Early interactions of *Batrachochytrium dendrobatidis*

with amphibian skin

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The Red Queen said:
'It takes all the running you can do,
to keep in the same place."

Lewis Carroll, Through the Looking Glass (1871)

CONTENTS

AMP	antimicrobial peptide
AQP	aquaporin
AVT	arginine vasotocin
Bd GPL	*Batrachochytrium dendrobatidis* global panzootic lineage
CBM	chitin binding module
CF	calcium-free
CMFB	calcium- and magnesium-free Barth's solution
2,4-DAPG	2,4-diacetylphloroglucinol
dPBS	calcium-free Dulbecco's phosphate buffered saline
dpi	days post inoculation
EDTA	ethylenediaminetetra acid
EMA	ethidium monoazide
EPH	endemic pathogen hypothesis
FCS	fetal calf serum
FIM	frog integumentary mucin
FTE-explants	full-thickness epidermal explants
GE	genomic equivalents
GMS	gomori methenamine silver
HE	haematoxylin eosin
HPLC	high performance liquid chromatography
I3C	indol-3-carboxaldehyde
Ig	immunoglobulin
IPX	immunoperoxidase
IUCN	International Union for the Conservation of Nature
L-15	Leibovitz medium
LM	light microscopy
MHC	major histocompatibility complex
MIC	minimal inhibitory concentration
NPH	novel pathogen hypothesis
PCR	polymerase chain reaction
qPCR	quantitative PCR
SE-explants	stripped epidermal explants
SEM	scanning electron microscopy
T3	3,3',5-triiodo-L-thyronine
TEM	transmission electron microscopy
TGhL-broth	tryptone/gelatin hydrolysate/lactose-broth
UV-B	ultraviolet B

INTRODUCTION

Preface

Only ten years ago the fungus *Batrachochytrium dendrobatidis* was isolated for the first time from frog skin and described. This emerging pathogen causes chytridiomycosis, a lethal skin disease in amphibians. Since its discovery intensive ecological studies have mapped its distribution and have demonstrated the devastating impact of this disease on amphibian populations worldwide. However, the knowledge of this disease's pathogenesis is in its infancy and several fundamental questions remain open. The present thesis represents a series of studies, each focusing on some of the less well understood aspects of *B. dendrobatidis* infection, including the early interaction of the fungus with amphibian skin and the role of amphibian skin secretions in determining susceptibility to infection. This introductory chapter provides a general background on chytridiomycosis, with emphasis on host-pathogen interactions and pathogenesis, to fully comprehend the 'how and why' of the conducted research.

1. The host: Amphibia

1.1. Introduction to the Amphibia

Amphibians (Amphibia) are a class of vertebrates that includes frogs and toads (Anura), salamanders and newts (Caudata) and caecilians (Gymnophiona) (Frost, 2013). All amphibians are ectothermic, their body temperature varies with the environmental temperature (Crump, 2009). Almost all species are dependent on moist conditions, and many require freshwater habitats in which to breed (Crump, 2009). Most amphibians lay aquatic eggs that hatch into gilled tadpoles, the larval stages. These larvae undergo metamorphosis to develop into terrestrial adults with lungs (Crump, 2009). Beside the lungs, adult amphibians also use their skin for respiration (Fox, 1994). However, salamanders of the family Plethodontidae, the largest salamander family with over 400 species, lack lungs and rely solely on respiration through their moist skin (Larson *et al.*, 2006; Amphibiaweb, 2012).

1.2. The amphibian skin

The integument of amphibians is one of the most important organ systems. It is not only involved in respiration, but also in osmoregulation, i.e. the regulation of the osmotic pressure of an amphibians' body fluid, thermoregulation, sex recognition and reproduction. The skin functions as a sensory organ and plays an important role in defense (Heatwhole &

Barthalamus, 1994). It is clear that the skin is crucial for the survival of amphibians, but as aptly put by Clarke (1997) it is at the same time their 'Achilles heel'.

1.2.1. Anatomy

In adult amphibians the skin consists of an epidermal and dermal layer, as shown in **Figure 1**. The epidermis comprises several layers: (1) the outer stratum corneum, usually consisting of a single layer of keratinized cells that is shed regularly, (2) the stratum granulosum, consisting of several cell layers and functioning as a replacement layer of the stratum corneum, (3) the stratum spinosum, with several cell layers and (4) an innermost stratum germinativum or basal germinative layer where new epithelial cells are generated by cellular division and migrate superficially to differentiate into the different cell types. The well-vascularized dermis consists of an outer stratum spongiosum and a more compact inner layer, the stratum compactum. Capillaries, nerves and smooth muscles are found in the dermis (Fox, 1994; Wells, 2007).
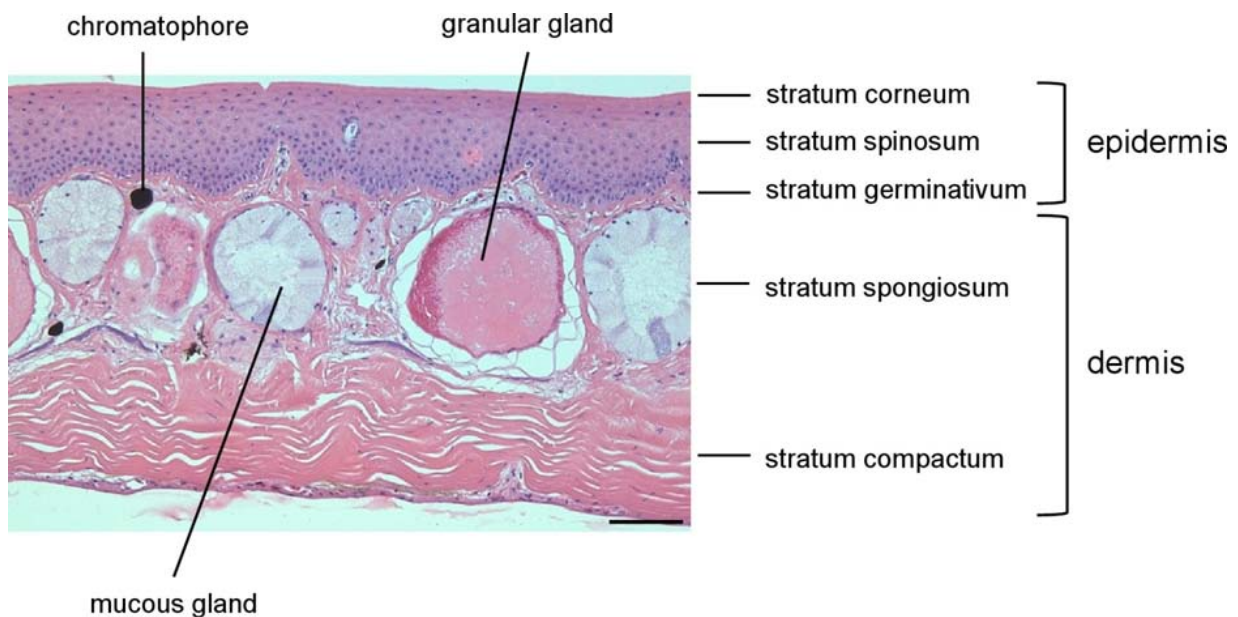
Figure 1. The anatomy of amphibian skin. Generalized features of the amphibian skin as observed in a haematoxylin and eosin stained skin section of *Xenopus laevis*; scale bar = 100 µm.

A variety of specialized secretory glands can be found within the epidermis and dermis. These glands consist of two types: serous glands, also known as granular or poison glands and mucous glands. Both types of glands are composed of a secretory compartment lined by a layer of myoepithelial cells (Clarke, 1997).

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1.2.2. Skin glands and their secretions

Mucous glands are usually distributed over the entire surface of the skin. These merocrine mucous glands continuously release visco-elastic mucous secretions or mucus (Wells, 2007). In most amphibians the epidermis is covered by a mucus layer that offers protection against mechanical damage, dehydration and microbial infection (Clarke, 1997). The main component of mucus are mucins or mucin glycoproteins. In amphibian mucus secretions, 3 types of frog integumentary mucins (FIM) have been characterized: FIM A1, B1 and C1. In general, FIM's consist of threonine-rich highly *O*-glycosylated repetitive domains and a cysteine-rich module. In amphibians, the carbohydrate portion of mucin glycoproteins consist principally of fucose, *N*-acetylgalactosamine, *N*-acetylglucosamine, *N*-acetyl neuraminic acid, galactose, mannose and sialic acid (Roussel & Delmotte, 2004; Meyer *et al.*, 2007). Due to their structure, mucins not only determine the elasticity and viscosity of mucus (Hoffman & Hauser, 1993) but are also are thought to bind pathogenic microorganisms and prevent adhesion to epidermal or epithelial cells as shown for mammalian mucins (Roussel & Delmotte, 2004; Alemka *et al.*, 2012). However, the protective function of amphibian skin mucins remains speculative (Probst *et al.*, 1992; Meyer *et al.*, 2007).

Granular glands may be scattered over the skin surface or arranged in compact glands or parotoid glands on the back, neck and shoulders. These latter are particularly well-developed in toads (genus *Bufo*) and fire salamanders (*Salamandra salamandra*) (Duellman & Trueb, 1986). In contrast with mucous glands, granular glands are holocrine. Upon irritation, stress or adrenergic stimulation these glands secrete an array of bioactive components, including biogenic amines, steroids, alkaloids, hormone-like peptides and antimicrobial peptides (AMP). The pharmacological effect of these molecules ranges from cardiotoxic, neurotoxic, myotoxic, hypotensive, hypertensive, hallucinogenic and anaesthetic to antimicrobial (reviewed by Barthalamus, 1994; Erspamer, 1994; Clarke, 1997). As such these compounds are an important source of novel therapeutic agents for human medicine as well as complements to conventional antibiotics (Erspamer 1994; Hancock & Sahl, 2006). Especially AMP's and their properties will be highlighted later (§ *4.1.1.1*). Notorious is the steroidal alkaloid batrachotoxin, an extremely potent cardio-, myo- and neurotoxin secreted by poison-dart frogs (Dendrobatidiae) (Toledo & Jared, 1995). Especially secretions of the African clawed frog *Xenopus laevis*, a model anuran for immunogenetic studies and developmental biology, have been studied extensively. An overview of peptides and biogenic amines in secretions of *X. laevis* is presented in **Table 1**.

Table 1. Overview of peptides and biogenic amines in granular gland secretions of *Xenopus laevis*

Active compounds	Classification	Pharmacological action
5-hydroxytryptamine (5-HT, serotonine)	biogenic amine, neurotransmitter	vasoactive/vasoconstrictor
Caerulein (CRL)	hormone-like peptide, neurotransmitter	neuroleptic, hypotensive, analgesic
Caerulein precursor fragments (CPF)	antimicrobial peptide	antimicrobial
Levitide	hormone-like peptide, neuropeptide	neuroleptic
Levitide precursor fragment (LPF)	antimicrobial peptide	antimicrobial
Magainin 1	antimicrobial peptide	antimicrobial
Magainin 2	antimicrobial peptide	antimicrobial
Magaininase	metalloendopeptidase	inactivation of peptide hormones
N-methylserotonin	biogenic amine, neurotransmitter	serotonin reuptake inhibitor
Peptide glycine-leucine amide (PGLa)	antimicrobial peptide	antimicrobial
Peptide glycine-leucine amide-H (PGLa-H)	antimicrobial peptide	antimicrobial
Thyrotropin-releasing hormone (TRH)	hormone-like peptide, neurotransmitter	neuroleptic
Trimethylammonium base (BUF, bufotenidine)	biogenic amine, toxin	neuromuscular blocking activity
Xenopsin (XN)	hormone-like peptide, neuropeptide	neuroleptic
Xenopsin precursor fragment	antimicrobial peptide	antimicrobial

Adapted from de Morais Carvalho *et al.*, 1992; Barthalamus, 1994; Erspamer, 1994; Toledo & Jared, 1995; Rollins-Smith *et al.*, 2009; Hou *et al.*, 2011.

1.2.3. Physiology

Amphibian skin plays a vital role in osmoregulation and respiration. The well-vascularized dermis and limitedly keratinized epidermis render amphibian skin highly permeable, allowing the exchange of respiratory gasses, water, and electrolytes with the environment (Fox, 1994; Lillywhite, 2004).

Exchange of respiratory gasses O_2 and CO_2 occurs across lungs, gills and skin (Boutilier *et al.*, 1992). However, the skin is the main site for both CO_2 excretion and O_2 uptake (Boutilier *et al.*, 1992). Gas movement in both directions of the skin depends on partial pressure gradients (PO_2) (Burggren & Vitalis, 2005).

Due to its architecture, amphibian skin shows little resistance for water evaporation. As such, efficient water absorption and utilization are critical for the survival of the amphibian (Lillywhite, 2006). For water uptake amphibians do not drink, but instead absorb water through the skin from standing water or moist substrates. The pelvic patch or 'drinking patch', an area of highly vascularized skin on the ventral abdomen of terrestrial amphibians, is the major site for water uptake from the environment (Pough, 2007). Aquatic amphibians are surrounded by a hypoosmotic environment and thus water is continuously absorbed through the skin driven by an osmotic gradient. The plasma osmolality or ion-water balance inside the amphibian is kept constant by a concerted action of the three major osmoregulatory organs,

i.e. the skin, kidneys and the urinary bladder (Boutilier *et al.* 1992; Wells, 2007). As such, aquatic amphibians can easily absorb water in excess and produce a large volume of urine to compensate (Shoemaker & Nagy, 1977; Pessier, 2009). Together with the urine, Na^+, Cl^- and other ions are lost. To counterbalance these important ion losses, Na^+ and Cl^- are taken up in excess across the skin (Boutilier *et al.*, 1992).

A key person in the study of ion transport across epithelia was the Danish scientist Hans H. Ussing. He was the first to describe the mechanism by which ions are actively transported across frog skin and found that the influx of Na^+ is correlated with a potential difference across the epithelium (Ussing, 1960). He got the idea to measure the rate of active ion transport across the epithelium as an electric current in frog skin, bathed apically and basolaterally in isotonic Ringer solution, by eliminating the transepithelial potential difference by an external current source. The amount of current necessary to eliminate this transepithelial potential difference is termed 'short-circuit current'. This led to the design of the Ussing chamber in which short-circuit current is measured as an indicator of ion transport taking place across the epithelium (reviewed in Larsen, 2002).

The ability to absorb water through the epithelium of skin, kidneys and urinary bladder is under control of several hormones such as arginine vasotocin (AVT), angiotensin II, aldosterone, prolactin and epinephrine (Boutilier *et al.*, 1992; Uchiyama & Konno, 2006; Pessier, 2009). Water transport across the epithelium is regulated by aquaporins (AQP). AQP's function as selective water channels and can be found in apical and basolateral cell membranes in the epithelia of the kidneys, urinary bladder and in the ventral pelvic skin. AQP's are stimulated by the neurohypophyseal hormone AVT (Suzuki & Tanaka, 2009).

1.3. Amphibian declines

The last 2 decades, amphibian populations worldwide have been declining at un-precedented rates or have gone extinct (Stuart *et al*, 2004; Wake & Vredenburg, 2008), even in protected or pristine areas (Lips, 1998; Puschendorf *et al.*, 2006; Ron *et al.*, 2003). The most recent assessment of the conservation status of all known amphibian species by the International Union for the Conservation of Nature (IUCN) found that at least 42% of all amphibian species are declining, 32% are threatened with extinction, with 159 species probably already extinct (IUCN, 2012). Especially in biodiversity hotspots like Central America, the Caribbean and Australia, amphibian populations have suffered 'enigmatic' declines that occurred without obvious cause as shown by **Figure 2** (Stuart *et al.*, 2004; IUCN, 2012).

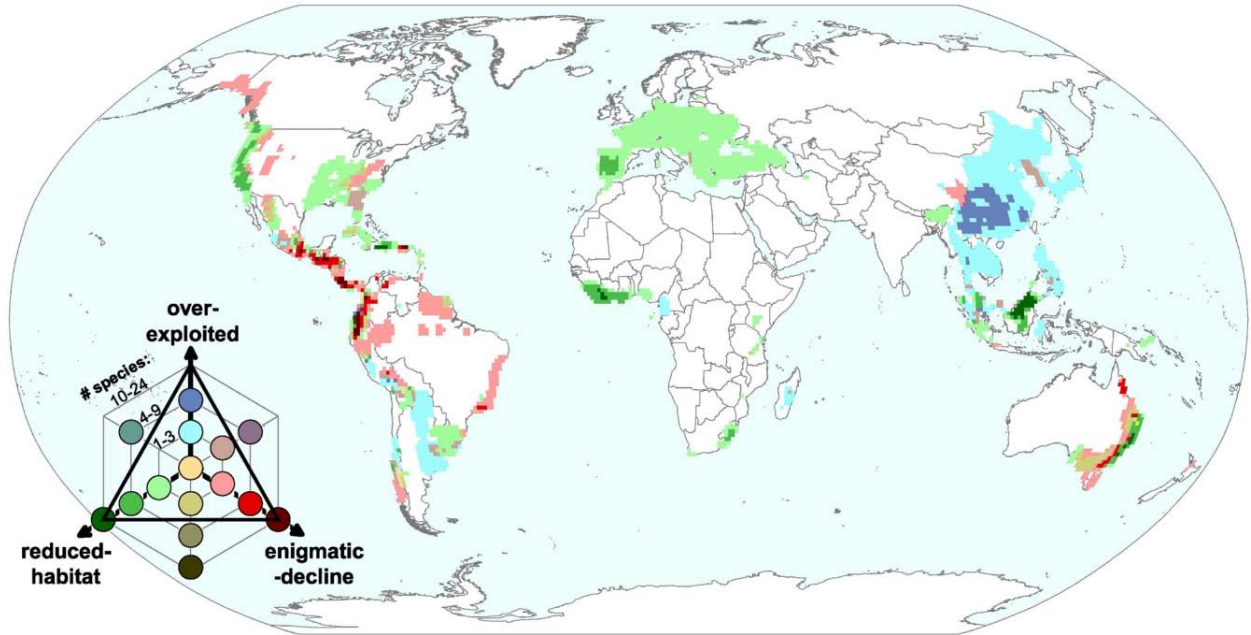**Figure 2**. **Geographical pattern of the dominant cause for rapid decline in amphibian species**. Over-exploited (blue), reduced-habitat (green) and enigmatic decline (red), with darker colors corresponding to higher numbers of rapidly declining species. Adapted from Stuart *et al.* (2004).
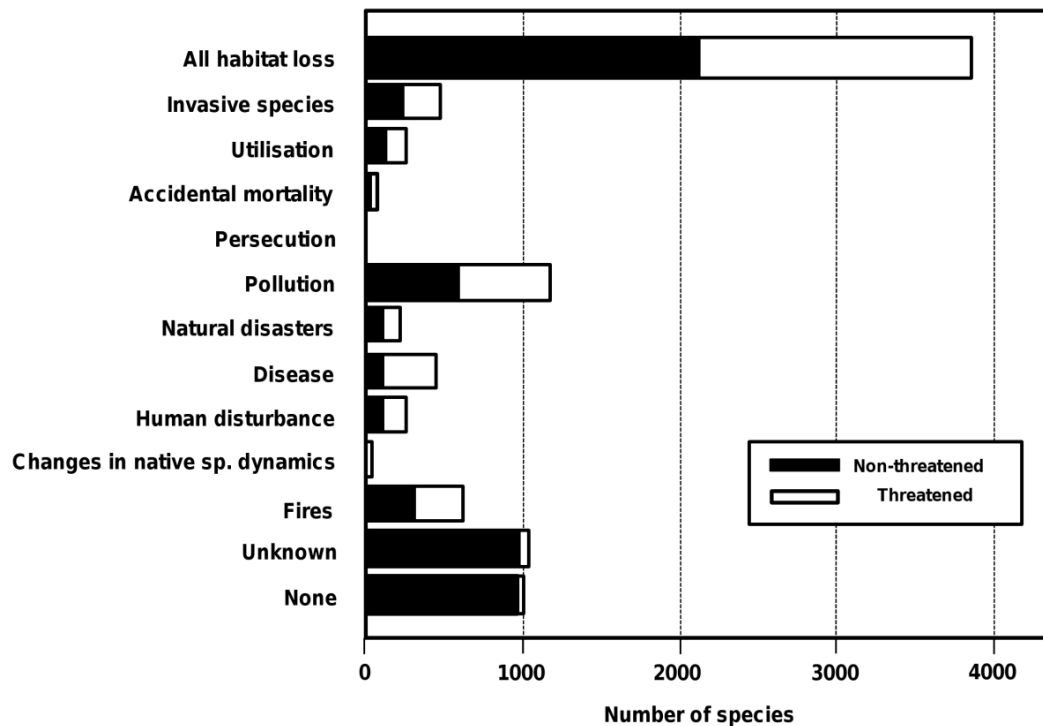

Figure 3. Major treats to amphibians. Summary of the number of species affected by each threatening process. Adapted from IUCN (2012).

Many factors contribute to these global declines and include habitat destruction, alteration and fragmentation, commercial over-exploitation for pet-trade and food, introduction of non-native species, environmental pollution, global climate change and emerging infectious diseases (reviewed by Blaustein & Kiesecker, 2002; Stuart *et al.*, 2004; Gascon *et al.*, 2007; Mendelson *et al.*, 2006; Wake & Vredenburg, 2008; Collins 2010; Wake 2012). It is not possible to indicate a single prevailing cause for these declines, instead several factors may interact and amplify their negative impact on amphibian populations as illustrated in **Figures 2** and **3** (reviewed in Blaustein & Kiesecker, 2002; Blaustein *et al.*, 2003; Rohr *et al.*, 2008; Rohr & Raffel, 2010).

A recent threat to amphibians is the emerging fungal disease chytridiomycosis, caused by *Batrachochytrium dendrobatidis*. This fungus was only discovered in 1997 (Berger *et al.*, 1998), strain isolation and species description followed in 1999 (Longcore *et al.*, 1999). Since then many enigmatic declines and extinctions of amphibian species in Central-America, North-America, Europe and Australia have been attributed to this fungus (Berger *et al.*, 1998; Bosch *et al.*, 2001; Muths *et al.*, 2003; Rachowicz *et al.*, 2006; Lips *et al.*, 2006; Lötters *et al.*, 2009; Cheng *et al.*, 2011). According to the IUCN Amphibian Conservation Action Plan (Gascon *et al*., 2007), "chytridiomycosis is the worst infectious disease ever recorded among vertebrates in terms of the number of species impacted, and its propensity to drive them to extinction". Anyhow, scientists argue that we are now in a sixth mass extinction event, with amphibians being hit the hardest (Wake & Vredenburg, 2008).

Despite the detection of *B. dendrobatidis* in a broad range of European amphibian species (Garner *et al*., 2005; Duffus & Cunningham, 2010; Spatial epidemiology, *Bd*-maps, www.bd-maps.net, 2012), only few chytridiomycosis-related declines and mortalities have been reported. The first record of amphibian population decline caused by *B. dendrobatidis* dates back from 2001, when infection in recently metamorphosed common midwife toads (*Alytes obstetricans*) in Peñalara Natural Park (Spain) led to a sharp decline of this species, disappearing from 86% of their known breeding-ponds (Bosch *et al.*, 2001). Since then, chytrid-driven declines of fire salamanders (*Salamandra salamandra*) and common toads (*Bufo bufo*) have been documented in Spain (Bosch & Martinez-Solano, 2006). Also the Mallorcan midwife toad (*Alytes muletensis*), a vulnerable species endemic to Mallorca (Spain), is affected by *B. dendrobatidis* which was unintentionally co-introduced to Mallorca in a conservation effort to recover wild populations by captive breeding and re-introduction of *A. muletentis* (Walker *et al*., 2008). On Sardinia (Italy), mortalities attributed to chytridiomycosis have been reported for the Sardinian newt (*Euproctus platycephalus*)

(Bovero *et al*., 2008) and Tyrrhenian painted frog (*Discoglossus sardus*) (Bielby *et al*., 2009). In Belgium, *B. dendrobatidis* has shown to be distributed among both endemic and introduced species (Spitzen-van der Sluijs, 2010; Martel *et al*., 2012). But so far, mortality due to chytridiomycosis has only been reported in a wild population of common midwife toads (*A. obstetricans*) (Pasmans *et al.*, 2010).

2. The pathogen: *Batrachochytrium dendrobatidis*

2.1. Taxonomy and ecology

B. dendrobatidis belongs to the Chytridiomycota, a phylum of 'lower fungi' (Longcore *et al.*, 1999). The taxonomic position of this phylum within the fungal kingdom is presented in **Figure 4.** These primitive microscopic fungi, often referred to as chytrid fungi or chytrids, are characterized by motile flagellated spores, termed zoospores (Barr, 2001). Most chytrid fungi are saprophytic or parasitic on plants, algae or invertebrates (Barr, 2001). Their habitat varies from moist soil to fresh water (Barr, 2001).

Host induced morphological variation is a substantive feature of many chytrid species. As such they can adopt a differential morphology between their parasitic and saprophytic state and change from endobiotic to epibiotic growth (Longcore, 1995; Barr, 2001). As illustrated in **Figure 5** endobiotic refers to a reproductive body in which asexual zoospores are produced (sporangium) inside the host cell, while epibiotic refers to a sporangium on the surface of the host cell.

B. dendrobatidis is currently placed in the order of the Rhizophydiales (Longcore *et al.*, 1999; James *et al.*, 2006). This water-borne fungus is the only member of the Chytridiomycota to parasitize on amphibian skin (Longcore *et al.*, 1999), is morphologically dissimilar from other known chytrid species (Longcore *et al.*, 1999) and has only one close relative i.e. *Homolaphlyctis polyrhiza* (Longcore *et al.*, 2011). The discovery of a large set of protease genes unique to *B. dendrobatidis*, indicate that it has recently evolved from a non-pathogenic ancestor to an amphibian pathogen (Rosenblum *et al.*, 2010; Joneson *et al.*, 2011).

2.2. Morphology and lifecycle

B. dendrobatidis reproduces exclusively asexually and has two main life stages as illustrated in **Figure 6**: a motile zoospore with a single posteriorly directed flagellum and the zoosporangium, in which asexual zoospores are produced (Berger *et al.*, 2005a).
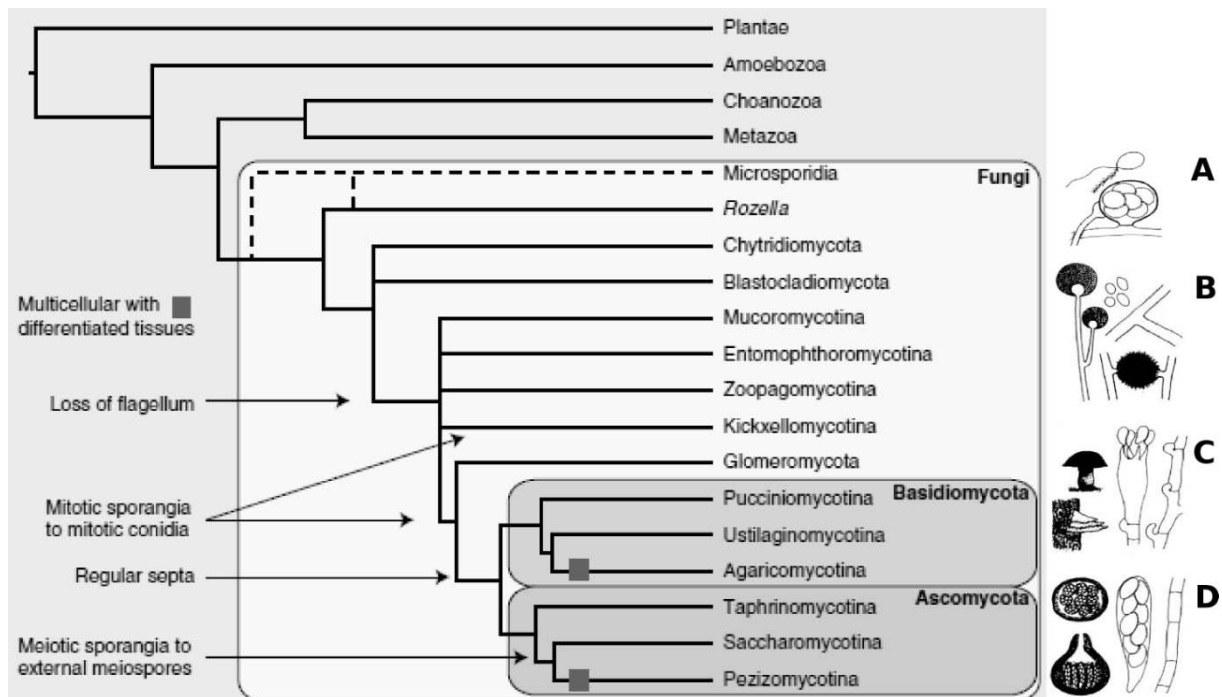
Figure 4. Taxonomic position of the Chytridiomycota within the fungal kingdom. Arrows at the left indicate changes in the fungal morphology. Line drawings at the right present most characteristic morphological features for zoosporic fungi **(A)**, zygosporic fungi **(B)**, Basidiomycota **(C)** and Ascomycota **(D)**. Adapted from Guarro *et al.* (1999) and Stajich *et al.* (2009).
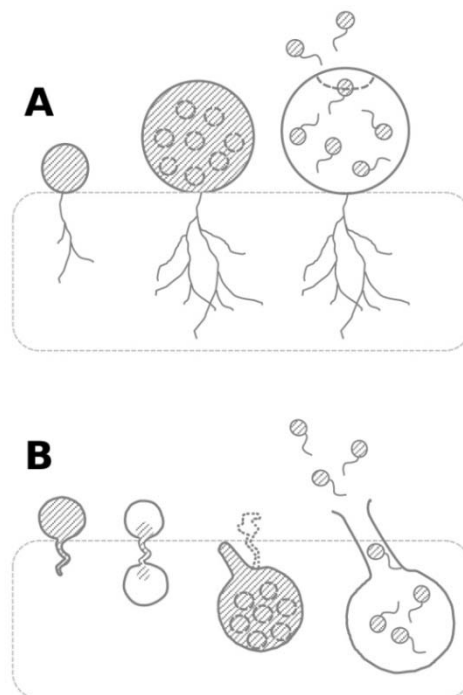
Figure 5. Main types of sporangium development in the Chytridiomycota. (**A**) Epibiotic sporangium development: the sporangium develops on the host cell, with a rhizoid system inside the host cell; (**B**) endobiotic sporangium development: the sporangium develops inside the host cell and zoospores are released by means of a discharge tube. Modified from Barr (2001).

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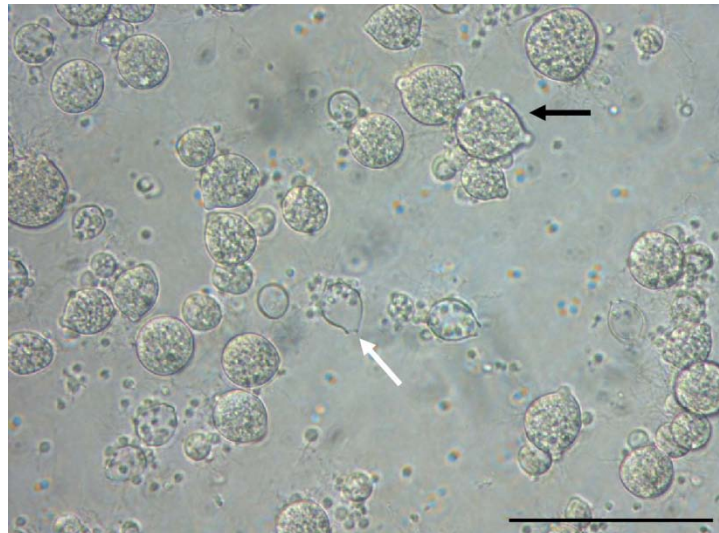
Figure 6. Morphology of *B. dendrobatidis* in culture. Three-days old culture of *B. dendrobatidis* on tryptone/gelatin hydrolysate/lactose (TGhL)-broth, showing abundant mature zoosporangia (black arrow) containing zoospores and empty, discharged sporangia (white arrow); scale bar = 100µm.
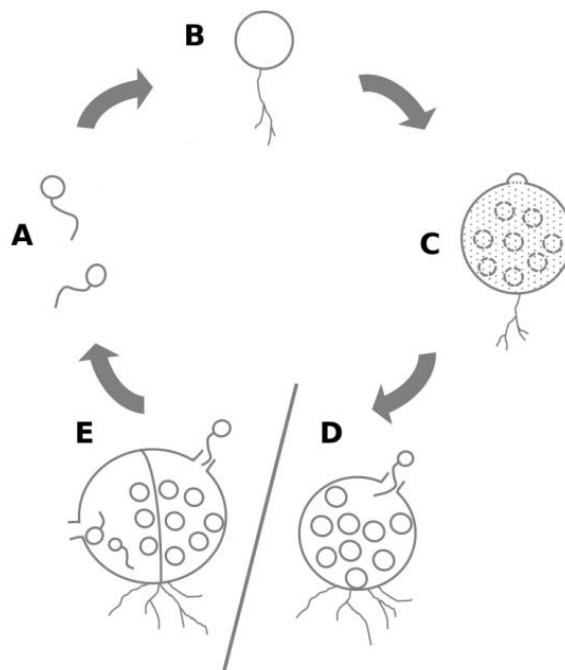
Figure 7. Schematic presentation of the life cycle of *B. dendrobatidis* in culture. After a period of motility zoospores **(A)** encyst, resorb their flagella and form germlings **(B)**; rhizoids appear from one or more areas; thalli grow larger and become mature sporangia over 4 to 5 days; contents of the enlarged thallus become multinucleate by mitotic divisions and the entire contents cleave into zoospores while discharge tubes form **(C)**; discharge tubes are closed by a plug that absorbs water and deliquesces when zoospores are ready to release **(D)**; some thalli develop colonially with thin septa dividing the contents into multiple sporangia, each with their own discharge tube **(E)**. Modified from Berger *et al.* (2005a).

The lifecycle of *B. dendrobatidis* in culture, from zoospore to zoosporangium takes 4 to 5 days at 22°C (Berger *et al.*, 2005a). As shown in **Figure 7**, first the zoospore encysts by developing a cell wall and absorbing its flagellum, to finally form a germling with fine tread-like rhizoids (Longcore *et al.*, 1999). The maturing germling develops into a zoosporangium in which the cytoplasm cleaves and forms new zoospores. Discharge papillae or tubes, blocked inside by a plug, are formed during the growth of the sporangium. At maturity the plug dissolves and the zoospores are released into the environment to continue their lifecycle (Longcore *et al.*, 1999; Berger *et al.*, 2005a).

To date, the lifecycle of *B. dendrobatidis* in amphibian skin is assumed to be the same as in culture. The infectious zoospores go actively in search of a suitable host, driven by chemotactic responses (Moss *et al.*, 2008). Upon colonization of the host epidermis, the zoospores encyst and develop zoosporangia within the cells of the stratum corneum and the stratum granulosum (Longcore *et al.*, 1999; Berger *et al.*, 2005a). Berger and co-workers (2005a) observed that *B. dendrobatidis* has its cycle tuned to the maturation of the epidermal cells. Immature sporangia are carried from the deeper skin layers to the skin surface by differentiating epidermal cells. At the time sporangia have developed discharge tubes and contain mature zoospores, they finally occur in stratum corneum where the zoospores are released in the environment.

2.3. Growth conditions and nutrients

Optimal growth of *B. dendrobatidis* is observed between 17 and 25°C and pH 6-7 (Piotrowski *et al.*, 2004). Growth and survival of *B. dendrobatidis* are strongly temperature dependent. Above 28°C and below 10°C growth ceases and zoospores are killed within 4 hours at 37°C (Johnson *et al.*, 2003). In addition, desiccation is poorly tolerated (Johnson *et al.*, 2003; Garmyn *et al.*, 2012) and 5% NaCl solutions are lethal for *B. dendrobatidis* (Johnson *et al.*, 2003).

In vitro, B. dendrobatidis grows on a variety of keratin containing substrates: autoclaved snake skin, 1% keratin agar, frog skin agar, feathers, geesepaws (Longcore *et al.*, 1999; Piotrowski *et al.*, 2004; Symonds *et al.*, 2008; Garmyn *et al.*, 2011). However, keratin is not an essential nutrient for *B. dendrobatidis* as it grows best in tryptone or peptonized milk (Longcore *et al.*, 1999), but most importantly the extracellular proteases secreted by *B. dendrobatidis* fail to degrade keratin *in vitro* (Piotrowski *et al.*, 2004). Very recently, also *in vitro* growth of *B. dendrobatidis* on chitinous crustacean carapaces has been reported (Mc Mahon *et al.*, 2013).

2.4. Origin and distribution of B. dendrobatidis

At present, *B. dendrobatidis* infects over 516 amphibian species in 54 countries (Spatial epidemiology, *Bd*-maps, www.bd-maps.net, 2012). But where does this fungus originate from and how did it spread? First, it is not clear whether *B. dendrobatidis* is an endemic pathogen that recently increased its host range or virulence due to environmental factors (endemic pathogen hypothesis, EPH) or a new emergent pathogen introduced into naïve host populations (novel pathogen hypothesis, NPH) (Rachowicz *et al.*, 2005). The presence of *B. dendrobatidis* in amphibians before declines occurred (Weldon *et al.*, 2004; Goka *et al.*, 2009; Soto-Azat *et al.*, 2010), the coexistence of *B. dendrobatidis* in endemic amphibian populations without marked declines (Spitsen-van der Sluijs *et al.*, 2010; Martel *et al.*, 2012; Tobler *et al.*, 2012) and climate change linked amphibian declines (Rohr & Raffel, 2010) support the EPH. Earlier population genetic studies showed a low genetic diversity among *B. dendrobatidis* isolates and favored the hypothesis of a novel pathogen undergoing rapid and recent range expansion (Morehouse *et al.*, 2003; Morgan *et al.*, 2007; James *et al.*, 2009). However recently, researchers found a much larger diversity in *B. dendrobatidis* strains than previously recognized (Farrer *et al.*, 2011; Rosenblum *et al.*, 2012a). Furthermore, resulting population genetic data suggest that recombination among genetically isolated allopatric[1] lineages of *B. dendrobatidis* have led to the diversification and the origin of highly virulent global panzootic lineage of *B. dendrobatidis* (*Bd* GPL) (Farrer *et al.*, 2011; Rosenblum *et al.*, 2012a). Especially isolates of the *Bd* GPL lineage seem to be linked with the onset of *B. dendrobatidis* epidemics in North and Central America, the Caribbean, Australia and Europe (Farrer *et al.*, 2011).

So far, the precise origin of *B. dendrobatidis* is unknown. Africa (Weldon *et al.*, 2004), North-America (James *et al.*, 2009) as well as Asia (Fisher, 2009; Goka *et al.*, 2009; Bai *et al.*, 2012) have been proposed as putative origin of *B. dendrobatidis*. Nevertheless, it is clear that global trade in amphibians has caused spread of *B. dendrobatidis* into naïve populations and species (Mazzoni *et al.*, 2003; Fisher & Garner, 2007; Picco & Collins, 2007). Especially the African clawed frog (*X. laevis*) and North American bullfrog (*Lithobates catesbeianus*) are notorious in this respect. *X. laevis* is widely traded for scientific research (Weldon *et al.*, 2004), whereas *L. catesbeianus* used to be sold in several EU countries, including Belgium and the Netherlands, to ornament garden ponds (Stumpel; 1992; Spitzen-van der Sluijs & Zollinger, 2010) and still is imported at large scale to mainly the US,

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[1] Having separate and mutually exclusive areas of geographical distribution.

South-America and France for consumption (Mazzoni *et al.*, 2003; Warkentin *et al*., 2008; Schloegel *et al.*, 2010). Both species not only act as reservoir for *B. dendrobatidis* (Daszak *et al.*, 2004; Weldon *et al*., 2004; Garner *et al.*, 2006) but are also highly invasive when introduced into new environments (Fisher & Garner, 2007). Moreover, recent studies demonstrate that *L. catesbeianus* is infected with a large diversity of *B. dendrobatidis* genotypes and corroborate the role of amphibian trade in facilitating intercontinental gene flow (Schloegel *et al.*, 2012).

Spread of *B. dendrobatidis* in the environment is also promoted by its ability to persist outside its amphibian host. This fungus can not only survive in water and moist soil for weeks up to several months (Johnson & Speare, 2003; 2005; Kirshtein *et al.*, 2007), but is also able to grow saprophytically on e.g. bird feathers, arthropod exoskeletons, keratinous paw scales of waterfowl and to survive in the gastrointestinal tract of crayfish (Longcore *et al.*, 1999; Johnson & Speare, 2003; 2005; Garmyn *et al.*, 2011; McMahon *et al*., 2013). So far, only waterfowl (Garmyn *et al.*, 2011), reptiles (Kilburn *et al.*, 2011) and crayfish (McMahon *et al*., 2013) have been identified as non-amphibian vectors contributing to the dissemination of *B. dendrobatids*.

3. The disease: Chytridiomycosis

3.1. Pathology

In metamorphosed amphibians*,* chytridiomycosis is diagnosed by the presence of immature fungal bodies*,* termed thalli or maturing sporangia of *B. dendrobatidis* found intracellularly in the keratinized layers of the skin as shown in **Figure 8** (Berger *et al.*, 1998; 2000; 2005a). Chytridiomycosis is mainly associated with a mild to severe irregular thickening of the stratum corneum or hyperkeratosis, increased tissue growth of the stratum granulosum or hyperplasia and erosion of the stratum corneum (Berger *et al.*, 1998; 2000). Other pathological changes in the epidermis adjacent to infected areas include mild focal necrosis, spongiosis, cytoplasmic degeneration with minimal to mild inflammation and vacuolation of the deeper cell layers (Nichols *et al.*, 1998; Berger *et al.*, 1998; 2000; 2005a). Occasional ulceration of the skin is observed (Berger *et al.*, 2000). In general, dissemination to the deeper layers of the skin or the internal organs does not occur (Pessier*,* 2008).

In anuran larvae, infection is limited to colonization of the keratinized mouthparts, i.e. tooth rows and jaw sheats and is absent in the non-keratinized epithelia, i.e. body, limbs tail, mouth and gills (Berger *et al.*, 1998; 2000; Marantelli *et al.*, 2004). Infection is accompanied

with minimal pathology, predominantly consisting of mild hyperkeratosis (Berger *et al.*, 2000).

Figure 8. Pathology of *B. dendrobatidis*-infected amphibian skin. Haematoxylin and eosin stained section of the ventral skin (drink patch) of *Litoria caerulea*. Chytrid infection is characterized by hyperkeratosis of the stratum corneum, with the presence of numerous zoosporangia at various stages and septate zoosporangia (arrow). Scale bar =10 µm.

3.2. Clinical signs

In larvae, clinical signs of chytridiomycosis are generally limited to depigmentation of the mouthparts, without morbidity and mortality (Berger *et al.*, 1998; Rachowicz & Vredenburg, 2004). In metamorphosed amphibians clinical signs are variable and range from sudden death without obvious disease to significant skin disorder (Pessier, 2008). Most common signs of chytridiomycosis are excessive shedding of the skin, erythema (redness) or discoloration of the skin (Pessier 2002; 2008). Skin of the ventral abdomen, especially the pelvic patch, feet and toes are predilected sites of infection (Berger *et al.*, 2005b; Puschendorf & Bolaños, 2006). Other clinical signs include lethargy, anorexia, abnormal posture (abduction of the hind legs), neurological signs such as loss of righting reflex and flee response (Pessier 2002; 2008). Particularly in bolitoglossine salamanders, chytridiomycosis can be associated with tail loss or tail autotomy (Pasmans *et al.*, 2004).

3.3. Pathogenesis
3.3.1. Colonization of amphibian skin

In a recent state of the art overview in *B. dendrobatidis* research, Voyles *et al.* (2011) conclude that the knowledge on the pathogenesis of *B. dendrobatidis* infections is still

fragmentary and urges further investigation. Yet, the non-existence of suitable *in vitro* models impedes advances in chytrid pathogenesis considerably and so far researchers had to fall back to *in vivo* infection experiments to study chytrid-related pathology and infectivity.

The early stages of infection, i.e. colonization with adhesion, invasion and proliferation are poorly studied and the mechanism of host cell entry remain speculative. Based upon observations in other chytrid genera, the development of *B. dendrobatidis* is hypothesized to be endobiotic (Longcore *et al.*, 1999). As such, zoospores would encyst upon the host cell of the stratum corneum and inject their nucleus and cytoplasm into the host cell via a germ tube. The germ tube forms a swelling inside the host cell and enlarges. Finally, the contents undergo mitosis, zoospores are formed and are released into the environment through discharge papillae (Longcore *et al.*, 1999).

3.3.2. Virulence factors

In contrast with the study of structural host-pathogen interactions, important advances have been made at molecular level that little by little add to our understanding of the pathogenicity of *B. dendrobatidis.* As such, several putative virulence factors, causing host damage, have been identified.

In pathogenic fungi causing skin infections such as *Candida albicans* (Naglik *et al.*, 2003) and dermatophytes (Baldo *et al.*, 2012) extracellular proteases e.g. serine-, aspartyl- and metallo-proteases play an important role in the invasion of the host skin. These digestive enzymes not only cause damage to host tissue but also impairment of host defenses (Yike, 2011). Rosenblum and colleagues (2008) identified two gene families in the *B. dendrobatidis* genome, encoding for a serine-type protease and a fungalysin metallopeptidase, two candidates aiding in host cell invasion and dissolution of cellular cytoplasma (Berger *et al.*, 2005a). Curiously, the same researchers found that amphibians infected by *B. dendrobatidis* show a surprisingly weak immune response. Only few immunity-related genes show up-regulation but only in a later stage of infection, indicating a possible suppression of the immune system by *B. dendrobatidis* (Rosenblum *et al.*, 2009; 2012b).

B. dendrobatidis appears to have several strategies to compromise the skin integrity. *B. dendrobatidis* not only secretes proteases capable of degrading casein, gelatin (a hydrolysed form of collagen) (Piotrowksi *et al.*, 2004; Moss *et al.*, 2010) and elastin (Moss *et al.*, 2010), but also triggers a decreased expression of host genes encoding for essential skin integrity components such as keratin, collagen, elastin and fibrinogen (Rosenblum *et al.*, 2012b). Additionally, Brutyn *et al.* (2012) found that *B. dendrobatidis* secretes a complex

mixture of virulence associated proteins including proteases, biofilm-associated proteins and lipases, affecting the integrity of the epidermis by rapid disturbance of the intracellular junctions.

Very recently, Abramyan & Stajich (2012) identified a chitin binding module (CBM18), unique to the genome of *B. dendrobatidis*. Chitin, a polymer of *N*-acetylglucosamine is the main component of the fungal cell wall. CBM's found in other pathogenic fungi function as competitor of, and limit access for foreign chitinases by binding to the chitin of their proper cell wall. In analogy, a key role of CBM18 in the pathogenesis and protection against host-derived chitinases is suggested. In addition, CBM18 would also allow attachment of *B. dendrobatidis* to non-host chitinous structures, such as insect or crustacean exoskeletons (Mc Mahon *et al.*, 2013), allowing vectored disease spread.

3.3.3. Pathophysiology

A fundamental question is how a superficial fungal skin infection can be so lethal to a wide range of amphibian species. First, it is generally accepted that mortality consistently occurs when a threshold of 10^5 *B. dendrobatidis* zoospore equivalents in amphibian skin is exceeded (Carey *et al.*, 2006; Vredenburg *et al.*, 2010). However, the outcome of infection is dictated by other factors than infection dose only (Garner *et al.*, 2009), as will be discussed later on in chapter 4.

Secondly, severe *B. dendrobatidis* infection with hyperkeratosis and hyperplasia as hallmarks compromises the osmoregulatory function of amphibian skin (Voyles *et al.*, 2009; Carver *et al.*, 2010; Rosenblum *et al.*, 2012b). Electrolyte transport across the skin is impaired, accompanied by a reduction in transepithelial resistance (Voyles *et al.*, 2009). Consequently, electrolytes become depleted (Voyles *et al.*, 2007) and rehydration becomes difficult (Marcum *et al.*, 2010). In fact, in blood samples taken from amphibians with clinical chytridiomycosis significantly reduced plasma Na^+, K^+ and Cl^- concentrations as well as reduced overall blood plasma osmolality are observed (Voyles *et al.*, 2007; 2009; Marcum *et al.*, 2010; Voyles *et al.*, 2012a). Especially, low plasma K^+ concentrations that are linked to abnormal cardiac electrical activity and cardiac arrest, are thought to be the proximate cause of death in amphibians with clinical chytridiomycosis (Voyles *et al.*, 2009; Campbell *et al.*, 2011).

Many fungal and bacterial pathogens are known to alter the structure and function of the host epidermis and induce changes in water and electrolyte transport by activation or by inhibiting ion channels and transporters (Kunzelmann & McMorran, 2004). Electrolyte

transport across the amphibian epidermis is partially accomplished by epithelial Na^+ channels (ENaC) and Na^+/K^+ pumps. Recently, Campbell *et al.* (2011) found that infection by *B. dendrobatidis* is likely to inhibit ENaC, leading to a severely reduced Na^+ absorption through the skin. Whether a toxin secreted by *B. dendrobatidis* or changes in enzyme function or protein expression induced by *B. dendrobatidis* cause disruption of normal skin functioning, requires further research.

4. Host susceptibility to chytridiomycosis

Not all amphibian species equally respond to *B. dendrobatidis* infection (Woodhams *et al.*, 2006; 2007a; Searle *et al.*, 2011). Moreover, within a single species some populations coexist with *B. dendrobatidis* without no evidence of disease while others go extinct (Pilliod *et al.*, 2010; Puschendorf *et al.*, 2011). Host response to infection can be roughly categorized as resistant (fast clearance of the infectious agent), tolerant (persistent infection in absence of disease) and susceptible (infection resulting in lethal disease) (Schneider & Ayres, 2008; Raberg *et al.*, 2009). However, in chytrid-literature the term resistant (pathogen-limiting) is often used for describing species that are actually tolerant (damage-limiting) and definitions may vary according to the author. More importantly, this classification is rather controversial as host susceptibility is more likely to fall along a continuum where the response of a species, population or individual host to *B. dendrobatidis* is determined by a multitude of factors inherent to host, pathogen and environment (Casadevall & Pirofski, 1999). In this chapter we will highlight some determinants of susceptibility to demonstrate the intricacy of this subject.

First, we will give some examples for each host response category. However, bearing in mind that susceptibility may vary within a species, this representation may oversimplify reality and the distinction drawn between resistant and tolerant may be disputable. Many frog species are highly susceptible to *B. dendrobatidis* infection, i.e. under laboratory conditions exposure to initial low doses (i.e. 100 zoospores) can lead to 100% mortality of the experimental animals (Nichols *et al.*, 2001; Carey *et al.*, 2006) and in the wild, exposure to *B. dendrobatidis* can lead to sharp declines of a given species. A striking example of this latter is the neotropical toad genus *Atelopus* (harlequin frogs) (Ron *et al.*, 2003; Stuart *et al.*, 2004; Lampo *et al.*, 2006). With at least 30 of the 97 species presumably extinct, *Atelopus* is by far the most threatened clade of amphibians (IUCN, 2012; Frost, 2013). Especially, the Panamian golden frog (*Atelopus zeteki*) has declined in numbers by over 80% over the past decade and is almost extinct in the wild (La Marca *et al.*, 2005; IUCN, 2012). Several chytridiomycosis

related declines have also been reported for neotropical plethodontid or lungless salamanders (Rovito *et al.*, 2009; Cheng *et al.*, 2011) and experimental infection has evidenced rapid colonization of the skin coupled to high mortality (Chinnadurai *et al.*, 2009; Vazquez *et al.*, 2009; Cheng *et al.*, 2011). As these salamanders rely exclusively on cutaneous respiration, their skin is of vital importance for survival. Therefor, failure of the skin function due to chytridiomycosis is critical. In sharp contrast, no chytridiomycosis associated declines have been reported for the sole European representative of the plethodontid family (genus *Speleomantes*) which is restricted to a small geographic range comprising North-West Italy, Sardinia and South-East France, regions where chytridiomycosis related declines do occur (Spatial epidemiology, *Bd*-maps, www.bd-maps.net, 2012).

Conversely, several amphibian species do not succumb to *B. dendrobatidis* infection neither in the wild, nor under laboratory conditions, although they may be persistently infected. These species coexist with *B. dendrobatidis* and act as carriers without developing lethal chytridiomycosis or associated declines, while sympatric[2] species experience catastrophic declines. These carrier species include the Northern leopard frog *Rana pipiens* (Woodhams *et al.*, 2008a), the Pacific chorus frog *Pseudacris regilla* (Reeder *et al.*, 2012) and the widespread invasive species, *L. catesbeianus* (Daszak *et al.*, 2004) and *X. laevis* (Weldon *et al.*, 2004). Some species, including *X. laevis* (Ramsey *et al.*, 2010) and the Eastern tiger salamander *Ambystoma tigrinum* (Davidson *et al.*, 2003) go one step further and are able to clear or reduce *B. dendrobatidis* infection to very low levels within about 40 to 60 days after initial exposure.

4.1. Host determined susceptibility

4.1.1. Innate immune defenses

At present, innate immune defenses are believed to be vital host factors against emerging pathogens such as *B. dendrobatidis*. The innate immune response serves as a first immediate non-specific defense and involves, among others, the secretion of AMP's from dermal granular glands as well as antifungal metabolites produced by symbiotic skin bacteria. These specific defenses will be discussed in the next paragraphs.

4.1.1.1. Antimicrobial peptides (AMP)

AMP's are generally small, comprising 12 to 50 amino acid residues, cationic and hydrophobic peptides that are widely distributed throughout the animal and plant kingdom

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[2] Species inhabiting the same or overlapping geographic areas.

and inhibit growth of a broad spectrum of bacteria, fungi, viruses and protozoa (Zasloff, 2002). Most AMP's can reorganize into an amphipathic[3] α-helix, as shown in **Figure 9,** when bound to charged residues on target cell membranes. Resulting peptide complexes interact with and penetrate into the cell membrane. So far, the precise mechanism of membrane penetration by these peptides is not exactly known, but ultimately membrane penetration disturbs the membrane permeability of the target cell, leading to cell lysis (Hancock, 2001; Zasloff, 2002).
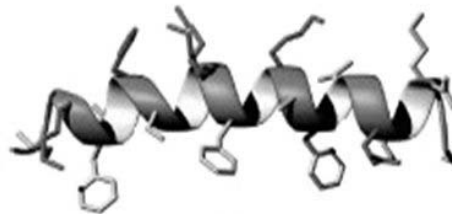

Figure 9. The α-helical structure of the antimicrobial peptide magainin-2, found in skin secretion of the African clawed frog (*X. laevis*). Adapted from Nguyen *et al.* (2011).

The ability to synthetize peptides offering protection appears to be limitedly distributed among amphibians and a vast number of well-studied species from the Bufonidae, Ceratophryidae, Dicroglossidae, Microhylidae, Pelobatidae, Pyxicephalidae, Rhacophoridae, and Scaphiopodidae families simply lack conventional AMP's (Conlon *et al.*, 2009). Several purified and natural mixtures of AMP's from amphibian skin secretions effectively inhibit *in vitro* growth of both *B. dendrobatidis* zoospores and sporangia at minimum inhibitory concentrations (MIC) as summarized in **Table 2** (reviewed in Rollins-Smith & Conlon, 2005; Woodhams *et al.*, 2007a; Rollins-Smith *et al.*, 2009; Ramsey *et al.*, 2010).

However, it is not clear to which extent these peptides provide protection against chytridiomycosis *in vivo*. Species with peptides active *in vitro* such as the mountain yellow-legged frog (*Rana muscosa*) may turn out to be very susceptible for infection in nature (Rollins-Smith *et al.*, 2006). Moreover, the efficacy of skin peptide defenses may vary at species and population level (Woodhams *et al.*, 2007a; Tennessen *et al.*, 2009). Also little is known about the activity of AMP's once they are secreted upon the skin. Degradation dynamics of skin peptide defenses in species of the *Pelophylax* complex (formerly *Rana*) and in the Northern leopard frog (*R. pipiens*) suggest that once peptides are secreted upon the skin they stay active up to 1 to 2 hours, but are then degraded by host proteases (Daum *et al.*, 2012; Pask *et al.*, 2012).

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[3] With both a hydrophobic and hydrophilic portion within the same molecule.

Table 2. Amphibian antimicrobial peptides active against zoospores of *B. dendrobatidis* with their respective minimal inhibitory concentrations (MIC)

Peptide	Species of origin	MIC (µM)
Aurein 2.1	*Litoria aurea, Litoria raniformis*	200
Brevinin-1BYa	*Rana boylii*	12.5
Brevinin-1BYc	*Rana boylii*	6.25
Brevinin-1TRa	*Rana tarahumarae*	12,5
Brevinin-2Ob	*Rana ornativentris*	6.25
Caerin 1.1	*Litoria caerulea, Litoria splendida, Litoria gilleni, Litoria ewingi*	25-50
Caerin 1.9	*Litoria chloris*	25-50
Citropin 1.1	*Litoria citropa*	100-200
CPF	*Xenopus laevis*	12.5
Dahlein 5.6	*Litoria dahlia*	100-200
Dermaseptin	*Phyllomedusa sauvagii*	23
Dermaseptin-L1	*Hylomantis lemur*	>100
Esculentin-1A	*Rana areolata*	12.5
Esculentin-2L	*Rana luteiventris*	12.5
Esculentin-2P	*Rana pipiens*	25
Fallaxin	*Leptodactylus fallax*	100
Frenatin 3	*Litoria infrafrenata*	100
Maculatin 1.1	*Litoria genimaculata*	25-50
Magainin I	*Xenopus laevis*	> 47
Magainin II	*Xenopus laevis*	162
Melittin-related peptide	*Rana tagoi*	25
Palustrin-3A	*Rana areolata*	6.25
PGLa	*Xenopus laevis*	50
Phylloseptin-L1	*Hylomantis lemur*	100
Ranalexin	*Rana catesbeiana*	12.5
Ranatuerin-1	*Rana catesbeiana*	12.5
Ranatuerin-2BYa	*Rana boylii*	25
Ranatuerin-2BYb	*Rana boylii*	12.5
Ranatuerin-2La	*Rana luteiventris*	50
Ranatuerin-2Ma	*Rana muscosa*	50
Ranatuerin-2Mb	*Rana muscosa*	25
Ranatuerin-2P	*Rana pipiens*	100
Ranatuerin-2TRa	*Rana tarahumarae*	50
Ranatuerin-6	*Rana catesbeiana*	>100
Temporin -1Ob	*Rana ornativentris*	25
Temporin A	*Rana temporaria*	66
Temporin-1M	*Rana muscosa*	100
Temporin-1P	*Rana pipiens*	50
Uperin 3.6	*Uperoleia mjobergii*	100

Adapted from Rollins-Smith & Conlon, 2005; Woodhams *et al.*, 2006; Rollins-Smith, 2009.

4.1.1.2. Antifungal metabolites

Another defense against *B. dendrobatidis* infections is provided by antifungal metabolites secreted by symbiotic bacteria present on amphibian skin. So far, only 3 inhibitory metabolites secreted by the skin bacterial species *Janthinobacterium lividum*, *Lysobacter gummosus* and *Pseudomonas fluorescens* have been in identified, i.e. 2,4-diacetylphloroglucinol (2,4-DAPG), indol-3-carboxaldehyde (I3C) and violacein (**Table 3).** These antifungal metabolites aid to maintain infection loads below a lethal threshold and exhibit a dual action. First, these metabolites can inhibit growth of *B. dendrobatidis* both *in vitro* and *in vivo* (Harris *et al.*, 2006; Woodhams *et al.*, 2007b; Brucker *et al.*, 2008a, b; Lam *et al.*, 2010; Flechas *et al.*, 2012). Moreover, Myers and co-workers (2012) recently showed that these metabolites work synergistically with AMP's to inhibit growth of *B. dendrobatidis in vitro*, at lowered MIC necessary for inhibition by either metabolites or AMP's. Second, Lam and colleagues (2011) observed that *B. dendrobatidis* zoospores more frequently move away from substrates containing either 2,4-DAPG or I3C and ascribed this effect to a repellent action of both metabolites.

Table 3. Metabolites of symbiotic skin bacteria active against zoospores of *B. dendrobatidis* with their respective minimal inhibitory concentrations (MIC)

Bacterial metabolite	MIC (µM)	Skin bacterium of origin	Amphibian host	Reference
2,4-diacetylphloroglucinol	136.13	*Lysobacter gummosus*	*Plethodon cinereus*	Brucker *et al.*, 2008a
		Pseudomonas fluorescens	*Rana muscosa*	Myers *et al.*, 2012
Indole-3-carboxaldehyde	68.9	*Janthinobacterium lividum*	*Plethodon cinereus*	Brucker *et al.*, 2008b
			Hemidactylum scutatum	Brucker *et al.*, 2008b
Violacein	1.82	*Janthinobacterium lividum*	*Plethodon cinereus*	Brucker *et al.*, 2008b
			Hemidactylum scutatum	Brucker *et al.*, 2008b

Like for AMP's, variation in pathogen susceptibility among populations is thought to result from differences in bacterial skin communities. By comparing bacterial communities on the skin of a declining *Rana muscosa* population and a population coexisting with *B. dendrobatidis*, researchers found a significantly higher number of individuals with culturable bacterial species displaying antifungal properties in coexisting populations than in those at decline (Woodhams *et al.*, 2007b; Lam *et al.*, 2010).

Alteration of the composition of the microbial community on amphibian skin, e.g. by environmental factors, can considerably increase susceptibility to disease (Woodhams *et al.*, 2007b; Brucker *et al.*, 2008a; 2008b; Becker *et al.*, 2009; Harris *et al.*, 2009a). Conversely, by adding *J. lividum* to the skin of individuals of a susceptible *Rana muscosa* population

before experimental exposure to *B. dendrobatidis*, the lab of Harris (2009b) found that addition of *J. lividum* alleviated symptoms of chytridiomycosis and prevented morbidity and mortality. Therefore, bio-augmentation and the use of probiotics may offer new perspectives to protect and safeguard declining amphibian populations in the wild (Harris *et al.*, 2009a).

4.1.2. Adaptive immune defenses

A second highly specific protection against pathogens is provided by the acquired or adaptive immune response. When infection cannot be prevented by non-specific innate defenses, pathogen specific antibodies or immunoglobulins (Ig) come into action. This has been described in detail for *X. laevis* where IgM, IgX (mammalian IgA-like) and IgY (mammalian IgG-like) antibodies are produced and secreted in the mucus after immunization. In certain amphibian species, a higher survival and even clearance is seen in frogs previously exposed to *B. dendrobatidis* than in immunologically naïve frogs (Richmond, 2009; Ramsey *et al.*, 2010; Shaw *et al.*, 2010). These antibodies are thought to play a role in neutralizing pathogens or presenting them to other components of the immune system for destruction (Rollins-Smith *et al.*, 2009; Ramsey *et al.*, 2010).

4.1.3. Impact of host genetics, life history and behaviour

The genetic make-up of the host also largely determines the outcome of *B. dendrobatidis* infection. In vertebrates, major histocompatibility complex (MHC) loci encode cell-surface glycoproteins regulating the acquired immune response. In amphibians, individuals with specific MHC genotypes, seem to benefit from a higher survival rate when infected by *B. dendrobatidis* (Savage & Zamudio, 2011). In contrast, low genetic diversity within a species or population and consequent reduced biological fitness, may complicate the ability of a species or population to withstand *B. dendrobatidis* infection (Luquet *et al.*, 2012).

Vulnerability to *B. dendrobatidis* may vary with the life history stage of the infected animal. Differential susceptibility is observed between larval, post-metamorphic, sub-adult and adult stages (Lamirande & Nichols, 2002; Rachowicz & Vredenburg, 2004; Garner *et al*, 2009). For example, tadpoles of several *Rana* species can be infected without clinical symptoms, while in post-metamorphic animals infection induces morbidity and mortality (Rachowicz & Vredenburg, 2004). Alternatively, *B. dendrobatidis* can negatively affect some species of amphibians at the larval stage and not others (Blaustein *et al.*, 2005).

Behavioural changes can influence the vulnerability of a species to chytrid infection. For example, the Panamian golden frog (*Atelopus zeteki*) can modify its thermoregulatory

behaviour, raising their body temperature and by doing so reduce the severity of infection (Richards-Zawacki, 2010).

4.2. Pathogen determined susceptibility

The virulence of *B. dendrobatidis* or its relative capacity to cause damage to its amphibian host, is isolate and genotype dependent (Berger *et al.*, 2005c; Retallick & Miera, 2007; Fisher *et al.*, 2009; Farrer *et al.*, 2011). Recently, 4 lineages of *B. dendrobatidis* were identified, including a hypervirulent global panzootic lineage (*Bd*GPL) (Farrer *et al.*, 2011). As such populations infected with non-GPL strains appear to be exempt from epidemic disease (Farrer *et al.*, 2011).

Also temperature seems to mediate virulence of *B. dendrobatidis*, by altering its generation time[4] and fecundity[5] (Woodhams *et al.*, 2008b; Voyles *et al.*, 2012b). As discussed earlier (§ *2.3*) optimal growth of *B. dendrobatidis* occurs with a temperature range of 17-25°C. Within this range, zoospores encyst and develop into zoosporangium faster than at low temperatures. However, at low temperatures a larger number of zoospores is produced per zoosporangium, with zoospores remaining active and thus infective for a longer period.

4.3. Environment determined susceptibility

Differential susceptibility to *B.dendrobatidis* observed in the field may be due to several abiotic, environmental factors such as season, temperature (Berger *et al.,* 2004; Kriger & Hero, 2006) and intensity of ultraviolet B (UV-B) radiation (Searle *et al.*, 2010; Ortiz-Santaliestra *et al.*, 2011). Plausibly, these environmental factors increase the vulnerability of species considerably, by either changing the virulence of *B. dendrobatidis* or by reducing the immunity of the amphibian host (Ortiz-Santaliestra *et al.*, 2011). For example, the optimal growth temperature for *B. dendrobatidis* is between 17 and 25 °C (Piotrowski *et al.*, 2004) and thus mortality of amphibians due to *B. dendrobatidis* increases experimentally with ambient temperatures below 25°C, and in the wild, in the cooler months of the year in tropical and subtropical areas (Berger *et al.*, 2004; Kriger & Hero, 2006). Alternatively, short exposure to warm microhabitats aids amphibians to keep infections below a lethal threshold (Daskin *et al.*, 2011).

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[4] Average time span between zoospore formation and development of a zoospore into a new sporangium.
[5] Reproductive rate, measured by the number of zoospores.

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SCIENTIFIC AIMS

The chytrid fungus *Batrachochytrium dendrobatidis*, etiological agent of chytridiomycosis, exerts a major impact on many amphibian species threatening amphibian biodiversity worldwide. The disease caused by *B. dendrobatidis,* chytridiomycosis, causes disruption of the skin function and thus compromises the survival of the amphibian. However, the effects of chytridiomycosis on amphibian species and even within one species are variable. While some tolerant amphibians are able to keep infection levels below a lethal threshold and function as carrier species, others are susceptible and develop severe disease and finally succumb to infection.

At the onset of this PhD research, our knowledge about the pathogenesis of *B. dendrobatidis* infections was scarce. In general, the first steps in fungal pathogenesis are considered to be adhesion to host surfaces, germination and invasion of host tissue, but none of these have been documented for *B. dendrobatidis*. Besides, the role of skin secretions in the development of chytridiomycosis have been poorly documented. Still, an improved understanding of host-pathogen interactions is a prerequisite to understand factors defining host susceptibility. In this regard, salamanders of the genus *Speleomantes* are of particular interest. The apparent absence of chytridiomycosis-related mortalities or declines in *Speleomantes* species, despite the omnipresence of *B. dendrobatidis* in its range, may suggest that this group of salamanders is resistant to *B. dendrobatidis* infection.

Consequently, the general aim of the present doctoral study was to gain insight into the early interactions between *B. dendrobatidis* and the amphibian skin with emphasis on skin invasion and the role of skin secretions.

The specific aims were:

1) to develop and validate suitable *in vitro* models as tools to model pathogenesis studies;
2) using these models, to characterize early interactions between *B. dendrobatidis* and the skin of tolerant and susceptible amphibian hosts;
3) to evaluate whether susceptibility to chytridiomycosis is associated with antifungal skin secretions, using European *Speleomantes* salamanders as model organisms.

STUDY 1

Development of *in vitro* models for a better understanding of the early pathogenesis of *Batrachochytrium dendrobatidis* infections in amphibians

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Summary

 Batrachochytrium dendrobatidis, the causal agent of chytridiomycosis, is implicated in the global decline of amphibians. This chytrid fungus invades keratinized epithelial cells, and infection is mainly associated with epidermal hyperplasia and hyperkeratosis. Since little is known about the pathogenesis of chytridiomycosis, this study was designed to optimize the conditions under which primary keratinocytes and epidermal explants of amphibian skin could be maintained *ex vivo* for several days. The usefulness of the following set-ups for pathogenesis studies was investigated: (1) cultures of primary keratinocytes, (2) stripped epidermal (SE) explants, (3) full-thickness epidermal (FTE) explants on Matrigel™, (4) FTE explants in cell culture inserts, and (5) FTE explants in Ussing chambers. SE explants proved most suitable for short-term studies, since adherence of fluorescently-labeled zoospores to the superficial epidermis could be observed within one hour of infection. FTE explants in an Ussing chamber set-up are most suitable for the study of the later developmental stages of *B. dendrobatidis* in amphibian skin up to five days post-infection. These models provide a good alternative for *in vivo* experiments, and reduce the number of experimental animals needed.

1. Introduction

The chytrid fungus *Batrachochytrium dendrobatidis* is the etiologic agent of chytridiomycosis. The impact of this fungal disease on amphibian populations is devastating (Berger *et al*., 1998; Daszak *et al*., 1999; 2003; Lips *et al*., 2006; Skeratt *et al*., 2007; Fisher *et al.*, 2009). Worldwide, populations are declining or are already extinct (Stuart *et al*., 2004; Spatial epidemiology, *Bd*-maps, http://www.bd-maps.net, 2012). Infection is confined to the stratum corneum (superficial epidermal layer) and stratum spinosum of adult skin, and to the keratinized layers of larval mouthparts (Berger *et al*., 1998; Pessier *et al*., 1999; Marantelli *et al.*, 2004). It is associated with epidermal hyperplasia and hyperkeratosis (Berger *et al*., 1998). Zoospores of *B. dendrobatidis* have a single posterior flagellum (Longcore *et al*., 1999; Berger *et al*., 2005). To infect a host, zoospores rely on motility and chemotactic responses toward certain nutritional cues in the environment (Moss *et al*., 2008). Upon colonization of amphibian skin, zoospores presumably encyst within keratinized tissues. After the flagellum is resorbed, a cell wall forms. Zoospores develop into sporangia with discharging tubes, containing asexually produced zoospores that can either reinfect the host, or be released into the surrounding environment to infect other amphibians (Berger *et al*. , 2005).

Several studies have demonstrated that *B. dendrobatidis* can readily invade the epidermis of experimentally infected susceptible amphibians, with clinical manifestation of chytridiomycosis (Nichols *et al*., 2001; Daszak *et al*., 2004; Carey *et al*., 2006; Chinnadurai *et al.*, 2009; Vazquez *et al*., 2009). Recently, the studies of Voyles *et al.* (2007, 2009) have shown that chytridiomycosis is associated with disruption of the osmoregulatory function of the skin, leading to electrolyte imbalance and death due to cardiac arrest.

Relatively little is known about the underlying mechanisms of invasion, e.g. the initial contact of the zoospores with the stratum corneum, and the subsequent events that take place before the development of hyperplasia, hyperkeratosis and lesions. Until now, all studies concerning infectivity and pathogenicity of *B. dendrobatidis* have been performed on *in vivo* infected amphibians (Berger *et al*., 2005; Voyles *et al*., 2009). The nature of interactions between chytrids and skin cells requires further investigation. Therefore, a well-defined *in vitro* infection model could be a critical tool, providing a valuable alternative for *in vivo* experiments, with the use of fewer experimental animals.

This study aimed to develop and evaluate several *in vitro* models for the investigation of the early stages of the pathogenesis of chytridiomycosis. In the initial part of the study, several set-ups were established, involving the use of cultures of primary keratinocytes as

well as *ex vivo* explants of amphibian skin. In the subsequent part of the study, the optimized models were inoculated with zoospores of *B. dendrobatidis*, in order to determine whether chytrid infection could be successfully established *in vitro*.

2. Materials and Methods

2.1. Experimental animals

Post-metamorphic and adult wild type *Xenopus laevis* (South-African clawed frog) and *Silurana tropicalis* (Western clawed frog) were obtained from the European *Xenopus* Resources Centre (Portsmouth, UK). Captive-bred *Alytes cisternasii* (Iberian midwife toad) were obtained from a private breeder in Germany. For the setting-up and fine tuning of the infection models, *X. laevis* was selected as tissue source, since this species is commercially available and easily maintained in the laboratory, and one single adult animal (10–15cm) can provide sufficient skin for testing several conditions. Although *X. laevis* is relatively resistant to clinical *B. dendrobatidis* infection due to the secretion of inhibitory skin peptides (Rollins-Smith & Conlon, 2005; Rollins-Smith, 2009), it is considered to be one of the main reservoirs of *B. dendrobatidis* with persistent infections (Parker *et al*. 2002; Weldon *et al.* 2004). Interestingly, whole genome sequences of *S. tropicalis* and a large number of gene sequences of *X. laevis* are available, permitting the study of transcriptional responses after infection with *B. dendrobatidis*. Upon arrival, the animals were pre-screened for the presence of *B. dendrobatidis*. Swab samples were taken by rubbing the pelvic region and hind limbs with a cotton-tipped swab.

2.2. In vitro *culture of amphibian skin*
2.2.1. Culture media and supplements

Ca^{2+}-free medium 154 (medium 154 CF; Cascade Biologics, Portland, OR, USA) was diluted to 70% (v/v) with distilled water, to effect osmotic adjustment to the amphibian cellular environment, and was supplemented with 0.2% (w/v) insulin (Sigma, St Louis, MO, USA), 10μg/ml amphotericin B (SPRL Bristol-Myers Squibb, Brussels, Belgium), 100U/ml penicillin, 100μg/ml streptomycin sulphate (penicillin/streptomycin; Gibco, Invitrogen, Grand Island, NY, USA), and 100μg/ml kanamycin sulphate (Gibco, Invitrogen). In order to determine an optimal formulation for the *in vitro* culture of amphibian skin, this starting medium was supplemented with either: (1) 5% (v/v) fetal calf serum (FCS, Hyclone, Cramlington, UK), (2) 0.02 mM Ca^{2+} (Cascade Biologics), (3) 0.05 mM Ca^{2+}, necessary for

growth and differentiation, respectively (Nishikawa *et al*., 1990), or (4) 10 nM 3,3´,5-triiodo-L-thyronine (T3, Sigma), necessary for the induction of keratin gene expression during *X. laevis* development (Mathisen & Miller, 1989).

2.2.2. Primary keratinocytes

The animals were euthanized with intracoelomically-injected T61® (Intervet, Mechelen, Belgium), and washed according to the protocol of Nishikawa *et al.* (1990). By washing, skin mucus, which possibly contains antimicrobial peptides and bacteria that play a role in protection from chytrid infection (Rollins-Smith & Conlon, 2005; Rollins-Smith, 2009), was removed. This facilitates the handling of skin tissue and reduces the risk of contamination. Full-thickness epidermal (FTE) skin, comprising epidermis and dermis, was excised and spread in a sterile Petri dish, dermis-side down. Both the apical and basal sides were rinsed with 70% medium 154 CF. From each donor animal, a skin sample was taken and tested for the presence of *B. dendrobatidis* by quantitative PCR (qPCR). The skin was cut into 10mm wide strips and incubated overnight at 4°C in MatriSperse™ Cell Recovery Solution (BD Biosciences, Bedford, MA, USA) for the isolation of stripped epidermal (SE) samples. This non-enzymatic solution, initially designed to isolate epithelial cells grown on Engelbreth-Holm-Swarm (EHS) biomatrices, also allows detachment of the integral epidermal layer from the underlying dermal layer in amphibian skin explants. The following day, the epidermis could be gently peeled off the dermis by using sterile needles. The resulting epidermal strips were treated for 2 to 3 hours with 10 U/ml dispase (Gibco, Invitrogen), in 70% medium 154 CF, at 20°C. The cells were suspended by repetitive pipetting and centrifuged for 10 minutes at 350 g. The supernatant was discarded, and the pellet was washed in 70% medium 154 CF, centrifuged for 10 minutes at 350 g, and resuspended in 70% medium 154 CF. The cells were seeded in 24-well tissue culture dishes (Cellstar®, Greiner Bio-One, Wemmel, Belgium) containing plastic coverslips (Thermanox®, NUNC, Rochester, NY, USA) coated with 0.1% (w/v) bovine plasma fibronectin (Sigma), at 2.5×10^5 cells per well (in 1ml of medium) and incubated at 20°C, 5% CO_2. The medium was replaced daily. After 1, 4 and 7 days of incubation, the viability of the primary keratinocytes was assessed microscopically by Trypan blue staining (Merck, Darmstadt, Germany) and proliferation was determined by using the WST-1 cell proliferation assay (Roche Molecular Biochemicals, Mannheim, Germany).

2.2.3. Stripped epidermal (SE) explants

SE explants were isolated as described above, with the exception that, for this purpose the skin was harvested with a sterile 4 mm biopsy punch (Kai Europe GmbH, Solingen,

Germany). In this way, epidermal keratinocyte layers are minimally disrupted and a standard surface for testing is provided. SE explants were placed individually into wells of a 24-well tissue culture dish coated with 100 μl Matrigel™ basement membrane matrix (BD Biosciences) or 0.1% (w/v) bovine plasma fibronectin. After waiting 1 hour for the skin to adhere to the plate, a 100 μl-aliquot of medium was added to each well. The plates were incubated at 20°C, in 5% CO_2. The medium was replaced daily, and after 1, 2, 3, 4 and 5 days of incubation, explants were processed for histology.

2.2.4. Full-thickness epidermal (FTE) explants

FTE explants were obtained as described above. For the *in vitro* culture of the FTE explants, the following set-ups were used: (1) a Matrigel™-based model, (2) a cell culture insert-based model, and (3) Ussing chambers. For the Matrigel™-based model, the same procedure was used as that described for SE explants. After 1, 2, 3, 4 and 5 days of incubation, explants were processed for histology. For the cell culture insert-based model, FTE explants were harvested with a 6 mm biopsy punch. The explants were spread out over the entire surface of sterile, 6.5 mm-diameter cell culture inserts (Transwell® Permeable Supports, polyester membrane, pore size 3.0 μm, Corning, Lowell, MA, USA), and placed in 24-well tissue culture dishes. A 100 μl-aliquot of distilled water was added to the apical compartment of each insert to prevent dehydration, and 100 μl of medium were added to each well. The plates were sealed with Parafilm® and incubated at 20°C, in 5% CO_2, for 7 days. The medium was replaced daily, and after 7 days, the explants were processed for histology. For the Ussing chamber-based model, FTE explants of 15 x 25 mm were collected, placed in acrylic inserts with an aperture size of 14 x 2 mm (exposed surface area of 0.28 cm^2), and mounted into a modified Ussing chamber (Muβler Scientific Instruments, Aachen, Germany) within 20 minutes after euthanasia. Chamber openings intended for the insertion of agar bridges were closed with custom-made silicone plugs to prevent leakage. Compartments on the apical side of the skin fragments were filled with 7ml of distilled water, and at the basolateral side with medium 154 CF supplemented with 0.05 mM Ca^{2+}. The solutions were circulated by gas lift (95% O_2, 5% CO_2), and maintained at 20°C by water-jacketed reservoirs. The cell culture medium was replaced daily, and after 5 days, the skin preparations were removed from the chambers and the exposed skin surface area was excised and processed for histology. For each model, morphological characteristics and structural changes in the skin architecture were studied on histological slides, and compared with freshly-isolated skin preparations.

2.3. Experimental inoculation of in vitro *models of amphibian skin with* B. dendrobatidis

2.3.1. Inoculum

Inoculations were carried out with zoospores of *B. dendrobatidis* isolate IA042, isolated from a dead *Alytes obstetricans* involved in a mass mortality event (Ibon Acherito, Spanish Pyrenees) (Garner *et al.*, 2009). This strain was proven to colonize *A. cisternasii* to a high extent (Martel *et al.*, 2010). Strain IA042 was cultured in tryptone/gelatin hydrolysate/lactose (TGhL) broth, in 25 cm^2 cell culture flasks, at 20°C for 5 days. A 2ml-aliquot of a 5-day old broth culture was transferred onto a TGhL agar plate, and incubated for 5-7 days at 20°C. Zoospores were collected by flooding the agar plate with 2 ml of distilled water, and were immediately counted in Lugol's solution by using a haemocytometer. The zoospore suspension was adjusted to a concentration of 2-3 x 10^7 zoospores per ml.

2.3.2. Fluorescent quantitative adherence assay in SE and FTE explants

SE explants from *X. laevis* and FTE explants from *A. cisternasii* were placed on cover slips, epidermis side-up, in 24-well plates, and 70% medium 154 CF was added to each well, just enough to submerge the skins. The explant tissue was labeled with 3 μM CellTracker™ Green CMFDA (from 100 μM stock, diluted in 60% Dulbecco's phosphate buffered saline (dPBS); 1ml per well; Invitrogen). A zoospore suspension was labeled by adding an equal volume of 3 μM CellTracker™ Red CMTPX (from 100 μM stock, diluted in distilled water; Invitrogen). Finally, the explants were inoculated with 20 μl of stained zoospore suspension (at 1 x 10^7 zoospores/ml, in distilled water). After 1 hour of incubation at 20°C, in 5% CO$_2$, the explants were washed three times with 60% (v/v) dPBS, to remove non-adhered zoospores, fixed in 3.7% (w/v) paraformaldehyde (Fluka, Seelze, Germany), and mounted in glycerol/DABCO® (1,4-diazabicyclo[2.2.2]octane, Sigma). Adhesion was studied by fluorescence microscopy (Leica DMRB, Leica Microsystems GmbH, Wetzlar, Germany). For each assay, zoospores in four microscopy fields were counted in triplicate. The assay itself was performed in duplicate.

2.3.3. Infection assay in FTE explants

In an initial experiment, the infection rate of *B. dendrobatidis* in the pelvic skin of *X. laevis* and *S. tropicalis* on Matrigel™ was studied at 1 day and 4 days post-infection (dpi), in triplicate and on two occasions. In a second experiment, the infection rates of *B. dendrobatidis* in pelvic skin and dorsal skin from three *X. laevis* and three *A. cisternasii* on Matrigel™ were compared at 1 dpi, in triplicate. For this purpose, FTE explants of *X. laevis*, *S. tropicalis* and *A. cisternasii* were inoculated with 25 μl of cell suspension (3 x 10^7

zoospores/ml). The culture conditions were as described above, with the exception that the explants were incubated for 3 hours before the addition of the culture medium. In a third infection experiment, the infection rate of *B. dendrobatidis* in pelvic skin from *A. cisternasii* and *X. laevis*, maintained in cell culture inserts, was studied at 7 dpi. Explants were inoculated apically with 100 µl of cell suspension (2×10^7 zoospores/ml). The culture conditions were as described above. For each of these experiments, one portion of explant tissue was processed for histology, while the other portion was washed four times in 70% medium 154 CF, then subjected to DNA extraction and qPCR. In a fourth experiment, FTE explants in the Ussing chamber set-up were apically infected with 7 ml of inoculum (2×10^7 zoospores/ml), and incubated for 5 days. The culture conditions were as described above. At 5 dpi, the skin preparations were removed from the chambers, and the exposed skin surface area was excised and processed for histology. For each infection model, histological slides were inspected microscopically for the presence of histopathological lesions characteristic for chytridiomycosis.

2.4. DNA extraction and qPCR

DNA extraction from explant tissue was performed by using a proteinase K digestion, following the protocol of Bandi *et al*. (1994). DNA from swabs was extracted in 100 µl PrepMan Ultra (Applied Biosystems, Foster City, CA, USA), according to the method of Hyatt *et al*. (2007). The DNA samples were diluted 1:10 and stored at -20°C until required. QPCR assays were performed on a CFX96 Real Time System (BioRad, Hercules, CA, USA), with amplification conditions, primer and probe concentrations according to Boyle *et al*. (2004). The samples were run in duplicate.

2.5. Histology and immunohistochemistry

The explants were fixed in 10% (v/v) neutral buffered formalin, embedded in paraffin, and 5µm sections were stained with haematoxylin and eosin (HE). Selected slides from inoculated explants were further subjected to immunoperoxidase (IPX) staining for *B. dendrobatidis*, as described by Berger *et al*. (2002).

3. Results

3.1. Optimal conditions for in vitro cultures of primary keratinocytes and ex vivo epidermal explants

Based on histological observations, 0.05 mM Ca^{2+}- supplemented serum-free medium 154 CF was chosen as the culture medium for primary keratinocytes, and SE and FTE explants. In this culture medium, isolated primary keratinocytes remained viable for 1-4 days. After 4 days, viability decreased abruptly. No significant proliferation was observed. In **Figure 1**, an overview is given of the histological characteristics of freshly isolated skin (**Fig. 1A**) and skin explants cultured *ex vivo* in the different models (**Figs. 1B-E**). SE explants (**Fig. 1B**) remained intact for only a very short time, and thus are most suitable for applications such as short-period adherence studies. Given the short incubation time, the additional coating of cell culture plates was considered to be unnecessary. In almost all the set-ups with FTE explants, epidermal hyperplasia, most probably caused by hypoxia, occurred. This was followed by coagulation necrosis of the epidermis after 1 and 4 days of incubation, respectively. Despite the occurrence of mild hyperplasia and occasional apoptosis, a rather good preservation of the epidermis was achieved when using Matrigel^{TM} coating (**Fig. 1C**). However, on some occasions, separation of the basal epidermal layer and the dermis occurred. Necrosis was most pronounced in FTE explants incubated in cell culture inserts. The overall impression of the resulting skin morphology was poor, but no epidermal hyperplasia was observed (**Fig. 1D**). In the more labor-intensive Ussing chamber-based set-up, mild to moderate epidermal hyperplasia was seen after 5 days of incubation, and dermal tissue and muscle cells were still viable (**Fig. 1E**).

3.2. Defining an optimal model for the study of chytrid–keratinocyte interactions

3.2.1. Fluorescent quantitative adherence assay in SE and FTE explants

The adherence of zoospores to *X. laevis* SE explants and *A. cisternasii* FTE explants occurred within one hour. In both experiments, the observed average number of adhered zoospores in explants of *A. cisternasii* (128 in Experiment 1; 124 in Experiment 2) was approximately 20% higher than in explants of *X. laevis* (103 in Experiment 1; 100 in Experiment 2).

3.2.2. Infection assay in FTE explants

A comparison of infection rates of *B. dendrobatidis*, expressed as mean detected genomic equivalents (GE) with standard errors (SE), in pelvic skin of *X. laevis* and *S.*
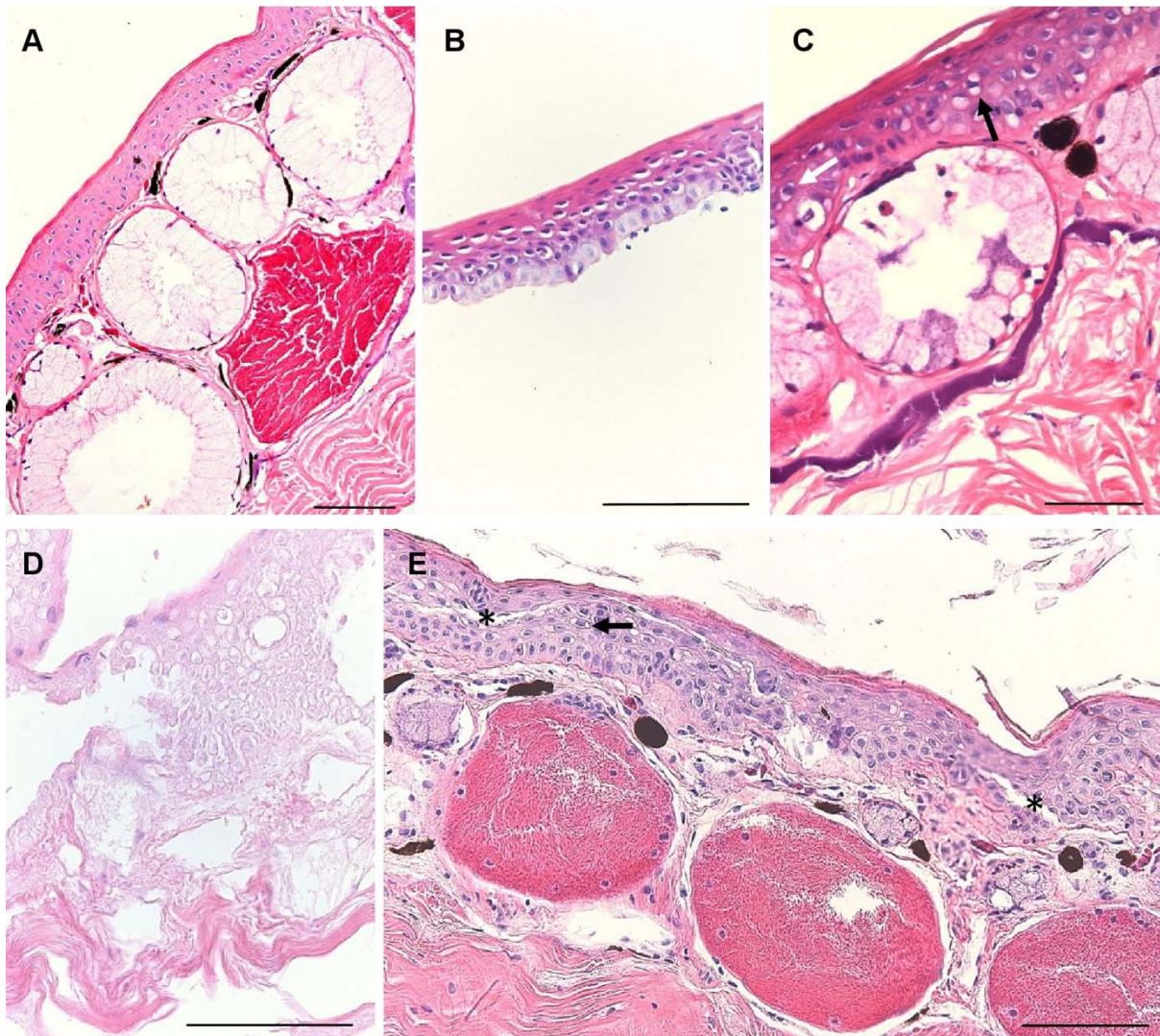
Figure 1. HE stained histological sections of amphibian skin explants under different conditions of isolation and inoculation. (A) FTE explant at day 0; **(B)** SE explant after overnight treatment with Matrisperse™; **(C)** FTE explant on Matrigel™ at day 4, showing a relatively well preserved epidermis, joining tightly with the adjacent dermis; morphological changes in the keratinocytes range from cytoplasmic vacuolation (white arrow) to frank apoptosis (rather scarce; black arrow), and a mild increase in epidermal thickness; **(D)** FTE explant on cell culture inserts at day 7, showing an overall coagulative necrosis; **(E)** FTE explant in maintained in an Ussing chamber at day 5, showing mild to moderate epidermal hyperplasia, keratinocytes which are multifocally mildly vacuolated with increased cytoplasmic eosinophilia and nuclear condensation (black arrow), possibly indicating an early stage of apoptosis; at various levels of the epidermis there is cleft formation (*), and focally there is dermo-epidermal separation (not shown here); however, the dermal tissue and muscles are still viable. Scale bar **figs. A**, **E** = 100 μm; scale bar **figs. B**, **C**, **D** = 50 μm.

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tropicalis on Matrigel^TM coating, at 1 and 4 dpi, is presented in **Table 1**. The mean GE's were higher for *X. laevis* skin at 1 dpi than for *S. tropicalis* skin. At 4 dpi, the mean GE's for both species are approximate. Adhesion of zoospores to the stratum corneum was observed on rare occasions, in histological slides of *S. tropicalis* skin at 1 dpi and *X. laevis* at 4 dpi.

Table 1. Average genomic equivalents (GE) of *B. dendrobatidis* in *in vitro* infected FTE explants of pelvic skin of *X. laevis* and *S. tropicalis*

	Genomic equivalents (mean ± SE)				
	1 dpi			4 dpi	
	Exp. 1	Exp. 2		Exp. 1	Exp. 2
Xenopus leavis	123 ± 44	67 ± 37		205 ± 272	25 ± 20
Silurana tropicalis	25 ± 10	26 ± 33		217 ± 267	23 ± 20

Explants were maintained on Matrigel^TM and GE values were determined at 1 and 4 dpi. The experiment was carried out in triplicate and on two occasions. Data are presented as mean GE values ± SE.

A comparison of infection rates of *B. dendrobatidis* in pelvic skin and dorsal skin of *X. laevis* and *A. cisternasii* on Matrigel^TM coating, at 1 dpi, is shown in **Table 2**. For both *X. laevis* and *A. cisternasii*, the mean GE's were higher in dorsal skin than in pelvic skin.

Table 2. Average genomic equivalents (GE) of *B. dendrobatidis in vitro* infected FTE explants of pelvic skin and dorsal skin from *X. laevis* and *A. cisternasii*

	Genomic equivalents (mean ± SE)				
	Xenopus laevis			*Alytes cisternasii*	
	Pelvic region	Dorsal skin		Pelvic region	Dorsal skin
Specimen 1	174 ± 23	389 ±132		32 ± 18	86 ± 56
Specimen 2	514 ± 214	783 ± 200		60 ± 13	627 ± 121
Specimen 3	292 ± 37	612 ± 245		135 ± 60	839 ± 98

Explants were maintained on Matrigel^TM, and GE values were determined at 1 dpi. The experiment was warried out with three animal specimens of each type. Data are presented as mean GE values ± SE.

However, in none of the histological slides from infected *X. laevis* skin, adhesion was observed. Slides from infected *A. cisternasii* pelvic skin explants revealed adhesion of zoospores to the stratum corneum at 1 dpi. Limited adhesion was seen in infected dorsal skin.

At 7 dpi, adhesion of zoospores to a sloughed piece of stratum corneum was observed in *X. laevis* skin infected in cell culture inserts. Extensive adhesion, limited to a patch of sloughed stratum corneum, was observed in *A. cisternasii*. Structures ranging from thin-walled zoospores to encysted zoospores, and mature and post-discharge sporangia, were attached to the epidermal surface (**Figs. 2A-B**). At 5 dpi, large-scale attachment of zoospores, encysted zoospores and zoosporangia to the stratum corneum was observed in pelvic skin of *X. laevis* infected in a Ussing chamber. The stratum corneum was partially shed and severely eroded. In dorsal skin, albeit only in a small patch of stratum corneum, the adhesion of numerous zoospores and encysted zoospores was observed, with zoospore cysts occasionally merged into the stratum corneum (**Figure 2C**).
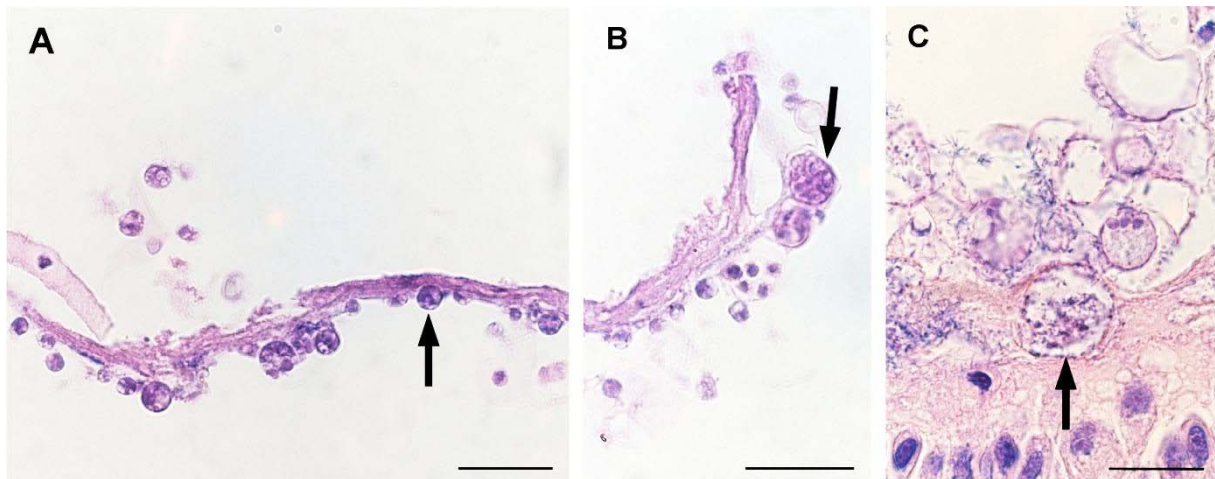

Figure 2. **HE stained sections of *in vitro* infected FTE explants**. **(A)** pelvic skin of *A. cisternasii* infected in a cell culture inserts based model, at 7 dpi, showing evident adhesion of *B. dendrobatidis* zoospores and zoospore cysts to the sloughed stratum corneum; arrow indicates a zoospore cyst; **(B)** pelvic skin of *A. cisternasii* infected in a cell culture inserts based model at 7 dpi, arrow indicates mature sporangium containing zoospores; **(C)** dorsal skin of *X. laevis* infected in a Ussing chamber based model at 5 dpi, showing several post discharge sporangia; arrow indicates sporangium merged into stratum corneum, with at both sides of the arrow moderate hyperkeratosis. Scale bars = 20 µm.

4. Discussion

A number of *in vitro* infection models, intended for the study of early stages of pathogenesis of *B. dendrobatidis*, were elaborated. The initial challenge was to maintain amphibian epidermis *ex vivo/in vitro* for several days, in the knowledge that a complete lifecycle of *B. dendrobatidis*, from zoospore to fertile sporangium, takes approximately 4-5

days at 22°C (assuming that this is the same in culture as it is *in vivo*; Berger *et al.*, 2005). Here, a short overview of the developed models with their advantages, limitations and potential applications, is given.

Cultures of primary keratinocytes are usable for 1-4 days, and therefore could find an application in the field of cytotoxicity testing. However, given the incompatibility of commonly-used culture media and the motility of *B. dendrobatidis* zoospores (data not shown), this method is not suitable for studies on the interaction between *B. dendrobatidis* and amphibian keratinocytes. On the contrary, the infection model which involved the use of SE explants could be adopted for the study of adhesion over a short time-period, and is easy to prepare. Nevertheless, as stripping of epidermis and dermis was only achieved with the skin of *X. laevis* and *S. tropicalis*, and not with the skin of *A. cisternasii*, this method cannot be applied universally. Unlike *X. laevis* and *S. tropicalis*, *A. cisternasii* possesses a pelvic patch, as is the case for the majority of amphibians. This ventral skin of the pelvic region constitutes a prominent site of water uptake, and is significantly thinner than the dorsal skin.

FTE explants are more suitable for long-term studies and the study of different developmental stages of *B. dendrobatidis* in amphibian skin. However, in all the models, except for the cell culture insert model, epidermal hyperplasia and hyperkeratosis occurred. Consequently, no conclusions can be drawn concerning the induction of these phenomena after experimental infection with *B. dendrobatidis*. When performing an infection assay with FTE explants on Matrigel^{TM} provided with cell culture medium, the explanted tissue stayed relatively intact for up to 4 days. Taking into account the immobility of zoospores upon contact with this culture medium, a 3-hour interval was left between the time of infection and the time of addition of the culture medium. After 1 and 4 dpi, adhesion of spores to the stratum corneum of *S. tropicalis*, *X. laevis* and *A. cisternasii* was observed. Zoospores of *B. dendrobatidis* were found not to invade amphibian skin within 4 dpi.

In theory, an application of this model could be the prediction of species susceptibility toward *B. dendrobatidis*. However, prudence is necessary when interpreting qPCR results regarding the differential susceptibility of *X. laevis*, *S. tropicalis* and *A. cisternasii*. Since the animals were washed prior to the isolation of epidermal explants, skin mucus was partially removed. This could lead to a biased view on species susceptibility, since defense against chytridiomycosis appears to result partially from a combined interplay between the antimicrobial peptides, skin bacteria and antifungal metabolites present in this mucus (Rollins-Smith, 2009; Woodhams *et al.*, 2007; Becker *et al.*, 2009).

The high inter-experimental and intra-experimental variability of the infection assays, makes these infection models unsuitable for quantitative statements concerning species susceptibility. The same applies for the infection site preference of *B. dendrobatidis*. The detected mean GE ± SE of *B. dendrobatidis* in *in vitro* infected pelvic skin and dorsal skin, highlights the dorsal skin as the preferred infection site. However, *in vivo*, the ventral abdominal surface, or pelvic region, of anurans is more predisposed to chytrid infection (Berger *et al*., 2000; 2005). Furthermore, in clear qPCR-positive samples, histological slides were often negative for the presence of *B. dendrobatidis*, despite the fact that infected explants were washed thoroughly prior to DNA extraction.

In anticipation of the inhibition of zoospore motility on contact with cell culture medium, infection assays with FTE explants were performed in cell culture inserts. In this way, only the apical side of explant tissue is in contact with the inoculum, and only the basolateral side is in contact with the cell culture medium. Here, adhesion to the stratum corneum was observed in tissue samples from *A. cisternasii* and *X. laevis*. A major drawback of this model is the suboptimal preservation of explanted tissue, as evidenced by the presence of necrosis in all cell layers.

Given the promising initial results, a similar experiment was carried out on a larger scale in Ussing chambers. These chambers confer the advantage that the tissues maintained within them are continuously gassed, which improves the capacity for maintenance of the explanted tissues. At 5 dpi, the exposed tissues demonstrated mild hyperkeratosis, and advanced adhesion of *B. dendrobatidis*. Interestingly, thick-walled zoospores or encysted zoospores adhered to the stratum corneum, and occasionally merged into the stratum corneum, were observed. This is in accordance with the findings of Di Rosa *et al.* (2007), who described the presence of encysted zoospores upon the skin of *Pelophylax lessonae* (formerly *Rana)*. Furthermore, the merit of this model is that, once the time point for invasion is determined for a given species, measurements of transepithelial potential difference and electrolyte transport can be performed by analogy with the experiments of Voyles *et al.* (2009). One drawback, however, is the relatively large pieces of skin required.

The proposed *in vitro* models comply with the Three R's principles, as defined by Russell and Burch (1959). They are good alternatives to *in vivo* experiments and animals do not need to suffer lethal chytridiomycosis. Although these *in vitro* models still require the use of animals to provide skin, several conditions can be tested with the skin from one animal. One adult *X. laevis* can provide six FTE explants of 1.5x2.5mm, and six different conditions can be tested during an *in vitro* experiment in an Ussing chamber. When performing the

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experiment in triplicate, three frogs are needed. A similar experiment *in vivo*, carried out in triplicate, would require at least 18 animals.

5. Conclusion

These *in vitro* models are promising tools for the study of the interactions of *B. dendrobatidis* with amphibian skin. They represent good alternatives to *in vivo* experiments, and reduce the number of experimental animals required. SE explants are most suitable for short-term studies, and adherence can readily be visualized by the fluorescent labeling of zoospores. For the study of later developmental stages of *B. dendrobatidis* in amphibian skin during the infection process, FTE explants in an Ussing chamber are the most appropriate model.

6. Acknowledgements

7. References

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STUDY 2

Germ tube mediated invasion of *Batrachochytrium dendrobatidis* in amphibian skin is host dependent

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Summary

Batrachochytrium dendrobatidis is the causative agent of chytridiomycosis, a fungal skin disease in amphibians and driver of worldwide amphibian declines. We focussed on the early stages of infection by *B. dendrobatidis* in 3 amphibian species with a differential susceptibility to chytridiomycosis. Skin explants of *Alytes muletensis*, *Litoria caerulea* and *Xenopus leavis* were exposed to *B. dendrobatidis* in an Ussing chamber during 3 to 5 days. Early interactions of *B. dendrobatidis* with amphibian skin were observed using light microscopy and transmission electron microscopy. To validate the observations *in vitro*, comparison was made with skin from experimentally infected frogs. Additional *in vitro* experiments were performed to elucidate the process of intracellular colonization in *L. caerulea*.

Early interactions of *B. dendrobatidis* with amphibian skin are: attachment of zoospores to host skin, zoospore germination, germ tube development, penetration into skin cells, invasive growth in the host skin, resulting in the loss of host cell cytoplasm. Inoculation of *A. muletensis* and *L. caerulea* skin was followed within 24h by endobiotic development, with sporangia located intracellularly in the skin. Evidence is provided of how intracellular colonization is established and how colonization by *B. dendrobatidis* proceeds to deeper skin layers. Older thalli develop rhizoid-like structures that spread to deeper skin layers, form a swelling inside the host cell to finally give rise to a new thallus. In *X. laevis*, interaction of *B. dendrobatidis* with skin was limited to an epibiotic state, with sporangia developing upon the skin. Only the superficial epidermis was affected. Epidermal cells seemed to be used as nutrient source without development of intracellular thalli. The *in vitro* data agreed with the results obtained after experimental infection of the studied frog species. These data suggest that the colonization strategy of *B. dendrobatidis* is host dependent, with the extent of colonization most likely determined by inherent characteristics of the host epidermis.

1. Introduction

Chytridiomycosis is a lethal skin disease in amphibians caused by the fungal pathogen *Batrachochytrium dendrobatidis*. Causing widespread amphibian declines, this disease constitutes a major threat to amphibian biodiversity and conservation (Stuart *et al*., 2004; Wake & Vredenburg, 2008; Lötters *et al*., 2009; Crawford *et al*., 2010). *B. dendrobatidis* colonizes the keratinized layers (stratum corneum) of amphibian skin or larval mouthparts (Berger *et al*., 1998; Pessier *et al*., 1999; Marantelli *et al*., 2004). Clinical infection is characterized by epidermal hyperplasia, hyperkeratosis and excessive shedding of the epidermis (Berger *et al.*, 1998; 2005a). Extensive colonization gives rise to a series of physiological effects such as disruption of the osmoregulatory function of the skin, leading to dehydration, electrolyte imbalance and death due to cardiac arrest (Berger *et al*., 1998; Voyles *et al*., 2007; 2009; Carver *et al*. 2010; Marcum *et al*., 2010; Campbell *et al*., 2012).

The lifecycle of *B. dendrobatidis* in culture and the pathology in skin from diseased animals are well documented (Longcore *et al*., 1999; Berger *et al.*, 1998; 2005a). Infection is established by zoospores, the motile flagellated stage of *B. dendrobatidis* (Longcore *et al*., 1999; Berger *et al.*, 2005a). Zoospores display chemotactic responses in search of a suitable host to infect (Moss *et al*., 2008). Upon colonization of the host epidermis, the zoospores encyst (Berger *et al*., 2005a). The flagellum is absorbed and a cell wall is formed (Berger *et al.*, 2005a). Based on observations in infected *Litoria gracilenta,* an intracellular development of *B. dendrobatidis* was described by Berger *et al.* (2005a). As such, the fungus proliferates within the epidermal cells and has its cycle tuned to the maturation of the epidermal cells. Immature fungal bodies, termed thalli or sporangia are carried to the skin surface by differentiating epidermal cells. Mature sporangia containing zoospores finally occur in the sloughing stratum corneum.

Early stages of infection have hitherto been poorly studied (Voyles *et al.*, 2011). As such it is still not clear how host cell entry is achieved. In analogy with other pathogenic fungi e.g. *Candida albicans* (Naglik *et al*., 2003) and dermatophytes (Vermout *et al*., 2008), most probably a range of digestive enzymes capable of degrading skin components enable penetration of *B. dendrobatidis* into the host cells (Rosenblum *et al*., 2008; Moss *et al*., 2010; Voyles *et al*., 2011).

The main objective of this study was to find out how *B. dendrobatidis* infection is established. The early interaction between *B. dendrobatidis* and anuran skin was characterized using an *in vitro* infection model. Amphibian skin explants were inoculated with *B.*

dendrobatidis and incubated in an Ussing chamber. To determine how *B. dendrobatidis* infection is established and to what extent infection strategies of *B. dendrobatidis* are host dependent, host-pathogen interactions were evaluated in 3 species with a differential susceptibility to *B. dendrobatidis*: the African clawed frog (*Xenopus laevis*), the Mallorcan midwife toad (*Alytes muletensis*) and the green tree frog (*Litoria caerulea*). *X. laevis* generally does not show clinical signs associated with chytridiomycosis, nor have population declines due to chytridiomycosis been reported (Weldon *et al.*, 2004). *A. muletensis* is a vulnerable European species restricted to Mallorca (Balearic Islands, Spain) (IUCN, 2011). Since *B. dendrobatidis* has been detected in reintroduced captive-bred populations this species is currently threatened by decline (Walker SF *et al.*, 2008; IUCN, 2011). *L. caerulea* is a common Australasian species (IUCN, 2011), but has proven to be highly susceptible to chytridiomycosis in the wild (IUCN, 2011; Berger *et al.*, 2005b) as well as under laboratory conditions (Woodhams *et al.*, 2007; Berger *et al.*, 2009).

In a first experiment adhesion, invasion and the development of *B. dendrobatidis* in skin of *A. muletensis*, *L. caerulea* and *X. laevis* were studied during 3 to 5 consecutive days of *in vitro* infection, using light microscopy (LM) and transmission electron microscopy (TEM). In parallel, *A. muletensis*, *L. caerulea* and *X. laevis* frogs were experimentally infected to assess the validity of the observations

In a second experiment, skin of *L. caerulea* was exposed to *B. dendrobatidis* for 1, 2, 4, 8, 16 and 24 hours to further characterize the process of intracellular colonization. The morphology of the infecting fungal elements during invasion of the skin was followed by LM and TEM. The time-points of exposure found most critical for intracellular colonization were repeated in triplicate.

2. Materials and methods

2.1. Experimental animals

Postmetamorphic wild type *X. laevis* were purchased from the European *Xenopus* Resources Centre (Portsmouth, UK) and adult outbred *X. laevis* from *Xenopus* Express (Le Bourg, France). Subadult *A. mulentesis* and *L. caerulea* were captive bred. Upon arrival and before the start-up of all experiments skin swabs from all animals were examined for the presence of *B. dendrobatidis* by the quantitative PCR (qPCR) of Boyle *et al.* (2004).

2.2. **B. dendrobatidis** *strains and culture conditions*

Inoculations were carried out with the virulent *B. dendrobatidis* strain IA042, a representative of the *B. dendrobatidis* global panzootic lineage (Farrer *et al*., 2011), isolated from a dead *Alytes obstetricans* (Fisher *et al*., 2009). Cultures were maintained on tryptone/gelatine hydrolysate/lactose (TGhL) broth in 25 cm² cell culture flasks at 20°C for 5 days. Two ml of a 5 days old broth culture were inoculated on TGhL agar and incubated for 5 to 7 days at 20°C. Zoospores were harvested by flooding the agar plates with 2 ml of distilled water and were immediately counted in lugol with a haemocytometer.

2.3. *Isolation* **in vitro** *culture and infection of anuran skin*

For a detailed study of the early interaction between zoospores and host epidermis, full-thickness epidermal (FTE) explants of *A. mulentensis*, *L. caerulea* and *X. laevis* were experimentally infected in an Ussing chamber based model. Isolation and treatment of FTE explants, *in vitro* culture and infection procedures have been described in detail by Van Rooij *et al.* (2010).

Early interactions of *B. dendrobatidis* zoospores with amphibian epidermis and the *in situ* development of zoospores to sporangium were observed during 5 consecutive days in *X. laevis* and 3 days in *A. muletensis* and *L. caerulea*. Immediately after euthanasia with intracoelomically-injected T 61® (Intervet, Mechelen, Belgium) frogs are washed according to the protocol of Nishikawa *et al.* (1990) to facilitate the handling of the tissue and to reduce the risk of contamination. Briefly, frogs were washed in plastic containers containing respectively 70% ethanol, Leibovitz L-15 medium 70% (3 times; Gibco, Life technologies Europe, Gent, Belgium), Ca^{2+}/Mg^{2+}-free Barth's solution (CMFB), 1.25 mM ethylenediaminetetraacetic acid (EDTA; Sigma, St. Louis, MO, USA) in CMFB for 5 min and 70% L15 medium (twice) at 4°C. FTE explants (10 x 25 mm for *A. muletensis* and 20 x 25 mm for *L. caerulea* and *X. laevis*) were excised and were mounted in an Ussing chamber (exposed surface area of 0.28 cm^2 for *A. muletensis* and 1.07 cm^2 for *L. caerulea* and *X. laevis*). From each donor animal a skin sample was tested for the presence of *B. dendrobatidis* by qPCR (Boyle *et al*., 2004). Explants were apically exposed to 7 ml inoculum (2.8 x 10^7 zoospores/ml distilled water). In the course of the subsequent 3 to 5-days incubation period at 20°C, skin samples were removed at 1 to 3-5 days post infection (dpi) and the exposed skin surface area was excised and processed for histology and transmission electron microscopy (TEM).

Additional experiments in skin of *L. caerulea* were performed under the same conditions as described above. In short, FTE skin explants were mounted in an Ussing chamber with an exposed surface area of 1.07 cm^2. Explants were exposed to an inoculum of 2.8 x 10^7 zoospores/ml distilled water. In the course of a 24 hours incubation period at 20°C, skin samples were removed after 1, 2, 4, 8, 16 and 24 hours of exposure. The experiment was then repeated in triplicate, with sampling after 8, 16 and 24 hours of exposure.

For light microscopic (LM) studies, skin samples were fixed in 10% neutral buffered formaldehyde for at least 72 hours prior to embedding in paraffin. Five µm sections were stained with haematoxylin and eosin (HE) and Gomori methenamine silver (GMS). For TEM, skin samples were fixed in 4% formaldehyde containing 1% CaCl$_2$ (w/v) in 0.121 M Na-cacodylate adjusted to pH 7, for 24 hours. The samples were washed and postfixed in 1% OsO$_4$ (w/v). Subsequently the skin samples were dehydrated through a graded series of alcohol and embedded in LX-112 resin (Ladd Research Industries, Burlington, Vermont, USA). Semi-thin sections (2 µm) were cut and stained with toluidin blue to select regions for ultrathin sectioning (90 nm) with an ultratome (Ultracut E; Reichert-Jung, Nussloch, Germany). The ultrathin sections (90 nm) were stained with uranyl acetate and lead citrate solutions and examined under a JEOL EX II transmission electron microscope (JEOL Ltd, Zaventem, Belgium) at 80 kV. Measurements of all structures are given as (Min.)-Av.-(Max.), with Min.= minimum value for the measured collection of structures (n), Av.= average value and Max.= maximum value.

2.4. In vivo *infection of* A. muletensis*,* L. caerulea *and* X. laevis

To assess the validity of the *in vitro* results, *A. mulentensis*, *L. caerulea* and *X. laevis* frogs were experimentally infected. All animal experiments were approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (EC2008/120, EC2010/98). Experiments were performed following all necessary ethical and biosecurity standards.

For inoculation, respectively three subadult *A. mulentensis*, *L. caerulea* and *X. laevis* frogs were individually housed for 24 hours in plastic containers (18 x 13 x 4 cm) containing moistened paper tissue, terracotta flower-pots as shelter and a petri-dish filled with dechlorinated tap water for bathing. Frogs were inoculated by topical application of 2 x 10^6 zoospores/100 µl distilled water (*B. dendrobatidis* strain IA042). Twenty-four hours after exposure all frogs were transferred to fresh containers. *X. laevis* frogs were housed in plastic containers (32 x 17 x 21 cm), containing dechlorinated tap water (water depth 10 cm) at 20°C.

Terracotta flower-pots were provided as shelter. Frogs were fed twice weekly with trout pellets (Skretting, Cheshire, UK). Water was changed three times weekly. *A. muletensis* and *L. caerulea* frogs were housed in plastic containers (18 x 13 x 4 cm), with moistened paper tissue, terracotta flower-pots as shelter and a petri-dish filled with dechlorinated tap water for bathing. Containers were regularly sprayed with dechlorinated tap water to maintain humidity. Tissue and bathing water was changed three times weekly. Ambient temperature varied between 19 and 20 °C. *A. muletensis* and *L. cearulea* frogs were fed calcium powdered crickets, fruit flies (*Drosophila melanogaster*) or buffalo worms (*Alphitobius laevigatus*) *ad libitum*. The photoperiod of the experimental animal facilities followed natural ambient conditions (12-15 h light). At day 12 post inoculation all animals were sampled by passing a sterile synthetic swab (160 C, Copan Italia S.p.A., Brescia, Italy) along the pelvic region 10 times, fore- and hind limbs 5 times. Gloves were changed between handling of each animal. Swabs were examined for the presence of *B. dendrobatidis* by qPCR (Boyle *et al*., 2004). At day 14 post inoculation all animals were sacrificed by intracoelomically-injected T61® (Intervet, Mechelen, Belgium). Skin samples were collected from the pelvic region, fixed in 10% neutral buffered formalin, processed for histology and stained with HE and GMS.

3. Results

An overview of the early pathogenesis in *X. laevis* skin as observed by light microscopy is given in **Figure 1**. At 1 day post infection (dpi) numerous encysted zoospores had settled in clusters upon the epidermis or were situated in glandular pores (**Fig. 1A**). Zoospore cysts were spherical and had doubled in size (n=30, (5)-6.1-(7.5) µm diameter) when compared to zoospores (n=10, (2.0)-2.35-(3.5) µm diameter). From 1 dpi on, zoospore cysts germinated (termed germlings) and developed a short tubular structure of (0.5)-0.58-(0.86) µm diameter, further called germ tube (**Fig. 1B**). Germ tubes had elongated over the epidermal surface or had protruded into the cells of the stratum corneum. In heavily colonized cells the germ tubes growed into a profusely branched, fuzzy mesh work of rhizoids that spread out in the entire cell and was most clearly demonstrated by GMS stain (**Fig. 1B**). At 2 dpi germlings had increased in size (n=30, (5)-8.8-(13.2) µm diam.) and were developing into maturing zoosporangia. From 2 dpi on, invasion of the host cells of the stratum corneum resulted in loss of their cytoplasm and only their cell membrane persisted (**Fig. 1C**). Both at 3 and 4 dpi, the germlings upon the epidermal surface had matured into zoosporangia, containing zoospores. Several post-discharge zoosporangia were observed upon the epidermal
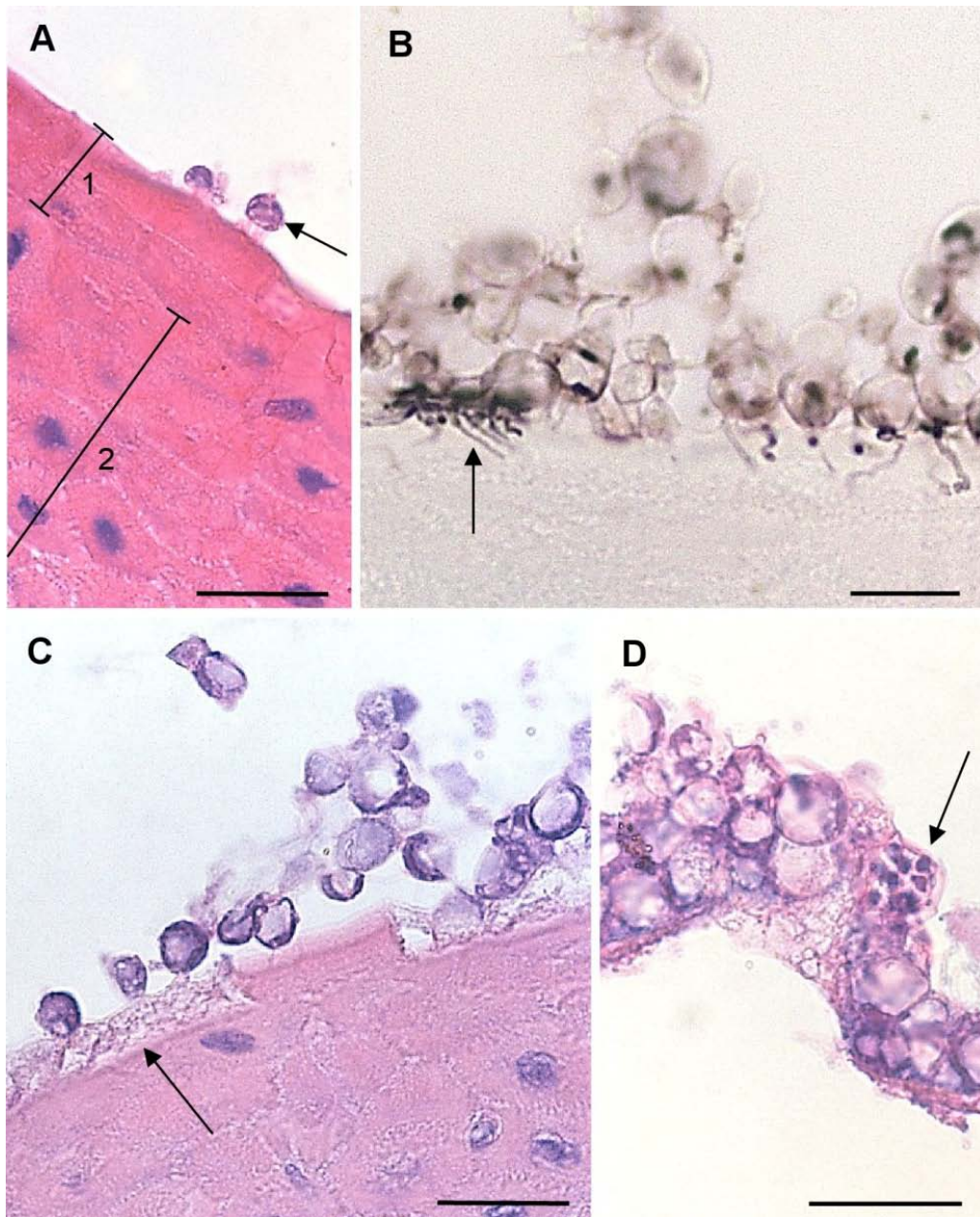
Figure 1. Light microscopical overview of the development of *B. dendrobatidis* in skin explants of *Xenopus laevis*. (A) adhesion of encysted zoospores (arrow) to the host epidermis at 1 dpi; (1) stratum corneum, (2) stratum spinosum; haematoxylin and eosin (HE) stain; scale bar = 20 µm; **(B)** at 1 dpi *B. dendrobatidis* germlings have developed germ tubes, that penetrate the stratum corneum and develop into a branched mesh work of rhizoids (arrow) in heavily infected epidermis; Gomori methenamine silver stain; scale bar = 10 µm; **(C)** at 2 dpi the infected host cells have lost their cytoplasm (arrow) subsequent to invasion by *B. dendrobatidis*, only the cell membrane remains; HE stain; scale bar = 20 µm; **(D)** at 4 dpi germlings have developed into mature zoosporangia (arrow), the upper layer of the stratum corneum is shed; HE stain; scale bar = 20 µm.
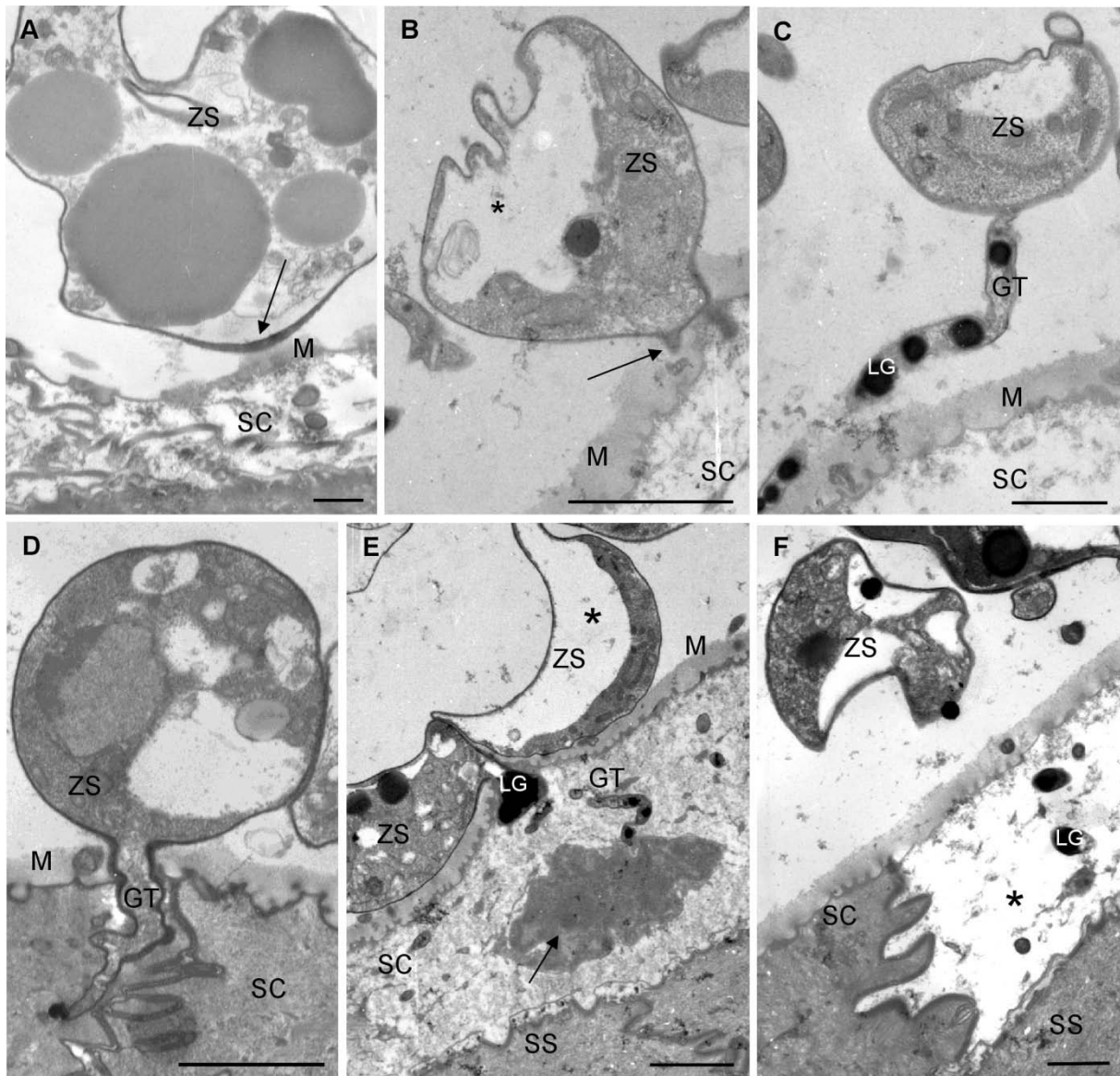
Figure 2. TEM overview of the development of *B. dendrobatidis* in skin explants of *Xenopus laevis*. (A) adhesion of an encysted zoospore (ZS) to the superficial mucus layer (M) on top of the stratum corneum (SC); at the site where adhesion occurs the cell wall of the encysted zoospore is remarkably thickened (arrow); scale bar = 500 nm; **(B)** initiation of germ tube development (arrow); note the polarisation of the cell cytoplasm (*); scale bar = 2 µm; **(C)** germ tube (GT) elongating upon the epidermis of *X. laevis*, with the presence of numerous lipid globules (LG) in the germ tube; scale bar = 1µm; **(D)** a growing germ tube protruding the stratum corneum; scale bar = 2 µm; **(E)** invasion of a host cell resulting in the loss of cell cytoplasm; remnants of the host cell cytoplasm (arrow) are seen at the tip of a protruded germ tube; note the presence of a collapsed sporangium (ZS) due to cell polarisation (*); (SS): stratum spinosum; scale bar = 2 µm; **(F)** infected epidermal cell with digested cell content (*) alternated by an uninfected normal epidermal cell; note the presence of lipid globules in the infected host cell; scale bar = 1µm.

skin surface. Sporangia were shed together with the affected upper layer of the stratum corneum (**Fig. 1D**).

TEM provided more detailed information on ultrastructural changes (**Fig. 2**). At 1 dpi, encysted zoospores were attached to a thin residual superficial mucus layer on top of the stratum corneum and adhesion to this layer was characterized by a conspicuous thickening of the fungal cell wall (a 3 to 6 fold increase, from 0.05-0.1 µm to 0.2-0.3 µm) (**Fig. 2A**). The initiation of a germ tube, as shown in **Figure 2B,** started as a pointed outgrowth of the thickened cell wall**.** Cross-sections of a germ tube by TEM show prominent osmiophilic rounded structures inside the germ tube (**Fig. 2C**). These structures without a membrane and of variable size resembled the lipid globules in the zoospores of *B. dendrobatidis*. Analogous structures were seen in the cytoplasm of the affected keratinocytes (**Figs. 2E,F**). No mitochondria or nuclei could be discerned in these germ tubes. **Figure 2D** shows a growing germ tube that had protruded in the stratum corneum. The affected epidermal cells seemed to have partially or completely lost their cytoplasm and only their cell membrane persisted (**Figs. 2E,F**). Remnants of the host cell cytoplasm were observed at the tip of an invading germ tube (**Fig. 2E**). In un-inoculated skin samples, incubated during 5 days under the same conditions as described above, the stratum corneum was still intact and no altered keratinocytes were observed.

Another striking feature seen from 1 dpi onwards was the presence of collapsed sporangia. This was observed in LM and TEM preparations (**Fig. 2E**). A polarisation of the sporangial cytoplasm was observed. The cytoplasm was concentrated at one side of the sporangium, lined by an empty space most probably to be considered as a vacuole.

Figure 3 illustrates the development of *B. dendrobatidis* in skin explants of *A. muletensis* and *L. caerulea*. Compared to *X. laevis*, a similar initial infection process was seen in *A. muletensis* and *L caerulea*. Likewise, zoospore cysts adhered to the stratum corneum, host cells were invaded by germ tubes that developed into rhizoidal axes spreading out in the entire cell (**Figs. 3A,B**). Invasion of the keratinocytes by germ tubes and loss of the cellular cytoplasm was most obvious by TEM (**Figs. 4A,B**). From 2 dpi on, maturing sporangia were observed upon the infected skin surface. However, the development of *B. dendrobatidis* in *A. muletensis* and *L. caerulea* was clearly distinct in the respect that besides superficial colonization, intracellular chytrid thalli were observed in superficial and deeper layers of the epidermis. Within 24 hours after inoculation, marked intracellular colonization was seen in *L. caerulea* by LM (**Fig. 3B)** and TEM (**Fig. 4B**) and occasional intracellular colonization in *A. muletensis***.**
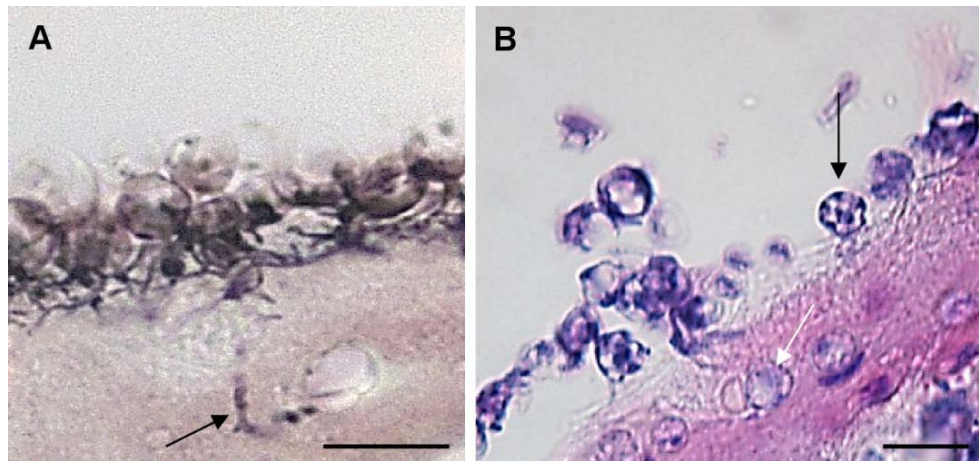
Figure 3. Light microscopical overview of the development of *B. dendrobatidis* in skin explants of *Alytes muletensis* and *Litoria caerulea*. (A) at 1 day post infection (dpi) germlings have developed germ tubes (arrow) that invade the epidermis of *A. muletensis*; Gomori methenamine silver (GMS) stain; scale bar = 10 μm; **(B)** at 1 dpi both *B. dendrobatidis* germlings (black arrow) attached upon the epidermal surface as intracellular chytrid thalli (white arrow) in the stratum corneum of *L. caerulea* are observed; haematoxylin and eosin stain; scale bar = 10 μm.
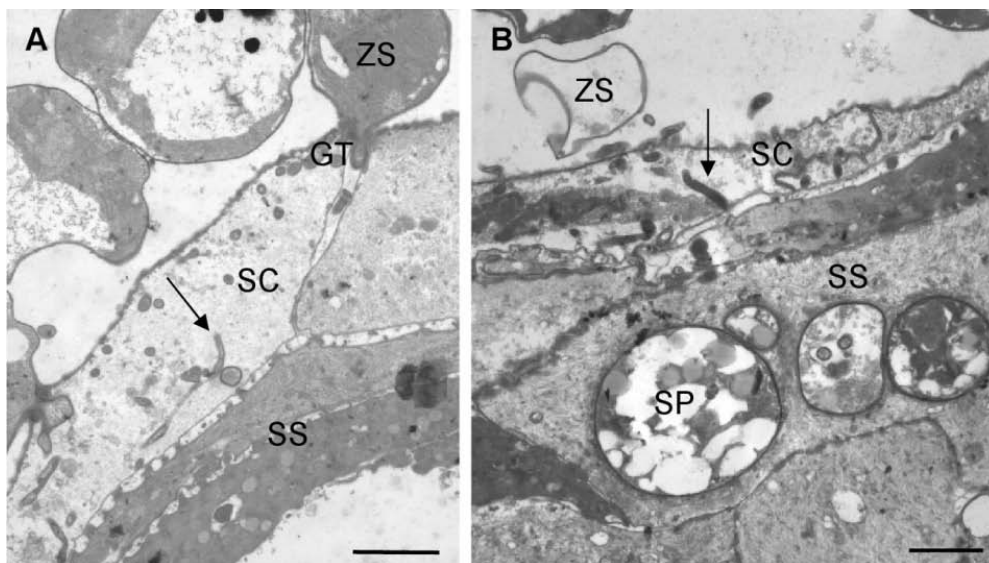
Figure 4. TEM overview of the development of *B. dendrobatidis* in skin explants of *Alytes muletesis and Litoria caerulea*. (A) infected epidermis of *A. mulentensis* at 1 dpi, with loss of the host cell cytoplasma and the presence of germ tube fragments inside the infected cell in cross and longitudinal section (arrow); scale bar = 2 μm; **(B)** infected epidermis of *L. caerulea* at 2 dpi showing colonization of the stratum corneum, loss of the host cell cytoplasm and the presence of germ tube fragments (arrow); intracellular chytrid sporangia are observed in the stratum spinosum; scale bar = 2 μm; GT; germ tube, SC: stratum corneum, SP: sporangium, SS: stratum spinosum, ZS: encysted zoospore.

To support the validity of the observations made *in vitro*, *A. muletensis, L. caerulea* and *X. laevis* frogs were infected *in vivo*. During the course of the infection trial no clinical signs were observed in *A. muletensis* (n=3). In 1 out of 3 *L. caerulea* excessive shedding of skin and erythema of the hind limbs occurred. In all *X. laevis* frogs (n=3) only excessive shedding of skin was observed. At 12 days post infection, all *A. muletensis* and *L. caerulea* frogs were infected, with mean genomic equivalents of *B. dendrobatidis* ± standard error detected by qPCR of 517 ± 636 for *A. muletensis* (n=3) and 350 ± 589 for *L. caerulea* (n=3). All *X. laevis* frogs tested negative (n=3).

In skin samples taken at 14 days after exposure, the epidermis of experimentally infected *X. laevis* frogs was still intact. No adhering zoospores nor sporangia could be observed. In contrast, in all infected *A. muletensis* (n=3) (**Fig. 5A**) and *L. caerulea* frogs (n=3) the stratum corneum was colonized with intracellular sporangia. Germlings or developing sporangia adhering to the epidermis were not observed. Colonization was more abundant in *L. caerulea*. One out of 3 infected *L. caerulea* frogs carried a high infection load (1020 GE), was colonized to broad extent (**Fig. 5B**) but did not show any clinical signs. The other *L. caerulea* individuals were infected to the same and lesser extent (9 and 11 GE), with only one individual presenting clinical signs.
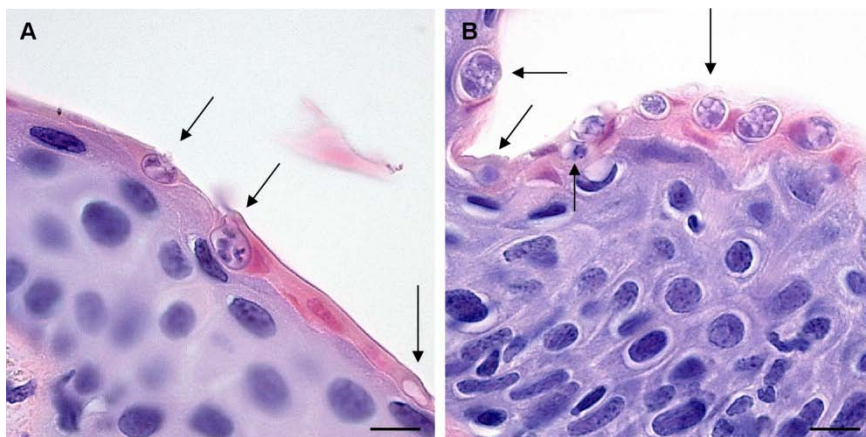
Figure 5. Skin sections of *Alytes muletensis* and *Litoria caerulea* experimentally infected with *B. dendrobatidis* at 14 days post infection. Exclusively intracellular chytrid thalli (arrows) are observed by light microscopy in the stratum corneum of *A. muletensis* **(A)** and *L. caerulea* **(B)**; haematoxylin and eosin stain; scale bar = 10 µm.

A more detailed study of the invasion process in *L. caerulea* showed that at earliest, frog skin was invaded by germ tubes 2 hours after exposure to *B. dendrobatidis* (**Fig. 6A,B**). Eight, 16 and 24 hours of exposure to *B. dendrobatidis* were defined as most critical time-
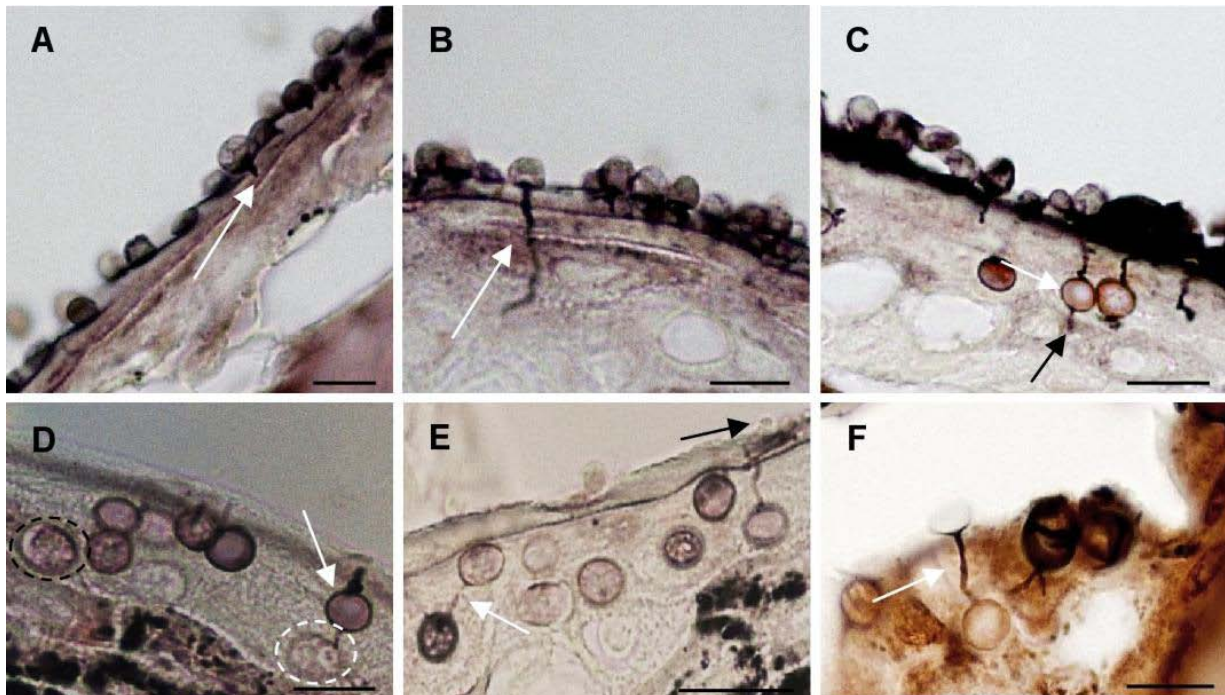
Figure 6. Intracellular colonization of *Litoria caerulea* skin by *B. dendrobatidis*. (A-E): *in vitro,* **(F)**: *in vivo.* **(A)** invasion of the stratum corneum by a germ tube (white arrow) at 2 hour post infection (hpi); **(B)** strong elongation of the germ tube (white arrow) into the stratum spinosum at 8 hpi; **(C)** development of intracellular chytrid thalli (white arrow) at the end of a germ tube at 24 hpi; rhizoid-like structures (black arrow) arise from newly developed chytrid thalli; **(D)** development of a new chytrid thallus at 24 hpi; a swelling is formed at the end of a rhizoid-like structure, a thin cell wall is formed and the cell content of the mother thallus (white arrow) is transferred into the new daughter thallus (white circle); a new thallus in a later developmental stage (black circle); **(E)** thalli connected by a rhizoid-like structure (white arrow); remnants of a germling, after having injected its cell content into a new intracellular thallus (black arrow); **(F)** mother thallus connected to a newly formed daughter thallus by a rhizoid-like structure (white arrow) at 14 days post infection. Gomori methenamine silver stain, scale bar= 10 μm.

points to study intracellular colonization and were repeated in triplicate during additional *in vitro* assays. Chytrid thalli developing intracellularly were observed at 16 to 24 hours after exposure. In one out of the 3 repeats, intracellular colonization occurred 8 hours after exposure. In this experiment the stratum corneum had already detached from the stratum spinosum, probably rendering the epidermis more accessible.

GMS staining showed that both superficially and deeper localized intracellular chytrid thalli were often connected to a tubular rhizoid-like structure, stretching out to the deeper layers of the epidermis or either to the epidermal surface (**Figs. 6C,D**). In rare cases, remnants of empty zoospore cysts were found at the skin surface (**Fig. 6E**). On several occasions, both *in vitro* (**Fig. 6E**) and *in vivo* (**Fig. 6F**) intracellular chytrid thalli apparently connected by a rhizoid-like structure were noticed. As such, older thalli were connected to newly formed thalli. **Figure 6D** shows a clearly stained older thallus giving rise to a new thallus, outlined by a faintly stained thin cell wall.

4. Discussion

The present results provide a missing link in the infection process of *B. dendrobatidis*. Until now established *B. dendrobatidis* infections had been described with zoospore development occurring in a zoosporangium inside the host cell and the intracellular zoosporangium forming discharge papillae through which zoospores exit (Berger *et al*., 2005a). Our results provide a novel insight into the early interaction of *B. dendrobatidis* zoospores with amphibian skin.

The early pathogenesis consists in the first place of an epibiotic development, upon the host skin and was observed in the 3 species studied. Zoospores matured into thick-walled cysts on the host epidermis and were clustered in foci of infection. Subsequently, invasion of amphibian skin was established by germ tube development. A tubular extension or germ tube arised from the zoospore cysts and penetrated into the epidermal host cells. GMS stained sections showed most clearly that in heavily infected cells germ tubes grew into an irregularly branched mesh work of rhizoids. Histological sections and TEM images strongly suggest an extracellular digestion of the host cytoplasm, followed by an uptake by the germ tubes. A similar effect has been described by Berger *et al.* (2005a), who observed dissolution of cellular cytoplasm in infected epidermis of *L. gracilenta*. However, this was not associated with the presence of germ tubes.

In vitro, *X. laevis* skin does become infected but the development of *B. dendrobatidis* in *X. laevis* skin is apparently limited to an 'epibiotic' stage, with epidermal cells solely being used as nutrient source for the growing sporangium upon the epidermis. The typical histological picture of chytridiomycosis with chytrid sporangia developing intracellularly was not observed. Upon examination of stained skin sections from *in vivo* infected *X. laevis* frogs, 14 days after exposure to *B. dendrobatidis,* the skin was still intact and no colonization was observed.

X. laevis is considered tolerant to clinical chytridiomycosis as defined by Schneider & Ayers (2008), i.e. this species can be colonized by *B. dendrobatidis* but is able to limit the impact of *B. dendrobatidis* on its health and to maintain a low-level infection (Weldon *et al.*, 2004; Fisher (2009). Unfortunately, reports of chytrid infections in *X. laevis* rely merely on PCR-detection (Weldon *et al.*, 2004; Hill *et al.*, 2010; Solis *et al.*, 2010) and there is no conclusive histological evidence of how chytrid infections manifest in this species under natural conditions.

Recently, Ramsey *et al.* (2010) found that the level of infection in *X. laevis* is likely to be determined by both innate and adaptive components of the immune system. As such antimicrobial peptides (Rollins-Smith & Conlon, 2005; Rollins-Smith, 2009) and antifungal metabolites (Becker *et al.*, 2009) provide a non-specific protection to potential pathogens, while antibodies in skin secretions of previously exposed frogs provide specific anti-*B. dendrobatidis* protection. A combined action of these defenses is likely to limit colonization of *X. laevis* by *B. dendrobatidis* to mild and non-lethal infections. To which extent epibiosis occurs in other chytrid tolerant species remains to be determined. For example, though the American Bullfrog (*Lithobathes catesbeianus*) is considered a notorious carrier of *B. dendrobatidis*, there is solid evidence of *B. dendrobatidis* developing intracellularly in the skin of this species (Garner *et al.*, 2006; Green *et al.*, 2007).

In *A. muletensis* and *L. caerulea* epibiotic development was followed by extensive intracellular colonization of the stratum corneum. Intracellular growth of chytrid thalli was established within 24 hours in *L. caerulea* and in the later stages of infection in *A. muletensis*. Additional infection assays in *L. caerulea* confirm that colonization propagates to the deeper skin layers within 24 hours, at earliest at 16 hours after exposure to *B. dendrobatidis*. Both in the wild (IUCN, 2011; Walker *et al.*, 2008; Berger *et al.*, 2005b) as in experimental infection trials (Woodhams *et al.*, 2007; Berger *et al.*, 2009; Martel *et al.*, 2011) *A. muletensis* and *L. caerulea* can be severely colonized by *B. dendrobatidis*.

Especially GMS staining proved its usefulness in visualising fungal cell walls and revealing structures that were overlooked using HE staining. In *A. muletensis* and *L. caerulea* intracellular chytrid thalli with rhizoid-like structures stretching out either to the epidermal surface or to the deeper layers of the epidermis were observed. On several occasions, both *in vitro* and *in vivo*, intracellular chytrid thalli apparently connected by a rhizoid-like structure were noticed. These observations provide consistent evidence of how intracellular colonization is established, as summarized in **Figure 7**.
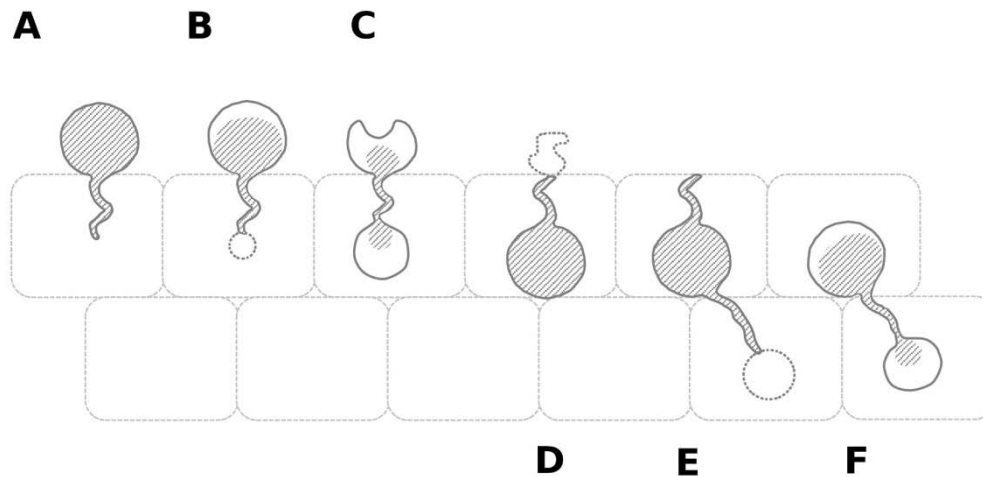

Figure 7. Schematic summary of the intracellular colonization process by *B. dendrobatidis* in amphibian skin. (A) Germination of a zoospore cyst or germling is followed by the development of a germ tube that invades an epidermal cell; **(B)** at the end of the germ tube a swelling is formed, that gives rise to a new thallus; **(C)** cell contents of the germling migrate into the newly formed thallus; **(D)** the emptied germling evanesces; **(E)** the new intracellular thallus forms a rhizoid-like structure that extends to a deeper epidermal layer and develops a swelling at its end; **(F)** a new intracellular thallus is formed.

Together with the presence of germ tubes these data confirm the hypothesis of an endobiotic development of *B. dendrobatidis* as formulated by Longcore (1999). During endobiotic development zoospores encyst upon the host cell and inject their nucleus and cytoplasm into the host cell via a germ tube. The germ tube forms a swelling inside the cell and enlarges. Finally, the contents undergo mitosis, zoospores are formed and are released into the environment through discharge papillae (Longcore *et al*., 1999). Analogous invasion mechanisms are seen in chytrids parasitizing plants and algae, e.g. *Entophlyctis* spp. (Longcore *et al.*, 1995; Shin *et al.*, 2001). In addition, our observations indicate how colonization by *B. dendrobatidis* proceeds to deeper skin layers. It seems that older mother

thalli develop rhizoid-like structures that spread to the deeper skin layers and form a swelling inside the cell. This swelling enlarges and gives rise to a new daughter thallus.

Genetic material of *B. dendrobatidis* was probably injected into epidermal cells in order to establish intracellular sporangia. As we did not observe any mitochondria or nuclei in the germ tubes, additional observations are desirable. However, migration of lipid globules through these germ tubes into the host cell was seen on several occasions. Most probably the lipids function as a source of concentrated energy for the zoospores and as an energy source for the young thallus while it grows into an epidermal cell (JE. Longcore, *pers. comm.*).

Host induced morphological variation is peculiar in many chytrids (Barr, 2001), i.e. they exhibit morphological differences between their parasitic and saprophytic state and are able to change from an endobiotic growth to an epibiotic growth depending on nutrients and the substrate (Longcore *et al.*, 1995; Barr, 2011). In this perspective, our data suggest that the colonization strategy of *B. dendrobatidis* is host dependent. The extent of *B. dendrobatidis* invasion clearly differed between the 3 host species used: from near absence in *X. laevis*, to moderate in *A. muletensis* and high in *L. caerulea*. Moreover, the ability of *B. dendrobatidis* to enter amphibian skin and to spread in the skin, or its invasiveness, appears to coincide with the susceptibility of the studied species to chytridiomycosis, i.e. low in *X. laevis* (Weldon *et al.*, 2004; Fisher, 2009; Kielgast *et al.*, 2010), moderate in *A. muletensis* (Walker *et al.*, 2008; Martel *et al.*, 2011) and high in *L. caerulea* (Berger *et al.*, 2005b; 2009; Woodhams *et al.*, 2007).

Why colonization is limited to epibiosis in *X. laevis* and what makes *A. muletensis* and *L. caerulea* more 'receptive' to *B. dendrobatidis* infection remains speculative. However, the influence of certain factors on the outcome of the experiments was minimized. *In vitro* colonization experiments were carried out under the same conditions. Prior to the isolation of skin explants *A. mulentensis*, *L. caerulea* and *X. laevis* frogs were washed to facilitate the handling of skin tissue and to reduce the risk of bacterial contamination. By washing, skin mucus and skin secretions were also partially removed. Especially *X. laevis* skin is covered with a prominent mucus layer and skin mucus in itself can be considered as a mechanical barrier and an obstacle for colonization. In addition, skin secretions containing e.g. antimicrobial peptides, antifungal metabolites, are thought to provide protection against chytridiomycosis. However, since the activity of residual skin secretions in skin explants is not yet studied, one must be cautious in assuming a reduced defensive action.

Consequently, we hypothesize that the degree of invasiveness of *B.dendrobatidis* and amphibian susceptibility to chytridiomycosis is determined by inherent characteristics of the

host skin. However, more observations are required to draw definite conclusions about species susceptibility and pathogenesis patterns. The challenge ahead will be to identify which factors mediate these variations in the pathogenesis of *B. dendrobatidis* infections.

5. Acknowledgements

6. References

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STUDY 3

Detection of *Batrachochytrium dendrobatidis* in Mexican bolitoglossine salamanders using an optimal swabbing protocol

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Summary

The role of the chytrid fungus *Batrachochytrium dendrobatidis*, which is the causal agent of chytridiomycosis, in the declines of Central American bolitoglossine salamanders is unknown. Here we establish a swabbing protocol to maximize the detection probability of *B. dendrobatidis* in salamanders. We then used this protocol to examine captive and wild Mexican bolitoglossine salamanders of 14 different species for the presence of *B. dendrobatidis.* Of the seven body parts sampled, the pelvic region, hindlimbs, forelimbs and the ventral side of the tail had the most *B. dendrobatidis* per surface area and thus might provide the best sampling regions of salamanders to detect *B. dendrobatidis* infections. Sixteen out of 33 (48%) of the dead captive salamanders had *B. dendrobatidis* infections and epidermal hyperkeratosis, whereas none of the 28 clinically healthy captive animals were infected. Nine out of 17 (53%) of the wild salamanders carried low zoospore loads of *B. dendrobatidis* but had no clinical signs of disease. The high prevalence of *B. dendrobatidis* in dead captive salamanders, its absence in clinically healthy living ones and its presence in wild salamanders is consistent with *B. dendrobatidis* being involved in recent bolitoglossine population declines, but further studies would be required to draw a causal link.

1. Introduction

Since the 1960's, significant declines and extinctions of amphibians have been observed worldwide (Stuart *et al*., 2004) and potential causes include overexploitation, habitat loss, climate change, and infectious diseases (Stuart *et al*., 2004; Rohr *et al*., 2008; Lötters *et al.*, 2009; Rohr & Raffel, 2010). Several population declines have been linked to the presence of a pathogenic chytrid fungus, *Batrachochytrium dendrobatidis* (La Marca *et al*., 2005; Lips *et al*., 2006), which invades keratinized epithelial cells and can cause increased tissue growth (hyperplasia) and thickening of the cornified skin layers (hyperkeratosis) (Berger *et al*., 1998).

Salamanders of the family Plethodontidae, commonly called "lungless salamanders", contribute significantly to the biological diversity of Central America (Larson *et al.*, 2006; Wake and Vredenburg, 2008), but are experiencing significant declines that might be caused by chytridiomycosis (Parra-Olea *et al*., 1997; Lips *et al*., 2006; Rovito *et al*., 2009). For instance, *B. dendrobatidis* was recently detected in plethodontid salamander populations that are declining in Mexico and Guatemala (Cheng *et al*., 2011). Here we establish a swabbing protocol to maximize the detection probability of *B. denderobatidis* in salamanders and then used this protocol to examine captive and wild Mexican bolitoglossine salamanders of 14 different species for the presence of *B. dendrobatidis*.

2. Materials and methods

Thirty-three dead captive adult bolitoglossine salamanders (Bolitoglossinae, Plethodontidae, Caudata), each preserved in 70% ethanol in a separate container, were used to establish an efficient swabbing protocol for *B.dendrobatidis*. The specimens had not been fixed in formalin prior to storage in ethanol. To determine whether these animals were infected with *B. dendrobatidis*, a pelvic tissue sample was taken from each specimen. To prevent possible cross-contamination, disposable gloves and dissection material were changed between containers. For each sample, DNA was extracted from the tissue using proteinase K digestion, following the protocol of Bandi *et al.* (1994), with 1:10 dilutions stored at -20°C. Quantitative PCR (qPCR) assays were performed on a CFX96 Real Time System (BioRad Laboratories, Hercules, CA, USA), with amplification conditions and primer and probe concentrations according to Boyle *et al.* (2004). For each sample, qPCR assays were performed in duplicate. Amplification standards of 1000, 100, 10, 1 and 0.1 zoospore genomic equivalents (GE) were included within each assay, as well as 3 negative and one

positive control sample. A result was considered positive when values higher than 0.1 GE's were obtained twice.

Animals with high *B. dendrobatidis* loads were selected for the sampling of 7 selected body sites: chin, plantar side of forelimb and hindlimb, dorsum, dorsal and ventral sides of the tail, and the abdomen (**Fig. 1**, **Table 1**). Each of these sites was rubbed 5 times with separate sterile synthetic swabs (160 C, Copan Italia S.p.A., Brescia, Italy). DNA was extracted according to Hyatt *et al.* (2007) and *B. dendrobatidis* was quantified as described above. To standardize the surface area sampled, surface area morphometry of each body part was performed using Optimas 6.5 image analysis software (Media Cybernetics Inc., Bethesda, MD, USA) so that GE's of *B. dendrobatidis* could be quantified per cm^2 of skin. To validate the qPCR results, the selected body sites were examined histologically for the presence of infection. For each salamander, skin from the sampling sites with the lowest and the highest GE's was excised, fixed in 10% neutral buffered formalin, embedded in paraffin and stained with hematoxylin & eosin (HE) and an immunoperoxidase (IPX) stain for *B. dendrobatidis,* as described by Berger *et al*. (2002). Histological slides were scored for the presence of *B. dendrobatidis* (-: no sporangia observed, +: few sporangia present, ++: sporangia abundant).
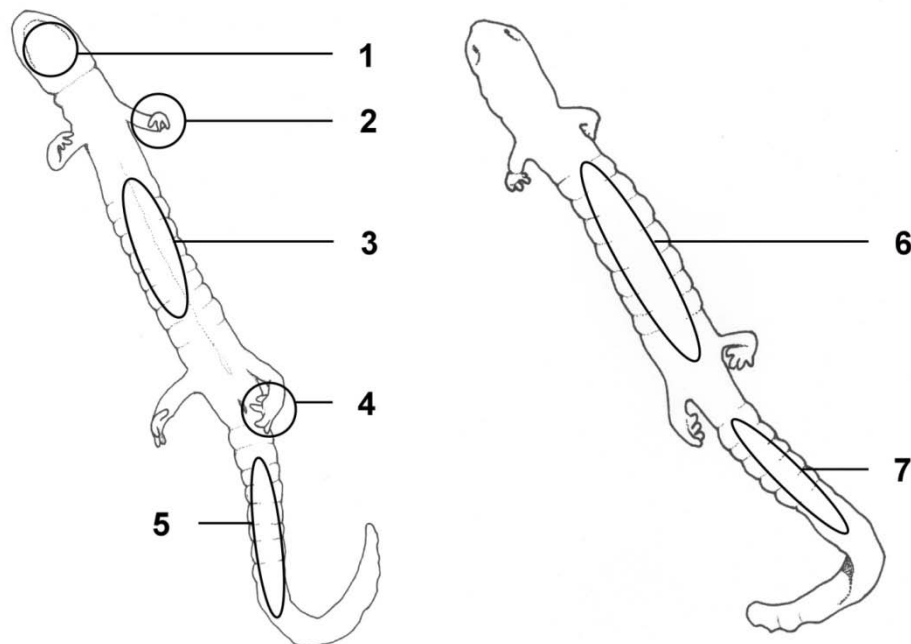
Figure 1. Assessing the effect of the body part sampled on the detection of *B. dendrobatidis* in Caudata. Schematic presentation of the body sites that were swab sampled: (1) chin, (2) plantar side forelimb, (3) ventral side abdomen, (4) plantar side hindlimb, (5) ventral side tail, (6) dorsum, (7) dorsal side tail.

Table 1. Density of *Batrachochytrium dendrobatidis* in different locations on the bodies of infected bolitoglossine salamanders

Sampling site	Sample type	Mean GE ± SE/cm^2 (log 10)	Min. GE/cm^2 (log 10)	Max. GE/cm^2 (log 10)	No. of positives	No. of samples	Prevalence (%)	Significance level (P)
Chin	Swab	2.31 ± 3.70	Neg.	4.23	9	11	82	0.046*
Plantar side forelimb	Swab	3.45 ± 3.58	1.76	4.04	11	11	100	0.920
Dorsum	Swab	2.55 ± 2.77	Neg.	3.28	10	11	91	0.002*
Dorsal side tail	Swab	3.06 ± 3.30	Neg.	3.76	11	11	100	0.034*
Ventral side abdomen	Swab	2.92 ± 3.36	Neg.	3.89	9	11	82	0.002*
Plantar side hindlimb	Swab	3.89 ± 4.14	Neg.	4.68	10	11	91	0.818
Ventral side tail	Swab	3.06 ± 3.38	Neg.	3.92	11	11	100	0.038*
Pelvic region	Tissue	4.43 ± 4.49	2.45	5.01	11	11	100	N/A

Neg.: samples where *B. dendrobatidis* was not detected; N/A: not applicable.

*Significant difference between a swabbed site compared to the pelvic tissue samples.

Data were log transformed to correct for non-normality. A one-way ANOVA was used to determine whether the abundance of Bd (GE/cm^2) differed according to the body part sampled. A post-hoc Tukey HSD test was used to identify which body parts were significantly different from one another. All tests were performed in SPSS (Version 17; SPSS Inc., Chicago, IL, USA).

Thirty-three dead adult and 28 healthy adult captive Mexican plethodontid salamanders from two private collections were screened for the presence of *B. dendrobatidis*. Then, the swabbing protocol was used on 17 healthy (no clinical signs of chytridiomycosis) adult wild bolitoglossine salamanders during an opportunistic field survey conducted in the Véracruz and México regions of Mexico in August 2010 (**Fig. 2**). These regions were selected because they represent the natural habitat of the examined captive species. All of the animals were sampled with a sterile synthetic swab over the 7 aforementioned body regions as described above. An overview of all the animals sampled, together with their species designation and sampling localities is given in **Table 2**.

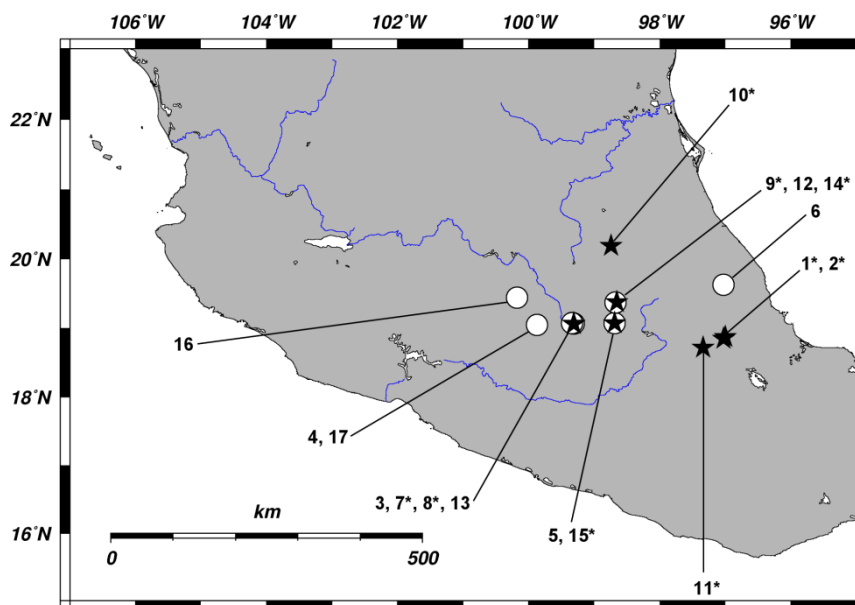**Figure 2. Localities in Mexico surveyed for the presence of *Batrachochytrium dendrobatidis*.** The stars indicate localities where *B. dendrobatidis* was detected; the circles indicate localities where *B. dendrobatidis* was not detected. The numbers correspond with the species mentioned in **Table 2**; the numbers indicated with an asterisk (*) represent the species found to be positive for *B. dendrobatidis*.

Table 2. Overview of all bolitoglossine species examined for the presence of *Batrachochytrium dendrobatidis* using the swab protocol for Caudata

Species	Site	Latitude (°N)	Longitude (°W)	IUCN Red List Category (see footnote)	Wild/captive	No. infected/ No. examined [Prevalence % (95 % CI)]	GE
Bolitoglossa rufescens[1]	Cordoba, Veracruz	18 52.200	97 00.200	LC	wild	**1**/1	8
Bolitoglossa rufescens[2]	Orizaba, Veracruz	18 50.600	97 01.800	LC	wild	**1**/1	5
Chiropterotriton chiropterus[3]	Zempoala, Veracruz	19 03.892	99 18.814	CR	wild	0/1	
Chiropterotriton lavae[4]	La Joya, México	19 37.426	97 01.862	CR	wild	0/1	
Chiropterotriton orculus[5]	Rio Frio, México	19 04.400	98 41.300	VU	wild	0/1	
Chiropterotriton orculus[6]	Popocatepetl, México	19 02.800	99 52.500	VU	wild	0/1	
Pseudoeurycea cephalica[7,8]	Zempoala, Veracruz	19 03.900	99 18.800	NT	wild	**2**/2	4, 19
Pseudoeurycea cephalica[9]	Rio Frio, México	19 22.400	98 39.400	NT	wild	**1**/1	6
Pseudoeurycea cephalica[10]	Hidalgo, México	20 11.300	98 44.800	NT	wild	**1**/1	6
Pseudoeurycea firscheini[11]	Orizaba, Veracruz	18 42.900	97 20.500	EN	wild	**1**/1	18
Pseudoeurycea leprosa[12]	Rio Frio, México	19 21.873	98 40.651	VU	wild	0/1	
Pseudoeurycea leprosa[13]	Zempoala, Veracruz	19 04.219	99 20.430	VU	wild	0/1	
Pseudoeurycea leprosa[14]	Rio Frio, México	19 22.400	98 39.400	VU	wild	**1**/1	1
Pseudoeurycea leprosa[15]	Popocatepetl, México	19 04.400	98 41.300	VU	wild	**1**/1	12
Pseudoeurycea longicauda[16]	N/A	19 26.254	100 10.683	EN	wild	0/1	
Pseudoeurycea robertsi[17]	Nevado de Toluca, México	19 02.800	99 52.500	CR	wild	0/1	
Wild total						**9**/17 [52.9% (28.5-77.4)]	
Bolitoglossa platydactyla				NT	captive	0/2	
Bolitoglossa platydactyla†				NT	captive	**2**/2	379, 3191
Bolitoglossa rufescens†				LC	captive	**6**/7	1, 5, 68, 152, 386, 589
Chiropterotriton chiropterus				CR	captive	0/2	
Chiropterotriton chiropterus				CR	captive	0/6	
Chiropterotriton chiropteru s†				CR	captive	0/5	
Chiropterotriton multidentatus†				EN	captive	0/1	
Pseudoeurycea belli†				VU	captive	**2**/4	1, 9
Pseudoeurycea belli				VU	captive	0/2	
Pseudoeurycea cephalica				NT	captive	0/1	

Table 2. continued

Species	Site	Latitude (°N)	Longitude (°W)	IUCN Red List Category (see footnote)	Wild/captive	No. infected/ No. examined (Prevalence %)	GE
Pseudoeurycea cephalica†				NT	captive	**2**/3	**18, 117**
Pseudoeurycea leprosa				VU	captive	0/14	
Pseudoerycea leprosa†				VU	captive	**3**/3	41, 151, 1331
Pseudoeurycea longicauda†				EN	captive	**1**/4	**2**
Pseudoeurycea nigromaculata†				CR	captive	0/1	
Pseudoeurycea robertsi†				CR	captive	0/2	
Pseudoeurycea sp. nov.*				N/A	captive	0/1	
Thorius troglodytes†				EN	captive	0/1	
Captive total						**16**/61 [26.2% (15.1-37.4)]	

Species and numbers indicated in bold are qPCR positive for *B. dendrobatidis* and are presented with the corresponding genomic equivalents (GE); the indexed numbers refer to the localities were the wild species were sampled (**Fig. 2**); specimens from the species indicated with † were dead; species indicated with an asterisk (*) are under description by Parra-Olea & Wake. The abbreviations used for the IUCN Red List categories (for definitions and criteria, see www.iucn.redlist.org) are LC: least concern, NT: near threatened, VU: vulnerable, EN: endangered, CR: critically endangered, N/A: no data available.

3. Results

Based on the analysis of pelvic tissue, evidence of *B. dendrobatidis* infection was found in sixteen out of 33 (48%) dead captive bolitoglossine salamanders, comprising 6 species: *Bolitoglossa platydactyla*, *Bolitoglossa rufescens*, *Pseudoeurycea belli*, *Pseudoeurycea cephalica*, *Pseudoeurycea leprosa* and *Pseudoeurycea longicauda*. Tail loss or tail autotomy was observed in 8 out of the 16 infected salamanders belonging to the species *Bolitoglossa platydactyla*, *B. rufescens*, *P. cephalica* and *P. leprosa*, suggesting that infections might induce tail autotomy. Animals with GE's of *B. dendrobatidis* in pelvic skin samples equal to or higher than 20 were used to determine the effect of the body part sampled on the detection of *B. dendrobatidis* (**Table 1**). Eleven animals belonging to the species *B. platydactyla* (n= 2), *B. rufescens* (n= 6), *P. cephalica* (n=1) and *P. leprosa* (n=2) were included in this study. All seven sampling sites tested positive in the infected salamanders, except for 2 *B. rufescens* specimens (3/7 and 6/7 sites positive) and 1 *Pseudoeurycea leprosa* specimen (6/7 sites positive) (data not shown).

Figure 3. Histological section of infected skin of *Bolitoglossa rufescens*. Haematoxilin and eosin stained section of skin from a hindlimb. Chytrid infection is characterized by hyperkeratosis of the stratum corneum (superficial cornified layer), with the presence of several empty sporangia (black arrowheads), septate chytrid thalli (empty arrowhead) and maturing sporangia (black arrows) merged in the epidermis. Scale bar = 20 μm.

Based on the qPCR results, the plantar side of the forelimbs and hindlimbs and the ventral side of the tail consistently had the highest *B. dendrobatidis* densities, and the dorsum and chin had the lowest *B.dendrobatidis* densities. In 9 out of the 11 samples with high GE values of *B. dendrobatidis*, the histological slides (**Fig. 3**) showed few to abundant sporangia embedded in the upper keratinized layers of the epidermis coinciding with hyperkeratosis but

not epidermal hyperplasia. In 10 out of the 11 samples with low GE's, no histological lesions could be observed.

B. dendrobatidis was not detected in any of the 28 healthy, living Mexican bolitoglossine salamanders, whereas 48% (16/33) of the dead captive animals carried high zoospore loads of up to 3191 GE's (**Table 2**). Fifty-three percent of the wild animals (9/17) sampled were positive for *B. dendrobatidis,* and *B. dendrobatidis* was detected in 7 localities (**Fig. 2**). None of the wild animals showed obvious clinical signs of chytridiomycosis, and the positive wild animals carried rather low zoospore loads.

4. Discussion

We detected a significant main effect of body part on *B. dendrobatidis* density (P=0.025). However, Tukey's post-hoc multiple comparison test did not detect significant differences among the body parts. To determine whether swabbing might be a viable alternative to destructive tissue sampling, we compared *B. dendrobatidis* densities in skin samples from the pelvic region to *B. dendrobatidis* densities from swab samples of the seven body parts. For every body part, the mean *B. dendrobatidis* density was lower on the swab sample than it was in the tissue sample, suggesting that swabbing underestimates *B. dendrobatidis* densities. However, some caution should be used interpreting these results given that there is variation in *B. dendrobatidis* densities among body parts and all swab samples were compared to a single tissue sample from the pelvic region. Although all the swab samples had less *B. dendrobatidis* than the tissue sample, a multiple comparison test revealed that the *B. dendrobatidis* density in the tissue sample was not significantly different from *B. dendrobatidis* densities in the swab samples of the forelimbs (P=0.920) or hindlimbs (P=0.818). Hence, in salamanders, swab sampling of the limbs might provide a reliable, non-destructive alternative to tissue sampling for *B. dendrobatidis* quantification.

A high incidence of *B. dendrobatidis* infections was found in the sampled bolitoglossine salamanders, with the pelvic region, the forelimbs and hindlimbs and the ventral side of the tail having the highest *B. dendrobatidis* densities. To maximize the chances of detecting *B. dendrobatidis* in Caudata, we recommend sampling these body parts. In contrast, in Anura (frogs and toads) the ventral skin and the toes seem more predisposed to high *B. dendrobatidis* densities (Berger *et al.*, 2005; Puschendorf & Bolaños, 2006).

There is a paucity of data demonstrating susceptibility of plethodontid salamanders to *B. dendrobatidis* infection. We revealed that clinically healthy captive salamanders were free

of *B. dendrobatidis* infection, but that in half of the dead captive salamanders *B. dendrobatidis* was present, combined with hyperkeratosis, a hallmark of clinical *B. dendrobatidis* infections. This result is consistent with recent infection trials revealing high virulence of *B. dendrobatidis* to *B. rufescens* and *P. leprosa* (Cheng *et al*., 2011) and the report of Pasmans *et al.* (2004) demonstrating lethal *B. dendrobatidis* infection in captive *Bolitoglossa dofleini*. *B. dendrobatidis* was detected in half of the wild Mexican bolitoglossine salamanders sampled, but was not associated with any obvious clinical signs of disease. Although the high prevalence of *B. dendrobatidis* in dead captive salamanders, its absence in clinically healthy living ones, and its presence in wild salamanders is consistent with *B. dendrobatidis* being involved in recent bolitoglossine population declines, further studies will be necessary before *B. dendrobatidis* is conclusively demonstrated to be a causal factor in the decline of Mexican bolitoglossine salamanders.

5. Acknowledgements

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STUDY 4

Resistance to chytridiomycosis in European plethodontid salamanders

of the genus *Speleomantes*

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Summary

North America and the neotropics harbor nearly all species of plethodontid salamanders. In contrast, this family of caudate amphibians is represented in Europe and Asia by two genera, *Speleomantes* and *Karsenia*, which are confined to small geographic ranges. Compared to neotropical and North American plethodontids, mortality attributed to chytridiomycosis caused by *Batrachochytrium dendrobatidis* has not been reported for European plethodontids, despite the established presence of *B. dendrobatidis* in their geographic distribution.

We determined the extent to which *B. dendrobatidis* is present in populations of all eight species of European *Speleomantes* and show that *B. dendrobatidis* was undetectable in 921 skin swabs. We then compared the susceptibility of one of these species, *Speleomantes strinatii*, to experimental infection with a highly virulent isolate of *B. dendrobatidis* (*Bd*GPL), and compared this to the susceptible species *Alytes muletensis*. Whereas the inoculated *A. muletensis* developed increasing *B. dendrobatidis*-loads over a 4 week period, none of five exposed *S. strinatii* were colonized by *B. dendrobatidis* beyond 2 weeks post inoculation. Finally, we determined the extent to which skin secretions of *Speleomantes* species are capable of killing *B. dendrobatidis*. Skin secretions of seven *Speleomantes* species showed pronounced killing activity against *B. dendrobatidis* over 24 hours.

In conclusion, the absence of *B. dendrobatidis* in *Speleomantes* combined with resistance to experimental chytridiomycosis and highly efficient skin defenses indicate that the genus *Speleomantes* is a taxon unlikely to decline due to *B. dendrobatidis*.

1. Introduction

With more than 430 species, the family Plethodontidae comprises the majority of extant urodelan species and has experienced a marked evolutionary radiation in North, Central and northern South America (Vieites *et al*., 2011). In the rest of the world, this family is confined to the Maritime Alps, the central Apennine mountains in continental Italy and Sardinia in Europe (the genus *Speleomantes*, containing 8 species), and to South Korea (1 species, *Karsenia koreana*). The European plethodontids are closely related to the North American genus *Hydromantes* (Vieites *et al*., 2011) and occupy an area well known to be infected by the amphibian pathogenic fungus *Batrachochytrium dendrobatidis*, one of the known drivers underlying global amphibian declines (Garner *et al*., 2005; Bovero *et al*., 2008; Bielby *et al*., 2009; Fisher *et al*., 2009; Tessa *et al.*, 2012). In Italy and France, the two countries where the genus *Speleomantes* occurs, the aggressive lineage of the pathogen, the *B. dendrobatidis* global panzootic lineage (*Bd*GPL)*,* also occurs (Farrer *et al*., 2011). Both infection and mortality due to *B. dendrobatidis* has been reported for both countries, including localities where *Speleomantes sp.* are endemic (Bovero *et al*., 2008; Bielby *et al*., 2009; Walker *et al*., 2010; Tessa *et al*., 2012). Although habitat alteration has taken its toll on *Speleomantes* populations, enigmatic declines that would match chytridiomycosis driven declines witnessed elsewhere have not been reported. Indeed, these salamanders are among the most abundant vertebrates in suitable habitats (Lanza *et al*., 2005).

The skin is of vital importance to plethodontid salamanders, which rely exclusively on cutaneous respiration. Chytridiomycosis dramatically disturbs the skin function (Voyles *et al*., 2009; Brutyn *et al*., 2012) and thus compromises respiration. Therefore, chytrid infections in *Speleomantes* should result in rapid killing of the plethodontid host, as has been hypothesized to be the case for several declining neotropical plethodontids and demonstrated in some, but not all (Keitzer *et al.*, 2011), North American species (Chinnadurai *et al*., 2009; Vazquez *et al*., 2009; Weinstein *et al*., 2009; Cheng *et al*., 2011). Although different infection protocols have been used in these studies, they clearly demonstrate that some New World plethodontid species are easily colonized by the fungus. Hitherto no suspected chytridiomycosis associated declines in *Speleomantes* have been observed, even in a region where chytridiomycosis occurs. This leads us to hypothesize first that prevalence of lethal cutaneous infections, such as infections by *B. dendrobatidis*, are low in European plethodontid salamanders. For this purpose, we determined to what extent *B. dendrobatidis* is present in populations of all eight species of *Speleomantes*.

Susceptibility to clinical chytridiomycosis, however, varies greatly among plethodontid species. In amphibian hosts, differences in host susceptibility have been attributed to the presence of fungicidal skin microbiota (Brucker *et al*., 2008a; Harris *et al*., 2009; Becker & Harris, 2010), antimicrobial peptides (reviewed in Rollins-Smith, 2009), host genetics (Tobler *et al*., 2010; Savage & Zamudio, 2011; Luquet *et al*., 2012) and/or environmental factors (e.g. Walker *et al*., 2010), which may affect invasion of amphibian skin by the pathogen (Van Rooij *et al*., 2012). This leads us to hypothesize that resistance of European plethodontid salamanders is due to skin defenses that efficiently cope with *B. dendrobatidis* infection. Subsequently, we then examined susceptibility of *Speleomantes strinatii* to experimental infection with a global panzootic lineage strain of *B. dendrobatidis* (*Bd*GPL isolate IA2011, Farrer *et al*., 2011). Finally, we determined to what extent skin secretions of *Speleomantes* species are capable of killing *B. dendrobatidis*.

2. Materials and methods

All animal experiments were conducted according to biosecurity and ethical guidelines and approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University (EC2011-073). All species involved in this study are protected as defined in Annex IV of the EU Habitats Directive (Council Directive 92/43/EEC on the Conservation of natural habitats and of wild fauna and flora). The entire experiment was submitted and approved by the Italian Ministry of Environment that issued permits to SS (issue numbers: DPN-2010-0010807 and PNM-2012-0007331). In Italy, state permits are valid over the entire country, since wildlife is a public property (national law 157/92). In addition however, when salamanders were sampled inside Protected Areas, local permits were also obtained from the "Parco Regionale Frasassi and Gola della Rossa" (permit number 3774/2012) the "Parco Regionale delle Alpi Apuane" (permit number DD.5/2012) and "Parco Regionale Alpi Marittime" (permit DD.165a/2011 issued to DO and FO). Permit PNM-2012-0007331 was also valid to capture animals (outside Protected Areas) to be used in experimental infections at Ghent University (EC2011-073). Since the study did not involve work on living animals in Italian laboratories, authorisation from the Italian Ministry of Health was not required.

Between December 2004 and September 2012, we sampled 921 specimens including examples of all 8 recognized species of *Speleomantes* (*Speleomantes ambrosii*, *S. flavus*, *S. genei*, *S. imperialis*, *S. italicus*, *S. sarrabusensis*, *S. strinatii*, *S. supramontis*) at 65 localities in mainland Italy and southern France (351 samples) and Sardinia (570 samples) (**Fig. 1, Table**

1). Samples were collected by rubbing the abdomen, feet and the ventral side of the tail at least 10 times as has been described by Van Rooij *et al.* (2011) using a rayon tipped swab (160 C, Copan Italia S.p.A., Brescia, Italy). The sex of the adults was recorded for the three continental species (*S. italicus*, *S. ambrosii* and *S. strinatii*) by checking for the presence of the typical male mental gland (Lanza *et al.*, 2005) and the ratio females: males: juveniles was approximately 1:1:1.
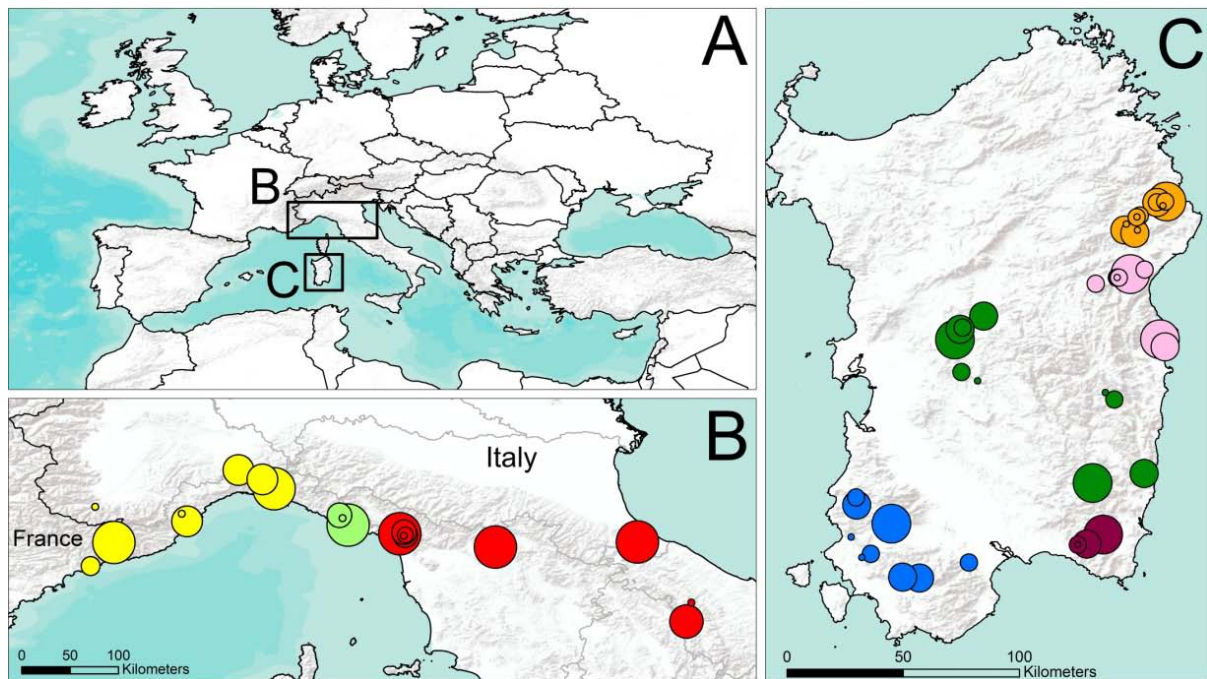
Figure 1. Overview of sampling localities for *B. dendrobatidis* in Sardinia (C), mainland Italy and southeastern France (B) in Europe (A). For map **(B)**, the colours represent the species *Speleomantes strinatii* (yellow), *S. ambrosii* (bright green) and *S. italicus* (red). For map **(C),** the colours represent the species *S. genei* (blue), *S. sarrabusensis* (purple), *S. imperialis* (dark green), *S. supramontis* (pink) and *S. flavus* (orange). Localities are indicated by symbols proportional to sample size.

DNA from the swabs was extracted in 100 µl PrepMan Ultra (Applied Biosystems, Foster City, CA, USA), according to Hyatt *et al.* (2007). DNA samples were diluted 1:10 and quantitative PCR (qPCR) assays were performed in duplicate on a CFX96 Real Time System (BioRad Laboratories, Hercules, CA, USA). Amplification conditions, primer and probe concentrations were according to Boyle *et al.* (2004). Within each assay, 1 positive control sample containing *B. dendrobatidis* DNA from a naturally infected and deceased Costa Rican *Eleutherodactylus* sp. as template and 3 negative control samples with high performance liquid chromatography water (HPLC) as template were included.

Table 1. Overview of the sampled *Speleomantes* species

Species	Coordinates*	Sample size	Date
S. flavus	N 40°35'; E 9°39'	9	30/12/2004
	N 40°31'; E 9°35'	3	28/12/2011
	N 40°31'; E 9°35'	2	28/12/2011
	N 40°35'; E 9°39'	5	28/12/2011
	N 40°35'; E 9°41'	3	28/12/2011
	N 40°30'; E 9°32'	2	21/01/2012
	N 40°28'; E 9°32'	7	21/01/2012
	N 40°32'; E 9°35'	4	29/12/2010
	N 40°28'; E 9°34'	6	8/02/2012
	N 40°28'; E 9°35'	1	8/02/2012
	N 40°34'; E 9°41'	1	8/02/2012
	N 40°35'; E 9°41'	26	8/02/2012
Subtotal		69	
S. genei	N 39°26'; E 8°29'	5	29/12/2011
	N 39°20'; E 8°38'	7	29/12/2011
	N 39°25'; E 8°29'	29	29/12/2011
	N 39°17'; E 8°28'	3	30/12/2011
	N 39°12'; E 8°31'	1	30/12/2011
	N 39°11'; E 8°56'	6	1/01/2012
	N 39°08'; E 8°44'	20	22/01/2012
	N 39°20'; E 8°38'	96	22/01/2012
	N 39°13'; E 8°33'	6	22/01/2012
	N 39°08'; E 8°40'	20	11/02/2012
	N 39°12'; E 8°31'	1	11/02/2012
Subtotal		194	
S. imperialis	N 40°03'; E 8°52'	25	4/12/2009
	N 40°03'; E 8°52'	9	26/12/2011
	N 40°03'; E 8°52'	3	27/12/2011
	N 39°49'; E 9°29'	6	31/12/2011
	N 39°51'; E 9°27'	1	31/12/2011
	N 39°30'; E 9°24'	58	1/01/2012
	N 39°53'; E 8°58'	3	21/01/2012
	N 39°32'; E 9°36'	9	6/02/2012
	N 40°08'; E 8°59'	12	9/02/2012
	N 40°05'; E 8°54'	1	9/02/2012
	N 40°06'; E 8°54'	10	9/02/2012
	N 40°03'; E 8°53'	7	9/02/2012
	N 39°55'; E 8°54'	5	10/02/2012
Subtotal		149	
S. sarrabusensis	N 39°15'; E 9°21'	2	28/12/2004
	N 39°18'; E 9°27'	62	14/05/2007
	N 39°15'; E 9°23'	5	31/12/2011
	N 39°15'; E 9°21'	1	12/02/2012
Subtotal		70	
S. supramontis	N 40°16'; E 9°25'	4	5/12/2009
	N 40°17'; E 9°30'	1	27/12/2011
	N 40°17'; E 9°30'	5	27/12/2011
	N 40°17'; E 9°30'	5	27/12/2011
	N 40°17'; E 9°30'	5	27/12/2011

Table 1. continued

Species	Coordinates*	Sample size	Date
S. supramontis	N 40°18'; E 9°33'	22	29/12/2011
	N 40°01'; E 9°41'	14	7/02/2012
	N 40°03'; E 9°40'	24	7/02/2012
	N 40°19'; E 9°36'	8	7/02/2012
Subtotal		88	
S. strinatii	N 44°38'; E 8°45'	20	4/10/2011
	N 44°33'; E 8°59'	20	6/10/2011
	N 44°17'; E 7°26'	8	9/08/2011
	N 44°09'; E 8°17'	20	8/10/2011
	N 44°27'; E 9°05'	22	9/11/2011
	N 43°57"; E 7°36"	21	31/05/2012
	N 44°13'; E 8°14'	7	30/07/2012
	N 43°44'; E 7°23'	17	30/08/2012
Subtotal		135	
S. ambrosii	N 44°13'; E 9°42'	22	12/10/2011
	N 44°11'; E 9°43'	16	12/10/2011
	N 44°07'; E 9°46'	30	18/07/2012
Subtotal		68	
S. italicus	N 44°02'; E 10°18'	13	2/07/2012
	N 44°02'; E 10°18'	10	2/07/2012
	N 44°01'; E 10°17'	3	2/07/2012
	N 44°03'; E 10°18'	12	2/07/2012
	N 44°02'; E 10°15'	26	20/08/2012
	N 43°55'; E 11°08'	28	25/08/2012
	N 43°58'; E 12°27'	30	15/09/2012
	N 43°24'; E 12°57'	6	23/09/2012
	N 43°13'; E 12°55'	20	23/09/2012
Subtotal		148	
Total		**921**	

*Seconds have been removed from coordinates to prevent illegal collection

Table 2. *Speleomantes* species sampled for collection of skin secretions and their respective coordinates

Species	Coordinates*
S. ambrosii	N 44°11'; E 9°43'
S. italicus	N 43°60'; E 10°18'
S. strinatii	N 44°33'; E 8°58'
S. genei	N 39°08'; E 8°44'
S. supramontis	N 40°18'; E 9°33'
S. flavus	N 40°28'; E 9°32'
S. sarrabusensis	N 39°18'; E 9°27'

*Seconds have been removed from coordinates to prevent illegal collection

Samples were considered positive for *B. dendrobatidis* when a clear log-linear amplification was observed, when the number of genomic equivalents (GE) of *B. dendrobatidis*, defined as the measure of infection, was higher than the detection limit of 0.1 GE and when amplification that met both of the previous criteria was observed in both replicates. In case of conflict between both replicates of the same sample, the sample was run again in duplicate. To control and estimate inhibition, a subset of samples negative for the presence of *B. dendrobatidis* (n= 84) was retested under the same conditions as described above, but with an exogenous internal positive control (VIC^{TM} probe, Life technologies, Austin, TX, USA) included as described by Hyatt *et al.* (2007). The Bayesian 95% credible interval for prevalence was estimated as described by Lötters *et al.* (2012).

We assayed susceptibility to chytridiomycosis in five male subadult *S. strinatii* by experimentally exposing them to a controlled dose of *B. dendrobatidis*. Ten captive bred juvenile *Alytes muletensis* were used as susceptible, positive control animals (Muijsers *et al.*, 2012). The animals were housed individually in plastic boxes (20 x 10 x 10 cm) lined with moist tissue, provided with PVC-tubes as shelter and kept at 18°C. Crickets were provided as food items *ad libitum*. All animals were sampled for the presence of *B. dendrobatidis* before inoculation using the method described above. A virulent isolate of the global panzootic lineage of *B. dendrobatidis* (*Bd*GPL IA2011) (Farrer *et al.*, 2011), isolated in 2011 in the Spanish Pyrenees and capable of causing severe chytridiomycosis in urodelans was used in this study. All animals were exposed to a single dose of 1 ml of distilled water containing 10^5 zoospores/ml. Skin swabs were collected weekly for 4 weeks and processed as described above to determine the infection load for *B. dendrobatidis* each animal exhibited over the course of the experiment. After termination of the experiment, all animals were treated with voriconazole (Martel *et al.*, 2011). The *S. strinatii* specimens are still kept following strict biosecurity guidelines at the clinic for Exotic Animals and Avian Diseases (Faculty of Veterinary Medicine, Ghent University) for further follow-up.

To determine the extent to which skin secretions of *Speleomantes* are capable of killing *B. dendrobatidis* zoospores, skin secretions were collected non-invasively from wild individuals of 7 of the 8 *Speleomantes* species (**Table 2**) and processed within 1h (*S. strinatii*), 48h (*S. ambrosii, S. italicus, S. flavus, S. supramontis, S. genei*) or 72h (*S. sarrabusensis*). For this purpose, a microbiological inoculating loop was gently rubbed over the dorsal tail until white skin secretions accumulated on the loop. Prior to sampling sterile loops were cut off, stored individually in sterile vials and the total weight of each vial was determined. Collected skin secretions were weighed to the nearest 0.1 mg by subtracting the

weight of the vial and the inoculation loop from the total weight. Varying amounts of skin secretions were collected per *Speleomantes* specimen, ranging from 3.1 to 32.7 mg. Collected skin secretions were not further diluted prior exposure to *B. dendrobatidis* zoospores. Loops with secretions were incubated in a zoospore suspension. To keep the ratio between the amount of skin secretions and *B. dendrobatidis* zoospores added constant, 10 µl of zoospore suspension containing 10^6 zoospores/ml distilled water was added per mg skin secretion. Samples were incubated for 24h at 20°C. At 0 and 24h of incubation, the number of viable zoospores was assessed using qPCR on the zoospores that were pretreated with ethidium monoazide (EMA, Sigma-Aldrich Inc., Bornem, Belgium) as described by Blooi *et al.* (2013). Viable/death differentiation is obtained by covalent binding of EMA to DNA in dead *B. dendrobatidis* by photoactivation. EMA penetrates only dead *B. dendrobatidis* with compromised membranes and DNA covalently bound to EMA cannot be PCR amplified (Rudi *et al.*, 2005). In brief, at 0 and 24h of incubation a 5 µl aliquot was taken from each sample, transferred into a 24-well plate and 195 µl TGhL broth (tryptone, gelatin hydrolysate, lactose) was added to each well for its protective effect on viable *B. dendrobatidis* zoospores during EMA treatment. Negative controls for skin secretion activity consisted of zoospore suspensions not exposed to skin secretions in order to quantify the 'natural' loss of viability in *B. dendrobatidis* zoospores, while positive controls were heat-killed zoospores. Five µl of a 1 mg/ml stock solution of EMA in dimethyl formamide (1 mg/ml) was added to 200 µl zoospore suspension in TGhL broth to obtain a final concentration of 25 µg/ml EMA, incubated for 10 minutes, protected from light and exposed to a 500 W halogen light at 20 cm distance for 5 minutes. Then, samples were washed by centrifugation (5000 rpm, 5 min, 20°C) and resuspended in HPLC water. In parallel, the total amount of *B. dendrobatidis* zoospores in all samples, including controls, was enumerated using exactly the same procedure as described above, only 5 µl HPLC water was added to each sample instead of EMA. DNA extraction and qPCR were then done as described above. Killing activity was expressed as log(10) reduction of viable spores in a given sample compared to the negative controls.

3. Results

None of the 921 skin swabs collected from any *Speleomantes* sp. tested positive for *B. dendrobatidis*. The Bayesian 95% credible interval for the observed prevalence of 0% is (0.0000, 0.0040). However, in 12 out of 84 samples tested (14 %) PCR-inhibition occurred

that could not be abolished by diluting the samples 1/100. Extrapolated to the total number of individual salamanders tested, this reduces the reliable number of *B. dendrobatidis*-negative salamanders to 789 with a corresponding 95% credible interval of (0.0000, 0.0047).

None of the experimental animals tested positive for *B. dendrobatidis* prior to the exposure. Over the four week infection period, 9/10 *Alytes muletensis* developed marked infection with increasing *B. dendrobatidis* loads. Of the 5 inoculated *Speleomantes strinatii*, 3 animals exhibited weak infections (small GE value) at 7 days post infection (dpi) with an average of 5.5 ± 1.8 GE per swab. At 14 dpi, one salamander was borderline positive (0.2 GE per swab) but at 21 and 28 dpi all salamanders tested negative for *B. dendrobatidis*. In contrast, *A. muletensis* exhibited median GE counts of 91 at 21 dpi and 3920 at 28 dpi (**Fig. 2**). No clinical signs were noticed in the infected salamanders. For animal welfare reasons, *A. muletensis* were treated at 4 weeks pi using voriconazole to clear infection, as described by Martel *et al.* (2011).

Skin secretions of all *Speleomantes* species were capable of efficiently killing *B. dendrobatidis* zoospores (**Fig. 3**). Exposure to skin secretions resulted in a 200 to 20000 fold reduction of the number of viable spores within 24h post exposure.

4. Discussion

B. dendrobatidis infections appear to be highly uncommon, if not absent, in adult and juvenile *Speleomantes* of all species and throughout their range. The genus *Speleomantes* occupies an ecological niche highly suitable to *B. dendrobatidis* colonization, persistence and spread due to relatively low preferred body temperatures (< 18.5°C, reviewed by Lanza *et al.*, 2005) and higher humidity environments. Contacts that would facilitate interspecific transmission are also common because at some locations salamanders occur outside of caves and are found under retreat sites with other species that are capable of carrying infections (F. Pasmans *pers. obs.*; Bovero *et al.*, 2008; Bielby *et al.*, 2009; Tessa *et al.*, 2012). Intraspecific transmission would also be highly likely, as courtship involves intimate contact, salamanders crowd together in summer retreats, and juveniles also exhibit highly aggregated distribution patterns at certain times of the year (Salvidio *et al.*, 2002). Thus, substantial opportunity exists for *B. dendrobatidis* for introduction into *Speleomantes* populations and to amplify rapidly once introduced, but neither of these seems to have occurred to any significant degree. Field studies of other amphibian species have reported low prevalence or absence of detectable
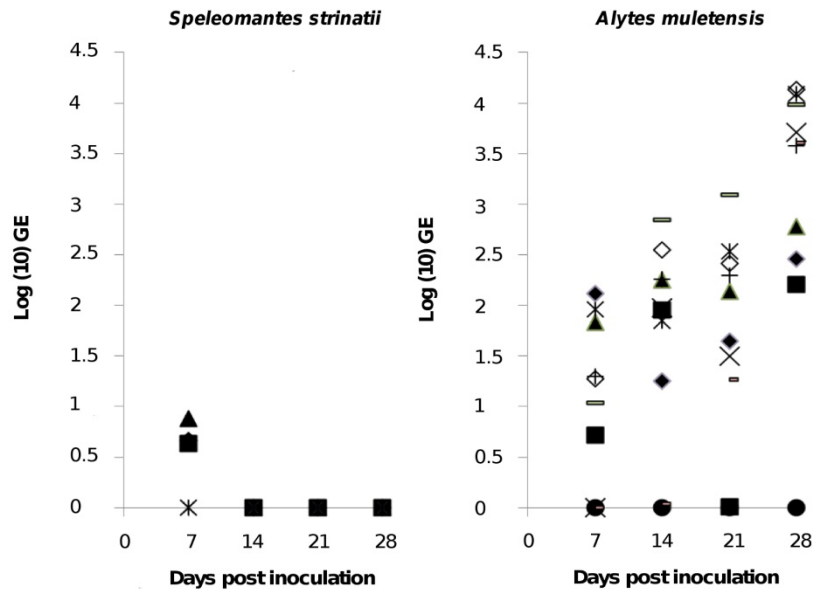
Figure 2. Experimental infection of *Speleomantes strinatii* with *B. dendrobatidis*. Infection loads are represented as log(10) genomic equivalents (GE) of *B. dendrobatidis* in skin swabs from *Speleomantes strinatii* (left panel) and compared with *Alytes muletensis* (right panel) serving as positive control animals, up to four weeks post experimental inoculation with *B.dendrobatidis*. Each symbol represents an individual animal.
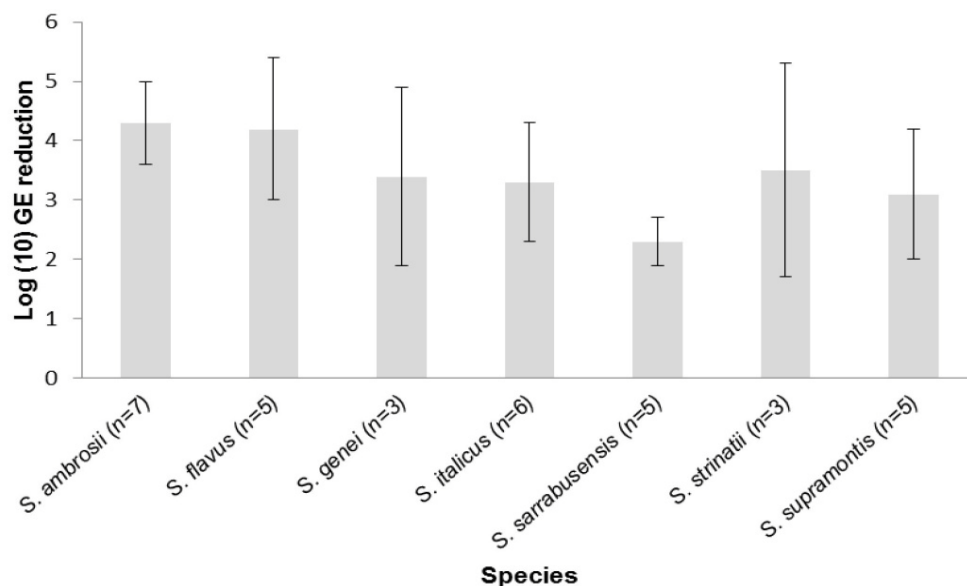
Figure 3. Killing activity of skin secretions of *Speleomantes* species against *B. dendrobatidis*. Killing activity of *Speleomantes* skin secretions at physiological concentrations is expressed as log(10) viable spores of *B. dendrobatidis* added to the skin secretions – log(10) viable spores recovered 24 h later. Results are presented as mean genomic equivalents of *B. dendrobatidis* (GE) ± standard error (SE); n = sample size.

infection in species that show seasonal fluctuations in prevalence (Kriger & Hero, 2007; Kinney *et al*., 2011; Martel *et al*., 2012). We find this an unlikely explanation for the observed absence of infection. Moreover, for the present study samples were taken from December to September and over multiple years. Seasonally mediated fluctuations are associated with strong variation in environmental metrics that influence *B. dendrobatidis* growth and reproduction (Kriger & Hero, 2007; Longo *et al*., 2010 Kinney *et al*., 2011) suggesting that more stable environments should result in more consistent patterns of infection (but see Chatfield *et al*., 2012). However, recent evidence suggests that variable temperatures may be most favorable for *B. dendrobatidis* driven declines, probably due to temperature drop induced zoospore release (Raffel *et al*., 2012). Variation of prevalence should be minimized in the cave-dwelling *Speleomantes sp.*, where relatively stable cave temperatures and moisture regimes should buffer against environmentally mediated changes in prevalence. Further, our experimental results indicate the infection is unlikely in at least one species and the majority of *Speleomantes* species are equipped with the tools to resist infection.

Exposing *S. strinatii* to a highly virulent *B. dendrobatidis* strain and in a manner that resulted in potentially lethal infection in a susceptible host did not result in persistent infection of the salamanders. It is possible that the low GE values detected until two weeks post inoculation in *S. strinatii* represent dead *B. dendrobatidis* cells or some form of *B. dendrobatidis* DNA contamination rather than active infection. Thus it is possible that *S. strinatii* is extremely efficient at blocking epidermal colonization by *B. dendrobatidis* even when exposed to a highly concentrated and strong dose of *B. dendrobatidis* zoospores, but our experimental design prevents us from distinguishing between this and rapid clearing of infection. Since successful epidermal colonization by *B. dendrobatidis* requires keratinocyte invasion (Longcore *et al*., 1999; Berger *et al*., 2005; Van Rooij *et al*., 2012), we hypothesized that *Speleomantes* skin contains highly effective fungicidal properties that prevent skin invasion. Indeed, we showed that *Speleomantes* skin secretions were very efficient in killing *B. dendrobatidis* zoospores as assessed using a recently developed and highly reproducible assay (Blooi *et al*., 2013). Factors present in the skin secretions that account for the observed *B. dendrobatidis* killing need further identification but probably include antimicrobial peptides (AMP) (Woodhams *et al*., 2007; Ramsey *et al*., 2010; Rollins-Smith *et al*., 2011) and/or bacterially produced metabolites (Brucker *et al*., 2008a; 2008b, Harris *et al*., 2009). AMP's that play a defensive role against invasion by pathogenic microorganisms have been described for other Ambystomidae and plethodontid species (Fredericks *et al*., 2000; Davidson *et al.,* 2003; Sheafor *et al*., 2008) but not characterized. Hitherto, only the

antifungal metabolites 2,4-diacetylphloroglucinol, indol-3-carboxaldehyde and violacein have been identified that are secreted by symbiotic bacteria residing on the skin of plethodontid species *Plethodon cinereus* and *Hemidactylium scutatum* (Brucker *et al.*, 2008a; 2008b). Moreover, these metabolites may work synergistically with AMP's to inhibit colonization of the skin by *B. dendrobatidis* (Myers *et al.*, 2012). Further characterization of such AMP's and the composition of microbial skin communities, combined with the study of their assessment in plethodontid species and/or populations can open new perspectives for further understanding factors mediating resistance towards chytridiomycosis, its control and mitigation. In addition, a study of microbial skin communities has the potential to direct probiotic conservation strategies for susceptible species in the area.

The apparent absence of *B. dendrobatidis* and chytridiomycosis driven declines in *Speleomantes* throughout their range, lack of colonization or sustained infection in experimentally infected animals and pronounced *B. dendrobatidis* killing capacity of *Speleomantes* skin secretions together suggest the genus *Speleomantes* to be refractory to *B. dendrobatidis* infection and thus resistant to chytridiomycosis. Resistance to chytridiomycosis would at least in part explain the localized persistence of the genus *Speleomantes* in the presence of highly virulent *Bd*GPL strains in Europe. This situation differs markedly from some of the plethodontids in North America that are susceptible to infection and those in the Neotropics that underwent recent and sharp chytridiomycosis driven declines upon the arrival of *B. dendrobatidis* (Parra-Olea *et al.*, 1999; Gaertner *et al.*, 2009; Weinstein *et al.*, 2009; Rovito *et al.,* 2009; Hossack *et al.*, 2010; Cheng *et al.*, 2011; Caruso *et al.* 2012). While our results are preliminary evidence that the genus *Speleomantes* is a low-risk taxon for decline due to chytridiomycosis, we recommend additional studies that further investigate the risk *B. dendrobatidis* may pose to this unique amphibian taxon.

5. Acknowledgements

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GENERAL DISCUSSION

Many factors make *B. dendrobatidis* a significant concern, including its worldwide distribution, its rapid spread and high virulence, and the fact that it infects a broad diversity of amphibian host species leading to considerable losses in amphibian biodiversity (Daszak *et al.*, 1999; Skerratt *et al.*, 2007; Fisher *et al.*, 2009). The present research has added to an improved understanding of both *B. dendrobatidis* growth and development in amphibian skin and the role of skin secretions in determining the outcome of infection. In the following sections, the major findings of this research will be discussed in a broader context, with a glance at future research possibilities.

1. The Ussing chamber based explant model: a critical tool for pathogenesis studies only or is there more to it?

So far, infectivity and pathogenicity of *B. dendrobatidis* have been studied almost uniquely in *in vivo* infected animals and *in vivo* experimentation still is the 'golden standard' in chytrid research. However, to study the early pathogenesis of *B. dendrobatidis* in amphibian skin the development of an adapted *in vitro* model was essential for several reasons. For instance, serial sampling over short time-periods *in vivo* would require large numbers of experimental animals. Moreover, providing proper husbandry, with housing, food and care matching the physical and behavioral needs of each species, can be difficult (Pough, 1992).

Ussing chambers had previously been used by Voyles and colleagues (2009) to measure transepithelial potential differences in skin of naturally infected *L. caerulea* frogs. We adapted the set-up of these Ussing chambers for pathogenesis studies, allowing to infect and maintain amphibian skin explants for at least 4 days. Main advantage of these Ussing chambers is that, although the use of animals is required to provide skin, several conditions can be tested with the skin of one donor animal. In addition, a relatively small patch of skin can be exposed to very high dosages of *B. dendrobatidis* zoospores, resulting in a high number of invasion events. The elaboration and the fine-tuning of an Ussing chamber based *in vitro* model for pathogenesis studies met some obstacles, since several practicalities have to be taken into account when working with *B. dendrobatidis*. In contrast with most other pathogenic fungi, *B. dendrobatidis* is very susceptible to temperature changes, with temperatures above 25°C baleful for growth and viability. In addition, any factor immobilizing *B. dendrobatidis* during the initial infection process had to be ruled out.

Commonly used cell culture media, saline buffers and high centrifugation speed proved to adversely affect the motility of *B. dendrobatidis* zoospores.

It is clear that the Ussing chamber based *in vitro* model is meritorious for studying the development of *B. dendrobatidis* in amphibian skin, but can it be applied more widely? Recently, Brutyn *et al.* (2012) studied the effect of *B. dendrobatidis* zoospore supernatant on amphibian skin integrity using this model and successfully demonstrated disturbance of intercellular junctions by *B. dendrobatidis* secreted virulence related proteins. Several molecular and proteome-based studies have indicated metallo-, serine-, and aspartyl-proteases as putative *B. dendrobatidis* pathogenicity factors (Moss *et al.*, 2010; Brutyn *et al.*, 2012; Rosenblum *et al.*, 2012). In this regard, exposure of amphibian skin in an Ussing chamber to *B. dendrobatidis*, in combination with known protease inhibitors, could add substantially to the identification of effective pathogenicity factors. In addition, this model allows the study of cytoskeletal changes that take place in the host cell during invasion by *B. dendrobatidis*. Other applications include the identification of particular traits that make amphibian skin more or less receptive for infection such as skin structure, keratin composition, presence and composition of mucus secretions covering the epidermis. Likewise, this model has good prospects to determine to what extent innate host defenses limit skin colonization by *B. dendrobatidis*, but then it is imperative to know how long skin secretion stays active, once secreted on the skin and how long granular glands actively secrete host defense peptides in explanted skin. Finally, this model could find wide application in the study of 'invasiveness' of amphibian skin as predictor of susceptibility for infection. So far, no chytridiomycosis-related declines have been reported for native Belgian amphibian populations, with a prevalence of less than 0.2% recorded for *B. dendrobatidis* (Martel *et al.*, 2012). However, the susceptibility status of Belgian amphibians for *B. dendrobatidis* infection is not well-known. Studying invasiveness of *B. dendrobatidis* in these species, could help to identify highly susceptible species that need additional monitoring and draw up precise conservation strategies and priorities in case of chytrid epidemics.

2. Reconstructing the lifecycle of *B. dendrobatidis* in amphibian skin

Although the lifecycle *B. dendrobatidis* in culture has been well characterized, the development of *B. dendrobatidis* in amphibian skin was based on scarce data (i.e. mainly Longcore *et al.*, 1999; Pessier *et al.*, 1999; Berger *et al.*, 2005). The present research has elucidated the precise mechanism of host cell entry, intracellular development and spread

within the skin. Very recently, the findings of this research were partially confirmed by data obtained from experimentally infected bullfrogs (*Lithobathes catesbeianus*) and wood frogs (*Lithobathes sylvaticus*) (Greenspan *et al*., 2012). The observations of Greenspan *et al.* (2012) in these two Ranid species with a respectively low and high mortality risk for chytridiomycosis, corroborate encystment of *B. dendrobatidis* on the host skin, development of germ tubes that penetrate the host cell membrane, enabling transfer of the zoospore content into the host cell, finally giving rise to a new chytrid thallus at the distal end of the germ tube. Interestingly, on TEM images they clearly illustrate the formation of a septum segregating the chytrid thallus from the germ tube. Subsequently, immature thalli are carried to the epidermal surface by differentiating epidermal cells to appear in the stratum corneum at maturity. A synopsis of the lifecycle of *B. dendrobatidis* in amphibian skin, according Greenspan *et al.* (2012), in combination with the earlier work of Berger *et al*. (2005) is presented in **Figure 1**.
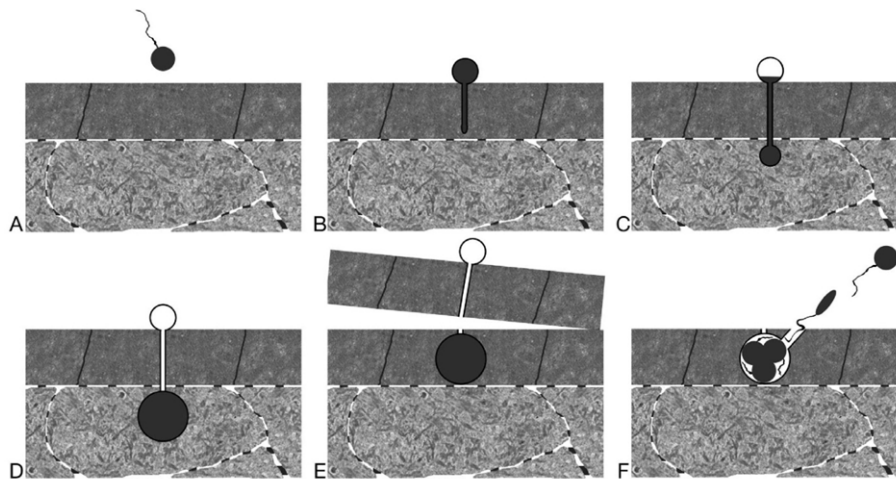

Figure 1. Schematic illustration of the *B. dendrobatidis* lifecycle in frog skin *sensu* Greenspan *et al.* (2012) (**A**) After a motile period, usually lasting ≤ 24 h, a zoospore attaches to the skin surface, retracts the flagellum, forms a wall of chitin around the spore body, and (**B**) produces a germination tube that penetrates the surface cell membrane of the host and usually extends through stratum corneum and (**C**) into deeper cell layers; cyst contents migrate into host tissue through the tube, and a single or colonial (not shown) zoosporangium arises from a portion of the tube within which cyst contents have accumulated; (**D**) a septum forms to segregate the zoosporangium from the tube; (**E**) host cells undergo maturation and outward movement as the zoosporangium develops, (**F**) maturation of the zoosporangium culminates in cleaving of zoosporangial contents into zoospores, formation of at least 1 zoospore-discharge papilla, and release of zoospores. Adapted from Greenspan *et al.* (2012).

In contrast with the findings of Greenspan *et al.* (2012) we did not observe any septa being formed at the junction between germ tube and zoosporangium. Our observations in *A. muletensis* and *L. caerulea* show that, at least for these species, the formation of an

intracellular thallus is not a final stage leading to maturing zoosporangia, as suggested in **Figure 1**. Instead, older intracellular thalli give rise to new intracellular thalli, spreading infection to the deeper layers of the epidermis. Berger *et al.* (2005) observed that *B. dendrobatidis* has its cycle tuned to the maturation of the epidermal cells. Immature sporangia are carried from the deeper skin layers to the skin surface by differentiating epidermal cells. At the time sporangia have developed discharge tubes and contain mature zoospores, they finally occur in stratum corneum where the zoospores are released in the environment. Consensus between both our observations, those of Berger *et al.* (2005) and Greenspan *et al.* (2012) leads to the reconstruction of the putative lifecycle of *B. dendrobatidis* in skin of susceptible amphibian species. During this endobiotic lifecycle, *B. dendrobatidis* develops intracellularly. A detailed overview is given in **Figure 2A**. The presence of intracellular chytrid thalli clearly contributes to the disease progression in susceptible animals, but the question remains whether 'internalization' of the chytrid fungus aids to evade the innate host defences. As such, more research is needed to define the biological advantage of spreading chytrid propagules to deeper skin layers.

Alternatively, **Figure 2B** presents the epibiotic lifecycle of *B. dendrobatidis* with sporangia developing on the skin, generally without intracellular sporangia. Hitherto, this type of development was only observed in *X. laevis* skin. Interestingly, encysted zoospore-like structures have been reported on the skin of pool frogs (*Pelophylax lessonae*), long before *B. dendrobatidis*-related declines occurred in Italy (Di Rosa *et al.*, 2007). It is clear that more observations are necessary before generalizing this saprophytic stage of *B. dendrobatidis* to other tolerant amphibian species and carriers.

3. Pathogenesis tactics of *B. dendrobatidis*: what is known and what do we need to know?

Although several studies, including the present research, have documented *B. dendrobatidis* growth and development at both morphological and ultrastructural level (Berger *et al.*, 2005; Greenspan *et al.*, 2012), our understanding of the whole infection process on molecular and cellular level, is limited. As outlined in **Figure 3** there are still several steps in the infection process that remain to be characterized. We will pick at random from the various possibilities.

Firstly, the kinetics of *B. dendrobatidis* adherence are unexplored. Specific attachment to a suitable host and induction of encystment are crucial for successful colonization and are
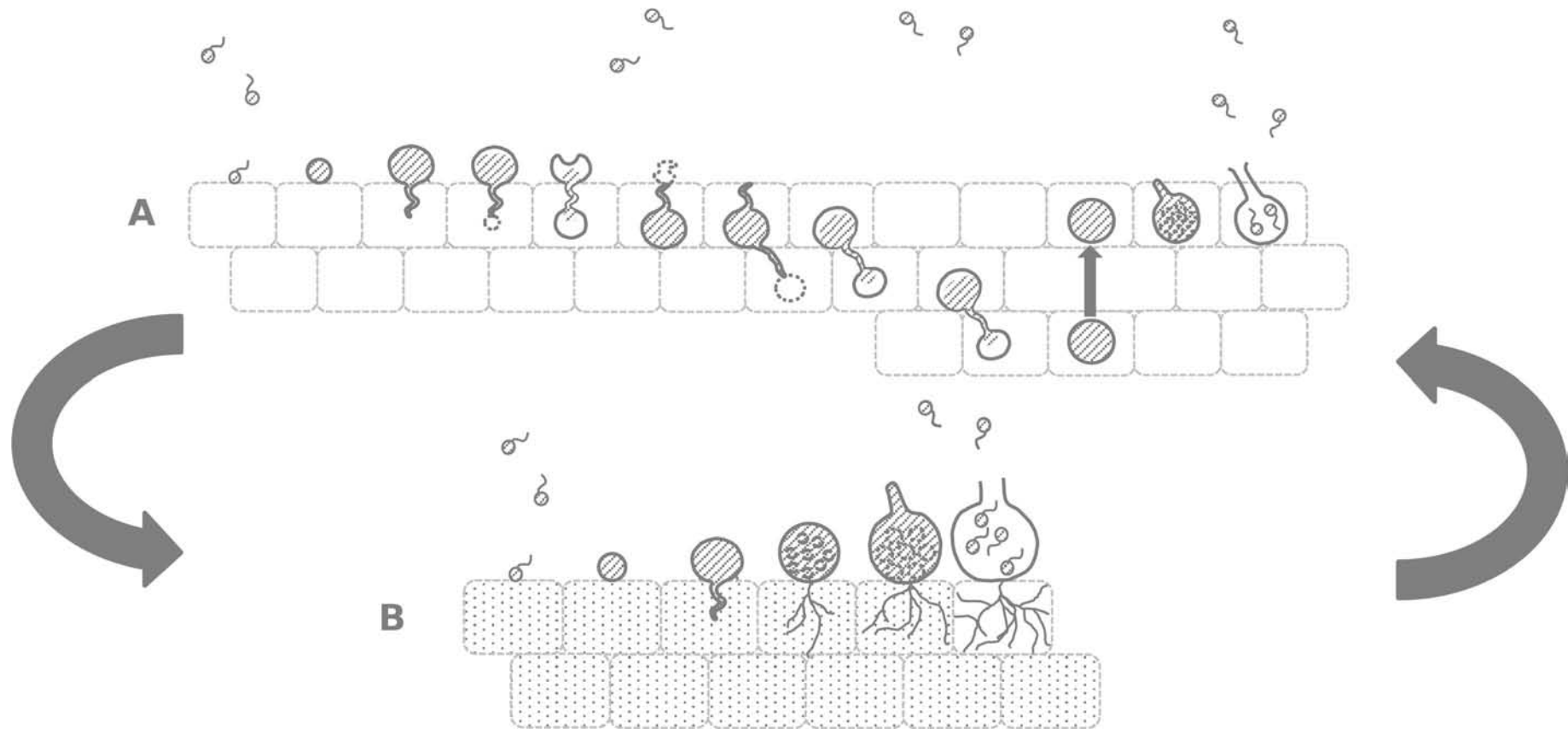
Figure 2. Schematic representation of the *B. dendrobatidis* lifecycle in susceptible host (A) and tolerant host (B). The endobiotic lifecycle in **(A)** includes successively germ tube mediated invasion, establishment of intracellular thalli, spread to the deeper skin layers, upward migration by the differentiating epidermal cell to finally release zoospores at the skin surface. The epibiotic lifecycle in **(B)** includes germ tube mediated invasion, outgrowth of a rhizoidal network, uptake of host cell cytoplasma as nutrient for the growing and maturing chytrid thallus upon the skin surface.

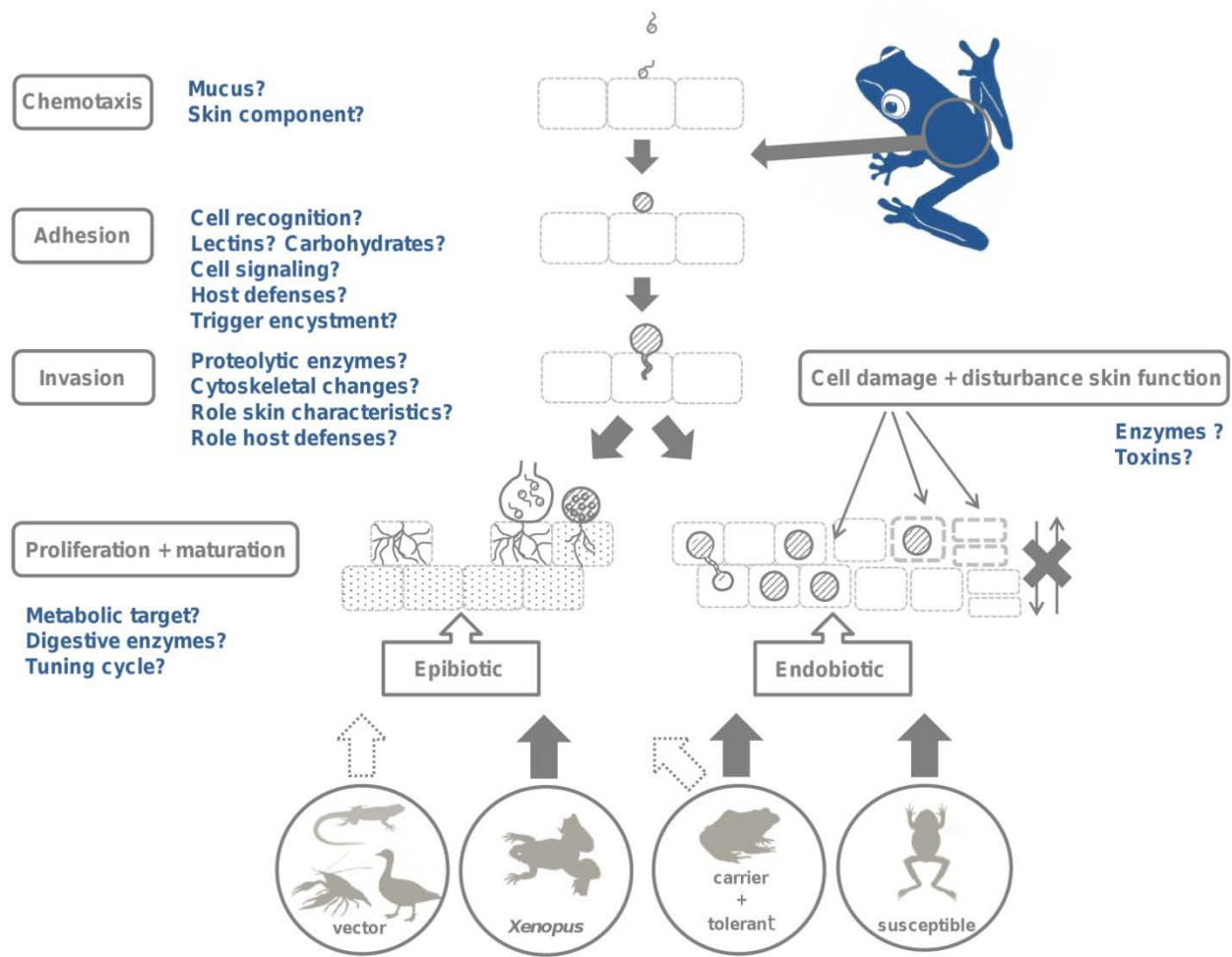**Figure 3**. **The proposed early pathogenesis of *B. dendrobatidis* in amphibian skin.** Factors involved in each pathogenesis step that remain to be fully characterized are indicated in blue; the relationship between the different host types and lifecycle types remaining to be specified are indicated with dotted arrows.

likely to involve receptors located at the cell surface of both host and pathogen. Adherence mechanisms are particularly well studied in *Candida albicans*, a human pathogenic yeast and include the action of mainly agglutinin-like and lectin-like proteins (reviewed in Tronchin *et al.*, 2008). Moreover, lectin mediated binding to specific host carbohydrates has been documented for numerous pathogenic bacteria (Boyle & Finlay, 2003), protozoa (Jacobsen & Boyle, 1996; Petri *et al.* 2002) and even zoosporic fungi (Deacon & Donaldson, 1993; Deacon & Saxena 1998, Gutman *et al.*, 2011). Further research could focus on *B. dendrobatidis* adhesins and their respective receptors on the host cell. Interestingly, identification and characterization of adherence mediators could open new perspectives for prevention and treatment of chytrid infection.

Secondly, the precise mechanism of host invasion is not fully understood. Both mechanical force exerted by the growing germ tubes and/or targeted secretion of substrate degrading enzymes germ tubes might aid *B. dendrobatidis* to penetrate the keratinized epidermis. Although several proteolytic enzymes secreted by *B. dendrobatidis* with high specificity for skin-related substrates have been identified (Rosenblum *et al.*, 2008; Moss *et al.*, 2010; Brutyn *et al.,* 2012) their precise role in the invasion process is to be examined. Cell damage including hyperkeratosis, hyperplasia, disturbance of intracellular junction and disruption of the vital skin function are thought to arise from fungal enzymes or metabolites including toxins that need further identification. Likewise, hitherto unidentified enzymes or metabolites secreted by *B. dendrobatidis* are found able to induce gill pathology and mortality in crayfish, a non-amphibian host for *B. dendrobatidis* (McMahon *et al.*, 2013). In analogy with dermatophyte infection it is also possible that besides fungal lytic enzymes, the host response to metabolic by-products from skin decomposition plays a substantial role in causing pathology (reviewed in Vermout *et al.,* 2008).

4. What is the key to tolerance for *B. dendrobatidis* infection?

Considering the rapid and global expansion of the chytrid fungus, there is a great need to identify mechanisms defining host resistance versus host susceptibility as these could open new perspectives on control and mitigation of *B. dendrobatidis* in susceptible amphibian species. As recently shown by Gervasi *et al.* (2013) intrinsic host traits alone can be important mediators of pathogen dynamics in the amphibian-chytrid fungus system. Indeed, our data suggest that the ability of *B. dendrobatidis* to rapidly invade the keratinized epithelium is related to host susceptibility. In the susceptible species studied, abundant intracellular

colonization of the skin occurred within 24 hours after infection with *B. dendrobatidis*, and at earliest within 8 hours after exposure. In contrast, in the tolerant *X. laevis*, sporangia develop upon the skin. These observations led to a first hypothesis that limitation of intracellular growth is a key to tolerance. Later experiments pointed out that intracellular thalli may sparsely occur in *X. laevis* skin. Consequently, we hypothesized that the degree of invasiveness of *B. dendrobatidis* and amphibian susceptibility is determined by inherent characteristics of the host skin. Yet, the properties of amphibian skin rendering it receptive to fungal colonization need to be determined.

There is a plethora of data underscoring the importance of anuran skin secretions as first line defense, reducing the infection load of *B. dendrobatidis* on frog skin to tolerable levels or even clearing frogs from infection (e.g. Woodhams *et al.*, 2006; 2007; Rollins-Smith, 2009; Ramsey *et al.*, 2010). We found that skin secretions of *Speleomantes*, a unique European representative of the plethodontid family, are capable of efficiently killing *B. dendrobatidis* zoospores and mediate resistance to *B. dendrobatidis* infection. Indeed, even despite the presence of an aggressive *B. dendrobatidis* lineage (*Bd*GPL) within the geographical range of *Speleomantes* (Farrer *et al.*, 2011) no chytridiomycosis-linked mortalities have been reported for *Speleomantes*, contrasting sharply with highly susceptible and declining neotropical and North American plethodontids (Rovito *et al.*, 2009; Cheng *et al.*, 2011; Caruso *et al.*, 2012). Future studies could extend this work in several ways. Antimicrobial peptides (AMP) present in these skin secretions most probably account for the marked fungicidal effect and need further identification as virtually nothing is known about both the diversity of the AMP arsenal in urodelan skin secretions. Identification of plethodontid AMP's could give interesting side-ways. AMP's, in general, seem excellent taxonomic and phylogenetic markers (Conlon *et al.*, 2004) and may be exploited for the study of adaptive evolution in plethodontid salamanders. In addition, AMP's represent potential templates for novel bioactive compounds with various therapeutic applications. Finally, the precise action of AMP's causing growth inhibition and killing of *B. dendrobatidis* remains to be defined. At present, the mechanisms of AMP action are under debate and both membrane disruption and cell internalization followed by disruption of intracellular targets have been proposed (Hancock & Rozek, 2002; Marcos & Gandia, 2009). Exposure of *B. dendrobatidis* zoospores to *Speleomantes* skin secretions shows an immediate effect on zoospore viability and thus the killing activity is hypothesized to be based on permeation of the zoospore's cell membrane and loss of cell integrity, but needs founded evidence.

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SUMMARY

Amphibians are currently facing massive and global declines, in part driven by the emerging infectious disease chytridiomycosis. The chytrid fungus *Batrachochytrium dendrobatidis*, etiological agent of chytridiomycosis, colonizes the superficial keratinized skin layers or keratinized larval mouthparts. Infection causes disruption of the skin function, compromising the amphibian's survival. A decade after the discovery of *B. dendrobatidis,* the knowledge of this disease's pathogenesis is in its infancy with fundamentals of host-pathogen interactions being poorly understood.

The general aim of the present research was to gain insight into the early pathogenesis of *B. dendrobatidis* in amphibian skin and host factors contributing to chytrid-susceptibility. The development and validation of several *in vitro* models as tools to model pathogenesis studies, was set as primary goal. Then, using the most convenient *in vitro* model, the early interactions between *B. dendrobatidis* and the skin of tolerant and susceptible amphibian hosts were characterized. As final goal, the role of skin secretions from European *Speleomantes* salamanders in determining susceptibility to *B. dendrobatidis* infection was studied.

At the onset of this research, neither existing *in vitro* models nor commercial amphibian epidermal cell lines were available, hampering detailed study of host-pathogen interactions. Thus, pathogenesis studies on *B. dendrobatidis* necessitated an adapted *in vitro* model using amphibian skin. In a first study, several set-ups were developed and included cultures of primary keratinocytes, stripped epidermal (SE) explants and full thickness epidermal (FTE) explants on Matrigel™, in cell culture inserts and Ussing chambers, respectively. Each model was evaluated based on its ability to maintain amphibian skin viability for several days and to mimic *B. dendrobatidis in vivo*. SE explants proved suitable for short-term studies, whereas FTE explants in an Ussing chamber could be maintained over a 4 to 5 day period. Due to its design, inoculum and tissue culture medium are strictly separated in an Ussing chamber, with the apical side of the skin exposed to the inoculum and the basolateral side exposed to nutrients from the tissue culture medium. The prolonged viability of tissues *ex vivo* allowing reproduction of the *B. dendrobatidis* life cycle *in vitro*, results in a marked reduction of the number of experimental animals to be sacrificed and makes the Ussing chamber based *in vitro* model a critical tool for pathogenesis studies.

In a second study, the early interactions of *B. dendrobatidis* with amphibian skin were compared between 3 host species from different anuran families, displaying a varying degree of susceptibility to *B. dendrobatidis* infection, i.e. the moderately susceptible Mallorcan midwife toad (*Alytes muletensis*), the highly susceptible green tree frog *(Litoria caerulea)* and the tolerant African clawed frog (*Xenopus laevis*). The lifecycle of *B. dendrobatidis* in the

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skin of these species was studied in an Ussing chamber based tissue explant model in which skin was exposed to *B. dendrobatidis* for 3 to 5 days and verified in experimentally inoculated frogs. Conventional light microscopy and transmission electron microscopy (TEM) of infected skin explants revealed the different steps in the infection process of *B. dendrobatidis* and included attachment of zoospores to the host skin, zoospore germination, germ tube development and penetration into the skin cells, followed by invasive growth in the host skin, finally resulting in the loss of the host cell cytoplasm. Within 24 hours after initial exposure, *B. dendrobatidis* developed endobiotically, with sporangia located intracellularly, in the skin of *A. muletensis* and *L. caerulea*. Additional *in vitro* experiments during which *L. caerulea* skin was exposed to *B. dendrobatidis* for 8, 16 and 24 hours showed that intracellular colonization is established via a germ tube, enabling transfer of genetic material into the host cell. Subsequently, the germ tube swells and develops into an intracellular chytrid thallus. The same tactics are used by *B. dendrobatidis* to spread to deeper skin layers, i.e. older thalli developed rhizoid-like structures spreading to deeper skin layers, form a swelling inside the host cell to finally give rise to a new thallus. Conversely, in the tolerant *X. laevis*, *B. dendrobatidis* generally only affected the superficial epidermis, with sporangia developing upon the skin (epibiotic), and using epidermal cells as a nutrient source. Based on these results, a host dependent colonization strategy of *B. dendrobatidis* is suggested, with the extent of colonization most likely determined by inherent characteristics of the host epidermis.

The last two studies focused on susceptibility to chytridiomycosis and related host factors in salamanders of the family Plethodontidae. These unique group of salamanders is marked by a particular species richness in North America and the neotropics. The only European plethodontid representatives of this family belong to the genus *Speleomantes*, which is restricted to a small geographic range comprising North-West Italy, Sardinia and South-East France. Plethodontid salamanders lack lungs, thus rely solely on skin respiration, which makes these salamander in theory highly susceptible for lethal chytridiomycosis. Compared to neotropical and North American plethodontids, mortality attributed to chytridiomycosis caused by *B. dendrobatidis* has not yet been reported for *Speleomantes* species, despite the omnipresence of *B. dendrobatidis* in their distribution area. It was necessary to first establish a non-invasive sampling protocol for salamanders that maximizes the detection probability of *B. dendrobatidis*. For this purpose dead captive plethodontid salamanders were sampled to determine the infection load of *B. dendrobatidis* per surface area for the chin, plantar side forelimb, dorsum, dorsal side tail, ventral side abdomen, plantar side hindlimb, and ventral

side of the tail. The pelvic region, hindlimbs, forelimbs and the ventral side of the tail came out as predilection sites for *B. dendrobatidis* and were considered best sampling regions to detect chytrid infections in salamanders. For validation of the resulting sampling protocol, 78 captive and wild Mexican bolitoglossine salamanders were examined for the presence of *B. dendrobatidis*. About 50% (17/33) of the dead captive salamanders tested positive for *B. dendrobatidis*, whereas none of the clinically healthy captive animals (n=28) were infected. Half of the wild salamanders (9/17) carried low zoospore loads without clinical signs of disease. Besides providing a useful swabbing protocol adapted for salamanders, these results added weight to the hypothesis of *B. dendrobatidis* being involved in recent bolitoglossine population declines, yet further studies are desirable to draw a causal link. Finally, the swabbing protocol was used to determine the extent to which *B. dendrobatidis* is present in wild populations of *Speleomantes* species in Europe. *B. dendrobatodis* was undetectable in all 8 *Speleomantes* species sampled, based on a total of 921 skin swabs. Subsequently, the susceptibility of *Speleomantes strinatii* to experimental infection with a highly virulent isolate of *B. dendrobatidis* (*Bd*GPL) was evaluated, using the susceptible species *A. muletensis* as positive control. Whereas *A. muletensis* developed increasing *B. dendrobatidis* loads over a 4 week period after initial exposure, none of the *S. strinatii* were colonized by *B. dendrobatidis* from 2 weeks after exposure onwards. Then, the extent to which skin secretions of *Speleomantes* species are capable of killing *B. dendrobatidis* was determined. *Speleomantes* skin secretions were markedly fungicidal for *B. dendrobatidis* and were likely to mediate resistance against chytridiomycosis in the salamanders of the genus *Speleomantes*. Factors present in *Speleomantes* skin secretions accounting for the fungicidal effect on *B. dendrobatidis* putatively involve antimicrobial peptides (AMP) but require further identification. The absence of *B. dendrobatidis* in wild populations of *Speleomantes*, combined with resistance to experimental infection and the highly efficient skin secretions, predict the unlikelihood of *Speleomantes* salamanders undergoing chytridiomycosis-related declines.

To conclude, the present research has provided new perspectives for the study of host-pathogen interactions and has led to new insights in the colonization process of the fungal pathogen *B. dendrobatidis* in susceptible and tolerant hosts. Especially the observation of germ tubes involved in the infection process is novel and the morphological process by which intracellular colonization is established and how colonization by *B. dendrobatidis* proceeds to deeper skin layers had yet not been reported. Studies in plethodontid salamanders evidenced a

sharp differential susceptibility between neotropical and European taxa and highlight the importance of *Speleomantes* skin secretions in averting *B. dendrobatidis* infection.

SAMENVATTING

Chytridiomycose is een opkomende infectieziekte die wereldwijd aanzienlijke schade toebrengt aan amfibieënpopulaties. De ziekteverwekker is de schimmel *Batrachochytrium dendrobatidis*, die de verhoornde monddelen van amfibieënlarven en de verhoornde huidlagen van volwassen amfibieën koloniseert. Als gevolg van deze huidaandoening wordt de normale werking van de huid verstoord, vaak met de dood tot gevolg. Veertien jaar na de ontdekking van *B. dendrobatidis* is er nog steeds weinig geweten over de pathogenese van chytridiomycose.

Het doel van deze thesis was te onderzoeken hoe infectie van de huid door *B. dendrobatidis* tot stand komt en waarom niet alle amfibieën even gevoelig zijn voor chytridiomycose.

In een eerste studie werden verschillende modellen op punt gesteld waarmee de interacties tussen schimmel en gastheer in detail bestudeerd kunnen worden. Zowel primaire celculturen van keratinocyten werden getest, als explantaten van enkel de verhoornde huidlagen. Bovendien werden explantaten van de volledige huiddikte getest, eerst op een Matrigel^{TM} coating, later in celcultuur inserts en uiteindelijk in Ussingkamers. Deze opstellingen werden beoordeeld op hun mogelijkheid om amfibieënhuid in leven te houden gedurende verschillende dagen én de levenscyclus van *B. dendrobatidis* te reproduceren. Explantaten van enkel de verhoornde huid bleken vooral geschikt voor kortetermijnstudies, terwijl explantaten van de volledige huiddikte 4 tot 5 dagen levensvatbaar konden gehouden worden in een Ussingkamer. Door de specifieke opbouw van de Ussingkamer zijn het inoculum en het weefselcultuurmedium strikt van elkaar gescheiden. Op die manier wordt de apicale zijde van de huid enkel blootgesteld aan het inoculum en de basolaterale zijde van huid enkel aan het weefselcultuurmedium. De langere levensduur van het geïsoleerde weefsel in de Ussingkamer laat toe de volledige levenscyclus van *B. dendrobatidis in vitro* te bestuderen. Bovendien vereist dit model slechts een beperkt aantal proefdieren om verschillende infectiecondities te testen. Voor de verdere studie van de pathogenese van chytridiomycose werd er bijgevolg gekozen voor het *in vitro* model dat gebruik maakt van Ussingkamers.

In een tweede studie werden de vroege interacties van *B. dendrobatidis* met amfibieënhuid vergeleken voor 3 gastheren van verschillende amfibieënfamilies, elk met een verschillende gevoeligheid voor *B. dendrobatidis* infectie: de matig gevoelige Mallorca vroedmeesterpad (*Alytes muletensis*), de hoog gevoelige koraalteenkikker (*Litoria caerulea*) en de ongevoelige Afrikaanse klauwkikker (*Xenopus laevis*). De levenscyclus van *B.*

dendrobatidis in de huid van deze soorten werd bestudeerd in het eerder ontwikkelde Ussingkamer *in vitro* model, waarin huid gedurende 3 tot 5 dagen blootgesteld werd aan *B. dendrobatidis*. Een simultaan experiment volgde de groei en ontwikkeling van *B. dendrobatidis* bij *in vivo* geïnfecteerde gastheren. Conventionele lichtmicroscopie en transmissie elektronenmicroscopie (TEM) lieten toe het infectieproces van *B. dendrobatidis* in de huid vast te leggen. Vijf opeenvolgende fasen werden daarbij waargenomen: de vasthechting van zoösporen aan de huid van de gastheer, het ontkiemen van de zoöspore, de uitgroei van een kiembuis die binnendringt in de huidcellen, verdere invasieve groei in de huid van de gastheer en het uiteindelijk verlies van het cytoplasma van de gastheercel. Het *in vitro* model toonde aan dat binnen de 24 uur volgend op inoculatie, *B. dendrobatidis* zich endobiotisch ontwikkelt in de huid van *A. muletensis* en *L. caerulea* en daarbij de typerende intracellulaire sporangia vormt. Bijkomende experimenten waarin de huid van *L. caerulea* kikkers gedurende 8, 16 en 24 uur werden blootgesteld aan *B. dendrobatidis*, toonden aan dat intracellulaire kolonisatie van de huid gebeurt door middel van een kiembuis, waarlangs genetisch materiaal in de gastheercel wordt binnengebracht. Deze kiembuis zwelt op en ontwikkelt zich vervolgens tot een intracellulaire chytrid thallus. *B. dendrobatidis* volgt dezelfde strategie om de dieper gelegen huidlagen te koloniseren. Hierbij vormen oudere thalli rhizoïde-achtige structuren die uitgroeien tot in de diepere huidlagen, vervolgens binnendringen in een nieuwe gastheercel, om daarin op te zwellen en uit te groeien tot een nieuwe thallus. In ongevoelige *X. laevis* kikkers echter, tast *B. dendrobatidis* enkel de bovenste verhoornde huidlaag aan. Hierbij vormt de schimmel sporangia bovenop de huid (epibiotisch) en dient de gastheercel als voedselbron. Deze resultaten suggereren dat de kolonisatiestrategie van *B. dendrobatidis* verschilt naargelang de gastheer. Hoogstwaarschijnlijk bepalen de inherente eigenschappen van de huid mee de mate waarin zij gekoloniseerd wordt.

In de derde en vierde studie staat de gevoeligheid van longloze salamanders (familie Plethodontidae) voor chytridiomycose centraal. Deze unieke groep van salamanders kent een bijzonder grote biodiversiteit in Noord-Amerika en de neotropen. De Europese vertegenwoordigers van deze familie behoren tot het geslacht *Speleomantes*, en kennen een beperkte geografische verspreiding in noordwest Italië, Sardinië en zuidoost Frankrijk. Zoals de soortnaam doet vermoeden zijn longloze salamanders volledig aangewezen op huidademhaling. Bijgevolg worden deze soorten bijzonder gevoelig beschouwd voor letale chytridiomycose. In tegenstelling tot neotropische en Noord-Amerikaanse longloze salamanders, werd sterfte door chytridiomycose nog niet waargenomen bij *Speleomantes*

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soorten. Nochtans is *B. dendrobatidis* alomtegenwoordig in het verspreidingsgebied van *Speleomantes*. Om de verspreiding en het voorkomen van *B. dendrobatidis* in Europese longloze salamanders te onderzoeken, was het noodzakelijk eerst een niet-invasief protocol voor staalname op punt te stellen. Het protocol moest de kans op detectie van *B. dendrobatidis* optimaliseren. Hiervoor werden longloze salamanders, gestorven aan de gevolgen van chytridiomycose, bestudeerd om na te gaan welke lichaamsdelen consistent met *B. dendrobatidis* geïnfecteerd waren. Zowel de kin, de palmaire zijde van de voorpoot en achterpoot, de rug, de dorsale en ventrale zijde van de staart en de ventrale zijde van het abdomen werden bemonsterd met een swab om de zoösporenaantallen van *B. dendrobatidis* per oppervlakte-eenheid huid te bepalen. Het bekken, de voor- en achterpoten en de ventrale zijde van de staart bleken bij voorkeur geïnfecteerd door *B. dendrobatidis* en zijn dus de beste plaatsen voor staalname bij deze salamanders. Voor validatie van het protocol voor staalname werden in totaal 78 in het wild en in gevangenschap levende neotropische longloze salamanders getest op de aanwezigheid van *B. dendrobatidis*. Van de dieren in gevangenschap bleek bij benadering 50% (17/33) van de dode salamanders positief voor *B. dendrobatidis*, terwijl geen enkele van de levende, klinisch gezonde dieren (n=28) geïnfecteerd was. Ongeveer de helft (9/17) van de wildlevende salamanders droeg een laag aantal zoösporen zonder tekenen van klinische ziekte. Naast het verstrekken van een specifiek protocol voor staalname bij salamanders, leveren deze resultaten bijkomend bewijs dat *B. dendrobatidis* kan betrokken zijn in de recente achteruitgang van neotropische salamanderpopulaties.

Als laatste doel werd het verband onderzocht tussen de huidsecreties van salamanders behorende tot het geslacht *Speleomantes*, en hun mogelijke gevoeligheid voor *B. dendrobatidis* infectie. Eerst werd het bovengenoemde protocol voor staalname gebruikt om te bepalen in welke mate *B. dendrobatidis* aanwezig is in de wilde populaties van Europese *Speleomantes* salamanders. In totaal werden 921 stalen genomen bij alle 8 gekende Europese *Speleomantes* soorten. *B. dendrobatidis* werd geen enkele keer aangetroffen in de stalen. Vervolgens werd de gevoeligheid van *Speleomantes strinatii* geëvalueerd na experimentele infectie met een hoog virulent isolaat van *B. dendrobatidis* (*Bd*GPL). Daarbij werd de gevoelige soort *A. muletensis* gebruikt als positieve controle. Tijdens de eerste weken volgend op inoculatie ontwikkelden *A. mulentensis* padden toenemende zoösporenaantallen van *B. dendrobatidis* op de huid. Alle *S. strinatii* salamanders testten 2 weken na inoculatie nog steeds negatief voor *B. dendrobatidis.* Hieropvolgend werd nagegaan of, en in welke mate *B.*

dendrobatidis afgedood wordt door de huidsecreties van *Speleomantes* salamanders. Deze huidsecreties toonden een duidelijk fungicide werking tegen *B. dendrobatidis* en bepalen waarschijnlijk in belangrijke mate resistentie van *Speleomantes* salamanders tegen chytridiomycose. Dit fungicide effect is vermoedelijk afkomstig van antimicrobiële peptiden (AMP) als onderdeel van het huidsecreet. Uit de afwezigheid van *B. dendrobatidis* in wilde *Speleomantes* populaties, de resistentie van deze salamanders tegen experimentele infectie en hun hoogst efficiënte huidsecreties, kan besloten worden dat sterfte van *Speleomantes* salamanders ten gevolge van chytridiomycose weinig waarschijnlijk is.

Het huidige onderzoek heeft geleid tot een betere kennis van het kolonisatieproces van *B. dendrobatidis* in gevoelige en ongevoelige gastheren. De betrokkenheid van kiembuizen in het infectieproces was tot nu toe onbekend alsook de manier van intracellulaire kolonisatie en de verspreiding van de infectie tot in de diepere huidlagen. De studie van *B. dendrobatidis* infecties in longloze salamanders getuigt van een schril contrast in de gevoeligheid tussen neotropische en Europese taxa en benadrukt het belang van huidsecreties in de resistentie van *Speleomantes* salamanders tegen infecties met *B. dendrobatidis*.

CURRICULUM VITAE

Pascale Van Rooij werd geboren op 22 september 1978 te Borgerhout. Na het behalen van het diploma secundair onderwijs, richting Grieks-Latijn aan het Sint-Jozefinstituut te Antwerpen, startte ze in 1996 de studies Biologie aan de Universiteit Gent. In 2001 behaalde ze het diploma van Licentiaat in de Biologie (optie Plantkunde) met onderscheiding.

Na een korte zijsprong in het onderwijs als leerkracht Fysica en Chemie aan het Sint-Lievenscollege te Antwerpen, trad zij in dienst als wetenschappelijk attaché bij de dienst Mycologie van het Wetenschappelijk Instituut Volksgezondheid (WIV). Daar verrichtte zij onderzoek naar de incidentie en epidemiologie van tinea capitis in België, gefinancierd door het Federaal Wetenschapsbeleid (BELSPO). Achtereenvolgens was zij werkzaam als curator-assistent en curator van de BCCM/IHEM cultuurcollectie van biomedische schimmels en gisten.

In september 2008 verliet zij het WIV om haar doctoraatsonderzoek aan te vatten bij de vakgroep Pathologie, Bacteriologie en Pluimveeziekten aan de Faculteit Diergeneeskunde, Universiteit Gent. Deze studie werd gefinancierd door het Bijzonder Onderzoeksfonds (BOF) van de universiteit. Onder begeleiding van Prof. dr. F. Pasmans en Prof. dr. A. Martel, voerde zij gedurende 4 jaar onderzoek naar de vroege pathogenese van *Batrachochytrium dendrobatidis* infecties bij amfibieën.

Haar wetenschappelijk onderzoek leidde tot meerdere wetenschappelijke publicaties in internationale tijdschriften. Ze nam tevens deel aan verschillende nationale en internationale congressen.

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