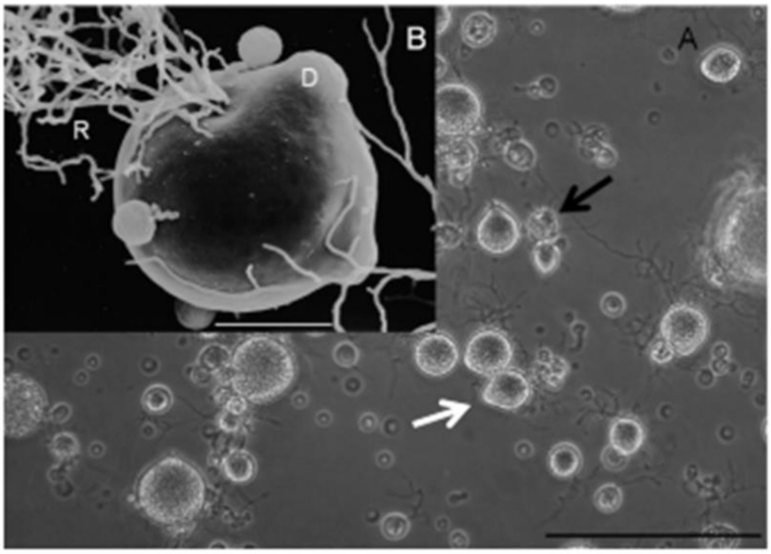

Figure 2. In vitro culture of *B. salamandrivorans* in TGhL broth at 15 °C. (A) Monocentric thalli predominate, with the rare presence of colonial thalli (black arrow). Sporangia develop discharge tubes (white arrow) to release zoospores (Scale bar, 100 µm.) (B) Scanning electron microscopic image of a mature sporangium with rhizoids (R), discharge tubes (D), and germ tube formation (arrow) (Scale bar, 10 µm.)

Taxonomy. *Batrachochytrium salamandrivorans* Martel, Blooi, Bossuyt and Pasmans sp. nov. MycoBank accession no. MB803904.

In vitro (tryptone-gelatin hydrolysate-lactose broth). Thalli predominantly monocentric, although some colonial. Development exogenous with sporangia forming at tip of germ tube. Rhizoids fine, isodiametric, extending from a single or several areas, lacking subsporangial swelling; Sporangium diameter 15.7–50.3 µm (average, 27.9 µm). One to several discharge papillae; cell wall at tip discharge papillae forms plugs that deliquesce resulting in release of motile zoospores. Motile zoospores roughly spherical, with highly irregular surface and cell surface projections; diameter 4.0–5.5 µm (average 4.6 µm). Resting spore not observed. Growth at 5, 10, 15, 20, and 22°C, but not at temperatures ≥24°C. Death of thalli after 5 d at 25°C. Five-day generation time at 15°C.

In Vivo. In epidermis of amphibians; forming predominantly colonial thalli that contain several walled sporangia. Thalli located inside keratinocytes; diameter 6.9–17.2 µm (average 12.2 ± 1.9 µm, n=50).

Zoospore Ultrastructure. Ultrastructure highly similar to that of *B. dendrobatidis*. Nucleus located outside the ribosomal mass, multiple mitochondria and numerous lipid globules. Position of the nonflagellated centriole in free swimming zoospores varies from angled to parallel to kinetosome.

rDNA Sequences. Partial nucSSU rDNA GenBank accession no. KC762294, partial nucLSU rDNA GenBank accession no. KC762293, partial ITS1-5.8S-ITS2 rDNA GenBank accession no. KC762295.

Holotype. Isolate AMFP13/1 (CBS 135744) from a fire salamander (*Salamandra salamandra*), kept in liquid nitrogen at Ghent University.

Etymology. The species epithet salamandrivorans (sa.la.man.dri.vo'rans. L. n. salamandra, salamander; L. part. adj. vorans, eating, devouring; N.L.part. adj. salamandrivorans, salamander-devouring) refers to the extensive skin destruction and rapid mortality observed in infected salamanders.

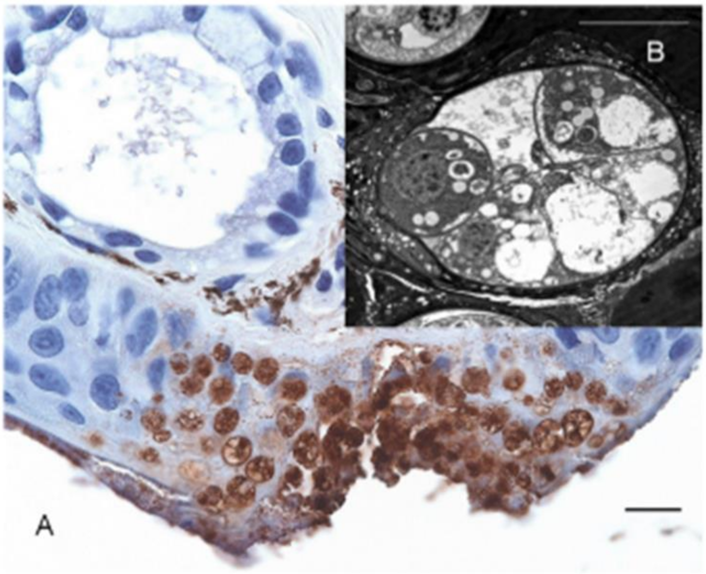

Figure 3. Microscopy of the skin of a fire salamander that died due to infection with *B. salamandrivorans*. (A) Immunohistochemical staining of a 5-µm skin section. Intracellular colonial thalli abound throughout all epidermal cell layers and are associated with erosive lesions. (Scale bar, 20 µm.) (B) Transmission electron microscopy picture of an intracellular colonial thallus of *B. salamandrivorans* inside a keratinocyte (Scale bar, 4 µm.)

Materials and Methods

Postmortem Examination of Fire Salamanders. Six *S. salamandra* that died in captivity between November and December 2012 were subject to gross necropsy, histopathology, and routine bacteriological, mycological, and virological examinations. Histological examination of liver, spleen, kidney, lung, gonad, midgut, and skin was done using microscopic examination of paraffin-embedded, 5-µm tissue sections stained with H&E, Ziehl Neelsen, or periodic acid shift. A 1:10 (vol:vol) tissue suspension of these organs in PBS was inoculated on sheep blood and tryptic soy agar and incubated at 20°C and 30°C. A liver suspension was inoculated on IgH2 and RTG cells. PCRs were performed to detect the presence of herpesviruses (Doszpoly et al. 2008), adenoviruses (Wellehan et al. 2004), iridoviruses (Mao et al. 1997), Chlamydiales (Martel et al. 2012), and Bd (Annis et al. 2004; Boyle et al. 2004). Immunohistochemistry was performed on all skin samples to detect Bd antigens (Hyatt et al. 2007). Transmission electron microscopy of epidermal samples was performed with glutaraldehyde fixation in 0.05 M sodium cacodylate buffer, 1% osmium tetroxide postfixation, and en bloc staining for 1 h in a 1% solution of uranyl acetate. Five *S. salamandra* specimens were found dead in the field during 2010 and 2011. Due to the severe autolysis of these animals, the postmortem examination was limited to skin histopathology and PCR for the detection of herpesviruses, adenoviruses, iridoviruses, Chlamydiaceae, and Bd.

***B. salamandrivorans* Strain Isolation and Culture Conditions**. Chytrid isolation on tryptone-gelatin hydrolysate-lactose (TGhL) agar plates containing penicillin/streptomycin (200 mg/L) at 20°C was attempted from the dead *S. salamandra* as described previously for the isolation of Bd (Longcore et al. 1999). Skin samples without contaminating bacterial or fungal growth were transferred to TGhL broth once zoospores were seen on the agar plates. The isolate was subsequently subcultured in TGhL broth in cell culture flasks at 15–20°C. A 10-d-old subculture was frozen in liquid nitrogen (Boyle et al. 2003). To obtain zoospores, 1 mL of a culture growing in TGhL broth was transferred to a TGhL agar plate and incubated for 5–10 d at 15°C. Zoospores were obtained by washing the agar plate with 2 mL of 0.2-μm filtered pond water. The number of zoospores in the suspension was determined using a hemocytometer.

To determine thermal growth conditions, 200 μL of a 5-d-old Bsal culture in TGhL broth at 15°C was transferred to the wells of a 24-well plate, and 0.8 mL of TGhL broth was added. The plates were incubated at 5°C, 10°C, 15°C, 20°C, 22°C, 23°C, 24°C, 25°C, and/or 30°C ± 1°C for 10 d. Growth was defined as a significant increase of the surface of the well covered by the fungus compared with wells incubated at 30°C (which is above the lethal temperature for Bsal) and the presence of motile zoospores. The surface coverage was determined by image analysis (GNU Image Manipulation Program) of pictures, taken through an inverted light microscope (Nikon Eclipse ts100, 20× magnification). Each condition was tested in triplicate. If no growth was seen after 10 d of incubation, the plates were further incubated at 15°C. Cultures were considered dead if no growth occurred within 10 d.

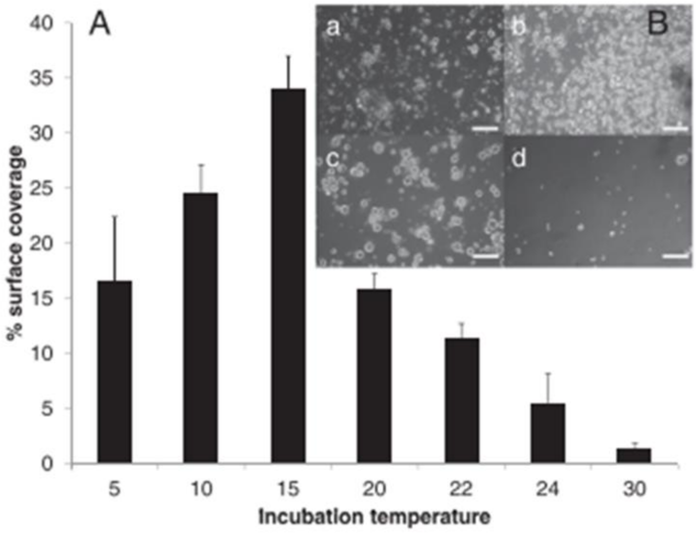

Figure 4. Growth of *B. salamandrivorans* in TGhL broth at different temperatures. (A) Growth was quantified by calculating the average percentage ± SD of the surface area of three wells covered by the fungus after 10 d of incubation at a given temperature. Motile zoospores were present at 5–20°C, but not at 22, 24, and 30°C. (B) *B. salamandrivorans* growth after 10 d at 4°C (a), 15°C (b), 20°C (c), and 30°C (d) (Scale bar, 200 μm.)

***B. salamandrivorans* Molecular Characterization and Diagnostic PCR Development.**

PCRs were done on the chytrid culture obtained to amplify the 18S, 28S, and the 5.8S rRNA genes and the flanking ITS regions ITS1 and ITS2 (White et al. 1990). Based on the ITS1-5.8S-ITS2 sequence, the primer set (STerF 5′TGCTCCATCTCCCCCTCTTCA3′ and STerR 5′TGAACGCACATTGCACTCTAC3′) was developed and used to detect the 5.8S rRNA gene of Bsal in skin samples from the six *S. salamandra* found dead in the field, six animals that died in captivity, and 33 swabs collected from *S. salamandra* in Bunderbos in 2010. Amplification reactions consisted of 10 ng DNA, 1 µM of each primer, 1.5 mM MgCl2, 1× Taq buffer, 0.2 mM of each dNTP, and 0.8 units of Taq polymerase in a volume of 20 µL. PCR amplification was performed under the following conditions: 10 min at 93°C, followed by 30 cycles of 45 s at 93 °C, 45 s at 59°C, 60 s at 72°C, and 10 min at 72°C. DNA of a pure culture of *B. salamandrivorans* was used as a positive control. Using primer set STerF and STeR, we assessed whether DNA of nine *B. dendrobatidis* strains would be amplified—Cape lineage (BdCAPE) isolates: SA1D, TF5a1, and CCB1; Swiss lineage (BdCH) isolates: Con2A, APEP, and 0739; and the global panzootic lineage (BdGPL) isolates: MAD, IA042, and JEL197. All derived amplicons were sequenced.

Phenotypic Characterization. The morphology of the chytrid isolate in TGhL agar and broth was examined using inverted, phase contrast, and scanning (Garmyn et al. 2012) and transmission electron microscopy (Lechter and Powell, 2005). Zoospores were collected from growth on TGhL agar plates and fixed for transmission electron microscopy with s-collidine buffer followed by osmium tetroxide (Lechter and Powell, 2005).

Experimental Infection of Fire Salamanders and Midwife Toads. The animal experiment was performed with the approval of the ethical committee of the Faculty of Veterinary Medicine (Ghent University, EC2013/10) under strict BSL2 conditions. Ten captive bred fire salamanders (*S. salamandra*) and midwife toads (*Alytes obstetricans*) were housed individually at 15 ± 1°C on moist tissue, with access to a hiding place and a water container. All animals were clinically healthy and free of Bd as assessed by sampling the skin using cotton-tipped swabs and subsequent performing qPCR (Boyle et al. 2004). Using the PCR described above, all swab samples were negative for the presence of DNA of Bsal. After 1 wk of acclimatization, 1 mL of a zoospore suspension in filtered (0.2 µm) pond water, containing 5,000 zoospores/mL, was dripped on the five animals of each species. Animals were fed twice weekly with crickets and followed up by clinical examination and weekly collection of skin swabs until 3 wk after exposure. The skin swabs were examined for the presence of Bsal DNA as described elsewhere.

Skin Swabs from Declining and Stable *S. salamandra* Populations. Skin swabs were collected from 33 *S. salamandra* from the Dutch fire salamander population experiencing the decline during 2010. For comparison, skin swabs were collected from 51 clinically healthy fire salamanders from a population without a history of decline (N50°57′13″; E3°43′15″, Merelbeke, Belgium). DNA from the swabs was extracted in 100 µL PrepMan Ultra (Applied Biosystems) (Hyatt et al. 2007). Samples were examined for the presence of DNA of Bd using qPCR and for the presence of DNA of Bsal using the PCR described above.

Phylogeny. In addition to the unique chytrid fungus, our taxon sampling consisted of three *B. dendrobatidis* strains and 27 species representing a broad evolutionary range of Chytridiomycota. In addition, *Rozella allomycis* and two Blastocladiomycota (*Allomyces arbuscula* and *Catenaria anguillulae*) were used as outgroup taxa. Alignment was done with ClustalX 2.0.10 (Thompson et al. 2002), and ambiguously aligned fragments were excluded for further analysis, resulting in a 1,513-bp reliably aligned data matrix. Maximum parsimony (MP) and maximum likelihood (ML) analyses were performed using PAUP* 4.0b10 (Swofford 2002). Heuristic MP searches were executed in 10,000 replicates, with all characters unordered and equally weighted, and using tree bisection reconnection (TBR) branch swapping. The strict consensus tree of 81 equally most parsimonious trees (tree length = 1,471) supported the (Bd, Bsal) sister relationship and received an MP bootstrap support of 100. Bayesian and likelihood analyses were performed with the GTR + G + I model of DNA substitution. For the likelihood analyses, heuristic searches were performed with substitution rates, γ-shape parameter, and proportion of invariable sites estimated from neighbor joining trees. These parameters were reestimated from the best ML tree found thus far, and the tree was submitted to additional rounds of TBR swapping; this procedure was repeated several times. These maximum likelihood analyses resulted in a single best tree [−ln L = 9,562.04266; pinvar = 0.301311; shape parameter α = 0.60887]. ML bootstrapping was done in 1,000 replicates with fixed parameters. Bayesian analyses were done with MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). Two runs of four Markov chain Monte Carlo (MCMC) chains each were executed in parallel for 5,000,000 generations, with a sampling interval of 500 generations and a burnin corresponding to the first 1,000,000 generations. Posterior probabilities for clades were obtained by combining the post–burn-in trees from parallel runs in a single consensus tree. Convergence of the parallel runs was confirmed by split frequency SDs (<0.01) and potential scale reduction factors (approximating 1.0) for all model parameters.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1307356110/-/DCSupplemental.

Supporting Information

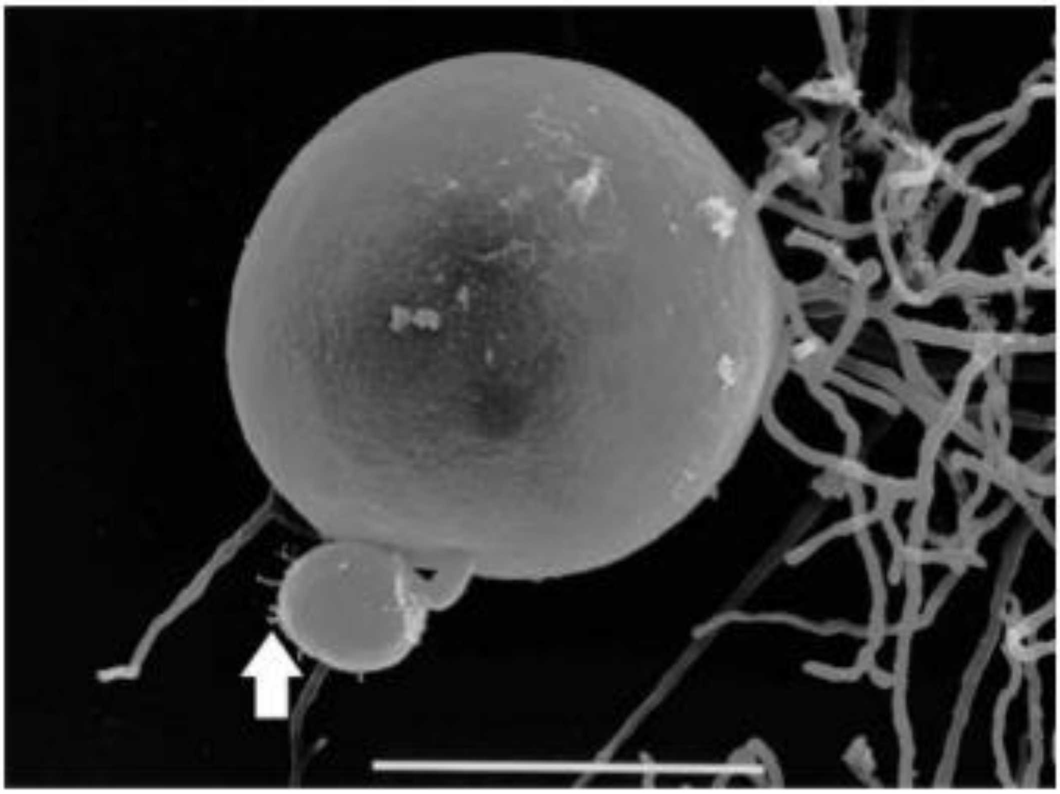

Figure S1. Scanning electron microscopic image of a *Batrachochytrium salamandrivo*rans sporangium showing rhizoid formation on a germ tube–derived developing sporangium (arrow). (Scale bar, 10 μm.)

Table S1. Taxon sampling for phylogenetic analysis of 30 isolates

Taxon	Strain	GenBank accession no.	
		18S	28S
Arkaya lepida	JEL93	AF164278	DQ273814
Batrachochytrium dendrobatidis	JEL423	DS022300	DS022300
Batrachochytrium dendrobatidis	JEL197	AF051932	AY546693
Batrachochytrium dendrobatidis	JAM81	GL882879	GL882879
Boothiomyces macroporosum	PLAUS21	DQ322622	DQ273823
Chytridium confervae	ATCC24931	NSA	AY349065
Chytriomyces hyalinus	MP4	DQ536487	DQ273836
Cladochytrium replicatum	JEL180	AY546683	AY546688
Entophlyctis sp.	JEL174	AY635824	DQ273782
Entophlyctis helioformis	JEL326	AY635826	DQ273784
Entophlyctis luteolus	JEL129	AH009064	AY442957
Gaertneriomyces semiglobifer	UCB-91-10	AF164247	DQ273778
Homolaphlyctis polyrhiza	JEL142	AF164299	EF634247
Kappamyces laurelensis	PL98	DQ536478	DQ273824
Kochiomyces dichotomus	BR269	FJ804151	FJ804155
Lacustromyces hiemalis	JEL31	AH009039	NSA
Lobulomyces angularis	JEL45	Af164253	DQ273815
Mesochytrium penetrans	X-10	FJ804149	FJ804153
Monoblepharis macrandra	M53B	AY349029	AY349061
Obelidium mucronatum	JEL57	AH009056	AY439071
Oedogoniomyces sp.	CR84	AY635839	DQ273804
Olpidium brassicae	SS218	DQ322624	DQ273818
Polychytrium aggregatum	JEL109	AY601711	AY546686
Rhizoclosmatium globosum	JEL06	AH009057	AY349063
Rhizophlyctis harderi	JEL171	AF164272	DQ273775
Rhizophlyctis rosea	JEL318	AY635829	DQ273787
Spizellomyces punctatus	ATCC48900	AY546684	AY546692
Synchytrium decipiens	DUH0009362	DQ536475	DQ273819
Synchytrium macrosporum	DUH0009363	DQ322623	DQ273820
Triparticalcar sp.	JEL555	FJ827658	FJ827683
Outgroup			
Allomyces arbuscula	Brazil2	AY552524	AY552525
Catenaria anguillulae	PL171	FJ804150	FJ804154
Rozella allomycis	UCB-47-54	AY635838	DQ273803

NSA, no sequence available.

Chapter 6

Expanding Distribution of Lethal Amphibian Fungus *Batrachochytrium salamandrivorans* in Europe

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Emerging fungal diseases can drive amphibian species to local extinction. During 2010–2016, we examined 1,921 urodeles in 3 European countries. Presence of the chytrid fungus *Batrachochytrium salamandrivorans* at new locations and in urodeles of different species expands the known geographic and host range of the fungus and underpins its imminent threat to biodiversity.

Introduction

Amphibians provide an iconic example of disease-driven global loss in biodiversity. The recently described chytrid fungus *Batrachochytrium salamandrivorans* (Bsal) is an emerging pathogen that is driving amphibian populations to local extinction (Martel et al. 2013; 2014). This highly pathogenic fungus causes a lethal skin disease that has so far been restricted to urodele amphibians (newts and salamanders); the fungus was most likely introduced from East Asia into Europe via the pet trade (Martel et al. 2014). In Europe, Bsal infection has led to dramatic declines of fire salamander (*Salamandra salamandra*) populations in the Netherlands and Belgium (Martel et al. 2014). Within 7 years after the supposed introduction of the fungus, a population in the Netherlands declined by 99.9% (Spitzen-van der Sluijs et al. 2013; Goverse and de Zeeuw, 2014). In the United Kingdom and Germany, Bsal has been detected in captive salamanders and newts (Cunningham et al. 2015; Sabino-Pinto et al. 2015). Infection trials suggest that Bsal represents an unprecedented threat to diversity of Western Palearctic urodeles (Martel et al. 2014); nevertheless, reports of deaths among salamanders and newts in their naturalized ranges have been restricted to a few populations of a single salamander species in the southern Netherlands and adjacent Belgium (Martel et al. 2013; Spitzen-van der Sluijs et al. 2013). Using data from field surveillance, we examined the hosts and the geographic range of Bsal in Europe.

The Study

During 2010–2016, we collected samples of free-living populations of newts and salamanders from 48 sites in the Netherlands, Belgium, and adjacent regions of the Eifel region in Germany (near the border with the Netherlands and Belgium) (Figure; online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/22/7/16-0109-Techapp1.pdf). Site selection was based on reported amphibian deaths, apparent negative amphibian population trends, preventive Bsal surveillance in susceptible populations, or geographic proximity to known outbreak sites. Samples were also collected at 6 additional sites in Germany and 1 in the Netherlands, which were located >100 km from the nearest known outbreak (online Technical Appendix Table 2). Sampling was conducted by swabbing skin (Hyatt et al. 2007; Van Rooij et al. 2011) of live animals and collecting skin samples from dead animals. All samples were kept frozen at -20°C until they were analyzed for the presence of Bsal DNA via real-time PCR, as described (Blooi et al 2013).

Across all 55 sites, we tested 1,019 fire salamanders (43 dead, 976 skin swab samples); at site 14, skin swab samples instead of tissue samples were collected from 16 dead salamanders. We also collected samples from 474 alpine newts (*Ichthyosaura alpestris*; 18 dead, 456 skin swab samples), 239 smooth newts (*Lissotriton vulgaris*; 2 dead, 237 skin swab samples), 80 palmate newts (*Lissotriton helveticus*; all skin swab samples), 79 crested newts (*Triturus cristatus*; all skin swab samples), and 30 Italian crested newts (*Triturus carnifex*; all skin swab samples). To obtain a Bayesian 95% credible interval for prevalence (online Technical Appendix), we used the computational methods of Lötters et al. (2012). We ran 3 parallel Markov chains with 20,000 iterations each and discarded the first 5,000 iterations as burn-in; chains were not thinned.

Bsal was found at 14 of the 55 sites; infected amphibians were fire salamanders, alpine newts, and smooth newts. Our results demonstrate that the range of Bsal distribution may be up to ≈10,000 km^2 (measured as the surface of a minimum convex polygon encompassing the outermost points) across Germany, Belgium, and the Netherlands (Figure 1). The presence of

Bsal in wild alpine newts and smooth newts shows distinct expansion of the known host range in the wild (online Technical Appendix Table 1). Furthermore, we document that Bsal is present in natural fire salamander populations in Germany (confined to the Eifel region). At some sites, because of our sample sizes, the upper limit of the 95% credible interval for Bsal prevalence was as high as 0.7; therefore, we may have failed to detect Bsal at these sites (online Technical Appendix Table 1). In addition, the fungus may have been present at several sites before first detection. For example, Bsal was detected at site 4, where population-monitoring efforts in the years before detection (2000–2013) showed declines in 4 newt species (http://www.ravon.nl/EID_SI_Spitzen_et_al_2016). However, because no samples were collected before 2015, we have no evidence for a causal relationship between the presence of Bsal and the declines. We have also recorded the presence of Bsal in populations with no evidence of population change so far, such as the incidental findings of dead Bsal-positive newts in fyke nets at sites 5 and 11, and the incidental findings of dead Bsal-positive fire salamanders at sites 12 and 14. Clinical signs of mycosis, such as lethargy and skin shedding (Martel et al. 2013), were observed at some Bsal-positive sites (1, 2, 7, 8, 14) but not at others.

Conclusions

Our study provides evidence that Bsal among wild amphibians in Europe is more widely distributed and affects a wider host range than previously known, which can either indicate recent spread of the fungus or point to historically infected sites that hitherto remained undetected. The presence of Bsal in wild populations can easily remain unnoticed because the lesions develop only near the final stage of the disease (Martel et al. 2013). This information is crucial for the design of field surveys for Bsal surveillance. Our data might be used to inform a management strategy and to implement the recommendation of the Bern Convention (Council of Europe, 2015) to halt the spread of Bsal in Europe. Research to search for molecular evidence that the outbreak locations are connected is under way. Chytrid disease dynamics are affected by multiple factors (e.g., temperature regimes (Martel et al. 2013)), and yet undetermined environmental determinants might be essential for disease outbreaks (Van Rooij et al. 2015). Untangling these factors, as well as the modes of Bsal spread and its geographic distribution, are points for further research to fully map the problem and identify populations and species at risk.

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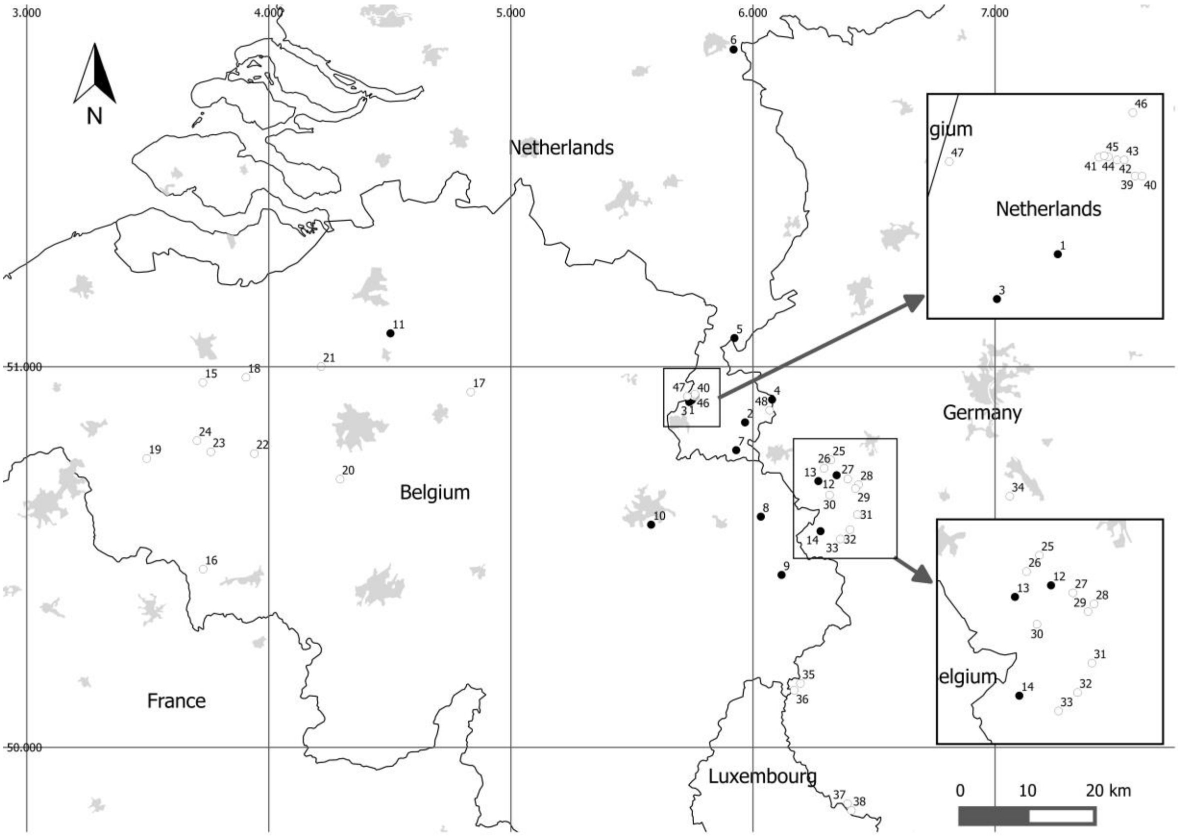

Figure 1. Study sites for collection of amphibians in Western Europe, 2010–2016. Numbers correspond to field sites at which amphibians were collected and examined for *Batrachochytrium salamandrivorans* (Bsal) (online Technical Appendix, http://wwwnc.cdc.gov/EID/ article/22/7/16-0109-Techapp1.pdf). Solid circles, Bsal detected; open circles, Bsal not detected. Larger cities are indicated in light gray. Note that there are additional sites where the fungus remained undetected (not shown).

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Technical Appendix

Technical Appendix Table 1. Field sites where Bsal was detected, sampled species, numbers of Bsal-positive and total sampled specimens*

Site no., location, and amphibian collected	No. *Bsal*-positive/total tested (year)	Observed prevalence (Bayesian 95% credible intervals)	Remarks
The Netherlands			
1, Bunderbos, deciduous forest			
Fire salamander	3/3 (2010)	1.00 (0.42-1.00)	Past mass mortality; 99.9% population decline (1997 - 2014)
	1/1 (2011)	1.00	
	1/1 (2012)	1.00	
	0/3 (2014)	0.00 (0.00-0.61)	
	2/14 (2015)	0.14 (0.04-0.40)	
	0/1 (2016)	0	
Alpine newt	1/1 (2013)	1.00	Possibly declining (monitoring started in 2013)†
	1/39 (2014)	0.03 (0.01-0.13)	
	1/10 (2015)	0.10 (0-0.43)	
	0/6 (2016)	0 (0-0.43)	
2, Putberg, deciduous forest			
Smooth newt	0/2 (2014)	0 (0-0.70)	Possibly declining†
Alpine newt	0/10 (2014)	0 (0-0.31)	Possibly declining†
	1/1‡ (2014)	1.00	
	1/1‡ (2015)	1.00	
3, Meerssen, garden pond			
Fire salamander	0/1 (2015)	0	No evidence of decline §
Smooth newt	4/43(2015)	0.09 (0.04-0.21)	No evidence of decline §
Alpine newt	0/9 (2015)	0 (0-0.30)	No evidence of decline §
4, Wormdal, clusters of natural ponds in nature conservation area¶			
Smooth newt	1/22 (2015)	0.05 (0.01-0.21)	87% decline (2000-2013) †#
Alpine newt	0/12 (2015)	0 (0-0.26)	96% decline (2000-2013) †#
5, Pepinusbeekdal, extensive agriculture			
Smooth newt	1/2‡ (2014)	0.50 (0.09-0.88)	No evidence of decline †
6, Berg en Dal, garden pond			
Alpine newt	12/12‡ (2015)	1.00 (0.74-1.00)	Yearly mass deaths; species still present §
7, Vijlenerbosch , deciduous forest			
Alpine newt	0/1 (2013)	0	No evidence of decline §
	0/30 (2014)	0 (0-0.11)	
	1/18 (2015)	0.05 (0.02-0.24)	
Smooth newt	0/8 (2014)	0 (0-0.31)	No evidence of decline §
	0/11 (2015)	0(0-0.26)	
Palmate newt	0/1 (2014)	0	No evidence of decline §
	0/9 (2015)	0(0-0.30)	
Belgium			
8, Eupen, deciduous forest			
Fire salamander	1/2 (2013)	0.50 (0.09-0.88)	Deaths, probably fire salamanders severely declining, no monitoring trend available
9, Robertville , deciduous forest			
Fire salamander	16/30 (2014)	0.53 (0.36-0.69)	Deaths, severe decline,

Site no., location, and amphibian collected		No. *Bsal*-positive/total tested (year)	Observed prevalence (Bayesian 95% credible intervals)	Remarks
				monitoring ongoing
10,	Liège, deciduous forest			
	Fire salamander	5/5 (2014)	1.00 (0.55-1.00)	Deaths
11,	Duffel, garden pond			
	Alpine newt	2/30‡ (2015)	0.07 (0.02-0.22)	2 dead in fyke; no evidence of decline
	Smooth newt	0/16 (2015)	0 (0-0.20)	No deaths; no evidence of decline
Germany				
12,	Weisse Wehe, deciduous forest			
	Fire salamander	4/11‡ (2015)	0.36 (0.15-0.65)	No evidence of decline†
13,	Solchbachtal, mixed forest			
	Fire salamander	0/2 (2014) 1/51 (2015)	0 (0-0.70) 0.02 (0.01-0.10)	Decreased newts and salamanders§
	Palmate newt	0/19(2014)	0 (0-0.18)	Decreased newts and salamanders§
	Alpine newt	0/5(2014)	0 (0-0.44)	Decreased newts and salamanders§
14,	Belgenbachtal, mixed forest			
	Fire salamander	21/22‡ (2015)	0.96 (0.79-0.99)	Remarkable deaths (16 dead), noted only since Nov 2015†

*Bsal, *Batrachochytrium salamandrivorans*. Data provide an overview of novel information and previously published data. Site numbers correspond to those on map (Figure).
†Population monitored.
‡Includes individual(s) found dead by chance.
§Anecdotal reports.
¶At this site, crested newts and smooth newts decreased with similar percentages over the same period (-96%; -94%, respectively).
#http://www.ravon.nl/EID_SI_Spitzen_et_al_2016.

Technical Appendix Table 2. Field sites studied where Bsal was not detected, number of sampled species and specimens*

Site no., location, and amphibian collected		Number of specimens tested (year)	Observed prevalence (Bayesian 95% credible intervals)	Remarks
Belgium				
15,	Nerenbos , deciduous forest			
	Fire salamander	30 (2015)	0 (0-0.11)	No evidence of decline†
16,	Heilig Geestgoed, deciduous forest			
	Fire salamander	30 (2015)	0 (0; 0.11)	No evidence of decline†
17,	Kasteel van Horst, deciduous forest			
	Fire salamander	30 (2015)	0 (0-0.11)	No evidence of decline†
18,	Smetledebos, deciduous forest			
	Fire salamander	30 (2015)	0 (0-0.11)	No evidence of decline†
19,	Kluisbos ,deciduous forest			
	Fire salamander	30 (2015)	0 (0-0.11)	No evidence of decline†
20,	Hallerbos, deciduous forest			
	Fire salamander	30 (2015)	0 (0-0.11)	No evidence of decline†
21,	Buggenhoutbos - deciduous forest			
	Fire salamander	30 (2015)	0 (0-0.11)	No evidence of decline†
22,	Raspaillebos - deciduous forest			
	Fire salamander	30 (2015)	0 (0-0.11)	No evidence of decline†
23,	Haeyesbos- deciduous forest			
	Fire salamander	30 (2015)	0 (0-0.11)	No evidence of decline†
24,	t Burreken - deciduous forest			
	Fire salamander	30 (2015)	0 (0-0.11)	No evidence of decline†
Germany				
25,	Lamersiefen - deciduous forest			
	Fire salamander	17 (2014) 32 (2015)	0 (0-0.19) 0 (0-0.11)	No evidence of decline†
26,	Fischbach, deciduous forest			
	Fire salamander	36 (2014) 51 (2015)	0 (0-0.09) 0 (0-0.07)	No evidence of decline; 3 dead-found specimens tested negative for *Bsal* via histology (2014)†
	Alpine newt	1 (2015)	0	Live-studied specimen by chance, no evidence of decline
	Palmate newt	1 (2015)	0	Live-studied specimen by chance, no evidence of decline
27,	Kallerbach , deciduous forest			
	Fire salamander	24(2015)	0 (0-0.15)	No evidence of decline†
28,	Rosbach, deciduous forest			
	Fire salamander	47 (2015)	0 (0-0.07)	No evidence of decline†
29,	Zweifallshammer, deciduous forest			
	Fire salamander	41 (2015)	0 (0-0.08)	No evidence of decline†
30,	Peterbach, mixed forest			
	Palmate newt	12 (2014)	0 (0-0.26)	No evidence of decline
	Alpine newt	4 (2014)	0 (0-0.52)	No evidence of decline
31,	Haftenbach , deciduous forest			

Site no., location, and amphibian collected		Number of specimens tested (year)	Observed prevalence (Bayesian 95% credible intervals)	Remarks
	Fire salamander	46 (2015)	0 (0-0.08)	No evidence of decline†
32,	Sauerbach, deciduous forest			
	Fire salamander	22 (2015)	0 (0-0.15)	No evidence of decline†
	Alpine newt	1 (2015)	0	No evidence of decline
33,	Härtgessief, deciduous forest			
	Fire salamander	15 (2014)	0 (0-0.19)	Strong evidence of decline†
34,	Kottenforst, deciduous forest			
	Fire salamander	51 (2015)	0 (0-0.07)	No evidence of decline
35,	Großkampenberg, mixed forest			
	Alpine newt	4 (2015)	0 (0-0.52)	No evidence of decline
	Palmate newt	1 (2015)	0,00	No evidence of decline
36,	Lützkampen, mixed forest			
	Alpine newt	8 (2015)	0 (0-0.31)	No evidence of decline
37,	Ferschweiler, mixed forest			
	Alpine newt	2 (2015)	0 (0-0.70)	No evidence of decline
	Palmate newt	8 (2015)	0 (0-0.31)	No evidence of decline
38,	Ernzen, mixed forest			
	Fire salamander	4 (2015)	0 (0-0.52)	No evidence of decline†
The Netherlands				
39,	Moerveld surroundings (A), Bunderbos vicinity			
	Alpine newt	13 (2015)	0 (0-0.22)	No evidence of decline‡
40,	Moerveld surroundings (B), Bunderbos vicinity			
	Alpine newt	2 (2014)	0 (0-0.70)	No evidence of decline‡
41,	Snijdersbergweg 21, garden pond			
	Alpine newt	30 (2015)	0 (0-0.11)	No evidence of decline‡
42,	Mevr van der Meijstraat 12, garden pond			
	Alpine newt	14 (2015)	0 (0-0.20)	No evidence of decline‡
43,	Mevr van der Meijstraat 20, garden pond			
	Alpine newt	17 (2015)	0 (0-0.19)	No evidence of decline‡
44,	Snijdersbergweg 20, 2 garden ponds			
	Alpine newt	20 (2015)	0 (0-0.15)	No evidence of decline‡
45,	Snijdersbergweg 23b, garden pond			
	Alpine newt	15 (2015)	0 (0-0.19)	No evidence of decline‡
46,	Broekhoven, garden pond			
	Fire salamander	2 (2015)	0 (0-0.70)	No evidence of decline‡
47,	Meerssen, deciduous forest			
	Fire salamander	57 (2013) 43 (2014) 29 (2015) 2 (2016)	0 (0-0.06) 0 (0-0.08) 0 (0-0.11) 0 (0-0.70)	No deaths; no evidence of decline†
48,	Carisberg, deciduous forest			
	Alpine newt	8 (2014)	0 (0-0.31)	No information available
	Palmate newt	23 (2014)	(0-0.14)	No information available
	Smooth newt	2 (2014)	0 (0-0.70)	No information available
Additional far-out sites (Germany)				
N.S.	Solling, deciduous forest			
	Fire salamander	23 (2015)	0 (0-0.14)	No evidence of decline‡
N.S.	Ilsenburg, deciduous forest			
	Fire salamander	8 (2015)	0 (0-0.31)	No evidence of decline‡
N.S.	Lelm, deciduous forest			

Site no., location, and amphibian collected		Number of specimens tested (year)	Observed prevalence (Bayesian 95% credible intervals)	Remarks
	Alpine newt	57 (2015)	0 (0-0.06)	No evidence of decline‡
	Palmate newt	6 (2015)	0 (0-0.43)	No evidence of decline‡
	Smooth newt	16 (2015)	0 (0-0.20)	No evidence of decline‡
	Crested newt	29 (2015)	0 (0-0.11)	No evidence of decline‡
N.S.	Kleiwiesen, exposed ponds surrounded by deciduous forest			
	Alpine newt	27 (2015)	0 (0-0.13)	No evidence of decline‡
	Smooth newt	117 (2015)	0 (0-0.03)	No evidence of decline‡
	Crested newt	27 (2015)	0 (0-0.13)	No evidence of decline‡
N.S.	Waldecker Schlossgrund , deciduous forest			
	Fire salamander	22 (2015)	0 (0-0.15)	No evidence of decline‡
N.S.	Closewitz - exposed ponds surrounded by deciduous forest			
	Crested newt	23 (2015)	0 (0-0.14)	No evidence of decline‡
Additional far-out site (the Netherlands)				
N.S.	Veluwe, deciduous forest			
	Italian crested newt	30 (2015)	0 (0-0.11)	No evidence of decline‡

*Bsal, *Batrachochytrium salamandrivorans*; N.S., not shown on map (Figure). Data provide an overview of novel information and previously published data. Site numbers correspond to those on map (Figure).
†Population monitored.
‡Anecdotal report.

Technical Appendix – Supplementary file

https://www.ravon.nl/EID_SI_Spitzen_et_al_2016

Information on monitoring efforts in the years 2000–2013 at site 4

Four species of newts (great crested newts (*Triturus cristatus*), smooth newts (*Lissotriton vulgaris*), palmate newts (*L. helveticus*) and alpine newts (*Ichthyosaura alpestris*)) occur in natural populations at site 4 (Wormdal, The Netherlands). All newt populations have shown severe population declines over the period 2000–2013. A direct causal relationship between the presence of the chytrid fungus *Batrachochytrium salamandrivorans* and the decrease of the newt populations at this site has not yet been demonstrated.

All newts at this site are monitored yearly according to the Dutch national standard (Groenveld and Goverse, 2011). Population trend calculation was conducted following Spitzen-van der Sluijs et al. (2013), alpine newts and smooth newts have retrospective decreases of -96.6% and -87% in the period 2000–2013. The great crested newt population decreased by 96% as well, and the palmate newt population decreased by 94% (Figure 1).

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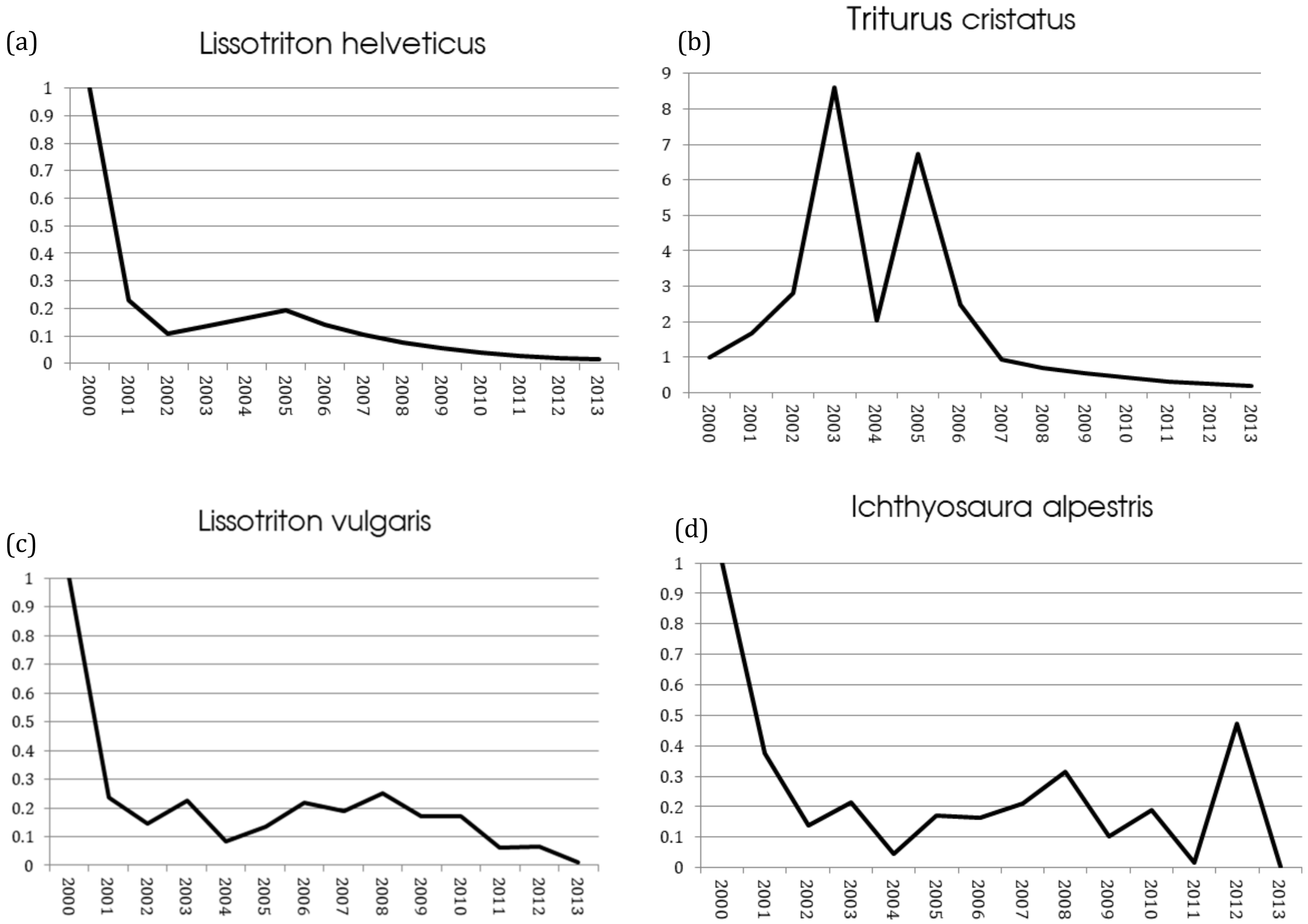
	Lissotriton helveticus	*Triturus cristatus*	*Lissotriton vulgaris*	*Ichthyosaura alpestris*
Year	Computed Model Index	Computed Model Index	Computed Model Index	Computed Model Index
2000	1	1	1	1
2001	0.2283	1.6792	0.2357	0.3764
2002	0.1104	2.8197	0.1462	0.1407
2003	0.1348	8.5967	0.227	0.2143
2004	0.1646	2.0418	0.0843	0.0473
2005	0.1951	6.7271	0.134	0.1707
2006	0.1422	2.4949	0.2198	0.164
2007	0.1036	0.9253	0.1889	0.2093
2008	0.0755	0.7088	0.2529	0.3165
2009	0.055	0.5429	0.1727	0.102
2010	0.0401	0.4159	0.1717	0.1909
2011	0.0292	0.3185	0.0613	0.0181
2012	0.0213	0.244	0.0641	0.474
2013	0.0155	0.1869	0.011	0.0012



Population trend of (a) *L. helveticus* (Lh), (b) *T. cristatus* (Tc), (c) *L. vulgaris* (Lv) and (d) *I. alpestris* (Ia) at site 4 over the period 2000 – 2013 (X-axis). The index (Y-axis) is set at 1 at the start of the monitoring programme and shows dramatic and continuing declines (Lh: -94%, Tc: -96%, Lv: -87% and Ia: -96.6%).

Chapter 7

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

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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* (Bsal) 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 Bsal 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, Bsal-driven collapse of susceptible hosts at the source site. Although the exact cause remains unclear, the aggregate evidence suggests that Bsal may be a poorer disperser than previously hypothesized. The lack of Bsal dispersal between neighbouring salamander populations opens perspectives for disease management and stresses the necessity of implementing biosecurity measures preventing human-mediated spread.

Introduction

Emerging infectious disease of wildlife are a leading cause of biodiversity loss worldwide (Daszak et al. 2000). Because successful mitigation of epizootics 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 northwestern Europe, the recently detected chytrid fungus *Batrachochytrium salamandrivorans* (hereafter: Bsal) (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 Bsal from infected wild populations or to reduce its impact (Canessa et al. 2018). Previous studies have hypothesized that Bsal should be able to spread rapidly, similarly to *B. dendrobatidis* (hereafter: Bd) (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 Bsal-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 Bsal 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 Bsal 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 Bsal.

To clarify the potential factors determining this failure of Bsal spread between two neighbouring sites, we carried out field surveys at both the Broek and Bunderbos subpopulations to estimate fire salamander abundance, to quantify Bsal 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 Bsal vector sympatric with fire salamanders (Stegen et al. 2017)), in which we determined the ability of Bsal to spread autonomously when host contact is physically impeded. We present the results of these analyses and discuss their implications for Bsal dispersal and potential mitigation strategies.

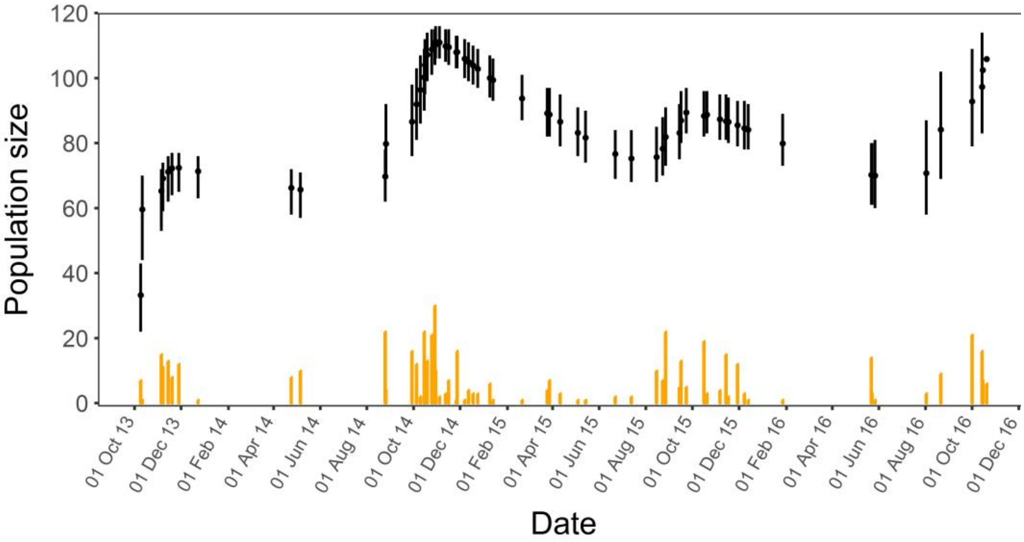


Figure 1. Population size of fire salamanders at the new site, estimated from open-population Jolly-Seber model. Bars indicate 95% credible intervals. Orange bars indicate the total count of captures on a given survey.

Results

Persistence of a stable Bsal-free salamander subpopulation in the vicinity of a Bsal outbreak site

To quantify the Bsal 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 (Supplementary Information), we estimated the Broek subpopulation size to have fluctuated between 75 and 115 individuals over the study period (Figure 1), showing a seasonal pattern consistent with the breeding season of fire salamanders (juveniles emerging between August and October). 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 Bsal. 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 Bsal. 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 Bsal. 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 $5.3 \cdot 10^3$ 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 Bsal (0/7 and 0/74 respectively).

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 (Figure 2).

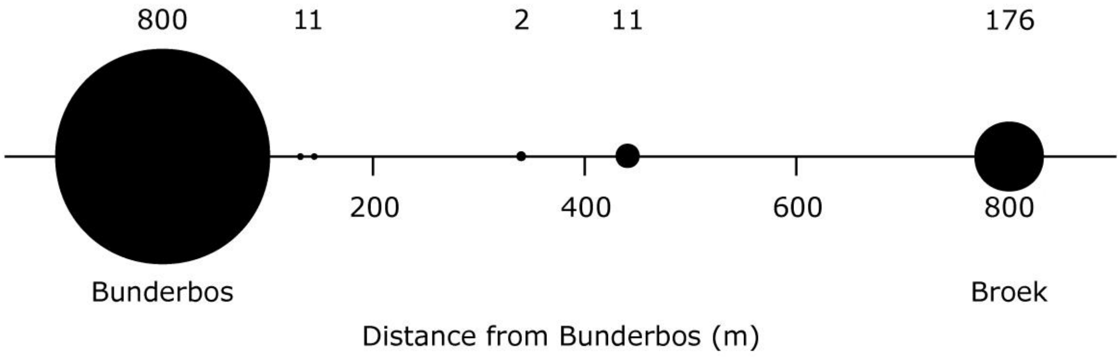
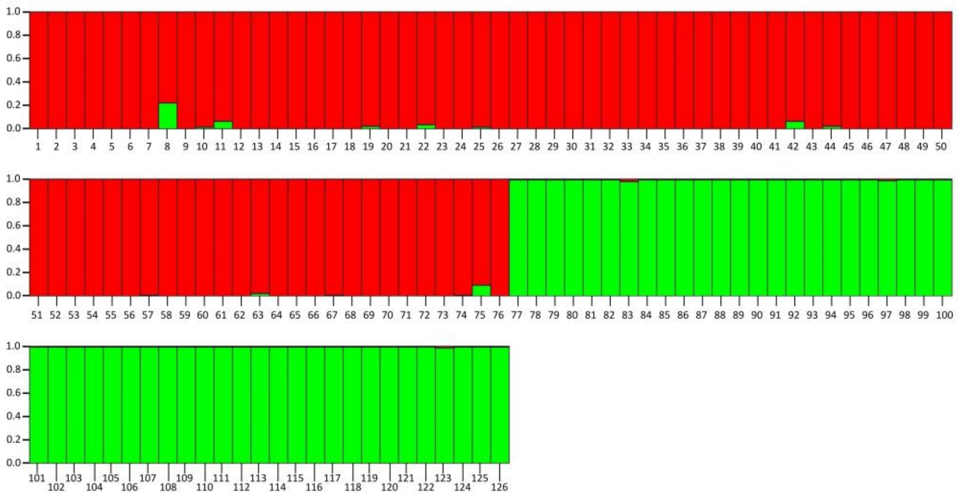


Figure 2. Schematic representation of the distance (in meters) between the Bunderbos and Broek subpopulations and in the matrix in between the two subpopulations. The size of circles corresponds to the number of fire salamanders observed from 2007–2017 (indicated above each circle).

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) (Figure 3a, Supplementary Information Table S1, Fig. S1a,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) (Figure 3b, Supplementary Information Table S1, Figure S2a,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).


a Broek and Bunderbos (1-76) subpopulations and Kottenforest (77-126) population

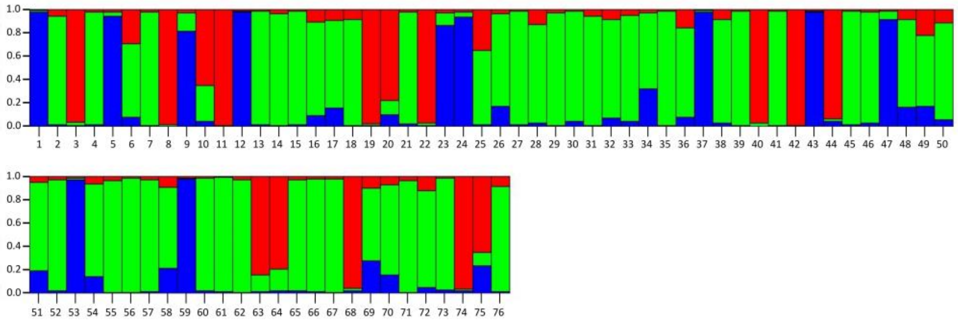

b Broek (22-34) and Bunderbos (1-21; 35-76) subpopulation

Figure 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**).

Physical barriers prevent Bsal transmission We conducted a laboratory experiment with alpine newts (*Ichthyosaura alpestris*) to test if Bsal 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 × 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 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; Figure 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 Bsal.

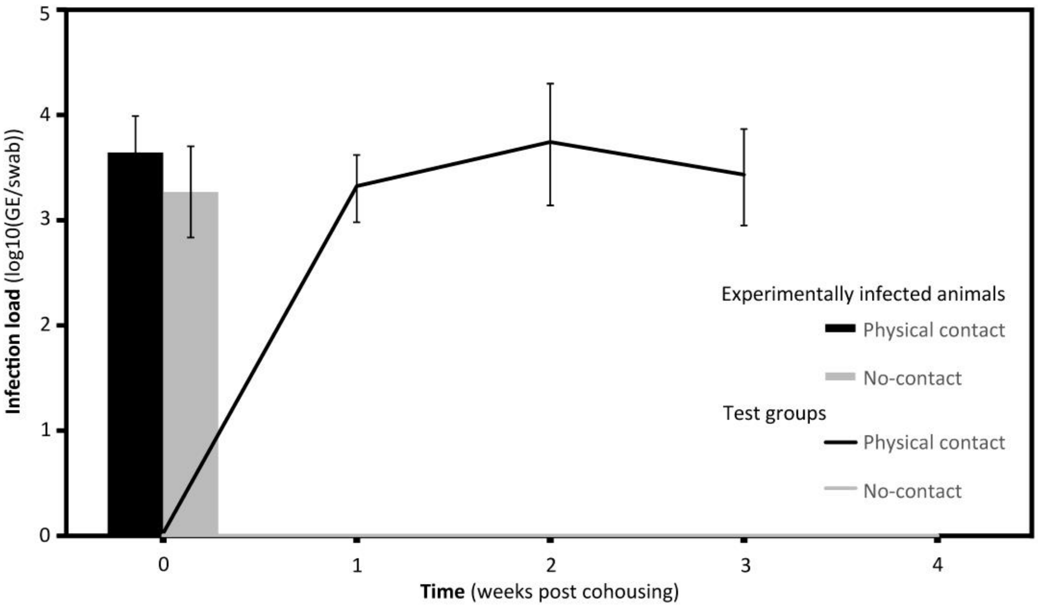


Figure 4. In vivo infection experiment with alpine newts (*Ichthyosaura alpestris*). Average infection load for infected newts in each group (contact vs no-contact group). In the group where physical contact was possible (black), 5 out of 7 newts developed chytridiomycosis while none of the newts in the group where contact was prevented (grey) tested positive for Bsal 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.

Discussion

We found no evidence of Bsal 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 Bd (Lips et al. 2008) and previous hypotheses about the imminent threat of a rapid Bsal spread (Schmidt et al. 2017; Yap et al. 2017). The Broek subpopulation has so far persisted in a Bsal-refuge in the vicinity of the Bsal index site, although the exact reason remains unclear. In the remainder of this discussion, we consider the possible scenarios and the implications for future Bsal mitigation.

Autonomous dispersal by Bsal 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 Bsal by infected hosts is arguably the most intuitive. In the surroundings of a known Bsal outbreak site near Liège in Belgium (Spitzen-van der Sluijs et al. 2016), Bsal-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 (Figure 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 (Suppl. Inf. Table S2a, b). Additionally, there is an indication of recent or ongoing gene flow between the two subpopulations (but see Suppl. Inf. 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 Bsal-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 Bsal, 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 Bsal 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 Bsal 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 Bsal 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 Bsal epizootic 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 Bsal 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 Bsal 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 Bsal 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 Bd (Lips et al. 2008). In turn, this information has important implications for Bsal mitigation. Although mitigation is likely to prove highly challenging during the epizootic 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 Bsal is indeed a weaker disperser than originally hypothesized further reinforces the need to prevent its human-mediated dispersal. The currently known distribution of Bsal 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 Bsal arrival within their ranges.

Methods

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 Bsal 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 (www.ndff-ecogrid.nl; accessed 16 Nov. 2017). Elevation of the Broek subpopulation ranges between 40 m and 56 m above sea level (www.ahn.nl; accessed 7 Sept. 2017), and the vegetation consists of poplar trees, shrubs, bushes and grassland.

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 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 between surveys. We fitted the model in JAGS (Kéry and Schaub, 2011; Plummer, 2003) using uninformative priors for all parameters (model code in Supplementary Information). 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.

Detection of Bsal 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 Bsal 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 Bsal infection on dead salamanders.

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).

Physical barriers act to restrain Bsal transmission Although soil can act as a vector for Bsal (Stegen et al. 2017), it is unknown whether the fungus can spread actively over short distances. We studied the role of physical barriers in Bsal 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 × 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 Bsal type strain (AMFP13/1) suspended in 1 ml distilled water following Martel et al. (2014) to ensure 100% Bsal 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 Bsal 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 Bsal 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 Bsal, the animal was removed from the experiment and treated as described by Blooi et al. (2015). Each container (19 × 12 × 7 cm) was filled with a layer of unsterilized and moisturized forest soil – from a Bsal-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 Bsal, Bd and ranavirus – two other infections causing major amphibian diseases.

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/75 A/2016/015 issued by the Netherlands Enterprise Agency.

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Supplemental Information

Imperfect barriers promote post-epizootic salamander persistence in disease free refugia

Annemarieke Spitzen, Gwij Stegen, Sergé Bogaerts, Stefano Canessa, Sebastian Steinfartz, Nico Janssen, Wilbert Bosman, Frank Pasmans and An Martel. *Scientific Reports* (2018).

More detailed methods, results and discussion section on the analysis of genetic analyses

Supplemental Information Table S1. Evanno table identifying the most probable hypothetical K within the dataset.

Supplemental Information Table S2a. Mitochondrial D-loop analysis.

Supplemental Information Table S2b. Comparison of the nucleotide sequences

Supplementary Information Table S3. Genetic diversity indices for Bunderbos

Supplementary information Figure S1. Selection of the most likely number of K

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

References

More detailed methods, results and discussion section on the analysis of genetic analyses

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 and Smouse, 2012) and ARLEQUIN ver3.5.1.2. (Excoffier and Lischer, 2010) to determine deviations from Hardy–Weinberg equilibrium in the Bunderbos subpopulation for each locus, which provides an exact probability value (Guo and Thompson, 1992). We then estimated the populations' inbreeding coefficients (F_{IS} and F_{ST}).

Results

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) (Supplementary Table S2a), 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 (Supplementary Table 2b).

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 (Supplementary Table 3), 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. The mean number of effective alleles was 2.40 (range: 1.16-3.88). Mean observed heterozygosity (H_o=0.48) was lower than expected (H_e=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 (H_o=0.55) was also lower than expected (H_e=0.57). Mean inbreeding coefficient was (F_{IS}) 0.05. The genetic distance (F_{ST}) between the two populations (Kottenforst and Bunderbos) was estimated to 0.20.

Discussion

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.

Similar genetic constitution of the subpopulations

The outcome of the STRUCTURE analysis indicating the presence of three genetic clusters (K=3; see Supplementary Figure S2b), 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 Bsal. 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.

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 (FIS) 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-Ureña 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.

Supplemental Information Table S1.

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

Supplemental Information Table S2a.

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	No. of individuals analysed for the D-loop	Haplotypes assignment and 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%)

Supplemental Information Table S2b.

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

D-loop haplotype	Nucleotide site										
	1-26	27	28-274	275	276-336	337	338-428	429	430-589	590	591-724
Type I	IDENTICAL	C	IDENTICAL	A	IDENTICAL	-	IDENTICAL	A	IDENTICAL	C	IDENTICAL
Type Ia		C		A		-		A		-	
Type Ib		C		A		T		A		C	
Type I-like		C		T		-		A		C	
Type II		T		A		-		A		C	
Type IIa		T		A		-		G		C	

Supplementary Information Table S3.

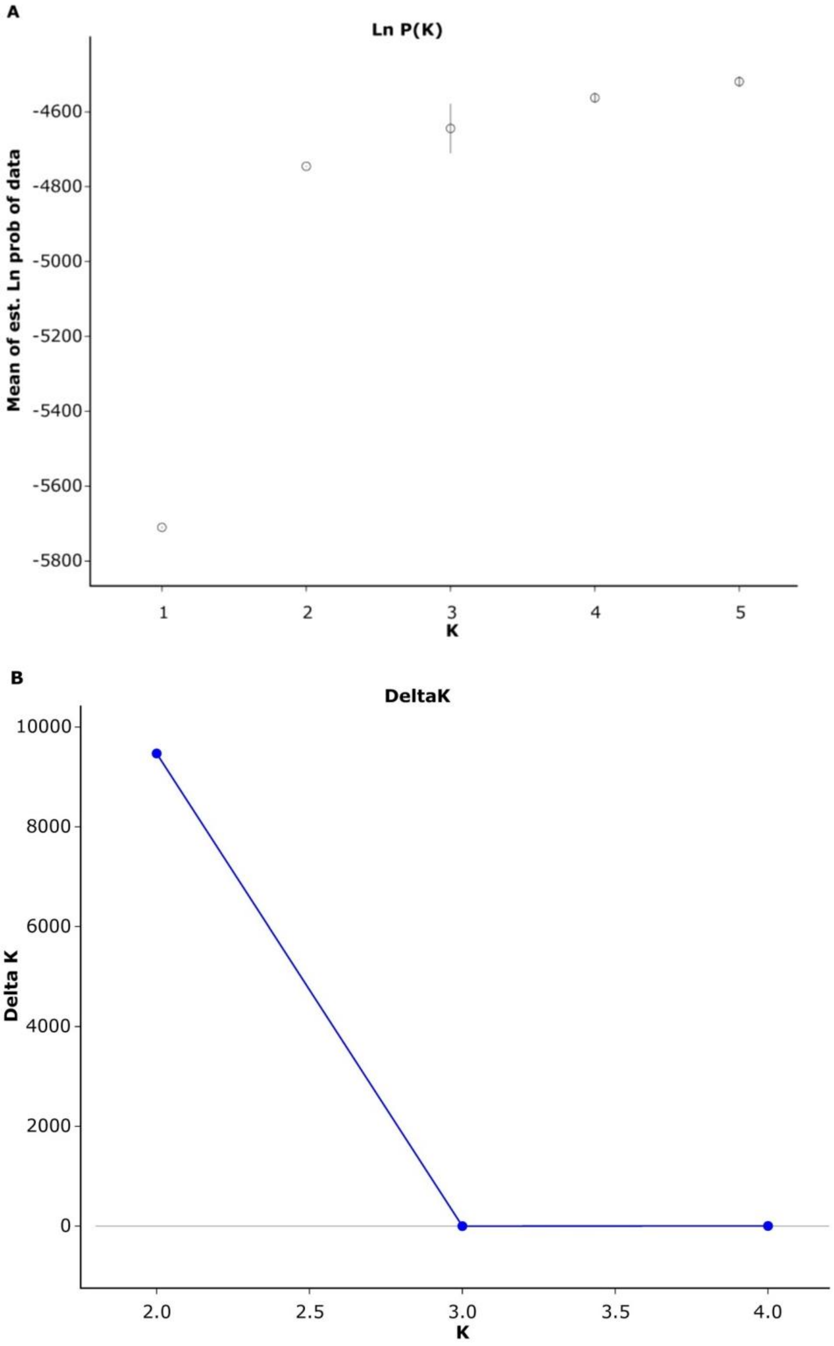
Genetic diversity indices for Bunderbos based on 18 microsatellite loci. Significance testing was corrected for multiple comparisons by the Bonferroni correction (adjusted *P*-value = 0.0028). N: number of samples, N_a: number of alleles, Ne: effective number of alleles, I: Shannon's information index, Ho: observed heterozygosity, He: expected heterozygosity, χ^2: Chi-square test for Hardy-Weinberg equilibrium, Signif: *P*-value for χ^2 test, Post Bonferroni corr: significance of *P*-value after Bonferroni correction (ns: not significant; SIGNIF: significant), FIS: 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	3.9	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	-	-	-	0.05
	SE	0.71	0.35	0.19	0.08	0.05	0.04	-	-	-	0.06

* adjusted *P*-value: 0.0028

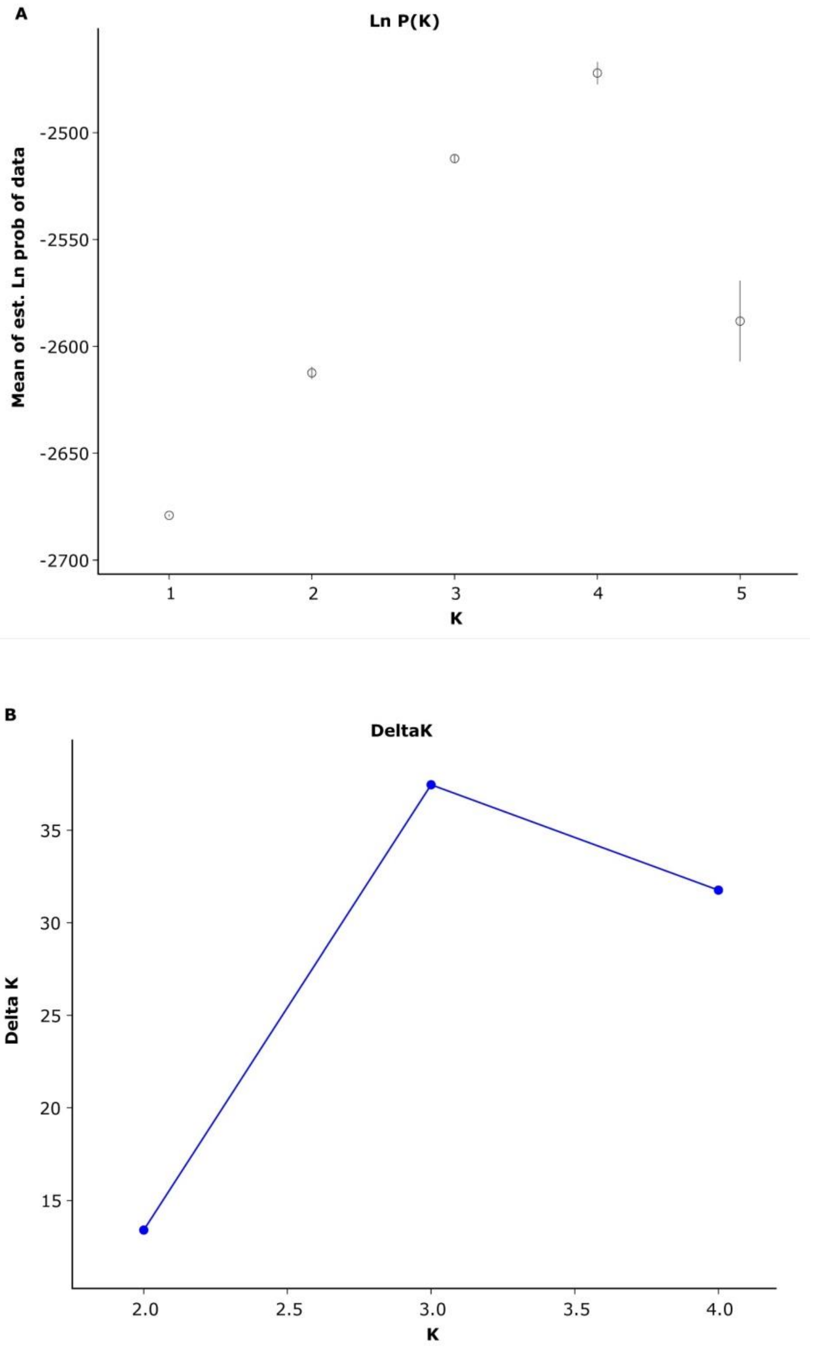
Supplementary information Figure S1.

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.



Supplementary information Figure S2.

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 Kéry and 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[])


}
```

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Discussion

Discussion

The quest for the key-factors that determine amphibian disease dynamics –and which could potentially be targeted– is complicated. The search is complex because of the potential synergistic effects of the multiple interacting stressors, and because of the strong context-dependency of these stressors on amphibian communities (e.g. Blaustein et al. 2011; Daskin and Alford, 2012; Doddington et al. 2013; Scheele et al. 2015; Chapter 3). The presence of a virulent pathogen, a susceptible host and a conducive environment facilitate amphibian disease outbreaks. Sometimes the outcome can be quite unequivocal as has been observed in the case of the collapsed fire salamander (*Salamandra salamandra*) populations in the Netherlands (Chapter 4). Here the emergence of a pathogen (*Batrachochytrium salamandrivorans* (Bsal); Chapter 5) has severely reduced a population fire salamanders, and spreads over Europe (Chapter 6). Sometimes the impact of a potentially lethal pathogen is less apparent as shown by the complex situation of the yellow-bellied toad (*Bombina variegata*) and Bd in the Netherlands (Chapter 2, 3). Here, Bd poses a threat to its amphibian host, but due to the current environmental conditions and the possibility of additive recruitment, an endemic situation remains (Chapter 3). Long-term monitoring studies can elucidate the interactions between host, environment and pathogen (Chapter 3), and these studies are key to amphibian conservation. By quantifying the relative contributions of the diverse processes we can guide resources to the appropriate tailored sets of measures.

The general aim of this thesis was '*to elucidate the presence and impact of chytridiomycosis on amphibians in the Netherlands and define practical conservation tools*'. The discussion follows this aim by discussing the possible conservation management options per invasion-stage. The required conservation management actions are summarized in Figure 8.

1. Invasion-stage specific management actions

Invasion-stage appropriate management actions implemented at the right epidemic stage (pre-arrival, invasion, epidemic (or epizootic) and established (or enzootic)) are important for successful disease management (Langwig et al. 2015; Canessa et al. 2018). The identification of the invasion stage of the pathogen at various spatial scales is key for cost-effective management, and requires data on host abundance and pathogen prevalence, which makes initial collection of these data a critical part of disease management (Langwig et al. 2015).

Implementing the appropriate -effective- conservation measures in response to outbreaks of emerging infectious diseases is a complex process in which many actors participate. Scientists, politics, landowners, nature conservationists and the public all have their own views and opinions on the appropriate allocation of resources. Yet, the development of context-dependent invasion stage-appropriate management actions are key (Figure 1). Especially during the first two stages of invasion (pre-arrival and invasion) there is a strong lack of knowledge about nearly everything of the pathogen involved. Remarkably, also during the last stage of invasion (establishment of the pathogen, or the enzootic phase) knowledge can be limited, even to such an extent that making an informed management decision seems impossible. Potentially, a presumed stable system might not be as sustainable as previously considered (Chapter 3), and disease management in seemingly commensal cases or enzootic areas remains important (Woodhams et al. 2011).

Host-pathogen interactions are complex and dependent on many ecological and evolutionary processes that are driven by biotic and abiotic factors. If we want to manage the challenges of EIDs, this requires a clear understanding of the full ecological context of infection and transmission, and forces us to look beyond the interaction between a single host and a single pathogen. For instance, comprehending the modes of spread of Bsal will prevent unwanted 'ecological surprises' (Johnson et al. 2015), such as the fungus popping up in small ranged endemic salamander populations. So, in order to understand what is going on and what we can do about this (and if we need to do something about it) we must look at the entire community and at all the relevant processes. However, this is a bulky task and with every question answered, another one is raised.

The fundament of management decisions, the identification of the invasion stage and of defining management actions is data. Obtaining more data to reduce uncertainties with regard to the best and most efficient allocation of resources is naturally strived for, but is a task that never ceases. Having the destructive global impact of Bd in our mind, we cannot afford post-hoc crisis management (Grant et al. 2017) with regard to Bsal. This means we need to be pragmatic and translate the available scientific work into practical management tools. Infectious diseases often demand rapid decision-making in the face of scarce knowledge, limited time for learning, and challenges turning the available scientific knowledge into actions (Grant et al. 2017). Yet, complexity is not an excuse for inaction (Lindgren et al. 2012) and much uncertainty can be reduced by the information provided in the current thesis.

2. Current situation in the Netherlands

The huge level of detailed information that is required to disentangle the relation between host, pathogen and environmental conditions confirms the necessity to identify the local factors that may exacerbate or reduce the impact of an infectious disease. This emphasizes the need for the analyses of host-pathogen relationships at micro-scale, for long-term monitoring, and shows that it might be unwise to extrapolate the findings of a study one on one to other species or regions. Despite some strong knowledge gaps, relevant information is available that allows for immediate implementation, such as the urgency to prevent pathogen pollution by spill-over of non-endemic Bd strains (Urbina et al. 2018), the safeguarding of high quality terrestrial and aquatic habitat (Langwig et al. 2015; Garner et al. 2016; Chapter 3) and halting global warming or construct disease refugia (Chapter 3).

2.1 Batrachochytrium dendrobatidis (Bd)

The host-pathogen system in the Netherlands (Chapter 2 and 3) currently seems balanced for Bd, albeit a fragile equilibrium, dictated by environmental conditions. Interestingly, Bd-GPL is endemic, which is counterintuitive as it is widely accepted as a hypervirulent Bd lineage causing epizootics (O'Hanlon et al. 2018). If, for instance, the environmental conditions change, this could imply a risk of shifting the system towards an alternative epidemiological scenario. In our amphibian-Bd system, Bd-GPL coincides with isolated, range-constricted, susceptible species of high conservation concern like the midwife toad, yellow-bellied toad and fire salamander. These populations are susceptible to stochastic events like periodic recruitment failure, which implies that populations can easily be lost when either one of the parameters changes (host susceptibility, pathogen virulence or environmental conditions), which emphasizes the value of good habitat management.

The presence of endemic Bd still requires measures to prevent pathogen pollution and ensure that the two other corners of the disease triangle ('environmental conditions' and 'host') are able to preclude an outbreak. Amphibian hosts in our study region may have an innate or acquired immune response to a specific Bd-GPL isolate, but not to another isolate. Possibly, Bd-GPL has attenuated in our region, and less virulent isolates dominate in our study region. Furthermore, it is unknown if there is a similar cost of infection as found for yellow-bellied toad in our study region in other amphibian species that were not studied in great detail. If these dynamics are more common, the impact of Bd on native amphibians is currently severely underestimated.

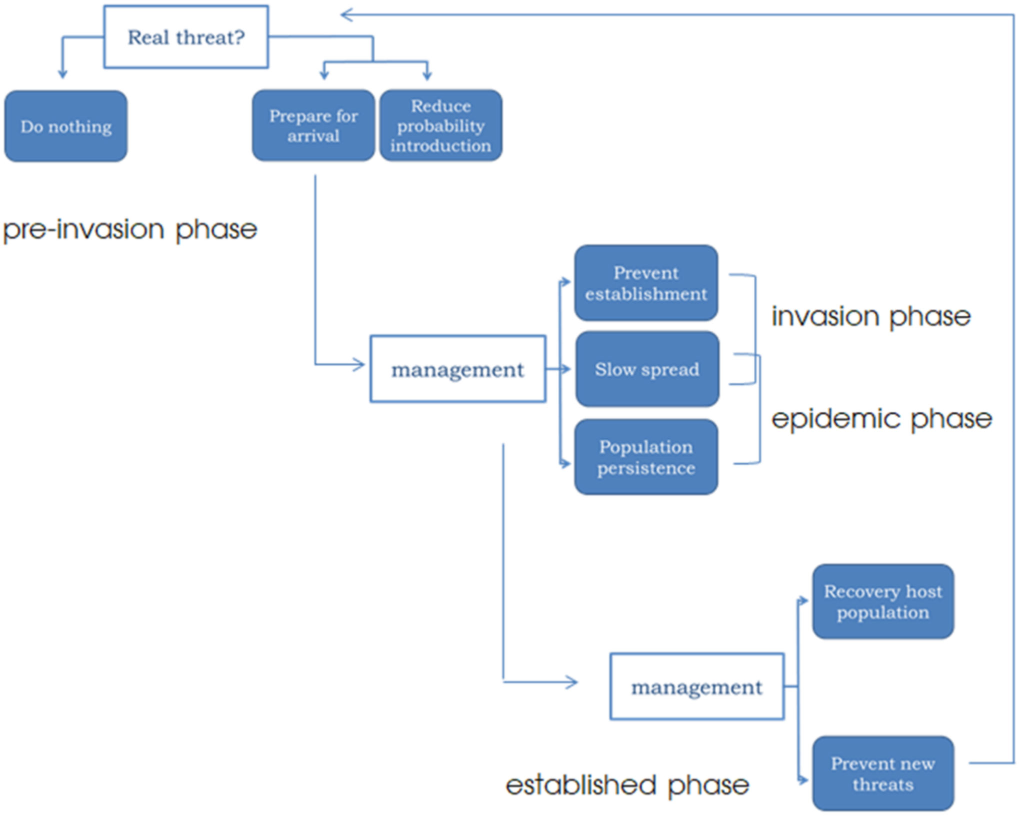

Figure 1. The invasion stages (Langwig et al. 2015). The first question is if the pathogen that is due to arrive poses a conservation threat. If it does, then measures to reduce the probability of introduction should be taken, and additional preparations such as the set-up of an Action Plan. If the pathogen does arrive, the main management goals during the invasion phase are the prevention of establishment and the limitation of spread of the pathogen. During the epidemic phase, management focuses on reducing the dispersal rate of the pathogen, as well as on population persistence, either in-situ or ex-situ. Then, in the established phase, the management focus lies on the recovery of the affected populations or species, as well as the prevention of the introduction of novel threats.

For instance, experimental work suggests that Bd could incur a cost to palmate newts (*L. helveticus*). Wild-caught adult palmate newts were shown to suffer significant mass loss due to a sub-lethal infection with Bd in an experimental set-up (Cheatsazan et al. 2013), and in another experimental set-up it was concluded that Bd has an impact on the ventral rehydration capacity of palmate newts (Wardziak et al. 2013). Kolenda et al. (2017) suggest that the pool frog (*Pelophylax lessonae*) may have suffered severely from clinical chytridiomyocosis at a site in Poland, but this needs to be further investigated. Earlier, Woodhams et al. (2012) hypothesized that the edible frog (*P. esculentus*) and the pool frog were tolerant to infection with Bd in the laboratory. Finally, synergistic effects of multiple pathogen interactions and other environmental stressors have not been addressed and are therefore a black box.

2.2 Batrachochytrium salamandrivorans (Bsal)

Figure 2. Fire salamander with severe skin lesions due to infection with *B. salamandrivorans* (photo: F. Pasmans)

Within a very short time period, *Batrachochytrium salamandrivorans* (Bsal) is able to reduce a healthy population fire salamanders to the level of functional extinction (Chapter 4; Stegen et al. 2017). The optimal growth temperature of the Bsal-strain found in the Dutch and Belgian populations is much lower (10-15°C) than that of Bd (17-25°C), but Laking et al. (2017) found Bsal-infected salamanders in natural waterbodies in Vietnam with temperatures up to 26.4°C. After infection, fire salamanders quickly die, and over the whole body erosive lesions in the skin and ulcerations can be found (Chapter 5; Figure 2). Infected fire salamanders do not mount any protective immune response, and death is irrevocable regardless dose. This currently excludes vaccination as a mitigation strategy (Stegen et al. 2017). In contrast with Bd, Bsal has a dual transmission strategy. The fungus not only has motile aquatic spores, but also non-motile environmentally resistant spores (Stegen et al. 2017). Pathogen transmission through contaminated soil is possible even 48 hours after the soil has been in contact with a Bsal-infected animal (Stegen et al. 2017). Ex-situ treatment of infected caudates is possible (Blooi et al. 2015a,b). Common midwife toads and alpine newts (*Ichthyosaura alpestris*), both sympatric species with fire salamanders, are able to asymptomatically carry the infection at low infection levels and hereby enable pathogen persistence in the system (Stegen et al. 2017).

The fungus' origin lies in Eastern Asia, where it diverged from Bd between 115–30 million years ago, which suggests that Asia has been a natural reservoir for Bsal for the past 30 million years (Martel et al. 2014). In China, Thailand, Vietnam and Japan, Bsal has been detected in the absence of disease, and given the discontinuity of the global distribution of Bsal, the fungus was presumably transported to Europe via pet traded newts and salamanders (Martel et al. 2014;

Yuan et al. 2018). Asian salamander species like *Cynops pyrrhogaster*, *C. cyanurus* and *Paramesotriton deloustali* are asymptotic carriers of the fungus and may function as reservoir hosts, whereas the majority of Western Palearctic salamanders (families Plethodontidae and Salamandridae) are highly susceptible (Martel et al. 2014). In this there is observed variance in susceptibility (Stegen et al. 2017). Although the outcome of Bsal-infection in fire salamanders is dose- and temperature independent, the disease-course in alpine newts is dose-dependent. Both species do not show an immune response after previous infection, but low dose inoculation of the latter species may result in total clearance of the infection.

2.2.1 Current situation in the Netherlands

In 2017 the number sighted alive fire salamanders seemed to increase, with 0.6 fire salamanders seen per site visit (Table 1). In 2016 (166 swabs samples) and 2017 (135 swab samples) no Bsal has been detected in the Bunderbos area. However, early 2018, three dead fire salamanders were observed with severe lesions. Of these three animals, one, a subadult male, was collected and this animal tested positive for Bsal (unpublished data). In April 2018, three Bsal-positive alpine newts (two subadults and one adult male; terrestrial phase) and one smooth newt (adult male, aquatic phase) were detected spread over the forest (unpublished data, see also Box 2). These findings show the unpredictable course of a Bsal-outbreak and suggests both a density dependent and frequency dependent transmission mechanism, as well as the presence of an environmental reservoir for Bsal zoospores. The non-motile environmentally resistant spores may allow for a random spread of the pathogen, regulated by host density, whereas the mating behaviour (fighting between males and copulation between males and females), allows for frequency dependent transmission, regardless –to a certain degree– fire salamander density. A drop in fire salamander density below a certain, yet unknown, number animals/hectare, leads to encounter reduction as animals are simply too far away from each other to encounter each other. It has been calculated that a reduction of 85% of the initial density is required to achieve $R_0<1$[5] and prevent a disease outbreak (Canessa et al. 2018). Here however, the presence of asymptomatic hosts as alpine newts may allow disease sustenance. There is however some food for thought, as the infected fire salamanders in 2018 were found in January, February and in April, whereas mating and territorial fighting occurs mostly between March – October. How, where and when did these animals become infected with Bsal? Possibly, the animals contracted Bsal prior to winter dormancy, and the fungus did not proliferate due to suboptimal environmental conditions. This latter hypothesis however seems unlikely as a long-term study of several fire salamander hibernation sites (old mineshafts) in Germany showed that humidity and temperature fluctuated between 92-99% and 6-12°C respectively (Feldmann, 1987; Balogová et al. 2017). Although these data are absent for other underground shelters than caves and mines, it seems plausible that similar humidity and temperature ranges apply in underground refuges too (Leeb, 2013), which would enable Bsal to survive and in case of physical inter- or intraspecific contact, pathogen-transfer is then possible. Several studies describe how fire salamanders overwinter concurrently with alpine newts and midwife toads (Feldmann, 1987; Leeb et al. 2012; Leeb, 2013). Remarkably, despite the listed mechanisms that could enhance the spread of Bsal, a spread of the fungus to other parts of the Netherlands has yet not been observed. The limited intrinsic dispersal capacity of the fungus may even have prevented the spread to a nearby fire salamander population (Chapter 7).

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[5] R_0 is the Bsal basic reproduction number. If $R_0 < 1$, the disease dies out and $R_0 > 1$ implies a disease outbreak (Canessa et al. 2018)

Box 2. Succinct overview of the chain of events and studies conducted in the Netherlands following Bsal discovery.

Bsal in the Netherlands

From 2008 onwards dead fire salamanders were seen by the volunteers who monitor this species in the Netherlands for the national monitoring scheme. The volunteers reported their findings to RAVON, and the deceased animals that were found were collected and sent to Ghent University for post-mortem examination. In 2012 it was decided to collect a subset of the remaining animals and keep them in quarantine to safeguard the genetic material. Nearly half of these animals died in quarantine, and this led to the discovery of the pathogen *Batrachochytrium salamandrivorans* (Bsal). The detailed monitoring data before, during and after the outbreak, as well as the continuous Bsal surveillance, have provided useful information. This research has shown that after the observed absence of Bsal for 2-years in the Bunderbos, Bsal resurfaced and infected alpine newts and fire salamanders. This finding suggests the presence of an environmental reservoir. The finding of a Bsal-free novel population in the vicinity of the Bunderbos showed that isolation is a possible effective conservation tool, and suggests that the scattered European Bsal distribution is human-facilitated.

The discovery of Bsal has not led to conservation measures in the field, apart that for a few years, the mycological working group and several other organisations were denied access for excursions in the Bunderbos (the focal outbreak site in the NL). The area did remain freely accessible for others, no warning signs were placed.

European level
Within the European project (www.BsalEurope.com), which works towards the set-up of a European Early Warning System (EWS), develops Emergency Action plans and works on effective mitigation strategies, the NL has been able to participe and invest in public awareness, syndromic surveillance in an international team of experts.

National level
Immediately after the discovery of Bsal, the national government provided funding for a multiple-year project to study mitigation possibilities, innovative detection techniques, national Bsal distribution and disease surveillance and the population development of the fire salamander. They also ensured the persistence of the assurance colony. Support is guaranteed until at least 2020. This project has allowed RAVON to initiate an EWS and set-up a national syndromic surveillance network. Ensuring commitment from people requires intensive communication from our side. This project has also allowed RAVON to broadly communicate the relevance of preventive biosafety measures, and now- after a few years - this is slowly becoming accepted.

Local level
Additional budget to monitor the population development of the fire salamander was provided at provincial level, but this might not be prolonged after 2019. The terrestrial habitat of the fire salamander is unchanged over the years, but the reproductive habitat is less than marginal at the moment (§ 3.1.7 in the Discussion). The Province Noord-Brabant has (pre-arrival) financed a three-year project for pathogen surveillance, and to set-up a regional EWS and syndromic surveillance network.

Trade
A project was initiated by the NL Food and Consumer Product Safety Authority (NVWA) to study Bsal prevalence in traded amphibians, as well as to study the volume of trade in amphibians. An EWS was set up in collaboration with the Salamandervereniging, OFI and Dibevo. Samples were collected at wholesalers, private persons and in pet shops. Private keepers were offered to have their animals tested if they suspected disease. The sector was willing to sign a covenant for a disease-free trade, but this was never launched. This project might end December 2018.

Table 1. From Chapter 4 and new data. The total number of metamorphosed fire salamanders sighted during nightly transect counts per site (Bunderbos (8 transects) and Broek (1 transect)) from 2013-2017.

	total nr. fire salamanders/all transects/year (nr. site visits)		mean nr. (sub)adult fire salamanders/transect/visit	
	Bunderbos	Broek	Bunderbos	Broek
2013	13 (49)	70 (9)	0.3	7.8
2014	15 (45)	239 (33)	0.3	7.2
2015	14 (87)	151 (34)	0.2	4.4
2016	9 (82)	9 (21)	0.1	0.4
2017	34 (59)	92 (20)	0.6	4.6

2.2.2 Distribution in the Netherlands and Europe

After detection of Bsal in the Netherlands, Bsal-induced deaths of wild salamanders and newts were recorded in Belgium (alpine newt and fire salamander) and in Germany (fire salamander, smooth newt, great crested newt (*Triturus cristatus*), palmate newt) (Chapter 6; Dalbeck et al. 2018). In parallel, the fungus was found in captive collections in the Netherlands (Vietnamese crocodile newt; *Tylotriton vietnamensis*) (Martel et al. 2014; Fitzpatrick et al. 2018), Belgium (Fitzpatrick et al. 2018), Germany (confirmed Bsal infection in nine subspecies of *S. salamandra* and *S. algira*) (Sabino-Pinto et al. 2015), Spain (Fitzpatrick et al. 2016) and in the UK (Cunningham et al. 2015; Fitzpatrick et al. 2016) and also in a German pet-store in an anuran host (*Bombina microdeladigitora*; Nguyen et al. 2017). Up-to-date overviews of the distribution of Bsal in wild amphibians are online on www.BsalEurope.com and www.sossalamander.nl. Up until now, both in the Netherlands and in Belgium no Bsal-infected great crested newts and palmate newts have been found. Yet in Germany, in 2016 and 2017, Bsal-infected palmate newts were observed, albeit with a low infection intensity (no higher than 29GE). Additionally at 2 sites Bsal-infected great crested newts were found with a variable infection intensity (range: < 1–6148 GE) (Dalbeck et al. 2018). Despite the high diagnostic sensitivity and specificity of Bsal-specific realtime PCR, it is suggested to use a detection limit of 1.0 GE/reaction for post-mortem diagnostics to prevent false positive results (Thomas et al. 2018). In Dalbeck et al. (2018) a detection limit of 0.05 GE was used. Standardization should be agreed upon to ease the interpretation of results. It is known

Figure 3. Dedicated volunteers formed the basis of Bsal discovery

Figure 4. The Bunderbos, the NL. Index site of Bsal. Photo: A. Kloor

that Bsal is able to invade the skin of palmate and great crested newts, and during experimental inoculation of both species, all palmate newts survived (3/3), whereas all (5/5) great crested newts died (Martel et al. 2014). It is therefore yet unknown if palmate newts can be considered reservoir hosts, equal to alpine newts. Could the discrepancy between high susceptibility of caudates to Bsal in the laboratory (Martel et al. 2014), but the near absence of infection in for instance smooth newts (*L. vulgaris*) and crested newts in the wild, be caused by the pathogen (different strain virulence; Stegen et al. 2017) or by a certain environmental context which currently precludes mass mortality events in these species, or is it host specific behaviour that prevents infection or allows clearing?

2.2.3 Transmission and persistence

The optimal temperature for Bsal persistence more or less equals the optimal temperature for fire salamander activity. In our study region, this nocturnal species avoids high temperatures and shelters below ground during dry and warm spells (Seidel and Gerhardt, 2016). This behaviour ensures that fire salamanders do not raise their body temperature to temperatures high enough to clear an infection. In brief, host and pathogen seemingly share the same niche with regard to temperature and humidity, ensuring sustenance of infection. In the short time that Bsal has been discovered, a lot of work has been done, but how and where Bsal persists in the environment yet remains to be discovered. Like Bd, Bsal is able to attach and grow on keratinous tissue of bird feet (Van Rooij et al. 2015). Passive adherence of floating encysted spores enables pathogen persistence in aquatic systems (Stegen et al. 2017), and in terrestrial systems, pathogen persistence and dispersal is facilitated by survival of Bsal in forest soil. Successful transmission of Bsal is possible even up to 48 hours after an infected salamander has shed zoospores in forest soil, enabling pathogen transfer between caudate, or alternative, hosts (Stegen et al. 2017). Possibly Bsal is, like Bd, also able to grow on chitinous exoskeletons of arthropods (McMahon et al. 2013), which would ensure pathogen persistence in the environment. Alpine newts are sympatric to fire salamanders and as they can remain infected for months whilst shedding zoospores, these newts function as reservoir hosts (Stegen et al. 2017). The finding that the common midwife toad may function as vector and reservoir host (Stegen et al. 2017), allows pondering on the role of vector by common toads and other sympatric anuran amphibians to fire salamanders and alpine newts. However, infection of midwife toads with the Dutch Bsal strain (AMFP13/1) did not lead to sufficiently high infection loads in the toads that were capable of transmitting Bsal to co-housed fire salamanders (Stegen et al. 2017). Hence, the role of anuran vectors in the sustenance and spread of Bsal might be very restricted in the Dutch situation.

The finding of Bsal at the focal site (Figure 4) after a two-year-concealment is suggestive of the presence of an environmental reservoir, and indicates that the conditions in the forest are suitable for long-term persistence. Simultaneously, the environmental conditions seem to have precluded infection load build-up in alpine newts. Discovery of the exact whereabouts of the chytrid in the environment is clearly highly relevant, as is the finding of the in-situ mechanism of disease transmission dynamics. It is currently assumed that Bsal is intolerant to drought, as is Bd, but the exact response of Bsal on desiccation is yet unclear. The role of waterbodies in pathogen persistence and possibly pathogen transfer remains to be elucidated. Fire salamanders are a mainly terrestrial species. Male salamanders, subadults and juveniles do not go near the water, and female fire salamanders only migrate to the water to deposit larvae. The species may use stagnant waterbodies or fast flowing streams for reproduction, the latter of which provides unsuitable habitat for newts. Experimental inoculation of fire salamander larvae with Bsal did not lead to infection (Van Rooij et al. 2015), and hence it is hypothesized that also other urodelan larvae are resistant to Bsal. All this suggests that the probability of Bsal entering a waterbody is restricted to either infected metamorphosed newts in stagnant water bodies and/or Bsal-infected female fire salamander that deposit larvae. Distribution by water might however not be a key dispersal route for Bsal (Chapter 7). Conversely, shallow puddles of rainwater could provide Bsal refuges through which wandering salamanders/newts could easily be infected as they walk through and pick up the floating encysted spores (Stegen et al. 2017). However, one would expect that a high prevalence and infection intensity is required before sufficient rain puddles contain sufficient numbers of zoospores to ensure transfer is possible and significant. Stegen et al. (2017) showed that medium infection loads were not sufficient to transfer Bsal from soil to a sentinel animal.

2.2.4 Management implications

As Bsal was only recently discovered, many unknowns impede conservation management. The application of radical interventions in Bsal mitigation is required to hinder the spread of Bsal and prevent population declines (Canessa et al. 2018). Clearly, Bsal has lethal capacities and strain-specific differences in virulence are suggested by Stegen et al. (2017). The outbreaks in Belgium, the Netherlands and in a captive German collection were caused by different Bsal isolates and the environmental envelope of Bsal might be broader than assumed up till now (Laking et al. 2017). This means that the currently observed, or perceived, absence of disease incidence in newts in the Netherlands, could be reversed by the appearance of another isolate, or by altering environmental conditions. As it is known that the fungus is present in traded amphibians, albeit at a low prevalence (Yuan et al. 2018), the risk of new focal introductions remains, imaginably of novel isolates that may instigate a similar disastrous effect of Bsal infection in alternative hosts. Hence, the prevention of the introduction of novel strains remains a pressing issue. The presence, and perhaps interaction, of three lethal multiple-host amphibian pathogens in the Netherlands is of great concern. Multiple-pathogen interactions could lead to an even more unpredictable disease outcome. Co-infection dynamics of the concurrent presence of Bd (Chapter 2 and 3), Bsal (Chapter 5-7) and ranavirus (Kik et al. 2011; Spitzen-van der Sluijs et al. 2016a,b; Saucedo et al. 2018), could trigger amplified or cumulative effects in amphibian communities (Rosa et al. 2017). Up until now, there is only a single report of the co-occurrence of Bd and ranavirus in the Netherlands (Saucedo et al. 2018), which presumably indicates more strongly towards a research omission than a true absence.

3. Pre-invasion stage

Management should be considered before a disease has been detected in populations, and actions should preferably be based on scientific evidence. Proactive management actions are suggested for the 'pre-arrival phase', and these actions are key in the prevention of novel pathogen introductions. This phase is perhaps the most complex of all, information should be collected about pathogens yet to arrive, and decisions made on their potential impact (Figure 1). Information requirements are plentiful in this phase. Depending on the interpretation of the available information about for instance virulence, host specificity, pathways and pathogenicity it can be decided that either no measures are required, or that resources should be allocated for the prevention of introduction and preparations in case arrival is inevitable.

Some actions linked to the specific epidemic phases such as ‘targeted biosecurity’ and ‘monitoring’ in the pre-arrival phase and ‘ecosystem management’ in the established phase, are not unique for these phases, but should all be part of integral disease management. Appropriate habitat management may prevent the introduction of invasive species and/or pathogens and may ascertain the required microenvironment for amphibian hosts to avoid pathogen exposure.

3.1 Reduce probability of introduction

Taking preventive actions to avoid pathogen influx are more cost effective than the subsequent responses to disease outbreaks (Langwig et al. 2015). Simultaneously, adverse and irreversible environmental consequences could be precluded. Hence, preventive management actions are the most important ones to be implemented at all levels, including -amongst others-; customs, site managers, pet owners and fieldworkers. In the Netherlands where preventive actions were absent and Bsal was successfully introduced in a susceptible population, preventive management is still relevant to ensure that Bsal remains in the current isolated situation and is not further spread to other sites. Simultaneously, the prevention of the introduction and spread of novel Bd and Bsal strains, or perhaps other pathogens, is important and requires effort at all aforementioned levels. A key-question in this is to define the transmission pathways. This knowledge allows for a targeted approach to reduce the probability of introduction. Tools that reduce the probability of introduction, and should ideally all be implemented are: i) create awareness, ii) make the right political choices, iii) initiate an Amphiban Task-Force, iv) implement biosecurity at all levels, v) enforce restrictions in amphibian trade, vi) proper habitat management and vii) allow for early detection, which includes an Early Warning System (EWS), monitoring, syndromic surveillance and innovation.

3.1.1 Awareness

Often overlooked is the issue of problem awareness. At various levels, from international to regional political levels, conservation managers, private keepers, commercial breeders, the general public, people need to be aware of the relevance of the problem of introducing emerging infectious diseases for wildlife and their role in it. If all these people are oblivious to the matter, then there will be no commitment to implement certain rules, and no-one will report dead animals for syndromic surveillance, and captive animals might be released in the field. Targeted communication should unveil the relevance of the problem, and make it less academic.

3.1.2 Political choices

Ultimately, conservation is a political choice (Muradian and Rival, 2012). Generally, regional, national and international political choices are made within and for a limited time frame and are strongly anthropocentric. The ‘EU biodiversity strategy to 2020’ states that: “*biodiversity is both our life insurance and natural capital, providing us with food, clean air, medicine and ecosystem services*” (EC, 2011), and the Dutch ‘Natuurwet’ aims to: “*strengthen biodiversity and its sustainable use*” (Natuurwet, 2017). A first example of the anthropogenic inclusion of biodiversity conservation is one of the key actions in the EU 2020 biodiversity strategy which aims to ‘value nature’. Financially valuing nature quantifies the loss of ‘ecosystem services’ which endangers our own wellbeing (EC, 2011; WNF, 2015; Natuurwet, 2017). A second example is the inclusion of wildlife diseases in the OneHealth approach (Rostal et al. 2013). OneHealth is the all-encompassing concept that recognizes the inextricable links between the health of people, animals (wild and domestic), and the environment, but also in this context the role of wildlife is usually discussed as a source of emerging diseases infecting cattle or humans. Within this anthropocentric political climate, issues regarding zoonotic diseases, humans and livestock often derive more attention than the intrinsic value of natural diversity. In short, the intrinsic conservation of biodiversity is not high-listed as a political priority, which limits conservation efforts usually to policy-relevant species such as those listed on Annex IV of the Habitat Directive.

In the Netherlands, the national government (Ministry of Agriculture, Nature and Food Quality) has responded promptly to the discovery of Bsal in the NL and has warranted a multiple year study on the impact and spread of Bsal in the Netherlands. This project ends early 2019. The responsibility of the sustainable management of wildlife species has been delegated to the provinces (Ministry of Economic Affairs, 2013). Currently, the province responsible for the fire salamander (as this species is only native in one province), considers the threat of amphibian infectious diseases to be a national issue, and points at the landowners and conservation organisations to address the issue. However, without a clear local (provincial) policy with regard to, for instance fire salamander conservation, these organisations do not have the financial possibilities to act. This political unwillingness to claim responsibility threatens the prolongation of the population monitoring and pathogen prevalence monitoring, and provides a case-example of the difficulty of scientists and conservationists to collect information on even a disastrous pathogen that genuinely threatens the persistence of a species in the Netherlands as well as the persistence of all European caudates, but does not have a direct commercial impact.

3.1.3 Amphibian Task-Force

Recovery planning requires brave, effective governance, leadership and decision making in the face of uncertainty. Importantly, decisions should be made when there’s still an opportunity to act (Martin et al. 2012) and therefore, ideally, a knowledgeable task-force ‘amphibian diseases’ that has legal competency should be set in place. This task-force has political support (at áll levels), a long-term financial base, and also the legal strength/possibility to enforce measures to be taken (such as the quick removal of animals from a site and the set-up of a captive assurance collection). It is a task-force with authority that has the capacity to initiate actions and promote research. It will provide advice for management decisions that need to be taken, even when concrete evidence-based information is scarce or unavailable. Not acting on the evidence of rapid and consistent population declines may lead to the extinction of species (Martin et al. 2012), and in order to prevent such an impasse, well-informed, empowered, and responsive governance and leadership is essential.

3.1.4 Early Detection

When the prevention of pathogen introduction is unsuccessful, early detection (reporting of dead animals, their collection and examination) of new invasions can increase the likelihood of control and reduce control costs and damages (Epanchin-Niell and Hastings, 2010). In collections, the regular testing of captive animals will aid in the detection of Bd and Bsal, and in their elimination. A critical factor in the assessment of the invasion stage and risk, is the taxonomic, geographic, and temporal completeness of data in the region of concern (Stohlgren and Schnase, 2006). A long-term investment in citizen science is key to obtain sufficient sightings at a wide geographic national level over a prolonged period of time. Early detection of the introduction of novel strains of Bd and Bsal remains important, even when an EID is already established at national, regional or population level. The risk of the introduction of another, perhaps more virulent strain, remains. Hence, vigilance is required at all times.

Syndromic Surveillance

The reporting and collection of animals with disease syndromes by the public plays an important role in the early detection of novel, unexpected arrivals of pathogens and also in the mapping of pathogen distributions. The clinical symptoms of amphibian diseases may be difficult to observe in the field by lay-men. Therefore a general call for reporting dead animals to a fixed contact point (task-force, reference lab) by the public facilitates the detection and recovery of amphibian carcasses (Lawson et al. 2015). An illustrative example of the priceless contribution of volunteers to the study of amphibian emerging infectious diseases is the detection of Bsal. It is only because of the long-term voluntary monitoring of fire salamanders in the Bunderbos, the Netherlands, by two elderly volunteers that the first dead animals were found ánd reported to the relevant organization (Chapter 4). Their finding led to a cascade of events and the discovery of a novel chytrid fungus. If these volunteers had not been in the field, if they had not been so vigilant and if they had not known where to report their findings, action would not have been taken –or perhaps at a much later time.

Early Warning System

Clearly, an Early Warning System (EWS) is multifaceted and encompasses the involvement and collaboration of lay-men, landowners, veterinarians, volunteers, scientists, pet owners, importers and NGO's. Open communication, no competition between parties, easy online data importation and local contact persons are key to obtain as many observations as possible. This network of people is not only important at the early stages of pathogen emergence, but also has added value during disease outbreaks. National or regional EWSs should be led by a task-force 'amphibian diseases' (Discussion § 3.1.3). Partners should commit long-term to an EWS. An EWS requires sufficient long-term capacity and budget for diagnostics and coordination of all observations. In 2016 an EWS was set-up for a three-year period to specifically detect Bsal in captive collections and traded amphibians, but the continuance of this project is not guaranteed post 2018.

Monitoring

If you don't know what you have, you don't even know what you had, 'till it's gone (Mitchell, 1970). The objective, standardized monitoring of amphibian numbers forms the basis of all scientific work on disease dynamics under natural conditions. Prior to disease emergence, the long-term data on population dynamics help to recognize changes in dynamics and decreasing population trends (Chapter 4). During a disease outbreak, monitoring and syndromic surveillance provide information on the actual impact of a disease on a population, as well as the scale of the problem (Lawson et al. 2015; Spitzen-van der Sluijs et al. 2016a,b; Rijks et al. 2016; Chapter 7). Available long-term information on natural dynamics prior to a disease outbreak helps elucidating cause and effect. The availability of monitoring data and absence/presence data predating a disease outbreak is needed to single out the impact of disease from that of other forces influencing population dynamics (LaDeau et al. 2007).

Informed management decisions depend on data (Langwig et al. 2015), and the lack of monitoring data may delay the onset of conservation or mitigation actions where they preferably should be taken immediately upon disease emergence. Not all impacts of emerging infectious diseases are instantly obvious, and here the availability of long-term data sets are extremely valuable (Chapter 3). In the pre-arrival phase it is important to establish a robust dataset on the actual status of amphibian populations. This dataset facilitates the recognition of the invasion stage by providing insight in (decreasing) local population trends. In the epidemic phase, the scale of the problem can be meticulously calculated, because of the available 'prior-' data and eventually, recovery and the effect of conservation actions can be measured in the last phase. Additionally, the information on the spread of an emerging infectious disease (Chapter 6), allows for predictions on the speed of the spread and predictions can be made when the pathogen may reach a particularly susceptible population (Beukema et al. 2018). The availability of monitoring data facilitates the realistic modelling of disease dynamics which supports the decision making of the appropriate management actions for mitigation or recovery (Canessa et al. 2018).

Long-term monitoring has elucidated relationships between host and pathogen with direct relevance for conservation (Chapter 3 and 7). Unfortunately, despite the frequent call for, and emphasizing of the importance of long-term monitoring (e.g. Zampiglia et al. 2013; Whitfield et al. 2017; Techangamsuwan et al. 2018), not many long-term studies combining population monitoring and prevalence exist and much of our knowledge on the interaction of host and pathogen is based on short-term in-situ or ex-situ experimental studies.

Innovation

Besides the investment in a network of volunteers and an EWS, the development of technical tools for the swift, non-invasive and reliable surveillance of diseases is important. The detection of viable Bd- and Bsal-zoospores in the aquatic and terrestrial environment aids in surveillance and in the rapid detection of the pathogens (Hyman and Collins, 2012). An on-site 'predictor' test would be most practical, and currently investing in the development of using environmental DNA (e-DNA; Taberlet et al. 2012) for reliable and immediate detection of the presence of Bd and/or Bsal in water or soil is a good step forward. Additionally the on-site detection of the infection status -also at low infection loads- of an animal is valuable. Dillon et al. (2016) have shown the potential of on-site diagnostics to detect infection with Bd and Bsal in the field, but the technique using a monoclonal antibody has not yet been refined to satisfaction. With the immediate verification of the infection status of the host, direct intervention is possible and it can be decided to remove the salamander, frog or toad from the population, either to reduce infection pressure on the population and/or treatment of the individual. Finally, with the known differences in strain-virulence for Bd and Bsal, the availability of a PCR-tool to distinguish chytrid strains would be highly valued.

3.1.5 Biosecurity

An easy-to-implement, low-budget and highly efficient technique to limit the spread of EIDs is the implementation of disinfection procedures. Actions that aim at the prevention of the entry of pathogens remain a priority, especially given the limited ability to mitigate epidemics. At the local level, besides mandatory biocontrol precautions, more radical options could involve restriction of access by humans or isolation of host populations in-situ, for example through quarantine fences (Canessa et al. 2018).

The mechanism of spread of Bd and Bsal is unknown, but the potential for human involvement in transmission (passing of disease from an infected to an uninfected individual) and spread (movement of disease geographically) of the fungi, within and among amphibian populations, appears to be significant (St. Hilaire et al. 2009). Bd and Bsal have probably been introduced into new countries and regions by the movement of infected amphibians, or in contaminated water or soil containing zoospores (Daszak et al. 2000; Martel et al. 2014; Chapter 6). Bsal and Bd may remain viable in aquatic and moist terrestrial habitat for a prolonged period (Johnson and

Speare, 2003; Stegen et al. 2017) and both fungi may be sustained by asymptotic hosts (Brannelly et al. 2017; Stegen et al. 2017). Natural transmission via waterways and animal-animal contact might be hard to prevent, but it is possible to reduce the spread of pathogens through human activities (St-Hilaire et al. 2009). People are often not aware of the potential for animal pathogen transfer via their activities, either because they do not know pathogens are present in the area, and/or the mechanisms by which pathogens can be transferred. This ignorance should be tackled at all possible levels. Conservation professionals, site managers, customs, volunteers, amphibian pet owners, shops, government agencies, and policy makers should be aware of the importance of the prevention of pathogen spread via human activities.

During all epidemic phases, the prevention of pathogen introduction is essential, and preventive measures should be administered during fieldwork, the translocation of amphibians, landscape management, during the exchange of individuals between collections and for the prevention of pathogen spill-over from captive collections to wild amphibians. Per epidemic phase, the aim could be different, but the preventive measures are alike. In the pre-arrival phase, taking measures to prevent or minimize human-mediated spread of Bsal or Bd to naïve populations or regions is of paramount importance (Van Rooij et al. 2017). In the other phases, prevention of the introduction of novel, non-endemic strains can be key. The introduction of exotic virulent strains may cause an epizootic in a previously enzootic situation, and is surely a realistic scenario considering the size of trade and the prevalence of Bd in these collections (Chapter 1; Discussion § 3.1.2; O'Hanlon et al. 2018).

Effective disinfection protocols to prevent transmission of Bd and Bsal are needed in the laboratory, in captive husbandry and in the field (Johnson et al. 2003). The recommended disinfection protocol includes the use of 1% Virkon S®, 4% sodium hypochlorite and 70% ethanol for disinfecting equipment in the field, lab or captive setting, with a minimal contact time of 5 minutes for 1% Virkon S® and 1 minute for the latter disinfectants. These conditions efficiently target Bsal, Bd and ranavirus (Van Rooij et al. 2017). Finally, the responsible disposal of waste water and dead amphibians from captive collections prevents pathogen spillover and should be actively advocated (Pasmans et al. 2014). See also § 4.3 in the introduction.

3.1.6 Trade

Amphibian trade appears to be driving the emergence of chytridiomycosis by (1) spreading infected animals worldwide, (2) introducing non-native infected animals into naïve populations and (3) amplifying infection of amphibians by co-housing, followed by untreated discharge of infectious zoospores into water supplies (Fisher and Garner, 2007). Current diagnostic tests for Bd and Bsal are reliable, but time-consuming, and do not target all potential unknown invasive pathogens. Also, through amphibian trade, previously isolated lineages may come into contact which elicits the potential for the spread of more virulent recombinant genotypes of pathogens (Farrer et al. 2011; Schloegel et al. 2012). As it only takes one infected individual to initiate an epizootic (Kriger and Hero, 2009a), banning the transport of amphibians is the ultimate preventive measure, and the most effective disease mitigation measure. A transport ban removes the threat of the introduction of known and novel diseases altogether (Kriger and Hero, 2009a; Garner et al. 2016).

Ideally, there should be no more trade in amphibians for other purposes than conservation or science. At a minimum, amphibian trade should be disease free and be limited to the sale of captive-bred individuals with health certificates (Kriger and Hero, 2009b). Non-CITES listed amphibians should be traceable by an amphibian specific unique identifier code in TRACES, and the sale of amphibians in garden centers should be prohibited. A concordant advantage is the reduction of the pressure on wild populations. Simultaneously, awareness campaigns should be launched to inform owners on the importance of biosafety and the undesirableness of disposing amphibians in nature.

B. salamandrivorans

A ban on the import, and restriction of the trade of all salamanders have been suggested as the sole most effective mitigation action against Bsal (Grant et al. 2017). The risk of Bsal invasion is compounded by the magnitude of international salamander trade. In the years 2010–2014 nearly 780,000 live salamanders entered the United States. Of these, about 99% originated from Asia, and 98% were species native to Asia, and 91% consisted of species in the genera *Cynops* and *Paramesotriton*. The major sources were Hong Kong, mainland China, Singapore, and Japan (Yap et al. 2015). It is estimated that between 2005–2015 around 620,000 individual caudates were imported into the EU (Balàž et al. 2017). In the Netherlands an estimated 21,000 individual salamanders/newts were being imported into, or via, the Netherlands, mainly *Paramesotriton chinensis*, *Notophthalmus viridescens* and *Cynops* sp. (Spitzen-van der Sluijs et al. 2015).

Recent sampling for Bsal in wild caudate species in China revealed a true Bsal prevalence between 2%-4%, with a maximum of 50% in a population of *P. hongkongensis* (Yuan et al. 2018). Even at this overall relatively low Bsal prevalence, the exportation of millions of animals renders Bsal introduction in naïve, importing countries a near certainty, which calls for the urgent implementation of proper biosecurity in the international wildlife trade. There is conclusive evidence that Bsal is transported by amphibian trade (Martel et al. 2014; Nguyen et al. 2017), and Bsal has been found in captive collections in the UK, Germany and in Spain (Cunningham et al. 2015; Sabino-Pinto et al. 2015; Fitzpatrick et al. 2016). A high risk of pathogen spill-over is the possibility of anurans asymptomatically carrying Bsal, such as the small-webbed fire-bellied toads (*Bombina microdeladigitora*), and possibly the closely related, but heavily traded, *B. orientalis* too (Nguyen et al. 2017).

The possibility for garden centers to sell anurans and caudates that are either tolerant or susceptible to Bd or Bsal is another worrying aspect of the unregulated trade in salamanders and newts (Spitzen-van der Sluijs et al. 2015). The release of non-native animals in a garden pond involves a high risk of pathogen spill-over to native amphibians, and also to other vertebrate classes (ranaviruses; Price et al. 2017). The sale of animals and plants, both native and non-native, for outdoor ponds or terraria constitute a high risk of disrupting ecological balances, of which the problems with the plant *Crassula helmsii* (CABI, 2018) and the Italian crested newt (*T. carnifex*) are exemplary (Meilink et al. 2015).

Swift and decisive management has been shown to be possible. Switzerland, the USA and Hungary have proclaimed a temporary ban on salamander trade (Schmidt, 2015; USFWS, 2010; 2016; Korm. rendelet 199/2017). The USA prohibits both the importation and interstate transport of 20 genera of salamanders, and in Switzerland the importation of all salamander species is banned by the Swiss Federal Food Safety and Veterinary Office (Schmidt, 2015). In Hungary it is "*prohibited to keep, breed or market any individual species of Salamandridae and of any species of Asian salamander (Hynobiidae) and of the Korean crevice salamander (Karsenia koreana) and no further specimens may be obtained for that purpose*." (Korm. rendelet 199/2017). The World Organisation for Animal Health (OIE) has added Bsal to the list of notifiable diseases (OIE, 2017). Although Bsal has already been introduced in the EU, a ban of intra-EU movements of salamanders could limit the spread of the fungus to new areas and Member States (More et al. 2018; EC, 2018).

Both Pet Industry Joint Advisory Council (PIJAC) and Ornamental Fish International (OFI) have requested in 2015 that all exporters, shippers, importers and buyers of *Cynops orientalis* and *Pachytriton labiatus* immediately establish a voluntary moratorium on their import (Likins, 2015; Willis, 2015). In the USA, Hungary and Switzerland this measure has been overtaken by national legislation (Schmidt et al. 2015; USFWS, 2016; Korm. rendelet 199/2017. (VII.10.). The member states and the EU were pressed by the Standing Committee of the Bern Convention to take measures to prevent novel introduction and the further spread of Bsal (Bern Convention 2015; 2017). Temporary animal health protection measures have been issued by the European Commission in March 2018, which should apply at least until December 2019 (EC, 2018). This regulation includes both intra-Union trade and the introduction of salamanders into the EU from third countries and is based on a 'no, unless...' approach. Member states shall prohibit the dispatch of consignments of salamanders (all amphibians of the order Caudata) to another Member State, and prohibit the introduction into the Union of consignments of salamanders from a third country, except where the consignments comply with certain conditions (EC, 2018). This has led to the prohibition of selling caudates at Terraristika, the largest European fair for amphibians and reptiles (Figure 5). The restriction of trade, the enforcement of preventive screening and biosafety measures are welcomed, however the trade in Bsal vectoring anuran species is not regulated, and the lack of a unique identifier (CN-code) for amphibians makes it impossible to trace the flow of traded cold-blooded non-CITES listed species. Detection of consignments containing caudates therefore remains problematic.

Terraristika Hamm

31 juli om 13:27

Terraristika setzt Handel mit Schwanzlurchen bis auf weiteres aus!
Bei Schwanzlurchen (alle Amphibien der Ordnung Caudata) hat sich der Pilzerreger Batrachochytrium salamandrivorans (abgekürzt Bsal) stark ausgebreitet.
Um die Schwanzlurchbestände zu schützen, hat die EU-Kommission Maßnahmen erlassen, die die Bundesrepublik Deutschland umgesetzt hat. Danach dürfen Schwanzlurche innerhalb der EU und aus Drittländern nicht mehr gehandelt werden. Es gibt Ausnahmen, wenn strenge, amtliche Tiergesundheitsbedingungen erfüllt werden. Dies kann auf der Terraristika nicht erfüllt werden. Daher dürfen ab sofort auf der Terraristika in Hamm keine Schwanzlurche (alle Tiere der Ordnung Caudata wie Salamander, Molche, Axolotl usw.)mehr gehandelt, angeboten, mitgebracht oder übergebenwerden.
Wir bedauern diesen Schritt, aber es erscheint uns, im Sinne der Tiere und Halter, im Moment unumgänglich.

Figure 5. Screenshot from the Facebookpage of the largest European fair in amphibians and reptiles, Terraristika in Germany. From July 2018 onwards, the sale of salamanders and newts at this fair is prohibited.

Strangely the commercial trade of the vast majority of the world's amphibian (non-CITES) species has not been regulated (Auliya et al. 2016), which makes it impossible to derive the exact size of the trade in salamanders and newts. A case example of the urgent need for CN-codes is a survey in 2013 for salamander shipments. During this survey for the volume of imported caudates via Schiphol Airport in the Netherlands, the packing lists of shipments registered in the VGC-system (Veterinarian Border Control System) were checked for the presence of salamanders and newts. Searches for shipments with the term 'amphibia, anura, urodela or ranidae' revealed 11 shipments of which 9 contained amphibians, but no caudates. A search with the term 'squamata' showed 80 shipments and hereof 21 of the packing lists were checked. All 21 packing lists contained amphibians, and thereof 4 contained salamanders/newts. The term 'Ornamental Fish' derived most shipments (n=972) of which 267 packing lists were checked; 49 shipments contained amphibians of which 37 contained salamanders/newts (Spitzen-van der Sluijs et al. 2015). Remarkably, hardly any American salamanders, such as *Ambystoma* sp., were registered via the VGC-system as imports, but these species are widely for sale in pet shops. The US Fish and Wildlife Service reported 933 shipments of salamanders from the USA to the Netherlands in 2013 and 2014 totaling 2230 individuals of mostly wild-caught specimens (Spitzen-van der Sluijs et al. 2015). This example illustrates the current impossibility of a species-specific trade ban, and supports the suggestion by EFSA (Balàž et al. 2017), which has been adopted by the EU (EC, 2018) to implement a more effective movement ban of taxonomic order.

A ban on the trade in caudates has been suggested to constitute a risk in stimulating increased underground trade (Garner et al. 2016; OFI et al. 2017). Despite the plausibleness of this reasoning, this added risk is presumably negligible in terms of an increased risk of pathogen pollution. Underground, illegal trade is something of all times, and reducing the availability of newts and salamanders will preclude impulse purchases of uniformed consumers. Instead, people will need to obtain their animals from captive collections where knowledge on keeping and breeding is available and transferable when animals are sold. Finally, it is assumed that if someone is forced to put time and effort in obtaining either a legal or illegal animal, this person is less likely to simply dispose the animal outside. So, the risk of increased underground trade is real and should be monitored, it is however not a valid argument to refrain from implementing a movement ban.

B. dendrobatidis

In captive collections and in the wild, Bd is widespread in Europe and lethal infections occur (Chapter 1 and 2; Pasmans et al. 2004; Kik et al. 2012; Olson et al. 2013). Therefore, the necessity of implementing trade bans or at least movement restrictions seems ‘too little, too late’. Nonetheless, the prevention of pathogen pollution and pathogen spillover into naïve areas and populations is the best and cost-efficient mitigation strategy possible. Both the cross-over of strains into hypervirulent ones (Farrer et al. 2011) can be prevented as well as the introduction of novel strains (O’Hanlon et al. 2018) and pathogens. The voluminous unsustainable trade threatens many species such as *Paramesotriton hongkongensis*, *Mantella aurantiaca* and *Ranitomeya vanzolinii* by overexploitation (Lau and Chan, 2004; Vences and Raxworthy, 2008; IUCN SSC Amphibian Specialist Group, 2014), and closer to home, in Belgium and the Netherlands, the invasive American bullfrog (*Lithobates catesbeianus*) not only threatens native species (Lowe et al. 2000), but also acts as a vector for Bd (prevalence rate of 21%; Chapter 2; Urbina et al. 2018). Altmann and Kolby (2017) estimated that nearly 27 million live amphibians were imported into the USA in nine years, of which nearly 12% is infected with Bd (Kolby et al. 2014). The commercial trade in amphibians for biomedical, educational, research, consumptive or pet trade purposes, directly (overexploitation of local populations, invasion of alien species) and indirectly (pathogen pollution) impacts on amphibian species and populations. As member states are committed to prevent the transboundary spread of both Bd and Bsal (OIE, 2017), a robust and traceable health screening should be mandatory for all shipments of live amphibians. The call for regulating the live trade and screen the trafficked amphibians for infections known to cause population declines, becomes louder and more urgent.

3.1.7 Habitat management

Proper habitat management is key during all invasion stages. During the pre-arrival phase, a diverse and stable system may slow the natural invasion of a pathogen. Yet, habitat protection ‘an sich’ offers no barriers to threats such as climate change and infectious diseases (Bosch et al. 2018). Improving habitat quality is, with regard to the (perceived) uncertainty towards the effective possibilities of disease mitigation, under certain circumstances perhaps a simpler and more effective conservation tool (Garner et al. 2016); managing pathogens by managing habitats. In the pre-invasion stage, the local assessment of the risk of disease emergence (to ascertain if a threat actually exists), and the definition of the subsequent custom-tailored measures are important. The improvement of habitat quality might enable compensatory recruitment or increase the survival probability of a specific cohort and hereby support population persistence (e.g. Scheele et al. 2015; McDonald et al. 2016; Chapter 6). A large, genetically diverse, demographically balanced healthy population has reduced sensitivity for perturbations and invasions and is more resilient. Prior to the invasion of Bsal in the Bunderbos, (NL), the nitrate concentrations were found to be very high in both the spring water and in the top layer of the forest soil (Chapter 4). Although there is no causative link between environmental nitrate concentrations and Bsal emergence, and the impact of high background nitrate levels on fire salamander fitness are unknown, reducing environmental pollution might increase host condition and herewith population persistence. A second example that illustrates how habitat management could contribute in population persistence despite the presence of an EID, is the current yearly loss of nearly all fire salamander larvae in the same forest (Bunderbos) in the Netherlands. Because of the changed land-use on the top of the plateau, rainwater during heavy rainfall gushes down and washes away the streams and small ponds where larvae are deposited. In this situation, Bsal threatens the adult fire salamanders in their survival, and recruitment is severely diminished due to these recurrent floods.

3.2 Prepare for arrival

When it is sufficiently likely that a specific pathogen is due to arrive, an Emergency Action Plan (EAP) should be launched in which biosecurity (§ 3.1.5) and early detection (§ 3.1.4) are prominent, and if required, the set-up of a captive assurance colony is initiated. An important question to address in the EAP is the cost of infection for all native species. As we have seen, the impact of Bsal, and also that of Bd, varies considerably between, and even within, species.

Figure 6. Ex-situ colony in GaiaZOO (NL) of the Dutch fire salamanders from the Bsal index site. Photo credits: GaiaZOO

3.2.1 Assurance colonies

As Bsal spreads erratically over Europe (Chapter 6), many small-ranged -highly susceptible-salamander species, such as *Calotriton arnoldi* and *Salamandra lanzai* have a high risk of extinction if Bsal arrives (Martel et al. 2014). It is therefore sensible to draft the required protocols for ex-situ captive assurance colonies in advance. To enable smooth and clear decision-making when it is clear the pathogen has arrived, or is likely to arrive soon, sufficient budget for multiple years should be reserved. Setting up a breeding colony post disease emergence may lead to inbreeding depression which is one of the most frequent causes of failure of recovery programs (Albert et al. 2014). Proper captive genetic management intends to minimize changes in a population's gene pool. For this, it is best to have a genetically diverse and healthy captive population prior to disease emergence. A pro-active approach to the ex-situ conservation of species at threat, additionally aids in the development of the right level of husbandry knowledge. Combining the currently sole known effective mitigation measure of population removal (Canessa et al. 2018), which inevitably leads to the complete extirpation of the population in-situ, with ex-situ conservation is pragmatic and solid. Extensive experience is available on keeping sound collections of a multitude of species (e.g. Bogaerts et al. 2012; Pasmans et al. 2014), and because caudates are generally long-lived, provide large clutches, and are usually easy-to keep (cheap) low maintenance species (Figure 6), a pro-active approach is therefore within reach and encompasses a small financial risk.

Alternatively, establishing gene banks, in concert with fine-tuning amphibian reproductive technologies prior to population declines, allows for a cost-effective and large scale possibility of species assurance and genetic management assurance (Kouba et al. 2013). Hormone therapy and IVF (*in vitro* fertilization) are already being used in amphibian management. For instance in the Southern Corroboree frog (*Pseudophryne corroboree*) hormone treatment successfully induced spermiation and ovulation (Byrne and Silla, 2010). Nonetheless for most species refinement of the techniques is required before ART (Assisted Reproductive Technologies) can be applied routinely.

4. Invasion and epidemic phase

The focus for management during the invasion phase lies on the prevention of establishment and on reducing the dispersal rate of the pathogen (Figure 1). If this is unsuccessful, then the invasion becomes epidemic, and the main focus shifts to delaying the spread of the pathogen and to ensuring population persistence. These actions, listed in the EAP, already require a lot of knowledge, as well as a rapid response to the events.

With the current state of knowledge, we know that it is unlikely that a population susceptible hosts can sustainably persist after Bsal emergence (Chapter 7; Stegen et al. 2017; Canessa et al. 2018). Therefore Bsal management should focus on ex-situ population persistence and on the interruption of the spread of the pathogen to other populations by effectively interrupting transmission and minimizing host movements (Canessa et al. 2018). Management actions may target either host (remove hosts/vectors) or environment (isolate the outbreak) to prevent pathogen spread and establishment. A current uncertainty is the definition of the perimeter at which preventive or reactive measures are required. An important aspect to be considered during all stages of the conservation management process are the ecological, ethical and legal constraints of certain strategies. Eventually, the applied methods are the resultant of the consideration of various (local) conservation priorities.

4.1 Prevent pathogen establishment

The actions listed in the pre-arrival phase remain important. Additional measures to prevent the establishment of the pathogen are required in this phase and can either target the habitat, the host and environmental reservoirs.

4.1.1 Habitat management

Recently Grant et al. (2017) concluded that there are only few effective options for post-emergence control. They listed an extensive overview of potential mitigation actions at various stages of Bsal emergence. The three most effective actions suggested were to ban all import of salamanders, to restrict trade in salamanders and the third; destroy the habitat at infected sites. This latter suggestion manipulates the habitat quite rigorously, and is impossible under the current situation in the Netherlands. Nonetheless, making the habitat unsuitable, either physically or chemically (environmental disinfection), for the pathogen is an option. It could be considered to chemically manipulate the habitat (for instance, the repeated chemical treatment of a pond (Bosch et al. 2015)) or physically (for instance the draining a pond, or removal of the top soil layer to eliminate the environmental reservoir). For this, a thorough understanding of the pathogen's disease ecology is useful, but not mandatory. Especially in situations with limited knowledge about the pathogen, the full clearance of a site (and the proper waste disposal) can be a 'quick and firm' successful solution, preventing the spread and losing control. Again, the ecological, ethical and legal constraints should be considered.

Habitats that provide a non-conducive environment for the growth of *Batrachochytrium* sp., either in the host or in the environment, may suppress the severity of the outbreak during the invasion phase. Habitat management might also create pockets of disease refugia for susceptible hosts, that could function as safe havens (Schmeller et al. 2014; Garner et al. 2016; Chapter 7). These disease-free refuges are equally important in the epidemic phase in which the focus lies on reducing the impact on affected populations. Reducing non-disease sources of mortality might also facilitate population persistence in the presence of disease (Langwig et al. 2015; Chapter 3), such as the frequent drainage of breeding ponds to kill-off predators of amphibian larvae. This might increase tadpole survival which leads to increased juvenile recruitment.

Observational evidence suggests that the frequent drying of ponds is not an adequate tool for Bd eradication. Two study ponds in the Netherlands with common midwife toads (*Alytes obstetricans*) completely dried up. The first pond was dry in July 2011 and Bd prevalence in the 10 collected juvenile toads was 0%. The next month 3 out of 5 collected juveniles from that same pond were infected with Bd. Likewise for the second pond. This one dried up completely in July and August 2011 (prevalence 0% in 7 collected *Lissotriton vulgaris*, *Bufo bufo* and *A. obstetricans*), but in October 2011, 6 out of the 10 sampled *A. obstetricans* tested positive for Bd (Spitzen-van der Sluijs unpubl. data).

4.1.2 Host management

The habitat management can be combined with the removal (or culling) or treatment of hosts and vectors, as well as the removal of environmental reservoirs (if they are known). At an early stage of invasion, outbreaks might be relatively small-scale, and nonorthodox measures might prevent much more devastating outbreaks. In brief, for Bsal host management, currently the only effective management action to be considered is the quick and complete removal of all hosts from the infected site.

Host removal

Actively removing a substantial part of the host population might interrupt the spread, and herewith locally eradicate the fungus. The feasibility of this management strategy depends on the presence of reservoirs (with a specific focus on supershedder hosts), the spatial dynamics of the host and if transmission is frequency dependent or density dependent (Keesing et al. 2006; Canessa et al. 2018). The transfer of Bsal zoospores occurs either via direct contact between individuals (Martel et al. 2014; Chapter 7) or via floating encysted zoospores (Stegen et al. 2017). As fire salamanders actively search partners, partial reduction in density won't alter the frequency dependent transmission rates, whereas for instance reducing alpine newt density, a species that gathers (sometimes in great numbers) in breeding ponds, will reduce density dependent transmission rates. The immediate -upon disease emergence- and complete removal of all available susceptible and tolerant hosts in the absence of a non-caudate Bsal-reservoir was shown to be the sole action that would minimize the spread of Bsal to adjacent populations (Canessa et al. 2018). This already challenging management action is severely complicated when environmental reservoirs or non-amphibian reservoirs are demonstrated. As this control measure is only effective if executed at the appropriate scale, and within three days, policy makers and conservationists need to be prepared to take this action and plan ahead on the question what to do with the removed animals.

Host treatment

For species like the fire salamander, mitigation actions at the Bsal invasion front and during a Bsal epidemic event are unlikely to be effective (Canessa et al. 2018). Treatments need to be capable of a perfect interruption of transmission and need to cover >80% of the population treated within three days (Canessa et al. 2018). At this moment the sole treatment that has the desired probability of clearing infection is heat treatment or heat treatment in combination with the application of an antifungal treatment (Blooi et al. 2015a,b). However, the functional application of this treatment in the field remains complex, as a single treatment per individual salamander takes 10 days. But even if this practical issue can be overcome, fire salamanders do not acquire immunity (Stegen et al. 2017), so the application of even a highly effective treatment prolongs the survival of the individual host, but as re-infection is possible, it creates a larger pool of infectious individuals which increase the probability of pathogen spread to nearby populations. The modelling exercise by Canessa et al. (2018) showed that if a treatment that reduces transmission probabilities by 'only' 50% would actually worsen the situation and allow for the eradication of a population. Alternative treatments include the inoculation of the host with probiotics, to apply an antifungal treatment or vaccinate individuals (Grant et al. 2016; 2017; Canessa et al. 2018; Bletz et al. in prep.). None of these techniques have yet been developed for field applications that account for imperfect treatment in salamander hosts.

4.2 Prevent spread of the pathogen

4.2.1 Habitat management

Apart from the measures listed in the pre-arrival phase (§ 3) and in the measures to prevent establishment of the pathogen (§ 4.1) the isolation of an infected site can reduce the transmission rates between amphibian hosts/populations.

Isolating an infected population to prevent pathogen spill-over to yet naïve populations can be achieved by either the physical isolation of an infected population by for instance a quarantine fence, increasing the landscape resistance of the matrix between the populations, or by removing all potential vectors. Both approaches should be almost perfect to interrupt transmission (Canessa et al. 2018). This would theoretically imply that physical isolation of an infected site should not only prevent the dispersal/migration of infected amphibian hosts, but also of possible non-amphibian vectors, such as hikers with fomites on their shoes, birds that could transport the fungus, or invertebrates. It was estimated that an infected fire salamander would be able to cover on average 100 m before succumbing to disease (Canessa et al. 2018). However, in Chapter 7 it is shown that Bsal has poor autonomous dispersal capacities and has not spread from the index site to a nearby fire salamander population, despite a semi-permeable matrix. This might indicate that blocking transmission is a feasible conservation tool. In large continuous forested habitats this is not likely to hold. Here more radical measures might be required to block pathogen transmission via fomites (for instance by hikers on long-distance tracks) and dispersing individuals.

4.2.2 Host management

To prevent the spread of the pathogen by host manipulation the same management tools (treatment – if effective tools are available – of the hosts and vectors, culling or removing hosts/vectors from the population, and the removal or treatment of environmental reservoirs) can be applied as in the previous phase (§ 4.1).

4.3 Ensure host population persistence

4.3.1 Habitat management

Bsal specifically impacts on the adult life stages (Stegen et al. 2017), and increased recruitment rates may offset this reduced adult survival, allowing for population persistence (Scheele et al. 2015). However, as the only effective measure to mitigate Bsal, is the complete removal of the population, ensuring host population persistence in-situ is per definition problematic during

Figure 7. An overgrown breeding pond of *Bombina variegata* in sept. 2016 (Wahlwiller, the NL).

this phase. With the current knowledge, an increase in host densities may only lead to recurrent outbreaks and mortalities.

Fieldwork on endemic Bd showed that the younger life stages had the highest prevalence (Chapter 2 and 3), and as amphibian population growth rates are highly sensitive to survival rates of post-metamorphic juveniles (e.g. Hels and Nachman, 2002; Middleton and Green, 2015), controlling infection in these life stages has priority. Disease mitigation in the Netherlands should focus on the augmentation of demographic rates, which implies proper habitat management. A focus on the maximisation of reproductive output has multiple advantages, as increased recruitment might additionally shorten the severity of the bottleneck (reduction in population size and genetic variability) during the epidemic phase, it may ensure population persistence and allow for host-pathogen co-evolution.

In practice, this means that the focus for amphibian habitat management should lie on the construction and proper maintenance of a network of small ponds because compensatory recruitment is vital in population persistence. Both the aquatic and terrestrial habitat should provide sufficient possibilities for amphibians to shelter from increasing temperatures as rising temperatures increase the probability for adult yellow-bellied toads of becoming infected with Bd. A conservation concern for these toads specifically is that, due to poor site management, breeding ponds have become more and more unsuitable (Figure 7). Ultimately this lack of management will lead to local population declines. Instead of allocating budget for the introduction of the species at new sites, resources should be spent on the cheaper, and more effective management of their breeding habitat.

So, for host population persistence with an endemic Bd-infection, habitat management might ensure population persistence. For the situation with a hypervirulent Bd strain, and for Bsal, there is currently no proof-of-concept for – apart from the isolation of the outbreak site – habitat management as mitigation action.

4.3.2 Host management

Theoretically, the animals surviving an initial outbreak of Bsal could have a protective innate or acquired immune response. Selective breeding with these specific individuals could improve host resistance and herewith increase population persistence (Garner et al. 2016). With regard to Bd, some species respond through natural selection on immunity (Savage et al. 2016; Voyles et al. 2018) and selective breeding for resistant or tolerant genotypes could be a management strategy. In a recent paper Voyles et al. (2018) have shown that despite persistent Bd presence in Panama, amphibian species reappeared whilst Bd pathogenicity had remained equal. A shift in host resistance in some affected species within 5–13 yrs after the epizootic, had driven the disease dynamics towards an enzootic situation. Currently no studies have yet been conducted on heritable Bsal tolerance, nor has there been any evidence for this in the field. If salamanders happen to have the evolutionary potential to adapt to Bsal, then this could be facilitated by selective ex-situ breeding, or promoted via head-starting larvae either ex-situ or in-situ. Selective breeding for resistance against Bd or Bsal could be feasible, but will require extensive investment and is highly complicated by the context-dependent interaction of Bd/Bsal and its host (Garner et al. 2016). In brief, the only currently known host management action for persistence of the host in a situation with Bsal, is an ex-situ assurance colony.

5. Established phase

The Bsal post-epidemic response varies between persisting species; the fire salamander has a lower abundance and still suffers significant mortality, whereas clinical disease has only been observed in 2014 and 2015 in four infected alpine newts and in one infected smooth newt. An unmitigated Bsal-outbreak is expected to lead to the effective extirpation of a fire salamander population within three months (Canessa et al. 2018), which corresponds with the findings in the Netherlands and Belgium (Chapter 4; Stegen et al. 2017; Goverse and De Zeeuw, 2017). Current management should therefore focus on the eradication of Bsal from the system, and increasing host resistance (Figure 8). However, at this moment these are pressing research questions.

5.1 Host population recovery

Management strategies for endemic Bd that target the amphibian host are plentiful and are listed in the introduction of this thesis. Altering disease susceptibility of the amphibian host or reducing transmission rates, have proven to be successful in some situations, but the translation of treatments that work in captivity to actual field interventions is not straightforward (Garner et al. 2016). In the current situation in the Netherlands, there is no need to manipulate the amphibian host. Endemic Bd impacts the host, but here the key to long-term population persistence is the maintenance of high-quality habitat to reduce stress induced by pollution, climatic changes or alien invasive species, and ensure sufficient recruitment to compensate for Bd-induced mortality. Yet, vigilance and monitoring of the actual situation in the field remain key for the implementation or required adjustment of the proper disease management.

Theoretically, maximising the reproductive output of the surviving fire salamanders in an endemic Bsal situation could allow for evolutionary processes, eventually increasing population persistence, but this is not what is observed in the field and in the laboratory; fire salamanders' susceptibility to Bsal remains unabated. It is therefore – at this moment – pointless to boost the population fire salamanders by for instance repopulation as long as Bsal is not fully eradicated.

5.2 Prevent new threats

With this final phase, the circle is round and all measures listed in § 3 (Discussion) are applicable. Continuing severe and realistic threats for amphibian populations are plentifold and include habitat destruction, the focal introduction of current Bsal or novel Bsal/Bd strains in naïve populations, and global climate change. The synergistic effects of these threats may be even more detrimental. There is an urgent need for a study which encompasses all these factors to elucidate the key relationships and identifies actions.

6. Conclusions

The translation of scientific information into conservation actions is scarce, and nearly non-existent (Canessa et al. in prep.), yet to get things done in the field, this translation is important and deserves more attention. Figure 8 lists the conservation measures and questions per invasion phase. With regard to Bsal, the sole reactive effective management possibilities at this moment are to set-up an assurance colony, quickly and effectively remove all hosts and vectors from an infected site, eliminate the environmental reservoirs, or completely isolate the outbreak site. For endemic Bd, the proof-of-concept management tool is habitat management to allow for compensatory recruitment. The introduction of a highly virulent Bd isolate could however potentially require equally fierce interventions as for Bsal. An adequate response to an outbreak requires proper preparation and an ready-to-use Emergency Action Plan.

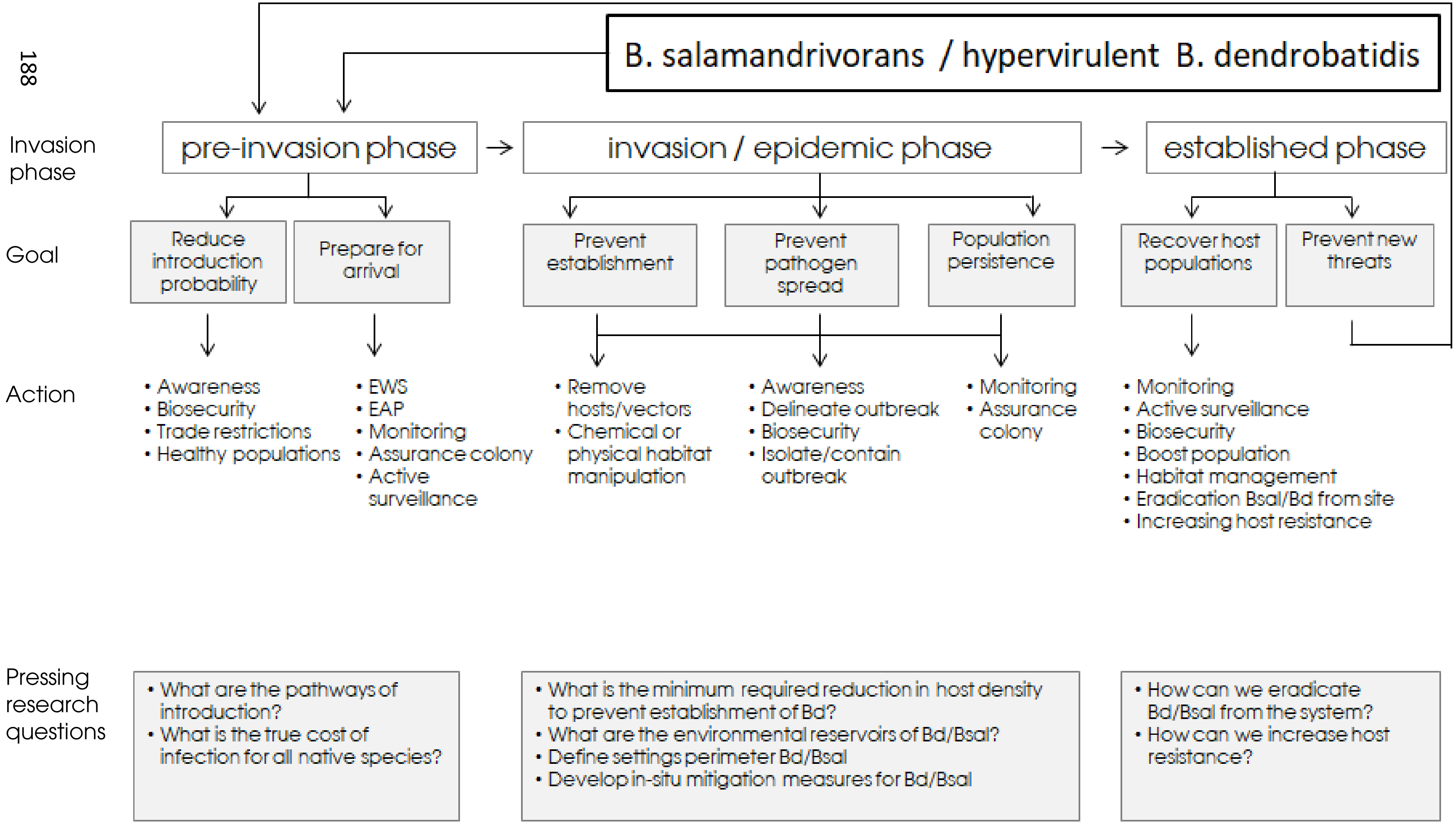

Figure 8. Listing of all conservation management actions and important research questions in the presence of Bsal or a hypervirulent Bd strain (= mas mortality or population declines are associated with Bd) per invasion phase (classification by Langwig et al. (2015). EWS (Early Warning System encompasses syndromic surveillance and passive surveillance. EAP= Emergency Action Plan.

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Summary

Globally, so also in the Netherlands, amphibians compose a species group with a high conservation urgency. Habitat destruction is a key issue in amphibian conservation, but the impact of emerging infectious diseases (EIDs) on amphibian population survival should not be disregarded. EIDs may cause mass mortality events and may have severe negative impact on population dynamics. The amphibian disease chytridiomycosis can cause epizootic outbreaks that may threaten the persistence of populations, and even that of amphibian species with limited geographic ranges.

The aim of this thesis was to elucidate the presence and impact of chytridiomycosis on amphibians in the Netherlands, and define practical conservation tools. For this purpose I first inventoried the presence of the chytrid fungus *Batrachochytrium dendrobatidis* (Bd) in captive collections (Study 1) and in wild amphibians in the Netherlands (Study 2). Second, I studied the long-term effects of this chytrid fungus on the rare yellow-bellied toad (*Bombina variegata*) (Study 3). A sudden collapse of the native fire salamander (*Salamandra salamandra*) population drove this species to the edge of extinction in the Netherlands (Study 4) and it was found that a novel chytrid fungus *B. salamandrivorans* (Bsal) was the causative agent (Study 5). This fungus originates in Asia and was presumably transported to Europe via the pet trade. In Europe, the fungus spreads and is currently found in wild amphibian populations in Belgium and Germany (Study 6). However, the dispersal capacity of this fungus seems to be dependent on vectors, which provides possibilities for disease management (Study 7).

Despite reports of large scale amphibian declines due to Bd in other parts of the world, it was yet unknown if Bd occurred in the Netherlands. In **Study 1** we therefore collected skin swabs from captive amphibians at a pet fair in the Netherlands, and from private collections, zoos and laboratories in the Netherlands, Belgium, France and Germany. Overall, 3.4% of the 971 collected swabs tested positive for Bd (spread over six of the 44 (13.6%) sampled collections). In one collection, four months after the initial detection of Bd, animals were still infected. Additionally, an illegally collected – and confiscated yellow-bellied toad - tested positive as well. These examples clearly show that chytrid infections are present in captive anuran and urodelan amphibians, and underline the necessity for implementing the appropriate biosecurity measures to prevent pathogen spillover and the introduction of the pathogen in captive breeding collections for conservation purposes.

In **Study 2** I describe that Bd is not only widespread in captive collections, but also in wild amphibian populations in the Netherlands and in Belgium. Sampling in museum collections showed that Bd is present at least since 1999. This oldest record concerns a natterjack toad (*Epidelea calamita*) from Amstelveen, and the first two chytrid-induced casualties in the Netherlands were reported. Two juvenile common midwife toads (*Alytes obstetricans*) died due to clinical chytridiomycosis. Nearly 5% of all sampled individual amphibians tested positive for Bd. Interestingly, Bd-prevalence in native species was considerably higher in 2009 than in 2010; in the common midwife toad prevalence dropped 93%, in the yellow-bellied toad Bd 72% and in the alpine newts (*Ichthyosaura alpestris*) prevalence dropped with 78%. The mechanism behind these fluctuations yet remain unclear, as Bd prevalence did inconsistently respond to the weather covariates. For instance, for the yellow-bellied toad and alpine newt, Bd prevalence seemed unaffected by the weather conditions, but for the common toad (*Bufo bufo*) and the midwife toad the weather conditions respectively 7 and 30 days prior to sampling affected prevalence. In this study we hypothesize that Bd is endemic in this region, and that currently the context (climatic conditions) preclude the build-up of Bd to lethal levels.

Yet, even endemic chytridiomycosis can lead to mortalities, which was shown in **Study 3**. Here, the apparent coexistence of the yellow-bellied toad and Bd in the Netherlands was studied over a 7-year period in two populations. We found that Bd does incur an actual cost to the survival of both adult and juvenile toads, and survival decreased considerably with increasing infection load. The condition of the host and environmental temperature did not influence survival probability, but the probability for an adult toad to become infected did increase with increasing temperatures. The two populations that were studied were not connected with each other, but did show remarkably similar patterns in adult survival, recruitment and Bd prevalence. The fluctuations at population level suggest compensatory recruitment, and the current equilibrium is probably maintained by environmental mediation.

The above work on Bd shows that conservation tools for this enzootic situation should focus on the prevention of pathogen spillover from captive held amphibians and non-native species to natural situations, and that even though Bd is widespread and endemic, the maintenance of quantitatively and qualitatively sufficient breeding habitat is key for the long-term persistence of populations. Keeping a close watch on both population trends and prevalence is essential to observe changes that could be indicative of a disrupted balance.

The sudden collapse of the native fire salamander population in the Netherlands with 96% is described in **Study 4**. Volunteers observed dead fire salamanders in the field, the monitoring trend crashed, but necropsies yielded no conclusive result on the cause of death of these animals. The incapability of finding the causative agent of the enigmatic fire salamander decline was not odd, as it appeared to have been caused by a second chytrid fungus that was previously unknown, which is described in **Study 5**. This novel chytrid Bsal represents a previously undescribed lineage that forms a clade with Bd. Bsal has a lower optimal growth temperature (10°C - 15°C) than Bd (17°C - 25°C), it causes erosive skin lesions and induces 100% mortality in fire salamanders within 18d after inoculation.

After the discovery of Bsal in the Netherlands, there was a great urgency to detect the spread of the fungus in other parts of Europe. In **Study 6** the results are presented of sampling activity over a 6-year period (2010 – 2016) in the Netherlands, Belgium and Germany. The fungus was detected at six other locations in the NL (fire salamanders, alpine newts (*Ichthyosaura alpestris*) and smooth newts (*Lissotriton vulgaris*)), in four sites in Belgium (fire salamanders and alpine newts) and at three sites in Germany (all fire salamanders), and our results show that the range of Bsal distribution may be up to 10,000 km^2.

With the increasing distribution of Bsal over Europe, it is to be expected that also within the Netherlands the fungus would have expanded, especially after it's been present for nearly ten years. We therefore describe a case in **Study 7** that shows the absence of Bsal spill-over between the index site and a nearby population fire salamanders, despite a permeable landscape matrix. In this study we also describe a laboratory experiment with alpine newts, which shows that Bsal has a low autonomous dispersal capacity. These findings suggest that the observed spread as described in Study 6 is largely human-mediated and that biosecurity measures are urgently required, ánd useful, to prevent the spread to adjacent, yet naive areas.

The work presented in this thesis provides key information on the distribution and impact of Bd and Bsal on native amphibians, as well as on the environmental conditions that currently preclude disease outbreaks of Bd. This work also shows that detailed, longitudinal and regional studies are required to reveal the context-dependency of Bd and Bsal disease dynamics. Because of the latter, conservation management should be tailored and basic information on the biology of especially Bsal, is urgently required.

Samenvatting

Amfibieën worden sterk bedreigd, niet alleen in de tropische delen van de wereld, maar ook in Nederland gaat het niet goed met minstens de helft van de inheemse soorten. De belangrijkste reden hiervoor is het verloren gaan van leefgebied. Door verstedelijking, vervuiling en versnippering wordt het leefgebied van amfibieën steeds marginaler en van lagere kwaliteit. Echter, de impact van opkomende infectieziekten op amfibieën populaties mag ook in Nederland, ondanks de afwezigheid van frequente massale sterfte, niet worden genegeerd. De amfibieziekte chytridiomycose kan een epidemie veroorzaken die het duurzaam voortbestaan van populaties, en zelfs van soorten met een beperkte geografische verspreiding, kan bedreigen.

Het doel van dit proefschrift was om inzicht te krijgen in de aanwezigheid, verspreiding en impact van chytridiomycose op amfibieën in Nederland, en op basis van deze kennis suggesties te doen voor de praktische bescherming in-situ. Met het oog op dit doel heb ik eerst de aanwezigheid van de schimmel *Batrachochytrium dendrobatidis* (Bd) (die de ziekte chytridiomycose kan veroorzaken) geïnventariseerd bij gehouden (Studie 1) en wilde amfibieën (Studie 2) in Nederland. Daarna is gekeken naar de lange termijn effecten van Bd op twee populaties van de inheemse geelbuikvuurpad (*Bombina variegata*) (Studie 3). Het plotseling ineenstorten van de grootste populatie vuursalamanders (*Salamandra salamandra*) in Nederland bracht deze soort op het randje van uitsterven (Studie 4). De reden van deze sterke achteruitgang bleek een tot nu toe onbekende chytride schimmel te zijn: *B. salamandrivorans* (Bsal). Deze schimmel heeft zijn oorsprong in Azië en is waarschijnlijk via de handel in amfibieën naar Nederland gekomen (Studie 5). In Europa breidt het verspreidingsgebied van Bsal zich uit. Momenteel komt de schimmel ook voor bij wilde amfibieën in België en Duitsland (Studie 6). In de laatste studie van dit proefschrift wordt o.a. beschreven dat Bsal een beperkte intrinsieke mogelijkheid heeft tot verspreiding, wat betekent dat de verspreiding die we zien zeer waarschijnlijk komt door menselijk handelen (Studie 7), hetgeen openingen biedt voor beheer en beheersing.

Ondanks dat uit andere delen van de wereld gerapporteerd werd over grootschalige sterfte bij amfibieën was onbekend of Bd ook in Nederland voorkomt. In **Studie 1** hebben we om die reden huidswabs verzameld van gehouden amfibieën op een beurs in Nederland en bij dieren uit privé collecties, dierentuinen en laboratoria in Nederland, België, Frankrijk en Duitsland. Van de 971 verzamelde swabs testte 3,4% positief voor Bd, verspreid over zes van de 44 (13,6%) van de bemonsterde collecties). Vier maanden nadat Bd in een collectie was geconstateerd, testte deze collectie wéér (nog) positief, en een in beslag genomen, want illegaal verzamelde, geelbuikvuurpad testte ook positief voor Bd. Deze voorbeelden illustreren dat Bd aanwezig is bij amfibieën in gevangenschap, en het onderstreept de noodzaak voor het goed op orde hebben van hygiënemaatregelen om te voorkomen dat ziekteverwekkers vanuit gehouden dieren in de natuur terecht komen, of in kweekprojecten die zijn opgezet voor beschermingsdoeleinden.

In **Studie 2** laten we zien dat Bd niet alleen bij gehouden amfibieën in Nederland voorkomt, maar ook in wilde populaties in Nederland en België, en dat tenminste sinds 1999. Door te bemonsteren in museumcollecties werd het tot nu toe oudste besmette dier gevonden, een rugstreeppad (*Epidalea calamita*) uit Amstelveen. Dit onderzoek beschrijft ook de eerste twee bewezen sterfgevallen van inheemse, wilde amfibieën (vroedmeesterpadden) in Nederland als gevolg van chytridiomycose. Bijna 5% van de bemonsterde amfibieën testte positief op aanwezigheid van Bd. Interessant genoeg was de prevalentie van Bd in 2009 vele malen hoger dan in 2010. Waarom dit zo was, is niet duidelijk. De weersomstandigheden hadden een variabele en inconsistente invloed op de prevalentie van Bd. Zo hadden de weersomstandigheden geen invloed op Bd prevalentie bij de geelbuikvuurpad en de Alpenwatersalamander (*Ichthyosaura alpestris*), maar hadden de weersomstandigheden 7 en 30 dagen vóór het bemonsteren wel impact op Bd prevalentie bij respectievelijk de gewone pad (*Bufo bufo*) en de vroedmeesterpad. In deze studie geven we aan dat Bd endemisch is in de studieregio en dat momenteel de context (omgevingsparameters) voorkomen dat Bd zich kan opbouwen tot lethale niveaus.

Ook endemische chytridiomycose kan leiden tot sterfte, zoals is beschreven in **Studie 3**. Het ogenschijnlijke probleemloze samen voorkomen van Bd en de geelbuikvuurpad in Nederland werd gedurende 7 jaar bestudeerd in 2 populaties. We vonden dat Bd wel degelijk invloed heeft op de individuele overleving van zowel volwassen als juveniele padden, en dat de overlevingskansen aanmerkelijk daalden als de infectiegraad toenam. De conditie van de pad en de omgevingstemperatuur hadden geen invloed op kans dat een geelbuikvuurpad het volgende jaar zou halen, maar de kans dat een volwassen pad een besmetting met Bd opliep steeg wel met toenemende temperaturen. De twee onderzochte populaties waren niet met elkaar verbonden, maar ze vertoonden opmerkelijk gelijke patronen in de overlevingskansen van de volwassen padden, voortplantingssucces en Bd-prevalentie. De fluctuaties in populatiedynamiek suggereren ‘compensatie door voortplanting’ (sterfte door Bd wordt gecompenseerd door meer nakomelingen te krijgen). Waarschijnlijk zorgen de huidige omgevingsomstandigheden ervoor dat er geen massale sterfte optreedt als gevolg van Bd.

Het hierboven beschreven werk laat zien dat 1) beheersmaatregelen voor deze endemische situatie moeten zich richten op het voorkomen van de overdracht van ziekteverwekkers van gehouden dieren en uitheemse soorten naar natuurlijke situaties, en 2) het onderhouden en aanleggen van kwalitatief goede voortplantingswateren is essentieel voor het behoud van populaties op de lange termijn. Het blijft cruciaal om zowel het voorkomen (verspreiding en prevalentie) van Bd te monitoren, als de populatie trends van amfibieën om zo veranderingen in de balans tijdig te detecteren.

Het plotselinge ineenstorten van de inheemse vuursalamander populatie in Nederland wordt beschreven in **Studie 4**. Vrijwilligers van RAVON zagen dode vuursalamanders in het bos, de monitoring trend stortte in, maar onderzoek aan de dode dieren in het laboratorium leverde in eerste instantie geen inzicht in de doodsoorzaak. Dat dit niet lukte bleek achteraf logisch, want uiteindelijk bleek het te gaan om een –tot dan toe– onbekende chytride schimmel *Batrachochytrium salamandrivorans* (Bsal). Deze schimmel is beschreven in **Studie 5**. Bsal heeft een lagere optimale groei temperatuur (10°C - 15°C) dan Bd (17°C - 25°C) en veroorzaakt bij geïnfecteerde salamanders erosie van de huid (salamandrivorans betekent letterlijk: salamander-eter) en 100% sterfte bij besmette vuursalamanders binnen 2-3 weken na besmetting.

Na de ontdekking van Bsal in Nederland was de vraag of Bsal zich verder heeft verspreid. In **Studie 6** staan de uitkomsten van een brede screening van salamanders in Nederland, Duitsland en België gedurende 6 jaar (2010-2016). De schimmel werd op zes andere locaties in Nederland gevonden. Niet alleen bij vuursalamanders, maar ook bij Alpenwatersalamanders en kleine watersalamanders (*Lissotriton vulgaris*). In België werd de schimmel op vier locaties gevonden bij vuursalamanders en Alpenwatersalamanders en in Duitsland in drie gebieden, allemaal vuursalamanders. Het grove verspreidingsgebied van Bsal is ongeveer 10.000 km^2.

Wetende dat de schimmel zich naar andere landen heeft verspreid, was het ook de verwachting – zeker 10 jaar na de eerste detectie – dat Bsal zich ook in Nederland verder zou hebben uitgebreid. In **Studie 7** beschrijven we dat Bsal zich, ondanks het tussenliggende landschap, zich niet heeft verspreid vanuit het Bunderbos naar een nabijgelegen andere populatie vuursalamanders. Het is mogelijk dat Alpenwatersalamanders (die als vector kunnen fungeren), of ook vuursalamanders, via het tussenliggende habitat van de ene naar de andere locatie kunnen migreren. Door een experiment in het laboratorium met Alpenwatersalamanders laten we zien dat Bsal zelf een slechte verspreider is. Verspreiding lijkt gekoppeld te zijn aan vectoren en dat suggereert dat de waargenomen uitbreiding van Bsal (Studie 6) door de mens wordt gefaciliteerd. Het nemen van hygiëne maatregelen is dus een belangrijke manier om verspreiding te voorkomen.

Het werk dat in dit proefschrift wordt gepresenteerd draagt bij aan de kennis over de verspreiding en impact van Bd en Bsal op inheemse amfibieën. Het laat zien dat meerjarige, gedetailleerde studies belangrijk zijn om uiteindelijk de impact van context op ziekte dynamiek te ontrafelen en inzichtelijk te maken. Dit levert handvatten op om beschermingsmaatregelen te kunnen definiëren. Door de sterke context-afhankelijkheid van het verloop van een infectie met Bd of Bsal, is maatwerk vereist in de bescherming van soorten. Monitoring in het veld van pathogeen en gastheer, én kennis van de biologie van de chytride schimmels is hiervoor essentieel.

Curriculum Vitae

Annemarieke Spitzen-van der Sluijs was born on August 7th, 1977 in Rotterdam (NL). In 1999 she obtained her bachelor in Animal Management, profile Wildlife Management at the Van Hall Institute, Leeuwarden (NL). After a few years of work in New Zealand for the Department of Conservation, she obtained her Master of Science in Forest and Wildlife Management, profile Animal Ecology at Wageningen University, Wageningen (NL) in 2003 (graduated cum laude). She started a doctoral research project in 2011 at the Wildlife Health Ghent research group at the Department of Pathology, Bacteriology and Poultry diseases from the Faculty of Veterinary Medicine at Ghent University, whilst working as a researcher and senior project manager at Reptile, Amphibian and Fish Conservation the Netherlands (RAVON). This doctoral study was financed by RAVON and Ghent University. Annemarieke Spitzen is author or co-author of 29 scientific publications and she presented at 12 international conferences.

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Het was pas in Nieuw Zeeland na mijn HBO studie, dat een fascinatie voor reptielen en amfibieën werd gewekt. Het meewerken aan het beschermingsproject voor de grand en Otago skink (*Oligosoma* grande en *O. otagense*), de belangenconflicten tussen agrariërs, natuurbeschermers en huisdier-eigenaren, en de problematiek van exotische fauna en flora in Nieuw Zeeland hebben ervoor gezorgd dat ik na deze reis bos- en natuurbeheer ben gaan studeren in Wageningen. Gedurende die studie heb ik aan twee mooie projecten mogen werken ('landscape resistance for *Rana temporaria*' bij Claire Vos en een ecologisch onderzoek aan de hazelworm (*Anguis fragilis*) bij Ton Stumpel – dat moeten we nog maar eens opschrijven Ton ;-)). In die periode was ik aan het werk bij de Zoogdiervereniging in Arnhem, maar na drie jaar kon ik de overstap naar RAVON maken.

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