Mitigation of chytridiomycosis in amphibians

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LIST OF ABBREVIATIONS

AMP	antimicrobial peptide
B.	*Batrachochytrium*
Bd	*Batrachochytrium dendrobatidis*
Bs	*Batrachochytrium salamandrivorans*
BSA	bovine serum albumin
C_q	quantitation cycle
Cy5	cyanine 5
DNA	deoxyribonucleic acid
EMA	ethidium monoazide
EPH	endemic pathogen hypothesis
FIC	fractional inhibitory concentration
FICI	fractional inhibitory concentration index
GE	genomic equivalent
GLMM	generalised linear mixed model
µg	microgram
H&E	haematoxylin and eosin
Ig	immunoglobulin
ITS	internal transcribed spacer
IU	international units
IUCN	the International Union for Conservation of Nature
L	litre
µl	microlitre
M	molar
Ma	million years ago
mg	milligram
MGB	minor groove binder
MHC	major histocompatibility complex
MIC	minimal inhibitory concentration
ml	millilitre
MP	maximum parsimony
µm	micrometer
µM	micromolar
ng	nanogram
nov	novarum
NPH	novel pathogen hypothesis
PCR	polymerase chain reaction
qPCR	real-time polymerase chain reaction
RNA	ribonucleic acid
rpm	rotations per minute
rRNA	ribosomal ribonucleic acid
SD	standard deviation
sp.	species
TGhL	tryptone, hydrolysed gelatine and lactose
UV-B	ultraviolet B

General introduction

1. Chytridiomycosis

1.1 History and origin

Chytridiomycosis is caused by the chytrid fungus, *Batrachochytrium dendrobatidis*. Taxonomically, *B. dendrobatidis* is placed in the phylum of Chytridiomycota (1), which are fungi characterized by production of motile flagellated spores (2). Most Chytridiomycota, or chytrids, have adopted saprophytic or parasitic lifestyles, living on plants, algae or invertebrates and inhabit aquatic environments varying from moist soil to fresh water (2). At the start of this thesis, *B. dendrobatidis* was the only chytridiomycete taxon known to infect vertebrate hosts. The complete life cycle of *B. dendrobatidis* takes 5 – 7 days, starting with flagellated zoospores, which develop into sporangia with discharge tubes in the amphibian skin, from which new zoospores are released. The first description of the amphibian disease chytridiomycosis dates from 1998, when mass mortality events in Australia and Panama were linked to skin changes occurring due to the presence of a chytridiomycete fungus (3). Since then, research into amphibian declines and extinctions has shown that *B. dendrobatidis* is present on all continents with existing amphibians (4) (Figure 1), and chytridiomycosis is recognized as a major threat to amphibian diversity and driver of declines and extinctions of amphibian populations worldwide (4, 5).

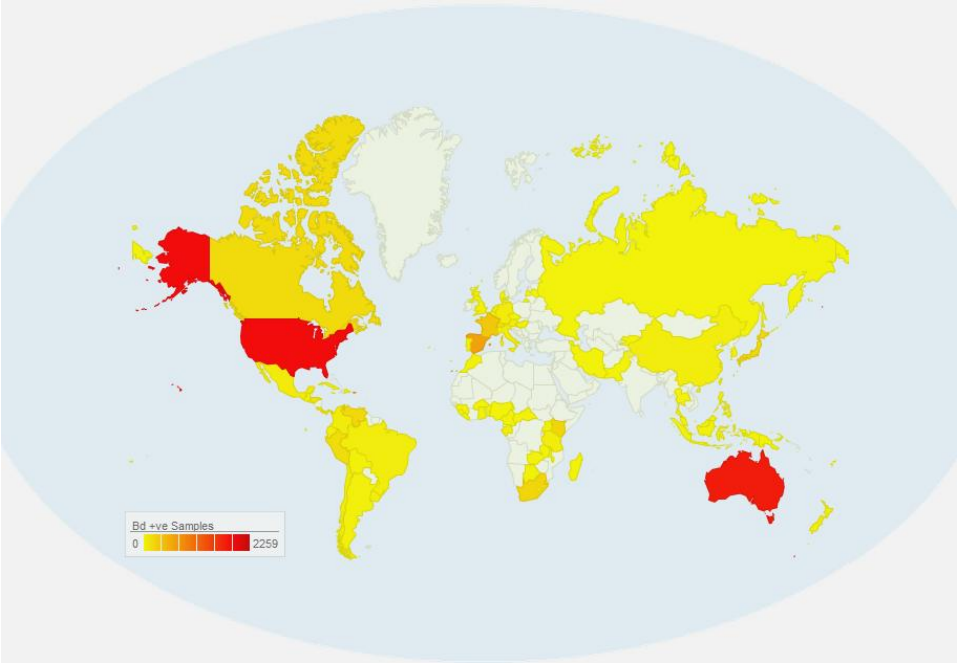

Figure 1. Map showing number of positive *Batrachochytrium dendrobatidis* samples per country in 2015. Adapted from www.bd-maps.net.

Two competing hypotheses exist regarding *B. dendrobatidis*' origin. Firstly, the novel or spreading pathogen hypothesis (NPH) (5) is supported by evidence of epidemics in amphibian populations (6-8) and species that serve as vector for *B. dendrobatidis* (9-17). Africa (18, 19), North-America (20) and Asia (21) have been proposed as possible foci from where *B. dendrobatidis* was potentially spread. Secondly, the endemic pathogen hypothesis (EPH) proposes that the pathogen has been present in the environment but alterations in ecological, immunological and/or behavioural parameters regarding host and/or parasite have resulted in a more pathogenic relationship (22). This hypothesis is supported by evidence that *B. dendrobatidis* has been present in amphibian populations well before amphibian population declines had occurred (18, 23), co-existence of *B. dendrobatidis* and susceptible amphibian species in absence of disease (24, 25) and by the correlation between environmental conditions/shifts and chytridiomycosis disease dynamics (26-29).

1.2 Impact on amphibian diversity

The class of amphibians (Amphibia) is composed out approximately 6500 species, subdivided in the orders of Anura (frogs and toads, approximately 5800 species), Caudata (salamanders and newts, approximately 580 species) and Gymnophiona (caecilians, approximately 170 species) with new amphibian species being described regularly (30-33). Amphibians inhabit all continents with the exception of Antarctica, with over half of the species occurring in the New World (North, South and Central America) (34) and highest diversity of amphibian species found in tropical South America and sub-Saharan Africa (35) (Figure 2). We are currently heading towards (or are even already in) the sixth mass extinction event the world has known, with amphibians a major group at risk (28, 36); 30,5% of the world's amphibian species are threatened according to the IUCN Red List Criteria (35) and recent amphibian extinction rates are suggested to be 211 times the background extinction rate (37). Chytridiomycosis is considered as one of the main drivers of extinctions and declines of amphibian populations (3, 5, 7, 26, 38-40), with presence of *B. dendrobatidis* confirmed in 350 amphibian species (4). Despite *B. dendrobatidis*' association with amphibian population declines, its global spread and its broad spectrum of susceptible hosts, the classic scenario with ongoing amphibian population declines is not observed everywhere. Chytridiomycosis disease dynamics in natural host-pathogen systems are considered to be steered by variability in factors associated with the host, pathogen and environmental context. Examples of factors steering outcome of infection with *B. dendrobatidis* are differential susceptibility to *B. dendrobatidis* observed between amphibian species (41-43) and amphibian life stages (44),

differences in pathogenicity between *B. dendrobatidis* strains (45) and variable local chytridiomycosis disease dynamics associated with climatic conditions and host diversity (27, 46, 47). Throughout Europe for instance, virulent strains of *B. dendrobatidis* and highly susceptible amphibian species are present, but declines of amphibian populations due to presence of *B. dendrobatidis* are limited to specific areas (12, 24, 38, 48). Altogether, this illustrates that instead of looking at single factors influencing *B. dendrobatidis* infections in a particular amphibian species, it is important to take into account the complete array of cofactors that influence and determine eventual outcome of infectious disease when looking at local disease dynamics (49).

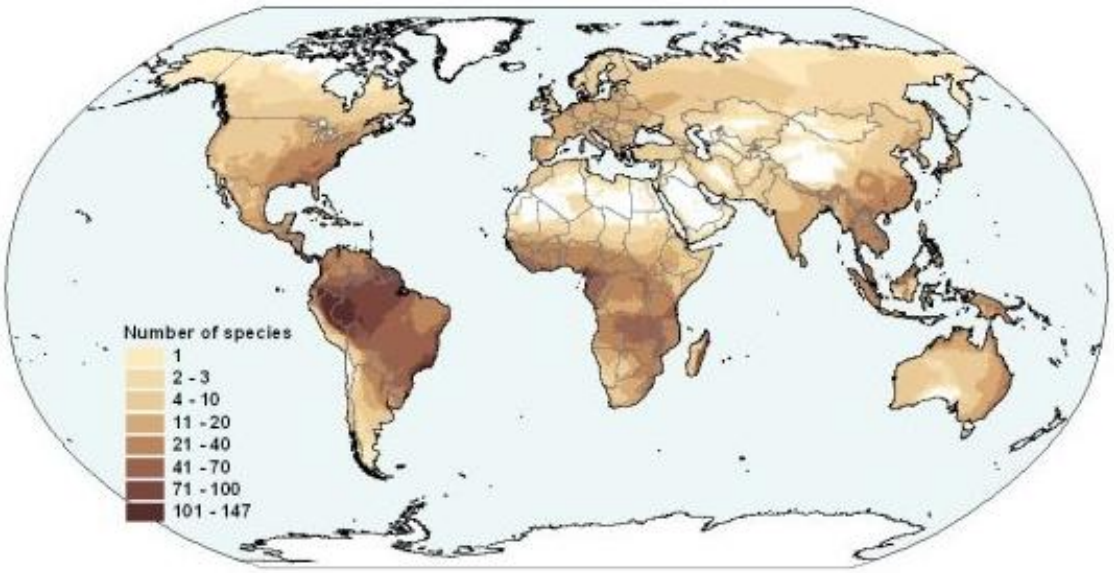

Figure 2. Map showing global pattern of amphibian diversity. Adapted from www.iucnredlist.org.

1.3 Clinical signs, pathology and pathogenesis

Common clinical signs observed in amphibians affected by chytridiomycosis are excessive and abnormal sloughing, erythema of the skin and (mass) die-offs. Furthermore non-specific signs such as lethargy, anorexia and loss of righting reflex can be observed (50, 51). Macroscopic findings at necropsy of adult amphibians affected by chytridiomycosis can vary from none (3) to severe dysecdysis and ulceration of the skin (52). Examination of haematoxylin and eosin stained histological preparations reveals that pathologic changes are restricted to the skin. Pathological changes comprise fungal thalli or sporangia associated with focal areas of hyperkeratosis and ulceration of the stratum corneum, epidermal hyperplasia and focal necrosis of epidermal cells (3, 52, 53) (Figure 3). In amphibian larvae, *B.*

dendrobatidis sporangia can be found solely in the keratinised parts of the mouth with minimal pathology, characterized by depigmentation and abnormal keratinisation of the oral disc (3, 52, 54, 55).

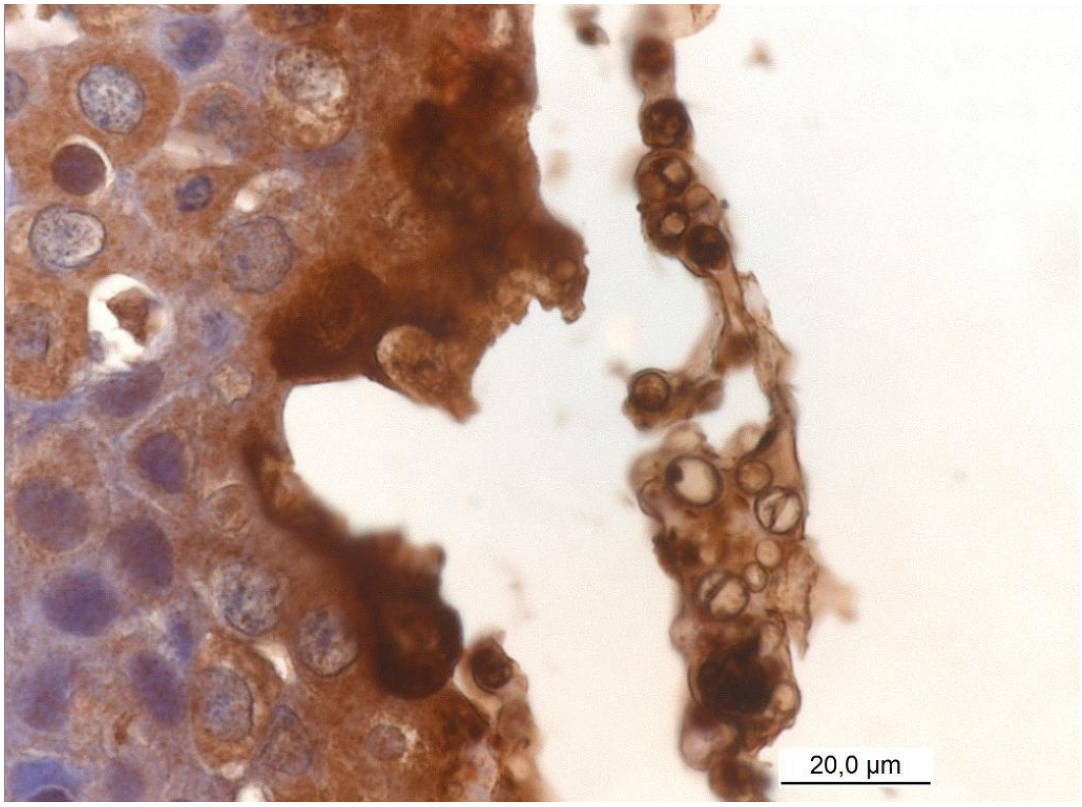

Figure 3. Immunoperoxidase stained histological section of the skin of a common midwife toad (*Alytes obstetricans*) severely affected by chytridiomycosis. Pathological changes are presence of thalli and sporangia of a chytrid fungus in the keratinised epidermal layers of the skin.

Chemotaxis towards several attractants present in the amphibian skin aids the motile zoospores of *B. dendrobatidis* in finding susceptible hosts (56). After coming into contact with the skin of a suitable host, the zoospore resorbs its flagellum, encysts (53) and subsequently penetrates deep into the hosts' epidermal cells by deploying germ tubes (57). Maturation of *B. dendrobatidis* thalli into sporangia coincides with the epidermal sloughing rate of the host. Pathological epidermal alterations are thought to be induced by production of virulence associated proteins and proteases (58-60), reduced expression of host genes encoding for essential skin components (61) and disruption of intercellular junctions (60). The mechanism by which the described skin pathology leads to fatality in infected amphibians is

thought to be an electrolyte depletion-induced cardiac arrest due to disruption of osmoregulatory functioning of the skin and impairment of electrolyte transport (61-65).
Before impairment of normal skin functioning occurs, an infection intensity threshold commonly set at 10000 genomic equivalents of *Bd* zoospores needs to be surpassed (66-69).

1.4 Diagnosis

Histology of ethanol or formaldehyde preserved skin samples has been the golden standard for diagnosis of chytridiomycosis in amphibians (3, 52, 70). Most routinely, haematoxylin and eosin stained preparations of skin are used, but since this technique is not specific for detecting *B. dendrobatidis*, expertise in recognizing *B. dendrobatidis* organisms is required. An immunoperoxidase assay with polyclonal antibodies against *B. dendrobatidis* allows specific detection of *B. dendrobatidis* in skin samples (71). Cross-reactivity with other members of the Chytridiomycota with this immunoperoxidase assay does not interfere with chytridiomycosis diagnosis as long as *B. dendrobatidis* is the only Chytridiomycota member known to infect amphibian skin. Although histological examination allows identification of *B. dendrobatidis* organisms together with detection of pathological changes, it can be hard to detect low levels of infection. Skin changes associated with chytridiomycosis are focal (52) and numbers of thalli and sporangia present in the skin vary due to correlation with the sloughing rate of the host (72, 73). Although quantitative histopathology is possible (72) it is too labour-intensive to use routinely. Apart from histopathology, molecular tests like PCR and real-time PCR are used in chytridiomycosis diagnosis (74, 75). These assays are used to detect RNA sequences present in non-functional internal spacer (ITS) regions, which are highly genetically preserved regions in the DNA of *B. dendrobatidis*. Major advantages of the real-time PCR are that it allows detection of *B. dendrobatidis* in non-invasive skin swab samples and that it allows quantification of infection intensity with low detection limits (75). Comparisons between histopathology and real-time PCR in detecting *B. dendrobatidis* show that the molecular assay is more sensitive in detecting *B. dendrobatidis* (76, 77). An important drawback of the molecular techniques is that these tests only detect presence of *B. dendrobatidis* and do not necessarily indicate disease. A drawback specific for the real-time PCR is that its target has variable copy numbers depending on the *B. dendrobatidis* strain. Copy numbers of the ITS target can vary from 10 up to 144 copies per single *B. dendrobatidis* zoospore, which makes comparing infection loads difficult when dealing with multiple *B. dendrobatidis* strains (78).

2. Susceptibility to chytridiomycosis

Although the term 'disease triangle' is derived from the field of phytopathology, it also helps in illustrating the interacting components in infectious diseases in humans and animals, taking into account host, environment and pathogen as determinants of disease. During the last decades, research has shown that the disease dynamics of chytridiomycosis are also influenced by and dependant on these three factors. A brief introduction of the factors influencing chytridiomycosis disease dynamics associated with the host, the environment and the pathogen will be given.

2.1 Host

The major host associated factor that influences disease dynamics is the hosts' immune system, subdivided in innate and adaptive components. The innate immune system serves as an important first line of defence against pathogens, providing immediate protection by means of humoral and cell-mediated components (79, 80), but without conferring long-lasting protective immunity. The innate immune system in amphibians can protect its host by secretion of antimicrobial peptides from granular glands in the skin and digestive tract (81) with activity against bacteria, fungi and yeasts (82, 83). Not all amphibian species secrete these antimicrobial peptides however (84). The other components of the innate immune system known to confer protection in amphibians are phagocytic cells, natural killer cells and a complement system (85-88). Furthermore, symbiotic bacteria present on the skin contribute to the innate immune system of amphibians by producing potent antimicrobial substances (89). The adaptive immune system provides amphibians with an important and specific second line of defence against pathogens. In general, the amphibian adaptive immune system is comparable to the adaptive immune system of mammals, possessing B- and T-lymphocytes, Ig isotype heterogeneity, leukocyte-derived cytokines and major histocompatibility class (MHC) I and II genes (90-96). It should be noted though that our current knowledge on functioning of the amphibian adaptive immune system is mostly derived from studies conducted on the African clawed frog (*Xenopus laevis*) and the axolotl (*Ambystoma mexicanum*). Although the components of the anuran and urodelan adaptive immune system are similar, a far less robust antibody response to antigens was observed in urodelans in comparison to anurans (97). The clinical relevance of this difference has yet to be determined.

2.1.1 Innate immune system and chytridiomycosis

The amphibian innate immune system plays an important role as defence mechanism against chytridiomycosis. In particular, differences in production and effectiveness of skin antimicrobial peptides (AMP's) and differences in composition of symbiotic microbial barriers present on the skin are likely contributing elements in differential susceptibility to chytridiomycosis between and within amphibian species. Although skin AMP's have been shown to inhibit growth of *B. dendrobatidis* zoospores and sporangia *in vitro* (98-104), the effectiveness of the peptides *in vivo* varies (89, 102, 105). Furthermore, differential chytridiomycosis disease dynamics may be associated with variable efficacy of skin peptides at species (99) and population levels (103).

Symbiotic antimicrobial barriers form another defensive mechanism against chytridiomycosis by secreting antifungal metabolites with a high degree of inhibitory activity against *B. dendrobatidis* both *in vitro* and *in vivo* (89, 106-109).In addition, secreted antifungal metabolites are able to potentiate the effectiveness of the amphibian skin AMP's in inhibiting *B. dendrobatidis* (110). The importance of the skin microbiome as defence mechanism against chytridiomycosis is further illustrated by the fact that alterations and variations in the composition of bacterial community of the skin are correlated to variable susceptibility to chytridiomycosis (89, 107, 108). Recently, an important correlation between the amphibian skin mucosome function, the micro-ecosystem of the mucus encompassing interdependent host factors and microbial-community factors, and prevalence of *B. dendrobatidis* infections in natural amphibian populations was demonstrated (111). Furthermore differential efficacy of the mucosome functioning under different immunological and environmental settings was shown, which could help to make probiotic treatment of *B. dendrobatidis* infections in amphibians successful.

2.1.2 Adaptive immune system and chytridiomycosis

Recently, important advances regarding the role of the adaptive immune system in chytrid infection dynamics have been made. Previous exposure of amphibians to *B. dendrobatidis* resulted in higher survival rates at subsequent exposure to *B. dendrobatidis* (100, 112, 113) and the number of previous exposures to *B. dendrobatidis* was a negative predictor of *B. dendrobatidis* infection loads and mortality in frogs while being a positive predictor for lymphocyte abundance and proliferation (114). These results indicate that amphibians are able to generate an adaptive immune response after *B. dendrobatidis* exposure and furthermore that the resulting immune response is effective in counteracting *B. dendrobatidis*. This latter

result is of particular interest, as other studies have proposed that although a *B. dendrobatidis* specific adaptive immune response in amphibians can occur, it is ineffective in conferring protection against *B. dendrobatidis* due to pathogen-induced immune suppression (115, 116). These novel insights into the role of the adaptive immune system as protection against chytridiomycosis could prove very useful, opening up opportunities for vaccination mitigation measures, as the robust immune response was observed both after exposure to live and dead *B. dendrobatidis* organisms (114).

2.2 Environment

A conducive environment is required before establishment of disease can occur. For instance, abiotic factors like temperature and humidity greatly influence prevalence and severity of important infectious diseases in humans and animals (117, 118) and anthropogenic changes to the environment have been shown to drive disease emergence (119).

Amphibians are considered to be the first species affected by environmental stressors and are therefore considered as important indicators of environmental health (120). In the disease dynamics of chytridiomycosis, several abiotic factors such as temperature (121-125) and UV-B radiation (126, 127) have been identified as important determinants of disease, able to affect host fitness and pathogen virulence.

2.2.1 Abiotic factors

Several abiotic environmental factors like rainfall, temperature and UV radiation influence local chytrid infection dynamics. Due to the complex relationship between pathogen, host and environment, heterogeneity in disease dynamics caused by environmental differences can be brought forth on several levels. For instance, *B. dendrobatidis*' growth, survival and reproduction rate are heavily dependent on temperature *in vitro*, with optimal growth occurring at environmental temperatures between 17 and 25 °C, and partial fungal mortality occurring at exposure to 30 °C for a period of 8 days (58). Studies looking at spatial and temporal patterns of chytridiomycosis outbreaks seem to follow the trend that problems arise in cooler periods and localities (121, 128, 129), which is what to be expected looking solely at the thermal niche of *B. dendrobatidis*. On the other hand, the amphibian immune system is also influenced by environmental temperature, with an overall decreased response during colder periods (29, 130), which could also determine the temporal and spatial pattern of amphibian declines and chytridiomycosis outbreaks. The difficulties that are met when comparing local chytrid infection dynamics is further illustrated by the described relationship

between the pattern of spread of *B. dendrobatidis* and chytridiomycosis-associated mortality on one hand and weather conditions on the other in areas were *B. dendrobatidis* has become endemic (131), while this relationship could not be observed in another region were *B. dendrobatidis* is endemic (24). As stated before, all cofactors that influence and determine eventual *B. dendrobatidis* infection dynamics should be taken into account when looking at local disease dynamics.

Studies investigating the influence of UV-B radiation on the relationship between amphibians and infections between *B. dendrobatidis* also show contrasting results. On one hand a positive correlation between UV-B radiation and chytridiomycosis can be found by an UV-B influenced impaired immune function of amphibians (132, 133). On the other hand exposure to environmentally relevant UV-B levels resulted in decreased prevalence of *B. dendrobatidis* infections in amphibian larvae (127).

2.2.2 Biotic factors

Relatively limited research has been conducted on interactions between biotic factors and chytridiomycosis disease dynamics. One important biotic factor influencing *B. dendrobatidis* prevalence that did receive attention is that of species serving as *B. dendrobatidis* carriers. Amphibian species resistant to chytridiomycosis, birds, reptiles and crawfish have been identified as carriers of *B. dendrobatidis* (14, 15, 41, 134), and could therefore help in spreading *B. dendrobatidis*, introducing it into populations of susceptible amphibian species and sustaining presence of *B. dendrobatidis*. On the other hand increased amphibian species richness reduces chytridiomycosis disease risk and underlines the importance of looking at community structure when studying chytridiomycosis disease dynamics (47, 135).

Another biotic factor that could influence local chytridiomycosis disease dynamics is the community structure of zooplankton (136). The zooplankter *Daphnia magna* feeds on *B. dendrobatidis* zoospores, and is therefore able to reduce *B. dendrobatidis* infection pressure in the environment (136-138). This is in line with the role other chytrid fungi fulfil in aquatic food web systems, were zoospores are considered to be important food sources for zooplankton and other filter feeders (139) and were predation on zoospores has been shown to impact disease dynamics (140).

2.3 Pathogen

Lastly, next to a susceptible host and a conducive environment a virulent pathogen is required before establishment of disease can occur. The major pathogen derived factor determining disease dynamics is the ability to cause disease and the associated intensity of disease, in other words its pathogenicity. Different strains with associated different genotypes and phenotypes of *B. dendrobatidis* show variability in virulence, greatly affecting chytrid infection dynamics (4, 45, 141-143). The exact mechanisms underpinning differential virulence of *B. dendrobatidis* strains are currently poorly understood.

3. Mitigation

The devastating impact of chytridiomycosis on amphibian diversity has resulted in considerable attention for development of chytridiomycosis mitigation measures. Although success has been made in these studies, the resulting measures are mostly (if not all) targeted at *ex situ* mitigation, and are not suitable for combating chytridiomycosis in nature. While *ex situ* chytridiomycosis mitigation measures are important (for example for generating disease free captive colonies), effective *in situ* mitigation measures are urgently needed.

3.1 *Ex situ*

3.1.1. Treatment of chytridiomycosis with antimicrobial compounds

A vast amount of studies exist describing treatment of chytridiomycosis in captive amphibians using antimicrobial compounds. However, the majority of these studies describe empirical treatments, based on small sample size studies and lack clinical trials necessary to ascertain treatment effectivity (144). One important class of antifungal drugs used to treat chytridiomycosis in amphibians is that of the imidazole, triazole and thiazole, or “azole” group. Azole antifungals exert their antifungal effect by selectively interfering with fungal enzymes of the ergosterol synthesis pathway, resulting in an arrest of growth due to a change in the intracellular sterol composition (145). The most widely used azole antifungal in treating chytridiomycosis is itraconazole. Presenting a clear overview of the effects of itraconazole treatments is challenging, as variable treatment protocols (with ambiguous results) are used throughout studies (146). In general, most itraconazole treatment protocols are based on a study performed by Nichols *et al*. (147) describing successful treatment of chytridiomycosis by bathing *B. dendrobatidis* infected amphibians in a 0.01% solution (100 mg/L) of

itraconazole diluted in 0.6% saline, daily for 5 minutes during 11 days (147-151). Studies describing successful treatment of chytridiomycosis with minor adaptations to this protocol (other concentration, other diluting agent, longer exposure time or treatment period) also exist (150, 152-154). However, treatment failure and adverse side-effects due to itraconazole toxicity at this concentration (and even lower concentrations) are also reported for some amphibian species (155, 156). Only very recently, the minimum inhibitory concentration of itraconazole for *B. dendrobatidis* was described together with an evaluation of the effect of the frequency of exposure to itraconazole (157). Results of this study showed that variable outcome of itraconazole treatment in clearing *B. dendrobatidis* infections might be explained not only by the used concentration but also by the frequency of exposure to itraconazole. Other antifungals belonging to the azole group used to treat chytridiomycosis are miconazole, fluconazole and voriconazole. For miconazole and fluconazole only single studies exist describing their effect. In the study using miconazole, treatment comprised miconazole baths at a concentration of 100 µg/ml, once daily for 5 minutes during 8 days was effective in clearing *B. dendrobatidis* infections (147). Although fluconazole did show inhibitory activity for *B. dendrobatidis in vitro*, the highest concentration used (25 µg/ml) in a clinical treatment trial did not result in clearance of infection (158). Voriconazole has been shown to have potent *B. dendrobatidis* inhibitory effects both *in vitro* and *in vivo* (157, 159, 160), with successful clearance of *B. dendrobatidis* in experimentally and naturally infected amphibians using a treatment protocol composed of topically spraying voriconazole once daily during 7 days at a concentration of 1.25 µg/ml (160).

Terbinafine hydrochloride, an antifungal from the allylamines class which also exerts antifungal activity through interfering with the synthesis of ergosterol, has described effectiveness in inhibiting *B. dendrobatidis in vitro (157)* and *in vivo* (161). The *in vivo* treatment was however, not a controlled trial, and an inhibiting effect caused by ethanol which was the diluting agent cannot be ruled out.

Some antibacterial compounds are also able to inhibit growth of fungi. Placing amphibians, critically ill due to chytridiomycosis, in chloramphenicol baths (20 mg/L, continuous exposure, 14 days) resulted in recovery of all treated animals. Again, the number of treated animals was low, and furthermore aggressive supportive electrolyte therapy together with increased ambient temperature were instituted in conjunction with the chloramphenicol baths, which could have influenced the success of treatment (162). In another study, *in vitro* sensitivity of *B. dendrobatidis* for chloramphenicol was shown, but a clinical treatment trial with chloramphenicol at a concentration of 200 µg/ml failed to clear *B. dendrobatidis* from

infected amphibians after 28 days of continuous exposure (163) similar to florfenicol (10 µg/ml for 14 days) (159). Other antibiotics with shown *in vitro* efficacy are sulfamethoxazole and sulfadiazine alone and both in combination with trimethoprim (159). However, an empirical treatment with trimethoprim-sulfadiazine failed to clear *B. dendrobatidis* infections from infected amphibians (147).

Other chemical compounds that have been used for treating chytridiomycosis are a combination of formalin and malachite green (70, 164), copper sulphate (164) and benzalkonium chloride (158, 164). These treatments are not recommended, as it was shown that these compounds were unable to clear *B. dendrobatidis* from amphibians and are potentially toxic.

3.1.2. Treatment of chytridiomycosis with physical therapy

As discussed earlier, *B. dendrobatidis'* infection and disease dynamics are heavily dependent on environmental temperature (29, 121), and *B. dendrobatidis* cultures die at exposure to 30 °C for 8 days (58). Even in nature, individual amphibians show a decreased probability of infection when they spent more time above *B. dendrobatidis'* upper thermal maximum (165). This dependency on temperature has led to the development of treatment protocols consisting of raising the ambient temperature. Short exposure to relatively high ambient temperatures (37 °C less than 16 hours (166) and 30 °C, 10 day exposure (167)) and longer exposure to lower ambient temperatures (27 °C, clearance after 98 days (121)) have been able to clear *B. dendrobatidis* infections from adult amphibians. Exposure to 26 °C during 5 days was able to clear *B. dendrobatidis* infections in midwife toad (*Alytes obstetricans*) larvae (168). The main disadvantages linked to temperature treatment of *B. dendrobatidis* infections is that elevated temperature might not be endured by all amphibian species, and that thermal shock might occur (especially when taking into account that the treatment is applied on sick individuals) and furthermore that a *B. dendrobatidis* strain dependent thermal tolerance might exist. Further research to determine optimal exposure temperature and period will need to be instigated (156).

3.1.3. Treatment of chytridiomycosis with biotherapy

As mentioned earlier, the innate immune system defends amphibians against chytridiomycosis by secreting potent AMP's, and antifungal metabolites produced by symbiotic skin microbiota aid in this defence mechanism. Since variation in the production of AMP's between amphibian species exists (84), treatment of *B. dendrobatidis* infected amphibians with AMP's

could theoretically ameliorate the effects of chytridiomycosis. However, in a treatment trial, addition of AMP's from edible frogs (*Pelophylax esculentus)* which are considered to be chytridiomycosis-resistant, did not result in increased survival of common toads (*Bufo bufo*) infected with *B. dendrobatidis* (156). Addition of symbiotic bacteria which secrete potent antifungal metabolites with activity against *B. dendrobatidis* did not improve survival of *B. dendrobatidis* infected amphibians in one study (156), while positive effects in terms of less morbidity and mortality due to chytridiomycosis have been described (169, 170).

A recent study found that the antifungal activity of probiotic bacterial isolates used as biotherapy is strongly associated with environmental context and the immunological context of the amphibian host (111). For instance, a change in environmental temperature from 18 to 25 °C was shown to cause the antifungal activity of *Serratia plymuthica*, a bacterial symbiont of common midwife toad's (*A. obstetricans*) eggs and skin, to shift from inhibition of *B. dendrobatidis* growth to enhancing *B. dendrobatidis* growth.

Another study once again underlines that it is important to consider multiple cofactors and take into account community levels, as interactive effects between bacterial isolates from amphibian skin with antifungal activity occur (171). Co-culturing of bacterial species isolated from the skin of red-backed salamanders (*Plethodon cinereus*) with antifungal activity resulted in additive and synergistic effects in inhibiting *B. dendrobatidis*. Furthermore, co-culturing of bacterial isolates resulted in the production of potent emerging antifungal metabolites with greater inhibitory activity in comparison to the metabolites produced by monocultures of the isolates (171).

3.2 *In situ*

Mitigating the impact of chytridiomycosis in nature viewed from a conservational perspective has a different endpoint compared to treatment of captive amphibians. In nature, complete eradication of a pathogen (on a local scale) is virtually impossible due to pathogen reintroduction and presence of reservoir species. Furthermore, to prevent disease and associated declines, elimination of the pathogen is not always necessary (172). At the start of this thesis, mitigation strategies applicable in nature were very limited and most were speculative. One mitigation measure that has been trialed in nature is that of microbiome bioaugmentation. In short this encompassed adding a bacterium (*Janthinobacterium lividum*) that produces an antifungal metabolite (violacein) with anti-*B. dendrobatidis* activity to frogs susceptible to chytridiomycosis (173). Although short-term follow up indicated that untreated control frogs had relatively high infection loads, no long-term data of this study are available.

Propositions on how to select effective probiotic isolates that are able to inhibit *B. dendrobatidis* under ecologically relevant conditions are present (174), and together with recent discoveries that show the importance of the environmental, immunological and bacterial community context (111, 171), this might bring us closer to developing effective *in situ* mitigation measures. Other conceptual chytridiomycosis mitigating strategies exist and a brief overview of the thorough review by Woodhams *et al.* (172) will be given. First of all mitigation could theoretically be achieved by limiting pathogen transmission and infectious doses by reducing host density. This management tool has been applied (with variable success) in other infectious diseases and is primarily based on reducing the chances of the pathogen encountering susceptible hosts (172). Secondly, limiting prevalence of infection and reducing infection pressure can be achieved by treating individual hosts and/or habitats (or by modifying habitats). Examples of this strategy are treatment of animals translocated for *ex situ* conservation programs, application of agricultural fungicides in the environment and drainage of wetland systems. A strategy that has been trialed, but has resulted in variable success is that of assisted reintroduction of amphibian species. Frequently encountered problems linked to this mitigation approach (such as sustained presence of the pathogen in the environment) can theoretically be overcome, especially when other areas of chytridiomycosis mitigation (such as biotherapy of amphibians or habitat modification) are becoming more usable. Apart from assisting controlled reintroduction of amphibian species, habitat bioaugmentation and biocontrol using predators of *B. dendrobatidis* could prove successful mitigation approaches on their own. As stated before, recent advances in our understanding of the complex interplay of cofactors could bring us closer to establish effective *in situ* mitigation measures. Lastly, the promising recent discovery of a protective immune response occurring in amphibian species repeatedly exposed to live and dead *B. dendrobatidis* organisms might be translated in effective immunisation strategies of wild amphibian species at risk of developing chytridiomycosis (114). In conclusion, although currently no *in situ* chytridiomycosis mitigation measure has been trialed with success in nature, recent advances regarding chytridiomycosis mitigation are providing solid frameworks based on different mitigation approaches, that show promising potential.

4. References

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Scientific aims

Although northwestern European countries appear to have been spared until recently from disease driven amphibian declines, mysterious mass mortality events in Dutch fire salamander (*Salamandra salamandra*) populations have been suggestive for the emergence of infectious diseases like chytridiomycosis (Spitzen-van der Sluijs *et al*., 2013). The only known chytrid fungus parasitizing amphibians, *Batrachochytrium dendrobatidis*, however, fails to explain these events. Its impact in northwestern European countries appears to be limited to erratic mortality in the absence of population declines. The emergence of novel, highly pathogenic chytrid strains, however, could alter our current view on chytrid epidemiology.

The general aim of this PhD thesis was to broaden our insights into the contribution of chytridiomycosis to amphibian declines in northwestern Europe and to develop diagnostic and treatment protocols to mitigate the impact of the disease *in situ* and *ex situ*.

Specific aims were:

I. to identify and characterise the agent causing fire salamander population declines in the Netherlands
II. to predict the threat of this agent to amphibian diversity
III. to expand diagnostics to include the novel agent
IV. to design physical and chemical treatments for chytridiomycosis *ex situ*
V. to explore reducing the environmental chytrid infection pressure using aquatic micropredators as an option for *in situ* mitigation of the impact of chytridiomycosis on amphibian populations

The first specific aim is adressed in the first study using molecular diagnostics, histopathology, microscopy, phylogeny and challenge experiments in fire salamanders and midwife toads. The second study adressess whether the identified novel agent poses a threat to amphibian diversity in general based on assessment of its pathogenicity for different amphibian taxa and the novel agent's current range based on global presence screening. Furthermore, a hypothesis on the possible origin of the agent is presented. The third aim is adressed in the third study, describing the development of a molecular diagnostic tool that incorporates the detection of the novel agent next to the classical cause of chytridiomycosis, *B. dendrobatidis*, based on highly conserved DNA regions. The fourth and fifth study describe the development of a physical and chemical *ex situ* treatment respectively, able to clear infections with the novel agent from fire salamanders. The *B. dendrobatidis* viability assay described in the sixth study is applied together with *B. dendrobatidis* challenge experiments in the seventh study to adress the last specific aim, showing that aquatic microzooplankton steer local *B. dendrobatidis* infection and disease dynamics in nature.

Experimental studies

Batrachochytrium salamandrivorans sp. nov. causes lethal chytridiomycosis in amphibians

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Abstract

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*. 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., 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 *B. dendrobatidis*, this taxon forms a well-supported chytridiomycete clade, adapted to vertebrate hosts and highly pathogenic to amphibians. However, the lower thermal growth preference of *B. salamandrivorans*, compared with *B. dendrobatidis*, and resistance of midwife toads (*Alytes obstetricans*) to experimental infection *with B. salamandrivorans* suggest differential niche occupation of the two chytrid fungi.

Introduction

Amphibians have become an icon of the global biodiversity crisis (1). 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 (2, 3). Chytridiomycosis is currently considered to be caused by a single species of fungus, *Batrachochytrium dendrobatidis*, which is the only chytridiomycete taxon known to parasitize vertebrate hosts. However, *B. dendrobatidis* and other factors known to cause amphibian decline fail to explain several recent amphibian population losses (4, 5).

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 (5). 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 (5). The recent start-up 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 *B. dendrobatidis*, in these salamanders yielded negative results (5). Instead, we found, isolated, and characterized a second, highly pathogenic chytrid fungus from this decline event that occupies a different niche compared with *B. dendrobatidis*.

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 *B. dendrobatidis* (Fig. 1; Table S1). Its considerable genetic distance from *B. dendrobatidis* (3.47–4.47% for the 1,513 18S + 28S rRNA base pairs) compared with the shallow divergences between *B. dendrobatidis* isolates (6) warrants the description of a unique species within the chytridiomycote order Rhizophydiales (family *incertae sedis*): *Batrachochytrium salamandrivorans* spec. nov. 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 (Fig. 2A). The most obvious morphological differences, compared with the *B. dendrobatidis* type strain, are the formation of germ tubes in vitro (Fig. 2B; Fig. S1) and the abundant formation of colonial thalli both in vitro and in vivo (Fig. 3B). *B. salamandrivorans* 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 *B. dendrobatidis* (7) (Fig. 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 (8) (Fig. 3A). Transmission electron microscopic examination of the skin lesions confirmed the presence of intracellular structures consistent with the colonial thalli (Fig. 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 (9) nor quantitative PCR (qPCR) (10) suggested the presence of chytrid *B. dendrobatidis* DNA in the skin samples. Virological examination [including PCR for the detection of herpes viruses (11), adenoviruses (12), and ranaviruses (13) 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 (14), and bacterial isolation attempts did not yield any evidence of bacterial infections.

To further demonstrate that salamandrid mortality was caused by *B. salamandrivorans*, we performed infection experiments on healthy fire salamanders (n = 5) by exposing them to 5,000 zoospores of *B. salamandrivorans* 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 *B. salamandrivorans* 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 *B. salamandrivorans*, which matched the lesions found in wild animals. *B. salamandrivorans*– induced lesions are characterized by marked skin ulceration, opposed to those caused by *B. dendrobatidis*, which typically induces epidermal hyperplasia and

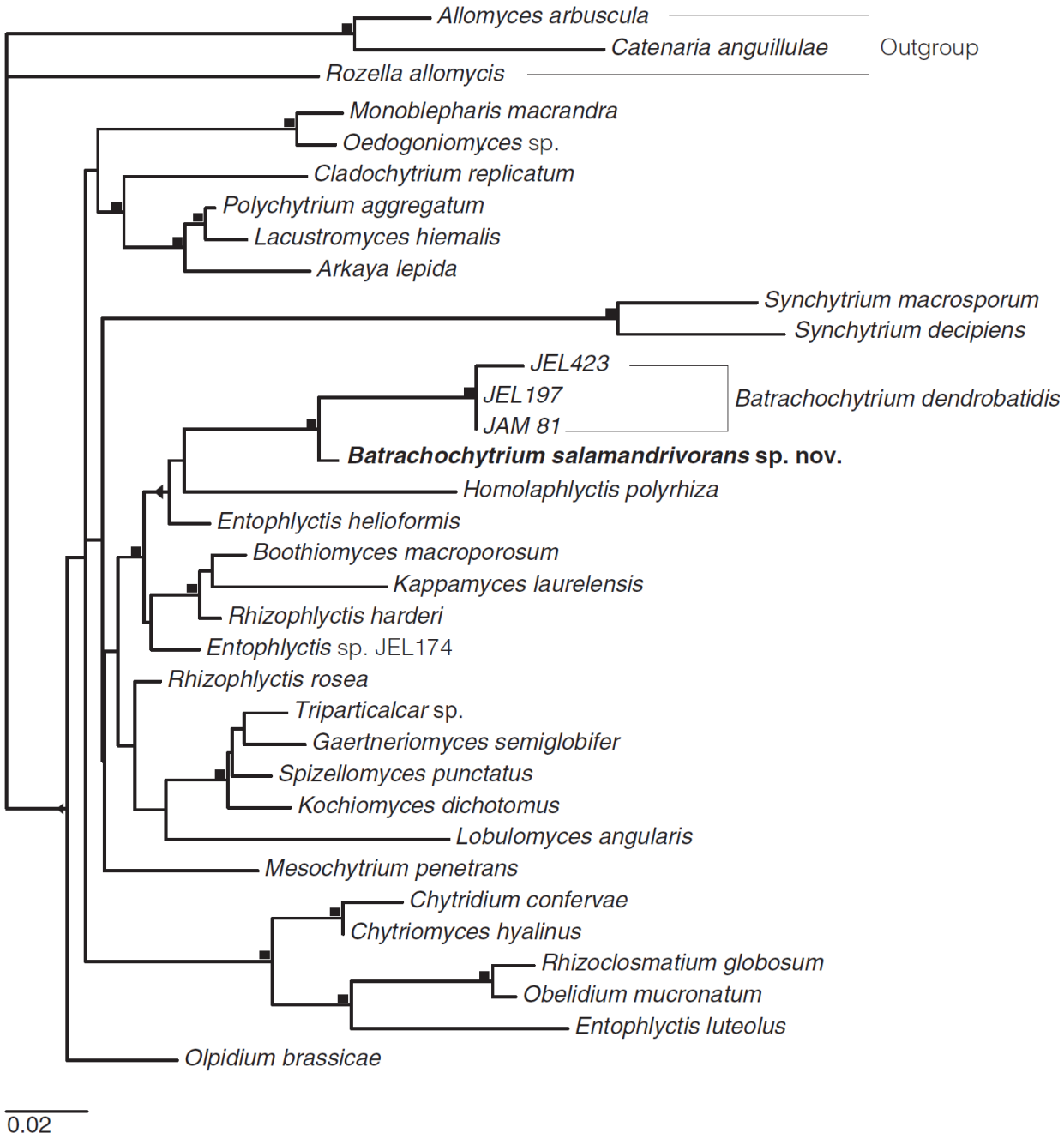

Fig 1. Maximum likelihood tree (−Ln *L* = 9,562.04266) for the analysis of a 1,513-bp data matrix of partial 18S + 28S rRNA genes. Together with *Batrachochytrium dendrobatidis*, *Batrachochytrium salamandrivorans* sp. nov. forms a well-supported clade [maximum parsimony bootstrap support = 100; maximum 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.

hyperkeratosis (15). 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 (8), and PCR demonstrated the presence of high numbers of *B. salamandrivorans* in their epidermal layers, with lesions identical to those described above. Cohousing on damp toweling effectively transmitted *B. salamandrivorans* 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 (16, 17), did not show any signs of colonization by *B. salamandrivorans*, 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 *B. salamandrivorans* 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 *B. salamandrivorans* in all positive tissues examined. Importantly, these primers did not amplify any of the nine tested strains from all three *B. dendrobatidis* lineages known to infect Europe and therefore provide a rapid noninvasive method for detecting of *B. salamandrivorans* infections. Furthermore, by using the newly developed PCR primers, we were also able to detect *B. salamandrivorans* 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. *B. salamandrivorans* 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 *B. salamandrivorans*–induced chytridiomycosis.

Chytridiomycosis in amphibians can no longer be attributed to a single species of chytrid, but can be caused by either *B. dendrobatidis* for *B. salamandrivorans*. Our results reveal striking similarities and differences between *B. salamandrivorans* and the behaviour of the hypervirulent global pandemic lineage of *B. dendrobatidis* (18). Both fungal species share

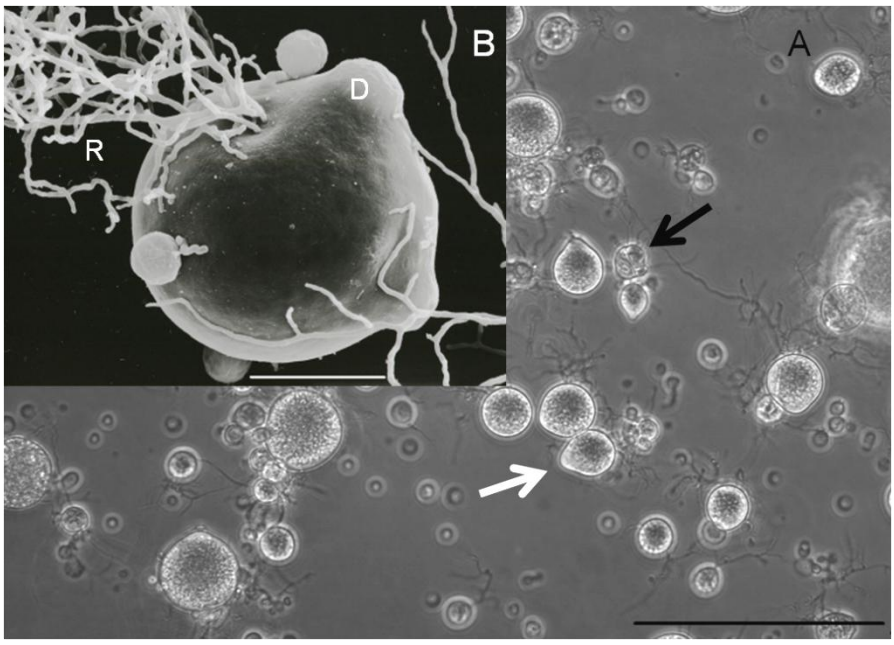

Fig 2. In vitro culture of *Batrachochytrium salamandrivorans* in TGhL broth at 15 °C. (A) Monocentric thalli predominate, with the rare presence of colonial thalli (black arrow). Sporangia develop discharge tubes (white arrow) to release zoospores (Scale bar, 100 μm.) (B) Scanning electron microscopic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

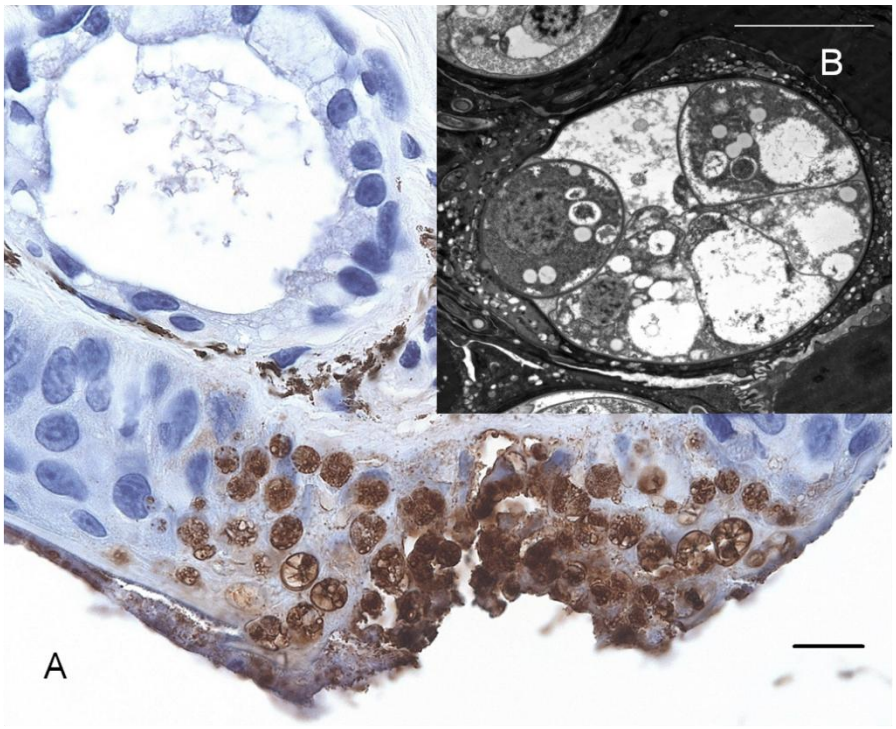

Fig 3. Microscopy of the skin of a fire salamander that died due to infection with *Batrachochytrium salamandrivorans*. (A) Immunohistochemical staining of a 5-μm skin section. Intracellular colonial thalli abound throughout all epidermal cell layers and are associated with erosive lesions. (Scale bar,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

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 *B. salamandrivorans* is capable of infecting a broad amphibian host range, as is the case for *B. dendrobatidis* (3). However, development of erosive vs. hyperplastic/hyperkeratotic skin lesions, failure to experimentally infect midwife toads, and relatively low thermal preferences of *B. salamandrivorans* suggest differential host specificity of the two pathogens and possibly a differential effect on amphibian assemblages. Because the majority of recent *B. dendrobatidis* surveillance worldwide is based on the *B. dendrobatidis*–specific qPCR (10), it is currently impossible to estimate the extent and impact of *B. salamandrivorans* on amphibian populations worldwide using the *B. dendrobatidis* mapping framework (19). However, the emergence of the pathogenic *B. salamandrivorans* chytrid fungus is worrying and warrants close monitoring, urgent risk analysis, and its inclusion in any monitoring program assessing amphibian population health.

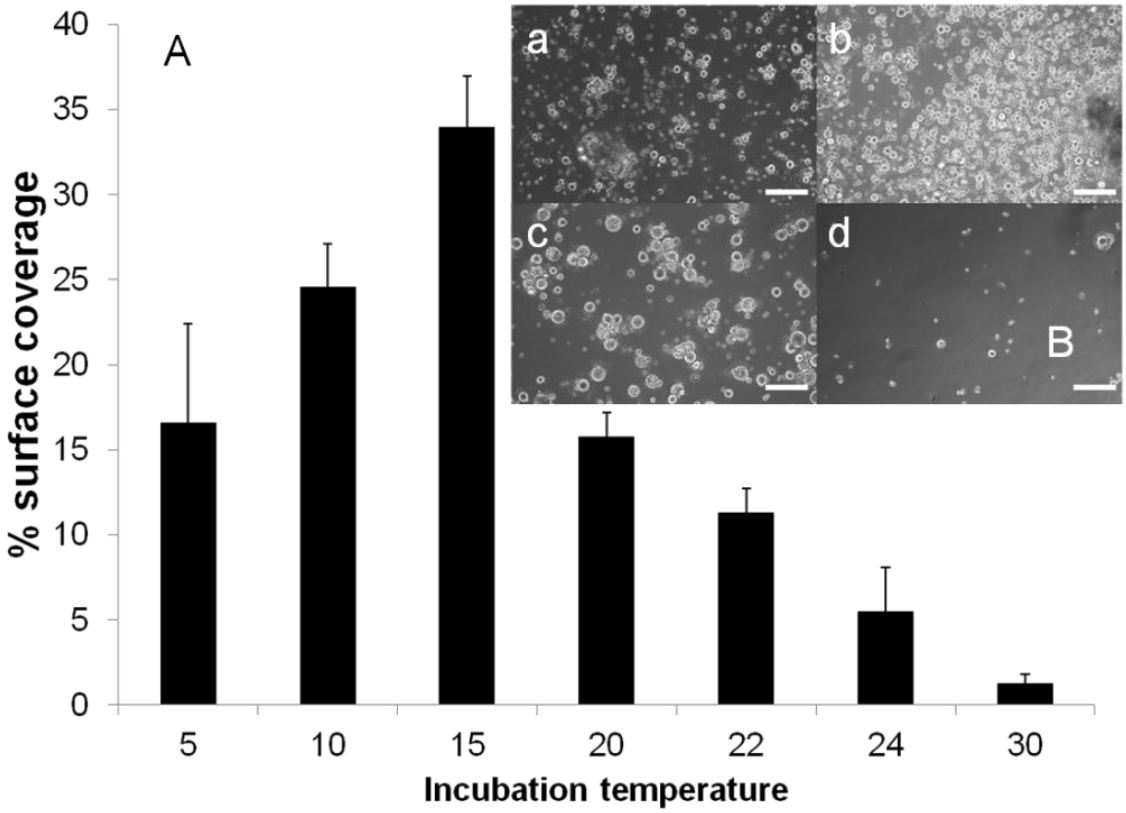

Fig 4. Growth of *Batrachochytrium salamandrivorans* in TGhL broth at different temperatures. (A) Growth was quantified by calculating the average percentage ± SD of the surface area of three wells covered by the fungus after 10 d of incubation at a given temperature. Motile zoospores were present at 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.)

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.

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 (11), adenoviruses (12), iridoviruses (13), Chlamydiales (14), and *B. dendrobatidis* (9, 10). Immunohistochemistry was performed on all skin samples to detect *B. dendrobatidis* antigens (8). Transmission electron microscopy of epidermal samples was performed with glutaraldehyde fixation in 0.05 M sodium cacodylate buffer, 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 *B. dendrobatidis*.

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 *B. dendrobatidis* (7). 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 (20). 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 *B. salamandrivorans* 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 *B. salamandrivorans*) 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.

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 (21). 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 *B. salamandrivorans* 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 mMof 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 (22) and transmission electron microscopy (23). Zoospores were collected from growth on TGhL agar plates and fixed for transmission electron microscopy with s-collidine buffer followed by osmium tetroxide (23).

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 *B. dendrobatidis* as assessed by sampling the skin using cotton-tipped swabs and subsequent performing qPCR (10). Using the PCR described above, all swab samples were negative for the presence of DNA of *B. salamandrivorans*. 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 *B. salamandrivorans* 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) (8). Samples were examined for the presence of DNA of *B. dendrobatidis* using qPCR and for the presence of DNA of *B. salamandrivorans* 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 (24), 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 (25). 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 (*B. dendrobatidis*, *B. salamandrivorans*) 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 (26). Two runs of fourMarkov 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.

Acknowledgments

The technical assistance of M. Claeys, M. Couvreur, and C. Adriaensen is appreciated. We thank Dr. J. Z. Euzeby for his kindness in helping with the Latin for the species name. We thank the editor and two anonymous reviewers for their constructive comments, which improved the manuscript. I. Van Bocxlaer assisted with phylogenetic analyses. M.B. was supported by a Dehousse grant provided by the Royal Zoological Society of Antwerp. F.B. was supported by European Research Council Starting Grant 204509 [project Tracing Antimicrobial Peptides and Pheromones in the Amphibian Skin (TAPAS)]. M.C.F. was supported by the Biodiversa project Risk Assessment of Chytridiomycosis to European amphibian Biodiversity (RACE).

Author contribution statement

Author contributions: A.M. and F.P. designed research; A.M., M.B., and F.P. performed research; A.M., A.S.-v.d.S., W.B., R.D., M.C.F., A.W., W.B., K.C., and F.P. contributed new reagents/analytic tools; A.M., F.B., and F.P. analyzed data; A.M., M.C.F., F.B., and F.P. wrote the paper; A.S.-v.d.S., A.W., and W.B. contributed field data; R.D. and K.C. performed histopathology; M.C.F. delivered DNA and genetic data; F.B. performed phylogenetic analysis; and A.M. and F.P. discovered the fungus.

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Supplementary materials

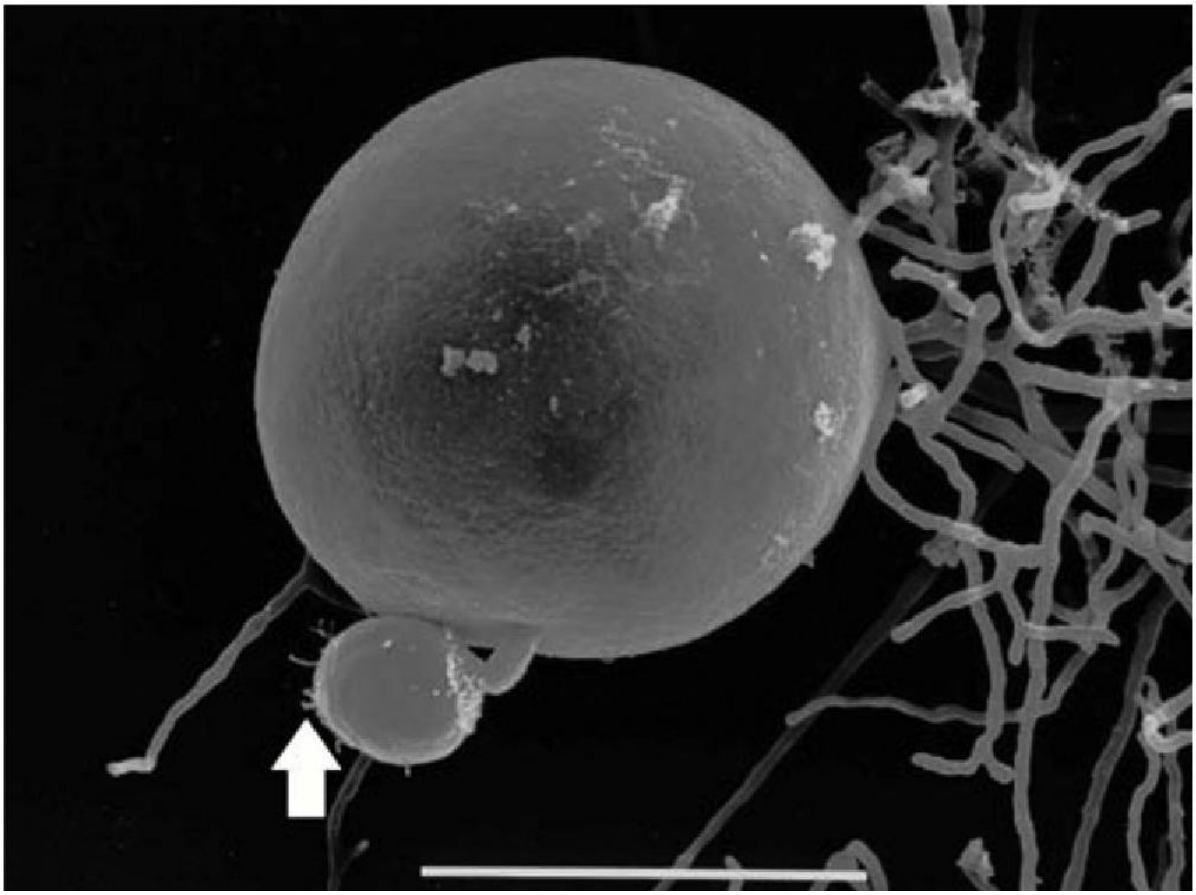

Fig S1. Scanning electron microscopic image of a *Batrachochytrium salamandrivorans* sporangium showing rhizoid formation on a germ tube–derived developing sporangium (arrow). (Scale bar, 10 µm.)

		GenBank accession no.	
Taxon	Strain	18S	28S
Arkaya lepida	JEL93	AF164278	DQ273814
Batrachochytrium dendrobatidis	JEL423	D2022300	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
Entophlytcis helioformis	JEL326	AY635826	DQ273784
Entophlyctis luteolus	JEL129	AH009064	AY442957
Gaertneriomyces semiglobifer	UCB-91-10	AF164247	DQ273778
Homolaphlyctis polyrhiza	JEL142	AF164299	EF634247
Kappamyces lauremensis	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	AY6091711	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

Table S1. Taxon sampling for phylogenetic analysis of 30 isolates

Recent introduction of a chytrid fungus endangers Western Palearctic salamanders

A. Martel[1], M. Blooi[1,2†], C. Adriaensen[1†], P. Van Rooij[1†], W. Beukema[3], M. C. Fisher[4], R. A. Farrer[5], B. R. Schmidt[6,7], U. Tobler[6,7], K. Goka[8], K. R. Lips[9], C. Muletz[9], K. R. Zamudio[10], J. Bosch[11], S. Lötters[12], E.Wombwell[13,14] , T.W. J. Garner[14], A. A. Cunningham[14], A. Spitzen-van der Sluijs[15], S. Salvidio[16], R. Ducatelle[1], K. Nishikawa[17], T. T. Nguyen[18], J. E. Kolby[19], I. Van Bocxlaer[20], F. Bossuyt[20], F. Pasmans[1*]

[*] For author affiliations, see supplementary materials

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Abstract

Emerging infectious diseases are reducing biodiversity on a global scale. Recently, the emergence of the chytrid fungus *Batrachochytrium salamandrivorans* resulted in rapid declines in populations of European fire salamanders. Here, we screened more than 5000 amphibians from across four continents and combined experimental assessment of pathogenicity with phylogenetic methods to estimate the threat that this infection poses to amphibian diversity. Results show that *B. salamandrivorans* is restricted to, but highly pathogenic for, salamanders and newts (Urodela). The pathogen likely originated and remained in coexistence with a clade of salamander hosts for millions of years in Asia. As a result of globalization and lack of biosecurity, it has recently been introduced into naïve European amphibian populations, where it is currently causing biodiversity loss.

Emerging infectious diseases play an important role in the ongoing sixth mass extinction (1). Fungi comprise a greater threat relative to other taxonomic classes of pathogens and have recently caused some of the most severe die-offs and extinctions among a wide range of organisms (2). The classical cause of amphibian chytridiomycosis (*Batrachochytrium dendrobatidis*) has resulted in extensive disease and declines in a wide variety of amphibian species across the three orders [i.e., frogs and toads (Anura), salamanders and newts (Urodela), and caecilians (Gymnophiona)] (2). Recently, a second highly pathogenic chytrid fungus (*B. salamandrivorans*) emerged as a novel form of amphibian chytridiomycosis and extirpated fire salamander populations in northern Europe (3, 4) in a region where *B. dendrobatidis* is in a state of stable coexistence with the amphibian communities (5).

To predict the potential impact of *B. salamandrivorans* on amphibian diversity more broadly, we first estimated its host range by experimentally exposing 35 species from the three amphibian orders (10 anurans, 24 urodelans, and 1 caecilian) to controlled doses of 5000 zoospores for 24 hours (3) (table S1). Except for five urodelan taxa for which wild-caught specimens were used, all other experimental animals were captive bred. With the exception of four urodelan taxa, all experimental animals derived from a single source population. After exposure, animals were monitored daily for clinical signs until at least 4 weeks after exposure. Infection loads were assessed weekly using quantitative polymerase chain reaction (qPCR) on skin swabs (6), and histopathology was performed on all specimens that died. Our results show that colonization by *B. salamandrivorans* was limited to Urodela, whereas none of the anuran and caecilian species became infected (Fig. 1, squares). Alarmingly, 41 out of 44 of the Western Palearctic salamanders (Salamandridae and Plethodontidae) rapidly died after infection with *B. salamandrivorans*. The propensity of *B. salamandrivorans* to infect these species was confirmed by its ability to successfully invade the skin of several urodelan, but none of the anuran, species. This was demonstrated with an immunohistochemical staining of the abdominal skin of amphibians after exposure to 10,000 zoospores for 24 hours (table S1 and fig. S1).

To estimate the current range of *B. salamandrivorans* infections, we used qPCR to screen 5391 wild amphibian individuals from four continents for the presence of *B. salamandrivorans* DNA in their skin (6) (tables S2 and S3). In accordance with the results of the experimentally determined host range, infections were detected only in urodeles. Furthermore, the detection of *B. salamandrivorans* DNA (all sequences were 100% identical with GenBank accession number KC762295) was limited to East Asia (Thailand, Vietnam, and Japan) in the absence of obvious disease, and Europe (Netherlands and Belgium) where it

is associated with severe disease outbreaks [Netherlands, 2010 (3, 4), and Belgium, 2013 (Eupen, N50°37′23″; E6°05′19″) and 2014 (Robertville, N50°27′12″; E6°06′11″)]. These findings suggest long-term endemism in Asia and a recent incursion in Europe.

We used the results of our infection experiments as a proxy for classifying amphibians into four categories of response to *B. salamandrivorans*: resistant, tolerant, susceptible, and lethal (Fig. 1, squares). Although the limited number of source populations used does not allow estimation of within-species variation, responses to infection were highly consistent within a given population. Lethal responses were observed in specimens from both captive-bred (10 of 19 taxa) and wild (2 of 5 taxa) urodelans. Our infection experiments indicated three Asian salamanders (*Cynops pyrrhogaster*, *Cynops cyanurus*, and *Paramesotriton deloustali*) as potential reservoirs. Seven specimens of these species were capable of limiting clinical disease and either persisted with infection for up to at least 5 months with recurring episodes of clinical disease, or even totally cleared the infection (table S1 and fig. S2). The combined evidence of natural occurrence and experimental maintenance of *B. salamandrivorans* infections indicates that at least these three species may function as a reservoir in Asia.

To investigate whether these amphibian communities may have constituted a reservoir of infection in the past, we estimated when *B. salamandrivorans* diverged from *B. dendrobatidis* and used present-day patterns of susceptibility to reconstruct amphibian susceptibility through time. Our Bayesian estimates of divergence time with a broad prior calibration range resulted in a mean estimate of 67.3 million years ago (Ma) (fig. S3) and a 95% highest posterior density interval of 115.3 to 30.3 Ma, indicating that *B. salamandrivorans* diverged from *B. dendrobatidis* in the Late Cretaceous or early Paleogene (Fig. 1, gray bar). Maximum parsimony and maximum likelihood ancestral reconstructions (Fig. 1) of amphibian susceptibility suggest that the potential of being a reservoir evolved in the ancestors of modern Asian newts between 55 and 34 Ma in the Paleogene (Fig. 1, orange branch), shortly after the origin of their pathogen. These ancestors reached Asia after withdrawal of the Turgai Sea (7), suggesting that Asia has been a natural reservoir for *B. salamandrivorans* for the past 30 million years. Our detection of *B. salamandrivorans* in a >150-year-oldmuseum sample of the Asian newt *Cynops ensicauda* (table S4, RMNH RENA 47344) is consistent with this reservoir hypothesis.

Given the discontinuity of the global distribution of *B. salamandrivorans*, introduction from Asia into Europe must have been human-mediated. Asian salamanders and newts are being traded internationally in large numbers annually (for instance, more than 2.3 million

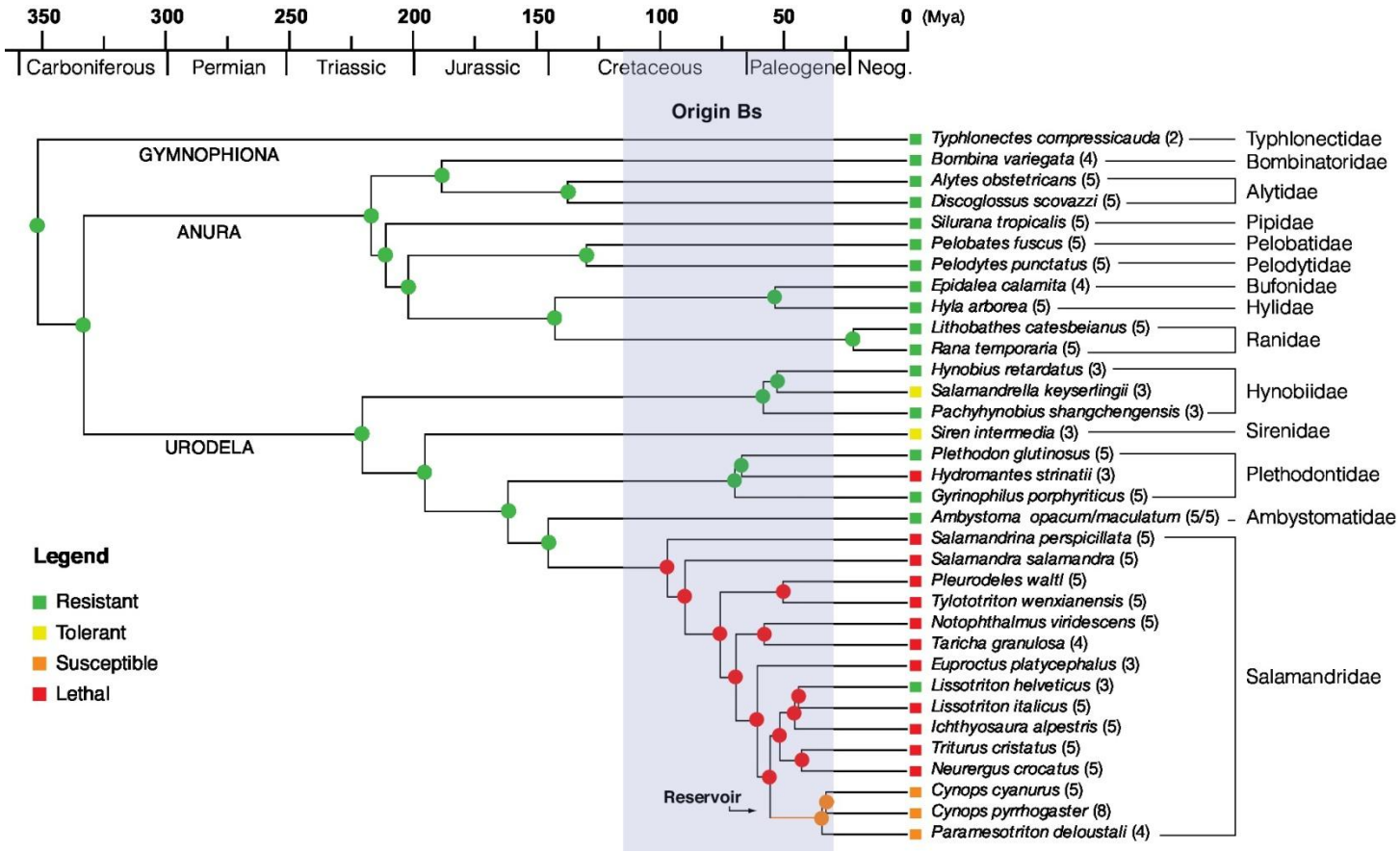

Fig 1. Amphibian susceptibility to *Batrachochytrium salamandrivorans* through time. Molecular time scale (millions of years ago) for 34 species; rectangles indicate the species category based on the experimental infection tests. Resistant: no infection, no disease; tolerant: infection in the absence of disease; susceptible: infection resulting in clinical disease with possibility of subsequent recovery; lethal: infection resulting in lethal disease in all infected animals. Colored dots on nodes indicate the results of the maximum likelihood ancestral reconstructions (P > 0.95).The clade of susceptible Asian salamanders that originated in the early Paleogene is indicated in orange. The 95% highest posterior density for time of divergence between *Batrachochytrium salamandrivorans* and *Batrachochytrium dendrobatidis* is indicated in gray.

individuals of *Cynops orientalis* were imported into the United States from 2001 to 2009) (8). To assess the potential of *B. salamandrivorans* spread by captive amphibians, we tested 1765 skin samples from amphibians in pet shops in Europe, London Heathrow Airport, and an exporter in Hong Kong (tables S5 and S6) and 570 samples from other captive amphibians (tables S7 and S8) for *B. salamandrivorans*. We found three positive samples from captive individuals of the Asian newt species *Tylototriton vietnamensis*, two of which were imported to Europe in 2010. Furthermore, our transmission experiments showed that *B. salamandrivorans* can effectively be transmitted across multiple urodelan species (e.g., from *Cynops pyrrhogaster* to *Salamandra salamandra*, fig. S4) by direct contact, demonstrating the potential for pathogen spillover.

Our infection experiments show that *B. salamandrivorans* is lethal to at least some of the New World salamandrid species (genera *Taricha* and *Notophthalmus*). Although these combined genera contain only seven species, together they have a widespread distribution and are often very abundant. The outcome of exposure of three lineages of plethodontids (a family comprising 66% of global urodelan diversity) to *B. salamandrivorans* ranged from a lack of any detectable infection (*Gyrinophilus*), to transient skin invasion (*Plethodon*) and lethal infection (*Hydromantes*), making it likely that other species in this large family are vulnerable.

Our study demonstrates that the process of globalization with its associated human and animal traffic can rapidly erode ancient barriers to pathogen transmission, allowing the infection of hosts that have not had the opportunity to establish resistance. Thus, pathogens, such as those we describe here, have the potential to rapidly pose a threat of extinction.

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Author contributions statement

A.M. and F.P. designed the research. A.M., M.B., C.A., P.V.R., W.B., I.V.B. and F.B. carried out the research. A.M., W.B., R.A.F., B.R.S., R.D., I.V.B., F.B., F.P. analysed the data. M.C.F., B.R.S., U.T., K.G., K.R.L., C.M., K.Z., J.B., S.L., E.W., T.W.J.G., A.S., S.S., K.N., T.T.N. provided samples. A.M., I.V.B., F.B. and F.P. wrote the paper with input from all other authors.

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Supplementary materials

Materials and methods

Animal infections

The infection experiments, approved by the ethical committee of the Faculty of Veterinary Medicine (Ghent University), were performed as described before (3). In total, 48 anurans, belonging to 10 species, 112 urodelans, belonging to 24 species and a single caecilian were used (Table S1). All animals were healthy and negative for *B. salamandrivorans*, *B. dendrobatidis* and Ranavirus. After infection, monitoring for clinical signs was performed daily and the microbiological status was assessed by weekly swabbing and qPCR analysis (6). Positive animals were kept until they died or returned negative. Negative animals were euthanized after three negative qPCR results obtained with one week intervals. Chytridiomycosis was diagnosed using histological examination on haematoxylin eosin staining of transverse sections through the body: behind the forelegs, before the hind legs and in the middle of the tail (only for urodela). Since most specimens were obtained from single source populations, in order to avoid lineage specific biases, conclusions were drawn on higher taxonomic levels rather than at the species level.

To determine *B. salamandrivorans*' invasive ability amphibians belonging to 7 anuran and 10 urodelan species (one animal per species) (Table S1) were exposed to a high amount (10,000) of zoospores for 24 hours and euthanized immediately after exposure. A piece of abdominal skin was taken and stained immunohistochemically to detect skin invasion (9). The experiment was performed in duplicate.

To determine interspecies *B. salamandrivorans* transmission between susceptible European urodelan species, six infected *Salamandra salamandra* (mean log(10) GE load per swab: 1.74 +/-0.12) were co-housed (1:1) with four uninfected *Ichthyosaura alpestris* or two uninfected *Pleurodeles waltl*. To determine transmission from a presumed reservoir species to a susceptible European species, three infected *Cynops pyrrhogaster* (mean log(10) GE load per swab: 1.68 +/-0.14) were co-housed (1:1) with three uninfected *Salamandra salamandra*. Co-housing lasted 8 hours at 5°C (two *Ichthyosaura alpestris*) and 15°C (two *Ichthyosaura alpestris*, two *Pleurodeles waltl* and three *Salamandra salamandra*). After co-housing, monitoring for clinical signs was performed

daily during 10 days and the microbiological status was assessed by weekly swabbing and qPCR analysis (6).

Screening

Toe clips from museum specimens (Supplementary Table S4) were taken from specimens deposited at in the Museum for Natural History - Naturalis, Leiden, The Netherlands. DNA was extracted using the QIAamp FFPE Tissue kit (Qiagen). Previously collected samples from amphibian assemblages, trade (pet shops in Europe, exporter in Hong Kong and Heathrow Airport) and captive kept amphibians (10-26) (Supplementary Table S2, S5, S7) were tested for the presence of *B. salamandrivorans* using qPCR (6).

Bayesian divergence time estimates for the origin of *B. salamandrivorans*

We combined protein sequences from three nuclear genes, RPB1, RPB2 and EF1a for a representative set of 12 ingroup fungal species and one fungal outgroup (*Rozella allomycis*). Alignment of the protein sequences was done with MAFFT (27) using the L-INS-i method and resulted in a data matrix of 1973 reliably aligned amino acids. Maximum Likelihood (ML) analyses were run in PAUP* (28), using a LG amino-acid rate matrix with empirical frequencies, estimated proportion of invariable sites (0.235325) and distribution of rates at variable sites following a gamma distribution with four categories and estimated shape parameter 0.69288. This resulted in a single ML tree with likelihood score -Ln L = 20836.93. Support was calculated as Bayesian posterior probabilities in MrBayes (29). To estimate the age of the divergence of *B. dendrobatidis* from *B. salamandrivorans*, we used a Bayesian relaxed molecular clock model implemented in Beast v1.7.5 (30). As a calibration point, we used the divergence of *Hyaloraphidium curvatum* from its sister clade, a relationship that is strongly supported by the literature (31) and in our ML tree. We implemented a broad range for this calibration based on Berney et al. (32), who estimated this divergence at 651.2 million years ago, with an upper bound of 860.0 and a lower bound of 477.4 for the 95% confidence interval. We used this information to set the prior distribution for our calibration point to a normal distribution with the same mean of 651.2 mya, and a standard deviation of 106.6, corresponding to a 95% highest posterior density (HPD) ranging from 860.1 till 442.3 mya. These settings for our calibration thus completely

encompass the broad range of Berney et al. (32). The MCMC chain was run for 10 million generations and trees were sampled every 1,000 generations. Convergence of parameters and a burn-in of 2,000 were determined with Tracer v1.3. The median and 95% HPD for fungal time estimates (Fig. S3) were therefore calculated from 8,000 sampled trees.

Amphibian timetree construction

The amphibian timescale was constructed using all but one species (i.e., 34 operational taxonomic units) that had been experimentally tested for their susceptibility against *B. salamandrivorans*. One *Ambystoma* species was excluded from the analyses because a divergence estimate between *Ambystoma opacum* and *Ambystoma maculatum* was not available. This however does not influence any of the results obtained. Dating estimates for amphibian diversification were based on previous work (7, 31, 33-37).

Ancestral reconstruction of amphibian susceptibility

We used the results of our infection experiments as a proxy to reconstruct the evolution of susceptibility against *B. salamandrivorans* in the three amphibian orders.

The results of the experimentally infected amphibians were classified into four categories:

1. Resistant (no infection, no disease), 2. Tolerant (infection in the absence of disease), 3. Susceptible (infection resulting in clinical disease with subsequent recovery) or 4. Lethal. The classification per species is listed in Table S9.

Ancestral state reconstructions were performed using Maximum Parsimony (MP) and Maximum Likelihood (ML) approaches on the timetree (see amphibian timetree construction) with Mesquite v2.6 (38). MP reconstructions where done with characters unordered and equally weighted. ML reconstructions where done under a single rate Mk likelihood model (mk1) for discrete characters (38), and the likelihood decision threshold was set to 2.0 (default). The results are listed in Table S10. Corresponding node numbers can be found in Fig. S5. Both analyses gave similar results.

Supplementary figures

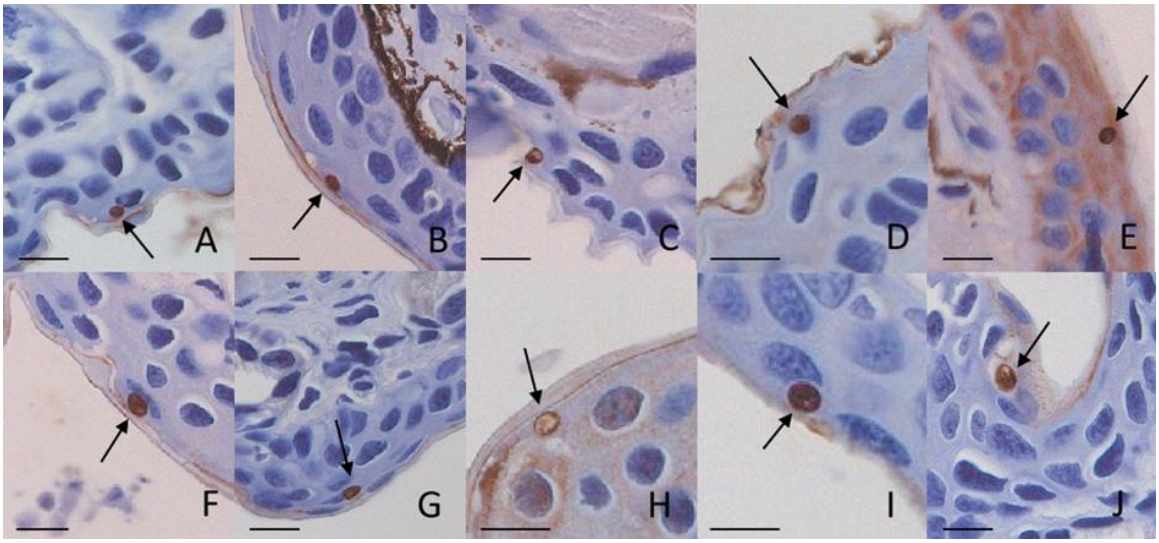

Fig S1. Invasion of *Batrachochytrium salamandrivorans* in the urodelan abdominal skin after 24 hours exposure. Immunohistochemical staining. Arrows point at *Batrachochytrium salamandrivorans* organisms. A. *Ichthyosaura alpestris*, B. *Triturus cristatus*, C. *Lissotriton helveticus*, D. *Notophthalmus viridescens*, E. *Pleurodeles waltl*, F. *Neurergus crocatus*, G. *Euproctus platycephalus*, H. *Salamandrella keyserlingii*, I. *Plethodon glutinosus*, J. *Salamandra salamandra*. Scale bar = 10 µm.

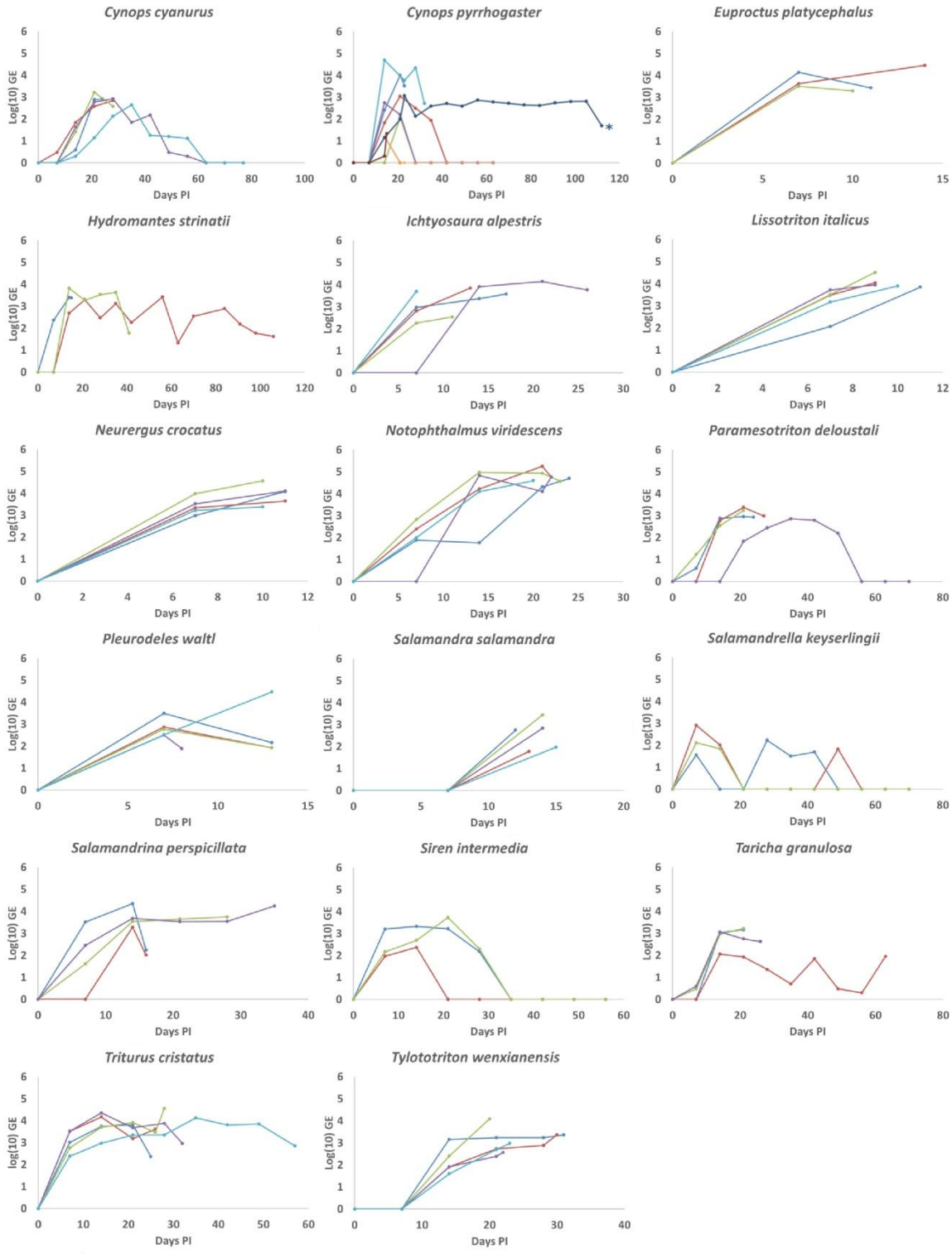

Fig S2. *Batrachochytrium salamandrivorans* infection course in infected amphibian species. Log (10) genomic equivalent (GE) values expressed per swab.

*** End of experiment with animal showing no clinical anomalies**

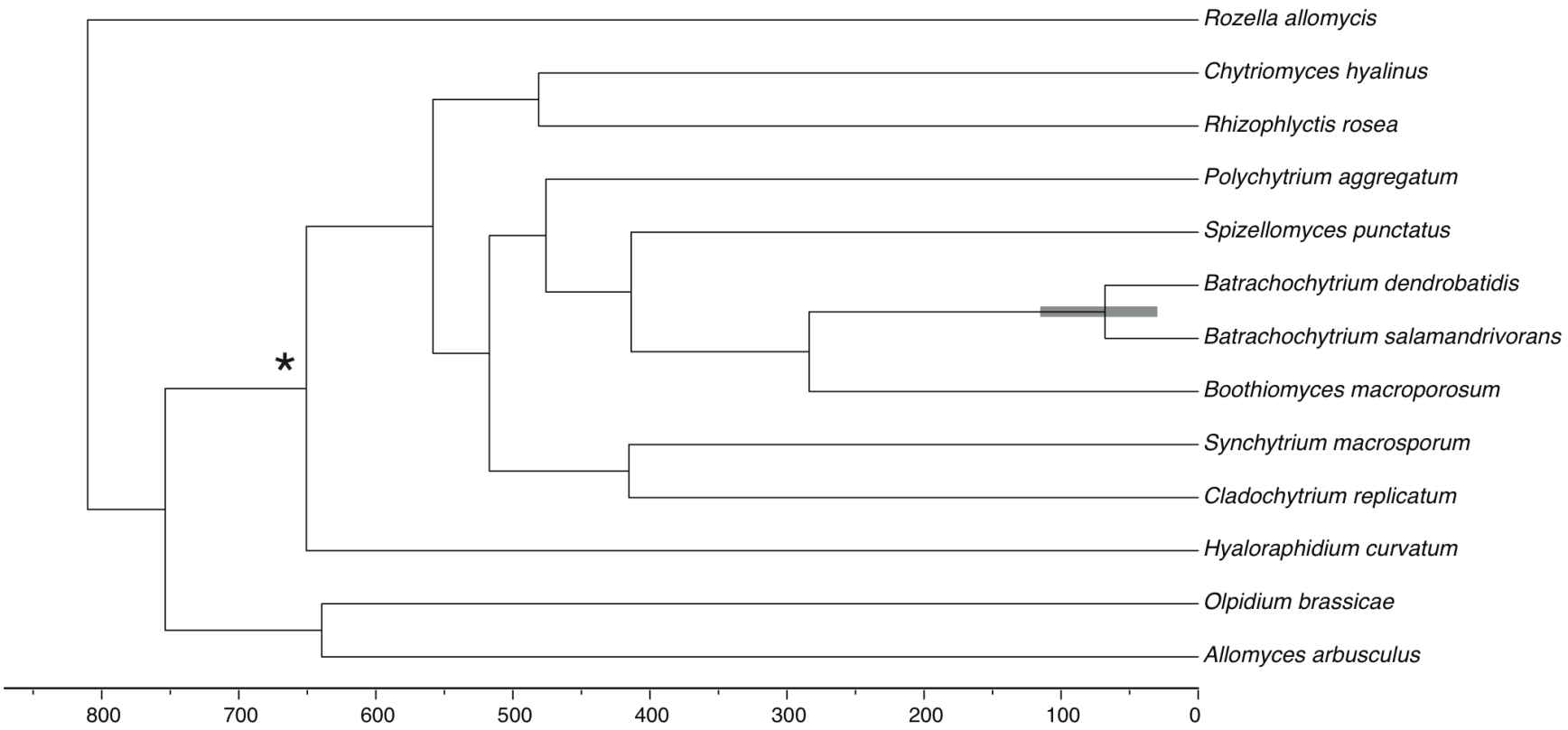

Fig S3. Divergence time estimates for fungi. The asterisk denotes the calibration point, the grey bar indicates the 95% HPD for the divergence of *Batrachochytrium salamandrivorans* from its sister species.

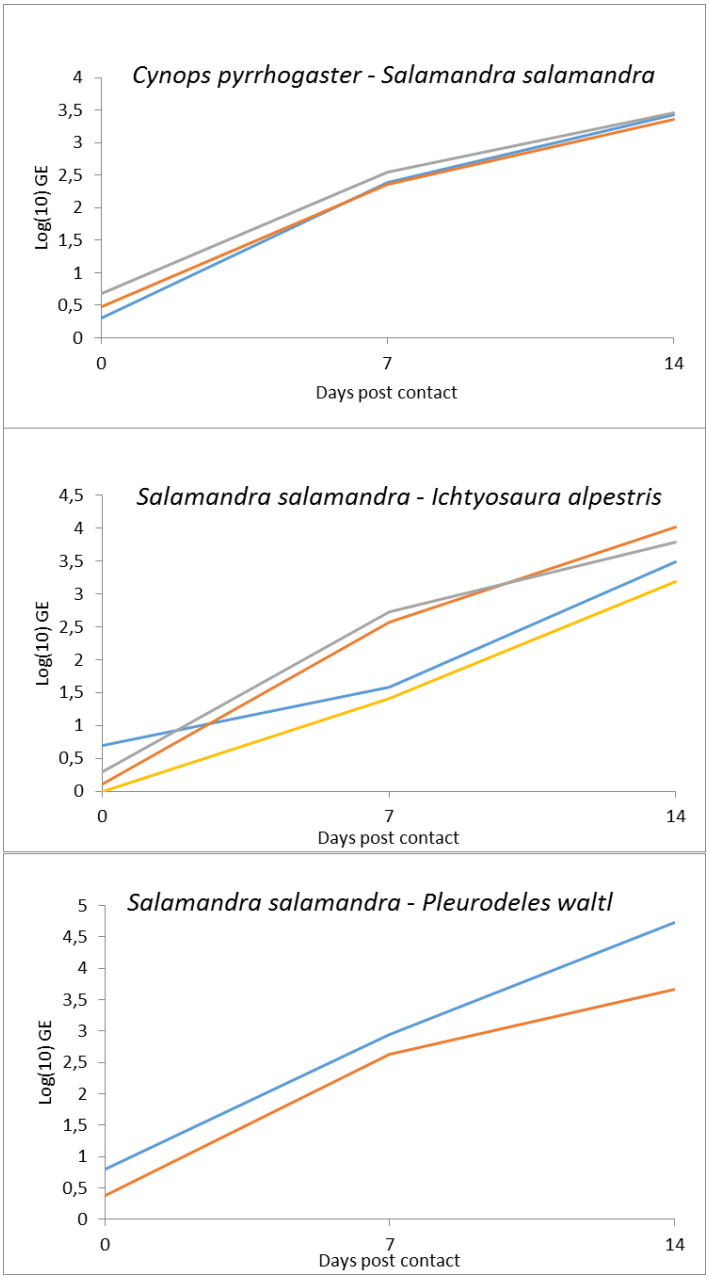

Fig S4. Interspecies transmission of *Batrachochytrium salamandrivorans*. *Batrachochytrium salamandrivorans* infected *Cynops pyrrhogaster* and *Salamandra iumalamandra* were co-housed with *Batrachochytrium salamandrivorans* negative *Salamandra salamandra* and *Ichthyosaura alpestris* or *Pleurodeles waltl*, respectively, for 8 hours. Immediately after the co-housing and one and two weeks later, the animals were swabbed (time point 0). Log (10) GE values expressed per swab.

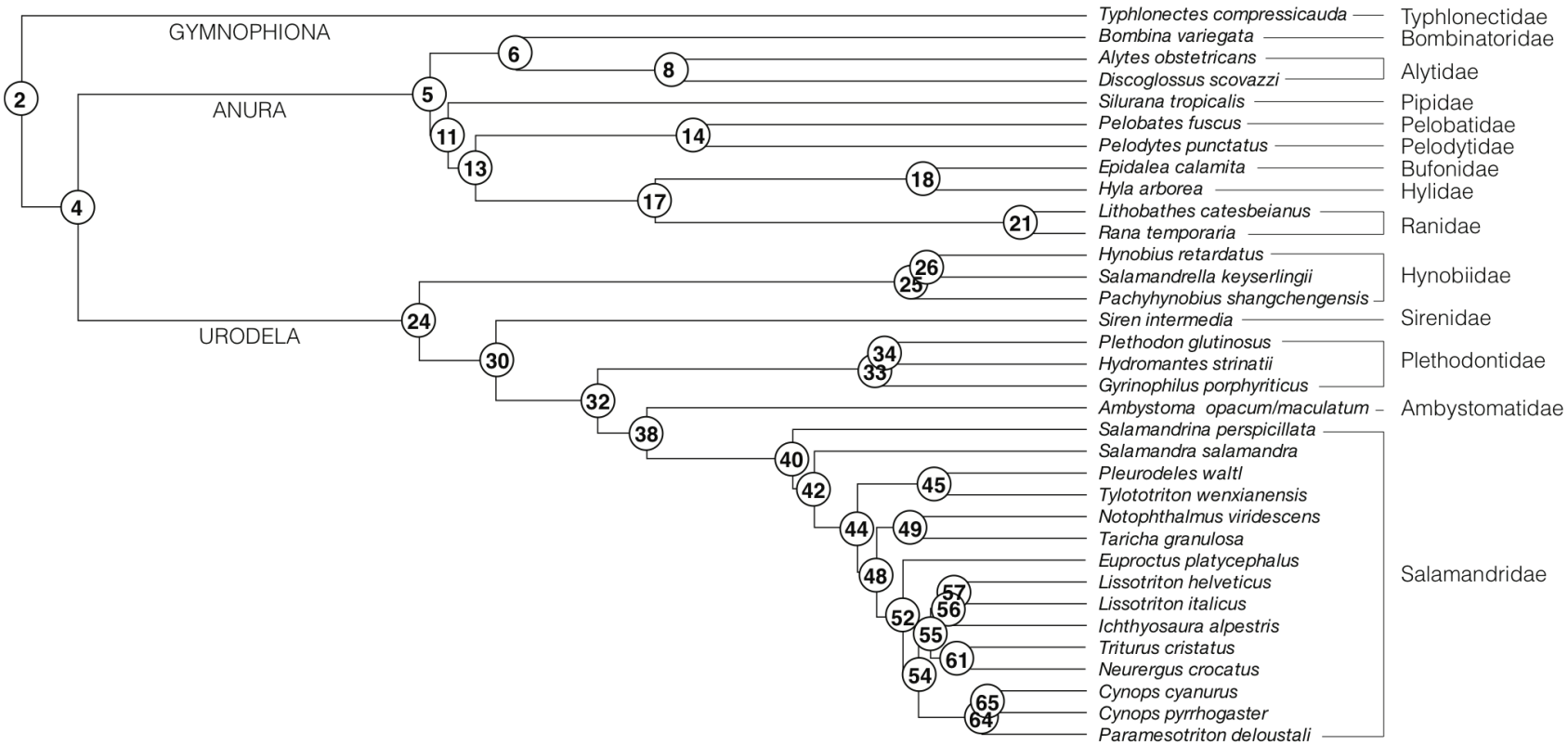

Fig S5. Tree of 34 amphibian species with node numbers that are cross-referenced in the ancestral state reconstructions of Table S10.

Supplementary tables

Table S1. Summary of the infection experiments. Responses to infection (in terms of presence or absence of *Batrachochytrium salamandrivorans* colonization after exposure) were always highly consistent within a given taxon, even if multiple sources of animals were used.

Amphibian species	Family	Age	Origin	Continent of origin	No used[a]	No Infected[b]	No Sick[c]	No Dead[d]	Average days to mortality (min – max)	Invasion[e]	Category[h]
Anura											
Alytes obstetricans	Alytidae	< 1 year	C1[f]	Europe	5	0	0	0	-	-	R
Bombina variegata	Bombinatoridae	> 1 year	C1	Europe	4	0	0	0	-	-	R
Discoglossus scovazzi	Alytidae	< 1 year	C1	Africa	5	0	0	0	-	-	R
Epidalea calamita	Bufonidae	< 1 year	C1	Europe	4	0	0	0	-	-	R
Hyla arborea	Hylidae	< 1 year	C1	Europe	5	0	0	0	-	-	R
Lithobathes catesbeianus	Ranidae	> 1 year	C1	North America	5	0	0	0	-	-	R
Pelobates fuscus	Pleobatidae	< 1 year	C1	Europe	5	0	0	0	-	Nt[g]	R
Pelodytes punctatus	Pelodytidae	< 1 year	C1	Europe	5	0	0	0	-	Nt	R

Amphibian species	Family	Age	Origin	Continent of origin	No used[a]	No Infected[b]	No Sick[c]	No Dead[d]	Average days to mortality (min – max)	Invasion[e]	Category[h]
Rana temporaria	Ranidae	< 1 year	C1	Europe	5	0	0	0	-	Nt	R
Silurana tropicalis	Pipidae	> 1 year	C1	Africa	5	0	0	0	-	-	R
Urodela											
Ambystoma maculatum	Ambystomatidae	< 1 year	C1	North America	5	0	0	0	-	-	R
Ambystoma opacum	Ambystomatidae	< 1 year	W1	North America	5	0	0	0	-	Nt	R
Cynops cyanurus	Salamandridae	> 1 year	W1	Asia	5	5	5	3	27 (24 – 28)	Nt	S
Cynops pyrrhogaster	Salamandridae	< 1 year	C2	Asia	8	8	8	4	44 (15 – 105)	Nt	S
Euproctus platycephalus	Salamandridae	> 1 year	C1	Europe	3	3	3	3	12 (10 – 15)	+	L
Hydromantes strinatii	Plethodontidae	> 1 year	W1	Europe	3	3	3	3	54 (15 – 106)	Nt	L
Gyrinophilus porphyriticus	Plethodontidae	> 1 year	C1	North America	5	0	0	0	-	Nt	R
Hynobius retardatus	Hynobiidae	> 1 year	C1	Asia	3	0	0	0	-	Nt	R
Ichthyosaura alpestris	Salamandridae	< 1 year	C2	Europe	5	5	5	5	15 (8 – 26)	+	L

Amphibian species	Family	Age	Origin	Continent of origin	No used[a]	No Infected[b]	No Sick[c]	No Dead[d]	Average days to mortality (min – max)	Invasion[e]	Category[h]
Lissotriton helveticus	Salamandridae	< 1 year	C2	Europe	3	3	0	0	-	+	R
Lissotriton italicus	Salamandridae	< 1 year	C1	Europe	5	5	5	5	10 (9-11)	Nt	L
Neurergus crocatus	Salamandridae	> 1 year	C1	Europe	5	5	5	5	11 (10-11)	+	L
Nothophthalmus viridescens	Salamandridae	> 1 year	W1	North America	5	5	5	5	22 (20 – 34)	+	L
Pachyhynobius shangchengensis	Hynobiidae	>1 year	C1	Asia	3	0	0	0	-	Nt	R
Paramesotriton deloustali	Salamandridae	> 1 year	C1	Asia	4	4	4	3	24 (21 – 27)	Nt	S
Plethodon glutinosus	Plethodontidae	> 1 year	W1	North America	5	0	0	0	-	+	R
Pleurodeles waltl	Salamandridae	< 1 year	C1	Europe	5	5	5	5	11 (8 – 13)	+	L
Salamandra salamandra	Salamandridae	< 1 year	C3	Europe	5	5	5	5	14 (12 – 15)	+	L
Salamandrella keyserlingii	Hynobiidae	> 1 year	C1	Asia	3	3	0	0	-	Nt	T
Salamandrina perspicillata	Salamandridae	< 1 year	C1	Europe	5	5	5	5	24 (16 – 35)	Nt	L

Amphibian species	Family	Age	Origin	Continent of origin	No used[a]	No Infected[b]	No Sick[c]	No Dead[d]	Average days to mortality (min – max)	Invasion[e]	Category[h]
Siren intermedia	Sirenidae	> 1 year	C1	North America	3	3	3	0	-	Nt	T
Taricha granulosa	Salamandridae	< 1 year	C1	North America	4	4	4	4	28 (21 – 42)	Nt	L
Triturus cristatus	Salamandridae	< 1 year	C1	Europe	5	5	5	5	34 (25 -57)	+	L
Tylototriton wenxianensis	Salamandridae	< 1 year	C1	Asia	5	5	5	5	25 (20 – 31)	Nt	L
Gymnophiona											
Typhlonectes compressicauda	Typhlonectidae	> 1 year	C1	South America	2	0	0	0	-	Nt	R

[a] number of animals exposed to *B. salamandrivorans*
[b] number of animals infected by *B. salamandrivorans*
[c] number of animals that developed clinical signs: anorexia, apathy and/or abnormal behaviour (preferring dry places)
[d] number of animals that died
[e] determined using skin from two animals per species, after 24h of exposure to *B. salamandrivorans*
[f] C captive bred; W wild caught animals, number reflects the number of source populations
[g] Nt not tested
[h] R = resistant (no infection, no disease), T = tolerant (infection in the absence of disease and mortality), S = susceptible (infection resulting in clinical disease with possibility of subsequent clinical recovery), L = lethal (infection resulting in lethal disease in all infected animals)

Table S2. Screening of amphibian assemblages for the presence of *Batrachochytrium salamandrivorans*.

Family	Species	Country	No tested	Years sampled	No positive (GE load)[a]
WESTERN PALEARCTIC					
Anura					
Alytidae	*Alytes obstetricans*	The Netherlands	15	2009	0
Bombinatoridae	*Bombina variegata*	The Netherlands	8	2009	0
Bufonidae	*Bufo bufo*	Belgium	100	2011	0
Bufonidae	*Bufo bufo*	The Netherlands*	19	2013	0
Bufonidae	*Epidalea calamita*	The Netherlands	24	2009	0
Hylidae	*Hyla arborea*	The Netherlands	22	2009	0
Ranidae	*Lithobates catesbeianus*	Belgium	100	2010-2011	0
Ranidae	*Rana temporaria*	Belgium	7	2010	0
Ranidae	*Rana temporaria*	The Netherlands	73	2013	0
Urodela					
Salamandridae	*Calotriton arnoldi*	Spain	5	2009-2012	0
Salamandridae	*Ichthyosaura alpestris*	Belgium	35	2009	0
Salamandridae	*Ichthyosaura alpestris*	France	5	2009	0
Salamandridae	*Ichthyosaura alpestris*	Switzerland	1239	2008-2013	0
Salamandridae	*Ichthyosaura alpestris*	The Netherlands*	44	2013	1 (13)
Salamandridae	*Lissotriton boscai*	Spain	5	2009-2012	0
Salamandridae	*Lissotriton helveticus*	Belgium	33	2009	0
Salamandridae	*Lissotriton helveticus*	Spain	6	2009-2012	0
Salamandridae	*Lissotriton helveticus*	Switzerland	323	2008-2009	0
Salamandridae	*Lissotriton vulgaris*	Belgium	6	2008	0
Salamandridae	*Lissotriton vulgaris*	Switzerland	62	2008-2013	0
Salamandridae	*Lissotriton vulgaris*	The Netherlands*	2	2013	0
Salamandridae	*Ichthyosaura alpestris*	Spain	5	2009-2012	0

Family	Species	Country	No tested	Years sampled	No positive (GE load)[a]
Salamandridae	*Mertensiella caucasica*	Turkey	8	2010	0
Salamandridae	*Ommatotriton ophryticus*	Turkey	3	2010	0
Salamandridae	*Salamandra algira*	Morocco	10	2011	0
Salamandridae	*Salamandra atra*	Austria	122	2010	0
Salamandridae	*Salamandra atra*	Germany	120	2011-2013	0
Salamandridae	*Salamandra atra*	Switzerland	120	2008-2013	0
Salamandridae	*Salamandra salamandra*	Belgium	233**	2012-2014	1*** (5)
Salamandridae	*Salamandra salamandra*	France	9	2011	0****
Salamandridae	*Salamandra salamandra*	Spain	132	2009-2013	0
Salamandridae	*Salamandra salamandra*	Switzerland	26	2008-2013	0
Salamandridae	*Salamandra salamandra*	The Netherlands	39	2010-2013	13 (17(4-139))
Plethodontidae	*Hydromantes imperialis*	Italy	79	2009-2012	0
Plethodontidae	*Hydromantes supramontis*	Italy	42	2009-2012	0
Plethodontidae	*Hydromantes flavus*	Italy	25	2004-2012	0
Plethodontidae	*Hydromantes genei*	Italy	173	2009-2012	0
Plethodontidae	*Hydromantes sarrabusensis*	Italy	5	2004-2012	0
Plethodontidae	*Hydromantes strinatii*	Italy	70	2012-2013	0
Salamandridae	*Pleurodeles waltl*	Spain	11	2009-2012	0
Salamandridae	*Triturus cristatus*	Belgium	2	2010	0
Salamandridae	*Triturus cristatus*	Switzerland	38	2008-2009	0
Salamandridae	*Triturus carnifex*	Switzerland	9	2008-2009	0
Salamandridae	*Triturus marmoratus*	Spain	54	2009-2012	0
Salamandridae	*Triturus pygmaeus*	Spain	5	2009-2012	0

EASTERN ASIA					
Anura					
Bombinatoridae	*Bombina maxima*	Vietnam	4	2010	0
Dicroglossidae	*Fejervarya limnocharis*	Vietnam	1	2010	0
Dicroglossidae	*Limnonectes* sp.	Vietnam	4	2010	0
Dicroglossidae	*Quasipaa* sp.	Vietnam	2	2010	0
Megophryidae	*Leptobrachium* sp.	Vietnam	2	2010	0
Megophryidae	*Leptolalax sungi*	Vietnam	3	2010	0
Megophryidae	*Megrophrys* sp.	Vietnam	2	2010	0
Megophryidae	*Ophryophryne* sp.	Vietnam	3	2010	0
Megophryidae	*Xenophrys major*	Vietnam	2	2010	0
Microhylidae	*Microhyla heymonsi*	Vietnam	3	2010	0
Microhylidae	*Microhyla pulchra*	Vietnam	2	2010	0
Microhylidae	*Micryletta ornate*	Vietnam	4	2010	0
Ranidae	*Amolops* sp.	Vietnam	3	2010	0
Ranidae	*Hylarana* sp.	Vietnam	6	2010	0
Ranidae	*Odorrana* sp.	Vietnam	8	2010	0
Rhacophoridae	*Kurixalus verrucosus*	Vietnam	1	2010	0
Rhacophoridae	*Polypedates leucomystax*	Vietnam	3	2010	0
Rhacophoridae	*Rhacophorus dennysi*	Vietnam	2	2010	0
Rhacophoridae	*Rhacophorus orlovi*	Vietnam	1	2010	0
Rhacophoridae	*Rhacophorus kio*	Vietnam	1	2010	0
Rhacoporidae	*Theloderma* sp.	Vietnam	1	2010	0
Urodela					
Salamandridae	*Cynops cyanurus chuxiogensis*	China	6	2013	0
Salamandridae	*Cynops ensicauda*	Japan	76	2009	8 (12 (2-27))
Salamandridae	*Cynops pyrrhogaster*	Japan	116	2009	1 (10)
Salamandridae	*Echinotriton andersoni*	Japan	3	2009	0

Salamandridae	*Paramesotriton deloustali*	Vietnam	30	2010-2013	2 (13 (9-16))
Salamandridae	*Paramesotriton chinensis*	China	3	2008	0
Salamandridae	*Paramesotriton hongkongensis*	China	1	2014	0
Salamandridae	*Paramesotriton granulosus*	Chinae	3	2008	0
Salamandridae	*Paramesotriton longliensis*	China	3	2011	0
Salamandridae	*Paramesotriton* sp.	China	3	2014	0
Salamandridae	*Paramesotriton yunwuensis*	China	2	2014	0
Salamandridae	*Tylototriton panhai*	Thailand	9	2003-2006	0
Salamandridae	*Tylototriton uyenoi*	Thailand	9	2001-2006	1 (13)
Salamandridae	*Tylototriton vietnamensis*	Vietnam	60	2010	0
Salamandridae	*Tylototriton verrucosus*	Laos	1	2013	0
Salamandridae	*Tylototriton ziegleri*	Vietnam	9	2010	1 (11)
Salamandridae	*Tylototriton* cf. shanjing	Thailand	3	2006	0
Salamandridae	*Tylototriton* sp.	Vietnam	3	2012	0
Hynobiidae	*Hynobius kimurae*	Japan	5	2009	0
Hynobiidae	*Hynobius lichenatus*	Japan	3	2009	0
Hynobiidae	*Hynobius naevius*	Japan	2	2009	0
Hynobiidae	*Hynobius nebulosus*	Japan	15	2009	1 (2)
Hynobiidae	*Hynobius nigrescens*	Japan	15	2009	0
Hynobiidae	*Hynobius retardatus*	Japan	3	2009	0
Hynobiidae	*Onychodactylus japonicas*	Japan	19	2009	1 (12)
Hynobiidae	*Salamandrella keyserlingii*	Japan	4	2009	2 (2 (2-2))
Cryptobranchidae	*Andrias japonicas*	Japan	26	2009	0
NEOTROPICS					
Anura					
Bufonidae	*Atelopus glyphus*	Panama	6	2007	0
Bufonidae	*Atelopus limosus*	Panama	1	2007	0
Bufonidae	*Incilius coniferus*	Panama	4	2007	0
Bufonidae	*Rhaebo haematiticus*	Panama	8	2007	0

Bufonidae	*Rhinella alata*	Panama	14	2007	0
Bufonidae	*Rhinella marina*	Panama	15	2008	0
Centrolenidae	*Centrolene ilex*	Panama	4	2007	0
Centrolenidae	*Cochranella albomaculata*	Panama	10	2007	0
Centrolenidae	*Cochranella euknemos*	Panama	5	2007	0
Centrolenidae	*Cochranella spinosa*	Panama	12	2007	0
Centrolenidae	*Cochranella pulverata*	Panama	1	2007	0
Centrolenidae	*Espadarana prosoblepon*	Panama	9	2007	0
Centrolenidae	*Hyalinobatrachium colymbiphyllum*	Panama	11	2007	0
Centrolenidae	*Hyalinobatrachium fleischmanni*	Panama	9	2008	0
Craugastoridae	*Craugastor bransfordii*	Panama	2	2007	0
Craugastoridae	*Craugastor crassidigitus*	Panama	10	2007	0
Craugastoridae	*Craugastor fitzingeri*	Panama	26	2007-2008	0
Craugastoridae	*Craugastor gollmeri*	Panama	3	2007	0
Craugastoridae	*Craugastor megacephalus*	Panama	1	2007	0
Craugastoridae	*Craugastor noblei*	Panama	3	2007	0
Craugastoridae	*Craugastor raniformis*	Panama	9	2008	0
Craugastoridae	*Craugastor tabasarae*	Panama	1	2007	0
Craugastoridae	*Craugastor talamancae*	Panama	22	2007	0
Dendrobatidae	*Allobates talamancae*	Panama	1	2007	0
Dendrobatidae	*Colestethus flotator*	Panama	1	2007	0
Dendrobatidae	*Colestethus panamensis*	Panama	4	2007	0
Dendrobatidae	*Colostethus pratti*	Panama	5	2007	0
Dendrobatidae	*Colostethus* sp.	Panama	2	2007	0
Dendrobatidae	*Dendrobates auratus*	Panama	8	2007	0
Dendrobatidae	*Silverstoneia nubicola*	Panama	5	2007	0
Eleutherodactylidae	*Diasporus diastema*	Panama	18	2007	0
Eleutherodactylidae	*Diasporus orange*	Panama	3	2007	0
Eleutherodactylidae	*Diasporus quidditus*	Panama	7	2007	0
Eleutherodactylidae	*Eleutherodactylus sp.*	Panama	1	2007	0

Hemiphractidae	*Hemiphractus fasciatus*	Panama	1	2007	0
Hemiphractidae	*Gastrotheca cornuta*	Panama	2	2007	0
Hylidae	*Agalychnis callidryas*	Panama	7	2007-2008	0
Hylidae	*Dendropsophus* sp.	Panama	2	2008	0
Hylidae	*Hyloscirtus colymba*	Panama	1	2007	0
Hylidae	*Hypsiboas boans*	Panama	1	2007	0
Hylidae	*Hypsiboas rufitelus*	Panama	1	2007	0
Hylidae	*Hypsiboas rosenbergi*	Panama	2	2007	0
Hylidae	*Scinax rostratus*	Panama	1	2008	0
Hylidae	*Scinax ruber*	Panama	7	2008	0
Hylidae	*Smilisca phaeota*	Panama	19	2007	0
Hylidae	*Smilisca sila*	Panama	2	2007	0
Hylidae	*Trachycephalus typhonius*	Panama	15	2008	0
Hylinae	*Hyloscirtus palmeri*	Panama	1	2007	0
Leptodactylidae	*Engystomops pustulosus*	Panama	30	2007	0
Leptodactylidae	*Leptodacytlus bolivianus*	Panama	1	2008	0
Leptodactylidae	*Leptodactylus fragilis*	Panama	1	2007	0
Leptodactylidae	*Leptodactylus labialis*	Panama	6	2007	0
Leptodactylidae	*Leptodactylus poecilochilus*	Panama	3	2008	0
Leptodactylidae	*Leptodactylus savagei*	Panama	2	2008	0
Microhylidae	*Elachistocleis panamensis*	Panama	9	2008	0
Ranidae	*Lithobates warszewitschii*	Panama	2	2007	0
Strabomantidae	*Pristimantis achatinus*	Panama	2	2008	0
Strabomantidae	*Pristimantis caryophyllaceus*	Panama	24	2007	0
Strabomantidae	*Pristimantis cerasinus*	Panama	23	2007	0
Strabomantidae	*Pristimantis cruentus*	Panama	24	2007-2008	0
Strabomantidae	*Pristimantis gaigei*	Panama	3	2007	0
Strabomantidae	*Pristimantis museosus*	Panama	1	2007	0
Strabomantidae	*Pristimantis pardalis*	Panama	8	2007	0
Strabomantidae	*Pristimantis pirrensis*	Panama	1	2007	0

Strabomantidae	*Pristimantis taeniatus*	Panama	2	2007	0
Strabomantidae	*Strabomantis bufoniformis*	Panama	27	2007	0
Urodela					
Plethodontidae	*Bolitoglossa* sp.	Panama	8	2007	0
NEARCTIC					
Anura					
Bufonidae	*Anaxyrus americanus*	Illinois	8	2008-2009	0
Hylidae	*Acris crepitans*	Illinois	8	2008-2009	0
Hylidae	*Hyla avivoca*	Illinois	8	2008-2009	0
Hylidae	*Hyla chrysoscelis*	Illinois	8	2008-2009	0
Hylidae	*Hyla cinerea*	Illinois	8	2008-2009	0
Hylidae	*Hyla versicolor*	New York	16	2011	0
Hylidae	*Pseudarcis crucifer*	Illinois	8	2008-2009	0
Hylidae	*Pseudacris crucifer*	New York	47	2011	0
Hylidae	*Pseudarcis feriarum*	Illinois	5	2008-2009	0
Hylidae	*Pseudarcis triseriata*	Illinois	8	2008-2009	0
Ranidae	*Lithobates areolatus*	Illinois	22	2008-2009	0
Ranidae	*Lithobates catesbeianus*	Illinois	8	2008-2009	0
Ranidae	*Lithobates catesbeianus*	New York	7	2011	0
Ranidae	*Lithobates clamitans*	Illinois	8	2008-2009	0
Ranidae	*Lithobates clamitans*	New York	8	2011	0
Ranidae	*Lithobates pipiens*	Illinois	16	2008-2009	0
Ranidae	*Lithobates pipiens*	New York	1	2011	0
Ranidae	*Lithobates septentriona*	New York	1	2011	0
Ranidae	*Lithobates sphenocephalus*	Illinois	8	2008-2009	0
Ranidae	*Lithobates yavapaiensis*	Arizona	38	2009	0
Urodela					
Ambystomatidae	*Ambystoma jeffersonianum*	New York	3	2011	0

Ambystomatidae	*Ambystoma maculatum*	New York	8	2011	0
Ambystomatidae	*Ambystoma texanum*	Illinois	9	2008	0
Plethodontidae	*Demognathus imitator*	Appalachians	24	2009-2011	0
Plethodontidae	*Desmognathus ocoee*	Appalachians	20	2009-2011	0
Plethodontidae	*Desmognathus wrighti*	Appalachians	26	2009-2011	0
Plethodontidae	*Eurycea wilderae*	Appalachians	11	2009-2011	0
Plethodontidae	*Plethodon cinereus*	Appalachians	343	2009-2011	0
Plethodontidae	*Plethodon cinereus*	New York	3	2011	0
Plethodontidae	*Plethodon cylindraceus*	Appalachians	15	2009-2011	0
Plethodontidae	*Plethodon glutinosus*	Appalachians	54	2009-2011	0
Plethodontidae	*Plethodon jordani*	Appalachians	35	2009-2011	0
Plethodontidae	*Plethodon jor x met*	Appalachians	47	2009-2011	0
Plethodontidae	*Plethodon jor x tey*	Appalachians	6	2009-2011	0
Plethodontidae	*Plethodon metcalfi*	Tennessee	8	2009-2011	0
Plethodontidae	*Plethodon raceus x glutinosus*	Appalachians	1	2009-2011	0
Plethodontidae	*Plethodon richmondi*	Appalachians	12	2009-2011	0
Plethodontidae	*Plethodon serratus*	Appalachians	14	2009-2011	0
Plethodontidae	*Plethodon teyahalee*	Appalachians	21	2009-2011	0
Plethodontidae	*Plethodon welleri*	Appalachians	8	2009-2011	0
Plethodontidae	*Plethodon yonahlossee*	Appalachians	8	2009-2011	0
Salamandridae	*Notophthalmus viridescens*	Illinois	19	2009-2011	0
Salamandridae	*Notophthalmus viridescens*	New York	12	2011	0

[a] (GE number per qPCR reaction (minimum GE number – maximum GE number); all positive samples were sequenced in triplicate and showed a 100% identity with Genbank accession number KC762295

[*] sampled in the outbreak area Bunderbos

[**] three samples from outbreak area in Eupen, Belgium, 60 samples from outbreak area in Robertville, Belgium.

[***] from outbreak area Eupen, Belgium.

[****] from outbreak area Robertville, Belgium

Table S3. Summary of the *Batrachochytrium salamandrivorans* survey data, for each area and taxonomic group based on the data of Supplementary Table 2. The table lists the number of individuals that were tested, the number that were *Batrachochytrium salamandrivorans*-positive, *Batrachochytrium salamandrivorans* prevalence (proportion infected), the Clopper-Pearson 95% confidence interval (CI) for prevalence and the probability of detecting at least one positive individual (assuming a prevalence of 1%).

Area	Taxonomic group	Number tested	Number positive	Proportion infected	Clopper-Pearson 95% CI	Probability of detecting at least 1 positive (assuming prevalence = 0.01)
Netherlands, Belgium	urodelans	394	39	0.0901	0.0648 – 0.1211	0.9871
Western Palaearctic (Netherlands and Belgium excluded)	urodelans	2711	0	0.0000	0.0000 - 0.0014	1.0000
Western Palaearctic	anurans	368	0	0.0000	0.0000 - 0.0100	0.9752
Eastern Asia	anurans	58	0	0.0000	0.0000 - 0.0616	0.4417
Eastern Asia	urodelans	432	17	0.0379	0.0222 – 0.0599	0.9890
Neotropics	anurans	472	0	0.0000	0.0000 - 0.0078	0.9913
Neotropics	urodelans	8	0	0.0000	0.0000 - 0.3694	0.0773
Nearctic	anurans	241	0	0.0000	0.0000 - 0.0152	0.9113
Nearctic	urodelans	707	0	0.0000	0.0000 - 0.0052	0.9992

Table S4. Presence of *Batrachochytrium salamandrivorans* in toe clips from Asian archived specimens.

Family	Species	Year of deposition	No tested	No positive
Salamandridae	*Cynops ensicauda*[*]	1861	10	1
Salamandridae	*Cynops pyrrhogaster*	unknown	3	0
Salamandridae	*Cynops pyrrhogaster*	1967	1	0
Salamandridae	*Cynops pyrrhogaster*	2009	1	0
Salamandridae	*Pachytriton sp.*	1928	1	0
Salamandridae	*Paramesotriton hongkongensis*	1957	1	0
Salamandridae	*Tylototriton verrucosus*	1965	2	0
Salamandridae	*Tylototriton verrucosus*	1966	2	0
Salamandridae	*Tylototriton verrucosus*	1967	2	0

***** all animals were kept in one container, 2 animals showed ulcerations.

Table S5. Presence of *Batrachochytrium salamandrivorans* in skins swabs from amphibians in trade.

Family	Species	Continent of origin	Trade origin[a]	Number of samples tested	Number of samples positive
Anura					
Alytidae	*Alytes obstetricans*	Europe	Pet shop	3	0
Arthroleptidae	*Leptopelis argenteus*	Africa	Airport	28	0
Bombinatoridae	*Bombina orientalis*	Asia	Pet shop	20	0
Bombinatoridae	*Bombina orientalis*	Asia	Airport	38	0
Bombinatoridae	*Bombina variegata*	Europe	Pet shop	9	0
Brevicipitidae	*Breviceps adspersus*	Africa	Airport	3	0
Bufonidae	*Anaxyrus debilis*	North America	Pet shop	4	0
Bufonidae	*Amietophrynus rangeri*	South America	Airport	12	0
Bufonidae	*Amietophrynus regularis*	Africa	Airport	61	0
Bufonidae	*Bufotes viridis*	Africa	Pet shop	2	0
Bufonidae	*Bufotes viridis*	Africa	Airport	37	0
Bufonidae	*Duttaphrynus melanostictus*	Asia	Pet shop	10	0
Bufonidae	*Incilius alvarius*	North America	Pet shop	4	0
Bufonidae	*Melanophryniscus stelzneri*	South America	Pet shop	15	0
Bufonidae	*Rhinella marina*	Americas	Airport	2	0
Bufonidae	*Schismaderma carens*	Africa	Airport	1	0
Ceratophryidae	*Ceratophrys cornuta*	South America	Airport	6	0
Ceratophryidae	*Ceratophrys cranwelli*	South America	Airport	81	0
Ceratophryidae	*Ceratophrys ornata*	South America	Airport	15	0
Ceratophryidae	*Lepidobatrachus laevis*	South America	Airport	15	0
Dendrobatidae	*Dendrobates tinctorius*	South America	Pet shop	5	0

Family	Species	Continent of origin	Trade origin[a]	Number of samples tested	Number of samples positive
Dendrobatidae	*Dendrobates tinctorius*	South America	Airport	2	0
Hemisotidae	*Hemisus marmoratus*	Africa	Airport	2	0
Hylidae	*Agalychnis callidryas*	South America	Pet shop	13	0
Hylidae	*Agalychnis callidryas*	North America	Airport	7	0
Hylidae	*Dendropsophus leucophyllatus*	South America	Airport	2	0
Hylidae	*Hyla chrysoscelis*	North America	Airport	7	0
Hylidae	*Hyla cinerea*	North America	Pet shop	37	0
Hylidae	*Hyla cinerea*	North America	Airport	34	0
Hylidae	*Hyla gratiosa*	North America	Airport	6	0
Hylidae	*Hyla versicolor*	North America	Pet shop	19	0
Hylidae	*Hyla* sp.	unknown	Airport	14	0
Hylidae	*Hypsiboas calcaratus*	South America	Airport	2	0
Hylidae	*Litoria caerulea*	Australia/Asia	Airport	19	0
Hylidae	*Litoria infrafrenata*	Australia/Asia	Airport	8	0
Hylidae	*Litoria* sp.	unknown	Airport	29	0
Hylidae	*Phyllomedusa bicolor*	South America	Airport	2	0
Hylidae	*Phyllomedusa hypochondrialis*	South America	Airport	2	0
Hylidae	*Pseudacris crucifer*	North America	Airport	2	0
Hylidae	*Tachycephalus resinifinctrix*	South America	Airport	3	0
Hylidae	*Trachycepahlus resinifictrix*	South America	Pet shop	10	0
Hyperoliidae	*Afrixalus fornasini*	Africa	Airport	6	0
Hyperoliidae	*Heterixalus alboguttatus*	Africa	Airport	3	0
Hyperoliidae	*Heterixalus madagascariensis*	Africa	Airport	3	0
Hyperoliidae	*Heterixalus punctatus*	Africa	Airport	3	0

Family	Species	Continent of origin	Trade origin[a]	Number of samples tested	Number of samples positive
Hyperoliidae	*Hyperolius argus*	Africa	Airport	23	0
Hyperoliidae	*Hyperolius concolor*	Africa	Airport	17	0
Hyperoliidae	*Hyperolius guttulatus*	Africa	Pet shop	2	0
Hyperoliidae	*Hyperolius marmoratus*	Africa	Airport	26	0
Hyperoliidae	*Hyperolius parkeri*	Africa	Pet shop	3	0
Hyperoliidae	*Hyperolius picturatus*	Africa	Airport	6	0
Hyperoliidae	*Hyperolius puncticulatus*	Africa	Airport	20	0
Hyperoliidae	*Hyperolius tuberilingus*	Africa	Airport	5	0
Hyperoliidae	*Hyperolius viridiflavus*	Africa	Airport	19	0
Hyperoliidae	*Hyperolius* sp.	unknown	Airport	79	0
Hyperoliidae	*Kassina maculate*	South America	Pet shop	2	0
Hyperoliidae	*Kassina maculata*	South America	Airport	22	0
Hyperoliidae	*Kassina senegalensis*	Africa	Pet shop	2	0
Hyperoliidae	*Kassina senegalensis*	Africa	Airport	13	0
Mantellidae	*Mantella aurantiaca*	Africa	Airport	2	0
Mantellidae	*Mantella baroni*	Africa	Airport	8	0
Mantellidae	*Mantella betsileo*	Africa	Pet shop	4	0
Mantellidae	*Mantella betsileo*	Africa	Airport	4	0
Mantellidae	*Mantella laevigata*	Africa	Airport	1	0
Mantellidae	*Mantella madagascarensis*	Africa	Airport	4	0
Mantellidae	*Mantella nigricans*	Africa	Airport	4	0
Mantellidae	*Mantella pulchra*	Africa	Airport	6	0
Megophryidae	*Megophrys montana*	Asia	Airport	3	0
Megophryidae	*Megophrys nasuta*	Asia	Pet shop	9	0

Family	Species	Continent of origin	Trade origin[a]	Number of samples tested	Number of samples positive
Megophryidae	*Megophrys nasuta*	Asia	Airport	8	0
Microhylidae	*Dyscophus guineti*	Africa	Pet shop	10	0
Microhylidae	*Dyscophus guineti*	Africa	Airport	31	0
Microhylidae	*Dyscophus insularis*	Africa	Airport	4	0
Microhylidae	*Kaloula pulchra*	Asia	Pet shop	6	0
Microhylidae	*Kaloula pulchra*	Asia	Airport	9	0
Microhylidae	*Phrynomantis bifasciatus*	Africa	Airport	57	0
Microhylidae	*Scaphiophryne gottlebei*	Africa	Airport	2	0
Microhylidae	*Scaphiophryne madagascariensis*	Africa	Airport	2	0
Microhylidae	*Scaphiophryne marmorata*	Africa	Airport	2	0
Pipidae	*Hymenochirus boettgeri*	Africa	Pet shop	15	0
Pipidae	*Xenopus laevis*	Africa	Airport	63	0
Ptychadenidae	*Ptychadena mascareniensis*	Africa	Airport	4	0
Pyxicephalidae	*Pyxicephalus adspersus*	Africa	Pet shop	13	0
Pyxicephalidae	*Pyxicephalus adspersus*	Africa	Airport	37	0
Pyxicephalidae	*Tomopterna marmorata*	Africa	Airport	4	0
Rhacophoridae	*Chiromantis xerampelina*	Africa	Pet shop	8	0
Rhacophoridae	*Chiromantis xerampelina*	Africa	Airport	3	0
Rhacophoridae	*Polypedates leucomystax*	Asia	Pet shop	10	0
Rhacophoridae	*Polypedates otilopus*	Asia	Airport	9	0
Rhacophoridae	*Rhacophorus dennysi*	Asia	Pet shop	1	0
Rhacophoridae	*Rhacophorus dennysi*	Asia	Airport	6	0
Rhacophoridae	*Rhacophorus nigropalmatus*	Asia	Airport	2	0
Rhacophoridae	*Rhacophorus prominanus*	Asia	Airport	10	0

Family	Species	Continent of origin	Trade origin[a]	Number of samples tested	Number of samples positive
Rhacophoridae	*Theloderma asperum*	Asia	Pet shop	7	0
Rhacophoridae	*Theloderma corticale*	Asia	Airport	6	0
Rhacophoridae	*Theloderma corticale*	Asia	Pet shop	4	0
Urodela					
Ambystomatidae	*Ambystoma maculatum*	North America	Pet shop	11	0
Ambystomatidae	*Ambystoma maculatum*	North America	Airport	9	0
Ambystomatidae	*Ambystoma mexicanum*	South America	Pet shop	10	0
Ambystomatidae	*Ambystoma opacum*	North America	Pet shop	14	0
Ambystomatidae	*Ambystoma opacum*	North America	Airport	3	0
Ambystomatidae	*Ambytoma tigrinum*	North America	Pet shop	25	0
Plethodontidae	*Desmognathus auriculatus*	North America	Airport	4	0
Proteidae	*Necturus maculosus*	North America	Pet shop	7	0
Salamandridae	*Cynops cyanurus*	Asia	Pet shop	24	0
Salamandridae	*Cynops cyanurus*	Asia	Airport	5	0
Salamandridae	*Cynops orientalis*	Asia	Pet shop	68	0
Salamandridae	*Cynops orientalis*	Asia	Airport	5	0
Salamandridae	*Cynops orientalis*	Asia	Exporter	72	0
Salamandridae	*Cynops pyrrhogaster*	Asia	Pet shop	11	0
Salamandridae	*Ichthyosaura alpestris*	Europe	Pet shop	12	0
Salamandridae	*Notophthalmus viridescens*	North America	Pet shop	14	0
Salamandridae	*Paramesotriton hongkongensis*	Asia	Pet shop	11	0
Salamandridae	*Paramesotriton hongkongensis*	Asia	Exporter	72	0
Salamandridae	*Paramesotriton labiatus*	Asia	Pet shop	16	0
Salamandridae	*Paramesotriton labiatus*	Asia	Airport	2	0

Family	Species	Continent of origin	Trade origin[a]	Number of samples tested	Number of samples positive
Salamandridae	*Pleurodeles waltl*	Europe	Pet shop	41	0
Salamandridae	*Salamandra salamandra*	Europe	Pet shop	21	0
Salamandridae	*Salamandra salamandra*	Europe	Airport	12	0
Salamandridae	*Triturus marmoratus*	Europe	Pet shop	4	0
Salamandridae	*Tylototriton asperrimus*	Asia	Pet shop	8	0
Salamandridae	*Tylototriton kweichowensis*	Asia	Pet shop	11	0
Salamandridae	*Tylototriton shanjing*	Asia	Pet shop	45	0
Sirenidae	*Siren intermedia*	North America	Pet shop	1	0
Sirenidae	*Siren lacertina*	North America	Airport	2	0
Sirenidae	*Siren lacertina*	North America	Pet shop	2	0
Gymnophiona					
Dermophiidae	*Geotrypetes seraphini*	Africa	Pet shop	2	0

Table S6. Summary of the *Batrachochytrium salamandrivorans* survey of amphibians in the trade. The table lists the number of individuals that were tested, the number that were *Batrachochytrium salamandrivorans*-positive, *Batrachochytrium salamandrivorans* prevalence (proportion infected) and the Clopper-Pearson 95% confidence interval (CI) for prevalence.

Taxonomic group	Number tested	Number positive	Proportion infected	Clopper-Pearson 95% CI	Probability of detecting at least 1 positive individual (assuming prevalence = 0.01)
urodelans	542	0	0	0.0000-0.0068	0.9681
anurans	1221	0	0	0.0000-0.0030	0.9765

Table S7. Presence of *Batrachochytrium salamandrivorans* in skin swabs from captive kept amphibians.

Family	Species	Continent of origin	No tested	No positive
Anura				
Bufonidae	*Atelopus hoogmoedi*	South America	1	0
Bufonidae	*Barbarophryne brongersmai*	Africa	1	0
Bombinatoridae	*Bombina variegata*	Europe	17	0
Dendrobatidae	*Dendrobates auratus*	South America	6	0
Dendrobatidae	*Dendrobates leucomelas*	South America	2	0
Dendrobatidae	*Dendrobates tinctorius*	South America	33	0
Dendrobatidae	*Oophaga pumilio*	South America	14	0
Dendrobatidae	*Phyllobates terribilis*	South America	16	0
Dendrobatidae	*Ranitomeya reticulata*	South America	2	0
Discoglossidae	*Discoglossus pictus*	Africa	6	0
Hemiphractidae	*Gastrotheca riobambae*	South America	1	0
Hylidae	*Hyla eximia*	North America	2	0
Hylidae	*Agalychnis callidryas*	South America	1	0
Hylidae	*Hyla arborea*	Europe	19	0
Hylidae	*Litoria aurea*	Asia	3	0
Hylidae	*Litoria caerulea*	Asia	1	0
Hylidae	*Litoria infrafrenata*	Asia	1	0
Hylidae	*Trachycephalus resinifictrix*	South America	2	0
Hyperoliidae	*Hyperolius puncticulatus*	Africa	5	0
Leptodactylidae	*Eleutherodactylus montanus*	South America	2	0
Leptodactylidae	*Leptodactylus fallax*	South America	1	0
Microhylidae	*Dyscophus guineti*	Africa	1	0
Pipidae	*Silurana tropicalis*	Africa	5	0
Pipidae	*Xenopus laevis*	Africa	16	0
Rhacophoridae	*Rhacophorus dennysi*	Asia	1	0
Urodela				
Ambystomatidae	*Ambystoma andersoni*	North America	1	0
Ambystomatidae	*Ambystoma californiense*	North America	1	0
Ambystomatidae	*Ambystoma gracile*	North America	1	0
Ambystomatidae	*Ambystoma jeffersonianum*	North America	2	0
Ambystomatidae	*Ambystoma laterale*	North America	1	0
Ambystomatidae	*Ambystoma mexicanum*	North America	2	0
Ambystomatidae	*Ambystoma macrodactylum*	North America	4	0
Ambystomatidae	*Ambystoma maculatum*	North America	9	0
Ambystomatidae	*Ambystoma mavortium*	North America	5	0
Ambystomatidae	*Ambystoma opacum*	North America	2	0
Ambystomatidae	*Ambystoma ordinarium*	North America	3	0
Ambystomatidae	*Ambystoma rivulare*	North America	1	0

Family	Species	Continent of origin	No tested	No positive
Ambystomatidae	*Ambystoma tigrinum*	North America	3	0
Ambystomatidae	*Ambystoma velasci*	North America	1	0
Cryptobranchidae	*Andrias davidianus*	North America	3	0
Cryptobranchidae	*Andrias japonicas*	North America	3	0
Dicamptodontidae	*Dicamptodon tenebrosus*	North America	3	0
Hynobiidae	*Hynobius boulengeri*	Asia	1	0
Hynobiidae	*Hynobius dunni*	Asia	3	0
Hynobiidae	*Hynobius naevius*	Asia	1	0
Hynobiidae	*Hynobius quelpartensis*	Asia	1	0
Hynobiidae	*Hynobius retardatus*	Asia	8	0
Hynobiidae	*Hynobius tokyoensis*	Asia	1	0
Hynobiidae	*Pachyhynobius shangchengensis*	Asia	10	0
Hynobiidae	*Paradactylodon gorganensis*	Asia	4	0
Plethodontidae	*Aneides lugubris*	North America	2	0
Plethodontidae	*Aneides flavipunctus*	North America	1	0
Plethodontidae	*Bolitoglossa platydactyla*	North America	5	0
Plethodontidae	*Bolitoglossa rufescens*	North America	8	0
Plethodontidae	*Chiropterotriton multidentatus*	North America	1	0
Plethodontidae	*Chiropterotriton sp.*	North America	6	0
Plethodontidae	*Chiropterotriton sp.*	North America	8	0
Plethodontidae	*Desmognathus carolinensis*	North America	3	0
Plethodontidae	*Desmognathus fuscus*	North America	1	0
Plethodontidae	*Desmognathus marmoratus*	North America	2	0
Plethodontidae	*Desmognathus ochrophaeus*	North America	1	0
Plethodontidae	*Desmognathus quadramaculatus*	North America	2	0
Plethodontidae	*Ensatina eschscholtzii*	North America	2	0
Plethodontidae	*Eurycea bislineata*	North America	2	0
Plethodontidae	*Gyrinophilus porphyriticus*	North America	1	0
Plethodontidae	*Plethodon cylindraceus*	North America	1	0
Plethodontidae	*Plethodon glorobrionus*	North America	1	0
Plethodontidae	*Plethodon glutinosus*	North America	8	0
Plethodontidae	*Plethodon shermani*	North America	1	0
Plethodontidae	*Pseudoeurycea bellii*	Central America	7	0
Plethodontidae	*Pseudoeurycea cephalica*	Central America	5	0
Plethodontidae	*Pseudoerycea leprosa*	Central America	18	0
Plethodontidae	*Pseudoeurycea longicauda*	Central America	5	0
Plethodontidae	*Pseudoeurycea nigromaculata*	Central America	1	0
Plethodontidae	*Pseudoeurycea robertsi*	Central America	3	0
Plethodontidae	*Pseudotriton ruber*	North America	2	0
Plethodontidae	*Thorius troglodytes*	Central America	1	0
Salamandridae	*Calotriton asper*	Europe	1	0
Salamandridae	*Cynops cyanurus*	Asia	3	0
Salamandridae	*Cynops ensicauda*	Asia	6	0

Family	Species	Continent of origin	No tested	No positive
Salamandridae	*Cynops pyrrhogaster*	Asia	17	0
Salamandridae	*Cynops orientalis*	Asia	3	0
Salamandridae	*Euproctus platycephalus*	Europe	11	0
Salamandridae	*Ichthyosaura alpestris*	Europe	16	0
Salamandridae	*Laotriton laoensis*	Asia	4	0
Salamandridae	*Lissotriton boscai*	Europe	1	0
Salamandridae	*Lissotriton montandoni*	Europe	1	0
Salamandridae	*Lyciasalamandra billae*	Asia	4	0
Salamandridae	*Lyciasalamandra fazilae*	Asia	2	0
Salamandridae	*Mertensiella caucasica*	Asia	8	0
Salamandridae	*Neurergus crocatus*	Asia	1	0
Salamandridae	*Neurergus kaiseri*	Asia	20	0
Salamandridae	*Neurergus strauchii*	Asia	6	0
Salamandridae	*Ommatotriton ophryticus*	Asia	3	0
Salamandridae	*Pachytriton* sp.	Asia	2	0
Salamandridae	*Paramesotriton caudopunctatus*	Asia	4	0
Salamandridae	*Paramesotriton chinensis*	Asia	12	0
Salamandridae	*Paramesotriton deloustali*	Asia	4	0
Salamandridae	*Paramesotriton fuzhongensis*	Asia	1	0
Salamandridae	*Paramesotriton hongkongensis*	Asia	7	0
Salamandridae	*Pleurodeles nebulosus*	Africa	2	0
Salamandridae	*Pleurodeles waltl*	Europe	14	0
Salamandridae	*Pleurodeles poireti*	Africa	1	0
Salamandridae	*Salamandra algira*	Africa	7	0
Salamandridae	*Salamandra corsica*	Europe	1	0
Salamandridae	*Salamandra infraimmaculata*	Asia	7	0
Salamandridae	*Salamandra salamandra*	Europe	5	0
Salamandridae	*Taricha granulosa*	North America	2	0
Salamandridae	*Taricha rivularis*	North America	1	0
Salamandridae	*Taricha sierrae*	North America	1	0
Salamandridae	*Taricha torosa*	North America	1	0
Salamandridae	*Triturus carnifex*	Europe	6	0
Salamandridae	*Triturus cristatus*	Europe	1	0
Salamandridae	*Triturus dobrogicus*	Europe	1	0
Salamandridae	*Triturus karelinii*	Europe	1	0
Salamandridae	*Triturus marmoratus*	Europe	1	0
Salamandridae	*Tylototriton asperrimus*	Asia	1	0
Salamandridae	*Tylototriton kweichowensis*	Asia	12	0
Salamandridae	*Tylototriton shanjing*	Asia	6	0
Salamandridae	*Tylototriton taliangensis*	Asia	1	0
Salamandridae	*Tylototriton vietnamensis*	Asia	18	3
Salamandridae	*Tylototriton verrucosus*	Asia	4	0
Salamandridae	*Tylototriton wenxianensis*	Asia	3	0

Family	Species	Continent of origin	No tested	No positive
Salamandridae	*Tylototriton ziegleri*	Asia	1	0
Sirenidae	*Siren intermedia*	North America	1	0
Gymnophiona				
Dermophiidae	*Geotrypetes seraphini*	Africa	1	0
Herpelidae	*Herpele* sp.	Africa	1	0

Table S8. Summary of the *Batrachochytrium salamandrivorans* survey of amphibians in captivity. The table lists the number of individuals that were tested, the number that were *Batrachochytrium salamandrivorans*-positive, *Batrachochytrium salamandrivorans* prevalence (proportion infected) and the Clopper-Pearson 95% confidence interval for prevalence.

Taxonomic group	Number tested	Number positive	Proportion infected	Clopper-Pearson 95% CI	Probability of detecting at least 1 positive individual (assuming prevalence = 0.01)
urodelans	408	3	0.0073	0.0015 - 0.0212	0.9834
anurans	159	0	0.0000	0.0000 - 0.0229	0.7977

Table S9. Species susceptibility category used for ancestral state reconstruction.

Species	Category
Typhlonectes compressicauda	Resistant
Bombina variegata	Resistant
Alytes obstetricans	Resistant
Discoglossus scovazzi	Resistant
Silurana tropicalis	Resistant
Pelobates fuscus	Resistant
Pelodytes punctatus	Resistant
Epidalea calamita	Resistant
Hyla arborea	Resistant
Lithobathes catesbeianus	Resistant
Rana temporaria	Resistant
Hynobius retardatus	Resistant
Salamandrella keyserlingii	Tolerant
Pachyhynobius shangchengensis	Resistant
Siren intermedia	Tolerant
Plethodon glutinosus	Resistant
Hydromantes strinatii	Lethal
Gyrinophilus porphyriticus	Resistant
Ambystoma opacum/maculatum	Resistant
Salamandrina perspicillata	Lethal
Salamandra salamandra	Lethal
Pleurodeles waltl	Lethal
Tylototriton wenxianensis	Lethal
Nothophthalmus viridescens	Lethal
Taricha granulosa	Lethal
Euproctus platycephalus	Lethal
Lissotriton helveticus	Resistant
Lissotriton italicus	Lethal
Ichthyosaura alpestris	Lethal
Triturus cristatus	Lethal
Neurergus crocatus	Lethal
Cynops cyanurus	Susceptible
Cynops pyrrhogaster	Susceptible
Paramesotriton deloustali	Susceptible

Table S10. Results of the Maximum Parsimony (MP) and Maximum Likelihood ancestral state reconstructions of amphibian susceptibility to *Batrachochytrium salamandrivorans.* Node numbers are cross-referenced on the tree in Figure S5.

Node N°	MP	Maximum Likelihood Probabilities			
		Resistant	Tolerant	Susceptible	Lethal
2	Resistant	0.988270	0.004081	0.003628	0.004021
4	Resistant	0.995242	0.001770	0.001282	0.001706
5	Resistant	0.999975	8.48E-06	7.91E-06	8.40E-06
6	Resistant	0.999792	6.94E-05	6.93E-05	6.94E-05
8	Resistant	0.999366	2.11E-04	2.11E-04	2.11E-04
11	Resistant	0.999986	4.87E-06	4.75E-06	4.85E-06
13	Resistant	0.999968	1.07E-05	1.07E-05	1.07E-05
14	Resistant	0.999248	2.51E-04	2.51E-04	2.51E-04
17	Resistant	0.999606	1.31E-04	1.31E-04	1.31E-04
18	Resistant	0.999846	5.12E-05	5.12E-05	5.12E-05
21	Resistant	0.999968	1.08E-05	1.08E-05	1.08E-05
24	Resistant	0.993609	0.003138	4.68E-04	0.002785
25	Resistant	0.995580	0.004139	1.28E-04	1.53E-04
26	Resistant	0.992494	0.007093	1.97E-04	2.17E-04
30	Resistant	0.985569	0.006714	7.29E-04	0.006988
32	Resistant	0.984866	0.001610	3.23E-04	0.013201
33	Resistant	0.991893	1.49E-04	1.13E-04	0.007845
34	Resistant	0.990246	1.98E-04	1.65E-04	0.009392
38	Resistant	0.956639	0.001879	9.90E-04	0.040492
40	Lethal	0.008573	2.55E-04	2.47E-04	0.990926
42	Lethal	8.28E-04	2.96E-05	2.89E-05	0.999113
44	Lethal	5.86E-06	6.66E-07	6.70E-07	0.999993
45	Lethal	1.21E-05	1.18E-05	1.18E-05	0.999964
48	Lethal	2.00E-07	1.42E-07	3.21E-07	0.999999
49	Lethal	7.10E-06	7.09E-06	7.12E-06	0.999979
52	Lethal	9.92E-07	9.66E-07	3.33E-05	0.999965
54	Lethal	7.13E-06	6.72E-06	5.65E-04	0.999421
55	Lethal	9.35E-07	1.34E-07	5.85E-06	0.999993
56	Lethal	8.93E-05	2.37E-06	3.31E-06	0.999905
57	Lethal	8.71E-04	2.18E-05	2.27E-05	0.999085
61	Lethal	3.01E-06	2.93E-06	3.53E-06	0.999991
64	Susceptible	2.52E-05	2.52E-05	0.997828	0.002122
65	Susceptible	7.54E-06	7.54E-06	0.999422	5.63E-04

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Duplex real-time PCR for rapid simultaneous detection of *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* in amphibian samples.

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Abstract

Chytridiomycosis is a lethal fungal disease contributing to declines and extinctions of amphibian species worldwide. The currently used molecular screening tests for chytridiomycosis fail to detect the recently described *Batrachochytrium* species *B. salamandrivorans* (*Bs*). In this study we present a duplex real-time PCR that allows simultaneous detection of *Bs* and *B. dendrobatidis* (*Bd*). With *Bd* and *Bs* specific primers and probes detection of both pathogens in amphibian samples is possible with a detection limit of 0.1 genomic equivalent of zoospores of both pathogens per PCR reaction. The developed real-time PCR shows high degrees of specificity and sensitivity, high linear correlation (r^2 > 0.995) and high amplification efficiency (> 94%) for both *Bd* and *Bs*. In conclusion the described duplex real-time PCR can be used to detect DNA of both *Bd* and *Bs* with highly reproducible and reliable results.

Introduction

Chytridiomycosis causes worldwide declines and extinctions of amphibian populations and is one of the most important infectious diseases in amphibians (1-3). *Batrachochytrium dendrobatidis* (*Bd*) was the sole chytridiomycete taxon known to infect vertebrate hosts and able to cause this devastating disease (4) until a second chytrid species was isolated from a mortality event, which drove the Dutch fire salamander (*Salamandra salamandra*) population to near extinction (5, 6). This novel species, *Batrachochytrium salamandrivorans* (*Bs*), cannot be detected with the *Bd*-specific PCR described by Annis et al. (7) or the *Bd*-specific real-time PCR described by Boyle et al. (8). Because both *Bd* and *Bs* are able to cause amphibian chytridiomycosis, the development of a test that would allow fast and reliable detection and quantification of both pathogens is necessary. This test could aid in rapid diagnosis of chytridiomycosis in diseased amphibians but could also be used to map the worldwide distribution of the novel pathogen. Therefore the aim of this study was to develop a duplex real-time PCR that allows detection of *Bd* and *Bs* in amphibian samples with a high degree of sensitivity and specificity.

Materials and Methods

Chytrid strains & culturing conditions

Bd and *Bs* were grown in TGhL broth (16 g tryptone, 4 g gelatin hydrolysate, 2 g lactose per liter of distilled water) in 25 cm^3 cell culture flasks and incubated at 20 °C for *Bd* and at 15 °C for *Bs*. *Homolaphlyctis polyrhiza*, *Gaertneriomyces semiglobifer*, *Geranomyces variabilis*, *Rhizophlyctis rosea*, *Rhizoclosmatium globosum*, *Polychytrium aggregatum*, *Monoblepharis polymorpha* and *Podochytrium dentatum* (Table 1) were grown in PmTG broth (0.5 g peptonized milk, 0.5 g tryptone, 2.5 g glucose per liter of distilled water) in 25 cm^3 cell culture flasks and incubated at 23 °C. To obtain zoospores of *Bd* and *Bs*, 2 ml of a 5-day-old culture was transferred to TGhL with 1% agar plates and incubated for 5 – 7 days at 20 °C for *Bd* and at 15 °C for *Bs*. Zoospores were subsequently collected by flooding the agar plates with 2 ml of filtered (0.2 µm) pond water followed by collection of the fluid. The number of zoospores present in the suspension was determined using a haemocytometer.

Quantitation standards and DNA extracts

Suspensions containing standardized numbers of *Bd* and *Bs* zoospores were prepared as described by Boyle et al. (8). Tenfold serial dilutions series ranging from 1000 to 0.01

genomic equivalents of zoospores (GE) per real-time PCR reaction were prepared for *Bd* and *Bs*. DNA of the other described Chytridiomycota (Table 1) was prepared from growing cultures with DNA extraction in 100 µl Prepman Ultra (Applied Biosystems, Foster City, CA, USA) following the DNA extraction method described by Hyatt et al. (9).

Primer and probe design

The previously described forward primer STerF (5'TGCTCCATCTCCCCCTCTTCA3') and reverse primer STerR (5'TGAACGCACATTGCACTCTAC3') were used to detect the 5.8S rRNA gene of *Bs* (6) (Gen-Bank database accession number KC762295). The *Bs* specific Cy5-probe STerC (5'ACAAGAAAATACTATTGATTCTCAAACAGGCA3') based on the 5.8S rRNA gene of *Bs* was developed using Kodon (Applied Maths, Kortrijk, Belgium) (Figure 1). The primer set ITS1-3 Chytr (5'CCTTGATATAATACAGTGTGCCATATGTC'3) and 5.8S Chytr (5'TCGGTTCTCTAGGCAACAGTTT3') and the Taqman probe Chytr MGB2 (5'CGAGTCGAACAAAAT'3) described by Boyle et al. (8) were used to detect the ITS-1 rRNA gene of *Bd*. All primers and probes were checked with BLASTN analysis to ensure that amplification of genes from other organisms or species was unlikely. For *Bs* the specificity of the primer set was tested in a SYBR green real-time PCR on DNA extracts of pure *Bs* culture and negative controls with melting curve analysis and gel electrophoresis of the real-time PCR products (see below).

A.

SterF

271 cattatctgctccatctccccctcttcatccctaaccctatttttagtaatagacgaggtagaggggagaagtagggattgggataaaaat

317 tatcacttttagatgatataaaaagacaaggaaatgaattaaaaaatagtgaaaaatctactatatttttctgttcctttacttaatttttt

SterC

363 aagaaaaataga**acaagaaaatactattgattctcaaacaggca**ttattttatcttgttcttttatgataactaagagtttgtccgtat

409 ctctacaaagtagagtgcaatgtgcgttcaaagattcgatgagagatgtttcatctcacgttacacgcaagtttctaagctact

SterR

B.

Sequences	
***B. salamandrivorans* forward primer: SterF**	**21 bases**
5'-TGCTCCATCTCCCCCTCTTCA-3'	
***B. salamandrivorans* reverse primer: SterR**	**21 bases**
5'-TGAACGCACATTGCACTCTAC-3'	
***B. salamandrivorans* Cy5-Probe: SterC**	**32 bases**
5'-/Cy5/ACAAGAAAATACTATTGATTCTCAAACAGGCA/IAbRQSp-'3	

Fig 1. Specific primers and probe for *Batrachochytrium salamandrivorans*.
A. rDNA sequence of ITS-1, 5.8S and ITS-2 regions used for the *Batrachochytrium salamandrivorans* primer and probe design.
B. *Batrachochytrium salamandrivorans* primer and probe sequences. The used sequence has been deposited in the Gen-Bank database (Accession number KC762295).

Bs SYBR Green real-time PCR

The *Bs* SYBR green assay was performed on a CFX96 Real Time System (BioRad Laboratories, Hercules, CA, USA). A reaction mixture composed of 12.5 µl SYBR green PCR mix (1x SensiMix™ SYBR No-ROX, Bioline reagents Ltd., London, UK), *Bs* forward primer STerF at a concentration of 0.3 µM, *Bs* reverse primer STerR at a concentration of 0.3 µM, 5 µl template and a volume of RNase and DNase free water to a total of 25 µl was used per reaction. Amplification conditions consisted of 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 62 °C for 15 seconds. A temperature gradient ranging from 60 °C to 95 °C with plate reads at every temperature increment of 0.5 °C was used to generate melting curve data.

Duplex Real-Time Taqman PCR assay optimisation

Both the *Bd* and *Bs* real-time PCR's were first optimized as simplex assays. Assays were performed on a CFX96 Real Time System (BioRad Laboratories, Hercules, CA, USA). Amplification conditions for both simplex and the duplex assays consisted of 10 minutes at 95 °C followed by 40 cycles of melting (95 °C for 15 seconds) and annealing/extension (62 °C for 1 minute). Primer concentrations were optimized in a checkerboard system with a standard probe concentration of 250 nM. Subsequently, the probe concentrations were optimized with the previously determined optimal primer concentrations. After optimalization of the simplex assays, both PCR's were combined to form the duplex real-time PCR. Precision of the developed duplex real-time PCR assay was evaluated by determining intra- and inter-assay variability expressed as the mean coefficient of variation. For the intra-assay variability three replicates of the quantitation standard were run in three separate assays, while for the intra-assay variability three replicates were run in one assay. The specificity of the duplex real-time PCR was evaluated by assaying DNA extracts of a wide range of Chytridiomycota (Table 1). Real-time PCR efficiency, slope and r^2 were calculated with Bio-Rad CFX Manager v1.6 (BioRad Laboratories, Hercules, CA, USA) with the baseline subtracted curve fit setting. Slope and r^2 were calculated with the standard curves made with the quantitation standard described earlier. Efficiency was calculated as $10^{-1/\text{Slope}} - 1$. After the optimalization of the duplex real-time PCR, a protocol that includes adding bovine serum albumin (BSA) to the PCR mixture was validated as an alternative to diluting samples in order to alleviate PCR inhibition that could arise due to the nature of amphibian samples(9, 10). BSA (Sigma-Aldrich Inc., Bornem, Belgium) was added to the PCR mixture at a concentration of 400 ng μl^{-1}, as this is the optimal concentration to relief PCR inhibition (11). Four replicates of the

described quantitation standards of *Bd* and *Bs* with added BSA and four replicates without added BSA were run. Generated mean C_q values of both conditions were compared to evaluate any significant effect of adding BSA on basic PCR results.

Amphibian samples

To validate the real-time PCR to detect *Bd* and *Bs* in skin samples from amphibians, we applied the optimized protocol to samples from 1) ten *S. salamandra* experimentally inoculated with *Bs* 2) forty-one *S. salamandra* from the declining population in the Netherlands 3) fifty-one *S. salamandra* from a stable Belgian population and 4) twenty-seven Yellow-bellied toads (*Bombina variegata*) from a healthy Dutch population with known *Bd* infection (Table 4). The *Bs* infection experiment with fire salamanders was carried out with approval of the ethical committee of the Faculty of Veterinary Medicine (Ghent University, EC2013/10). Skin swabs were collected by gently rubbing a sterile cotton-tipped swabs 10 times across the ventral abdomen, inner thigh and hind limb digits (9, 12). From dead amphibians, pieces of skin taken from the ventral abdomen with an approximate size of 0.25 cm^2 were collected for analysis. DNA was extracted from skin swabs in 100 µl Prepman Ultra (Applied Biosystems, Foster City, CA, USA) (9). DNA was extracted from skin tissue using proteinase K digestion, following the protocol of Bandi et al. (13). After DNA extraction, 1:10 dilutions were prepared to minimize possible PCR inhibition (9) and stored at -20 °C until further use. All tested samples were run in both simplex and duplex real-time PCR to be able to compare variability in C_q values between the simplex and duplex runs. Samples that did not generate a signal were assigned a C_q value of 40 corresponding to the maximum number of cycles run in this real-time PCR setup. For positive samples, the number of GE per swab or total skin tissue was calculated with the quantitation standards. A sample was considered positive when the number of GE per swab/skin tissue exceeded 20, which, because of the dilution of the sample in the process of DNA extraction, corresponds to the detection limit of 0.1 GE per real-time PCR reaction.

Results & Discussion

Assay optimization

The primer and probe concentrations used in the duplex real-time PCR were the lowest concentrations that yielded the highest ΔR_n and the lowest quantification cycle (C_q) value respectively in a checkerboard system. This resulted in a PCR reaction mixture of 25 µl per

reaction composed of 12.5 µl Taqman PCR mix (1x iQ™ Supermix, BioRad Laboratories, Hercules, CA, USA), *Bs* forward primer STerF at a concentration of 0.3 µM, *Bs* reverse primer STerR at a concentration of 0.3 µM, *Bs* Cy5-probe STerC at a concentration of 0.1 µM, *Bd* forward primer ITS 1-3 Chytr at a concentration of 0.9 µM, *Bd* reverse primer 5.8S Chytr at a concentration of 0.9 µM, *Bd* FAM-probe Chytr MGB2 at a concentration of 0.15 µM and 5 µl template. The amplification conditions are identical to the conditions used in the *Bd*-specific real-time PCR (8), with the exception of an increase in annealing/extension temperature from 60 °C to 62 °C. No differences between PCR results were found when assaying serial tenfold dilution series of *Bd* DNA in triplicate with standard and elevated annealing/extension temperature. All *Bs* positive samples, as determined with the *Bs* PCR (6) or by immunohistochemistry (9), and positive controls generated a single peak in the SYBR green real-time PCR melting curve analysis with a constant melting temperature (Tm) of 75.5 °C. Gel electrophoresis of the PCR product of *Bs* positive samples and positive controls always generated a single DNA band at the expected length of approximately 160 base pairs. Negative samples and negative controls did not generate a peak in the melting curve analysis nor did they generate a visible band in gel electrophoresis, indicating that no aspecific binding of the primers occurred.

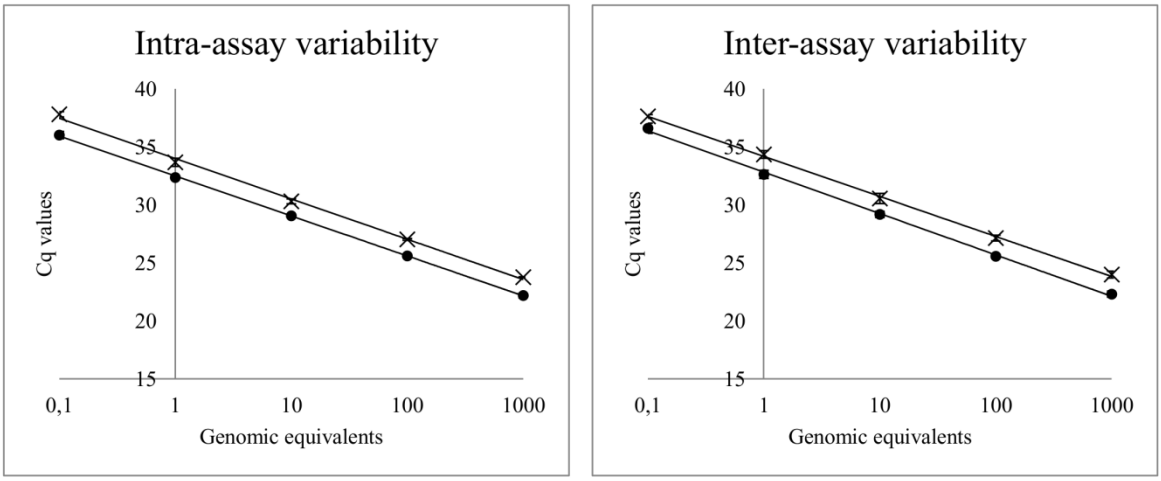

Figure 2. Standard curves for *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* generated with the duplex real-time PCR. Standard curves for *Batrachochytrium dendrobatidis* (×) and *Batrachochytrium salamandrivorans* (•) generated by assaying triplicates of quantitation standards. Error bars represent the standard deviations of the assayed quantitation standard triplicates.

Sensitivity and specificity

The sensitivity of the duplex real-time Taqman PCR was tested with the described quantitation standards of *Bd* and *Bs*. Triplicates of serial dilution series ranging from 0.01 GE to 1000 GE of zoospores of both pathogens were assayed with the duplex real-time PCR (Figure 2). Although some of the 0.01 GE samples did generate C_q values, these were not consistent. The remaining concentrations of the tenfold dilution series of both pathogens were detected with the duplex real-time PCR in all replicates. This demonstrates that the limit of detection of the *Bs* component of this duplex real-time PCR is 0.1 GE per PCR reaction which is similar to that for *Bd* in the *Bd*-specific real-time PCR (8). A detection limit lower than 1 *Bs* GE suggests the presence of a high copy number of the ITS-1 region, as was already demonstrated for certain *Bd* strains (14, 15). This ability of the duplex real-time PCR to detect both pathogens at low levels makes it ideal for early pathogen detection in environmental screening and in the diagnosis of chytridiomycosis. To assure that no interference occurs when DNA of both *Bd* and *Bs* is present in a sample, quantitation standards with DNA of both pathogens were assayed. This resulted in C_q values highly similar to the values obtained with single strain standards, indicating that an accurate quantification can be done in samples containing DNA of both pathogens. To verify the specificity of the duplex real-time PCR, a total of 10 different isolates belonging to the class of Chytridiomycota, including the *Bd* and the *Bs* type strain, were assayed (Table 1). The real-time PCR only amplified *Bd* and *Bs* of all included isolates indicating a high degree of specificity.

Assay performance and precision

Assay performance and precision were evaluated with the described quantitation standards of *Bd* and *Bs*. High linear correlation ($r^2 > 0.995$) and amplification efficiency (> 94%) for *Bd* and *Bs* in intra- as well as in inter-assay variability experiments together with low (< 1%) intra- and inter-assay variability demonstrate that the developed duplex real-time PCR has a good performance over the tested quantitation range with highly reproducible results (Table 2). These traits make the duplex real-time PCR highly suitable for use in screening surveys and aiding in disease diagnostics.

PCR Protocol with bovine serum albumin

Adding BSA to the duplex real-time PCR mixture at a final concentration of 400 ng μl^{-1} did not significantly affect the generated C_q values (T-test, $p > 0.05$) (Table 3). This allows BSA to be used as an alternative method to sample dilution in order to alleviate influence of PCR inhibitors present in amphibian samples.

Amphibian samples

To validate the developed duplex real-time PCR amphibian skin swabs and skin tissue were assayed (Table 4). The samples included known negative and positive *Bd* and *Bs* samples. All tested samples were run in both simplex and the duplex real-time PCR to compare variability in C_q values between the simplex and duplex runs (Figure 3). Very little variation was found between the results of the duplex real-time PCR and both simplex real-time PCR's as indicated by a high degree of correlation ($r^2 > 0.995$) for the C_q values of the simplex and duplex runs for both *Bd* and *Bs*. In the setup used in this study the lowest detectable number of GE per swab was 20 which corresponds to 0.1 GE per PCR reaction. Dilution occurring in the process of DNA extraction and in preventing PCR inhibition account for this difference in detection limit between swab and reaction. All samples that tested negative in the *Bd* and *Bs* simplex PCR's also tested negative in the duplex PCR. The samples that tested positive in the *Bd* or *Bs* simplex PCR also tested positive for the corresponding pathogen in the duplex PCR. The non-invasive sampling technique (skin swabbing) resulted in overall lower GE numbers when compared to the invasive technique (skin tissue collection) (Table 4). In the *S. salamandra* samples taken from the declining Dutch population, skin swabs were collected from live and apparently healthy animals, while skin tissues were collected from animals found dead on site. A feasible explanation for the higher number of GE found in the skin tissue samples could therefore be due to a further progressed disease state of the animals accompanied by increased infection intensity. The smaller difference between GE numbers in the skin swab and skin tissue samples from the *Bs* positive *S. salamandra* in the *Bs* infection experiment could be explained by the short period of time between swabbing and the animals dying due to the infection with *Bs*. For *Bd*, a threshold in infection intensity (mean GE >10000 per swab) predicts if amphibian populations will decline due to *Bd* infection (16). In the *Bs* infection experiment mean *Bs* GE per swab is comparable to this threshold (mean GE 6920 per swab), indicating that this could also be the case for *Bs*.

Species	Class	Order	Isolate	Amplification
Batrachochytrium dendrobatidis	Chytridiomycetes	Rhizophydiales	JEL423	Yes (*Bd*)
Batrachochytrium salamandrivorans	Chytridiomycetes	Rhizophydiales	AMFP13/1	Yes (*Bs*)
Homolaphlyctis polyrhiza	Chytridiomycetes	Rhizophydiales	JEL142	No
Rhizophlyctis rosea	Chytridiomycetes	Rhizophlyctidales	JEL532	No
Gaertneriomyces semiglobifer	Chytridiomycetes	Spizellomycetales	JEL384	No
Rhizoclosmatium globosum	Chytridiomycetes	Chytridiales	JEL791	No
Podochytrium dentatum	Chytridiomycetes	Chytridiales	JEL30	No
Polychytrium aggregatum	Chytridiomycetes	Polychytriales	JEL109	No
Geranomyces variabilis	Chytridiomycetes	Spizellomycetales	JEL518	No
Monoblepharis polymorpha	Monoblepharidomycetes	Monoblepharidales	JEL486	No

Table 1. An overview of the Chytridiomycota isolates used to verify the specificity of the real-time duplex PCR for *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans*.

The component of the duplex real-time PCR that showed amplification in positive samples is indicated between brackets in the amplification column.

	Batrachochytrium dendrobatidis		*Batrachochytrium salamandrivorans*	
	Intra-assay	Inter-assay	Intra-assay	Inter-assay
Efficiency (%)	94.1	99.4	95.7	96.0
Linear correlation (r^2)	0.997	0.996	0.999	0.997
Coefficient of variation (%)	0.56 (±0.34)	0.99 (±0.39)	0.39 (±0.48)	0.98 (±0.27)

Table 2. Assay precision, efficiency and linear correlation of the *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* real-time duplex PCR.

	Batrachochytrium dendrobatidis		*Batrachochytrium salamandrivorans*	
	With BSA	Without BSA	With BSA	Without BSA
1000 GE	24.95 (± 0.02)	23.96 (± 0.04)	23.00 (± 0.04)	22.90 (± 0.10)
100 GE	27.22 (± 0.03)	27.21 (± 0.07)	26.32 (± 0.06)	26.34 (± 0.17)
10 GE	30.48 (± 0.10)	30.55 (± 0.11)	29.69 (± 0.15)	29.67 (± 0.17)
1 GE	33.53 (± 0.10)	33.67 (± 0.32)	33.32 (± 0.62)	32.99 (± 0.19)
0.1 GE	37.33 (± 0.17)	37.43 (± 0.23)	36.18 (± 0.23)	36.18 (± 0.16)

Table 3. Effect of adding BSA (400 ng μl^{-1}) to the duplex real-time PCR mixture on C_q values generated with *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* quantitation standards. Four replicates with and four replicates without added BSA were assayed in order to evaluate the effect on standard dilutions of *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* DNA. No significant difference was found between the two conditions for any of the dilutions for both pathogens (T-test, $p > 0.05$).

Amphibian species	Origin	Health status	Sample type and amount	PCR positive	Mean GE value positives (Min – Max)
Fire salamander (*Salamandra salamandra*)	Bunderbos, the Netherlands (N50°55', E5°45'), 2010	Declining	Skin swab (33) Skin tissue (8)	13 (*Bs*) 4 (*Bs*)	219.8 (60 - 1750) 2398.3 (242 – 10180)
	Merelbeke, Belgium (N50°57'; E3°43'), 2012	Healthy	Skin swab (51)	0	N/A
	Bs infection experiment (Martel et al., PNAS), 2013	Diseased	Skin swab (5) Skin tissue (5)	5 (*Bs*) 5 (*Bs*)	6920 (1572 – 10740) 10915 (3420 – 19380)
	Bs infection experiment (Martel et al., PNAS), 2013	Healthy	Skin swab (5) Skin tissue (5)	0 0	N/A N/A
Yellow-bellied toad (*Bombina variegata*)	't Rooth, The Netherlands (N50°50', E5°47'), (Spitzen-van der Sluijs et al., 2013)	Healthy	Skin swab (27)	14 (*Bd*)	175.4 (20 – 1488)

Table 4. An overview of the amphibian samples used to validate the real-time duplex PCR for *Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrivorans* (*Bs*).
Mean values of genomic equivalents of zoospores (GE) per swab of *Bd* and *Bs* of the positive samples were calculated with the included quantitation standards for *Bd* and *Bs*.

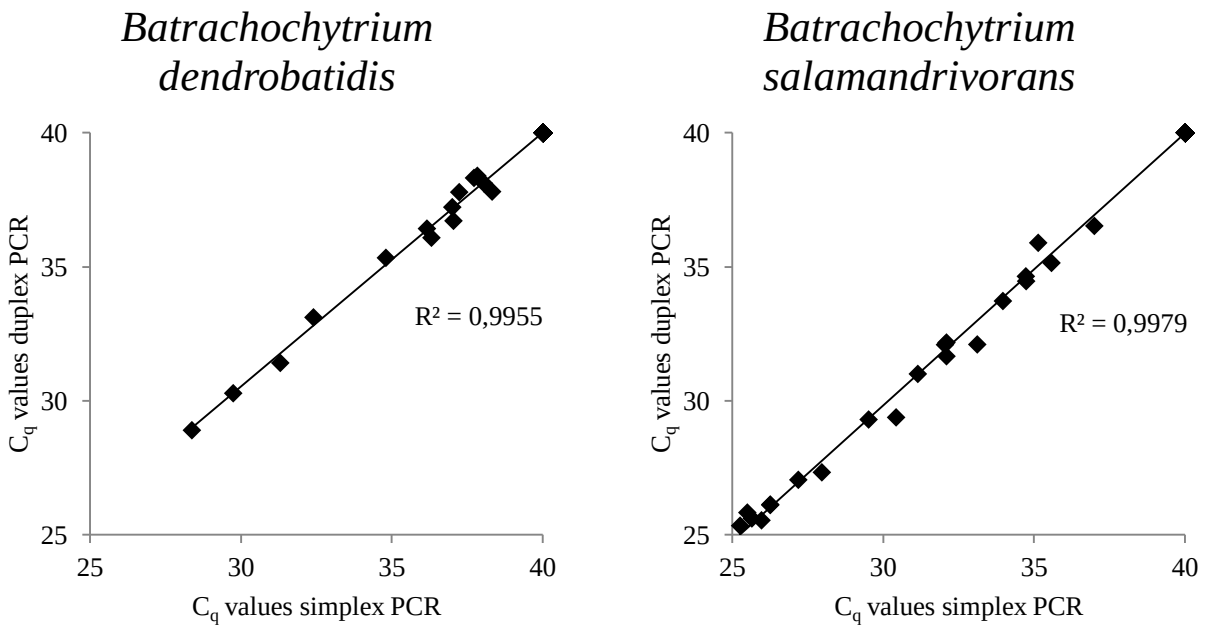

Figure 3. C_q values generated by assaying amphibian samples with the *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* duplex real-time PCR and both simplex real-time PCR's. An overview of all assayed amphibian samples is presented in Table 1. Samples that did not generate a signal have been assigned a C_q value of 40 corresponding to the maximum number of cycles run in this real-time PCR setup.

Conclusion

The described *Bd* and *Bs* duplex real-time PCR can be used to accurately and reliably detect both pathogens in amphibian samples. The real-time PCR can be used to aid in chytridiomycosis disease diagnosis and in mapping worldwide distribution of both *Bd* and *Bs*.

Acknowledgements

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Author contributions statement

M.B. contributed in the design of the duplex real-time PCR. M.B. performed all optimization and validation experiments. M.B. contributed in writing and reviewing of the manuscript.

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Treatment of urodelans based on temperature dependent infection dynamics of *Batrachochytrium salamandrivorans*

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Abstract

The recently emerged chytrid fungus *Batrachochytrium salamandrivorans* currently causes amphibian population declines. We hypothesized that temperature dictates infection dynamics of *B. salamandrivorans*, and that therefore heat treatment may be applied to clear animals from infection. We examined the impact of environmental temperature on *B. salamandrivorans* infection and disease dynamics in fire salamanders (*Salamandra salamandra*). Colonization of salamanders by *B. salamandrivorans* occurred at 15 °C and 20 °C but not at 25 °C, with a significantly faster buildup of infection load and associated earlier mortality at 15 °C. Exposing *B. salamandrivorans* infected salamanders to 25 °C for 10 days resulted in complete clearance of infection and clinically cured all experimentally infected animals. This treatment protocol was validated in naturally infected wild fire salamanders. In conclusion, we show that *B. salamandrivorans* infection and disease dynamics are significantly dictated by environmental temperature, and that heat treatment is a viable option for clearing *B. salamandrivorans* infections.

Introduction

In the decades following the identification of *Batrachochytrium dendrobatidis* in 1999 (1) it became apparent that this chytrid fungus was one of the biotic drivers of declines and extinctions of hundreds of amphibian species worldwide (2-5). However, the impact of *B. dendrobatidis* varies regionally from a dramatic decrease of amphibian diversity to a state of host-pathogen equilibrium. In one such region characterized by the co-existence of *B. dendrobatidis* with local amphibian communities (6), another recently described chytrid fungus, *Batrachochytrium salamandrivorans* (7), caused amphibian population declines. The reason for this obvious difference in disease dynamics between both chytrid fungi is not known. Disease dynamics are dictated by pathogen virulence, host factors and environmental determinants. Virulent strains of both chytrids, as well as susceptible host species are present in the affected regions (8-11). For *B. dendrobatidis*, temperature is considered a key environmental factor (12-14). One major difference between both chytrids is their different thermal growth characteristics, which is probably due to differences in host spectrum, *B. salamandrivorans* being restricted to urodelan hosts (15). Knowledge of the infection dynamics of *B. salamandrivorans* at different temperatures may help to develop treatment protocols (16-18). These are urgently needed as current therapies developed against *B. dendrobatidis* (19) fail to eliminate *B. salamandrivorans* from infected amphibians (unpublished results). We hypothesized that temperature dictates infection and disease dynamics of *B. salamandrivorans* in salamanders, which may be applied to develop a heat treatment protocol to clear infection in animals.

Results & Discussion

Only after exposure at 15 °C or 20 °C but not at 25 °C, the salamanders were colonized by *B. salamandrivorans*. If a 10000 GE infection load per swab is considered indicative for a clinical threshold (20-22), this threshold was reached two times earlier at 15 °C than at 20 °C (on average 15 ± 4 (SD) days and 31 ± 12 days (SD) respectively, independent *t*-test $p < 0.05$) (Fig. 1). The faster buildup of *B. salamandrivorans* infection loads coincided with earlier mortality at 15 °C than at 20 °C (22 ± 8 (SD) days and 35 ± 14 (SD) days respectively, Cox regression analysis, $\chi^2 = 3.941$, df = 1, $p < 0.05$) (Fig. 1 and 2). Besides preventing infection of salamanders with *B. salamandrivorans*, exposure of infected salamanders to a temperature of 25 °C during 10 days completely eliminated the infection and resolved *B.*

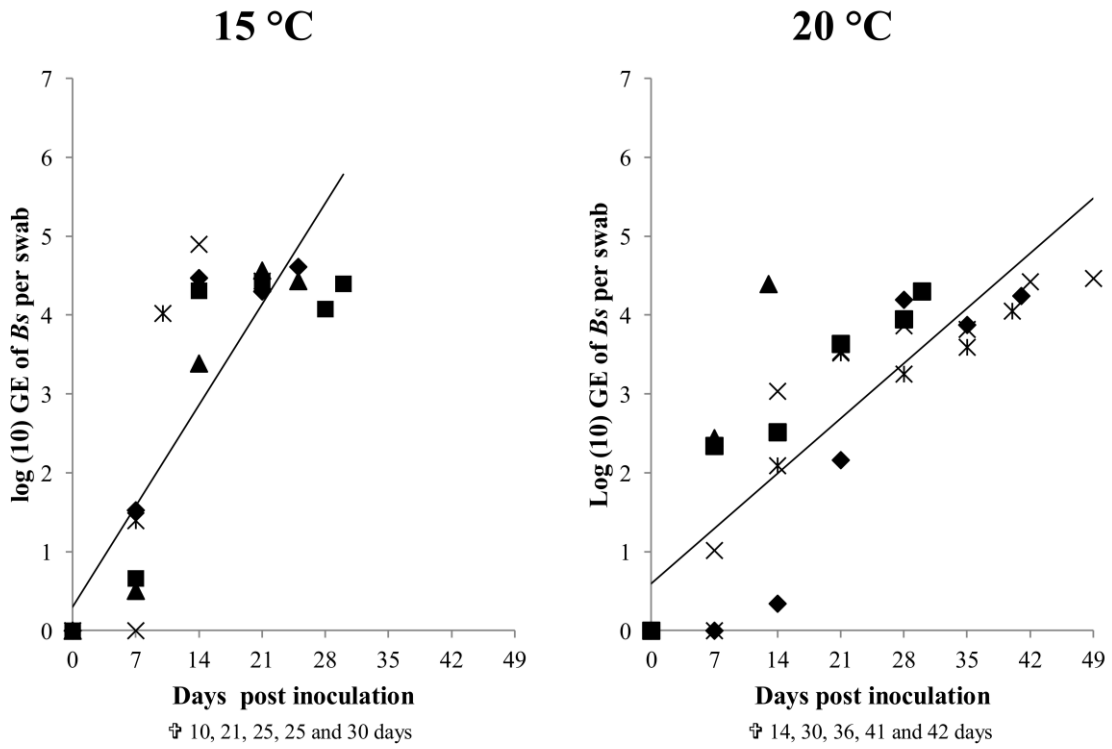

Fig 1. The course of *Batrachochytrium salamandrivorans* infection in fire salamanders at 15 and 20 °C. Each symbol represents the course of infection of an individual animal. Time of death of all animals is depicted beneath the graphs. The line represents the average increase in infection intensity in all tested individuals based on a repeated measure regression analysis.

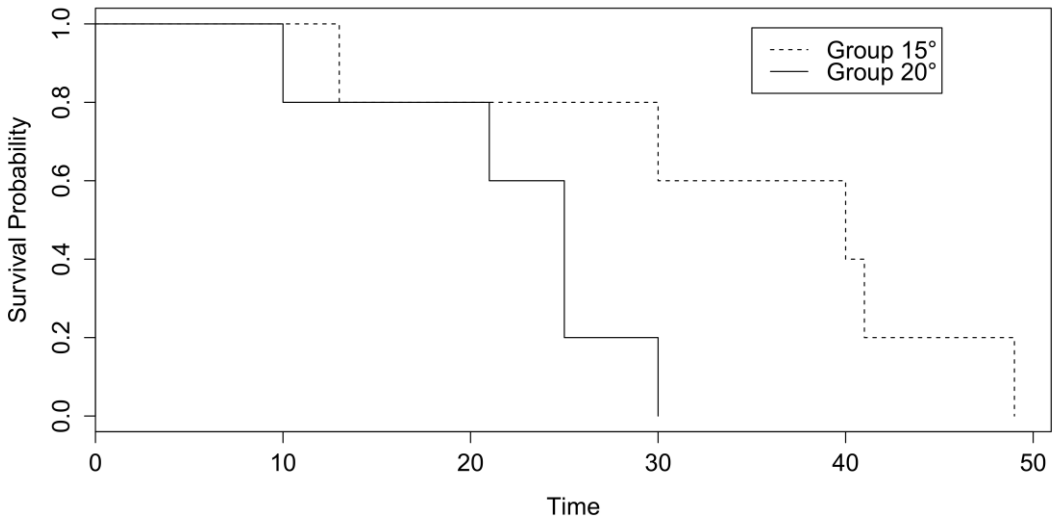

Fig 2. The probability of survival of salamanders housed at 15 or 20 °C after infection with *Batrachochytrium salamandrivorans*. Survival probability was plotted based on a Cox regression analysis (χ^2 = 3.981, df = 1, $p < 0.05$). Time is displayed in days after initial infection. The dotted line represent survival probability of *Batrachochytrium salamandrivorans* infected salamanders housed at 15 °C and the full line those housed at 20 °C.

salamandrivorans lesions from all infected animals (Fig. 3 and 4). However, 7 days exposure at 25 °C did not result in fungal clearance since recrudescence of infection was observed in all these salamanders within 1-3 weeks after transferring them to an ambient temperature of 15 °C (Fig. 4). This is remarkable since cultures of the fungus are killed *in vitro* within 5 days of incubation at 25 °C (7) and shows that *B. salamandrivorans* is capable to persist in an urodelan host experiencing temperatures that temporarily surpass the fungal thermal maximum for up to one week. Exposure of the relapsing animals to 25 °C for 10 days eliminated the infection. Our results reflect *B. salamandrivorans* growth curves obtained *in vitro*, with an optimal growth range around 15 °C (7). In contrast, the pattern of temperature-dependent growth of *B. dendrobatidis* at 15, 20 and 25 °C on frogs was opposite to the pattern of temperature-dependent growth at these temperatures in culture (23), and time until death in frogs infected with *B. dendrobatidis* at 27 °C, which is above *B. dendrobatidis'* thermal preference (24), was shorter when compared to time until death of infected frogs kept at lower temperatures (14). The suitability of raised ambient temperature as treatment option was validated by keeping 30 wild-caught *B. salamandrivorans* infected fire salamanders at 25 °C during 10 days. Twenty-six animals were cured of *B. salamandrivorans* infection after this treatment period, 2 died early during treatment, and 2 needed an additional treatment period of 2 days in order to completely clear the infection (Fig. 5). This shows that heat treatment is a viable treatment option for *B. salamandrivorans* infected amphibians when the clinical condition and the thermal tolerance of the animal is taken into account. In order to completely eliminate *B. dendrobatidis* infections higher temperatures, composed of short exposure to 37 °C (16) or extended exposure to 30 °C (17) are required. These protocols are not suitable for treating salamanders, as these temperatures surpass the upper thermal limit of most urodelans.

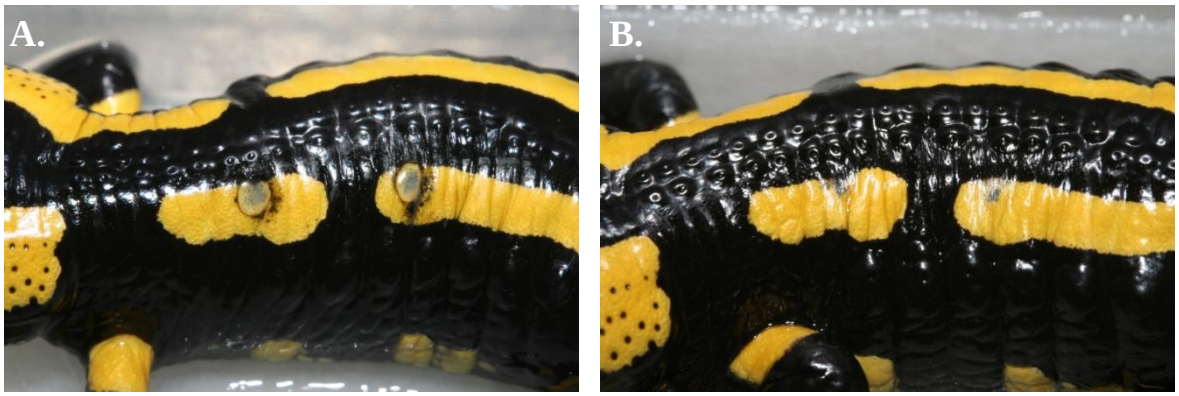

Fig 3. Heat treatment of amphibians infected with *Batrachochytrium salamandrivorans* clears infection and resolves associated lesions. *Batrachochytrium salamandrivorans* associated skin lesions (A) are clearly reduced after the heat treatment composed of keeping the animals at 25 °C during 10 days (B), and will eventually completely resolve.

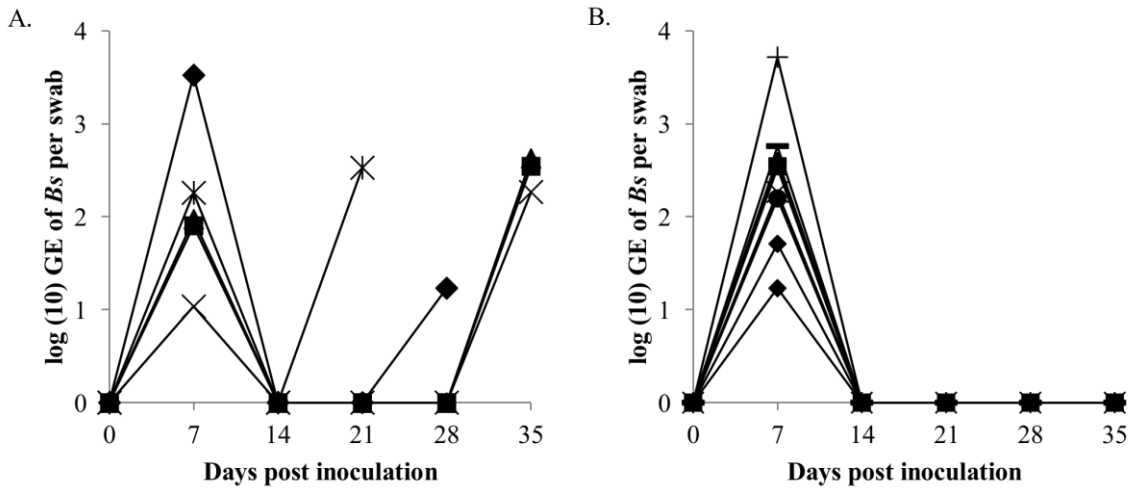

Fig 4. The effect of exposure to 25 °C for 7 and 10 days on the course of *Batrachochytrium salamandrivorans* infection in fire salamanders. After establishment of infection fire salamanders were subjected to an ambient temperature of 25 °C for 7 days (A), or 10 days (B). Each symbol represents the course of infection of an individual animal.

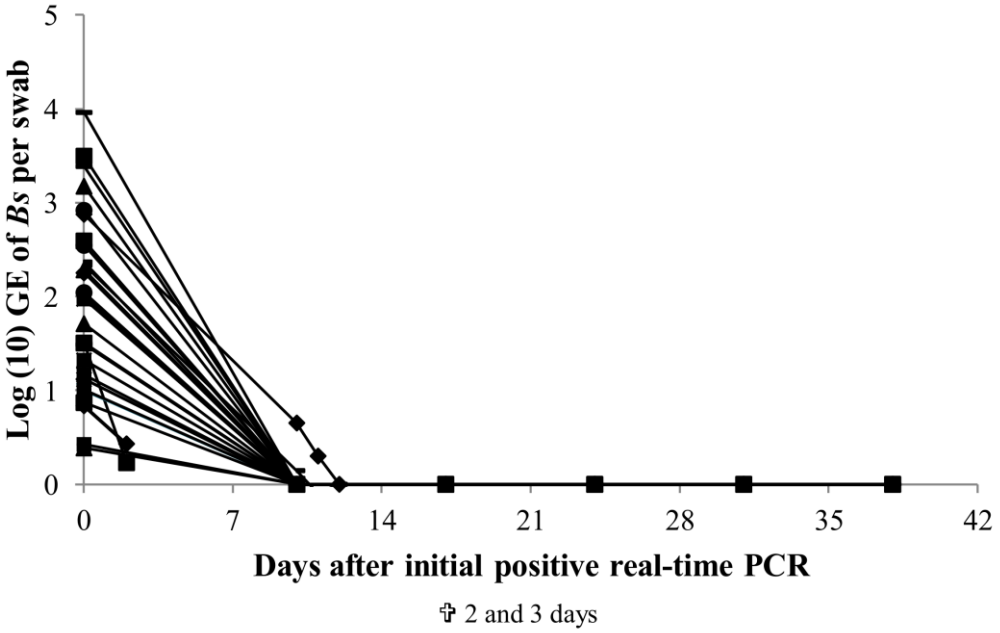

Fig 5. Heat treatment composed of exposure to 25 °C for 10 days of fire salamanders naturally infected with *Batrachochytrium salamandrivorans*. After ascertaining presence of *B. salamandrivorans* in all animals they were subjected to an ambient temperature of 25 °C for 10 days. Each symbol represents the course of infection of an individual animal. Time of death for the 2 deceased animals is displayed beneath the graph.

The 2 animals that died were in poor clinical condition at the start of the treatment period, and probably died due to thermal shock as *B. salamandrivorans* loads were low at time of death. This points out the narrow margin between the temperature able to eliminate *B. salamandrivorans* and the upper thermal limit most urodelans tolerate. Furthermore, these results show that the course of infection should be carefully monitored since not all animals tested negative for presence of *Bs* DNA after 10 days at 25 °C. Although we do not think that this is a result of an active infection but explained by presence of residual *B. salamandrivorans* DNA derived from dead *B. salamandrivorans* cells, this remains uncertain. This could have been further elucidated by transferring the animals back to 15 °C after 10 days but we chose to keep them at 25 °C until PCR results became negative. Thermal treatment of *B. salamandrivorans* infected amphibians would allow large groups of animals to be treated simultaneously at low costs and lacks the possible downsides linked to drug treatment like toxicity or development of acquired antimicrobial resistance.

In conclusion, these results demonstrate that infection and disease dynamics of *B. salamandrivorans* in urodelans are significantly dictated by environmental temperature. The inability of *B. salamandrivorans* to survive for more than 10 days at 25 °C inside its host, renders temperature treatment of infected urodelans a safe, effective and low-cost treatment option, when taking into account the host thermal tolerance.

Methods

All experiments were performed in accordance with the relevant guidelines and regulations. All experiments with experimental animals were carried out with approval of the ethical committee of the Faculty of Veterinary Medicine, Ghent University.

Batrachochytrium salamandrivorans strain, culture conditions and experimental inoculation

The *B. salamandrivorans* type strain was grown in TGhL broth (16 g tryptone, 4 g gelatin hydrolysate, 2 g lactose per liter of distilled water) in 25 cm^3 cell culture flasks and incubated at 15 °C. To obtain *B. salamandrivorans* zoospores, a 2 ml aliquot of a 5-day-old culture was inoculated on TGhL agar plates (16 g tryptone, 4 g gelatin hydrolysate, 2 g lactose, 10 g bacteriological agar per liter of distilled water) and incubated at 15 °C for 5 – 7 days. Zoospores were collected by flooding the agar plates with 2 ml of distilled water and subsequent collection of the fluid. A hemocytometer was used to count the number of zoospores present in the suspension and the concentration of the zoospore suspension was

adjusted to 5 x 10^3 zoospores per mL. Animals were inoculated with *B. salamandrivorans* by topically applying one mL of the inoculum on the intact skin.

Animals

Experimental animals

Fire salamanders (*Salamandra salamandra*) were experimentally infected with *B. salamandrivorans* to study temperature dependent infection dynamics. The animal experiment was performed with the approval of the ethical committee of the Faculty of Veterinary Medicine (Ghent University, EC2013/87). Twenty-five captive bred fire salamanders were housed individually in plastic containers in a climatized room with an ambient temperature of 15 °C. The animals were kept on a moist tissue, with access to a hiding place and water container. Crickets powdered with mineral and vitamin supplement were provided ad libitum as food source. All animals were clinically healthy and free of *B. dendrobatidis* and *B. salamandrivorans* as determined by duplex real-time PCR of skin swabs (20). An acclimatization period of 1 week was admitted before the start of the experiment.

Field outbreak animals

Heat treatment to clear *B. salamandrivorans* infections in amphibians was validated on 30 wild fire salamanders found to be infected with *B. salamandrivorans* as determined by real-time PCR. These animals originated from a population in Robertville Belgium (50°29'58.6"N 6°06'21.9"E) undergoing a *B. salamandrivorans* outbreak event and were translocated to the research facility with permission (2014/RS/nº23). Housing conditions of these animals were identical to the conditions described for the experimental animals.

Temperature dependency of *Batrachochytrium salamandrivorans* infection dynamics in salamanders

The experimental animals were randomly assigned to one of the 5 groups (5 animals per group, kept individually). The purpose of the 5 groups was to assess whether *B. salamandrivorans* was able to colonize the animals at different temperatures (groups 1 to 3), and whether temperature could be applied to clear *B. salamandrivorans* from colonized animals (groups 4 and 5). In group 1, animals were inoculated and subsequently kept at 15 °C, in group 2 kept at 20 °C and in group 3 kept at 25 °C (the animals kept at 20 and 25 °C were placed in incubators set at the corresponding temperature). The animals in group 4 and 5 were inoculated at 15 °C and put at 25 °C for 7 or 10 days respectively, after *B. salamandrivorans*

infection was established (determined as an increase in infection load between two subsequent samplings). To determine whether the infection would recrudesce after the 25 °C exposure, salamanders of groups 4 and 5 were put back at 15 °C afterwards and were followed up for another 3 weeks. In case of recrudescence of infection, the animals were put back at 25 °C for 10 days. During the experiment, all animals were checked daily for the presence of clinical signs. Skin swabs for *B. salamandrivorans* real-time PCR analysis were collected once every 7 days and/or at the time of death of the animals. An animal was considered negative for *B. salamandrivorans* infection after 4 consecutive negative real-time PCR results. Real-time PCR's were performed on a CFX96 Real Time System (Biorad, Hercules, California, USA) with amplification conditions, primer, and probe concentrations as described elsewhere (20). Infection loads are presented as genomic equivalents (GE) of *B. salamandrivorans* zoospores. Results were analyzed by means of independent *t*-test and Cox regression analysis using respectively the mass (25) and survival library in R (26). The censored response variable for the Cox regression analysis was time until death with temperature (15 or 20 °C) as explanatory variable.

Thermal treatment of *Batrachochytrium salamandrivorans* infected salamanders

Based on the results of the thermal infection experiments, the *B. salamandrivorans* infected field outbreak animals were treated by putting them at 25 °C for 10 days. Skin swabs for *B. salamandrivorans* real-time PCR analysis were collected after 10 days and subsequently every 7 days or at the time of death of the animals. Animals that remained positive after the heat treatment at 25 °C during 10 days were kept at 25 °C and swabbed daily to follow up remaining infection intensities until the first negative real-time PCR result and subsequently every 7 days. An animal was considered negative for *B. salamandrivorans* infection after 4 consecutive negative real-time PCR results. Real-time PCR's were performed as described above.

Additional information

Author contributions statement

M.B. contributed in the design of the experiments. M.B. performed the experiments. M.B. contributed in writing and reviewing of the manuscript.

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Successful treatment of *Batrachochytrium salamandrivorans* infections in salamanders requires synergy between voriconazole, polymyxin E and temperature

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Abstract

Chytridiomycosis caused by the chytrid fungus *Batrachochytrium salamandrivorans* poses a serious threat to urodelan diversity worldwide. Antimycotic treatment of this disease using protocols developed for the related fungus *Batrachochytrium dendrobatidis*, results in therapeutic failure. Here, we reveal that this therapeutic failure is partly due to different minimum inhibitory concentrations (MICs) of antimycotics against *B. salamandrivorans* and *B. dendrobatidis*. *In vitro* growth inhibition of *B. salamandrivorans* occurs after exposure to voriconazole, polymyxin E, itraconazole and terbinafine but not to florfenicol. Synergistic effects between polymyxin E and voriconazole or itraconazole significantly decreased the combined MICs necessary to inhibit *B. salamandrivorans* growth. Topical treatment of infected fire salamanders (*Salamandra salamandra*), with voriconazole or itraconazole alone (12.5 µg/ml and 0.6 µg/ml respectively) or in combination with polymyxin E (2000 IU/ml) at an ambient temperature of 15 °C during 10 days decreased fungal loads but did not clear *B. salamandrivorans* infections. However, topical treatment of *B. salamandrivorans* infected animals with a combination of polymyxin E (2000 IU/ml) and voriconazole (12.5 µg/ml) at an ambient temperature of 20 °C resulted in clearance of *B. salamandrivorans* infections. This treatment protocol was validated in 12 fire salamanders infected with *B. salamandrivorans* during a field outbreak and resulted in clearance of infection in all animals.

Introduction

The rate at which amphibian populations have been declining the past decades is alarming (1, 2). One of the factors in part responsible for these declines is the infectious disease chytridiomycosis caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*B. dendrobatidis*)(3-5). Recently, the related chytrid fungus *Batrachochytrium salamandrivorans* (*B. salamandrivorans*) has been identified as a novel threat to amphibian populations, with a potentially major impact on salamander diversity worldwide (6,7). For amphibian chytridiomycosis caused by *B. dendrobatidis*, topical antimycotic treatment using voriconazole at a concentration of 1.25 µg/ml during 7 days has proven highly successful and safe (8). However, applying this treatment to *B. salamandrivorans* infected salamanders is unable to clear infections (see Case report section). Thermal treatment consisting of exposure to the critical thermal maximum for *B. salamandrivorans* (25 °C) for 10 days was shown to be able to clear *B. salamandrivorans* infections from infected salamanders (9). However, this temperature approaches the critical thermal maximum of several urodelans (10), rendering this treatment of limited use for those species. Therefore the aim of this study was to develop an antifungal treatment protocol able to eliminate *B. salamandrivorans* in infected salamanders at temperatures below the critical thermal maximum of *B. salamandrivorans*, tolerated by most salamanders.

Case report

Thirty-nine fire salamanders (*Salamandra salamandra*) from a population in the Netherlands undergoing dramatic declines from 2008 onwards due to *B. salamandrivorans*(11) were included in an *ex situ* conservation program. Since the susceptibility of *B. salamandrivorans* to antimycotics was not known, the animals were treated with voriconazole (1.25 µg/ml, topical spray, twice a day for 7 days), based on the treatment protocol used to clear *B. dendrobatidis* infections from amphibians (8). Skin lesions, lethargy and inappetite did not resolve in the *B. salamandrivorans* infected animals.

Results & Discussion

In vitro susceptibility of *B. salamandrivorans* to antimycotic compounds

The results of the experiments to determine the MICs of the tested antimicrobials for *B. salamandrivorans* are summarized in Table 1. Florfenicol was the only compound tested that

was not able to limit growth or kill *B. salamandrivorans* at the concentrations tested. In contrast, florfenicol is capable of limiting growth of *B. dendrobatidis* at concentration of 0.5 – 1.0 µg/ml (12). Interestingly, the inhibitory concentrations of the other compounds against *B. salamandrivorans* differed noticeably from those against *B. dendrobatidis*. The mechanism underlying this difference remains unknown. Whereas polymyxin E did not show any inhibitory potential in *B. dendrobatidis* MIC tests at the concentrations used (12), *B. salamandrivorans* was inhibited by polymyxin E at a concentration of 8000 IE/ml (Table 1). Terbinafine limited *B. salamandrivorans* growth at a concentration of 0.2 µg/ml, which is in accordance with its activity against *B. dendrobatidis* at 0.063 µg/ml (19). Itraconazole, which is frequently used to treat amphibians infected with *B. dendrobatidis*, had a MIC against *B. salamandrivorans* 2.5 - 5 times lower (0.006 µg/ml) compared to its MIC against *B. dendrobatidis* (0.016 – 0.032 µg/ml) (19). Finally, the MIC of voriconazole for inhibiting *B. salamandrivorans* growth was 10 times higher (0.125 µg/ml) than the MIC for inhibiting *B. dendrobatidis* (0.0125 µg/ml) (8). This result at least partly explains the failed initial treatment of the wild fire salamanders using the voriconazole dosage for treating chytridiomycosis in amphibians infected with *B. dendrobatidis* (1.25 µg/ml sprays, twice a day for 7 days) (8). The concentrations to completely kill *B. salamandrivorans* cultures were all close to the MIC (Table 1, one dilution higher for all compounds).

Compound	2-fold dilution range	MIC value	100% killing concentration
Florfenicol	0.016 – 8 µg/ml	> 8 µg/ml	> 8 µg/ml
Voriconazole	0.016 – 8 µg/ml	0.125 µg/ml	0.25 µg/ml
Polymyxin E	1250 – 64000 IE/ml	8000 IE/ml	16000 IE/ml
Itraconazole	0.003 – 1.2 µg/ml	0.006 µg/ml	0.012 µg/ml
Terbinafine	0.1 – 12.5 µg/ml	0.2 µg/ml	0.4 µg/ml

Table 1. Susceptibility of *Batrachochytrium salamandrivorans* to antimicrobial compounds. An overview of the tested two-fold serial dilution range of the antimicrobial compounds, the minimum inhibitory concentrations (MICs) for *Batrachochytrium salamandrivorans* and the concentrations that completely killed *Batrachochytrium salamandrivorans* after an exposure period of 10 days.

Synergy between polymyxin E and voriconazole or itraconazole in inhibiting *B. salamandrivorans* growth

The three main techniques used for testing interactions between compounds in antifungal activity are Etest, time-kill methods and checkerboard dilution methods (20-23). In synergy testing for bacterial pathogens, the biggest disadvantage is that no two methods will produce comparable results, and therefore clinical applicability of results is under debate (16). These

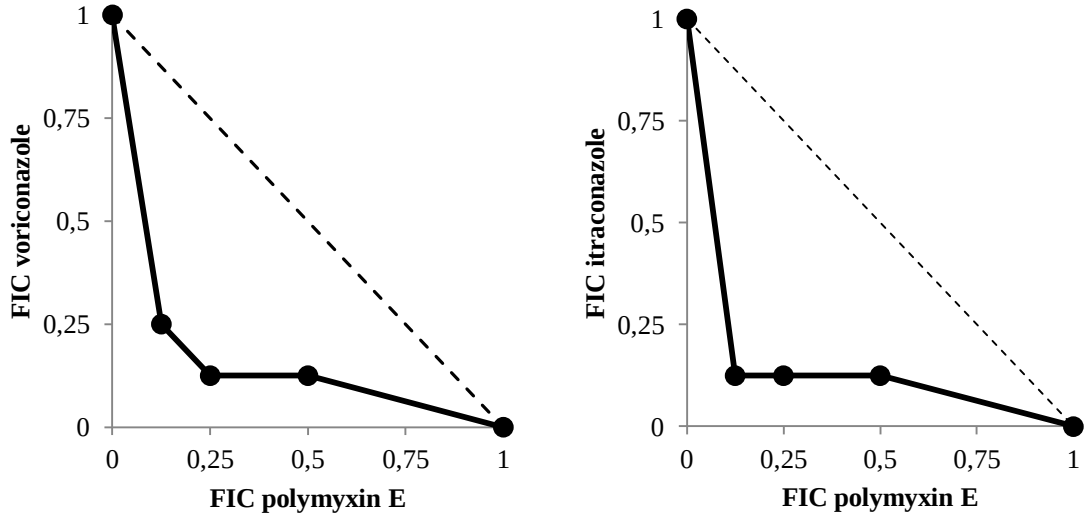

Figure 1. FIC Isobolograms showing the interactions between two antimicrobial agents in inhibiting *Batrachochytrium salamandrivorans* growth. FIC values derived from combinations of voriconazole, itraconazole and polymyxin E were used to plot the isobolograms. A FIC value of 1 corresponds to the MIC value of the particular antimicrobial agent. The dotted line represents the theoretical additive interaction between two agents (see text for our definition of the types of interactions).

limitations also apply for antifungal synergy testing (23). Furthermore, a vast amount of studies exist that describe *in vitro* synergy without linking (or being able to link) these results to a beneficial treatment outcome of combined treatment (16). The goal of this study was to evaluate potential synergy between antimycotic compounds in inhibiting *B. salamandrivorans* growth to allow development of an experimental treatment protocol using antifungal concentrations below toxicity levels. In this study, a checkerboard dilution method adopted from the method used to evaluate minimum inhibitory concentrations for *B. dendrobatidis* (8,12) was used, which in comparison to the time-kill method, is easier to carry out and interpret (23). The combinations of compounds tested both included an azole antifungal (voriconazole or itraconazole) and polymyxin E, which were already shown to be able to inhibit *B. salamandrivorans* growth (Table 1). Apart from polymyxins exerting antifungal activity on their own, combinations of polymyxins and azole antifungals showed synergistic

antifungal activity against infections with *Aspergillus spp*., *Candida spp*. and *Cryptococcus spp* (24-26). The bactericidal activity of polymyxin E against Gram-negative bacteria is an added advantage for treating *B. salamandrivorans* associated lesions, since histological preparations of skin samples of salamanders infected with *B. salamandrivorans* often revealed severe bacterial overgrowth of the skin in concordance with *B. salamandrivorans* infection (6). Secondary bacterial infections in immunocompromised amphibians are often caused by opportunistic Gram-negative bacteria (27,28). The azole antifungals voriconazole and itraconazole both have reported effectiveness in treating chytridiomycosis in amphibians caused by infections with *B. dendrobatidis* (8,29,30). Negative side effects of treating amphibians with itraconazole have been reported though (29-31), so a combination therapy that would allow concentrations of itraconazole to be used below toxicity levels to successfully treat chytridiomycosis could be a major advantage. The results of the experiments to determine the FICs of polymyxin E combined with voriconazole or itraconazole are graphically depicted in isobolograms (Figure 1). Two of the tested combinations of polymyxin E with voriconazole (2000 IE/ml + 0.02 µg/ml and 1000 IE/ml + 0.03 µg/ml) and two combinations of polymyxin E with itraconazole (2000 IE/ml + 0.0016 µg/ml and 1000 IE/ml + 0.0016 µg/ml) resulted in a FICI that demonstrates synergism (FICI $\leq$ 0.5, figure 1). All combinations that inhibited *B. salamandrivorans* growth also killed *B. salamandrivorans* completely after 10 days of incubation.

Effective treatment of *B. salamandrivorans* infections in fire salamanders based on synergy between polymyxin E, voriconazole and temperature

All initially tested treatment conditions, composed out of itraconazole or voriconazole alone and in combination with polymyxin E were unable to clear *B. salamandrivorans* infections from infected amphibians (Figure 2, panels A-E). Although the combination therapies of itraconazole or voriconazole with polymyxin E did reduce *B. salamandrivorans* infection loads to undetectable levels in 3 out of the 5 animals and 5 out of 5 animals respectively, recrudescence of infection did occur in all animals (Figure 2, panels C and E). The difference between *in vitro* and *in vivo* effects of the combination therapy at 15 °C might lie in the exposure to the compounds; in the *in vitro* experiments, *B. salamandrivorans* was exposed continuously to both compounds as opposed to the periodical exposure in the *in vivo*

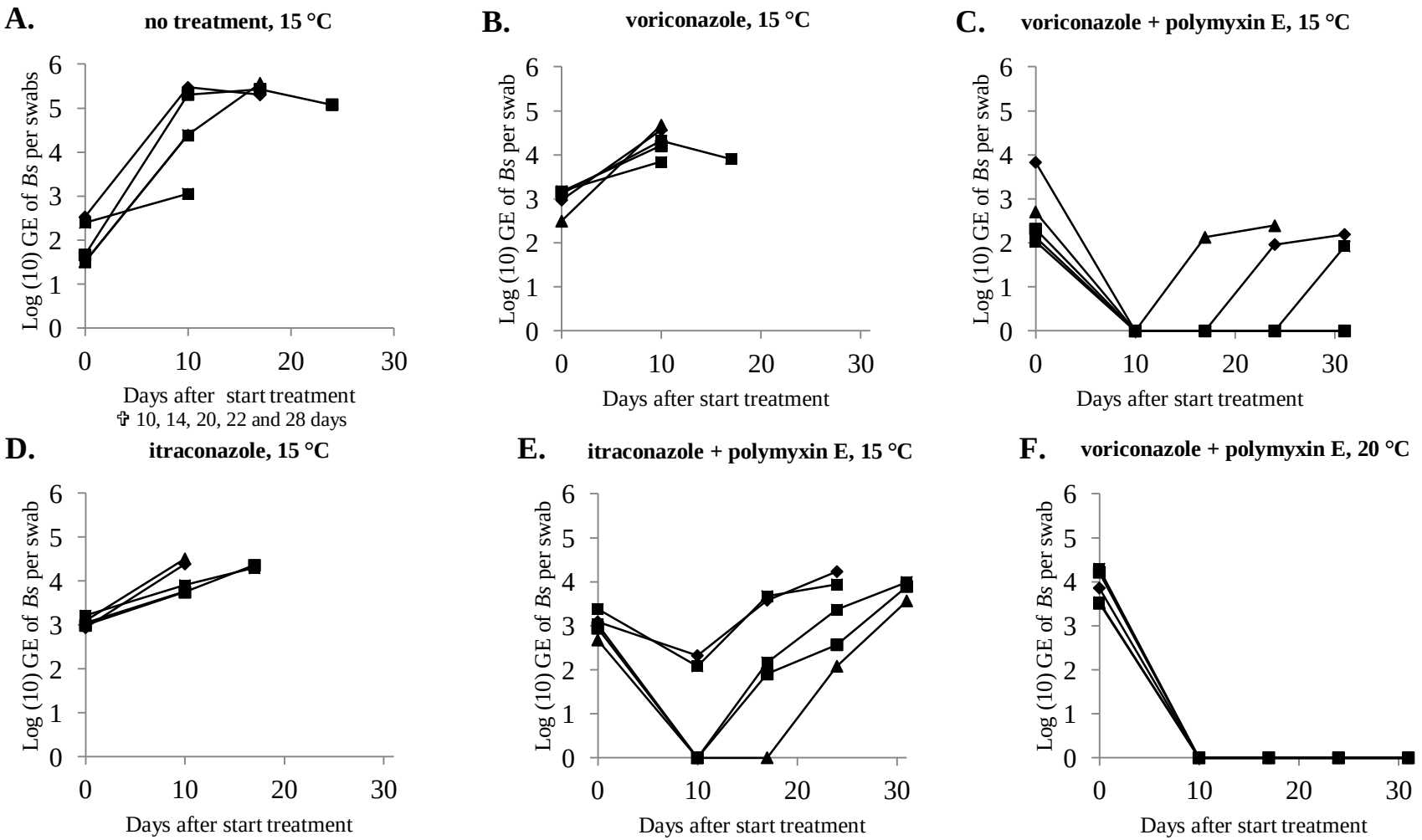

Figure 2. Results of five treatment protocols to clear *Batrachochytrium salamandrivorans* infections from experimentally infected fire salamanders. After ascertaining presence of *Batrachochytrium salamandrivorans* in all animals (day 0) they were either left untreated as control (A), treated twice a day for 10 days with voriconazole sprays (12.5 µg/ml) (B) or itraconazole sprays (0.6 µg/ml) (D) alone or with a combination of polymyxin E submersion baths (2000 IU/ml, 10 minutes) followed by spraying voriconazole (12.5 µg/ml) (C) or itraconazole (0.6 µg/ml) (E). Conditions A-E were all performed at an ambient temperature of 15 °C, condition F was performed at 20 °C.

experiments. At least for polymyxin E (2000 IU/ml) longer exposure times are unusable due to occurrence of toxicity (personal observations). The conditions of the additional treatment (Figure 2, panel F) instituted after failure of the initial conditions to clear *B. salamandrivorans* infections, were based on the *in vitro* synergy between voriconazole and polymyxin E in inhibiting *B. salamandrivorans* growth, the increased but suboptimal inhibition of *B. salamandrivorans in vivo* by combined exposure to voriconazole and polymyxin E (Figure 2, panel C) and the previously determined temperature dependent infection dynamics of *B. salamandrivorans* (9). Using the same concentrations of voriconazole and polymyxin E, but raising the temperature to 20 °C did result in successful elimination of *B. salamandrivorans* in all infected animals (Figure 2, panel F). The results of this study underline the key influence temperature plays in *B. salamandrivorans* infection dynamics, which already allowed development of a *B. salamandrivorans* temperature treatment protocol for amphibian species able to endure a continuous ambient temperature of 25 °C for 10 days (9). Ethical considerations allowed only one additional treatment condition to be tested. Therefore, the positive treatment effect could theoretically be attributed to the sole influence of either one of the compounds at 20 °C, as experimental treatments with individual compounds were only tested *in vivo* at a temperature of 15 °C. The results of this study show that synergy between voriconazole and polymyxin E together with the temperature dependent infection dynamics of *B. salamandrivorans* allow *B. salamandrivorans* infections to be eliminated in amphibian species with critical thermal maxima lower than that of *B. salamandrivorans*. The efficacy of the treatment protocol was validated by successful treatment of fire salamanders naturally infected with *B. salamandrivorans* during a field outbreak (Figure 3). In conclusion, *in vitro* synergy between antimycotic compounds in inhibiting *B. salamandrivorans*, together with the temperature dependent infection dynamics of *B. salamandrivorans* allowed development of a treatment protocol successful in eliminating *B. salamandrivorans* from experimentally and naturally infected amphibians.

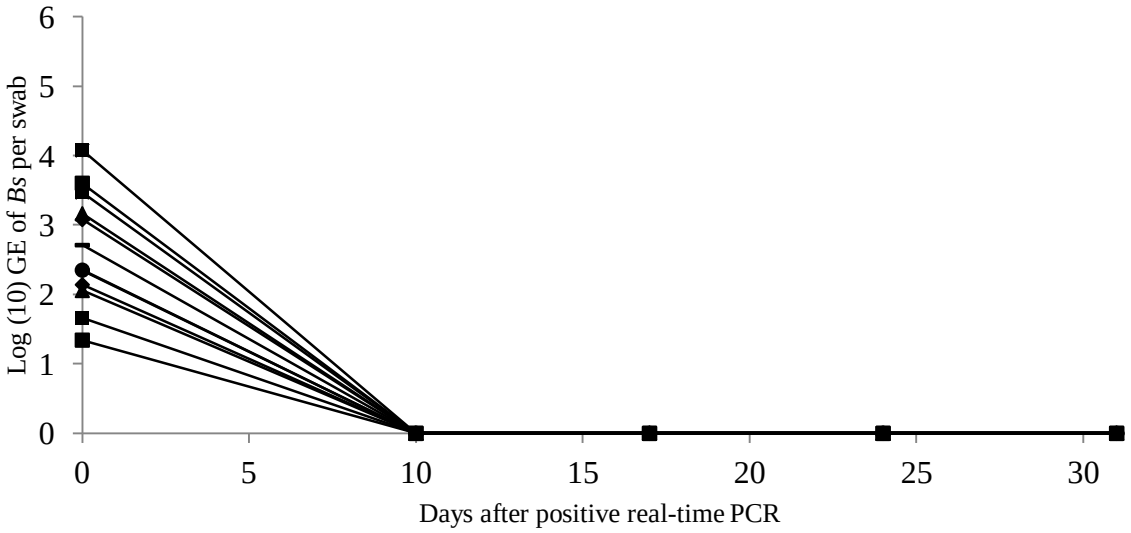

Figure 3. Treatment of fire salamanders naturally infected with *Batrachochytrium salamandrivorans*. After ascertaining presence of *Batrachochytrium salamandrivorans* in all animals (day 0), they were treated with polymyxin E submersion baths (2000 IE/ml, 10 minutes) followed by spraying voriconazole (12.5 µg/ml) twice a day for 10 days at an ambient temperature of 20 °C. Each symbol represents the course of infection of an individual animal.

Methods

All experiments were performed in accordance with the relevant guidelines and regulations. All experiments with experimental animals were carried out with approval of the ethical committee of the Faculty of Veterinary Medicine, Ghent University.

Chytrid strain & culture conditions

The *B. salamandrivorans* type strain (AMFP13/1) (6) was grown in TGhL broth (16 g tryptone, 4 g gelatin hydrolysate, 2 g lactose per liter of distilled water) in 25 cm^3 cell culture flasks and incubated at 15 °C. To obtain a suspension containing a mixture of zoosporangia and zoospores, the walls of a cell culture flask containing 5-day-old culture were scraped with a sterile cell scraper and the suspension subsequently collected.

Determination of the minimum inhibitory concentrations of antimicrobial agents against *B. salamandrivorans*

The minimum inhibitory concentrations (MICs) of florfenicol (20%), voriconazole (VFend IV, Pfizer, Kent, UK), itraconazole (Itrafungol, Elanco, Brussels, Belgium), terbinafine (Terbinafine hydrochloride, Sigma-Aldrich, Bornem, Belgium) and polymyxin E (Colistin

sulphate, VMD, Arendonk, Belgium) for *B. salamandrivorans* were determined using a broth macrodilution method used for MIC testing of *B. dendrobatidis* (8,12). In short, two-fold dilutions series of the antimicrobial agents were prepared in TGhL broth, and 200 µl of these prepared dilutions were added to wells of 24 well cell culture plates (Table 1). Two hundred µl of a suspension containing a mixture of *B. salamandrivorans* sporangia and zoospores (approximately 10^5 *B. salamandrivorans* organisms per ml) were added to all wells. Finally, 1600 µl of TGhL broth were added to all wells resulting in a final volume of 2 ml per well. Plates were incubated at 15 ºC (optimum growth temperature of *B. salamandrivorans* (6)) and checked for viability and growth daily for 10 days with an inverted light microscope. Wells containing TGhL broth with viable *B. salamandrivorans* sporangia and zoospores and wells containing heat treated (85 ºC, 10 minutes) *B. salamandrivorans* sporangia and zoospores served as positive and negative growth controls respectively. The MIC value was determined as the lowest concentration of the antimicrobial agent at which no growth could be observed after 10 days of incubation. To test which concentrations of the antimicrobial agents were lethal for *B. salamandrivorans* after 10 days of exposure, we removed the medium and replenished all wells with fresh TGhL broth without antimicrobial agents. Plates were incubated at 15 ºC and checked for viability and growth daily for an additional 14 days with an inverted light microscope. A concentration was considered to be lethal to *B. salamandrivorans* when no signs of growth could be observed after this incubation period of 14 days (Table 1). All conditions were tested in triplicate.

Determination of the fractional inhibitory concentrations of antimicrobial agents against *B. salamandrivorans*

To test for synergy in combinations of polymyxin E with voriconazole or with itraconazole in inhibiting *B. salamandrivorans* growth, fractional inhibitory concentrations (FICs) (13) were determined using a macrodilution broth checkerboard technique. Two-fold serial dilution series of all antimicrobial agents in TGhL broth were prepared. Polymyxin E was tested at final concentrations of 1000 – 64000 IE/ml, voriconazole at final concentrations of 0.016 – 1 µg/ml and itraconazole at final concentrations of 0.0007 – 0.05 µg/ml. All tested concentrations of the individual antimicrobial agents were included separately as controls for reproducibility of the earlier determined MIC values of the compounds. Twenty-four well cell culture plates were prepared with 1600 µl of TGhL broth, 200 µl of the respective compound or combination of compounds and 200 µl of a suspension containing *B. salamandrivorans* sporangia and zoospores including *B. salamandrivorans* positive and negative growth controls

as described earlier. Plates were incubated at 15 ºC and checked for signs of viability and growth daily for 10 days with an inverted light microscope. The FIC value for an individual antimicrobial agent is determined as the ratio of the MIC value of the antimicrobial agent used in combination (MIC_{combi}) to the MIC value of the antimicrobial by itself (MIC_{alone}) after 10 days of incubation:

$$1.\ \mathrm{FIC} = \frac{\mathrm{MIC}_{\mathrm{combi}}}{\mathrm{MIC}_{\mathrm{alone}}}$$

These FIC values are subsequently used to produce a single fractional inhibitory concentration index (FICI) as an indicator for the type of interaction between two antimicrobial agents (13):

$$2.\ \mathrm{FICI}_{1+2} = \mathrm{FIC}_1 + \mathrm{FIC}_2$$

We determined the possible interactions as synergistic (FICI ≤ 0.5), additive (FICI > 0.5 – 1.0), indifferent (FICI > 1.0 - 4.0) or antagonistic (FICI ≥ 4.0) (14-16). Isobolograms were used to graphically depict the FIC and FICI values of all tested combinations of antimicrobial agents (Figure 1) (17). To test which combination of concentrations of the antimicrobial agents was lethal for *B. salamandrivorans* after 10 days of exposure, we replaced the broth containing the compound(s) with fresh TGhL broth without antimicrobial agents. Plates were incubated at 15 ºC and checked for viability and growth daily for an additional 14 days with an inverted light microscope. A combination of concentrations was considered to be lethal to *B. salamandrivorans* when no signs of growth could be observed after this incubation period of 14 days. All conditions were tested in quadruplicate.

Treatment of experimentally infected fire salamanders

Fire salamanders (*Salamandra salamandra*) were inoculated with *B. salamandrivorans* in order to study *in vivo* efficacy of different antimicrobial treatment protocols. The animal experiment was performed with the approval of the ethical committee of the Faculty of Veterinary Medicine (Ghent University, EC2013/87 and EC2014/65). Thirty captive bred fire salamanders were housed individually in plastic containers in a climatized room with an ambient temperature of 15 °C. The animals were kept on a moist tissue, with access to a hiding place and water container. Crickets powdered with mineral and vitamin supplement were provided ad libitum as food source. All animals were clinically healthy and free of *B. dendrobatidis* and *B. salamandrivorans*, as determined with duplex real-time PCR

examination of skin swabs (18). An acclimatization period of 2 weeks was admitted before the start of the experiment. The experimental animals were randomly assigned to one of the 6 experimental treatment groups (5 animals per treatment group, kept individually). All salamanders were inoculated with *B. salamandrivorans* by topically applying one mL of inoculum containing 10^5 zoospores per ml on the skin. Animals were kept at 15 ºC (except for the animals in group F; details below) and skin swabs for *B. salamandrivorans* real-time PCR analysis (18) were collected every 7 days. Individual treatment commenced when *B. salamandrivorans* infection was established (determined as an increase in *B. salamandrivorans* infection load between 2 consecutive samplings). The different groups were untreated negative control (group A), voriconazole treatment (12.5 µg/ml) alone (group B), voriconazole and polymyxin E treatment (concentrations of 12.5 µg/ml and 2000 IU/ml respectively, group C), itraconazole treatment (0.6 µg/ml) alone (group D) and itraconazole and polymyxin E treatment (0.6 µg/ml and 2000 IU/ml respectively, group E). Initial failure to clear *B. salamandrivorans* infections in these experimental groups, led us to include one additional treatment condition composed of voriconazole and polymyxin E treatment (concentrations of 12.5 µg/ml and 2000 IU/ml respectively, identical to the treatment described for group C) but with an ambient temperature of 20 °C instead of 15 °C (group F). All experimental treatments were carried out twice a day for 10 days. Polymyxin E was administered through submersion baths (10 minutes) and voriconazole and itraconazole were administered through spraying the animals and tissue in their housing (after polymyxin E baths if applicable). After the treatment period, all animals were kept at 15 ºC. Skin swabs for *B. salamandrivorans* real-time PCR analysis were collected immediately after the treatment period and subsequently every 7 days for another 3 weeks. An animal was considered negative for *B. salamandrivorans* after 3 consecutive negative real-time PCR results. Development/progression of symptoms associated with *B. salamandrivorans* infections together with presence of *B. salamandrivorans* as determined with real-time PCR analysis in an animal was determined as experimental endpoint and resulted in withdrawal of the animal from the experiment. If an animal tested positive for the presence of *B. salamandrivorans* in the post-treatment follow-up phase (starting from day 10 in figure 2), the treatment was considered as failed. Remaining *B. salamandrivorans* infections in animals that were removed from the experiment due to reaching the described endpoint, and animals still positive for *B. salamandrivorans* at the last sampling time point were exposed to an ambient temperature of 25 °C during 10 days to clear the *B. salamandrivorans* infection (9).

Treatment of naturally infected fire salamanders

Thirty-five fire salamanders from the population from which *B. salamandrivorans* (strain AMFP13/1) was originally isolated (Bunderbos, Netherlands, N50°54′51″, E5°44′59″) were transferred to our research facility for treatment. Upon arrival, 12 of the translocated animals tested positive for presence of *B. salamandrivorans* DNA as tested with the *B. salamandrivorans* real-time PCR[18]. Based on the results of the treatments of experimental infections, the animals were treated with polymyxin E submersion baths (2000 IU/ml, 10 minutes) followed by spraying voriconazole (12.5 µg/ml) twice a day for 10 days at an ambient temperature of 20 °C. Housing conditions of the animals were identical to the conditions described for the experimental animals. After the treatment period all animals were put back at 15 ºC. Skin swabs for *B. salamandrivorans* real-time PCR analysis were collected directly after the treatment period and subsequently every 7 days for another 3 weeks. An animal was considered negative for *B. salamandrivorans* after 3 consecutive negative real-time PCR results.

Additional information

Author contributions statement

M.B. contributed in the design of the experiments. M.B. performed the experiments. M.B. contributed in writing and reviewing of the manuscript.

Competing financial interests statement

The authors declare no competing financial interests.

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Combining ethidium monoazide treatment with real-time PCR selectively quantifies viable *Batrachochytrium dendrobatidis* cells

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Abstract

Detection of the lethal amphibian fungus *Batrachochytrium dendrobatidis* relies on PCR-based techniques. Although highly accurate and sensitive, these methods fail to distinguish between viable and dead cells. In this study a novel approach combining the DNA intercalating dye ethidium monoazide (EMA) and real-time PCR is presented that allows quantification of viable *B. dendrobatidis* cells without the need for culturing. The developed method is able to suppress real-time PCR signals of heat-killed *B. dendrobatidis* zoospores by 99.9% and is able to discriminate viable from heat-killed *B. dendrobatidis* zoospores in mixed samples. Furthermore, the novel approach was applied to assess the anti-fungal activity of the veterinary antiseptic F10® Antiseptic Solution. This disinfectant killed *B. dendrobatidis* zoospores effectively within 1 minute at concentrations as low as 1:6400.

Introduction

Amphibian populations are currently facing declines on a global scale. One of the main causes of these declines is the amphibian disease chytridiomycosis, caused by the chytrid fungus *Batrachochytrium dendrobatidis* (1-4). In susceptible amphibian species *B. dendrobatidis* invades skin epithelium (5) and is able to cause hyperplasia and hyperkeratosis of the epidermis (1, 6). These changes attribute to a critical impairment of the normal functioning of the amphibian skin leading to dehydration, electrolyte imbalance and cardiac arrest (1, 7-10). Fast and reliable detection of *B. dendrobatidis* is therefore of the greatest importance. The most reliable techniques for detecting *B. dendrobatidis* are based on detecting and quantifying the amount of *B. dendrobatidis* DNA present in a sample (11-14). Although these methods can accurately detect and quantify the number of *B. dendrobatidis* genome equivalents (GE) present in samples, no distinction is made between viable and dead cells of *B. dendrobatidis*. While this is sufficient for the purpose of screening for presence of *B. dendrobatidis*, fast and selective quantification of viable *B. dendrobatidis* cells without the need for culturing would be a major advantage for other purposes. Stockwell *et al.* (15) already developed a technique to discriminate viable from dead *B. dendrobatidis* zoospores. However, the major drawback of this technique is the lack of specificity towards *B. dendrobatidis* since all cells with a compromised cell membrane will be stained. One method that has proven effective for selective quantification of viable cells is the use of the DNA intercalating dye ethidium monoazide (EMA) in conjunction with real-time PCR (16-19). The aim of this study is to develop a technique that allows quantification of viable *B. dendrobatidis* cells present in a sample by combining EMA treatment with the real-time PCR described by Boyle *et al.* (11). Furthermore the application of the developed EMA real-time PCR for the determination of the *B. dendrobatidis* killing capacity of a disinfecting agent is presented.

Materials and methods

Strain and culture conditions

The *B. dendrobatidis* strain JEL423 used in this study was kindly provided by Dr. J. Longcore. This strain was isolated from Lemur leaf frogs (*Phyllomedusa lemur*) involved in a mass mortality event (El Copé, Panama, 2004). Strain JEL423 was grown in TGhL broth (16 g tryptone, 4 g gelatin hydrolysate, 2 g lactose per liter distilled water) in 25 cm^2 flasks at 20° C for 5 days.

For collection of zoospores TGhL agar plates (16 g tryptone, 4 g gelatin hydrolysate, 2 g lactose, 10 g bacteriological agar per liter distilled water) were inoculated with a 2 ml aliquot of 5-day-old broth culture, incubated for 5 – 7 days at 20° C. Zoospores were collected by flooding each plate with 2 ml distilled water followed by collection of the fluid. The zoospores were washed three times in distilled water by centrifugation (1200 rpm, 20 °C, 2 minutes). The concentration of zoospores per ml was determined with a haemocytometer. Heat treatments (85° C, 15 minutes) of aliquots of zoospore suspensions were carried out to obtain dead zoospores. Successful killing of the zoospores was confirmed by plating the heat-treated zoospores on TGhL agar plates and checking for absence of growth during 10 days by light microscopy. Sporangia of *B. dendrobatidis* were harvested by gently scraping the inside of a 25 cm^2 flask that contained a 2-day-old broth culture.

Ethidium monoazide treatment and real-time PCR sample preparation

Ethidium monoazide (EMA) (Sigma-Aldrich Inc., Bornem, Belgium) was dissolved and diluted in dimethyl formamide (Sigma-Aldrich Inc., Bornem, Belgium) to a concentration of 1 mg/ml and stored at -20° C in 1.5 ml lightproof microcentrifuge tubes (Greiner Bio-One GmbH, Frickenhause, Germany). For the optimization of the EMA protocol, a zoospore suspensions containing approximately 10^7 zoospores per ml was prepared. During the optimization of the EMA protocol, different EMA treatment concentrations and light exposure times were tested. The tested EMA treatment concentrations were 10, 25 and 50 µg/ml. The effect of presence of TGhL broth during EMA treatment was assessed by adding a volume of TGhL broth equal to half the sample volume, while the same volume of sterile distilled water was added to the controls. The tested light exposure times were 1 and 5 minutes (500 Watt halogen light, 20 cm distance between samples and light). Samples were cooled on ice during incubation to avoid overheating. Samples were washed by centrifugation (5000 rpm, 5 minutes, 20° C) followed by resuspension of the pellet in 25 µl HPLC water. DNA extraction of these resuspended pellets was carried out by adding 100 µl Prepman Ultra (Applied Biosystems, Foster City, USA) and heating them to 100° C for 10 minutes. All samples were diluted 1:10 in HPLC water in order to minimize PCR inhibition, and stored at -20° C until further use. Details on the number of included samples per experiment can be found in the specific experiment subsections 2.3, 2.4 and 2.5. real-time PCR assays were performed on a CFX96 Real Time System (Biorad, Hercules, California, USA) with amplification conditions, primer and probe concentrations according to Boyle *et al.* (11). Every sample was run in triplicate in the real-time PCR assay. The method described by Hyatt

et al. (12) using the TaqMan Exogenous Internal Positive Control Reagents was used to make sure that PCR inhibition did not affect the real-time PCR results. real-time PCR signals (Ct-values) are converted to GE based on standards containing DNA of 1000, 100, 10, 1 and 0.1 *B. dendrobatidis* genome equivalents which are prepared as described by Boyle *et al.* (11). The GE values of the EMA treated samples are considered as the viable fraction of *B. dendrobatidis* cells, while the GE values of the untreated samples are considered as the sum of both viable and dead *B. dendrobatidis* cell fractions. With these assumptions both viable and dead fractions of *B. dendrobatidis* cells in a sample can be calculated.

In experiments described in subsection 2.3, 2.4 and 2.5 a final EMA concentration of 25 µg/ml was used. EMA treated samples were incubated shielded from light in 24 well plates (Greiner Bio-One GmbH, Frickenhause, Germany) for 10 minutes, followed by incubation in visible halogen light for 5 minutes. A volume of TGhL broth equal to half the sample volume was added for its protective effect on viable *B. dendrobatidis* organisms during EMA treatment.

Discrimination between viable and dead *B. dendrobatidis* zoospores in mixed samples

A zoospore suspension containing approximately 1.7 x 10^6 zoospores per ml was prepared. Mixed samples composed of viable and dead *B. dendrobatidis* zoospores were prepared. These samples had different ratio´s of viable and dead zoospores ranging from 0 to 100% viable zoospores and 100% to 0% dead zoospores respectively. Three replicates of each ratio were prepared. A 200 µl aliquot of each sample was treated with EMA according to the optimized protocol described in subsection 2.2. A 200 µl aliquot of each sample without EMA treatment was included as reference. The GE values for the EMA treated and untreated samples were used to determine the number of present viable and dead zoospores in each sample.

Discrimination between viable and *dead B. dendrobatidis* zoospores at different zoospore concentrations

Tenfold serial dilutions of a zoospore suspension (ranging from 10^6 to 10^1 *B. dendrobatidis* zoospores per ml) containing viable or heat-killed zoospores were prepared. Three replicates of each dilution were prepared. Two hundred µl aliquots of each dilution were treated with EMA according to the protocol described in subsection 2.2. A 200 µl aliquot of each sample without EMA treatment was included as reference. Again the GE values for the EMA treated

and untreated samples were used to determine the number of present viable and dead zoospores in each sample.

The killing capacity of F10® Antiseptic Solution evaluated by EMA real-time PCR

F10® Antiseptic Solution containing 5.4 g/100 ml benzalkonium chloride and 0.4 g/100 ml polyhexamethylene biguanide hydrochloride (Meadow´s Animal Healthcare, Loughborough, United Kingdom) was two-fold serial diluted (ranging from 1:100 to 1:6400) in distilled water. Three replicates of each dilution were prepared. A zoospore suspension containing approximately 1.5 x 10^6 zoospores per ml was prepared. One hundred fifty µl of this zoospore suspension was added to a 2 ml aliquot of each dilution, and after a contact time of 1 minute, 200 µl aliquots of all samples were diluted 10000 times in distilled water, centrifuged (1200 rpm, 20° C, 2 minutes) and brought back to the original volume with the purpose of diluting the F10® Antiseptic Solution to a negligible concentration. A 200 µl aliquot of each sample was treated with EMA according to the optimized protocol described in subsection 2.2. A 200 µl aliquot of each sample without EMA treatment was included as reference. The percentage of killed zoospores was calculated using the GE values of the EMA treated and untreated samples. In conjunction with the EMA real-time PCR the effect of the F10® Antiseptic Solution dilutions on the zoospore suspension was also evaluated by light microscopy and culturing. For each dilution of F10® Antiseptic Solution, the percentage of motile zoospores after a contact time of 1 minute was scored by counting 100 zoospores with an inverted microscope. To check the ability of growth of the zoospores after a contact time of 1 minute with the different dilutions of F10® Antiseptic Solution, the suspensions were further diluted a 10000 times in distilled water, centrifuged (1200 rpm, 20°C, 2 minutes) and resuspended in 10 ml TGhL broth in order to dilute the F10® Antiseptic Solution to a neglible concentration. Samples were incubated (20 °C, 10 days) and examined with an inverted microscope for growth on a daily basis.

Results and Discussion

EMA treatment optimization

EMA concentration and light exposure time were optimized to discriminate between viable and heat-killed *B. dendrobatidis* zoospores. In the first experiment a zoospore suspension was treated with final EMA concentrations of 10, 25 and 50 µg/ml and exposed to halogen light during 1 minute (Figure 1. A + B). All used EMA concentrations resulted in minor

differences in *B. dendrobatidis* GE values between EMA treated and untreated samples of viable and heat-killed zoospores. This indicates that in this setup EMA treatment has limited inhibitory effect on the real-time PCR results of both viable and dead zoospores. Since the chosen EMA concentrations were able to generate desirable signal reduction differences between viable and dead cells in other studies (16, 20, 21), the light exposure time was changed to 5 minutes without altering the EMA concentrations (Figure 1. C + D). This resulted in values that were comparable with the first setup for the EMA treated and untreated viable zoospores, while large differences between the values of EMA treated and untreated dead zoospores were seen. A final concentration of 25 µg/ml of EMA resulted in the largest difference in GE values between EMA treated viable and heat-killed zoospores. EMA treatment (25 µg/ml, 5 minute light incubation) of viable and heat-killed *B. dendrobatidis* sporangia resulted in GE differences that were comparable with the observed differences for zoospores (difference in log (10) GE between EMA treated and untreated viable and heat-killed sporangia of 2.69 (±0.01)). The PCR inhibition control described by Hyatt *et al.* (12) showed no indications of PCR inhibition. Based on these results the optimized protocol for discriminating between viable and dead *B. dendrobatidis* cells in samples is EMA treatment at a concentration of 25 µg/ml followed by incubation in halogen light during 5 minutes. It should be pointed out that the results of the optimization experiments were obtained from samples that were EMA treated with added TGhL broth. During optimization it became clear that viable zoospores died when EMA treatment was carried out without simultaneously adding TGhL broth. This was observed as large amounts of DNA derived from dead zoospores as indicated by the EMA real-time PCR in samples that contained only viable zoospores, but also by zoospore immobility observed by light microscopy directly after adding EMA to viable zoospores. In samples without added TGhL broth, a log (10) difference in GE between the EMA treated (25 µg/ml) and untreated viable zoospores of 1.16 (±0.00) was observed, while the difference in log (10) GE of the same samples with added TGhL broth (volume equal to half of the sample volume) was only 0.21 (±0.01). TGhL broth only has to protect the viable zoospores during the short EMA incubation period, so adding TGhL broth simultaneously with the EMA is sufficient. This way, the need to add TGhL broth to samples does not interfere with possible applications of the developed technique. For instance, the technique can still be used to develop *B. dendrobatidis* viability assays of environmental samples without risk of overgrowth of other fungi of bacteria due to added nutrients. Negative and positive controls, composed out of an EMA treated viable and heat-

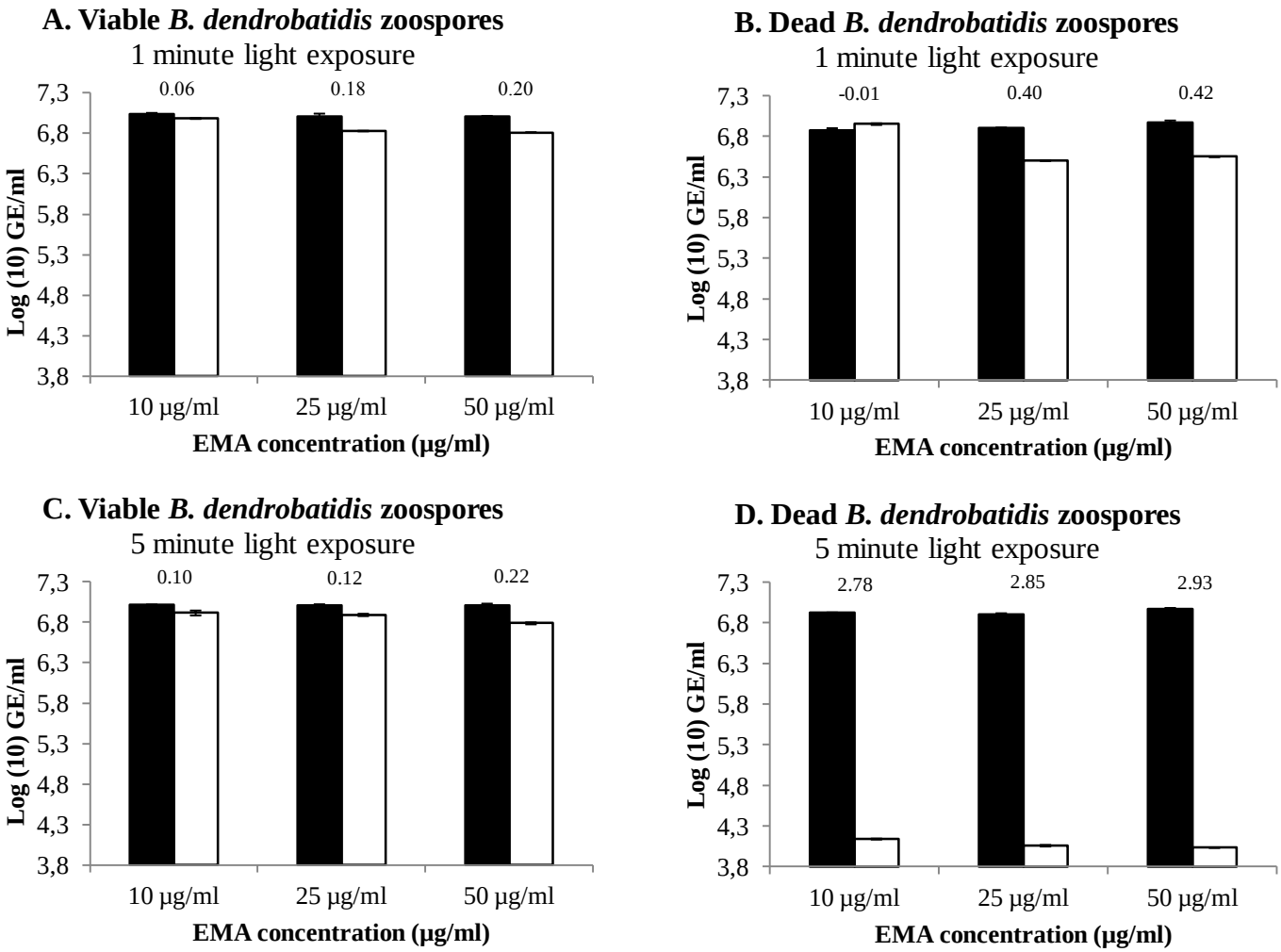

Fig 1. Optimization of EMA concentration and light exposure time. Viable (A + C) and heat-killed (B+D) *Batrachochytrium dendrobatidis* zoospore suspensions were treated with EMA concentrations of 10, 25 and 50 µg/ml before being exposed to halogen light (500 Watt) for the duration of either 1 minute (A+B) or 5 minutes (C+D). Bars represent the log (10) genomic equivalents (GE) of *Batrachochytrium dendrobatidis* detected by RT-PCR, either treated with EMA (white bars) or not (black bars). Three replicates of each sample were prepared. All replicates were assayed in triplicate in the RT-PCR. Error bars represent the standard deviations of mean GE values from three independent sample replicates.

killed zoospore suspension respectively, should be included in every EMA real-time PCR to assure that the EMA real-time PCR worked properly. The PCR inhibition control described by Hyatt *et al.* (12) can be applied to check for PCR inhibition.

Selective EMA real-time PCR detection of viable *B. dendrobatidis* zoospores

The optimized EMA protocol was used to selectively discriminate viable from heat-killed *B. dendrobatidis* zoospores in mixed samples (Figure 2). A good linearity, as indicated by the R^2 value of 0.91, was observed for the EMA treated samples. This indicates a strong predictive value of the EMA real-time PCR result for the number of viable zoospores present in the sample relative to the total number of zoospores. Very little variation in the GE values of the untreated samples was observed. This shows that the developed EMA real-time PCR method is able to selectively discriminate viable from dead zoospores. The capacity of the EMA real-time PCR to selectively detect and quantify viable *B. dendrobatidis* zoospores has major advantages, and allows the technique to be applied in several fields. For instance testing of anti-fungal activity of pharmaceuticals with the currently used methods is laborious and time-consuming (22).

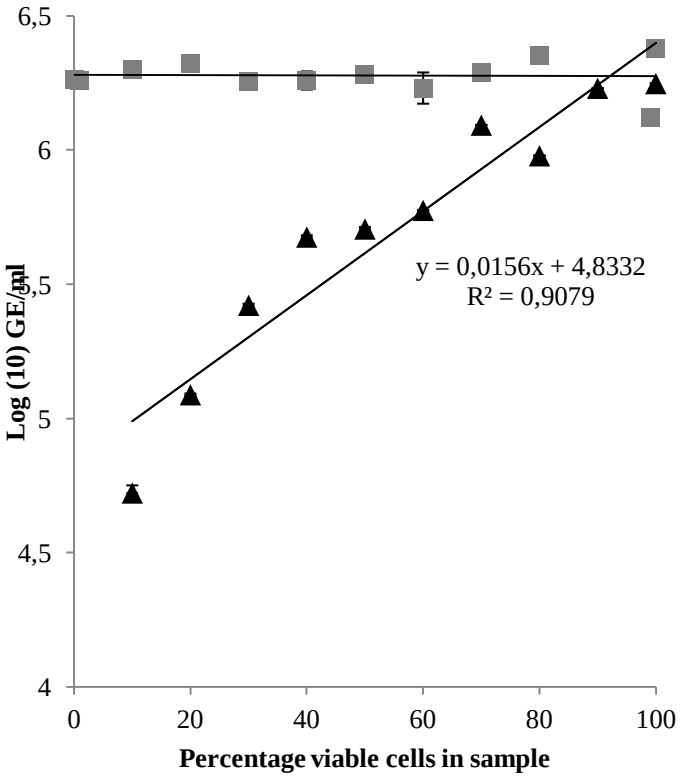

Fig 2. EMA RT-PCR and RT-PCR signals in samples containing a mixture of viable and heat-killed *Batrachochytrium dendrobatidis* zoospores. RT-PCR measured amounts of log (10) *Batrachochytrium dendrobatidis* genomic equivalents (GE) obtained from EMA (25 µg/ml) treated (▲) or untreated (■) samples composed out of different ratio´s of viable and heat-killed *Batrachochytrium dendrobatidis* zoospores. Three replicates of each sample were prepared. All replicates were assayed in triplicate in the RT-PCR. Error bars represent the standard deviations of mean GE values from three independent sample replicates.

The EMA real-time PCR can easily be applied as an alternative or supplement to these methods with only small adaptations of the described treatment protocol, as is demonstrated for testing the antifungal activity of F10® Antiseptic Solution. Another possible use of the developed technique could lie in *B. dendrobatidis* viability assays, for instance in environmental samples. The currently used methods to asses *B. dendrobatidis* viability resemble the methods used for anti-fungal activity testing (23) and share the same drawbacks. Furthermore, culture based viability testing of *B. dendrobatidis* organisms in environmental samples is currently made impossible due to overgrowth of other saprophytic fungi and bacteria (24, 25). This can be alleviated by taking specific measures or treatments in order to remove concurrent microbiota in experimental settings and samples, such as sterilization of samples, but this in turn could alter experimental outcome. The culture-independent EMA real-time PCR does allow fast and easy evaluation of *B. dendrobatidis* viability in presence of other microbiota.

Discriminatory range of the EMA real-time PCR between viable and dead *B. dendrobatidis* zoospores

In this experiment, the ability of the developed EMA real-time PCR to discriminate viable from dead *B. dendrobatidis* zoospores in samples with different starting concentrations of zoospores was evaluated (Figure 3). Good linearity, as indicated by the R^2 values, was observed for EMA treated and untreated viable and heat-killed zoospores. The difference in log (10) *B. dendrobatidis* GE between EMA-treated and untreated heat-killed zoospores was approximately 3 for the starting concentrations of 10^4, 10^5, 10^6 and 10^7 zoospores per ml, which was also the maximum difference during optimization. For this reason the EMA real-time PCR produced no signal for the starting concentrations of 10^3 and 10^2 heat-killed zoospores/ml. To be able to assess *B. dendrobatidis* viability regardless of zoospore concentrations we recommend processing samples both with EMA and regular real-time PCR. The GE value derived from viable *B. dendrobatidis* cells can then be expressed as percentage of total *B. dendrobatidis* GE in a given sample. This results in an assay that can estimate *B. dendrobatidis* viability from 0% up to 100% for samples below the concentration of 10^3 zoospores per ml, and 0.1% to 100% for samples that exceed this concentration. It is to be expected that most samples will have zoospore concentrations lower than 10^3 zoospores per ml. For example zoospore counts performed on environmental water and sediment samples yielded maximum *B. dendrobatidis* zoospore loads of up to 454 GE/liter [13, 14].

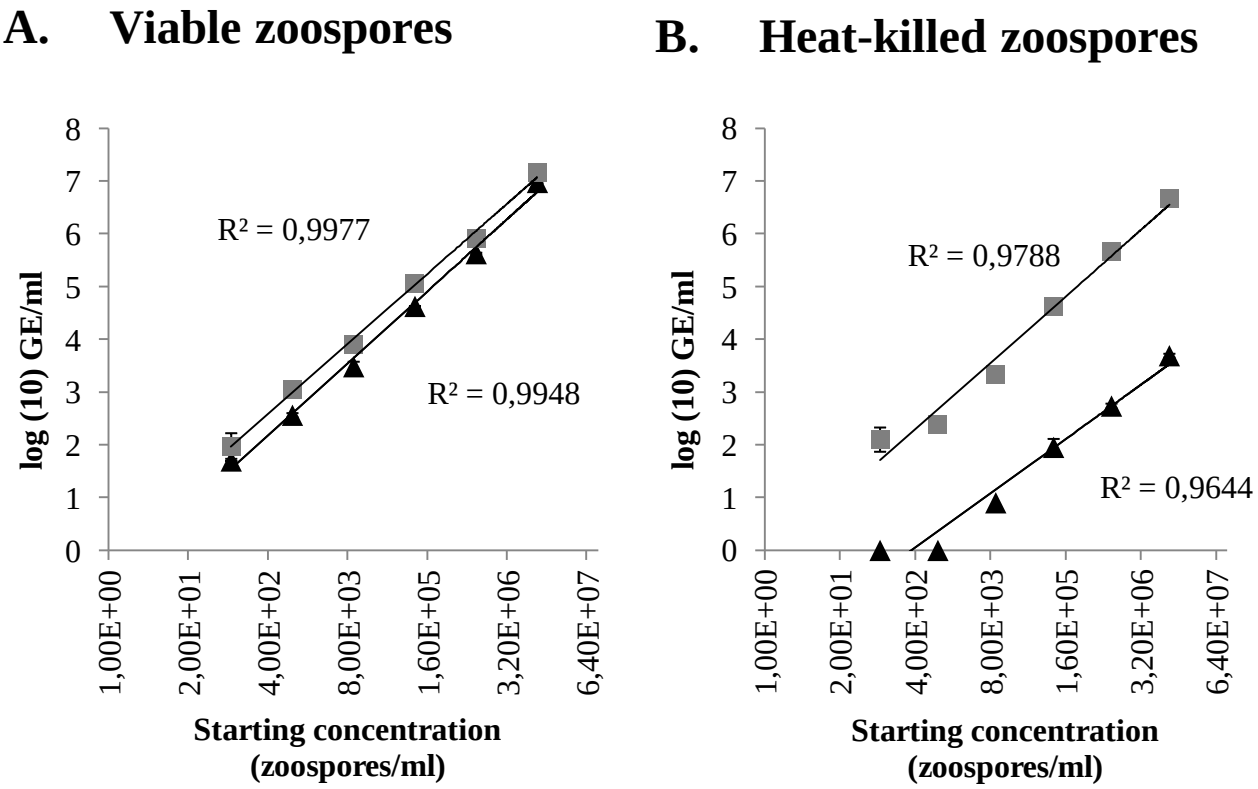

Fig 3. Linearity in EMA real-time PCR signals in samples with different concentrations of *Batrachochytrium dendrobatidis* zoospores/ml. Real-time PCR measured amounts of log (10) *Batrachochytrium dendrobatidis* genomic equivalents (GE) obtained from EMA (25 µg/ml) treated (▲) and untreated (■) samples composed out of different concentrations of viable (A) and heat-killed (B) *Batrachochytrium dendrobatidis* zoospores. Three replicates of sample were prepared. All replicates were assayed in triplicate in the real-time PCR. Error bars represent the standard deviations of mean GE values from three independent sample replicates.

The detrimental effect of F10® Antiseptic Solution on *B. dendrobatidis*

In this experiment the developed EMA real-time PCR was used to test the anti-fungal activity of F10® Antiseptic Solution against *B. dendrobatidis* zoospores (Table 1). Based on the results of the EMA real-time PCR, all tested concentrations of F10® Antiseptic Solution showed a anti-fungal activity of >95% after a contact time of 1 minute when compared to the real-time PCR signal for EMA treated viable zoospores. Light microscopy of the samples showed that for the F10® Antiseptic Solution concentrations of 1:800, 1:1600, 1:3200 and 1:6400 motility of some zoospores could still be observed for several minutes. However, subsequent growth and development of the zoospores were absent for all tested concentrations. A possible explanation for this could be that the integrity of the cell membranes of some zoospores is not affected at first, but enough damage is done to all

	% Viability (Based on GE values)	% Motility (Based on light microscopy)	Development (Based on culture)
Viable zoospores	100	98	Yes
Heat-killed zoospores	0.0 (±0.0)	0	No
F10® dilution 1:100	0.0 (±0.0)	0	No
F10® dilution 1:200	0.0 (±0.0)	0	No
F10® dilution 1:400	1.3 (±0.1)	0	No
F10® dilution 1:800	4.5 (±0.9)	2	No
F10® dilution 1:1600	3.1 (±0.1)	2	No
F10® dilution 1:3200	2.1 (±0.1)	3	No
F10® dilution 1:6400	3.1 (±0.0)	3	No

Table 1. Anti-fungal activity of F10® Antiseptic Solution measured by the EMA real-time PCR. The anti-fungal activity of a two-fold dilution series of F10® Antiseptic Solution was determined with the developed EMA real-time PCR. Differences in log (10) *Batrachochytrium dendrobatidis* genomic equivalents (GE) between EMA treated and untreated samples were used to determine the percentage of killed *Batrachochytrium dendrobatidis* zoospores. In addition, motility and development of the zoospores were evaluated by light microscopy and culturing respectively. Three replicates of each sample were prepared. All replicates were assayed in triplicate in the real-time PCR. Standard deviations are derived from mean GE values from three independent sample replicates.

zoospores to prevent further development. In comparison, investigation of several physiological indices in chlorine treated *Escherichia coli* showed that in this bacterium viable plate counts are affected before a change in cell membrane integrity is seen (26). Although obvious differences in physiology between fungal and bacterial cells exist, possibly the same applies to *B. dendrobatidis* zoospores. F10® Antiseptic Solution is a multi-purpose broad spectrum preparation which can be used as topical application to treat a variety of clinical situations in different animal species[1]. Webb *et al.* (24) already showed that low concentrations of F10® Antiseptic Solution (1:3300) were capable of inactivating *B. dendrobatidis* zoosporangia. They also point out that evaluating the effectiveness of disinfectants in field samples is hampered due to overgrowth of other fungi and bacteria in culture media. The developed EMA real-time PCR protocol however could allow testing of the effectiveness of disinfectants in field samples, as this technique is culture-independent. Altogether this experiment shows that the developed EMA real-time PCR can be effectively applied to test the *B. dendrobatidis* zoospore killing capacity of pharmaceuticals.

Conclusions

The EMA real-time PCR developed in this study allows fast, selective and accurate quantification of viable *B. dendrobatidis* organisms without the need for culturing. The optimized protocol for EMA treatment and light exposure time consist of adding EMA to a final concentration of 25 µg/ml, incubation of samples shielded from light for 10 minutes followed by incubation in visible halogen light (500 Watt) for 5 minutes. Simultaneously adding TGhL broth with EMA to a test sample will protect the viable *B. dendrobatidis* cells from detrimental effects of EMA. Adding a volume of TGhL broth equal to half the test sample volume is enough to alleviate these negative effects. By processing samples with both EMA and regular real-time PCR viability assays can be performed regardless of zoospore concentration. Negative and positive controls, composed out of an EMA treated viable and heat-killed zoospore suspension respectively, should be included in every EMA real-time PCR. The PCR inhibition control described by Hyatt *et al.* (12) can be applied to check for PCR inhibition.

Acknowledgments

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Author contributions statement

M.B. contributed in the design of the experiments. M.B. carried out the experiments. M.B. contributed in writing and reviewing of the manuscript.

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Microscopic aquatic predators strongly affect infection dynamics of a globally emerged pathogen

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Abstract

Research on emerging infectious wildlife diseases has placed particular emphasis on host-derived barriers to infection and disease. This focus neglects important extrinsic determinants of the host/pathogen dynamic, when all barriers to infection should be considered when ascertaining the determinants of infectivity and virulence of wildlife pathogens (1-3). Especially those pathogens with free-living stages, such as fungal pathogens causing catastrophic wildlife declines on a global scale (4), must confront lengthy exposure to environmental barriers before contact with an uninfected host (5-8). Hostile environmental conditions therefore have the ability to decrease the density of infectious particles, reducing the infection force and ameliorating the impact and the establishment probability of infection (9). Here we show that, in nature, risk of infection and infectious burden of the amphibian pathogen *Batrachochytrium dendrobatidis* (*B. dendrobatidis*) have a significant, site-specific component, and that these correlate with the microfauna present at a site. Experimental infections show that the presence of aquatic microfauna can rapidly lower the concentration of infectious stages by consuming *B. dendrobatidis* zoospores, resulting in a significantly reduced probability of infection in anuran tadpoles. Our findings offer new perspectives for explaining the divergent impacts of *B. dendrobatidis* infection in amphibian assemblages, and contribute to our understanding of the ecosystems resilience to colonization by novel pathogens.

Results

We investigated the infection dynamics of one of the most devastating wildlife pathogens, *Batrachochytrium dendrobatidis* (hereafter *B. dendrobatidis*) (10). While *B. dendrobatidis* is associated with species declines and mass mortalities of amphibians worldwide, prevalence varies significantly at local and regional scales (11-13). Even a single, highly susceptible host species, such as the European midwife toad *Alytes obstetricans*, may exhibit strong variation in prevalence of infection on small geographic scales. Mortality in this species caused by chytridiomycosis correlates positively with altitude, which is at least in part due to the effects of environmental temperature (11, 13, 14). However, this does not explain why sites with equivalent temperature regimes can still exhibit substantial variation in prevalence and mortality associated with infection (13), or why *B. dendrobatidis* positive sites were found to be more similar to each other than would be expected based on chance (15). We first used water sampled at amphibian breeding sites located in the Pyrenean Mountain range with known histories of presence of *B. dendrobatidis* in the sentinel amphibian host species, *A. obstetricans*, to experimentally examine the effect of water and the aquatic microbial community on the probability of infection. The majority of these sites (N = 23) contain populations of *A. obstetricans* that exhibit low prevalence (< 5%) or complete lack of infection (minimum sampling size = 30 individuals), and an absence of mortality in *A. obstetricans* across up to six years of field sampling, while a smaller number of populations (N = 9) have consistently exhibited high prevalence (usually $\geq$ 90%) through time (up to ten years). Mass mortalities of recently metamorphosed *A. obstetricans* were observed over the same time span at those sites exhibiting 97 to 100% *B. dendrobatidis* prevalence (Table S1).

Dynamics of motile and immotile Bd zoospores in environmental water

We investigated if unfiltered environmental water affected the motility of *B. dendrobatidis* zoospores based on the prevalence history of the source of the water (Exp. 1). We found that the number of motile zoospores varied significantly and in accordance with observed patterns of infection at the source of the water (GLMM; $F_{1,244} = 14.18$; $p < 0.001$). Motile zoospores decreased as early as 2h after exposure to water from low prevalence sites, while the number of motile zoospores in cultures that were exposed to water from high prevalence sites only declined 33h after exposure (Fig 1). Counts of immotile zoospores did not differ significantly between

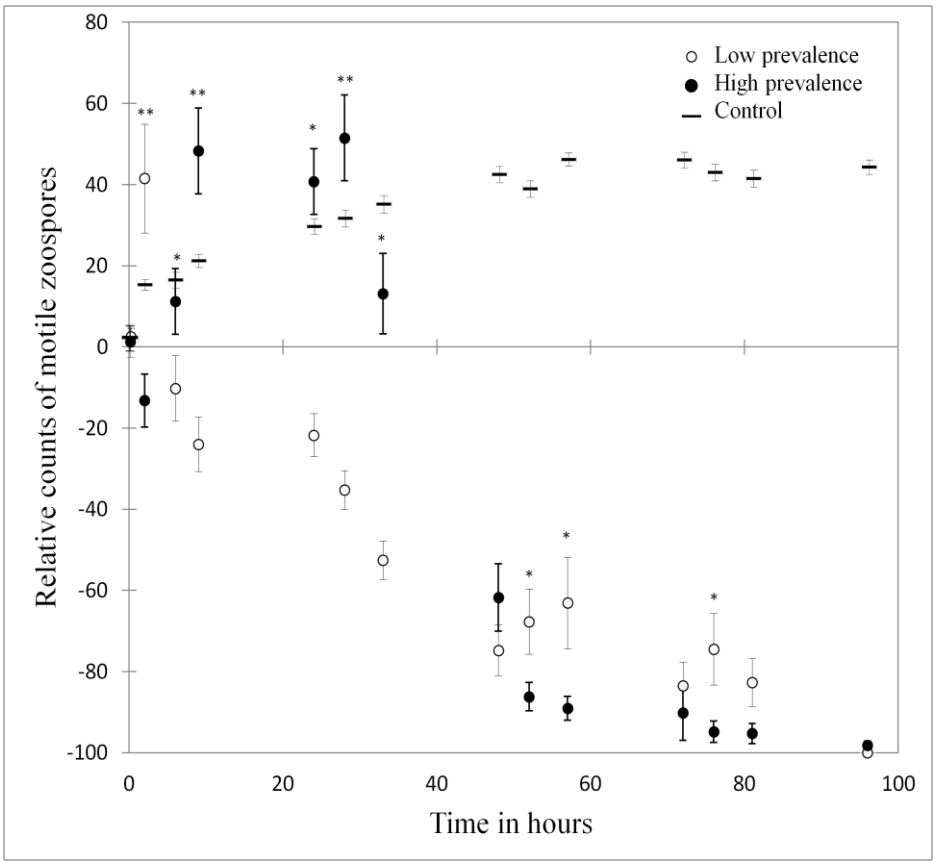

Fig 1. Dynamics of *Batrachochytrium dendrobatidis* zoospores under lab conditions in water of high and low prevalence sites. Relative counts of viable zoospores (Counts for each water sample were standardized relative to counts at t = 0) exposed to water from high prevalence (filled dots) vs low prevalence sites (open dots). Controls are counts of viable zoospores in treatments where distilled water was added to the culture. The error bars indicate the standard error of the mean across water samples in the same prevalence group. The asterisk indicate the significance level of the post-hoc test with * ≤ 0.05; ** ≤ 0.01; * ≤ 0.001.**

low and high prevalence sites (GLMM; $F_{1,244}$ = 0.35; p = 0.554; Fig. S1). To determine whether loss of motility was associated with zoospore death (Exp. 2), we assayed the viability of zoospores exposed for 24 hours to the two types of water using quantitative PCR. Zoospore survival was significantly greater in water from sites with high prevalence of infection (GLM; $F_{1,23}$ = 4.65; p = 0.042). Water from high prevalence sites also contained significantly reduced numbers of protozoans and microscopic metazoans compared to water from low prevalence sites (GLM; $F_{1,23}$ = 8.14; p = 0.004, Fig. S2). Further, the number of protozoans and microscopic metazoans was significantly negatively correlated with the observed *B. dendrobatidis* prevalence in 2012 (N = 25; *rs* = -0.430; p = 0.033), and positively correlated with the reduction in the number of viable *B. dendrobatidis* zoospores (N = 25; *rs* = 0.682; *p*

< 0.001; Table S1). *B. dendrobatidis* prevalence in 2012 positively correlated with the altitude of the site (N = 32; *rs* = 0.779; $p < 0.001$), while water acidity and conductivity had no apparent effect on zoospore survival (Table S1). The best model explaining the reduction in viable zoospores in our experiment included both the number of protozoans and microscopic metazoans and altitude, but not pH ($F_{1,23} = 8.267$; $p = 0.002$, AIC 43.087) and *B. dendrobatidis* prevalence in 2012 was best explained by altitude ($F_{1,23} = 18.976$; $p < 0.001$, AIC 180.765; Table S1). Filtering out microorganisms with 0.45µm cellulose acetate syringe filters (Exp. 3) significantly reduced the ability of water from low prevalence sites to cause mortality of *B. dendrobatidis* zoospores (GLMM; $F_{1,231} = 91.95$; $p < 0.001$, Fig. S3). Our observations in the first set of experiments suggest that the process through which *B. dendrobatidis* zoospore viability is affected is not determined by water quality, but rather by the resident aquatic microfauna.

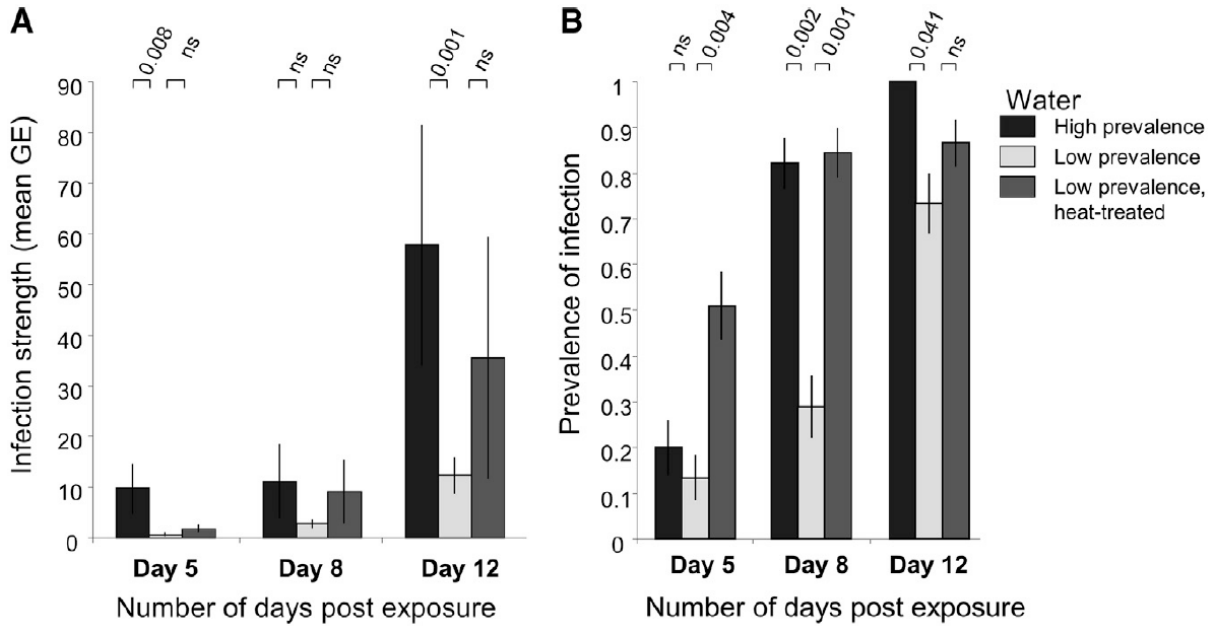

Fig 2. Infection probability and strength in different water treatments. The impact of water source and heat treatment on infection strength (A) and infection probability at 5, 8 and 12 days post exposure in *Alytes obstetricans* tadpoles (B). Bars are for different water types and treatments. Strength of infection is the uncorrected qPCR estimate of the average number of zoospores (genomic equivalents, GE) for each water category. We show pairwise *p*-values from Tukey's post-hoc tests.

Challenge experiments in environmental water

We directly tested infectivity of zoospores in the different water types by exposing uninfected *A. obstetricans* tadpoles to zoospores in water from the two prevalence categories (<5% and

>90% prevalence) and also in water from low prevalence sites that has been heat-treated (boiled for 15 minutes) to kill resident microfauna (Exp. 4). Infection was significantly greater 8 days after exposure in tadpoles that had been exposed in either water from high prevalence sites or from the heat-treated low-prevalence sites ($F_{2,93.1} = 17.14$; $p < 0.001$; Fig 2). Although prevalence in tadpoles exposed in water from low prevalence sites increased twelve days post exposure, infections of these animals were significantly weaker than those exposed in water from high prevalence sites (Tukey post-hoc test: $p = 0.041$), and trended in the same direction as experiments using heat-treated low prevalence water (Fig 2).

Effect of different microorganisms on Bd infection rate

To investigate if specific microorganisms could be responsible for the observed patterns, we exposed *B. dendrobatidis* zoospores to 14 freshwater protozoans and microbial metazoans and again quantified zoospore viability (Exp. 5). Two of these species (*Paramecium aurelia* and *Lecane stichaea*) were isolated from Pyrenean water samples and 12 were sister species of microorganisms commonly found in Pyrenean lakes. The viability of *B. dendrobatidis* zoospores varied substantially when exposed to different microorganisms [-0.04 ± 0.19 $\log_{10}$ GE for the species associated with the smallest reduction in viable *B. dendrobatidis* zoospores (*Stentor coeruleus*) to 2.02 ± 0.36 $\log_{10}$ GE for the species associated with the greatest reduction in viable zoospores, the rotifer *Notommatidae* spp., Fig. 3]. To determine the mechanism underlying this pattern, we observed the interactions between fluorescently stained zoospores and six microorganisms used in Exp. 5; two that had the weakest impact on zoospore viability (*Dileptus anser* and *S. coeruleus*), two that had the greatest impact (*P. caudatum* and *Notommatidae spp.*, Exp. 6), and the two species isolated from Pyrenean sites (*P. aurelia* and *L. stichaea*). Our observations suggest that the process through which viability is affected is in at least part due to ingestion of zoospores (Fig. S4). Interestingly, despite substantial differences in body size (lorica size in rotifers, length along longest axis in ciliates), some ciliates (size range 40 µm- 750µm) and rotifers (size range 20 µm – 180 µm) performed equally well in their ability to decrease the viability of *B. dendrobatidis*-zoospores under laboratory conditions.

In our final experiment, we tested whether microorganisms with different impacts on zoospore viability determined the probability of infection in the predicted manner. We exposed tadpoles (*Discoglossus scovazzi*) to *B. dendrobatidis* zoospores in water containing one of the three presumed predatory microorganisms (*P. aurelia* isolated from the Pyrenees, *P. caudatum*, the rotifer *Notommatidae* spp.; Exp. 7). The presence of microorganisms

significantly affected the probability of infection (GLMM; $F_{1,119}$ = 34.41, p<0.0001, Fig 4A; $F_{2,43}$ = 11.93, p=0.003, Fig. 4C). None of the tadpoles exposed with *Notommatidae* spp. developed infections, 3 of 15 tadpoles were infected in the presence of *P. caudatum*, and 16 of 60 tadpoles exposed with the Pyrenean *P. aurelia* were infected (Fig. 4). The *P. aurelia* isolated from the Pyrenees also reduced significantly the strength of infection (GE) compared to control tadpoles (GLMM; $F_{1,66}$ = 18.82, p=0.012, Fig. 4D), while this was not the case between control tadpoles and those housed with lab-reared *P. caudatum* treatments (GLMM; $F_{1,8}$ = 2.03, p=0.757, Fig. 4B).

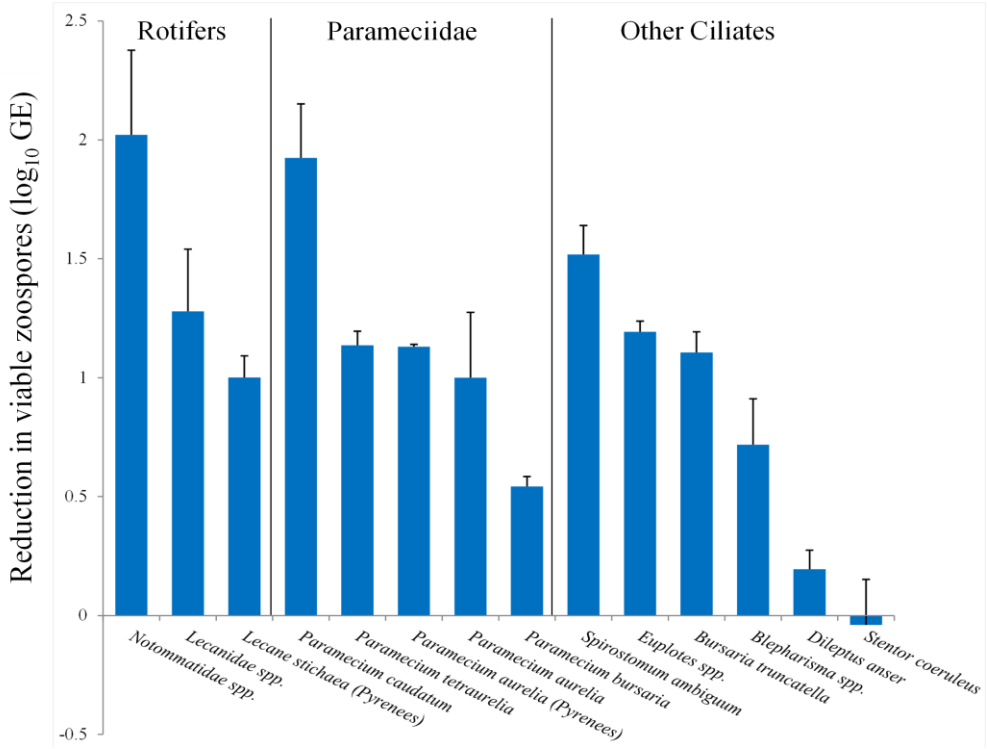

Fig 3. Reduction of zoospore viability associated with the presence of 14 protozoan/metazoan species. Reduction of viability is represented as log_{10} GE of viable *Batrachochytrium dendrobatidis* zoospores after 24 hours + 1 SD. The error bars indicate the standard error of the mean of the three replicates per species. Two species were bred from samples stemming from our Pyrenean sites, the Lecanidae rotifer *Lecane stichaea* and the Parameciidae ciliate *Parameciun aurelia*.

Discussion

The ability of microorganisms to forage on *B. dendrobatidis* zoospores has been postulated but never explicitly shown and linked to both field conditions and experimentally derived patterns of infection (16, 17). Here we show that ciliate and rotifer microorganisms are

effective consumers of *B. dendrobatidis* zoospores in the Pyrenean mountain lakes, reducing the number of free-swimming zoospores. These microorganisms also reduce the probability of infection in two amphibian species that are highly susceptible to *B. dendrobatidis*. Due to the dose-dependent impact of infection by *B. dendrobatidis* on host life-history traits, decreasing their infection burden will reduce the impact of *B. dendrobatidis* on larval development (18). *B. dendrobatidis* infection may result in host mortality only when a threshold density of sporangia (infection intensity) is reached (18), implying that control may be achieved by limiting the number of *B. dendrobatidis* zoospores. Our study raises hope that the rate and intensity of infection by *B. dendrobatidis* in amphibian populations can be manipulated by natural means, and that appropriate methods of natural augmentation of predatory microorganisms will significantly decrease the adverse effects of chytridiomycosis on amphibians and ecosystems. The results of our experiments show that both the prevalence and intensity of infection in larvae of *A. obstetricans* are site-dependent and correlate with the presence of indigenous predatory microorganisms in the water, this latter pattern was experimentally confirmed by us in a second susceptible species (*D. scovazzi*). We were able to show that rotifers and ciliates reduce the number of *B. dendrobatidis* zoospores in the environment, and that this reduction might occur in several different ways, such as concomitant predation, predation of free-living stages, or passive consumption and filtration (19). Predatory microorganisms such as the ciliates *P. caudatum* and *P. aurelia*, and the rotifers *Notommatidae spp.* and *L. stichaea* appear to have a higher foraging efficacy for *B. dendrobatidis* zoospores, ingesting *B. dendrobatidis* zoospores more efficiently than planktonic species with different foraging strategies, for example *D. anser* and *S. coeruleus*. Indeed, it is likely that only a few typical freshwater plankton species may be unable to ingest *B. dendrobatidis* zoospores. Microorganisms able to prey on *B. dendrobatidis* zoospores are expected to be numerous as aquatic environments are rich with species of chytrid that utilise zoospores as a dispersal unit and therefore represent a rich source of potential nutrition (20). This observation is confirmed by laboratory based studies showing that planktonic *Daphnia* species can consume *B. dendrobatidis* (16, 17). Therefore, we suggest that many indigenous plankton species are pre-adapted to predating *B. dendrobatidis* zoospores, as these are similar in size and form to endemic zoosporic aquatic fungi that likely form a key nutritional component of the microfaunal plankton diet (21).

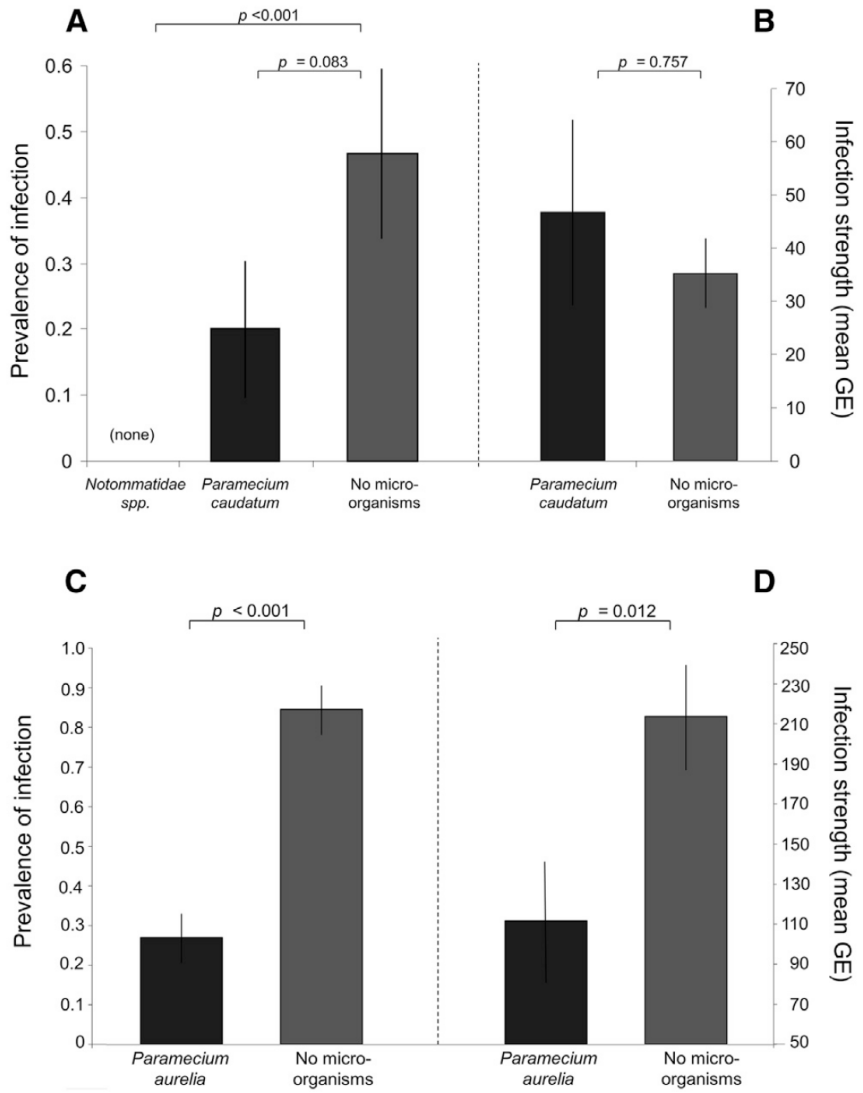

Fig 4. Challenge experiment under the influence of microfauna. Number of infected tadpoles (A + C) and intensity of infection (B + D) of *Discoglossus scovazzi* tadpoles exposed repeatedly to *Batrachochytrium dendrobatidis* zoospores and cohoused with *Paramecium caudatum*, *Notommatidae spp.* (A + B), *P. aurelia* from the Pyrenees (C + D), or housed in the absence of microorganisms.

Environmental factors have been identified that co-vary with the prevalence of infection and chytridiomycosis (11, 13). Most prominently, lower temperature regimes at higher altitudes are associated with higher *B. dendrobatidis* infection probability (13). Temperature may act to modify prevalence through direct and indirect pathways, either by directly influencing host immunity and pathogen growth rates, or by indirectly influencing the activity of microorganisms across infected sites. This goes some way to explaining why both prevalence of infection and mortality are more common in the Pyrenees when environmental

temperatures are very low, and when laboratory estimates of *B. dendrobatidis* growth rates and zoospore production indicate infection should be rare (13, 22). Environmental factors may also explain the composition, density and dynamics of the planktonic communities across seasons (23), with sites of high *B. dendrobatidis* prevalence being an enemy-free space for *B. dendrobatidis*, allowing the fungus to infect suitable host species rapidly and with a high intensity. Additional support for the generality of our findings comes from recent studies, showing that *B. dendrobatidis* positive ponds were more similar to each other than would be expected based on chance (15) and that the dilution effect hypothesis may apply to the amphibian-*B. dendrobatidis* system (24), suggesting links between pond ecology and local-scale epidemiological dynamics. More detailed ecological studies are now needed to better link abiotic variables to the composition, density and dynamics of the planktonic communities, and the outcome of the host/pathogen dynamic.

We here show the importance of predation in controlling infections in larvae of two amphibian species and provided direct evidence that zoospore ingestion is the mechanism through which infection is modified (19). Developing methods that facilitate natural augmentation of predatory microorganisms as a form of *B. dendrobatidis* biocontrol may hold promise as a field mitigation tool, one that lacks the downsides associated with introducing non-native biocontrol agents, such as the use of antifungal chemicals or release of non-native skin bacteria into the environment, or the reliance of unpredictable environmental temperature to 'cure' infections (25, 26). However, before biocontrol can be safely attempted, additional study is required.

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Author contributions statement

M.B. contributed in the design of the experiments. M.B. carried out the *B. dendrobatidis* viability and ingestion experiments. M.B. carried out the *B. dendrobatidis* challenge experiments. M.B. contributed in writing and reviewing of the manuscript.

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Supplementary materials

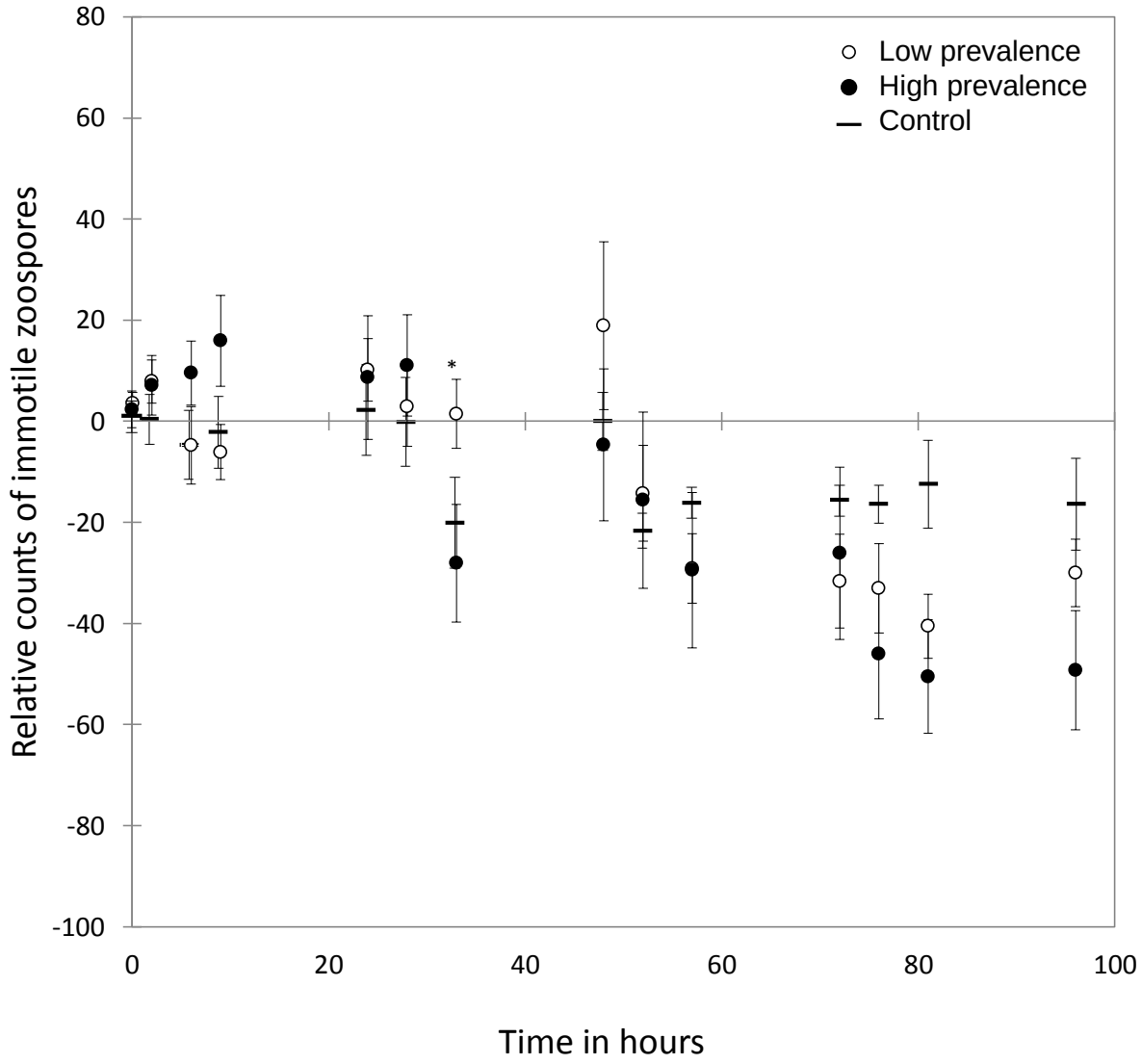

Fig S1. Dynamics of immotile *Batrachochytrium dendrobatidis* zoospores under lab conditions in water of high and low prevalence sites, related to Figure 1. Counts of immotile zoospores exposed to water from high prevalence (open filled dots) vs low prevalence sites (filled open dots). Counts for each water sample were standardized relative to counts at t = 0; relative counts). Controls are counts of immotile zoospores in treatments where distilled water was added to the culture. The error bars indicate the standard error of the mean across water samples in the same prevalence group. The asterisk indicate the significance level of the post-hoc test with * ≤ 0.05; ** ≤ 0.01; * ≤ 0.001.**

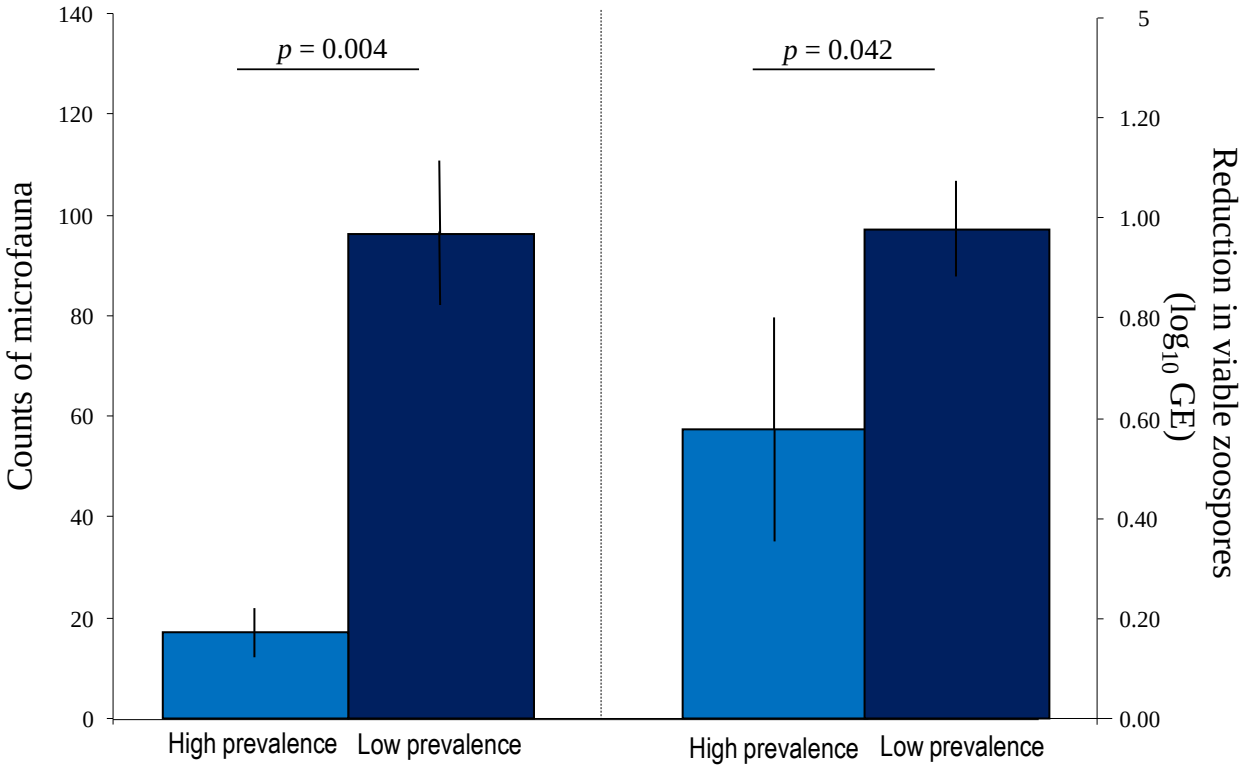

Fig S2. Microfauna and reduction in viable *Batrachochytrium dendrobatidis* DNA in water from high and low prevalence sites, related to Figure 2. Mean counts of microfauna detected in water collected from sites with different infection histories (left panel) and the impact of water source on the mean viability of *Batrachochytrium dendrobatidis* zoospores (right panel). Viability is represented as $\log_{10}$ of the number of living zoospores detected using qPCR (genomic equivalents, GE). Error bars are standard errors of the mean GE or number of microfauna counts per group of sites.

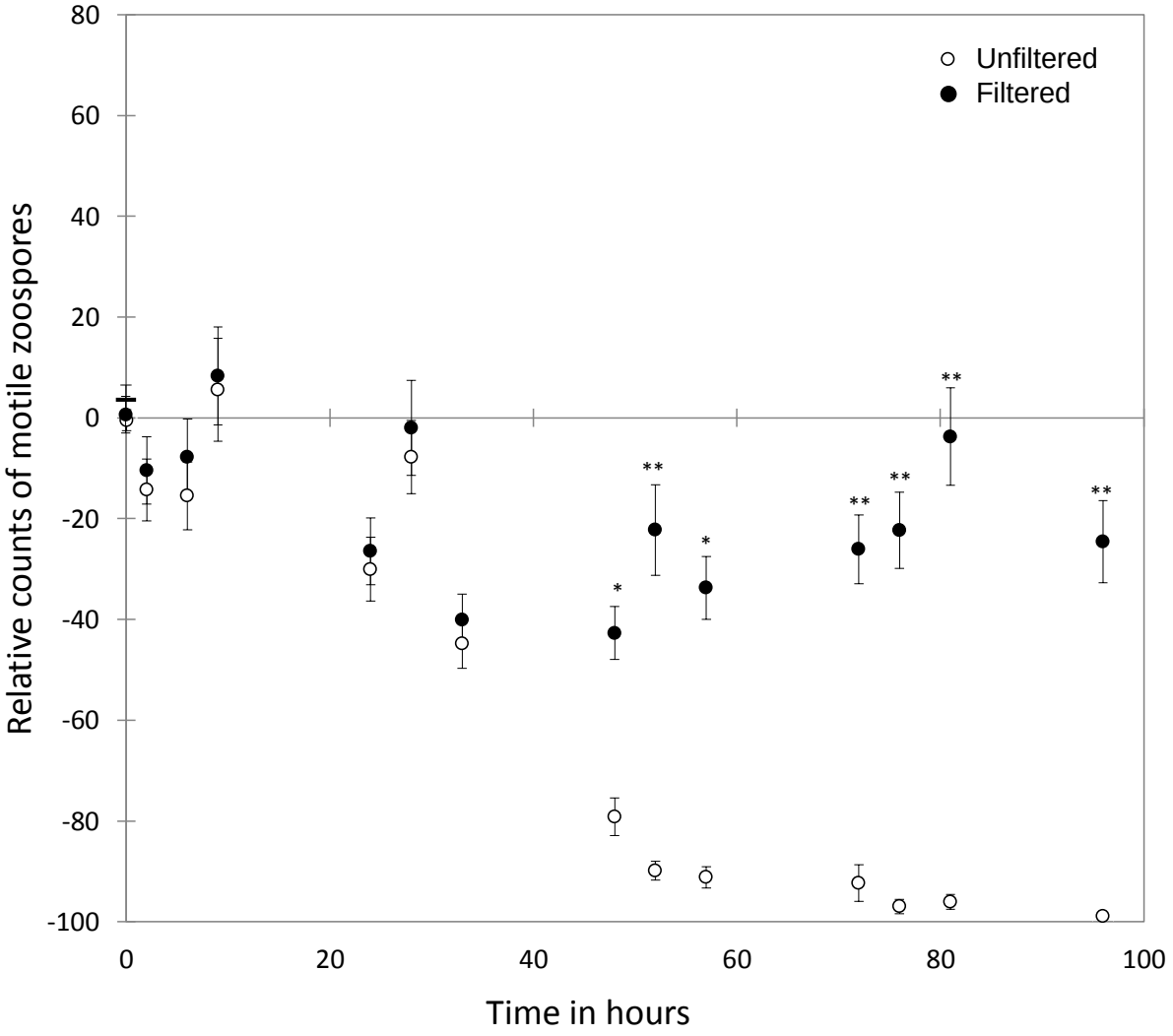

Fig S3. Dynamics of viable *Batrachochytrium dendrobatidis* zoospores under lab conditions in filtered (filled dots) and unfiltered (open dots) water, related to Figure 1. Counts for each water sample were standardized relative to counts at t = 0; relative counts). Controls are counts of viable zoospores in treatments where distilled water was added to the culture. The error bars indicate the standard error of the mean across the water samples in the same treatment group. The asterisk indicate the significance level of the post-hoc test with * ≤ 0.05; ** ≤ 0.01; * ≤ 0.001.**

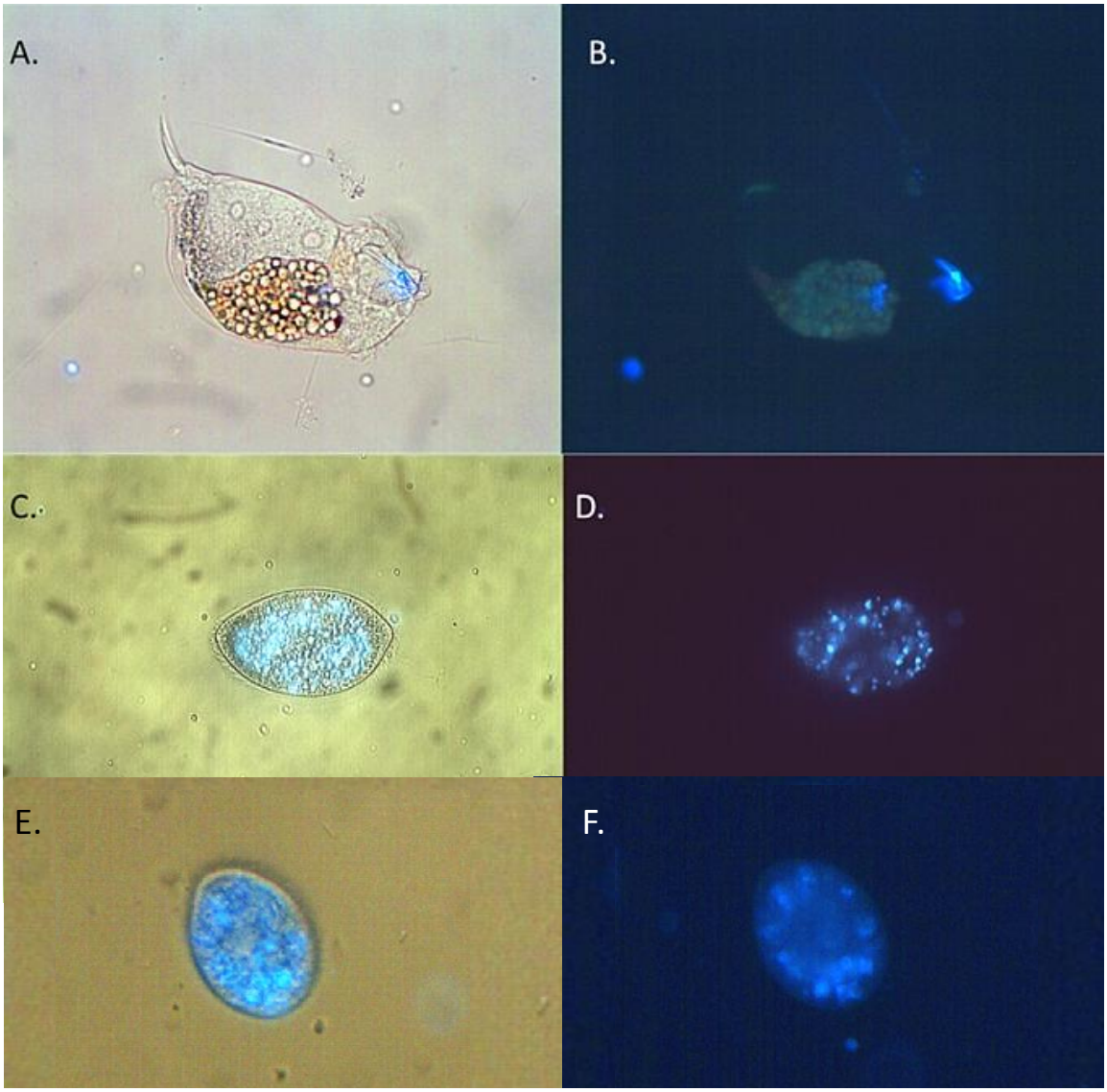

Fig S4. Ingestion of *Batrachochytrium dendrobatidis* zoospores by microfauna, related to Figures 3 and 4. Light (A + C + E) and fluorescence (B + D + F) microscopy images (100x magnification) 2 hours after adding CFW fluorescently labeled *Batrachochytrium dendrobatidis* zoospores to water with either *Notommatidae spp.* (A + B), *Paramecium caudatum* (C + D), or *Paramecium aurelia* (isolated from the Pyrenees; E + F) present.

Table S1: Sample sites and use in the different experiments. Acidity (pH) and Conductivity (µS) were not significantly different between low and high prevalence sites (pH: $U_{31} = 117$; $p = 0.584$; µS: $U_{31} = 64$; $p = 0.102$). Log_{10} GE was also best explained by the count of microorganisms in the water, and not by acidity or conductivity following from best model selection based on an AIC ($F_{1,23} = 11.25$, $p = 0.003$; AIC = 41.031; model with count and acidity: AIC = 40.785; model with count, acidity, and conductivity: AIC = 38.817). *Batrachochytrium dendrobatidis* prevalence in 2012 was best explained by altitude only (AIC 180.765; model with pH and altitude: AIC = 181.593; model with count, pH and altitude: AIC = 182.946).

Name	Longitude	Latitude	*B. dendrobatidis* prevalence over several years	*B. dendrobatidis* prevalence in 2012	Count	log_{10} GE	pH	µS	Altitude
Lac Acherito[1,2,3]	42.87825	-0.70920	high (>90%)	100	0	0.633291	8.0	117	1858
Lac Ansabere[1,2,3,4]	42.88778	-0.70885	high (>90%)	100	80	1.328100	8.5	121	1876
Lac du Lhurs[1,2,3]	42.92195	-0.70196	high (>90%)	77	0	1.239231	8.1	133	1697
Lescun[1,2,3]	42.93340	-0.63746	low (<5%)	0	0	0.478956	7.9	450	1000
Lac Puits d´Arious[1,2,3,4]	42.86403	-0.63343	high (>90%)	100	50	0.305379	8.1	14	1891
Lac Arlet[1,2,3,4]	42.84038	-0.61500	high (>90%)	100	30	1.549297	7.9	52	1974
Borce[2]	42.79622	-0.55572	low (<5%)	0	240	1.300827	8.3	98	1333
Andouste[1,3,4]	43.04441	-0.36261	low (<5%)	0		0.896113	7.8	306	1123
Lac de Paradis[2]	42.84915	-0.15998	low (<5%)	0	0	0.247464	8.7	67	1620
Lac Gaube[2]	42.83431	-0.13952	low (<5%)	0	115	1.096387	7.3	32	1729
Madaméte[1,2,3]	42.86840	0.14362	high (>90%)	97	0	-0.000260	7.2	16	2310
Lac de Madaméte[1,2,3]	42.86701	0.14369	high (>90%)	97	0	-0.031457	7.6	30	2306
Lac d´Aumar[2]	42.84576	0.14390	low (<5%)	5	60	0.311590	7.2	25	2186
Les Laquettes de madamete[1,2]	42.86342	0.14398	high (>90%)	97	0	0.032064	7.2	16	2400
Gourg de Rabas[1,2,3]	42.85250	0.14500	high (>90%)	100	0	0.051735	7.4	6	2395
Francazal[4]	43.01840	0.99816	low (<5%)	0		0.546851	8.2	436	410
Pradas Lavoir[1,2,3]	42.97042	0.99875	low (<5%)	0		0.233631	7.6	390	1009
Balagué[1,2,3,4]	42.96403	1.02503	low (<5%)	0		0.483944	7.5	520	803

Alas[1,2,3]	42.94993	1.04323	low (<5%)	0		0.486667	7.8	204	580
Etang d´Ayes[1,2,3]	42.84408	1.06479	low (<5%)	0	90	1.157742	7.6	43	1681
Caumont[1,2,3]	43.02630	1.07247	low (<5%)	0		0.567321	7.6	576	410
Lac de Bethmale[1,2,3,4]	42.86224	1.08435	low (<5%)	0		0.708633	7.7	112	1065
Estagnon[2]	42.80499	1.37213	low (<5%)	0	210	1.311571	7.3	121	1317
Lers brook[1,2,3]	42.80851	1.37399	low (<5%)	0	60	0.933718	7.3	72	1267
Etang de Lers[1,2,3]	42.80793	1.37486	low (<5%)	0	0	1.098308	7.2	70	1275
Arbu tourbiere[2]	42.80321	1.42116	low (<5%)	0	75	1.180261	8.0	10	1391
Arbu pond[2]	42.81935	1.43748	low (<5%)	0	25	0.992924	7.6	13	1729
Lac Arbu[2]	42.82038	1.43759	low (<5%)	0	25	0.982115	8.0	6	1729
Soula[2]	42.94325	1.69603	low (<5%)	0	45	0.969035	7.8	523	600
Plat Peyre[2]	42.64682	1.70218	low (<5%)	0	90	1.159868	7.5	28	1711
Plat Peyre Barrage[2]	42.66732	1.70763	low (<5%)	0	175	1.292168	7.9	28	1600
Leychart[2]	42.94416	1.72933	low (<5%)	0	290	1.347218	7.2	496	609

$\log_{10}$ GE = reduction in viable *Batrachochytrium dendrobatidis* zoospore DNA; Count = counts of protozoans and metazoans; Index in first column = experiment the sample was used in; pH = Acidity; µS = Conductivity

Supplementary Experimental Procedures

Sample sites

We collected water samples from the lenthic parts of 32 different amphibian breeding sites located in the Pyrenean Mountain range with known histories of presence of *B. dendrobatidis* in the sentinel amphibian host species, *A. obstetricans*, to experimentally examine the effect of water and the aquatic microbial community on the probability of infection. The majority of these sites (n = 23) have exhibited *A. obstetricans* populations with low prevalence (< 5%) or lack of infection (minimum sampling size = 30 individuals), and an absence of mortality in *A. obstetricans* for up to six years of field sampling, while a smaller number of populations (n = 9; Table S1) have consistently exhibited high prevalence (usually > 90%) through time (up to ten years) and in most cases mass mortality of recently metamorphosed *A. obstetricans* over the same time span [13].

The water sampling was conducted across all sites over a one week period in 2012 to minimize seasonal effects. The water was sampled in 50 ml sterile culture vessels. In the field, the samples were cooled by gel packs and transferred to a fridge at 6^{o}C as soon as possible, usually within 2hrs after sampling.

B. dendrobatidis strain & culture conditions

We used three different *B. dendrobatidis*-GPL isolates for experiments. We used IA043 for experiments 1 and 3, isolated in 2005 from a dead but recently metamorphosed *Alytes obstetricans* collected from a lake in the Spanish Pyrenees, Ibon Acherito. For experiments 2, 5, 6 and 7 we used JEL423, kindly provided by Dr. J. Longcore and isolated from Lemur leaf frogs (*Phyllomedusa lemur*) involved in a mass mortality event at El Copé, Panama, 2004. We used RAB3 for experiment 4, isolated in 2011 from the mouthparts of an *A. obstetricans* tadpole sampled at the Gourg de Rabas in the French Pyrenees. All strains were grown in TGhL broth (JEL423: 16 g tryptone, 4 g gelatin hydrolysate, 2 g lactose per liter distilled water; IA043 and RAB3: 10 g tryptone, 3.2 glucose and 1000 ml distilled water) in 25 cm^2 flasks at 20° C for 5 days before use. Experimental zoospores were harvested one of two ways. TGhL agar plates (16 g tryptone, 4 g gelatin hydrolysate, 2 g lactose, 10 g bacteriological agar per liter distilled water) were inoculated with a 2 ml aliquot of 5-day-old broth culture, and incubated for 5 – 7 days at 20°C. Zoospores were collected by flooding each plate with 2 ml distilled water and washed three times in distilled water followed by centrifugation (1200 rpm, 20 °C, 2 minutes) and resuspension. Zoospores were also harvested

from broth culture by centrifuging 200ml of culture four times (1500 rpm, 20°C, 2 minutes). Between each centrifugation step, we added 10ml of distilled water to further dilute the broth medium. The concentration of zoospores per ml was determined by visual counts using a haemocytometer.

Experiment 1

We collected water samples from 9 high prevalence and 10 low prevalence sites located in the French Pyrenees (Table S1) and stored them at 6°C for no longer than 72 hours before experimental procedures. We controlled for the presence of *B. dendrobatidis* zoospores in the water before the experiments by microscopically analyzing 1 mL of the sampled water. In no case did we observe any zoospores or zoosporangia in our water samples. Additionally, we filtered 2 liters of water in the field to test for the presence of *B. dendrobatidis* DNA in these samples, but also these samples were never *B. dendrobatidis*-positive. All exposures were done in a laminar flow safety cabinet with a Hepa14 filter. We exposed 5 mL of broth-cultured zoospores in sterile 6-well cell culture plates to 20µl of each water sample, replicated 3 times over different plates. Each plate included a negative control (20µl of commercially available purified water) and all plates were incubated for 96 hours at 18.0 +/- 0.1 °C. From each of our replicates/wells we took 2x 15µL aliquots to estimate the relative number of motile and inactive intact zoospores at hours 0, 2, 6, 9, 24, 28, 33, 48, 52, 57, 72, 76, 81, 96. For each aliquot we counted the number of motile (Fig. 1) and immotile (Fig. S1) intact zoospores in six haemocytometer cells each (12 cells in total per replicate) at a total magnification of 200X. These counts were then used to calculate the number of zoospores per ml following standard procedures for Malassez haemocytometers [S1]. For a given point in time, we obtained 6 counts per site. Counts for each water sample were standardized relative to counts at t = 0. Finally, we calculated the mean count per site and used this value for statistical analyses.

Experiment 2

One ml aliquots of environmental water samples (10 high prevalence sites, 15 low prevalence sites, Table S1) were transferred to wells of 48 well plates and we used inverted light microscopy (Nikon Eclipse ts100, 10 and 20x magnification) to count the microorganisms on the bottom of each well. We then added 200 µl of a 10^6 active zoospores per mL suspension prepared from plates inoculated with *B. dendrobatidis* strain JEL423 to each well. We measured the concentration of viable zoospores at t = 0 and after 24hrs using EMA-qPCR [S2] (Fig. S2). During qPCR and the EMA qPCR each sample was run in duplicate.

Experiment 3

Experiments 1 and 3 were run in parallel and we used a similar experimental design as of experiment 1 using 2 replicates of water from each site but filtered one replicate per site through a syringe filter (Millex sterile syringe filter, 0.45 µm, Millipore) before exposing zoospores. The filters consisted of a mixed cellulose ester grid that were nontoxic, ensuring sample integrity. Filter size was selected to remove microorganism without removing large chemical molecules. We standardized counts of zoospores after t = 0 for each replicate (n = 18; 8 high prevalence sites, 10 low prevalence sites) using the count at t = 0.

Experiment 4

We collected water from three high prevalence sites [(Arlet (N42.84048 W0.61483), Puits (N42.86403 W0.63343), Ansabère (N42.88778 W0.70885)] and three low prevalence sites [(Lac de Bethmale N42.86224 E1.08435), Balagué (N42.96403 E1.02503), Andouste (N43.04441 W0.36261)] located in the Pyrenees. Water samples (30 liters per site) were shielded from sunlight and kept cooled during transport and maintained at 6°C in the laboratory before the experiment. Tadpoles (n = 135) were collected from a population in Ariège (Francazal, N43.01843 E0.99809) and tested negative for *B. dendrobatidis* during the one week acclimatization period before the start of the experiment. Each experimental treatment (3, water from high prevalence sites and not heat-treated, water from low prevalence sites and not heat-treated and water from low prevalence sites and heat-treated for 15 min at 100 °C) was replicated 3 times with 5 tadpoles per replicate. Tadpoles were cohoused by replicate in 20 cm x 30 cm aquaria containing 3 liters of treatment water, with water changed at Day 1, Day 5 and Day 8, just before infection. Tadpoles were randomly assigned to treatment and tadpoles in different treatments did not differ in body weight (mean ± s.e., high prevalence treatment, 1.19 ± 0.12 g; low prevalence treatment, 1.19 ± 0.12 g; low prevalence and heat-treated treatment, 1.19 ± 0.11 g). The experiment was done in a constant temperature room (19°C) on 14hr:10hr light cycle (ZooMed Reptisun 2.0 fluorescent bulb). Tadpoles were fed twice a week with one tablet of Tetratabimin. On Day 1, Day 5 and Day 8 approximately 900,000 *B. dendrobatidis* zoospores were added to each aquarium just after the water change and food was added 5 hours after exposure to minimize any effect food addition might have had on initial contact between zoospores and tadpoles. Tadpoles were swabbed on Day 5, Day 8 and Day 12 before water changes and addition of zoospores. Swab samples were assayed for infection using the standard qPCR of Boyle et al [S3].

Experiment 5

Cultures of twelve species of common freshwater protozoans and metazoans were maintained according to standard culture conditions for these species, and two protozoan species were isolated from Pyrenean water samples (Lac de Bethmale, Francazal) and maintained according to culture conditions for sister species. Before exposure to *B. dendrobatidis* zoospores, cultures were pelleted using low speed centrifugation (1200 rpm, 20° C, 2 minutes), culture media was removed and each pellet resuspended in an equal volume of distilled water. Cultures were split (10 mL each) into filtered (5µm syringe filter, microorganism free solution) and unfiltered (approx. 500 microorganisms/mL) solutions, 1 ml of each was transferred to individual wells of 48 well plates and 200 µl of a zoospore suspension containing approximately 10^6 *B. dendrobatidis* was added to each well. Reduction of zoospore viability was calculated as the difference in the number of viable zoospores, estimated using EMA qPCR, between the filtered and unfiltered solutions (Fig. 3). The ability of each microorganism to reduce zoospore viability was tested 3 times.

Experiment 6

We labeled *B. dendrobatidis* zoospores with Calcofluor White (CFW; $C_{40}H_{44}N_{12}O_{10}S_2$, fluorescent brightener 28, Sigma-Aldrich Inc., Bornem, Belgium) using a stock solution (35 mg of CFW in 10 ml of sterile distilled water) diluted to a final concentration of 1% (v/v). This concentration has been used previously to selectively label other members of the Chytridiales [S4]. Thirty minutes after adding CFW to a *B. dendrobatidis* zoospore suspension containing approximately 10^5 zoospores per ml we pelleted the suspension using low-speed centrifugation (1200 rpm, 20 °C, 2 minutes) and re-suspended the resulting pellet in 10 mL of distilled water. We first examined 25 µl of the labeled *B. dendrobatidis* solution visually with an epifluorescence microscope equipped with a 340 to 380 nm filter (100 X magnification) and observed fluorescently labeled and viable zoospores. We attempted CFW labeling of six of the microorganisms from Exp. 5 [*Paramecium caudatum*, *Notommatidae* spp. (strong reduction in zoospore viability), *Dileptus anser* and *Stentor coeruleus* (little or no reduction in zoospore viability), *P. aurelia* and *L. stichaea* (originating from Pyrenean water samples)] following the same protocol and did not observe any evidence of autofluorescence, but did observe evidence of labeling of mouthparts of *Notommatidae* spp. (Fig. S4A, B) and *L. stichaea*. We then exposed 1 mL solutions of these six organisms for 2 hours at 20° C to 1 mL solution of labeled zoospores. Evidence of ingestion of *B. dendrobatidis* was observed by

direct epifluorescence microscopy (Fig. S4). Labeled *Notommatidae* spp. and *L. stichaea* mouthparts did not obscure ingested zoospores.

Experiment 7

We determined the difference in infection rate and infection intensity of *Discoglossus scovazzi* in the presence of *P. caudatum*, *Notommatidae* spp., *P. aurelia* originating from a Pyrenean site, or without microbiota. We conducted two experiments, one with *P. caudatum* and *Notommatidae* spp. (Exp. 7A), and one with *P. aurelia* (Exp. 7B) later in time. Forty-five (120 for Exp. 7B) 7-day-old *D. scovazzi* larvae (Gosner stages 26 – 30), provided through the captive breeding colony in the Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, were housed together for 7 days in a 6 l plastic container containing 3 l (4 l for Exp. 7B) of aged tap water prior to the infection experiment. Both before and after the start of the experiment, larvae were kept at 20 °C on a natural, 16hr:8hr light/dark cycle and fed commercial fish flakes (TetraMin by Tetra) daily *ad libitum*. Water was 2/3 changed every other day. For experiment 7A, on day 1 of the infection trial 5 tadpoles were randomly allocated to each of 9 plastic containers containing 200 ml of aged tap water. Replicates (containers) were assigned to one of three treatments (3 containers per treatment): water containing approximately 10 *P. caudatum* per mL of container water, water containing approximately 10 *Notommatidae spec.* per mL of container water, or water free of microbiota (control). For experiment 7B, on day 1 of the infection trial 20 tadpoles were randomly allocated to each of 6 plastic containers containing 500 ml of aged tap water. Replicates (containers) were assigned to one of two treatments (3 containers per treatment): water containing approximately 10 *P. aurelia* per mL of container water or water free of microbiota (control). In both experiments, microbiota were replenished after water changes (Day 2, 4 and 6) on Days 3 and 5, and *B. dendrobatidis* zoospores were added at Day 1, 3 and 5 to every container to a final concentration of 1000 *B. dendrobatidis* zoospores per ml. All larvae were euthanized on Day 7 of the infection trial by an overdose of benzocaine. Mouthparts were excised, stored in 70% ethanol and screened for infection with *B. dendrobatidis* following extraction [S5] and qPCR [S3].

Statistical analyses

Statistical analyses were performed using SAS 9.1.3 (Cary, USA). In experiments 1 and 3, we explored how source of water (infected vs uninfected sites or filtered vs unfiltered), day of zoospore counting and the interaction between the two (all fixed factors) affected the number of viable zoospores using generalized linear mixed models (GLMMs, proc glimmix). We did

run the statistical analysis on the means per site to avoid difficulties due to repeated sampling and double counting of samples. We examined the effect of the number of protozoans and metazoans on zoospore viability in experiment 2 using a generalized linear model (GLM, proc genmod) with a Poisson distribution of error terms (link function: log), the number of protozoans plus metazoans as a fixed factor and site identity as a repeated measure. We also used GLMs to investigate the impact of the site infection status on the number of protozoans and metazoans (proc genmod, distribution of error terms: Poisson, link function: log), and zoospore viability (proc glm, distribution of error terms: Gaussian, link function: identity), with the site infection status as a fixed factor. In experiments 4 and 7, we investigated the impact of treatment on infection status using GLMMs with a binomial distribution of error terms (0/1 = not-infected/infected tadpole, link function: logit). We included treatment (water from high prevalence, low prevalence and low prevalence and heat-treated for experiment 4, and water containing *P. caudatum*, *P. aurelia*, *Notommatidae* spp. and no microbiota for experiment 7) as a fixed factor and the aquarium/container identity as a random factor. In experiment 4, we also included the day of swab (5, 8 or 12) and the interaction between the treatment and time as covariates, and tadpole weight as an additional random factor. These GLMMs were followed by pairwise Tukey post-hoc tests. In addition, in these two experiments, we examined the impact of the treatment on *B. dendrobatidis* loads of infected individuals (GE) with a GLMM with a Poisson distribution of error terms (link function: log) and similar fixed and random factors as in the previous models. Pairwise *p*-values were obtained by Tukey post-hoc tests. When needed and when possible, we adjusted the degrees of freedom using the Satterthwaite correction which account for repeated sampling and can result in fractional degrees of freedom (Experiments 1, 3, and 4). We used a non-parametric Mann-Whitney U-test to test for differences in acidity and conductivity of high and low prevalence sites. We used a GLM (proc genmod, distribution of error terms: Poisson, link function: log) and best model selection based on AIC to test for explanatory power of microorganism count, acidity and conductivity on zoospore viability ($\log_{10}$ GE) and Spearman's Rank correlations for relationships between microorganism count, altitude and zoospore viability ($\log_{10}$ GE).

Supplemental References

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General discussion

As mentioned in the introductory chapter of this thesis, biodiversity loss is occurring at an alarming rate, with amphibians being the class of vertebrates most affected, and with infectious diseases identified as one of the main drivers for loss of amphibian diversity (1-3). The disease with the highest impact is without doubt the fungal skin disease chytridiomycosis, caused by the amphibian chytrid fungi *Batrachochytrium dendrobatidis* (4) and the recently emerged *Batrachochytrium salamandrivorans* (Chapter 2). Fungal agents of emerging infectious diseases are currently posing an increasing threat to plant and animal biodiversity, and are identified as drivers of loss of biodiversity (5). The proposed underlying mechanisms for the increased impact of these selected fungal pathogens on plant and animal diversity are their high virulence and generally high rate of reproductive potential, their broad host range, variability in the susceptibility to infection between host species and lifestages, effects associated with anthropogenically influenced dispersal of fungal pathogens and climate-change induced alterations to infectious disease systems (5). For chytridiomycosis, consensus among scientists is gathering that the combination of ecologically relevant cofactors determines the impact of chytridiomycosis on individual and population levels (6, 7). Therefore, instead of focusing on isolated parameters, attention should be turned towards interactions between hosts, pathogens and environments in order to understand and predict the impact of chytridiomycosis on local and global scales. The discovery of *B. salamandrivorans*, a novel chytrid fungus with the potential of becoming the next disaster for amphibian diversity (Chapter 3), calls for a quick response in terms of studies that address monitoring, control and disease dynamics of *B. salamandrivorans* on a species and population level. In this chapter I will discuss current issues of chytridiomycosis monitoring and disease mitigation, their future perspectives and research opportunities and the possible consequences associated with the discovery of *B. salamandrivorans*.

Chytridiomycosis monitoring

Effective disease surveillance is the essential first step that allows a quick response to be instigated after emergence of infectious diseases (8). Stringent biosecurity measures, reliable diagnostic assays and effective control implemented in the international trade in amphibians will reduce the chance of importing *B. dendrobatidis* (and other amphibian pathogens) (9, 10). Furthermore, a high degree of general biosecurity will create a barrier for currently unknown or unmonitored infectious agents. The necessity for disease surveillance in, and regulation of the trade in amphibians is also recognized by the IUCN SSC Amphibian Specialist Group (ASG) and the Amphibian Survival Alliance (ASA), which released the following statement

shortly after the discovery of *B. salamandrivorans*: "Unregulated and unmonitored global amphibian trade is considered a major mechanism for dispersal of invasive species, including non-native emerging infectious diseases (EID). There are currently no global safeguard standards to ensure that amphibians in the international trade are monitored and tested for amphibian diseases. This means that amphibian populations in unaffected areas are at a very high risk of being impacted by EIDs that may be transported by amphibian hosts in the pet trade." As we have encountered with *B. dendrobatidis*, and as is considered the case with all communicable wildlife emerging infectious diseases, preventing introduction is of the utmost importance, as after emergence of the disease has occurred, it is virtually impossible to control it. Lack of proper control measures and disease surveillance in the international trade in amphibians has led to the globalization of *B. dendrobatidis*, not only exposing naïve and susceptible amphibian populations to this pathogen, but also enabling recombination of different *B. dendrobatidis* strains creating the possibility of hypervirulent strains to emerge (11, 12). Apart from measures aimed at preventing introduction, attention should be focused on reducing chances of releasing pathogens in the environment and limiting spread of pathogens once they are released (9).

Although currently *B. salamandrivorans* seems to be present only in the environment in Asia and Europe, chances are that *B. salamandrivorans* infected animals are shipped around the globe (Chapter 3). Therefore, monitoring and control of *B. salamandrivorans* in the international trade should be given priority. The high degrees of sensitivity and specificity, the ability to detect the pathogens in non-invasively collected amphibian samples and the relative speed with which the samples can be processed, make the described duplex real-time PCR for detecting both *B. dendrobatidis* and *B. salamandrivorans* (Chapter 4) an ideal assay to implement for chytridiomycosis disease surveillance. There are however disadvantages linked to this technique that should be considered. First of all, although the duplex real-time PCR does detect al currently known etiological agents of chytridiomycosis, it does not imply a disease-free state of the animal. For disease surveillance and control in captive amphibians, assays designed to detect specific pathogens should always be used in conjunction with more general precautionary measures, like quarantine periods in which the clinical conditions of individuals can be observed, and strict biosecurity measures like general disinfection and proper disposal of biological material (9). Another drawback associated with using the duplex real-time PCR to screen for *B. salamandrivorans* presence is the apparent occurrence of false negative results under certain conditions. In three studies in which the real-time PCR was used to detect and quantify *B. salamandrivorans* infections in amphibians (Chapters 3, 5 and

6), initially negative results were obtained from animals that subsequently showed delayed increase in infection intensity or that showed relapse of infection. The exact cause for these false negative results remains unknown, but theoretically this could be explained by presence of *B. salamandrivorans* in the deeper layers of the skin where it is undetectable with non-invasively collected skin swabs, or by presence of low numbers of *B. salamandrivorans* organisms but occurrence of real-time PCR inhibition. The occurrence of false negative real-time PCR results implies a reduced sensitivity of the real-time PCR under certain circumstances and after a single test. An increase in the sensitivity of the real-time PCR can be achieved by repeated testing of the animal. Repeated testing is theoretically achievable for animals in the international trade, and the associated increase in costs of disease surveillance could be passed on to the consumer, without impacting economic benefits of wholesale and/or retail (13). The occurrence of false negative *B. salamandrivorans* results in wild amphibian assemblages poses another problem, as early disease detection is crucial in order to try to limit spread and impact of *B. salamandrivorans*. Detecting *B. salamandrivorans* in wild amphibian populations is further hampered by the fact that corpses of infected individuals deteriorate very rapidly resulting in the loss of detectable *B. salamandrivorans* DNA in the skin (< 24 hours) (unpublished results). In areas with confirmed presence of, and mortalities due to *B. salamandrivorans*, detection of *B. salamandrivorans* in the majority of dead animals is impossible due to rapid decay of the body and complete absence of the epidermis. Molecular testing (PCR and real-time PCR) and histological examination of tissues of these animals fail to identify *B. salamandrivorans* as causative agent (while the probability of death due to *B. salamandrivorans* infection is high). Advancements in molecular diagnostic machinery could prove promising for improving the detection of *B. salamandrivorans* both in wildlife and in the trade. Portable and battery-powered real-time PCR devices allow immediate on-site (even in remote areas) processing of small sample numbers for chytridiomycosis monitoring, removing the need for advanced laboratories and thus resulting in faster results at reduced costs (14, 15). Although other drawbacks such as storage of the reagents for the molecular assay and sterility of the work environment arise with these techniques, the possible benefits make it worth exploring.

Mitigation of chytridiomycosis

Ex situ mitigation of chytridiomycosis

Mitigation of an infectious disease *in situ* is far more challenging in comparison to mitigation of an infectious disease under controlled *ex situ* conditions, but *ex situ* conservation can form

an important part of, and sometimes last resort option for, preserving species from going extinct (16-18). For this strategy to work, safe and effective treatment regimes based on clinical trials are essential to eliminate the causative pathogen (19-22). Since the identification of *B. dendrobatidis*, many studies have been devoted to the *ex situ* treatment of chytridiomycosis. Variability in treatment outcome has been described for several pharmaceutical treatment protocols commonly used for treating *B. dendrobatidis* infections, showing that success of pharmaceutical treatment could be dependent on the amphibian species and lifestage at hand, and furthermore rely on apparent unidentified factors (19, 23). Secondly, the majority of *B. dendrobatidis* treatment studies are empirical, and lack essential *in vitro* susceptibility testing and fundamental clinical trials necessary to assure treatment efficacy, although more consensus surrounding these problems is gathering (19, 20, 24). An associated issue is the occurrence of drug related toxicity (20, 25-27). As these toxic side effects appear to be dosage dependent (20), *in vitro* susceptibility testing to determine minimal inhibitory concentrations and studies investigating mechanisms that allow drug concentrations to be lowered to achieve clearance of infection are of importance. In Chapter 7 of this thesis we describe the *in vitro* susceptibility of *B. salamandrivorans* for several pharmaceutical compounds together with a synergetic relationship between the azole antifungals voriconazole and intraconazole and the antibiotic polymyxin E. Where treatments with the isolated compounds failed to clear *B. salamandrivorans* infections in amphibians, a combination therapy of voriconazole (12.5 µg/ml) and polymyxin E (2000 IE/ml) administrated topically, twice a day during for 10 days, was able to clear the *B. salamandrivorans* infections. In this treatment protocol, the environmental temperature was a key variable, as clearance of infection was only observed at 20°C and not at 15°C. An advantage of synergy between pharmaceutical compounds is that it allows usage of reduced dosages of both compounds to achieve clearance of the pathogen, which in turn lowers possible dosage dependent drug-related toxicity Based on this study, it is worthwhile to explore possible synergistic relationships for inhibiting *B. dendrobatidis*, as similar inhibitory drug combinations might exist, allowing treatment of *B. dendrobatidis* infections with reduced drug dosages.

In situ mitigation of chytridiomycosis

As a result of chytridiomycosis conservation initiatives hundreds of threatened amphibians have been translocated from their *B. dendrobatidis* colonized habitats to captive facilities in order try and preserve them from going extinct (18). Ideally, successful *ex situ* treatment of *B.*

dendrobatidis and *B. salamandrivorans* infections in these captive amphibians as part of *in situ* mitigation could eventually result in assisted reintroduction of *B. dendrobatidis* and *B. salamandrivorans*-free amphibian species in their natural environment (or translocation to other suitable environments) (17). However, one major issue hampering reintroduction is the lack of suitable release sites due to omnipresence of *B. dendrobatidis* in the natural environment(28, 29). Theoretically, if we could augment the amphibian host and/or environment to increase the likelihood of populations to survive in presence of *B. dendrobatidis* (or *B. salamandrivorans*), this issue can be addressed (21, 29). Apart from assisting in reintroduction programs, bioaugmentation of host and/or environment could pose a promising method to reduce the impact of chytridiomycosis on a population level in nature on its own. One such bioaugmentation perspective is that of steering the aquatic environment towards conditions that limit the negative impact of chytrid infections on amphibians. This could be achieved by increasing the *B. dendrobatidis* predatory capacity of freshwater micropredator communities (Chapter 8). Chytrid infection dynamics determine the disease outcome, which ranges from asymptomatic infections (host-pathogen co-existence) to lethal disease and population crashes. Lower environmental *B. dendrobatidis* loads in the aquatic environment will lead to progression of disease being slowed down due to fewer reinfection events of the same host, reduced pathology and decreased zoospore release from infected individuals (30). Furthermore, mass mortality events and extinctions of amphibian species only occur after a *B. dendrobatidis* infection intensity threshold is surpassed (31). Measures and conditions that limit the environmental *B. dendrobatidis* load and prevent this threshold from being reached, could therefore be important mitigation strategies (21, 31). In an experimental setting, we were capable of reducing *B. dendrobatidis* prevalence and infection intensity in susceptible amphibian hosts by introducing predatory microzooplankton (heterotrophic protists and rotifers) in the water. This promising finding offers unprecedented opportunities in terms of mitigation strategies using bioaugmentation aimed at reducing the effects of chytridiomycosis in nature to counteract disease driven loss of biodiversity (21, 32). Composition and abundance of micropredator communities depend on biotic and abiotic factors, including physicochemical environmental parameter, which implies that these communities could possibly be steered (33-35). The aquatic environment is key in *B. dendrobatidis* epidemiology (36, 37). The small sized (5 µm) infectious *B. dendrobatidis* zoospores are exposed to the aquatic environment after leaving the infected host, and are therefore affected by environmental factors detrimental for zoospore survival, like for instance presence of aquatic micrograzers (38) (Chapter 8). Bioaugmentation of the aquatic

environment may thus be expected to have a major impact on *B. dendrobatidis* infection and disease dynamics of amphibian populations. Steering aquatic micropredator communities could form a promising method to lower aquatic *B. dendrobatidis* infection pressure and ultimately result in a reduction of the impact of *B. dendrobatidis* infection on amphibian populations. However, further research under controlled conditions regarding the applicability of bioaugmentation of the environment has to be undertaken before it can be applied in nature. Although this has to be confirmed, *B. salamandrivorans* infection and disease dynamics could be similarly affected by micropredator communities, as the zoospores of both *B. dendrobatidis* and *B. salamandrivorans* are likely to serve the same role in aquatic food web systems (39, 40).

Apart from creating environments that limit the environmental infection pressure of *B. dendrobatidis* and *B. salamandrivorans*, a reduced impact of chytridiomycosis on amphibian populations can also be achieved by increasing the amphibians' resistance towards *B. dendrobatidis* and *B. salamandrivorans* infections. Although generally vertebrate immune responses to fungal infections are relatively conserved, the amphibian immune system appears to play an important role in chytridiomycosis infection dynamics. More specifically, McMahon *et al*. (2014)(41) were able to induce behavioural and immunological resistance towards chytridiomycosis under laboratory conditions after repeated exposure to live and dead *B. dendrobatidis* cells, where other studies had so far failed to identify/induce a protective effect of the adaptive amphibian immune system against chytridiomycosis (42-44). Although the applicability of inducing resistance in amphibian assemblages (with different species and lifestages) against *B. dendrobatidis* under field conditions by means of exposure to dead *B. dendrobatidis* cells still has to be confirmed, development of an effective *in situ* mitigation strategy based on increasing the amphibian's resistance might be feasible. Another question that still needs to be investigated is whether the immunological resistance confers protection against all, or only selected lineages of *B. dendrobatidis*.

With the consensus that local chytridiomycosis infection and disease dynamics are steered by multiple cofactors (attributable to the host, pathogen and environment), mitigation aimed at one specific parameter might not suffice to combat chytridiomycosis at a global scale.

In my opinion a multifactorial mitigation approach, aimed at increasing the overall resistance against chytridiomycosis of amphibian assemblages together with reducing the build-up of environmental *B. dendrobatidis* infection pressures will have the highest probability of becoming successful in combating chytridiomycosis in nature.

What to expect from *B. salamandrivorans*?

Although *B. salamandrivorans* surveillance is only starting up, it appears that *B. salamandrivorans* has only emerged in Europe so far. Should this be further confirmed, attention should be focused on monitoring programs, preventing movement of *B. salamandrivorans* through international trade and effective mitigation measures as this early stage of discovery forms a major advantage in comparison to *B. dendrobatidis*, were in retrospect it was able to globalize due to lack of awareness of the importance of these aspects.

In chapter 3 of this thesis an estimation of the threat *B. salamandrivorans* poses to amphibian diversity is presented. Several worrying aspects about *B. salamandrivorans*' host range and infection dynamics are revealed, which call for immediate actions in order to avert a possible ecological disaster. First of all, experimental assessment of the pathogenicity of *B. salamandrivorans* for different amphibian taxa revealed that *B. salamandrivorans* is restricted to urodelans and highly pathogenic for 11 out of the 15 tested taxa belonging to the family of Salamandridae and 1 out of the 3 tested taxa belonging to the family of Plethodontidae. Therefore, the biggest threat lies in spread of *B. salamandrivorans* to areas with high diversity in naive amphibian species belonging to these families (just like the introduction of *B. salamandrivorans* in Europe). Although the exact route of entry of *B. salamandrivorans* into Europe remains uncertain, presence of imported *B. salamandrivorans* positive Asian salamanders in captive collections in Europe suggests the international trade in amphibians to be the likely source of introduction. Furthermore, for *B. dendrobatidis* several studies exist that correlate the international pet trade with the globalization of *B. dendrobatidis* (9, 45, 46). These aspects pose a serious threat to the United States, home to the largest diversity of salamanders in the world. Furthermore, with large numbers of Asiatic urodelan species being imported into North America on a yearly basis (47), chances are high that without stringent biosecurity measures it is just a matter of time before *B. salamandrivorans* will be introduced. Laws and legislations that put restrictions on the number of traded amphibians, and that enforce thorough monitoring for *B. salamandrivorans* presence and a high degree of general biosecurity in the international trade in amphibians, are therefore the first (and probably most effective) *B. salamandrivorans* mitigation measures, reducing the risk of introduction. Diminishing the number of exported Asiatic urodelans does not only reduce chances of vectoring *B. salamandrivorans* and other known and unknown amphibian diseases into other continents, but it would also protect wild Asiatic salamanders from overharvesting for the pet trade (48). The thermal treatment protocol described in chapter 5 could be implemented in conjunction with presence screening in traded amphibians. If the thermal tolerance of the

species at hand allow exposure to ambient temperatures of 25 °C for 10 days, this treatment can be implemented as part of a quarantine period of traded amphibians before animals are actually imported as a specific measure against *B. salamandrivorans*. It has to be noted however that the thermal treatment is currently only validated for one *B. salamandrivorans* strain. Should other *B. salamandrivorans* strains be discovered, the effectiveness of this thermal treatment protocol should be further validated.

As the study also reveals, Asiatic urodelan species exist that are able to harbour *B. salamandrivorans* infections without apparent signs of disease. Like in *B. dendrobatidis* infections, these species can significantly contribute to disease epidemiology, ascertaining sustained presence of *B. dendrobatidis* in environments and increasing chances of naive species to come into contact with the pathogen, without any signs of disease in themselves (49, 50). Therefore, extra attention should be given to monitoring and possibly restricting the trade amphibian species identified in the study as potential reservoirs of *B. salamandrivorans*.

As pointed out in the study, the conclusions on pathogenicity of *B. salamandrivorans* for the different amphibian taxa were mostly drawn from animals derived from single captive populations. Although this might hamper within-species comparisons in pathogenicity, little variation was observed in the response to exposure of *B. salamandrivorans* of individuals from the same amphibian species. Furthermore, most of the infection and disease dynamics of *B. salamandrivorans* in wild amphibian assemblages remains unknown. Although we believe that the subdivision of the amphibian taxa in the different disease categories is robust, cofactors present in nature might influence the disease dynamics of *B. salamandrivorans* after introduction. For instance, although the study reveals that *B. salamandrivorans* infections of alpine newts (*Ichtyosaura alpestris*) and great crested newts (*Trituris cristatus*) are associated with mortality of these species, national monitoring programs of amphibian species show no apparent sign of recent declines of these amphibian species in areas were presence of *B. salamandrivorans* is confirmed (51).

Although currently no indication of presence of *B. salamandrivorans* in North America exists (Chapter 3, (52, 53)), screening for *B. salamandrivorans* presence is only starting up and introduction might already have occurred. Furthermore, disease monitoring only offers snapshot views of current disease statuses of wild and traded amphibians, and since a rapid spread of disease can occur after initial introduction of *B. salamandrivorans* (Chapter 3), continued monitoring is required to allow a quick response to be initiated after introduction of *B. salamandrivorans*.

A unique basal Asian haplotype of *B. dendrobatidis* was demonstrated to be present in endemic Asian amphibians well before the globalization of *B. dendrobatidis* lineages (54), indicative of an ancient relationship between Asiatic amphibians and amphibian related chytrid fungi. The proposed ancient divergence of *B. salamandrivorans* from *B. dendrobatidis* is in concordance with this hypothesis (Chapter 3), and should it be further confirmed, it is not unlikely that Asia houses other, amphibian associated chytrid fungi (or novel strains of *B. salamandrivorans*). Should this theory be true, translocation of these chytrids to continents where amphibian species did not have the time to evolve resistance due to lack of a shared evolutionary history, could have the same catastrophic effects as introduction of *B. dendrobatidis* and *B. salamandrivorans*.

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Summary

Chytridiomycosis, the fungal skin disease in amphibians caused by the chytrid fungus *Batrachochytrium dendrobatidis*, is one of the major drivers of declines and extinctions of amphibian species worldwide. Effective *ex situ* treatment protocols for *B. dendrobatidis* exist, but measures to control chytridiomycosis in natural amphibian populations are lacking. Anthropogenically mediated dispersal has led to the globalization of *B. dendrobatidis*, and although the majority of amphibian species are considered to be susceptible to *B. dendrobatidis*, variability in the susceptibility between and within amphibian species exists. This variability is caused by factors associated with the amphibian host, the pathogen and the environment.

In northwestern European countries, presence of *B. dendrobatidis* is only associated with occasional, isolated mortalities of amphibians, and until recently no indication of disease driven declines of amphibian populations were observed. However, from 2008 onwards, rapid declines of stable populations of fire salamanders (*Salamandra salamandra*) in the Netherlands were observed, suggestive of emergence of an infectious disease.

The two-fold aim of the current thesis was therefore to gain insights in the contribution of chytridiomycosis in the decline of amphibian populations in northwestern Europe, and to develop chytridiomycosis diagnostic and treatment protocols, with emphasis on both *ex situ* and *in situ* applications.

In the **first study**, we isolated a novel chytrid fungus, *Batrachochytrium salamandrivorans*, from the aforementioned declining Dutch fire salamander population, and show that it is able to cause chytridiomycosis. Infections with this novel chytrid fungus were associated with erosive skin disease and rapid mortality in both naturally and experimentally infected fire salamanders. Striking differences between *B. salamandrivorans* and the closely related *B. dendrobatidis* (together forming a chytridiomycete clade adapted to vertebrate hosts) are *B. salamandrivorans'* lower thermal growth preference and resistance to *B. salamandrivorans* of midwife toads (*Alytes obstetricans*), an amphibian species considered highly susceptible to *B. dendrobatidis*. The threat posed by this novel disease entity to amphibian biodiversity, together with a hypothesis for *B. salamandrivorans'* origin, is presented in the **second study**. Results show that *B. salamandrivorans* poses a significant threat to salamanders and newts (Urodela); out of the 44 western Palearctic salamanders (Plethodontidae and Salamandridae) experimentally infected with *B. salamandrivorans*, 41 rapidly succumbed, while none of the exposed frogs, toads and caecilians were affected. Asia is proposed as possible focus of origin, based on detection of *B. salamandrivorans* in wild Asiatic amphibians in absence of

obvious signs of disease together with experimental evidence for Asiatic amphibian species that are able to limit the impact of, and maintain *B. salamandrivorans* infections. This tolerance for *B. salamandrivorans* in some Asiatic amphibian species is proposed to be brought forth by ancient co-evolution. It is likely that *B. salamandrivorans* was vectored into Europe by lack of biosecurity in the international trade in amphibians, where it is currently causing biodiversity loss. A novel diagnostic tool that allows detection of *B. salamandrivorans* is described in the **third study**. The presented duplex real-time PCR can be used to simultaneously detect the DNA of *B. salamandrivorans* and *B. dendrobatidis* on non-invasively collected skin swabs or tissue extracts. High degrees of sensitivity, specificity and reproducibility make this assay a suitable tool for accurate and reliable *B. dendrobatidis* and *B. salamandrivorans* disease monitoring.

Different options for *ex situ* treatment of *B. salamandrivorans* infections in amphibians were explored in the fourth and fifth study. Results of the **fourth study** reveal that environmental temperature significantly determines the infection dynamics of *B. salamandrivorans*; colonization of salamanders by *B. salamandrivorans* occurred at 15 °C and 20 °C, but not at 25 °C, with a significantly faster buildup of infection load and associated earlier mortality at 15 °C. Furthermore, we show that exposure of salamanders naturally and experimentally infected with *B. salamandrivorans* to 25 °C for 10 days can be used as an effective treatment protocol. In the **fifth study** we reveal that therapeutic failure of *B. salamandrivorans* infections in amphibians with antimycotic treatment protocols, used to clear *B. dendrobatidis* infections, is partly explained by different minimum inhibitory concentrations (MICs) of antimycotics against *B. salamandrivorans* and *B. dendrobatidis*. *B. salamandrivorans* growth is inhibited after exposure to voriconazole, polymyxin E, itraconazole and terbinafine but not to florfenicol. Furthermore, synergy between polymyxin E and voriconazole or itraconazole significantly decreased the combined MICs necessary to inhibit *B. salamandrivorans* growth. Based on these pharmaceutical sensitivity assays, an effective treatment protocol composed of exposure to a combination of polymyxin E baths (2000 IU/ml) and voriconazole sprays (12.5 µg/ml), administered twice a day for 10 days at an ambient temperature of 20 °C, was developed.

In the **sixth study**, a real-time PCR based *B. dendrobatidis* viability assay was developed in order to discriminate viable from dead *B. dendrobatidis* cells, and furthermore to quantify the number of viable *B. dendrobatidis* organisms in samples. Adding the DNA intercalating dye

ethidium monoazide (EMA) to samples before real-time PCR analysis results in selective amplification of DNA derived from viable *B. dendrobatidis* cells.

The developed method is able to suppress real-time PCR signals of heat-killed *B. dendrobatidis* zoospores by 99.9 % and is able to discriminate viable from heat-killed *B. dendrobatidis* zoospores in mixed samples. Furthermore, the application of the *B. dendrobatidis* viability assay in determining the antifungal activity of the veterinary antiseptic F10 Antiseptic Solution is presented. Another application of the viability assay is described in **the seventh**, and last study, in which the assay was used to determine *B. dendrobatidis* zoospore survival in different environmental water samples from the French Pyrenees and in samples containing different species of microzooplankton (heterotrofic protists and rotifers). The results of this last study show that in nature, the risk of infection together with the infectious burden of amphibians infected with *B. dendrobatidis* have a significant, site-specific component, and that these disease dynamics correlate with local microzooplankton communities. Experimental infections show that heterotrofic protists and rotifers can rapidly lower the abundance of *B. dendrobatidis* zoospores in the aquatic environment by means of ingestion, resulting in a significantly reduced probability of infection in anuran tadpoles. These results underline the need to take into account important extrinsic determinants of disease in order to try to understand the divergent impact of *B. dendrobatidis* on amphibian populations.

In conclusion, the present thesis has contributed to our understanding of the role of chytridiomycosis as a threat to amphibian diversity in northwestern Europe. More specifically, a novel chytrid fungus (*B. salamandrivorans*) able to cause chytridiomycosis in urodelans was found to be the driver of amphibian population declines in northwestern Europe, and shown to pose a serious threat to global amphibian diversity. The described duplex real-time allows simultaneous detection and quantification of *B. dendrobatidis* and *B. salamandrivorans*, and can be used to screen amphibian samples for presence of both pathogens. Physical and chemical *ex situ* treatment protocols used for clearance of *B. salamandrivorans* infections in amphibians were developed and validated in infected fire salamanders. Lastly, the importance of aquatic microzooplankton communities in *B. dendrobatidis* disease dynamics were demonstrated, opening up research possibilities aimed at developing effective *in situ* chytridiomycosis mitigation measures based on bioaugmentation of the amphibian's environment.

Samenvatting

Chytridiomycose, de dodelijke huidziekte van amfibieën veroorzaakt door de chytride schimmel *Batrachochytrium dendrobatidis*, is een van de belangrijkste bedreigingen voor het voortbestaan van amfibieën wereldwijd. Hoewel effectieve behandelingsprotocollen voor *B. dendrobatidis* in gevangenschap voorhanden zijn, bestaan er momenteel geen mogelijkheden om de gevolgen van chytridiomycose in natuurlijke amfibieën populaties te temperen. *B. dendrobatidis* is als gevolg van menselijke activiteit verspreid over de hele wereld. Ondanks dat de meerderheid van de amfibieën geacht worden gevoelig te zijn voor *B. dendrobatidis*, bestaan er verschillen in deze gevoeligheid tussen maar ook binnen amfibie soorten. Deze variabiliteit wordt veroorzaakt door factoren geassocieerd met de gastheer, het pathogeen en de omgeving.

In noordwestelijk Europa is de aanwezigheid van *B. dendrobatidis* enkel geassocieerd met occasionele, geïsoleerde gevallen van mortaliteit van amfibieën, en tot recent was er geen sprake van ziekte geïnduceerde afnamen van natuurlijke amfibieënpopulaties. Echter, vanaf 2008 werd in Nederland een snelle afname van vuursalamander (*Salamandra salamandra*) observaties opgemerkt met aanwijzingen voor de betrokkenheid van een infectieziekte.

De tweeledige doelstelling van deze thesis was daarom om inzicht te verkrijgen in de bijdrage van chytridiomycose in de afname van natuurlijke amfibieënpopulaties in noordwestelijk Europa Europa, en verder om diagnostiek en behandelings protocollen voor chytridiomycose op punt te stellen, zowel in gevangenschap als in de natuur.

In de **eerste studie** wordt de isolatie en karakterisatie van een nieuwe chytride schimmel, *Batrachochytrium salamandrivorans*, uit de hierboven vermelde Nederlandse populatie van vuursalamanders beschreven, en tonen de resultaten aan dat deze chytride schimmel in staat is chytridiomycose te veroorzaken. Infecties met deze nieuwe chytride schimmel zijn geassocieerd met erosieve huidletsels en acute sterfte in natuurlijk en experimenteel geïnfecteerde vuursalamanders. Een opvallend verschil tussen *B. salamandrivorans* en de nauw verwante chytride schimmel *B. dendrobatidis* (die samen een chytridiomycete groep vormen die zich hebben aangepast aan gewervelde gastheren) is de lagere voorkeurstemperatuur van *B. salamandrivorans*. Verder is het ook opmerkelijk dat vroedmeesterpadden (*Alytes obstetricans*), die als zeer gevoelig beschouwd worden voor de effecten van *B. dendrobatidis*, ongevoelig blijken te zijn voor de effecten van *B. salamandrivorans*. De dreiging uitgaande van dit nieuwe amfibieën pathogeen, samen met een hypothese over de mogelijke oorsprong van *B. salamandrivorans*, is beschreven in de **tweede studie**. Resultaten tonen aan dat *B. salamandrivorans* een significante dreiging vormt

voor salamanders (Urodela); van de 44 met *B. salamandrivorans* experimenteel geïnfecteerde westerse Palearctische salamanders (Plethodontidae en Salamandridae) bezweken 41 dieren in zeer korte tijd aan de gevolgen van de infecties, terwijl geen van de geïnfecteerde kikkers, padden of wormsalamanders nadelige gevolgen ondervonden. Azië wordt voorgesteld als mogelijke oorsprong van *B. salamandrivorans*, gebaseerd op aanwezigheid van *B. salamandrivorans* in gezonde wild levende Aziatische amfibieën samen met aanwijzingen dat de impact van *B. salamandrivorans* op bepaalde Aziatische amfibie soorten beperkt is en dat deze soorten drager kunnen zijn van *B. salamandrivorans*. Deze tolerantie voor *B. salamandrivorans* zou het resultaat zijn van co-evolutie. Het is waarschijnlijk dat *B. salamandrivorans* Europa is binnen gekomen via de internationale handel in amfibieën, waar onvoldoende bioveiligheidsmaatregelen worden getroffen. Een nieuwe diagnostische test die het mogelijk maakt *B. salamandrivorans* te detecteren en kwantificeren wordt gepresenteerd in de **derde studie**. De beschreven duplex real-time PCR kan ingezet worden om simultaan het DNA van *B. salamandrivorans* alsook van *B. dendrobatidis* te detecteren op huidswabs of weefselextracten van amfibieën. De hoge sensitiviteit, specificiteit en reproduceerbaarheid van de test maken hem uitermate geschikt om in te zetten voor monitoring van *B. dendrobatidis* en *B. salamandrivorans*.

Verschillende behandelingsopties voor *B. salamandrivorans* infecties in amfibieën in gevangenschap werden geëvalueerd in de vierde en vijfde studie. Resultaten van **de vierde studie** tonen aan dat de omgevingstemperatuur een bepalende invloed uitoefent op de infectie dynamiek van *B. salamandrivorans*; kolonisatie van salamanders door *B. salamandrivorans* vond plaats bij temperaturen van 15 °C en 20 °C, maar niet bij 25 °C, met een aanzienlijk snellere toename van infectie intensiteit en geassocieerde mortaliteit bij 15 °C. Verder toonden we aan dat blootstelling van met *B. salamandrivorans* (natuurlijk en experimenteel) geïnfecteerde salamanders aan 25 °C gedurende 10 dagen een effectief behandelingsprotocol vormt. Omdat het behandelinsprotocol op basis van verhoogde omgevingstemperatuur mogelijks niet toepasbaar is op alle amfibiesoorten vanwege soortspecifieke maximale temperatuurstolerantie, werden in een vijfde studie alternatieve behandelingsopties geevalueerd. In deze **vijfde studie** onderbouwen we dat het therapeutisch falen van behandelingsprotocollen van *B. salamandrivorans* infecties bij amfibieën met antimycotische middelen (gebruikt om *B. dendrobatidis* infecties te behandelen) ten dele veroorzaakt wordt door verschillen in minimale inhibitorische concentraties (MICs) van antimycotica tegen *B. salamandrivorans* en *B. dendrobatidis*. De groei van *B. salamandrivorans* wordt geïnhibeerd door voriconazole, polymyxine E, itraconazole en terbinafine, maar niet door florfenicol.

Synergie tussen polymyxine E en voriconazole of itraconazole verlagen significant de gecombineerde MICs benodigd voor inhibitie van groei van *B. salamandrivorans*. Gebaseerd op deze farmacologische gevoeligheidstesten werd een effectief behandelingsprotocol voor *B. salamandrivorans* infecties opgesteld, bestaande uit een twee maal daagse blootstelling aan een combinatie van polymyxine E baden (2000 IE/ml) en voriconazole sprays (12.5 µg/ml) gedurende 10 dagen bij een omgevingstemperatuur van 20 °C.

In de **zesde studie** werd een op een real-time PCR gebaseerde *B. dendrobatidis* afdodingstest ontwikkeld, die het mogelijk maakt een onderscheid te maken tussen levende en dode *B. dendrobatidis* cellen, en deze tevens kan kwantificeren. Toevoeging van een aan DNA bindende kleurstof (ethidium monoazide) aan stalen voorafgaande aan real-time PCR analyse resulteert in een selectieve amplificatie van DNA van levende *B. dendrobatidis* cellen.
De ontwikkelde techniek is in staat om 99.9% van het signaal afkomstig van dode *B. dendrobatidis* cellen te onderdrukken en om levende van dode *B. dendrobatidis* cellen te onderscheiden in gemengde stalen. Een mogelijke toepassing van de ontwikkelde techniek in het bepalen van de fungicide werking van een veterinair ontsmettingsmiddel (F10 Antiseptic Solution) wordt eveneens gepresenteerd. Een andere toepassing van de techniek staat beschreven in **de zevende** en laatste studie, waar deze werd ingezet om de overleving van *B. dendrobatidis* zoösporen te kwantificeren in verschillende omgevingswateren van de Franse Pyreneeën en in waterstalen met verschillende soorten microzoöplankton (heterotrofe protisten en rotiferen). De resultaten van deze laatste studie tonen aan dat de microzoöplankton samenstelling het risico op oplopen van *B. dendrobatidis* infecties en de infectie intensiteit van amfibieën geïnfecteerd met *B. dendrobatidis* significant beïnvloedt. Experimentele *B. dendrobatidis* infectieproeven toonden aan dat heterotrofe protisten en rotiferen in staat zijn de *B. dendrobatidis* infectiedruk in het water zeer snel te verlagen door ingestie van zoösporen. Dit resulteert op zijn beurt in een afname van de kans op *B. dendrobatidis* infecties in amfibie larven.
Concluderend heeft deze thesis bijgedragen aan onze kennis over de rol van chytridiomycose als dreiging voor de diversiteit van amfibieën in noordwestelijk Europa. Er werd een nieuwe chytride schimmel (*B. salamandrivorans*) gevonden die in staat is chytridiomycose te veroorzaken in salamanders, en die geassocieerd is met afname van aantallen amfibieën in noordwestelijk Europa. De beschreven duplex real-time PCR maakt het mogelijk *B. dendrobatidis* en *B. salamandrivorans* te detecteren en kwantificeren, en kan in gezet worden als monitoringstool voor beide pathogen in stalen van amfibieën. Fysische en chemische *ex*

situ behandelingsprotocollen voor bestrijden van *B. salamandrivorans* infecties in amfibieën werden op punt gesteld, en gevalideerd in geïnfecteerde vuursalamanders. Ten laatste werd de invloed van microzoöplankton op de ziektedynamiek van *B. dendrobatidis* vastgesteld, wat mogelijkheden biedt op vlak van bestrijdingsopties van *B. dendrobatidis* in de natuur gebaseerd op bioaugmentatie van de omgeving van amfibieën.

Curriculum Vitae

Mark Blooi werd geboren op 14 april 1983 te Arnhem, Nederland. Na het behalen van het diploma secundair ondewijs, richting natuur, techniek en gezondheid aan het Olympus College te Arnhem, startte hij de studies Diergeneeskunde aan de Universiteit van Utrecht. In 2010 studeerde hij af als dierenarts, optie Kleine Huisdieren, met onderscheiding.

In 2011 kwam hij op tijdelijke basis in dienst als assistent bij de vakgroep Pathologie, Bacteriologie en Pluimveeziekten aan de faculteit diergeneeskunde, Universiteit Gent. Aansluitend startte hij in datzelfde jaar zijn doctoraatsonderzoek bij de vakgroep Pathologie, Bacteriologie en Pluimveeziekten onder begeleiding van Prof. Dr. F. Pasmans, Prof. Dr. A. Martel en Dr. F. Vercammen. Deze studie werd gefinancieerd door de Konklijke Maatschappij voor Dierkunde Antwerpen (KMDA). Gedurende 4 jaar voerde hij onderzoek uit naar de bijdrage van chytridiomycose in de afname van natuurlijke amfibieënpopulaties in noordwest Europa, en naar diagnostiek en behandeling van chytridiomycose.

Mark Blooi is auteur en medeauteur van meerdere publicaties in internationale wetenschappelijke tijdschriften. Daarnaast nam hij actief deel aan internationale en nationale congressen.

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