Mitigating *Batrachochytrium salamandrivorans* infections in salamanders: strategies, intestinal health and host colonization

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LIST OF ABBREVIATIONS

AMPs	antimicrobial peptides
ASV	amplicon sequence variant
Bd	*Batrachochytrium dendrobatidis*
*Bd*GPL	*Batrachochytrium dendrobatidis* global pandemic lineage
*Bd*CAPE	*Batrachochytrium dendrobatidis* Cape lineage
BLAST	basic local alignment search tool
BSA	bovine serum albumin
Bsal	*Batrachochytrium salamandrivorans*
CBMs	carbohydrate binding modules
CI	confidence interval
Con A	concavalin A
CRN	crinkler-and-necrosis
CV	coefficient of variation
DGHT	Deutsche Gesellschaft für Herpetologieand Terrarienkunde
DNA	deoxyribonucleic acid
EAZA	European Association of Zoos and Aquaria
EID	emerging infectious disease
ELISA	enzyme-linked immunoassay
EPH	endemic pathogen hypothesis
FDR	false discovery rate
FPKM	fragments per kilobase million
GalNAc	N-Acetylgalactosamine
GC	guanine-cytosine
GE	genomic equivalents
GlcNAc	N-Acetylglucosamine
GLMM	generalized linear mixed models
GO	gene ontology
GPI	glycosylphosphatidylinositol
HPLC	high-performance liquid chromatography
IUCN	International Union for Conservation of Nature
IP	infection prevalence

LIST OF ABBREVIATIONS

IQR	Interquartile range
KEGG	Kyoto Encyclopedia of Genes and Genomes
LMM	linear mixed model
MHC	major histocompatibility complexes
ml	millilitre
µg	microgram
µl	microliter
NPH	novel pathogen hypothesis
OIE	office international des epizooties
OTU	operational taxonomic unit
PBS	Phosphate-buffered saline
PCR	polymerase chain reaction
PI	protease inhibitor
qPCR	quantitative polymerase chain reaction
RCA	*Ricinus communis* agglutinin
RDA	redundancy analyses
RIPA	radioImmunoprecipitation assay
RNA	ribonucleic acid
RNase H	ribonuclease H
RNA-seq	ribosomal ribonucleic acid sequencing
RPKM	reads per kilobase per million
rRNA	ribosomal ribonucleic acid
SAMs	steroidal alkaloids
SEM	standard error of the mean
SMI	scaled mass index
TGhL	tryptone-gelatin hydrolysate-lactose
UV	ultraviolet

GENERAL INTRODUCTION

GENERAL INTRODUCTION

1. The amphibian host

1.1 Introduction of Amphibia

The class of Amphibia is comprised of mostly four-limbed, ectothermic vertebrates, and includes the taxonomic orders of Anura (frogs and toads), Caudata (salamanders and newts) and Gymnophiona (caecilians) (Frost, 2021). Anura is the most diverse order of amphibians, accounting for 88% of species in the amphibian class, and encompassing at least 55 families (Frost, 2021). The order Caudata, also known as tailed amphibians, comprises about 700 species, representing all ten families of current living salamanders (Frost, 2021). Gymnophiona is the least known amphibian order, since their subterranean or streambed habitats make it very difficult to study them in detail. The order of Gymnophiona entails all currently living, as well as extinct, legless, worm- or serpentine shaped amphibians. It is also the least diverse order of amphibians, with only 200 species having been discovered to date (Goin, O.B and G.W, 1978; Frost, 2021). The order of Anura has many families with aquatic larvae stage, but also contains families with direct development (no tadpoles), such as family Brachycephalidae, Craugastoridae and Eleutherodactylidae. Some families from Caudata order are entirely aquatic at all life stages, such as Proteidae, Sirenidae, Amphiumidae and Cryptobranchidae, but also some families that are completely terrestrial at all life stages, such as the genera *Aneides*, *Ensatina*, and *Plethodon* in family Plethodontidae (lungless salamanders). Gymnophiona, in turn, have semi-aquatic larvae in some families but others already metamorphosed after hatch (Crump, 2009). Whether animals spend their larval and adult stage in aquatic or terrestrial environments depends on the specific species and many species are able to live both in water and on land after metamorphosis (Crump, 2009). In general, all amphibians require moist environments to keep their skin moisturized, in order to assist in oxygen uptake and to prevent desiccation, and therefore are often found close to rivers, ponds and lakes (Crump, 2009).

1.2 Life cycle of Amphibia

The typical development of amphibians consists of two distinct life stages, the larval and the adult stage. Most amphibians lay their eggs in the water or in moist environment and place them hidden under objects or stick to objects in their surroundings (Goin and Goin, 1962), but some species

give birth to live offspring, as for example the fire salamander (*Salamandra salamandra*), alpine salamander (*Salamandra atra*), and Tornier's forest toad (*Nectophrynoides tornieri*) (Frost, 2021). Amphibian eggs are protected by a transparent jelly capsule, which not only functions as an insulator for temperature regulation and gas exchange, but also plays a crucial role in the initial steps of fertilization (Shaver and Barch, 1960; Burggren, 1985; Seymour and Bradford, 1987). Amphibian egg jelly has been known to facilitate the chemoattraction and binding of sperm (Al-Anzi and Chandler, 1998; Olson and Chandler, 1999) and induce sperm motility and acrosome reaction (Simmons, Roberts and Dziminski, 2009). Larvae of anurans (tadpoles) have a distinct morphology compared with adults. Their main body mass is focussed on the front, which has a streamlined, rounded appearance, often lacking limbs, but possessing a laterally compressed tail, perfectly adapted for movement in their aquatic environment. Tadpoles have external gills after hatching, but these are subsequently internalized (Rugh, 1951). Salamander larvae look more like their adult counterparts. However, they also still possess a tailfin and external gills (Zug and Duellman, 2020). After hatching, most salamander larvae first develop forelimbs then followed by hindlimbs (Collazo and Marks, 1994). Tadpoles usually consume a wide variety of food, consisting of planktonic materials, anuran eggs, small insects and even other tadpoles, but most tadpoles are primarily herbivores (Kupferberg, Marks and Power, 1994; Kupferberg, 1997; Altig, Whiles and Taylor, 2007). Salamander larvae are carnivorous and eat small aquatic invertebrates (Zug and Duellman, 2020). At the end of the larval stage, many amphibian species undergo to some extent metamorphosis, to develop into the adult stage.

1.2.1 Amphibian metamorphosis

Metamorphosis is regulated by thyroid hormone levels in their blood (Rosenkilde, 1985; Galton, 1988), but is also affected by environmental factors, such as temperature (Gomez-Mestre and Buchholz, 2006), water abundance (Michimae and Emura, 2012), food quantity (Alvarez and Nicieza, 2002), pesticide exposure (Bridges, 2000), and genetic factors (Stolow *et al.*, 1997). Once metamorphosis starts, the biological process happens relatively fast, to limit the chance of exposing incompletely transformed amphibians to predators. During metamorphosis, amphibians undergo many dramatic changes in the aspect of morphology and physiology, such as alteration of gene expression, tissue restructuring, organ remodelling and programmed cell death, to prepare the

aquatic larvae for adaptation in a terrestrial environment (Tata, 1993; Wang and Brown, 1993; Nishikawa and Hayashi, 1994; Nakajima, Fujimoto and Yaoita, 2005). The extent of morphological changes varies between urodeles and anurans. In urodeles, the change in morphology is mostly limited to the resorption of the tail fin, remodelling of skin structure, and loss of external gills, as well as development of the lung and skin respiratory apparatus (Smirnov, 2005). Anurans exhibit more substantial morphological changes, as almost every organ is subject to change, including the development of four limbs, disappearing of the paddle tail, skull development from cartilage the bone, and reshaping of the mouth and jaw (Saha and Gupta, 2012).

Many amphibian species use the lungs as the primary organ for respiration. The formation of lungs is also a significant aspect of metamorphosis, but not all species will develop lungs. Some small terrestrial amphibian species, such as Bornean flat-headed frog (*Barbourula kalimantanensis*) and lungless salamanders absorb oxygen entirely through their skin and mucous membranes (Lanza, Vanni and Nistri, 1998; Bickford, Iskandar and Barlian, 2008). Although many amphibian species undergo metamorphosis, some species can bypass this process before maturity. This is called paedomorphosis or neoteny. For example, axolotl (*Ambystoma mexicanum*) and olm (*Proteus anguinus*) retain the larval external morphology, while reaching sexual maturity (Frost, 2021).

1.2.2. Amphibian postembryonic developmental stages

Based on the metamorphosis of anurans, the postembryonic development has been defined into four stages: premetamorphosis (PreM), prometamorphosis (ProM), metamorphic climax (Meta) and completion of metamorphosis (PostM) (Etkin, 1964). However, only three stages (PreM, ProM and Meta) were categorized for the development of fire salamander larvae (Sanchez *et al.*, 2018), by morphological analysis (Table 1, Figure 1).

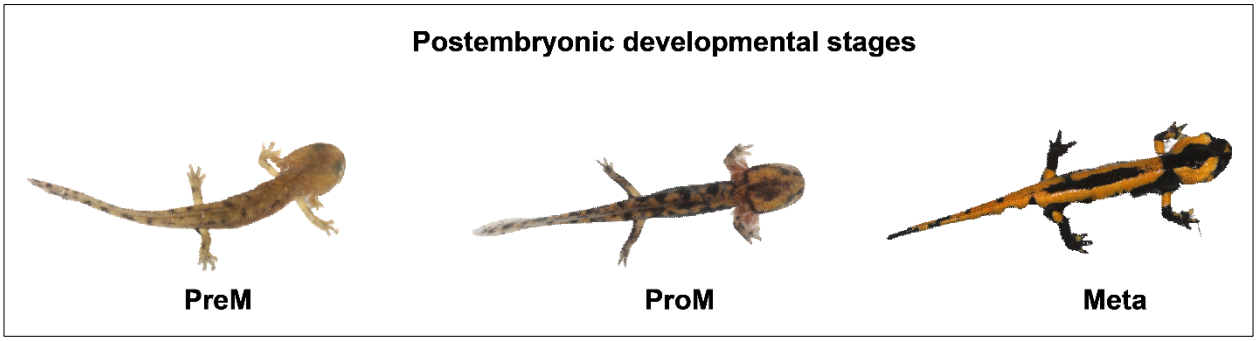


Figure 1. Phenotype of typical larvae in each postembryonic developmental stage. Developmental stages: PreM, premetamorphosis; ProM, prometamorphsis; Meta, metamorphic climax. Adapted from Sanchez *et al.*, (2018).

Morphological changes	Postembryonic developmental stages		
	PreM	ProM	Meta
Skin pigmentation	Slightly darker	Black and light brown color pattern	Black and yellow color pattern
Tail fins	Dorsal fin (aprox. 1.5 mm high) starts in the middle of the back. Ventral fin (aprox. 1.5 mm high) starts at the cloaca.	Show degression	Completely reduced
External gills	Same color with body	Dark pink color	Completely reduced

Table 1. Changes of morphological characters for different fire salamander postembryonic development. Developmental stages: PreM, premetamorphosis; ProM, prometamorphsis; Meta, metamorphic climax. Adapted from Sanchez *et al.*, (2018)

1.3 Amphibian integumentary system

The amphibian integument is the largest organ by size and plays a crucial role for amphibians, since it not only participates in respiration, but also contributes to water balance, temperature regulation, osmoregulation, ion transportation and sensorial perception (Wakeman and Ultsch, 1975; Mullen and Alvarado, 1976; Brattstrom, 1979; Feder and Burggren, 1985; Toledo and Jared, 1993; Eisthen, 2000).

1.3.1. Anatomy of amphibian skin

GENERAL INTRODUCTION

The adult amphibian skin consists of two principal layers, the outermost layer of skin, epidermis, and an underlying layer, dermis (Figure 2). The epidermal layer is in direct contact with the external environment and functions as the primary barrier from the body to the environment. The epidermis is subdivided into layers, as indicated, from outside to inside: stratum corneum, intermixed stratum granulosum and stratum spinosum, stratum germinativum, and the basement membrane. Stratum corneum is usually composed of one or two layers of keratinized cells. This layer of skin sheds on a regular basis and is often eaten by the animal itself, to recycle nutrients (Weldon, Demeter and Rosscoe, 1993). Sloughing of the stratum corneum is known to regulate cutaneous microbes, by periodically removing resident populations of bacteria and fungi on the skin surface (Meyer *et al.*, 2012; Cramp *et al.*, 2014; Colombo *et al.*, 2015). The stratum granulosum and stratum spinosum consists of three to five cell layers of cuboidal or polyhedral cells. The stratum germinativum, also called basal layer, contains cuboidal or columnar cells, and these cells migrate to the skin surface, past the stratum granulosum and stratum spinosum, to replace the stratum corneum. On the cell surface these cells, originating from the stratum germinativum, mature and eventually become keratinized. Below the stratum germinativum is the basement membrane, made up by protein fibres, which is made from both the epidermis and the dermis. The amphibian dermis is comprised of two layers: the upper stratum spongiosum layer and lower stratum compactum layer. The stratum spongiosum is made up of loose connective tissue, capillaries, smooth muscle tissue and exocrine glands (granular and mucous glands). The stratum compactum layer is formed by compactly organized connective fibres. The stratum compactum adheres loosely beneath connective tissue in anurans but is tightly attached in salamanders (Fox, 1994).

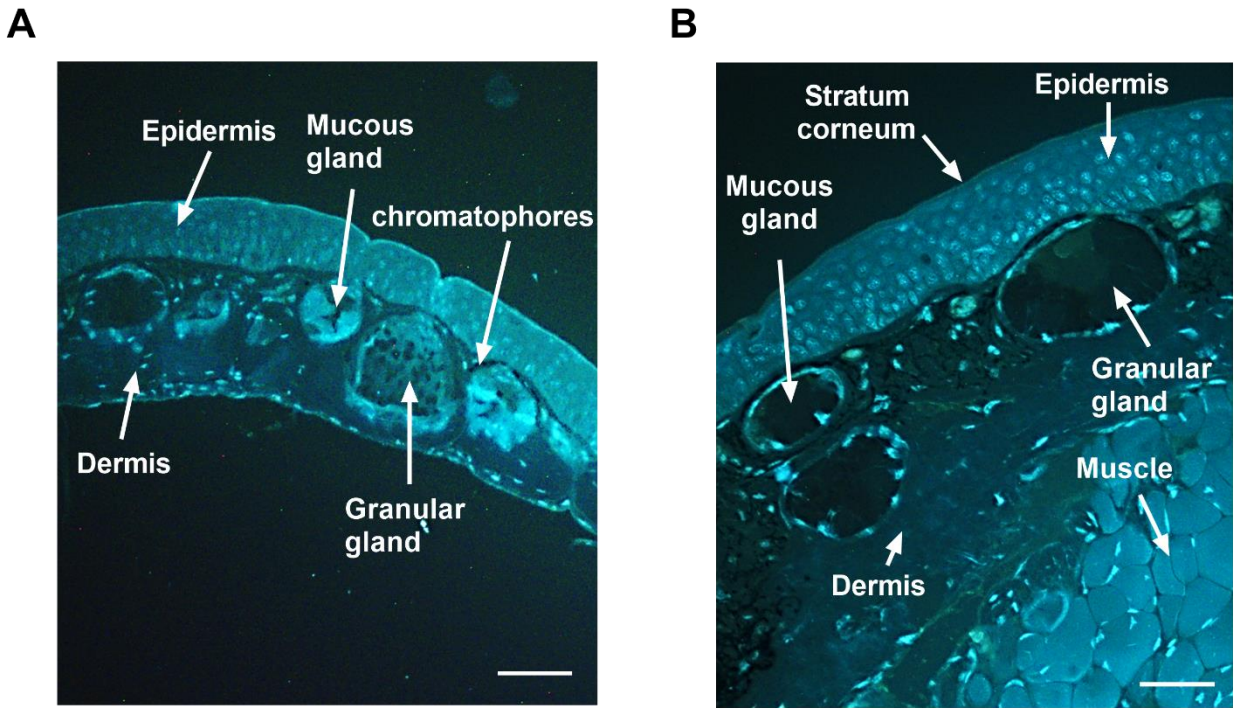


Figure 2. Anatomy of adult amphibian skin. Generalized features of Hoechst-stained ventral skin section of (A) *Alytes obstetricans* and (B) *Salamandra salamandra*; scale bar = 200 µl.

1.3.2. Exocrine glands and secretions of amphibian skin

Generally, two basic types of cutaneous glands are present in amphibians, namely mucous and granular glands (Clarke, 1997). The secretions of mucous and granular glands, together with the skin microbiome, make up the mucosome on the amphibian skin surface. Mucous glands are exocrine and distributed over the entire surface of the skin. These mucous glands produce polymeric, high molecular weight, *O*-linked glycoproteins, also called mucins (Thornton, Rousseau and McGuckin, 2008). The carbohydrate portion of mucin glycoproteins consists, but is not limited to: α-L-fucose, α-D-*N*-acetylgalactosamine, β-D-*N*-acetylglucosamine, *N*-acetylneuraminic acid, α-D-galactose and α-D-mannose (Roussel and Delmotte, 2004; Meyer *et al.*, 2007). The skin mucosal layer fulfils the same function, as it does in many other species, that is to say, as a primary frontier, or safety net, to saturate their skin with antigens, where bacterial, viral or fungal proteins harmlessly attach to and are being removed with mucins with their pathogenic receptors (Thornton *et al.*, 1990; Roussel and Delmotte, 2004; Dubaissi *et al.*, 2018). Therefore, the mucus layer is essential as a physical barrier to pathogens.

Granular glands are holocrine, and the localization and distribution varies among species. To name some examples, granular glands are uniformly distributed on both ventral and dorsal skin for western clawed frog (*Xenopus tropicalis*), evenly distributed along the body for eastern narrow-mouthed toad (*Gastrophryne carolinensis*), distributed on the back, neck and shoulders for toads (genus *Bufo*), distributed mainly around the head, tail and dorsal skin of fire salamanders. and no granular glands whatsoever are found in the skin of Angola river frog (*Rana angolensis*) (Toledo and Jared, 1995). Granular glands produce defensive and poisonous secretion, that are modulated by psychological conditions like irritation, stress, or adrenergic stimulation (Myers, Paolillo O and Daly, 1991; Smith *et al.*, 2000; Toledo, Sazima and Haddad, 2011), but the granular glands of non-stressed frogs were also found to release secretions in low amounts (Pask, Woodhams and Rollins-Smith, 2012). These secretions contain diverse bioactive compounds such as alkaloids, steroids, amines, antibodies and antimicrobial peptides (AMPs) (Roseghini *et al.*, 1989; Daly, Spande and Garraffo, 2005; Mebs and Pogoda, 2005; Rollins-Smith, 2009; Vences *et al.*, 2014), but the exact make-up of the excretions differs between species. These antimicrobial compounds, especially AMPs, are an important component of the amphibian innate immune response against pathogenic bacteria, viruses and fungi (Haney *et al.*, 2009; Lan *et al.*, 2010; Ramsey *et al.*, 2010). Granular glands also play a crucial role in predator defense. For example, fire salamanders can produce unique steroid alkaloid (such as samandarine and samadarone) (Table 2) through their granular glands and actively spray their predators (Brodie and Smatresk, 1990; Pezaro *et al.*, 2017; Lüddecke *et al.*, 2018). These neurotoxins can affect the central nervous system and even kill their predators (Habermehl, 1981; Bücherl, Buckley and Deulofeu, 2013).

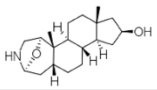
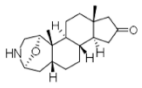
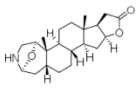
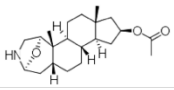
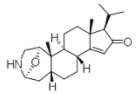
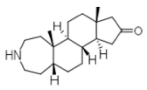
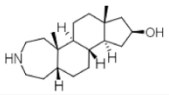
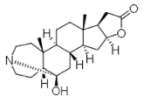
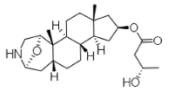
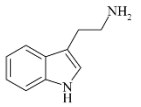
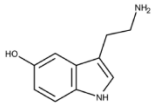
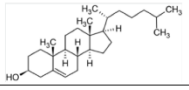
Chemical	Compound	Chemical formula	Chemical structure
SAMs	samandarine	$C_{19}H_{31}NO_2$	
	samandarone	$C_{19}H_{29}NO_2$	
	samandaridine	$C_{21}H_{31}NO_3$	
	O-acetylsamandarine	$C_{21}H_{33}NO_3$	
	samandenone	$C_{22}H_{33}NO_2$	
	samanone	$C_{19}H_{31}NO$	
	samanine	$C_{19}H_{33}NO$	
	isocycloneosamandaridine	$C_{21}H_{31}NO_3$	
	o-(S)-3-hydroxybutanoylsamandarine	$C_{23}H_{37}NO_4$	
Biogenic amines	tryptamine	$C_{10}H_{12}N_2$	
	serotonin	$C_{10}H_{12}N_2O$	
Steroids	cholesterol	$C_{27}H_{46}O$	
Peptides	Not specified		

Table 2. Overview of compounds detected in granular glands of fire salamander (*Salamandra Salamandra*), belonging to the main categories of steroidal alkaloids (SAMs), Biogenic amines, Steroids and Peptides. Adapted from Vences *et al.*, (2014); Lüddecke *et al.*, (2018) and Knepper *et al.*, (2019).

1.3.3. Amphibian skin changes during metamorphosis

GENERAL INTRODUCTION

Before metamorphosis, amphibian larvae have only a simplified skin, consisting of epidermis and premetamorphic dermis. The epidermis is mainly made up of apical and basal germinative cells, the two epidermal cell layers, with Leydig cells enclosed in between (Kelly, 1966; Ohmura and Wakahara, 1998). Other, more specialized, cells can also be found in the larval epidermis, although more sparingly, such as Merkel cells, which are associated with nerve differentiation and tactile perception (Merkel, 1875; Tweedle, 1978; Haeberle and Lumpkin, 2008; Severson *et al.*, 2017), and mucus secreting goblet cells, which are hypothesized to secrete mucus (Dubaissi *et al.*, 2014; Walentek *et al.*, 2014). Separating the epidermis and the dermis is the larval basement lamella, consisting of collagen fibrils, fulfilling the role of supportive and connective tissue (Ferris and Weiss, 1956). The dermis in premetamorphose larvae is also simplified, compared to their adult counterparts, containing chromatophores (pigment cells, such as melanophores, xanthophores or erythrophores) and mesenchymal cells (Bagnara, Frost and Matsumoto, 1978; Sm and Va, 1988; Epperlein and Löfberg, 1990).

During metamorphosis, depending on the species, a very complex process of skin changes takes place, which occurs in morphological and physiological levels. The most notable skin structural change in the epidermis is the cornification. Epidermal epithelial cells differentiate into keratinized cells, losing their nucleus and flattening into layers of hardened tissue (Budz and Larsen, 1973; French *et al.*, 1994; Alibardi, 2002). In the dermis, the most notable structural change is the appearance of mucous and granule gland cells, replacing the mucus-producing larval goblet and Leydig cells from the epidermis, which in turn go into apoptosis (Fox, 1981). Changes in skin pigmentation and patterns are also observed during metamorphosis. Physiological changes can also occur during metamorphosis, with more ATP-dependent sodium–potassium pumps being expressed in cells of the epidermis, to increase the animals capacity for skin osmoregulation (Bentley and Baldwin, 1980; Burggren, 1992). Carbohydrate residues also play an important role in balance cell membrane permeability (Montreuil, 1980), which has also been reported in several anuran species as a crucial factor in regulation the movement of water and ions through the skin (Navas, Villalba and Garcia-Herdugo, 1985; Villalba and Navas, 1989; Burggren, 1992; Faszewski and Kaltenbach, 1995; Choi *et al.*, 1997; Kaltenbach *et al.*, 2004).

1.4. Amphibian diet and gut microbiome

1.4.1 Amphibian diet

The study of the amphibian diet is an important tool in ecology for assessing food webs. Generally, amphibians are generalist and opportunistic predators (Avery, 1968; Griffiths, 1986; Toledo, Ribeiro and Haddad, 2007; Ferenti, David and Nagy, 2010). Adult amphibians are largely carnivorous, consuming mainly arthropods, mollusks, annelids, and small vertebrates (Solé and Rödder, 2010). Most amphibians only target moving and live prey items, using a variety of sensory organs for hunting, including visual, auditory and chemosensory organs (Fay and Simmons, 1999; Placyk and Graves, 2002; Miles, Williams and Hailey, 2004). Some amphibian species, however, might need only one sensory input for hunting. One such example is the olm, a blind cave salamander, which locates prey mainly by senses of taste and smell (Uiblein *et al.*, 1992). Fully terrestrial amphibians locate prey primarily by vision (Martin, Witherspoon and Keenleyside, 1974; Jaeger, Barnard and Joseph, 1982). Aquatic amphibians use a variety of methods: vision, electroreception, chemoreception and mechanoreception (Heatwole and Dawley, 1998). Most anurans and terrestrial caudates catch prey by their long, muscular and sticky tongue (Deban, O'Reilly and Nishikawa, 2001; Kleinteich and Gorb, 2015). But some fully aquatic species don't have a tongue and catch prey by creating negative pressure in opening their mouths, such as the clawed frogs (Pipidae) (Clayton, 2005; Hadfield, Clayton and Barnett, 2006; McWilliams, 2008).

The diet of adult amphibians is mostly influenced by their species, but also associated with extrinsic and intrinsic factors (Duellman and Trueb, 1994). Examples of extrinsic factors are the availability of food resources in the environment (Das, 1996; López *et al.*, 2007; Brenes-Soto, Dierenfeld and Janssens, 2018), seasons and climate change (Blaustein *et al.*, 2010). Examples of intrinsic factors are morphological traits of amphibians (such as body size or skull shape) and energy demand (Emerson, 1985; Grayson *et al.*, 2005; Sousa and Ávila, 2015). Prey size is usually correlated with individual predators' body size and head width (Biavati, Wiederhecker and Colli, 2004). One should also distinguish between two distinct hunting strategies, sit-and-wait and active foraging. The sit-and-wait strategy is employed mostly by anuran and urodele amphibians, which target more slow-moving prey, and the active foraging strategy, employed by some anuran amphibian species (such as dendrobatids), but also caecilian amphibians, which target less

chitinous and more mobile prey (Toft, 1980, 1981; Pough and Taigen, 1990; Duellman and Lizana, 1994).

Should the recommended nutrient intake for the respective species not be met, then nutritional deficiencies can occur. Common and observed deficiencies include vitamin deficiencies, such as Vitamin A deficiency, leading to squamous metaplasia and the characteristic short tongue syndrome (Pessier *et al.*, 2005; Pessier, 2014), as well as Vitamin B1, but also other B Vitamins, leading to neurological, muscular and skeletal defects and/or deformities (Wright and Whitaker, 2001).

1.4.2. Amphibian gut microbiome

The digestive system of vertebrates harbours a symbiotic microbial community (Ley *et al.*, 2008; Robinson, Bohannan and Young, 2010), that is essential for maintaining animal health and fitness, and for making sure that the intake of food is digested and the energy therein is harnessed at the highest possible efficiency. The mediation of the digestion and energy requisition by the gut microbiome includes, but is not limited to, the functions of supporting the production of vitamins (Gill *et al.*, 2006), the chemical processing of indigestible chyme components by bacterial fermentation (Pryor and Bjorndal, 2005), and degradation of toxic substances (Claus, Guillou and Ellero-Simatos, 2016). The gut microbiome also plays a role in the maturation of the immune system (Mazmanian *et al.*, 2005). A healthy gut microbial community is synergistic, helps in occupying the ecological niche and establishes a selective environment to prevent pathogenic bacteria to settle and cause illness. The exact make-up of gut microbiome populations is a dynamic balance, that is influenced by internal factors, namely host phylogeny and host immune system (Amato *et al.*, 2018; Bourguignon *et al.*, 2018; Woodhams, Bletz, *et al.*, 2020), and external factors, namely diet, climate and habitat (Rawls *et al.*, 2006; Sullam *et al.*, 2012; Kohl *et al.*, 2014; Carmody *et al.*, 2015a; Bletz *et al.*, 2016; Li *et al.*, 2020). Focussing on diet, new, non-resident, gut microbes are obtained through the ingestion of prey and their own skin sheddings (Wiggins *et al.*, 2011). These non-resident microbes are then further selected by the gastrointestinal environment (Feld *et al.*, 2008). Therefore, the total gastrointestinal microbiome is composed of resident intestinal microbes and allochthonous, non-resident microbes – some of which cannot permanently colonize the gut.

2. Chytrid fungi *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans*

2.1. History and origin

The chytrid pathogens *Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrivorans* (*Bsal*) belong to fungal phylum Chytridiomycota and genus *Batrachochytrium*. *Bd* was discovered in the late nineties after mass mortality with association of skin damage was observed in amphibian populations in Central America, the Caribbean and Australia (Longcore, Pessier and Nichols, 1999). Soon after, publications reported this fungus to be present and afflicting amphibians on all continents around the globe (Skerratt *et al.*, 2007; Fisher, Garner and Walker, 2009). Two hypotheses have been developed, to explain the sudden emergence of a previously unknown and unnoticed pathogen: The endemic pathogen hypothesis (EPH) and the novel pathogen hypothesis (NPH) (Rachowicz *et al.*, 2005). EPH states that the pathogen was previously already widespread, but that the disease prevalence has increased recently, caused by either environmental, immunological or behavioral alteration regarding either the host or pathogen. NPH, in turn, states that the pathogen was previously regionally limited and was only recently introduced to new areas and encountered local, immunulogical naïve amphibians, therefore causing mass mortality. The EPH hypothesis is supported by the fact, that *Bd* co-existed with endemic amphibian populations long before population declines were registered and no disease was previously observed (Ouellet et al., 2005; Tobler et al., 2012; Weldon et al., 2004). The NPH hypothesis, on the other hand, is supported by the fact that many amphibians or non-amphibian animals (e.g., waterfowl, reptiles and crayfish) can serve as vectors for *Bd*, and able to cause outbreaks in new areas due to animal migration or pet trade (Fisher and Garner, 2007; McMahon *et al.*, 2013; Burrowes and De la Riva, 2017). The NPH has recently become the most convincing hypothesis of the origin of *Bd*. A recent study using molecular techniques to analyse the phylogenetic trees of isolated strains, suggested that East Asia could be the most likely origin, since multiple panzootic lineages are that now distributed worldwide emerged in this region, such as *Bd*GPL (O'hanlon *et al.*, 2018).

Bsal was initially discovered in 2013 (Martel *et al.*, 2013), following a massive fire salamander population decline in the Bunderbos, Netherlands since 2008 (Spitzen-van der Sluijs *et al.*, 2013). East Asia, Vietnam, China, Thailand and Japan were suggested to be the origin of *Bsal*. *Bsal*

seems to be present in the amphibian populations in those countries but without disease. Several Asian salamander species show clearing or persisting with the *Bsal* infection, including Vietnamese salamander (*Paramesotriton deloustali*), Japanese fire belly newt (*Cynops pyrrhogaster*) and Chuxiong fire-bellied newt (*Cynops cyanurus*) (Martel *et al.*, 2014). Researchers suggested that the amphibians in East Asia may act as *Bsal* reservoir and have transmitted the disease to Europe through the pet trade (Martel *et al.*, 2014; Nguyen *et al.*, 2017).

2.2. Life cycle

The life cycles of both chytrid fungi includes two life stages, namely the flagellated motile spores (zoospores) and zoosporangium life stages. In general it can be said that *Bd* and *Bsal* share similar lifecycles. Motile zoospores are flagellated, unicellular, lack a cell wall, and are primed to infect host cells. After a suitable host is found, zoospores start encystation by resorbing its flagellum and forming a rhizoid-like germling. The fungal cells form a cyst underneath the host epidermis and the germling develops into zoosporangia, thereby going over to the zoosporangium life stage, in which the fungi asexually reproduces inside the host cell to create new zoospores (Longcore, Pessier and Nichols, 1999; Berger *et al.*, 2005; Martel *et al.*, 2013). When the new spores have matured, they are released into the environment through discharge tubes, which open on the surface of the sporangia (Longcore, Pessier and Nichols, 1999; Berger *et al.*, 2005; Martel *et al.*, 2013). Unlike *Bd*, *Bsal* zoosporangia have also been found to produce buoyant, encysted and non-motile spores, which are able to persist in the environment for at least 31 days without a host, during which these spores are still infectious (Stegen *et al.*, 2017).

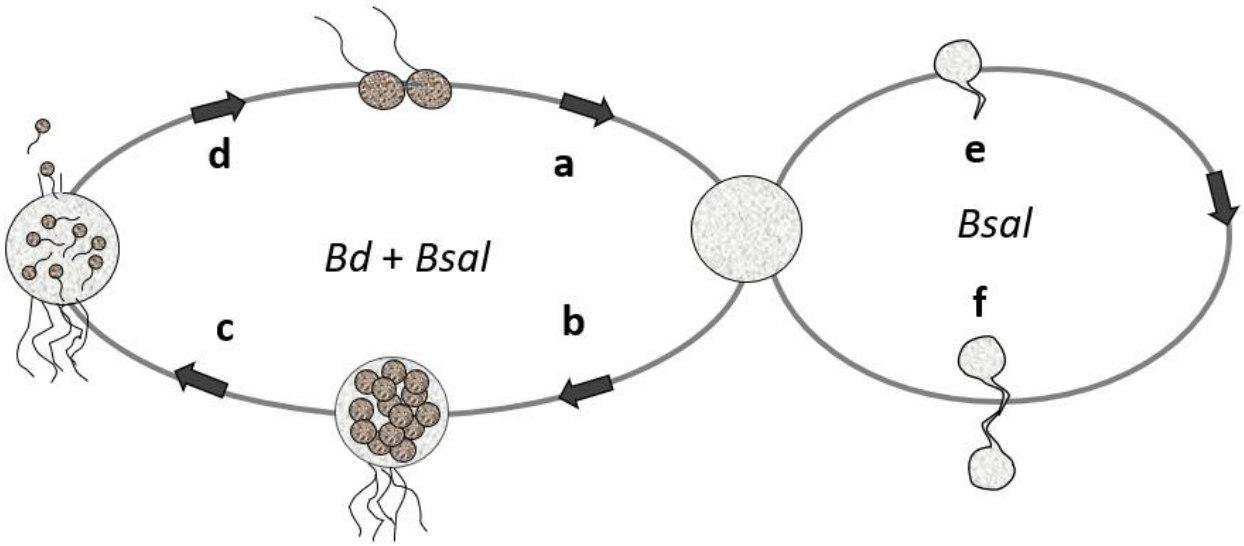


Figure 3. The lifecycle of *Bd* and *Bsal* in culture. Flagellate zoospores attach to suitable substrate and start encysting (a). Zoospores encystation through resorbing its flagellum and forming a rhizoid-like germling and first develop into immature sporangium (b), then form matured zoosporangium (c). Fully developed zoosporangium release zoospores into the environment through discharge tubes (d). Different with *Bd*, the germling of *Bsal* then develops a germtube (e), which transfers cell contents into the thallus (f). Adapted from Van Rooij *et al* (2015).

Typically, the complete life cycle takes 4 to 5 days for *Bd* and 5 days for *Bsal*. Temperature is a very important factor for growth and survival of chytrid fungi. The optimal temperature for *Bd* is between 17 to 25 °C (Piotrowski, Annis and Longcore, 2004) and the growth stops when the temperature reaches 28 °C. Once the temperature reaches 37 °C, it kills off the *Bd* spores (Johnson *et al.*, 2003). *Bsal* prefers a lower temperature, namely between 10 to 15 °C, whereas more than 25 °C is lethal (Blooi *et al.*, 2015).

3. Chytridiomycosis

3.1. Pathology and clinical symptoms

Chytridiomycosis is an emerging infectious skin disease of amphibians caused by both *Bd* and *Bsal* (Berger *et al.*, 1998; Martel *et al.*, 2013). *Bd* zoospores only infect the keratinized layers of amphibian skin. In anuran larvae, only the mouthpart is keratinized, therefore the infection is

limited to the mouth part, with symptoms of mouthparts depigmentation, without mortality (Berger *et al.*, 1998; Rachowicz and Vredenburg, 2004). However, symptoms in larvae include lethargy and decreased swimming ability, resulting in reduced foraging efficiency. This, in turn, can affect development, causing malformations and relatively small body size (Hanlon *et al.*, 2015). In adult anurans, the *Bd* preferential infection sites, where keratinized layers of skin are most susceptible to infection, are the ventral abdomen and feet (Berger, Speare and Skerratt, 2005). Salamander larvae have so far not been characterized in-depth regarding *Bd* infection. In adult salamanders, the ventral side of the tail, the fore- and hind limbs and pelvic region are most prone to infection (Van Rooij *et al.*, 2011). Both for adult anurans and adult salamanders, the observed epithelial histopathology of individuals infected with chytridiomycosis alternates between increase of epithelial keratinization (hyperkeratosis) and epithelial lesions, the latter representing considerable tissue damage of the stratum corneum (Berger, Speare and Skerratt, 2005). These significant changes in the skin structure can disrupt the normal skin functions, leading to impair skin-mediated electrolyte transport and osmoregulation, which can be lethal to the infected individuals (Voyles *et al.*, 2007, 2009; Campbell *et al.*, 2012). The most common clinical signs of adult amphibian *Bd* infection is excessive shedding of skin, erythema (redness) or discoloration of the skin, as well as other signs, such as lethargy, anorexia, abnormal posture, loss of righting reflex and flight response (Mutschmann, 2015) (Figure 4).

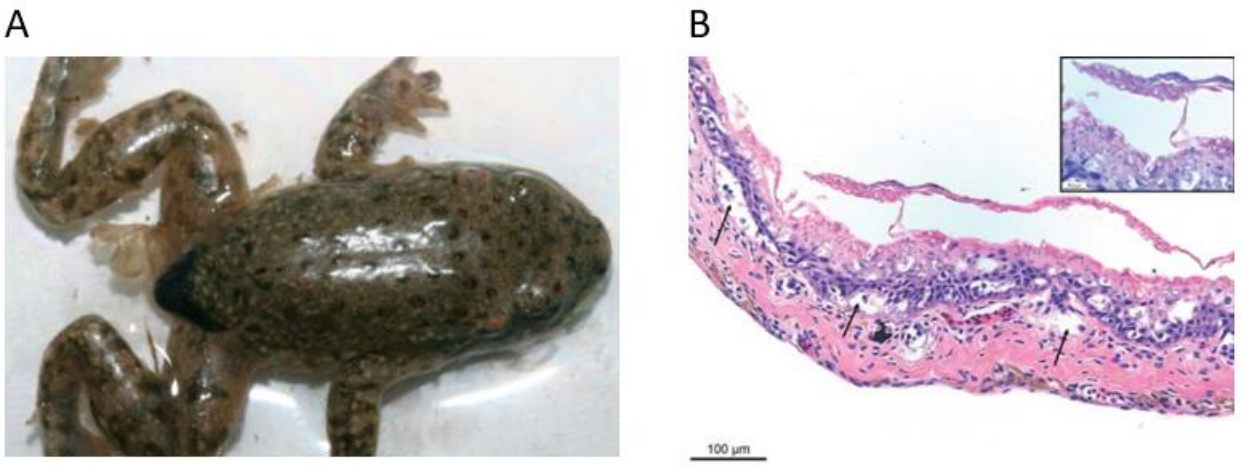


Figure 4. Midwife toad (*Alytes obstetricans*) infected with chytridiomycosis caused by *Bd*. (A) Macroscopic picture shows the clinical signs of loose skin shedding and abnormal posture. (B) Hematoxylin and eosin staining of ventral skin of infected midwife toad. Presence of epidermal

hyperkeratosis and hyperplasia are typical for *Bd* infection. Arrows indicate the intra-epidermal vesicle formation. Adapted from Pasmans *et al.* (2010).

Chytridiomycosis caused by *Bsal* has been proven to not affect fire salamander larvae (Van Rooij *et al.*, 2015). For adult urodelans, *Bsal* infection is characterized by all over the body (Figure 5), but the animals do not exhibit epithelial hyperkeratosis after *Bsal* infection. It has been shown that the severe skin damage caused by *Bsal* disrupts normal skin functions, which, together with septicemia induced by bacteria colonization, results in the death of the animals (Martel *et al.*, 2013; Bletz *et al.*, 2018).

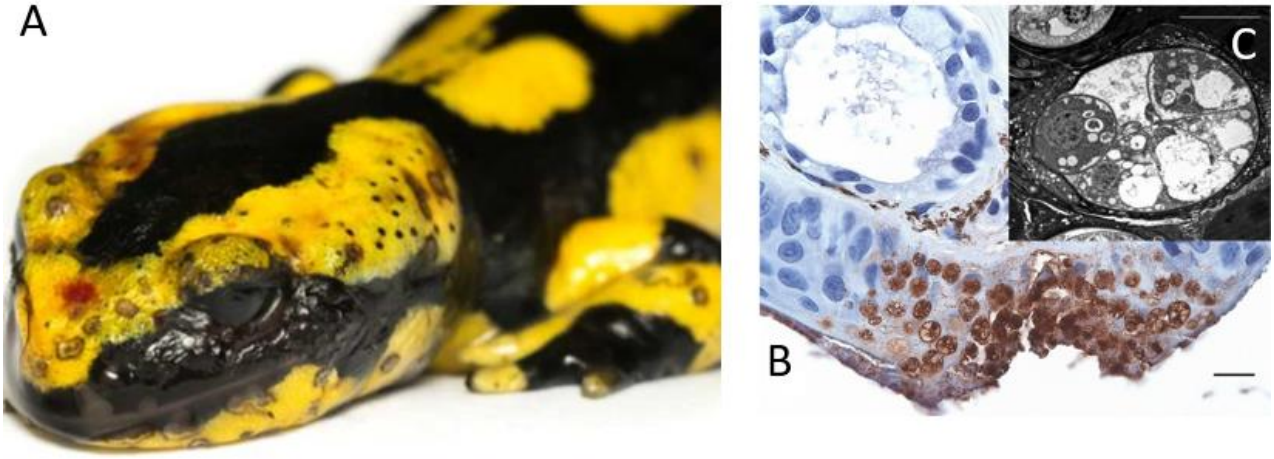


Figure 5. Fire salamander (*Salamandra salamandra*) infected with chytridiomycosis caused by *B. salamandrivorans*. (A) Macroscopic picture shows the clinical signs of epidermal ulcerations and erosive skin lesions throughout the body. (B) Immunohistochemical staining of skin lesion. Intracellular colonial thalli present in epidermal layer. (C) Transmission electron microscopic picture of an intracellular colonial thallus. B and C adapted from Martel *et al.* (2013).

3.2. Pathogenesis

The details of mechanisms of chytridiomycosis pathogenesis are still not fully elucidated and require further investigation. Studies regarding *Bsal* are scarce, whereas *Bd* has been studied more in-depth. *Bd* pathogenesis can be summarized as follows. It starts with chemotactic attraction of the zoospores to find a suitable host, using their flagellum to move in water. Once a host is found, the zoospores attach to the keratinized epidermis. After adhesion, the zoospores develop a germ tube to invade into host skin cells and starts endobiotic growth (Figure 6), which leads to loss of

host cell cytoplasm eventually (Longcore, Pessier and Nichols, 1999; Brutyn *et al.*, 2012; Van Rooij *et al.*, 2012). The germ tube generation has also been described for *Bsal* (Martel *et al.*, 2013). The mechanisms of *Bd* and *Bsal* endobiotic growth are still poorly understood. Quorum sensing has been found to regulate the growth of *Bd* and *Bsal* by producing tryptophol (Verbrugghe *et al.*, 2019).

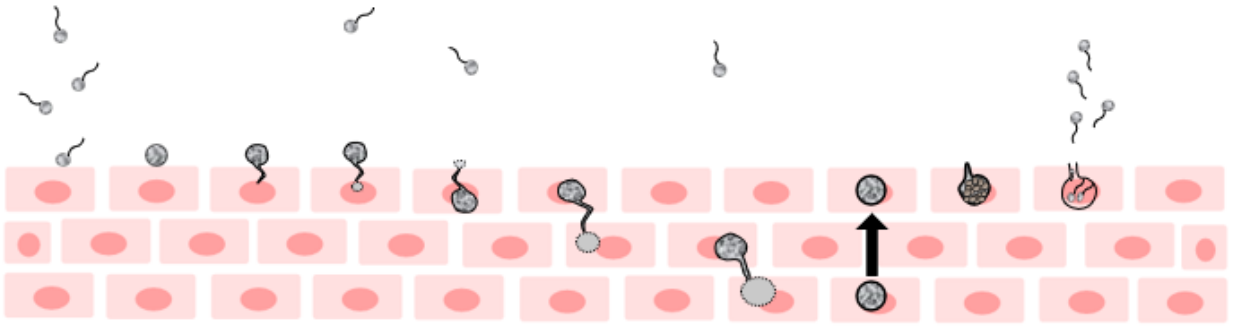


Figure 6. The endobiotic lifecycle of *Bd* in an infected host. *Bd* zoospore attaches to host skin and forms germtube to invade epidermal cells. Germ tube mediates invasion, establishes intracellular thalli and Bd spreads to the deeper skin layers. The differentiating epidermal cells migrate upward and to the surface, at which timepoint Bd releases its zoospores. Adapted from Van Rooij *et al* (2015).

3.2.1. Zoospore chemotaxis

Chemotaxis is an important feature of motile organisms, which helps them navigate towards a suitable nutrient source and avoid unfavourable environmental conditions (Lux and Shi, 2004). Studies prove that *Bd* exhibits positive chemotaxis towards a variety of cues, such as keratin, carbohydrates and amino acids (Moss *et al.*, 2008). It has also been shown that *Bd* spores exhibits positive chemotaxis towards keratinous toe scales from waterfowl (Garmyn *et al.*, 2012). As we described previously, amphibian skin is covered by a mucus layer of which mucin glycoproteins are the main component. A previous study found that *Bd* exhibits positive movement towards skin mucus isolated from *X. laevis* (Van Rooij *et al.*, 2015). The free carbohydrates in *X. laevis* mucus include, but are not limited to, α-l-fucose, α-d-N-acetylgalactosamine, β-d-N-acetylglucosamine, N-acetylneuraminic acid, α-d-galactose and α-d-mannose (Meyer *et al.*, 2007). These oligocarbohydrates were tested in a *Bd* chemotaxis assay and results show that attraction of *Bd*

zoospores to oligocarbohydrates was 3 to 9 times higher compared to water, but not significantly different between individual carbohydrates (Van Rooij *et al.*, 2015). Therefore, it can be said that amphibian skin mucus plays a dual role in pathogenesis, which not only serves as a defence barrier, but also attracts zoospores.

3.2.2. Virulence factors

Fungal pathogenicity in the host is subject to various virulence factors, which determine the ability of the pathogen to cause infection, such as adhesion, survive and persist in the host. For example by production of capsules (Iyalla, 2017), helping the fungal pathogens evade the host immune response. Fungal pathogens also can produce hydrolytic enzymes or toxins, which, in turn, exacerbates host tissue damage.

As the whole genome has been sequenced and published for both *Bd* and *Bsal* (Farrer *et al.*, 2017), subsequent genome-based studies building on the genome have led to an improved understanding of host-pathogen interactions. In chytrid fungi, several putative virulence factors have been identified. Studies found that the fungalysin metallopeptidase (M36) family and the serine-type peptidase (peptidase S41) were found highly expanded in both *Bd* and *Bsal*, compared to non-pathogenic chytrid fungi (Farrer *et al.*, 2017). M36 and S41 were known to be involved in the initial stages of zoospore colonization of amphibian skin and entry into host cells, by aiding in host cell invasion and dissolution of the cellular cytoplasma (Rosenblum *et al.*, 2008; Farrer *et al.*, 2013). Compared to the free-living chytrids, the lectin-like carbohydrate binding modules (CBMs) are markedly expanded in both *Bd* and *Bsal*. CBMs were known as chitin-binding molecules, which were suggested to play a role in host recognition and adhesion (Abramyan and Stajich, 2012; Farrer *et al.*, 2017). Crinkler-and-necrosis (CRN) genes have been identified as virulence factors in Oomycetes (Schornack *et al.*, 2010; Quiroz Velasquez *et al.*, 2014). A recent study found that the expression of CRN-like genes increased in *Bd* zoospores, after they were incubated with amphibian skin tissue, but *Bsal* zoospores show decreased expression of CRN-like genes, after being subjected to the same treatment (Farrer *et al.*, 2017). A unique effect, that we only found in *Bsal* during the same treatment was the upregulation of two large families of genes, known for secreting proteins (Tribes 1 and 4) (Farrer *et al.*, 2017), but the exact function or purpose of these proteins are still unknown.

3.3 Host susceptibility to chytridiomycosis

The host range of *Bd* encompasses over 700 species across three amphibian orders, whereas the host range of *Bsal* is highly restricted to the urodelan order (salamanders and newts). The host susceptibility of chytridiomycosis, however, varies greatly among different amphibian species. In general, animals are classified in their susceptibility as either resistant, tolerant or susceptible (Van Rooij *et al.*, 2015).

In resistant species no infection and no clinical signs occur. Examples of *Bsal* resistant species are most anuran and caecilian species, as well as a few urodelan species, such as the palmate newt (*Lissotriton helveticus*), that were tested so far (Martel *et al.*, 2014). Examples of *Bd* resistant species are less common. One example is the European cave salamanders (*Speleomantes*), which can quickly clear *Bd* infection in only one to two weeks (Bovero *et al.*, 2008; Pasmans *et al.*, 2013). Tolerant species maintain persistent infection, but without clinical signs of disease. These species co-exist with the pathogen and act as a long-term pathogen reservoir. Examples of *Bd* tolerant species are: *Xenopus laevis*, *Lithobates pipiens* and *Lithobates catesbeianus* (Solís *et al.*, 2010; Soto-Azat *et al.*, 2010; Urbina *et al.*, 2018). These animals carry high *Bd* loads but do not develop lethal chytridiomycosis or show a decline in their respective populations. For *Bsal*, *Salamandrella keyserlingii* and *Siren intermedia* have been reported as tolerant species (Martel *et al.*, 2014). In susceptible species, animals show clinical symptoms after infection. At this point the animal either clears the infection and recovers, or dies from the extent of the skin damage that chytridiomycosis inflicted. So far, all *Bsal* susceptible species that have been identified, belong to the urodelan order. Especially most Western Palearctic salamandrid species have been documented to be highly susceptible for *Bsal* infection, resulting in high mortality, except for the *Bsal* resistant species palmate newt (Martel *et al.*, 2014). However, some susceptible species have also been reported to have more favourable disease outcomes, such as Asian salamander species Japanese fire belly newt and Vietnamese salamanders (Martel *et al.*, 2014) (Figure 7).

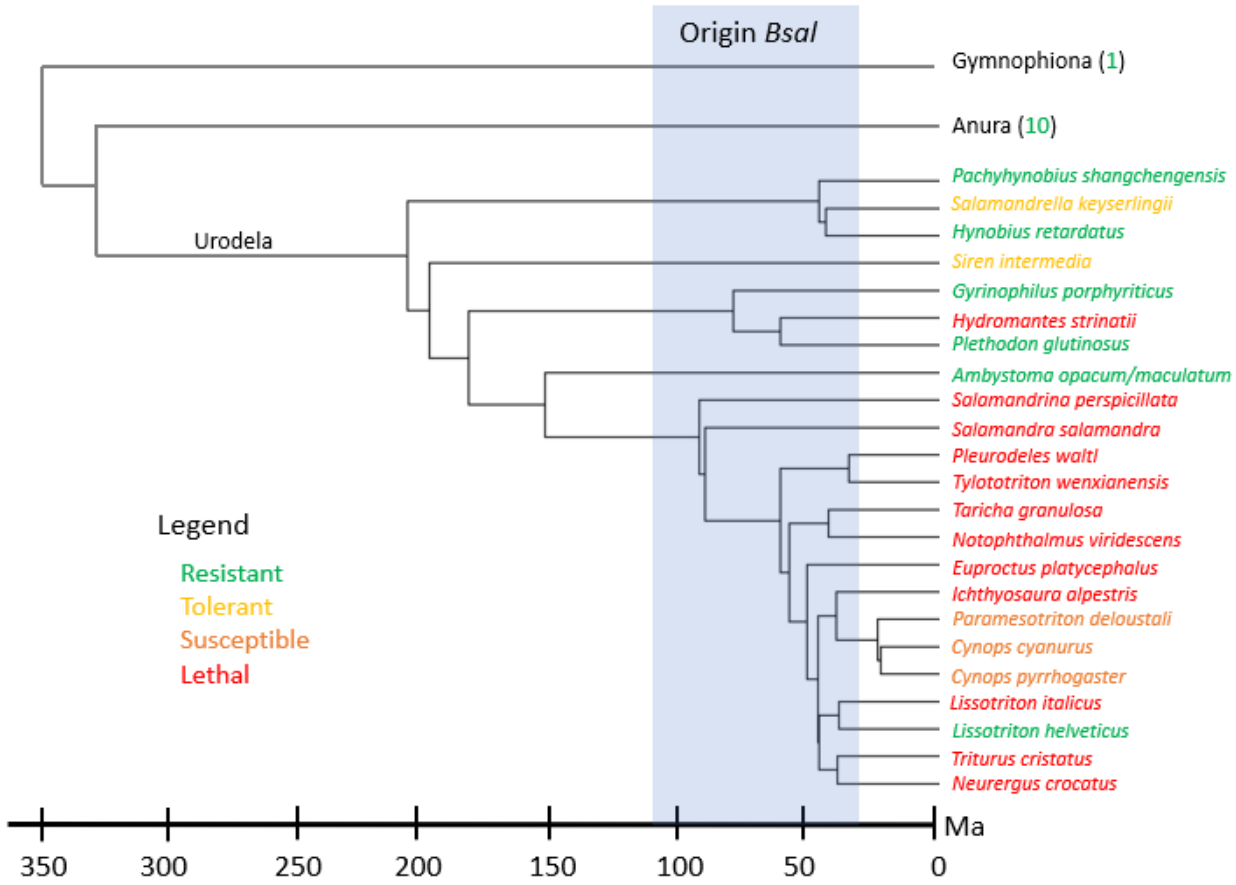


Figure 7. Species susceptibility of *Bsal* through time. Molecular time scale (Ma) for 34 amphibian species. Numbers in brackets are the number of species tested. Different colour indicate different species susceptibility of *Bsal* infection. The 95% highest posterior density for time of divergence between *Bd* and *Bsal* is indicated in light blue. Adapted from Martel *et al*. (2014).

The host susceptibility to *Bsal* infection not only varies between amphibian species, but also differs within the same species. This intra-species difference has been demonstrated in several urodelan species, such as the alpine newt (*Ichthyosaura alpestris*), Japanese fire belly newt, and Iberian ribbed newt (*Pleurodeles waltl*). Some with *Bsal* infected animals die after exposure to *Bsal*, whilst other individuals from the same species are capable of controlling and sometimes even clearing the same cutaneous *Bsal* load and surviving the infection (Martel *et al.*, 2014). These intra-species differences can be explained by many potential factors, including varying immune response, animal behaviour, environmental factors and co-infections. For inter-species differences, these factors, however, are likely to play a secondary role, as the success of the fungal

pathogens is primarily determined by the capacity of the fungus to bind and enter host cells, in other words, the initial colonization success of the fungal pathogen.

3.3.1. Fungal adhesion

Successful adhesion of pathogens to their host cells is a crucial stage for establishment of the infection. This adhesion is mediated by fungal adhesins, which are proteins located on the fungal cell wall, specialized on binding host receptors. Fungal adhesins are therefore important proteins to study the host-pathogen interactions, and an important virulence factor to consider. These proteins are often glycosylphosphatidylinositol (GPI)-anchored (Vogt, Essen and Mösch, 2020). Their N-terminal domain has a high complexity and is specialized on specific protein or carbohydrate antigens on the host receptors (de Groot *et al.*, 2013). These carbohydrate antigens are commonly part of host glycoproteins, on which the fungal adhesins can bind. As carbohydrate binding proteins, they often exhibit lectin-like characteristics. For example, the Agglutinin-Like Sequence (Als) protein family of *Candida albicans* and epithelial adhesin (Epa) family of *Candida glabrata* are typical GPI-anchored proteins (Frieman, McCaffery and Cormack, 2002; Chaffin, 2008). The N-terminal part of Als1 (N-Als1p) and Epa (N-Epa-p) have been found to have lectin-like properties, which mediates adherence to human epithelial and endothelial cells by recognizing glycans containing terminal fucose or galactose residues, respectively (Zupancic *et al.*, 2008; Donohue *et al.*, 2011).

So far, the adhesins of chytrid fungi have not been sufficiently studied. A previous publication found that cell adhesion related genes, such as vinculin, fibronectin and fasciclin, show increased expression in sporangia, compared to zoospores (Rosenblum *et al.*, 2008). As we described previously, CBMs found in both the *Bd* and *Bsal* genome could be potential adhesins. Therefore, the adherence mechanisms of chytrid fungi are likely to include agglutinin-like and lectin-like proteins as has been discovered for previous fungal pathogens.

3.3.2. Host determinants of susceptibility

3.3.2.1 Host immune response

The amphibian immune system is an important factor for determining the susceptibility of the host for chytrid infection. The innate immune system serves as a first defence line against chytrid pathogens. Innate defence mechanisms include physical barriers, an antimicrobial peptide arsenal and the skin microbiome. The amphibian skin is a crucial physical barrier for pathogen infection. Studies have shown that some species are able to increase the rate of skin sloughing during *Bd* infection, resulting in reduced infection loads, which may even lead to infection clearance (Meyer *et al.*, 2012; Cramp *et al.*, 2014; Ohmer *et al.*, 2015). As we described previously, granular glands in the amphibian skin release a variety of antimicrobial secretions, and they are known to be effective against chytrid fungal pathogens (Rollins-Smith and Conlon, 2005; Woodhams *et al.*, 2007; Pask, Woodhams and Rollins-Smith, 2012). Especially AMPs (antimicrobial peptides) are known to reduce *Bd* infection loads and help infection clearance (Rollins-Smith and Conlon, 2005; Rollins-Smith, 2009; Pask, Woodhams and Rollins-Smith, 2012; Holden *et al.*, 2015; McMillan and Coombs, 2020; Woodhams, Rollins-Smith, *et al.*, 2020). Amphibian AMPs are best studied in Anura, knowledge of AMPs in Urodela and their modulation of *Bsal* chytridiomycosis is still lacking. However, the proteins in skin secretion and mucosome of Urodelans have been reported to have the ability of killing both *Bd* and *Bsal* fungal pathogens (Smith *et al.*, 2018). The microbial community present on the amphibian skin surface also plays a role in innate immunity, protecting against chytrid fungi, by secreting antifungal metabolites (Lauer *et al.*, 2007; Rollins-Smith, 2009; Flechas *et al.*, 2012; Holden *et al.*, 2015; Woodhams *et al.*, 2018). For example, violacein and indole-3-carboxaldehyde are secreted by the bacterium *Janthinobacterium lividum* , which is isolated from the skin of the red-backed salamander (*Plethodon cinereus*), showing inhibition of *Bd* growth *in vitro* and *in vivo* (Brucker *et al.*, 2008). A study of the smooth newt (*Lissotriton vulgaris*) and great-crested newt (*Triturus cristatus*) found that the *Bsal* infection load is associated with changes in the composition of skin microbiome communities (Bates *et al.*, 2019). The skin microbiome of fire salamanders does not sufficiently protect them against *Bsal* in the wild. Nonetheless, artificially increasing the presence of anti-*Bsal* bacterial strains, such as *Stenotrophomonas* sp. and *Pseudomonas* sp. on the skin of fire salamanders in an experimental setup did inhibit *Bsal* disease progression (Bletz *et al.*, 2018).

The adaptive immune system is the second defence line against chytrid pathogens. The amphibian adaptive immune system is comprised of T- and B lymphocytes, leukocytes, cytokines and major

histocompatibility complexes (MHC) I and II, similar to mammals (Bleicher and Cohen, 1981; Watkins and Cohen, 1987; Sammut, Laurens and Tournefier, 1997; Grogan *et al.*, 2018). Some frog species repeatedly exposed to *Bd* zoospores have reportedly a higher survival rate, with reduction in the measured infection loads, as compared to immunologically naïve frogs (Richmond *et al.*, 2009; Ramsey *et al.*, 2010; McMahon *et al.*, 2014). This is a strong indicator for a targeted and acquired immune response, related to plasma cell antibody production, targeting the fungal pathogens, followed by Fc-mediated inhibition, destruction or phagocytosis of the zoospores (Grogan *et al.*, 2018). MHC class I and II genes are also upregulated during *Bd* infection (Savage and Zamudio, 2011, 2016), which is in line with the previously mentioned findings, and suggests that peptides from cytosolic and extracellular proteins are presented by all cells and antigen-presenting cells, respectively. MHC class I proteins presenting zoospore derived endogenous peptides would then result in cytotoxic T-cell activation of the affected cell. MHC class II proteins presenting zoospore derived extracellular peptides, that have been digested over phagocytosis by antigen-presenting cells (such as B cells or macrophages), would in turn increase T helper cell mediated activation of the immune response (Grogan *et al.*, 2018).

Some urodelan species, such as marbled newts, ribbed newts and Eastern newts can acquire immune response to *Bsal* infection after pre-exposure to low virulence *Bd* strains and increases their survival (Longo, Fleischer and Lips, 2019; Greener *et al.*, 2020). Fire salamanders, however, do not exhibit an immune response to *Bsal* infection, after pre-exposure to low virulence *Bd* strains or repeated exposure to *Bsal* zoospores (Farrer *et al.*, 2017; Stegen *et al.*, 2017; Greener *et al.*, 2020). Also in the wild it has been observed, that after outbreaks in certain populations, surviving animals would still be highly susceptible to the previous *Bsal* zoospore isolate, with experimental infection at a certain dose resulting invariably in death (Stegen *et al.*, 2017). In line with these observations, no MHC class I or II gene upregulation was observed in animals (*Tylototriton wenxianensis*) infected with *Bsal* (Farrer *et al.*, 2017).

3.3.2.2. Host behaviour

Host behavior is also known to mediate disease dynamics. Bacterial and viral agents have been shown to able to induce fever in amphibians (Sauer *et al.*, 2019). It has been reported that the

Panamian golden frog (*Atelopus zeteki*) increases its body temperature as a response to *Bd* infection, and thereby reduces the severity of the disease progression (Richards-Zawacki, 2010). Currently, there is no strong evidence for chytrid fungi inducing behavioral fever in amphibians, however, it has been found that thermal preference is able to inhibit the infection (Sauer *et al.*, 2018; Beukema *et al.*, 2021).

3.3.3. Environmental factors

Environmental factors determine outcomes of infection dynamics by affecting not only the host fitness, but also the virulence of chytrid pathogens. Several abiotic factors have been identified, such as climate and altitude (Clare et al., 2016; Kriger et al., 2007; Kriger & Hero, 2008), seasonality (Garner, Rowcliffe and Fisher, 2011; Raffel *et al.*, 2015), and ultraviolet exposure (Walker *et al.*, 2010; Ortiz-Santaliestra *et al.*, 2011). Studies show that at cooler climates or cooler seasons, susceptible host species experienced higher *Bd* infection prevalence and mortality (Berger *et al.*, 2004), which could be due to the optimal reproduction temperature of chytrid fungi being at lower temperatures (Piotrowski, Annis and Longcore, 2004; Blooi *et al.*, 2015), but also due to a decreased efficiency of the amphibian immune systems (Raffel *et al.*, 2006). Altitude can also be a factor, with amphibians living on mountain ranges remaining susceptible to *Bd* outbreaks for longer periods than populations residing at sea level (Kriger and Hero, 2008). A recent study shows that *Bsal* infected Eastern newts (*Notophthalmus viridescens*) died faster at 14 ^{o}C than at higher (22 ^{o}C) or lower (6 ^{o}C) temperatures (Carter *et al.*, 2021), which is in line with previous findings and also indicates that the optimal environmental temperatures plays a role in the epidemiology of *Bsal* infection.

Environmental UV-B radiation is another factor, that has potential effects on the host susceptibility of *Bd* infection. However, studies show conflicting results of the impact of UV-B on host susceptibility. For example, studies on juveniles of *Rana cascadae, Bufo boreas* and *Hyla regilla* found that exposure of animals to low level of UV-B had no effects on host susceptibility to *Bd* or mortality rates (Garcia, Romansic and Blaustein, 2006). One studies on *Bufo bufo* larvae observed a reduced *Bd* infection prevalence after radiation exposure, but no statistically significant difference on mortality rates was detected (Ortiz-Santaliestra *et al.*, 2011). In contrast, an increase of *Bd* susceptibility was observed among tadpoles of the green tree frog (*Litoria caerulea*) after

having been exposed to UV-B radiation (Cramp and Franklin, 2018). These conflicting results of different experiments could be explained by variation of the intensity of radiation, altitude, or host immune function (Cramp and Franklin, 2018). It has been reported that intense radiation not only can cause damage on the amphibian skin, but can also inhibit the host immune system, leading to an increased susceptibility to pathogens (Cramp and Franklin, 2018).

3.3.4. Diet and gut microbiome

It has previously been reported that the diet composition can have effects on amphibian health and their ability to resist chytridiomycosis infection. In southern leopard frog tadpoles it has been found that a low-protein diet resulted in decreased resistance of infection against Bd in the mouth region (Venesky *et al.*, 2012). Furthermore Ledón-Rettig *et al*. (2009) showed that couch's spadefoot (*Scaphiopus couchii*) exhibited higher corticosterone hormone levels, when they were not sufficiently fed, which could subsequently decrease the amount of circulating lymphocytes, as observed in *Xenopus laevis* (Rollins-Smith, 2017). Other foods have also found to be relevant for the amphibian immune system, such as pomegranates, Vitamin A and carotenoids. Overall, however, the effect on diet on the immune system of amphibians has not been studied in-depth, especially with regards to Chytridiomycosis.

Chytridiomycosis is an infectious disease of the skin. Therefore, it is only logical that most studies have so far focussed on the skin microbiome, and its effect on Chytridiomycosis (Rebollar, Martínez-Ugalde and Orta, 2020; Ruthsatz *et al.*, 2020). The gut microbiome is likely not a main player in conferring Chytridiomycosis resistance. Nonetheless, as it is a major factor in immune system maturation and health, an imbalanced gut microbiome could potentially decrease resistance against Chytridiomycosis, and as such needs to be studied to subsequently improve conservation efforts.

3.3.5. Co-infection

Many pathogens, including viruses, bacteria and fungi can cause disease in amphibians. Co-infection of *Bd* with other pathogens, such as Ranavirus, *Aeromonas hydrophila* and *Chlamydia pneumoniae* have been observed in wild amphibian populations (Reed *et al.*, 2000; Hill *et al.*,

2010; Kik *et al.*, 2012). How these different pathogens interact and affect the host during co-infections, and how co-infection modulates disease severity is still poorly understood. In *Craugastor fitzingeri* it has been observed that the *Bd* infected individuals showed a higher chance of also being infected by Ranavirus (Whitfield *et al.*, 2013). Lower survival rates were observed in larvae of *Pseudacris regilla* after being co-infected with the parasitic nematode *Ribeiroia* and the fungal pathogen *Bd*, as compared to being infected with only one of those pathogens (Romansic *et al.*, 2011). Co-infection with different *Bd* strains in the wild, such as *Bd*GPL and *Bd*CAPE have been observed in South Africa (Ghosh, 2020). Concurrent *Bd* and *Bsal* infections have also been observed in wild fire salamanders (Lötters *et al.*, 2018). A recently published study found that pre-exposure to some of the low virulence *Bd* isolates protects against disease following subsequent exposure to highly virulent *Bd*GPL isolate in midwife toads *(Alytes obstetricans)*. This study also observed that prior exposure to *Bd* reduces pathogenicity of *Bsal* in marbled newts and ribbed newts, but no significant impact was observed in fire salamanders (Greener *et al.*, 2020). A previous study shows that Eastern newts were co-infected with both *Bsal* and *Bd* zoospore in a sequential manner decreases the mortality, compared to *Bsal* infection alone. However, exposing animals under simultaneous co-infections of *Bd* and *Bsal* can decrease their probability of survival (Longo, Fleischer and Lips, 2019).

4. References

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SCIENTIFIC AIMS

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The emerging fungal pathogen *Batrachochytrium salamanderivorans* (*Bsal*) has become a serious threat to western Palaearctic amphibian diversity, bringing multiple native European salamander populations to the brink of extinction. The host susceptibility of *Bsal* infection varies greatly among different amphibian species. One of the highly susceptible and most severely affected species is fire salamander (*Salamandra salamandra*), of which more than 99.9% of the original population size has been wiped out in the Netherland within 6 years. Population collapses caused by *Bsal* have also been reported in Belgium and Germany, and there is no sign of recovery for those collapsed populations. Developing sustainable mitigation measures against *Bsal* will be necessary to help preserving a significant proportion of the world's amphibian biodiversity. Conservation efforts should be directed primarily towards the most susceptible amphibian species to ward off species extinction. These species will need be characterized in-depth, collecting all information regarding their health, including information on diet and their gut microbiota, since these factors are essential for maintaining animal health, disease resistance and adaptation to biotic and abiotic stressors. Moreover, understanding the host-pathogen interactions between the fungal pathogen *Bsal* and its hosts, is absolutely necessary for identifying and protecting susceptible species and developing long-term conservation strategies.

The general aim of this doctoral thesis is the scientific exploration of conservation strategies for urodelan species, by looking at both the host-pathogen interaction, and the diet and gut microbiome of fire salamanders in particular.

The specific aims were:

1. To provide an overview of potential *Bsal* mitigation methods (Chapter 1).

2. To profile the diet and intestinal bacterial composition of fire salamander populations, as well as the impact of the diet, sex and geographical location on the gut microbiome (Chapter 2).

3. To elucidate the initial step of pathogenesis for *Bsal*, and subsequently define a biomarker for determining infection susceptibility in amphibians (Chapter 3).

CHAPTER 1

CHAPTER 1

Mitigating *Batrachochytrium salamandrivorans* in Europe

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Abstract

The infectious chytrid fungus *Batrachochytrium salamandrivorans* (*Bsal*) has been responsible for severe population declines of salamander populations in Europe. Serious population declines and loss of urodelan diversity may occur if appropriate action is not taken to mitigate against the further spread and impact of *Bsal*. We provide an overview of several potential mitigation methods, and describe their possible advantages and limitations. We conclude that long-term, context-dependent, multi-faceted approaches are needed to successfully mitigate adverse effects of *Bsal*, and that these approaches should be initiated pre-arrival of the pathogen. The establishment of *ex situ* assurance colonies, or management units, for species threatened with extinction, should be considered as soon as possible. While *ex situ* conservation and preventive measures aimed at improving biosafety by limiting amphibian trade may be implemented quickly, major challenges that lie ahead are in designing *in situ* disease containment and mitigation post-arrival and in increasing public awareness.

Keywords: conservation, amphibians, salamanders, chytridiomycosis, chytrid fungus, emerging diseases, *Batrachochytrium salamandrivorans*, mitigation, biosafety, trade

CHAPTER 1

1. Introduction

Infection of an amphibian host with the chytrid fungi *Batrachochytrium dendrobatidis* (*Bd*) or *B. salamandrivorans* (*Bsal*), may cause clinical chytridiomycosis, an emerging infectious disease (EID) (Berger *et al*., 1998; Martel *et al*., 2013). *Bd* was first identified in the 1990s as the prevailing cause of worldwide enigmatic declines and local extirpations of amphibian populations (Berger *et al*., 1998; Longcore *et al*., 1999). In 2013, *Bsal* was described following a population collapse of European fire salamanders (*Salamandra salamandra*) in the Netherlands from 2010 onwards, of which less than 0.1% of the original population remained in 2016 (Spitzen-van der Sluijs *et al*., 2013, 2016). In-depth study of a similar outbreak in Belgium in 2014 demonstrated how the interplay between host, pathogen and environment is predicted to result in the extirpation of the affected fire salamander population (Stegen *et al*., 2017).

Both *Bd* and *Bsal* are highly contagious and are transmitted effectively by direct contact with pathogen shedding hosts or indirectly by contact with contaminated water or substrate (Bosch and Martinez-Solano, 2006; Garmyn *et al*., 2012; Kolby *et al*., 2014; Martel *et al*., 2014; Courtois *et al*., 2017; Stegen *et al*., 2017). Pathogen transmission for both amphibian chytrid fungi is via aquatic, motile zoospores which infect the epidermal cells of amphibian skin. Further, *Bsal* produces an infectious, non-motile, encysted spore that manifests increased environmental resilience (Stegen *et al*., 2017). Although not completely understood, the release of proteases by *Bd* zoospores and the growth of intracellular *Bd* and *Bsal*zoosporangia cause disruption of normal skin functioning which is vital to amphibian survival (Berger, Speare and Kent, 1999; Voyles *et al*., 2009; Brutyn *et al*., 2012; Martel *et al*., 2013; Van Rooij *et al*., 2015; Farrer *et al*., 2017).

Although *Bd* and *Bsal* belong to the same genus, they diverged an estimated 50 million years ago (Martel *et al*., 2014). *Bsal* is considered endemic in East Asia where it is widespread, at least in Vietnam, Japan and China, in species of the family Salamandridae (Laking *et al*., 2017; Yuan *et al*., 2018). Both species have an arsenal of virulence factors, which include a greatly expanded metalloprotease gene-family in *Bsal* (Farrer *et al*., 2017). Optimal growth temperatures for *Bd* range between 17°C and 25°C compared to 10-15°C for the *Bsal* type strain. Temperatures above 25°C and 30°C are lethal for *Bsal* and *Bd* respectively (Piotrowski *et al*., 2004; Martel *et al*., 2013; Blooi *et al*., 2015a). However, natural infections with *Bsal* were shown to occur in Asiatic

newts of the genus *Tylototriton* at water temperatures up to 26°C, suggesting variation in thermal tolerance between *Bsal* isolates and, possibly, lineages (Laking *et al*., 2017; Beukema *et al*., 2018).

Bd can infect the skin of, and cause lethal disease in, a large range of anurans, urodeles and caecilians, although population declines have been observed mainly in anurans (Skerrat *et al*., 2007). In comparison, disease caused by *Bsal* seems to be limited to urodeles (Martel *et al*., 2014), even though some anurans can be infected by this fungus (Nguyen *et al*., 2017; Stegen *et al*., 2017). The currently observed niche breadth of *Bsal* in Europe appears to be only partially filled, indicating a high potential of further spread of *Bsal* (Beukema *et al*., 2018). The international trade of Asian salamanders and newts is suspected to be the primary route for the intercontinental spread of *Bsal* (Martel *et al*., 2014; Nguyen *et al*., 2017; Yuan *et al*., 2018). However, in captive collections outside Asia, infection can spread to other species, which in turn, can spread *Bsal* when traded (Fitzpatrick *et al*., 2018; Sabino-Pinto *et al*., 2018b). Eliminating this captive reservoir of *Bsal* should be a key aim in order to curtail further spillover events into natural populations of naïve amphibians.

Given the high susceptibility of salamanders to *Bsal* (Martel *et al*., 2014), and the infectiousness, pathogenicity and host range of the pathogen in Europe (Spitzen-van der Sluijs *et al*., 2016; Stegen *et al*., 2017; Dalbeck *et al*., 2018), *Bsal* poses an unprecedented threat to non-Asian salamander species (Beukema *et al*., 2018). Also, the prevalence of *Bsal* can be low in Asian reservoir species in captivity (Martel *et al*., 2014; Fitzpatrick *et al*., 2018) rendering detection difficult. Such pathogen reservoirs pose a formidable challenge for effectively preventing the introduction of *Bsal*, or subsequently managing a disease outbreak (Canessa *et al*., 2018). The development of effective mitigation strategies and measures, therefore, is crucial to maintaining amphibian biodiversity both globally and locally (Woodhams *et al*., 2011; Garner *et al*., 2016). *Bsal* abatement options have been considered before (Grant *et al*., 2015) and are continuously under revision by the US *Bsal* taskforce. Here, we propose a set of options we deem most feasible and efficient for the European situation given the current state of knowledge.

Two decades of research on the amphibian chytrid fungi have not yielded a single, globally effective measure for controlling *Bd* (Garner *et al*., 2016). Despite this, knowledge gained from these efforts is informative and has guided the development of our proposed suite of actions that

are most likely to be effective in mitigating the effects of *Bsal* in Europe. An overview of potential *Bsal* mitigation methods show in supplementary fig S1.

2. Pre-exposure measures

Taking actions to prevent the introduction and spread of *Bsal* into naïve regions is currently considered as the most efficient control method available ("prevention is better than cure"; Langwig *et al*., 2015; Richgels *et al*., 2016; Grant *et al*., 2017; Roy *et al*., 2017). Within Europe, the potential threat posed by *Bsal* was first recognised by the standing committee of the Bern Convention (Convention on the conservation of European Wildlife and Natural Habitats, 1979). In December 2015, the Council of Europe released recommendation No. 176 which aims to reduce the likelihood of *Bsal* expanding its range throughout Europe. This recommendation states that the signatories develop a number of precautions, including i) imposing trade restrictions on salamanders until risk assessments and prevention/mitigation protocols have been developed, ii) pre-import screening for the pathogen in the live animal trade, iii) setting up and implementing monitoring, surveillance and early-warning systems to detect *Bsal* incursion into the wild as well as the expansion of its range following its introduction, and iv) requiring biosafety for field work, breeding sites and captive collections. In February 2021, the European commission issued a new legislation (2021/361) for commercial trade of salamanders between European Union Member States and for salamanders import into the European Union with disease spread prevention measures regards to *Bsal* infection. This legislation applies to all amphibians belonging to the order of Caudata. It implements animal health requirements and quarantine rules for salamanders being traded between Member States or imported into the Union.

2.1. Trade restrictions and import controls

Since the international trade of salamanders and newts is suspected to be the principal route for the international spread of *Bsal*, bans/restrictions on amphibian trade, alongside controls at import pathways, are likely to be the most effective precautionary measures for preventing the introduction of *Bsal* via amphibian vectors in *Bsal*-free countries (Fitzpatrick *et al*., 2018; O'Hanlon *et al*., 2018). Wildlife trade restrictions, improved quarantine and strengthened biosafety measures will also reduce the probability of introducing yet unknown pathogens and will thus have an impact beyond *Bsal*.

Such actions have already been implemented in several countries outside the European Union (EU) for example the United States (US Fish and Wildlife Service, 2016; EFSA, 2017a; Klocke *et al*., 2017) and Canada (EFSA, 2017a; Canada Gazette Part II, 2017; Wild Animal and Plant Protection Regulation of International and Interprovincial Act, 2017, updated May 12, 2018). The trade restrictions can be found summarised in supplementary table S1.

Within the European continent, import restrictions have been implemented in Switzerland and Hungary (199/2017. (VII. 10.) Korm. Rendelet, 2017; EFSA, 2018; Stark *et al*., 2018) and in 2018, the European Commission issued temporary legislation (2018/320) which establishes animal health protection measures for the trade of salamanders within the EU and the importation of salamanders from non-EU territories (EFSA, 2018; Stark *et al*., 2018). There are omissions that weaken this regulation's relevance (Auliya *et al*., 2016), for example, the non-inclusion of anurans, which can act as *Bsal* carriers (Nguyen *et al*., 2017; Stegen *et al*., 2017) and not regulating animal traffic between private individuals.

Although policy-making with the aim of curtailing the spread of *Bsal* has been conducted relatively quickly in the countries mentioned above, coordinated global measures are required to regulate both the formal (e.g. commercial) and informal (e.g. hobbyists, fairs) amphibian trade (Auliya *et al*., 2016) in order to mitigate the spread of pathogens such as *Bsal*.

2.2. Additional control measures

Import bans of caudates alone may create a false feeling of security. They are unlikely to be 100% effective and *Bsal* is already present in captive amphibians in European regions where no *Bsal* outbreaks in the wild have been reported yet (Fitzpatrick *et al*., 2018; Sabino-Pinto *et al*., 2018b). Within the EU, the trade in captive urodeles has been shown to contribute to the international spread of *Bsal* (Fitzpatrick *et al*., 2018). Thorough screening of captive collections for *Bsal* (e.g. carried out in Germany; Sabino-Pinto *et al*., 2018b; and France; Marquis *et al*., 2019) and immediate treatment of these captive collections upon detection, are urgently needed to eliminate this *Bsal* reservoir, preferably supported by legislation. Based on an estimation of the number of amphibian keepers and number of pet amphibians in Europe, a total initial screening cost of the European states would be well below 1 million Euros as presented in supplementary table S2. Clean trade, meaning the absence of known pathogens throughout the commercial chain, was promoted as a condition for sustainable exotic pet ownership (Pasmans *et al*., 2017). The sale

of caudates in garden centers and other retail outlets should be discouraged, since this suggests suitability for release in garden ponds, which could promote the release of contaminated animals. Several stakeholders have set up campaigns to raise awareness of *Bsal* (see supplementary fig. S2).

2.3. Biosafety measures

It is essential to curb anthropogenic spread of *Bsal* during fieldwork, laboratory research, trade, recreational activities and amphibian husbandry by educating all including the public on appropriate biosafety measures after use of amphibian habitats (Loyau and Schmeller, 2017). An effective measure to avoid spread of this pathogen during fieldwork is to ensure that proper disinfection protocols are utilised for hands, apparel, footwear, equipment and vehicles used in the field (EFSA, 2017b). *Bsal* can be killed using most common disinfectants (table 1) (Van Rooij *et al*., 2017). Individuals involved in amphibian husbandry should ensure that captive urodeles are not housed outdoors and that captive amphibians are not released into the wild. They should also ensure that all waste is properly disinfected and disposed of (EFSA, 2017b).

Virkon S (LANXESS Deutschland GmbH) is used widely, relatively safe and highly efficient, but its use in the field may require derogations from existing legislation. Ethanol (and probably methanol) based commercial disinfectants can also be used effectively. Bleach is also highly effective. Soaking equipment in 10% sodium chloride for 10 minutes is potentially an effective, nontoxic and cheap alternative and its use is worth exploring further. Unfortunately, the commonly used and relatively cheap disinfectant, hydrogen peroxide, has poor activity against *Bsal* (Van Rooij *et al*., 2017). The efficacy of these disinfectants in table 1 against cysts is unknown, however, it is expected to be lower than for spores and sporangia.

Heat treatment can kill all life stages of *Bsal* but its routine use as a disinfectant requires further study. The fungus tolerates high temperatures poorly: *Bsal* cultures are killed after incubation for 5 days at 25°C (Blooi *et al*., 2015a). If *Bsal* responds to heat in the same way as its sister species *Bd*, then exposing materials to 60°C for 5 minutes or 100°C for 1 minute should be an efficient disinfection procedure (Johnson *et al*., 2003). Drying may kill *Bsal*, however, since it is currently not known to what extent encysted *Bsal* spores tolerate drying, it is not recommended as the sole disinfection procedure.

In cases of *Bsal* incursion into the wild, drastic measures, such as closing areas to the public, might be required but such actions might not be compatible with local regulations, as was the case in the Netherlands and Belgium (EFSA, 2017b).

Table 1: Minimal exposure time for 100% killing of *Bsal* spores and sporangia in water and on fomites at room temperature (Van Rooij *et al*., 2017).

Disinfectant	**Concentration**	Minimal exposure time for 100% killing of Bsal
Ethanol (EtOH)	70%	**30 seconds**
Disolol®	undiluted	**30 seconds**
Hibiscrub®	0.25, 0.5, 0.75%	**30 seconds**
Chloramine-T®	0.5% 1%	**5 minutes** **2 minutes**
Bleach	4%	**30 seconds**
Kickstart®	0.05% 0.1%	**5 minutes** **2 minutes**
Potassium permanganate (KMnO4)	1% 2%	**10 minutes** **5 minutes**
Dettol medical®	1:20 dilution	**5 minutes**
Virkon S®	0.5% 1%	**5 minutes** **2 minutes**
Biocidal®	undiluted	**30 seconds**
Safe4®	undiluted	**30 seconds**
F10 ®	1:100 dilution 1:250 dilution 1:500 dilution 1:1000 dilution	**30 seconds** **30 seconds** **30 seconds** **30 seconds**
Sodium chloride (NaCl)	10%	**10 minutes**

Pre-emergence measures can reduce the likelihood of introducing *Bsal* into naïve locations at a relatively low cost. Isolated populations of *Bsal*-threatened species might be considered as disease refugia and be managed by limiting human interaction. However, precautionary measures alone may not be sufficient, particularly without a full understanding of transmission routes for, and potential vectors of, *Bsal*.

2.4. Increasing host resistance

Host resistance against *Bsal* is currently poorly understood. However, adhesion to, and invasion of, the salamander skin are key events that appear to determine the outcome of infection (Martel *et al*., 2014). Provoking a hereditary reduction in the susceptibility of highly susceptible urodelan species may be the only sustainable measure to avert further loss of biodiversity in the long term, given the high probability that *Bsal* will not be eliminated once it has invaded an ecosystem (Feldmeier *et al*., 2016; Schmidt *et al*., 2017; Stegen *et al*., 2017). If we decide to assist in decreasing host susceptibility, three options may be worth exploring: vaccination, bioaugmentation using pre- or probiotics and selective breeding. Based on their close genetic compositions we tend to expect similar responses from both pathogens and the various strains to these pre-exposure mitigation methods. *Bsal*'s genome is 32.6 Mb while Bd's is 23.7 Mb (Farrer *et al*., 2017). Therefore, though there are commonalities, there are still differences which are reflected in the two pathogens being separate species. For example, any intervention which relies on salamanders mounting an immune response is likely to be less successful against *Bsal* (Stegen *et al*., 2017) than other amphibians against *Bd.*

2.4.1 Vaccination

Although there is limited evidence that the development of a *Bd* vaccine might be possible (Woodhams *et al*., 2011; McMahon *et al*., 2014), similar trials with *Bsal* have not resulted in any protection against a challenge with virulent *Bsal* (Stegen *et al*., 2017). There are currently no proofs of concept available for vaccination against *Bsal*. This is probably because *Bsal* severely suppresses immune response in infected hosts (Farrer *et al*., 2017), negating the animal's ability to mount an effective response.

Developing a vaccine is likely to be costly and any vaccine would need to be useful in a range of species. In addition, the creation of vaccines for fungal agents has proven to be much more difficult than for viruses or bacteria, as evidenced by the lack of antifungal animal vaccines. For *Bsal*, there are currently no proofs of concept available. There may also be a need to develop appropriate policy and budget allocations to allow the vaccination of free-living wildlife (Garner *et al*., 2016). Finally, vaccination which requires application to individual wild salamanders would be logistically highly challenging *in situ* (Garner *et al*., 2016; Canessa *et al*., 2018) especially if booster doses were required.

In spite of such challenges, vaccination would be an appealing option in the event that a *Bsal* strain (or another chytrid/micro-organism) was isolated/designed that establishes self-sustaining populations in amphibian communities, is avirulent, safe for target and non-target species, yet evokes a protective response against virulent *Bsal* across host species and life stages and for different chytrid genotypes.

2.4.2 Bioaugmentation

Bioaugmentation is a method of inoculating beneficial probiotics into or on to the animal host or habitat to reduce host susceptibility by microbial defences (Woodhams *et al*., 2011). Probiotics have been isolated from soil, water and amphibian skin (Loudon *et al*., 2014). *Bd*-induced chytridiomycosis has been mitigated, although with variable success by bioaugmentation in the laboratory and in a field trial (Bletz *et al*., 2013), and probiotic therapy should be considered as a potential strategy for *Bsal* mitigation. Knowing any potential risks that probiotics pose to ecosystems and amphibian hosts is important prior to any application to wild populations. The risks of introducing probiotics in the wild are manifold, including disruption of nutrient cycling, which could have important cascade effects for the whole ecosystem (Schmeller *et al*., 2018).

In addition, a suitable probiotic for bioaugmentation should be effective across *Bsal* genotypes, should result in persistent colonization of the urodelan skin at densities that facilitate their antifungal activity, should preferably be transmissible to conspecifics (including offspring) and should be safe and espouse qualities which would allow it to be produced in large volumes. In order to understand the bacterial community on amphibian skin and identify the effect of probiotics on *Bsal* establishment, a much better understanding will be required (Bates *et al*., 2018), including of the host-pathogen-environment triangle (Schmeller *et al*., 2018). Recent work by Bletz *et al*. (2018) and Bates *et al*. (2018) has shown that *Bsal*-induced death coincides with significant perturbation of the bacterial community, resulting in increases of opportunistic bacteria that cause septicaemic events (Bletz *et al*., 2018). Besides, the composition of bacterial communities on urodele skin is highly dependent on their surrounding environment, raising the possibility that laboratory trials with *Bsal* may be influenced by the mere transition of the animals to captivity (Bates *et al*., 2018). Currently, there are no proofs of concept that bacteria or other microbes protect susceptible salamanders against *Bsal* infection at natural microbial densities. On

the contrary, Bletz *et al*. (2018) suggest that bioaugmentation might be impeded, at least in fire salamanders, as very low numbers of bacteria are maintained on their skin.

Although these bacterial communities on the salamander skin do contain bacterial lineages with pronounced *Bsal*-inhibiting capacity *in vitro*, only the repeated and consistent application of very high doses of these lineages was capable of attenuating *Bsal* infection (Bletz, *et al*., 2018).

2.4.3 Selective breeding

Increasing resistance against *Bsal* infection either by selective breeding (resembling natural selection by cross breeding the most resistant animals) or by genetic engineering could be an effective strategy in the mid- to long-term to permanently avert the risk of *Bsal*-induced population crashes. Based on their close genetic compositions we tend to expect similar responses from both pathogens to selective breeding. However, while there are commonalities, there are still difference in genetic composition and still a lot of important information on *Bsal* yet to be elucidated. While some frogs exposed to *Bd* and antifungals demonstrated a reduction in susceptibility (Garner, *et al*., 2016), salamanders previously exposed to *Bsal* did not demonstrate decreased susceptibility (reduced mortality) (Stegen *et al*., 2017).

Further, this would require extensive resources for training staff in genetic engineering, infrastructure and genetic management. Selective breeding requires the availability of markers for resistance. Genetic engineering requires the identification of the genetic basis underpinning host resistance (with relevance for the situation in the wild). While gene editing in amphibian eggs is commonplace, genetic engineering in viviparous species of the genus *Salamandra* presents an additional challenge. For *Bd*, susceptibility has been linked to several genetic markers and modifying several of the encoding genes to decrease disease susceptibility may result in difficult to predict, severe side effects. Since a targeted approach is hindered by a lack of knowledge of the determinants of susceptibility to *Bsal*, untargeted approaches may yield usable results, yet no proof of concept (neither for *Bd* nor *Bsal*, and in fact not for any infectious disease in vertebrates) exist. For selective breeding, the slow generation time of many urodeles (typically 3-4 years) precludes the rapid evolution of resistant populations. In the current absence of suitable markers, selecting for resistant individuals in captivity will require the use of large numbers of animals in (sub-)lethal animal experiments, which may raise ethical concerns. The European Union is currently reluctant to allow the use of genetically modified organisms in agriculture and targeted

modification of the urodele genome, while increasingly feasible, will have to deal with regulatory issues before any such animal can be released into the wild. In contrast with *Bd,* where response varies by species and sometimes populations (Bataille *et al*., 2015), there is no evidence of selection for individuals with increased disease resistance in infected, natural populations. For example, Stegen *et al*. (2017) demonstrated high susceptibility in the few remaining salamanders at an outbreak site and in 2018, several *Bsal*-infected salamanders were found dead at the index outbreak site in the Netherlands, where an estimated 0.1% of the animals has survived.

Selective breeding will probably be perceived by public opinion as more acceptable compared to genetic engineering (Garner *et al*., 2016) but 10 years of selective breeding of midwife toads has not resulted in any notable increase in their resistance against *Bd* (Bosch, unpublished). Genetic engineering could achieve the desired outcome of a more robust genetic lineage with decreased susceptibility, however, so far no genetic target has been identified. Both options could be explored but it will likely take decades before either could be shown as being successful – and probably only for a single species in that time frame. Selective breeding and genetic engineering, therefore, cannot be seen as short-term measures to address the urgency of *Bsal* mitigation, but at best as mid to long-term mitigation strategies. Moreover, these strategies also have significant drawbacks. Selective breeding could, if not done on a large enough scale, by including a sufficient population number, result in loss of species diversity and inbreeding depression, leading to an overall less robust genotype and an emergence of inbreeding diseases. The same would occur if genetic engineering is not conducted on sufficient animals to ensure a robust and diverse gene pool. On a grander timescale, animals that are not result of selective breeding or genetic engineering, are more susceptible to chytridiomycosis could potentially die off, thereby decreasing the overall genetic diversity of the species.

3. Post-exposure measures

Bd was already widespread and had decimated many amphibian populations in several countries before its diagnosis. Epidemiological investigations of *Bd,* causative agent of chytridiomycosis, were reported to have started 15 years after amphibian declines were initially observed, resulting in population declines, extirpations, and extinctions of approximately 200 species (Grogan *et al*., 2014). These measures have all been considered or research has been initiated for *Bd* mitigation. A few were implemented on various scales with varying levels of success (Woodhams *et al*., 2011;

Garner *et al*., 2016). In some populations and countries affected by *Bd,* host and pathogen have reached co-existence. *Bsal* was discovered much more recently, has not been detected in many countries and has been detected in relatively small regions in those affected. Therefore, the opportunity still exists to implement measures to avoid the incursion or delay the spread of this fungus. Also, if *Bsal* enters a naïve location, the efficacy of these methods may be high as the pathogen will likely be limited to a much smaller geographical area and fewer populations. However, the presence of two different forms of the fungus with one of them being the encysted environmentally resistant spore, makes success of these individual mitigation methods less likely. Once there has been a *Bsal* incursion to a novel site, mitigation methods should focus on: 1. reducing the impact of the pathogen on susceptible amphibian species 2. setting up conservation strategies to prevent population extirpation and 3. preventing further *Bsal* spread. In exceptional cases, elimination of *Bsal* from the system may be attempted. However, the presence of animal and environmental reservoirs will likely preclude eradication from most ecosystems (Stegen *et al*., 2017). These post-emergence approaches can be classified as measures to i) reduce the fungal load in the environment or host, and ii) safeguard populations from *Bsal*-induced extirpation. Such measures can be generally divided into *in situ* and *ex situ* approaches.

Short-term solutions are considered vital in temporarily preserving amphibian populations at risk (Garner *et al*., 2016). For example, as shown for *Bd*, interventions with antifungals during an epidemic can alter infection dynamics and alleviate disease (Hudson *et al*., 2016; Geiger *et al*., 2017). However, in the absence of long-term disease management *in situ*, any short-term measure is unlikely to result in significant conservation success. This underscores the importance of further research into potentially effective mitigation measures. Here, we will discuss captive assurance colonies, *in situ* treatment of animals and the environment, creating barriers to limit *Bsal* spread and bioaugmentation.

Some bacteria have been detected to decrease *Bd in vitro* and in the field (Bletz *et al*., 2013). In the case of *Bsal,* some bacteria found on the host's skin have been able to reduce *Bsal in vitro* (Bletz *et al*., 2018). Physical barriers appear to have reduced spread of both *Bd* and *Bsal* from infected populations to naïve populations located within close proximity (Rodríguez-Brenes *et al*., 2016; Spitzen-van der Sluijs *et al*., 2018). Captive assurance colonies have had mixed outcomes in the case of *Bd* (Woodhams *et al*., 2011) and we expect will be just as challenging for *Bsal,*

especially in terms of husbandry of such varied hosts and the host-pathogen-environment triangle. *In situ* treatment of the environment/animals has also been carried out for *Bd* and has some success (Woodhams *et al*., 2011; Garner *et al*., 2016). These *in situ* treatments of animals and the environment are expected to have some success in reducing the number of *Bsal* spores in the environment. However, they may not be as effective on the environmentally resistant spores. In addition, since less information is currently available on *Bsal*, these mitigation measures are not likely to work better than they have for *Bd*.

3.1 Reducing the impact of *Bsal*

3.1.1 Reduce fungal load

3.1.1.1 Decontaminating and manipulating environments

Manipulating *Bsal*-infected environments by applying *in situ* intervention measures can be implemented to limit the spread of infection, reduce the impact of the pathogen and, by extension, increase amphibian survival. Environmental manipulations may be biological, physical or chemical and applying environmental interventions, such as the use of natural predators, antibiotics, fungicides, pond-drying, disinfectants and changes in ambient temperature are the most common methods used for the veterinary treatment of fungal diseases in aquaculture (Woodhams *et al*., 2011).

Hitherto, no environmental treatment has been applied to mitigate *Bsal* infection, but a few interventions have been shown to be effective to control *Bd*. Using aquatic invertebrate 'micropredators' for the removal of *Bd* from the aquatic environment has been identified as a potential mitigation measure for aquatic or semi-aquatic species and may also be potentially used against *Bsal* spores (Buck *et al*., 2011; Searle *et al*., 2013; Schmeller *et al*., 2014a). However, it is unclear to what extent the availability of other food sources influences the capacity of these micropredators to remove spores from the environment. *Bd*-removing micropredators were found to contribute to creating refuges from chytridiomycosis (Blooi *et al*., 2017).

Eliminating the environmental reservoir of *Bsal* can be expected to contribute to controlling *Bsal* outbreaks. Crucial information is currently lacking about whether, how and to which extent *Bsal* (but equally *Bd*) can persist in the environment in the absence of amphibian hosts. Identifying and enhancing micropredators which are able to reduce the number of *Bsal* spores in the environment

may eventually lead to a reduction in the number of infected amphibians. The situation for *Bsal* is more complex compared to *Bd*, since *Bsal* produces two types of spores: zoospores and encysted spores. The latter, floating at the water-air interface, were shown to be less susceptible to predation (Stegen *et al*., 2017). Also, it is unclear whether a similar principle of predation is applicable to terrestrial systems. Currently, there is no proof of concept available of the impact of manipulating micropredator dynamics on amphibian chytrid dynamics in nature. Therefore, applying this approach to field situations requires caution since either selectively enhancing specific components or adding foreign organisms to ecosystems may alter foodwebs.

Physical methods, such as pond-drying and elevating the temperature of ponds, have been used to destroy *Bd* in the environment despite facing several challenges such as legal (protected species and habitats present), technical and epidemiological (for example: propensity of amphibians to escape from drying ponds, which may propagate pathogen spread). Physical methods are expected to have similar success in decreasing the *Bsal* zoospores in the environment while experiencing similar challenges as with *Bd*. *Bd* does not survive drying (Johnson *et al*., 2003) and the efficacy of pond-drying, in relation to *Bsal*, will depend on how *Bsal* spores respond to desiccation. The efficacy of pond-drying and elevating the temperature of ponds will also depend on the type of *Bsal* spores present in the environment. These methods are not expected to work as efficiently on the environmentally resistant encysted form of the *Bsal* spore (Stegen *et al*., 2017).

Subjecting the fungus to temperatures and conditions which are unfavourable for growth and persistence of aquatic and other life stages, will result in its reduction. Johnson *et al*. (2003) showed *in vitro* that *Bd* is sensitive to desiccation and is fully cleared within 1h of drying. However, in a field study by Bosch *et al*. (2015), pond drying combined with the application of itraconazole did not eliminate *Bd* but merely decreased infection intensities for a short period of time. When these were combined with environmental disinfection, later, *Bd* was eradicated. Also, if pond drying is not done at an appropriate time it could result in dispersal of infected individuals, the destruction of the local ecology, including the death of tadpoles, eradication of local benign nano-, micro- and mesoplankton, which could negatively affect amphibian populations and other biodiversity. Finally, pond drying is more difficult to apply to important urodele habitats such as streams. On the other hand, for species that reproduce in ephemeral ponds, strategic artificial desiccation may result in the elimination of *Bsal* and of predators of amphibian larvae thus

increasing juvenile survival and population persistence (Johnson *et al*., 2003; Woodhams *et al*., 2011). Any mitigation strategy that may potentially involve the degradation or destruction of habitat will require a careful and transparent cost-benefit analysis (where "costs" is used to encompass any side-effect, including environmental damage).

Increasing the water temperature of amphibian breeding ponds, which can be achieved by removing canopy cover, can provide an important refuge from *Bd* (Freidenburg and Skelly, 2004; Forrest and Schlaepfer, 2011; Savage, Sredl and Zamudio, 2011; Scheele *et al*., 2014). Decreased shading of ponds is linked to lower *Bd* infection intensities (Raffel *et al*., 2010; Heard, *et al*., 2014). While this is cost-effective and would be beneficial to amphibian species which are tolerant of or even prefer higher temperatures (Langton *et al*., 2001), the relevance for European urodeles can be questioned. Increasing water temperatures may be expected to be poorly tolerated by heat-sensitive species and its relevance for lotic ecosystems is very uncertain. Besides issues of feasibility, water temperatures should be higher than 25°C to kill *Bsal* (Blooi *et al*., 2015a), exceeding the thermal preferences of many European urodeles. While its efficacy has yet to be demonstrated, it may be worth considering the option of decreased shading of terrestrial habitats as a supportive action to reduce environmental *Bsal* loads through surface heating and desiccation. Again, competing objectives such as revegetation targets, the impact on other species and broader issues such as forestry interests will need to be taken into account.

The environmental application of chemical treatments is another option for fungal disease mitigation. Applying the disinfectant Virkon S 1% (as experimented by Bosch *et al*., 2015 at the breeding sites of *Alytes muletensis*) or adding sea salt to increase salinity (Stockwell, Clulow and Mahony, 2012; 2015) were able to eliminate or lower *Bd* infection in the aquatic environment and may be promising strategies for inhibiting *Bsal* growth. Fungicides have only been used in simple single-host systems and controlled, isolated habitats (Garner *et al*., 2016) and it remains to be demonstrated whether they could work in more complex habitats. In addition to these potential limitations to their *in situ* application, preliminary studies indicate that fungicides and disinfectants are ineffective in curbing *Bsal* (Van Rooij *et al*., 2017).

Creating saline refuges in amphibian environments has been suggested as a feasible conservation method to control *Bd* infections in anurans, being relatively cheaper than other methods. While this method functions by disrupting chytrid growth and motility (Stockwell, Clulow and Mahony,

2015), it has been shown to have deleterious effects in aquatic organisms (Karraker, Gibbs and Vonesh, 2008; Denoël *et al*., 2010; Karraker and Gibbs, 2011; Tollefsen *et al*., 2015; Jones *et al*., 2016). It will also be difficult to apply to lentic systems, and like with fungicides, its effects in terrestrial systems remain unknown.

The methods used in environmental manipulation may create tolerance to, or resistance against, *Bsal* among small isolated groups of amphibians and also provide sanctuaries for focal species deemed highly vulnerable and of particular conservation concern. However, they may be less effective mitigation measures for amphibians with large ranges and their effects may be variable in complex habitats. Environmental manipulation may face many legal barriers and may conflict with other conservation priorities. For example, manipulations in protected areas or with negative effects on protected species or habitats may require environmental impact assessment and public consultation.

3.1.1.2 In situ treatment of the amphibian host

There have been no studies to date that have investigated the *in situ* treatment of amphibians infected with *Bsal*. Hudson *et al*. (2016) and Geiger *et al*. (2017) evaluated the impact and feasibility of *in situ* treatment using the antifungal drug itraconazole to mitigate *Bd*-induced amphibian chytridiomycosis. Firstly, it is easier to treat *Bd*-infection than *Bsal* using itraconazole exclusively. The results from Hudson *et al*. (2016) and Geiger *et al*. (2017), indicated that itraconazole treatment decreased the probability of *Bd* infection and the mortality rate of infected animals, however, as soon as treatment was ceased, all benefits disappeared and the infection and mortality rate increased to those of untreated individuals. This suggests treating infection does not induce any protective immune responses to *Bd* (Hudson *et al*., 2016) and, when based on empirical data without proper toxicity assessment, may even have detrimental effects on the survival of a species (Loyau *et al*., 2016). This *in situ* treatment method, while labour-intensive and limited to amphibian species for which recapture rates are relatively high, could be used as a short-term conservation tool to reduce the mortality caused by *Bd* or *Bsal* during periods of high disease risk or to gain time during disease outbreaks while a more permanent solution is identified (e.g. Hudson *et al*., 2016; Geiger *et al*., 2017). Effectively treating a *Bsal*-infected fire salamander population would require an almost total coverage of the population, combined with a 100% effective treatment to interrupt transmission (Canessa *et al*., 2018). Anything less might result in

adverse effects: prolongation of the survival of infected animals would increase the potential for disease spread within and outside the focal population. Such an effective treatment can be done only *ex situ*, since it requires repeated and consistent application of either the use of relatively high temperatures (25°C) or a combination of the antimicrobial drugs polymyxin E and voriconazole (Blooi *et al*., 2015a; 2015b). In practice, this would mean removal of all infected animals from their habitat and release after treatment.

3.1.2 Safeguard Populations

3.1.2.1 Bioaugmentation and vaccination

For vaccination, see section 2.4.1. Provided a protective vaccine can be developed, this could be applied during an outbreak to limit losses. For bioaugmentation, see section 2.4.2. Besides being a preventative approach, micro-organisms, either alone or in mixtures, could potentially be used therapeutically during a *Bsal* outbreak to limit the impact of infection.

3.2 Preventing further *Bsal* spread

3.2.1 Reduce fungal load

3.2.1.1 Removal of hosts

The removal of infected or even of all susceptible hosts (including non-infected) from a population might be a mitigation strategy worth exploring. In susceptible species, the eradication of *Bsal* is likely to require the removal of a substantial proportion (> 90%) of the focal hosts as well as any other species in the same area that are acting as reservoirs (Canessa *et al*., 2018). Moreover, *Bsal* has been shown to persist in the environment in the absence of amphibian hosts. This possibly explains, at least in part, the high probability that a susceptible population will be extirpated by *Bsal* (Stegen *et al*., 2017). It also means that eradication from a site is unlikely, although the likelihood of this will be increased the longer the site is maintained free of amphibians. However, even if eradication cannot be achieved, removing infected animals reduces the probability of spillover of *Bsal* to neighbouring populations (Canessa *et al*., 2018; Spitzen-van der Sluijs *et al*., 2018). The reaction of the public to host removal may be expected to vary according to the fate of the animals removed. Translocation of these animals to other sites should be strongly discouraged and reintroduction at the original site is only acceptable after *Bsal* eradication has been demonstrated and maintained for a reasonable period of time. Otherwise, this

may result in flare-ups of infection, with the likelihood of further spread to neighbouring sites. While culling may well be the most rational option, and is well accepted in OIE disease control programmes, this is more likely to meet adverse reactions compared to transferring the animals to captivity with subsequent treatment.

3.2.2 Safeguarding populations

3.2.2.1 Creating barriers to the spread of Bsal

Simple mathematical models suggest that *Bsal* will spread rapidly in a homogeneous landscape (Schmidt *et al*., 2017). Yet, this is not what was observed near the *Bsal* index site. In fact, there are indications that the natural (autonomous) spread of *Bsal* is relatively slow and can be interrupted by barriers that limit dispersal of infected salamanders such as rivers, highways, unsuitable habitat and fences (Spitzen-van der Sluijs *et al*., 2018). That study did not identify biotic or abiotic vectors of *Bsal*, but its results suggest that the local movement of infected hosts may be crucial in the dispersal of *Bsal* over short distances, whilst human-mediated transmission will be the most important pathway of long-distance spread. Understanding the fundamentals of range expansion would offer opportunities for developing barrier-based strategies. This may be used to protect uninfected (sub)populations through isolation, or to contain outbreaks if caught at an early stage. Such measures may be effective in the short-term and could significantly reduce the risk of spread of *Bsal*, but their efficacy in the mid to long-term is unclear, given the non-continuous distribution pattern of *Bsal*. This pattern is characterized by often large distances between outbreak sites, which are highly unlikely to be bridged by infected salamander hosts within the observed timescales. Although human-mediated spread may at least in part explain the long distance dispersal of *Bsal*, between-site transmission is currently poorly understood and biotic (e.g. birds) and abiotic dispersers cannot be currently excluded. However, the persistence of an uninfected fire salamander population for over 8 years only 800m from the *Bsal* index outbreak suggests that managing landscapes, exploiting existing barriers and creating meaningful barriers may be a relatively low-cost option worth exploring.

3.3 Setting up conservation strategies to prevent population extirpation

3.3.1 Safeguarding populations

3.3.1.1 Reintroduction and captive breeding

Introductions to restore original populations require prior removal or management of the *Bsal* threat in the wild (IUCN, 2013; Muths and McCallum, 2016). Affected host species could be reintroduced, either with translocations from other wild populations or using individuals that have been captured and treated or bred in captivity. Also, reinforcement of extant populations may be implemented in combination with other mitigation actions that augment resistance to infection or disease. More radical options might also include the assisted movement of threatened species to areas of lower *Bsal* risk (Gagliardo *et al*., 2008). Experience shows that efforts to establish captive assurance colonies should be initiated early in the mitigation process (Martin *et al*., 2012). Given resource limitations, prioritisation is inevitable and conservation units (from population to species level) have to be defined (see further). Establishing captive assurance colonies is currently the only effective action to preserve species with small ranges, or otherwise valuable populations, following invasion by *Bsal*. Although this is a feasible option, any such action should be planned and executed carefully and conducted from the outset, with an explicit view to future reintroduction options (Canessa *et al*., 2016). This includes keeping animals under high levels of biosafety as necessary to prevent exposure to other pathogens that might eventually be released into the wild with the animals or their offspring, as was the case with the contamination of Mallorcan midwife toads by *Bd* (Walker *et al*., 2008). Also, captive assurance colonies need to have informed genetic and veterinary management - which often requires the involvement of multiple centres - and to be run in accordance with IUCN guidelines (Pessier *et al*., 2014). Protocols for such assurance colonies and resources should preferably be in place for all high-risk populations or species. Expertise to maintain and breed European urodeles is widely available, although largely limited to the private sector (e.g. DGHT, AG Urodela). Currently, only one European species is propagated consistently in the framework of a captive assurance colony, combined with reintroduction efforts: the Montseny brook newt (*Calotriton arnoldi*, LIFE-Tritó project, http://lifetritomontseny.eu/). Such captive assurance colonies would benefit from participation and collaboration of professional organisations (zoos, aquaria, represented by EAZA), research institutions and the private sector (Pasmans *et al*., 2017).

4. Supporting actions

The actions discussed above seek to achieve a conservation objective, namely to ensure the persistence of populations or species by preventing the introduction of *Bsal* or by mitigating its

effects if it is introduced. To be effective, such actions need to be informed by knowledge of *Bsal* host-pathogen dynamics and information on *Bsal* spread, host conservation status and outcomes of any previously implemented action. Moreover, mitigation strategies will require many decisions to be taken at different levels, from the global to the local scale, with widely differing levels of available resources. Here, we detail several actions that might assist the broader mitigation process. It must be noted that these actions are only useful in supporting the mitigation actions discussed above: for example, monitoring alone will not abate the negative impacts of *Bsal*, but the data collected are vital in understanding where and how to implement conservation interventions.

4.1 Early-warning system

An early-warning system is a valuable tool for rapid *Bsal* detection and response. It consists of *Bsal* notification points that are responsible for national or regional surveillance for, and the collection of, dead amphibians (by local volunteers) and the determination of the cause of death. Early warning systems were largely unimplemented for a long period during *Bd*'s spread since the cause of amphibian mortality remained elusive. Epidemiological investigations of *Bd* were reported to have started 15 years after amphibian declines were initially observed, resulting in population declines and extirpations, and extinctions of approximately 200 species (Grogan *et al*., 2014). A sensitive and specific diagnostic technique that shows high interlaboratory reproducibility of results is key to an efficient early-warning system and, for *Bsal*, consists of quantifying *Bsal* genome equivalents in non-invasively collected skin swabs (Blooi *et al*., 2013; Thomas *et al*., 2018). Once more information on the disease killing amphibians became known, a sensitive and specific test with interlaboratory reproducibility for detection of *Bd* was developed by Boyle *et al*. (2004). Presence of *Bd* was detected on museum specimens collected over a century prior to detection of chytridiomycosis infection. As part of a project, funded by the European Commission (Tender ENV.B.3/SER/2016/0028, Mitigating a new infectious disease in salamanders to counteract the loss of biodiversity, http://bsaleurope.com/), notification points have been set up in Belgium, France, Germany, the Netherlands, Spain, Italy, and the UK. The setup of an effective early-warning system requires informing, and active involvement of all stakeholders

(including the public), building sufficient diagnostic capacity and efficient data management, including proper reporting to the OIE (*Bsal* was listed in 2017 as a notifiable animal disease by the OIE).

Detecting environmental DNA of target organisms (eDNA) (Taberlet *et al*., 2012) is now widely used for biodiversity inventories, and recommended for use in the early detection of invasive species (Darling and Mahon, 2011). For *Bd*, eDNA detection in water was shown to be efficient in detecting occupancy of ponds by *Bd*, yet was demonstrated a laborious procedure (Schmidt *et al*., 2013). The applicability of eDNA for detecting *Bsal* is currently uncertain and would require detecting pathogen DNA in more complex matrices such as forest soil. This method would be useful for detection of *Bsal* during the aquatic phase of urodelan life. However, many salamanders in Europe are terrestrial, thus the testing for eDNA would need to be carried out in matrices more complex than water.

Regardless of the diagnostic method used, an efficient early-warning system should include active and passive disease surveillance. We here use the terms pathogen and disease surveillance as ongoing recordings of *Bsal* and *Bsal*-associated disease in wild amphibian populations. "Passive pathogen and disease surveillance" is used for the recording of *Bsal* and *Bsal* disease presence as they occur (reactive) and "active pathogen and disease surveillance" for targeting individuals to detect *Bsal* and *Bsal* disease presence (proactive). Active and passive surveillance was implemented for *Bd* pathogen and disease in several countries in Europe (Garner *et al*., 2005). *Bd* spread to many countries before information was available on the cause of amphibian mortality in those locations therefore, many measures which have been implemented as part of the early warning system against the incursion of *Bsal* were not able to be implemented in those environments but may still be implemented in countries or regions free of *Bd*.

4.1.1 Passive disease surveillance

Passive surveillance of *Bsal* outbreaks is currently done by the reporting of opportunistically observed suspect cases to a regional hotline for further examination. Observers can be professionals or lay people. Passive surveillance can enable the detection of disease across large spatial scales, but the likelihood of detection depends on many factors such as the mere detectability of the affected species (many urodele species are secretive), the degree of observer effort (e.g. number of observers and amount of time each observer spends looking for diseased animals), the ability of observers to identify disease and the likelihood that any diseased animals detected will be reported to the relevant authority (Buckland *et al*., 2010; Kéry and Schmidt, 2008; Lawson, Petrovan and Cunningham, 2015). Key example of public reporting leading to disease

detection in this context is detection of the index outbreak of *Bsal* in the Netherlands, the first signs of which were noted as a steep population decline in the framework of a long-term salamander monitoring campaign. Passive surveillance currently seems to be the most feasible approach for detecting the occurrence of *Bsal* disease outbreaks in Europe, at least in species with large ranges (EFSA, 2018). Through the European Union *Bsal* tender, passive surveillance for amphibian diseases recently initiated at the national level in Belgium, France, Germany, Italy, the Netherlands, Spain, Switzerland and has been ongoing at the national level in the United Kingdom since 1989 (Lawson, Petrovan and Cunningham, 2015) (http://bsaleurope.com/).

4.1.2 Active disease surveillance

Implementing a thorough system of active surveillance throughout Europe would be the most reliable way to determine the current distribution of *Bsal* infection in the wild; however, such a system would require enormous amounts of resources that may need to be diverted from other uses. It may be more efficient to concentrate active surveillance and monitoring within and around localities where a disease outbreak consistent with *Bsal* chytridiomycosis is detected (EFSA, 2018). In Austria, Belgium, Croatia, Czech Republic, France, Germany, Portugal, Slovenia, Spain, Switzerland, the Netherlands and the UK, non-systematic active surveillance has been carried out on an *ad hoc* basis (EFSA, 2018). Active surveillance for *Bsal* is currently done by proactively sampling amphibians for presence of *Bsal* infection or for *Bsal* disease itself in a quantitatively adequate number of populations. Since *Bsal* outbreaks are characterized by collapses of urodele populations, the least costly option is to monitor sentinel populations of susceptible host species for signs of population declines. Such actions can be designed as citizen science projects (Dickinson *et al*., 2012) coordinated by relevant scientific entities. Integration of professional and citizen-science monitoring schemes may broaden the coverage and amount of data collected, particularly if optimised spatially and temporally (Morán-Ordoñez *et al*., 2018). Longitudinal monitoring of amphibian populations is key to interpret disease findings and provides the necessary baseline information to evaluate disease impact.

4.2 Monitoring of ongoing population declines and past outbreak sites

Populations already in decline and adjacent ones, require special attention via monitoring (Grogan *et al*., 2014; Ficetola *et al*., 2018). Monitoring of the host population and the pathogen should continue well after host populations are ascertained to have declined or been extirpated, to provide

information about *Bsal*'s persistence in the environment and/or in alternative hosts. In the future, this will provide useful information for the development of post-outbreak restoration protocols, such as reintroductions.

4.3 Conservation prioritisation

Scientific evidence is essential to narrow knowledge gaps and inform the decision-making process as to which species are prioritised. However, clarifying the decision context (who decides whether a species should be allocated resources, who provides those resources, who implements the action) is just as important (Game, Kareiva & Possingham, 2013). Prioritisation of *Bsal* mitigation actions at the European level would need to follow these four steps: (1) definition of priorities, based on EU, state or local legislation, or criteria describing the importance of species and subspecies in terms of e.g. genetic diversity, ecosystem function or cultural values; (2) a complete risk assessment of the impacts of *Bsal* on all species; (3) evaluation of the benefits and costs of potential actions for each species by an expert panel including scientists, managers and policy-makers; (4) identification of priority species (selection and listing of specific species that fit the criteria for prioritisation per point 1 above). In the current situation, information about species-specific risks and actions is urgently needed.

Thirty-four urodele species occur across the 27 EU member states (European Red List, 2018). Given the limited resources available, it is unlikely that full protection against *Bsal* impacts could be provided to all those species in all those countries (also considering the intraspecific variants of conservation interest). Several quantitative methods for transparent conservation prioritisation have been developed (Brooks *et al*., 2006; Schmeller *et al*., 2008; Joseph, Maloney and Possingham, 2009; Moilanen, Wilson and Possingham, 2009; Grant *et al*., 2017; Gerber *et al*., 2017). Prioritisation is the result of a trade-off between the potential for successful conservation (the actions available and their chances of success, given the risk to a species) and the preferences and constraints of the decision makers, such as the conservation value attributed to a species, its distribution range, available resources, unwanted effects on ecosystems, and attitudes to risk (Joseph, Maloney and Possingham, 2009; Tulloch *et al*., 2015). Understanding these components and treating them appropriately is key to a transparent decision-making process (Game, Kareiva and Possingham, 2013).

These trade-offs are also relevant in the case of *Bsal*. First, priorities will inevitably depend on the decision context. For example, *S. salamandra* may not be considered a conservation priority at the EU level or in many countries in which it is common. Because of its restricted geographical range in the Netherlands and the fact that it has been severely affected by *Bsal, S. salamandra* is prioritised for conservation there (Spitzen-van der Sluijs *et al*., 2013). Many possible criteria for prioritisation have been suggested, from genetic representativeness (Isaac *et al*., 2007) to range-wide relevance of local declines (Schmeller *et al*., 2008; 2014b) to cultural values (Pollard *et al*., 2014). The object of prioritisation, is utilising feasible mitigation measures which are available for conserving species: if actions to mitigate *Bsal* are not available or feasible in practice, species priorities have little meaning (Brown *et al*., 2015). Also, the management of more common species that may for example serve as disease reservoirs needs to be implemented so that primary mitigation actions can be effective (Dobson, 2004; Stegen *et al*., 2017). This also applies to monitoring, where sentinel species might be prioritised for surveillance even though they are not conservation priorities (Halliday *et al*., 2007).

5. Conclusion: critical research gaps and future actions

Bsal mitigation is surrounded by a high level of uncertainty, however, this should not result in protracted decision-making periods or inaction as this will lead to certain biodiversity loss. From a pragmatic conservation perspective, the main objective of mitigating *Bsal*-induced chytridiomycosis should be to preserve susceptible amphibian species and populations and protect biodiversity, rather than the eradication of *Bsal* in the wild *per se*. In this sense, any single method is unlikely to accomplish the desired conservation outcome (Gagliardo *et al*., 2008; Garner *et al*., 2016). Each approach has its benefits and limitations; therefore, a combination of methods may have the best chance of success.

Given the lack of verified, reliable disease mitigation options, we advise that pre-emptive measures, aimed at reducing pathogen spread and further pathogen introductions by a combination of trade restrictions, biosafety measures and eliminating the captive *Bsal* reservoir are enacted as a matter of urgency. The set-up of a long-term population monitoring network is key in the early recognition of changes in population sizes, which allows estimating disease impact and evaluation of population recovery. Developing and maintaining a robust early warning system based on passive surveillance will be highly beneficial for the implementation of these *Bsal* control

measures. Another important supporting action is the monitoring of host population (size) and *Bsal*-infection dynamics (prevalence, mortality) in known outbreak areas with a view to making apropos conservation decisions. The final supporting action is the development of an evidence-based emergency action plan for at-risk species.

In case of a *Bsal* outbreak, actions that can be taken should focus on disease containment and preserving valuable populations or species where relevant. Disease containment may consist of a rigorous combination of:

1) limiting opportunities for pathogen dispersal, for example by fencing off areas and restricting access to prevent entry of humans, large mammals, waterbirds and anurans.

2) eliminating potential *Bsal* environmental reservoirs (drying and disinfection of ponds).

3) identifying and eliminating potential *Bsal* amphibian reservoirs by consistent and repeated removal of *Bsal* hosts.

4) delineating the outbreak by intensive monitoring of neighbouring populations for *Bsal* infection and population declines by repeated sampling using skin swabs and population monitoring.

Establishment of *ex situ* assurance colonies is the most immediately viable course of action and the only option available currently to preserve populations or even species at risk from *Bsal*. However, this must be implemented with the primary intention of developing a long-term protection strategy for effective and sustainable reintroduction. The latter needs applied conservation studies into sustainability, feasibility and effectiveness of mitigation actions (Table 2).

The implementation of current legislation and the above mentioned recommendations is likely to reduce introduction events of *Bsal* and may contain the disease at novel outbreak sites, but does not provide long-term, sustainable solutions for infected systems. This will require closing the following critical knowledge gaps:

1) introduction pathways: while it is currently assumed that amphibian trade is key in the global dispersal of amphibian-infecting chytrids (Martel *et al*., 2014; O'Hanlon *et al*., 2018), proven examples of this are rare (Walker *et al*., 2008). Identifying crucial components of amphibian-associated pathways for introducing chytrids (not *a priori* excluding any biotic or abiotic vector) would increase the efficacy of measures aimed at preventing further introductions.

2) understanding pathways of the dispersal of *Bsal* between populations. Preventing the further spread of *Bsal* in Europe from the existing outbreak sites requires knowledge of mechanisms underpinning this pathogen's spread. While dispersal through infected amphibian hosts seems important at short distances (Spitzen- van der Sluijs *et al*., 2018), human-mediated spread may be key on a larger spatial scale. However, the possible contribution of other biotic (e.g. migratory birds, large mammals) and abiotic (e.g. waterways, wind) vectors is not yet known.

3) understanding *Bsal* reservoirs is crucial to any *in situ* control programme: an eight year follow-up of the *Bsal* index outbreak demonstrates very low prevalence, with very low infection loads in the supposed reservoir host (Alpine newt), suggesting that the existence of a different, non-amphibian reservoir of *Bsal* may be necessary to maintain *Bsal* in this ecosystem. Identifying critical components in an affected ecosystem that allow *Bsal* persistence could greatly contribute to any eradication action.

4) understanding host susceptibility to *Bsal* infection. Any action aimed at increasing resistance against infection will benefit from a thorough understanding of the host-pathogen-environment interaction, knowledge of which is currently in its infancy. Understanding crucial events like adhesion and intra-epidermal pathogen proliferation from a host, pathogen and environment perspective could open opportunities for vaccination, bioaugmentation, environmental augmentation and the eventual creation of more resistant host lineages.

Table 2: Advantages and limitations of mitigation and support actions against *Bsal*

Mitigation action	**Advantages**	**Disadvantages**
Trade restrictions: importation of live urodeles into the EU	Likely to greatly reduce chances of further *Bsal* introduction in EU Associated costs low in case of ban Relative ease of implementation and control	May promote illegal trade As a stand-alone measure does not prevent *Bsal* spread within EU Associated costs are significant in case of implementing quarantine and entry control measures
Trade restrictions within EU	Likely to greatly reduce chances of *Bsal* spread between EU member states Relative ease of implementation Associated costs low in case of ban	May promote illegal trade Difficult to control As a stand-alone measure does not prevent *Bsal* spread within member

		states Associated costs significant in case of implementing quarantine and entry control measures
Eradication of *Bsal* from captive urodeles	Elimination of *Bsal* reservoir with reduced likelihood of pathogen pollution, part of a "clean trade" programme Improves animal welfare of captive urodeles	Costs associated with education, screening, diagnosis and treatment Depends on willingness of hobby sector to cooperate
Biosafety measures	Likely to reduce chances of spread of *Bsal* and other amphibian pathogens Protocols for disinfection already available Raises awareness Implementation of field protocols for working with amphibians already in place in many EU countries	May conflict with commercial interests Depends on willingness of all stakeholders to implement properly Considered a burden Use of chemicals may have adverse effects on humans and environment Costs associated with communication and implementation Cannot control for all potential routes of transmission (e.g. wildlife)
Vaccination	Could be used for prevention and during outbreaks Probably viewed as positive by public opinion	No vaccines available Vaccine development very expensive, long term and uncertain Proof of concept with wild type *Bsal* strain failed Costs associated with production and application Regulatory issues Generally not transferable across generations Imperfect treatments that only create tolerance while not interrupting transmission could have adverse effects by increasing spread May need to target multiple hosts in diverse amphibian communities
Bio-augmentation	Could be used for prevention and during outbreaks May be transferable across generations Probably viewed as positive by public opinion	Currently not available Development costly and uncertain No proof of concept Regulatory issues Imperfect treatments that only create tolerance while not interrupting

		transmission could have adverse effects by increasing spread May need to target multiple hosts in diverse amphibian communities
Selective breeding	Offers perspectives to long-term increased disease resistance	Currently no markers available for marker assisted breeding Genetic engineering fraught with regulatory and public opinion issues Development will take several generations, depending on species No proof of concept Possibly not transferable between species Requires intensive genetic population management Costs associated with producing breeding stock and genetic management
Environmental treatment with disinfectants / antimycotics	Proven effective in single host system for *Bd* May lower infection pressure and reduce likelihood of transmission	No proof of concept for *Bsal* *Bsal* may be less sensitive to disinfectants in terrestrial environments Efficacy questionable in complex systems Adverse effects on environment Not suitable for large scale application Regulatory issues Societal issue of antimycotic resistance Costs associated with products and application Feasibility dependent on application scheme Imperfect treatments that only create tolerance while not interrupting transmission could have adverse effects by increasing spread
Environmental manipulation	May reduce infection pressure and likelihood of transmission May allow host species to compensate *Bsal*-related mortality (e.g. by increased recruitment)	Potential adverse effects on environment No proof of concept for *Bsal* Environmental drivers for *Bsal*

		infections not known Costs associated with habitat manipulation + maintenance
In situ treatment	May eliminate *Bsal* from infected animals Probably viewed as positive by public opinion	Costs associated with treatment Labor intensive Unlikely that all infected animals are caught for treatment, which is necessary to curb infection at population level *Bsal* infection may recrudesce after treatment Imperfect treatments that only create tolerance while not interrupting transmission could have adverse effects by increasing spread
Reintroduction after *Bsal* eradication	Directly reinforces remnants of affected populations Probably positively viewed by public opinion	Requires *ex situ* captive assurance colonies (see below) Requires thorough follow up of reintroduction event, with associated costs of population and disease monitoring Risk of failure and increased pathogen proliferation if *Bsal* is not eradicated from the environment
Creating barriers to pathogen dispersal	Limits *Bsal* spread between sites May create disease free pockets Barriers (roads, canals) may be already present	Costs associated with installation and maintenance Barriers may have considerable failure rates and target only part of all potential vectors May conflict with local infrastructure May have adverse effects no non-target species Regulatory issues
Culling	May reduce *Bsal* dispersal Culling of reservoir hosts may reduce community-level epidemic and assist persistence of species of conservation priority	Costs associated with culling Unlikely that a sufficient proportion of animals can be captured High likelihood of adverse reactions of the public opinion *Bsal* may persist outside of managed hosts Regulatory issues May need to target multiple hosts in

		diverse amphibian communities
No action	No costs For broad range species with focal population declines if *Bsal* is spontaneously eradicated	Possibility of population extirpation or species extinction Potential conflict with Habitat's Directive High likelihood of adverse reactions of the public opinion
Support action	**Advantages**	**Disadvantages**
Early warning system	Allows rapid detection and response	Associated costs for efficient operation: diagnostic capacity, data management, communication
Passive surveillance	Large spatial scale possible Low associated costs	Likelihood of outbreak detection highly variable Requires intensive communication efforts and sufficient diagnostic capacity
Active surveillance pre-outbreak	Opportunity to collect data on host population size and distribution	Large-scale implementation problematic Associated costs of coordination, monitoring and sampling
Active surveillance post-outbreak	Monitoring infection dynamics highly informative to mitigation (e.g. pathogen persistence versus eradication)	Associated costs of coordination, monitoring and sampling in a contaminated environment Requires rigorous application of biosafety measures
Species prioritization	Allows efficient, evidence based allocation of resources for conservation Clarifies which species are conservation priorities (e.g. susceptible species), which are management priorities (e.g. reservoirs)	Requires the availability of detailed information on disease ecology for several urodele taxa that is currently lacking Inaccuracies may have far-reaching consequences for species conservation
Captive assurance colonies	Ensures species survival IUCN guidelines available Captive maintenance of urodeles relatively cheap	Costs associated with coordination, infrastructure and maintenance Requires genetic management Relevance questionable if no perspective for future re-introduction Regulatory issues Requires proper biosafety Most likely requires prioritization

		Husbandry techniques may need to be developed for some species
Monitoring	High likelihood of detecting mortality events and population declines Detects declines regardless of the causative agent Public involvement raises awareness	Costs associated with coordination and fieldwork For financial reasons, often involves the use of volunteers, which may reduce manageability

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Supplementary information

Table S1. Import restriction legislation to prevent *Bsal* introduction and spread.

Country	Restricted imports
Canada	The Canadian Wildlife Authorities prohibited the importation of all salamanders including dead Specimens, eggs, sperm, tissue culture or embryos, parts and derivatives without a permit
United States	The Lacey Act prohibits international trade of live or dead specimens and body tissue from 201 salamander species (US Fish and Wildlife Services, 2016; EFSA, 2017a; Klocke, 2017)
Hungary	Restrictions have been placed on specified species of high risk (EFSA, 2018; Stark, 2018) A modification has been made to decree 41/2010 which prohibits keeping, breeding, buying and selling of members of the family Salamandridae and Hynobidae and Karsenia koreana (Plethodontidae) (199/2017.(VII.10) Korm. Rendelet, 2017)
Switzerland	In December 2015, the Swiss Food Safety and Veterinary Office implemented import restrictions prohibiting the importation of all salamander species into Switzerland. After the implementation of EU decision 2018/320, Switzerland adapted its legislation accordingly
EU and Switzerland	Temporary animal health protection measures enacted for the trade of all amphibians in the order Urodela The European Commission issued decision (EU) (2018/320) which establishes animal health protection measures for the trade of salamanders within the EU and importation of salamanders from non-EU territories. These include rejection of any salamanders with obvious signs of illness (especially skin lesions) or originating from collections where there have been positive Bsal diagnoses, testing salamanders to ensure that they were free from Bsal, restricting movement of salamanders, implementing hygiene protocols and biosafety measures. (Commission Implementing Decision (EU) 2018/320)
Worldwide	The Convention on International Trade of Endangered Species of wild fauna and flora (CITES) was also explored as vehicle to restrict trade in Asian amphibians but not deemed appropriate

Table S2. Estimated cost of screening captive amphibian collections in Europe for *Bsal*.

1. Screening of captive collections		
Scenario 1		
298 members in Deutsche Gesellschaft für Herpetologieand Terrarienkunde (DGHT)	× 100 urodeles/keeper ≈ 30 000 urodeles	Pool samples in groups of 5 ≈ 6000 samples
With 298 urodele keepers and a theoretical 100 urodeles/keeper and pooling samples in groups of 5 (Sabino-Pinto et al., 2018a), screening would cost approximately 180 000 Euros		
Scenario 2		
1490 members in Deutsche Gesellschaft für Herpetologieand Terrarienkunde (DGHT)	× 100 urodeles/keeper = 149 000 urodeles	Pool samples in groups of 5 ≈ 30 000 samples
With 5 times the urodele keepers in scenario 1 (1490) and a theoretical 100 urodeles/keeper and pooling samples in groups of 5 (Sabino-Pinto et al., 2018a), screening would cost approximately 900 000 Euros.		

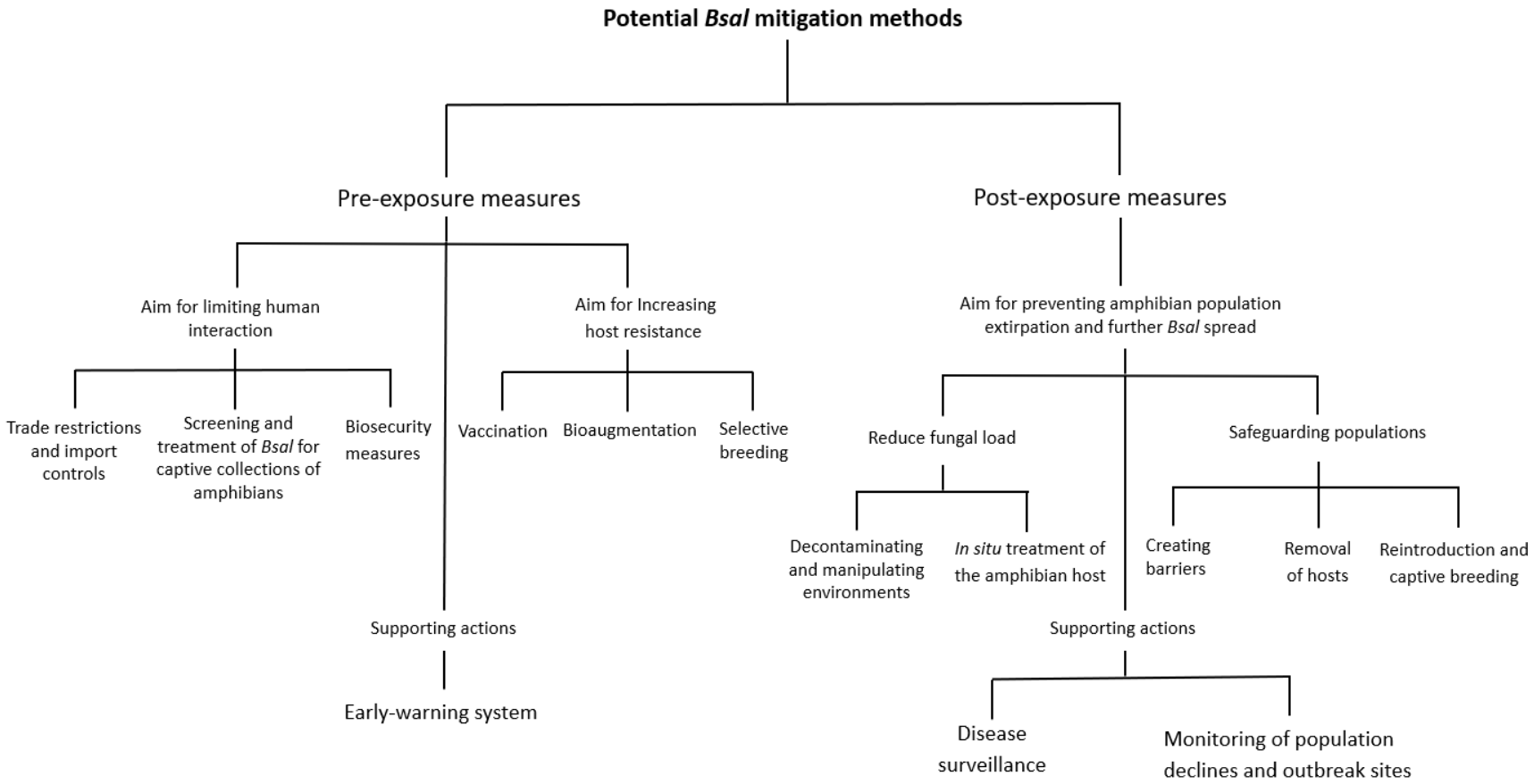

Figure S1. Overview of potential *Bsal* mitigation methods.

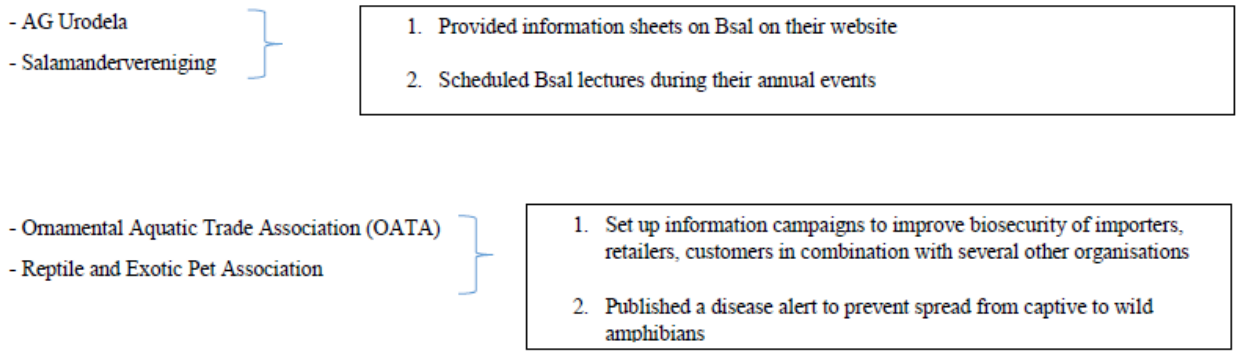

Figure S2. Stakeholders (urodelan keepers and associations) promoting *Bsal* awareness.

CHAPTER 2

CHAPTER 2

Diet diversity and environment determine the intestinal microbiome and bacterial pathogen load of fire salamanders

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Abstract

Diverse communities of symbiotic microbes inhabit the digestive systems of vertebrates and play a crucial role in animal health, and host diet plays a major role in shaping the composition and diversity of these communities. Here, we characterized diet and gut microbiome of fire salamander populations from three Belgian forests. We carried out DNA metabarcoding on fecal samples, targeting eukaryotic 18S rRNA of potential dietary prey items, and bacterial 16S rRNA of the concomitant gut microbiome. Our results demonstrated an abundance of soft-bodied prey in the diet of fire salamanders, and a significant difference in the diet composition between males and females. This sex-dependent effect on diet was also reflected in the gut microbiome diversity, which is higher in males than female animals. Proximity to human activities was associated with increased intestinal pathogen loads. Collectively, the data supports a relationship between diet, environment and intestinal microbiome in fire salamanders, with potential health implications.

Keywords: Amphibians, Urodela, *Salamandra salamandra*, diet, gut microbiome, molecular analysis

Introduction

The digestive system of vertebrates is home to a dynamic microbiome (Ley *et al.*, 2008; Robinson, Bohannan and Young, 2010), which forms a ubiquitous and complex symbiotic relationship with their host. This symbiosis has long been known to support the production of vitamins (Gill *et al.*, 2006), the chemical processing of indigestible chyme components by bacterial fermentation (Pryor and Bjorndal, 2005), and degrading toxic substances (Claus, Guillou and Ellero-Simatos, 2016). Moreover, the gut microbiome was also found to play a role in the maturation of the immune system (Mazmanian *et al.*, 2005) and even ecological adaptation (Alberdi *et al.*, 2016). The composition of the gut microbiome differs greatly among host taxa, as well as the primary factors determining it. For termites (Bourguignon *et al.*, 2018) and non-human primates (Amato *et al.*, 2018), the gut microbiome is strongly associated with host physiology and phylogeny. For fish (Sullam *et al.*, 2012; Bolnick *et al.*, 2014), birds (Grond *et al.*, 2018; Michel *et al.*, 2018), myrmecophagous mammals (Delsuc *et al.*, 2014), mice (Carmody *et al.*, 2015b) and amphibians (Kohl *et al.*, 2014; Vences, Lyra, Kueneman, *et al.*, 2016), host diet has been found to be one of the primary determinants in shaping microbial communities. Other factors, such as climate (Li *et al.*, 2020), habitat (Rawls *et al.*, 2006; Bletz *et al.*, 2016), and the host immune system (Woodhams, Bletz, *et al.*, 2020) also drive internal microbiome diversity of certain animal clades to some extent. Host diet not only serves as a source of potential gut colonists (Adlerberth and Wold, 2009), but also modulates gut microbiome dynamics (Wu *et al.*, 2011). Empirical studies on gut microbiomes and its relation to diet in additional species have the potential to further shed light on this delicate ecological balance.

Amphibians are among the world's most vulnerable groups of animals, with 40% of species in danger of extinction (Stuart *et al.*, 2004; Lips *et al.*, 2006; Bishop *et al.*, 2012) due to habitat destruction, climate change and emerging diseases (Kats and Ferrer, 2003; Chanson *et al.*, 2008; Rollins-Smith and Woodhams, 2012; Martel *et al.*, 2014; Birnie-Gauvin *et al.*, 2017; Scheele, Pasmans, Skerratt, Berger, Martel, Beukema, Acevedo, Burrowes, Carvalho, Catenazzi, De la Riva, *et al.*, 2019). Across the globe, amphibians are a key part of many ecosystems (Whiles *et al.*, 2006; Hocking and Babbitt, 2014), making up a large proportion of vertebrate communities in forest, tropical and wetland ecosystems both in terms of individual abundance and overall biomass

(Burton and Likens, 1975; Reagan and Waide, 1996; Stebbins and Cohen, 1997). Frogs and salamanders influence leaf litter decomposition as well as nutrient cycling, by preying upon omnivore and detritivore invertebrate populations (Flecker, Feifarek and Taylor, 1999; Beard, Vogt and Kulmatiski, 2002; Davic and Welsh, 2004; Reinhardt *et al.*, 2013). This also applies to fire salamanders (*Salamandra salamandra*), a species occurring throughout most of temperate deciduous forests in central and southern Europe (Buckley and Alcobendas, 2002). They can occur in high densities and are deeply woven into local food webs, contributing to ecosystem stability (Davic and Welsh, 2004). Understanding such food web data is important for drafting conservation strategies and understanding community ecology and ecosystem functioning (Fryxell and Lundberg, 1994; Deagle *et al.*, 2007; Birnie-Gauvin *et al.*, 2017).

In this study, we use DNA metabarcoding to profile both the diet and intestinal bacterial composition of fire salamander populations in Belgian forests. The aim is to provide detailed information on the diversity, relative abundance and prevalence of prey taxa found within fecal samples of fire salamanders, as well as the impact of diet, sex and geographical location on the gut microbiome.

Results

Diet analysis based on fecal DNA metabarcoding

A total of 20 prey taxa were identified, belonging to the phyla Mollusca, Annelida and Arthropoda.

Fire salamander ingested prey biomass was determined by the relative number of sub-OTU reads for each prey category. We used this as a proxy for the relative amount of ingested prey within all salamander fecal samples. More than 70% of the sub-OTU reads were identified as gastropods, followed by millipedes at 10.8%, centipedes at 5.8%, and soil mites at 4.4%. All other taxa were rare in fire salamander diet. Diet taxa prevalence (presence/absence) across all salamanders revealed gastropods to be the most prevalent taxonomic class at 40.6%. Both millipedes and annelids (earth worms) were found at 14.5%. Centipedes, true flies and crustaceans made up 5.8% of salamander diet. Relative abundance of prey sub-OTUs differed significantly between females (n = 12) and males (n = 21) (RDA F = 4.58, df = 2.28, p = 0.011). Prey ingested by females was made up largely of gastropods (73.3 %), with millipedes making up most of the remaining prey

(16.7 %). Males, on the other hand, had a more balanced diet, with a large portion of their ingested prey being divided between gastropods (36.4 %), millipedes (21.9 %), soil mites (16.7 %), and centipedes (11.6 %) (Figure 1a). Prevalence of prey taxa also differed significantly between both sexes ($\chi^2_{1,12} = 35.12$, $p < 0.001$) (Figure 1b).

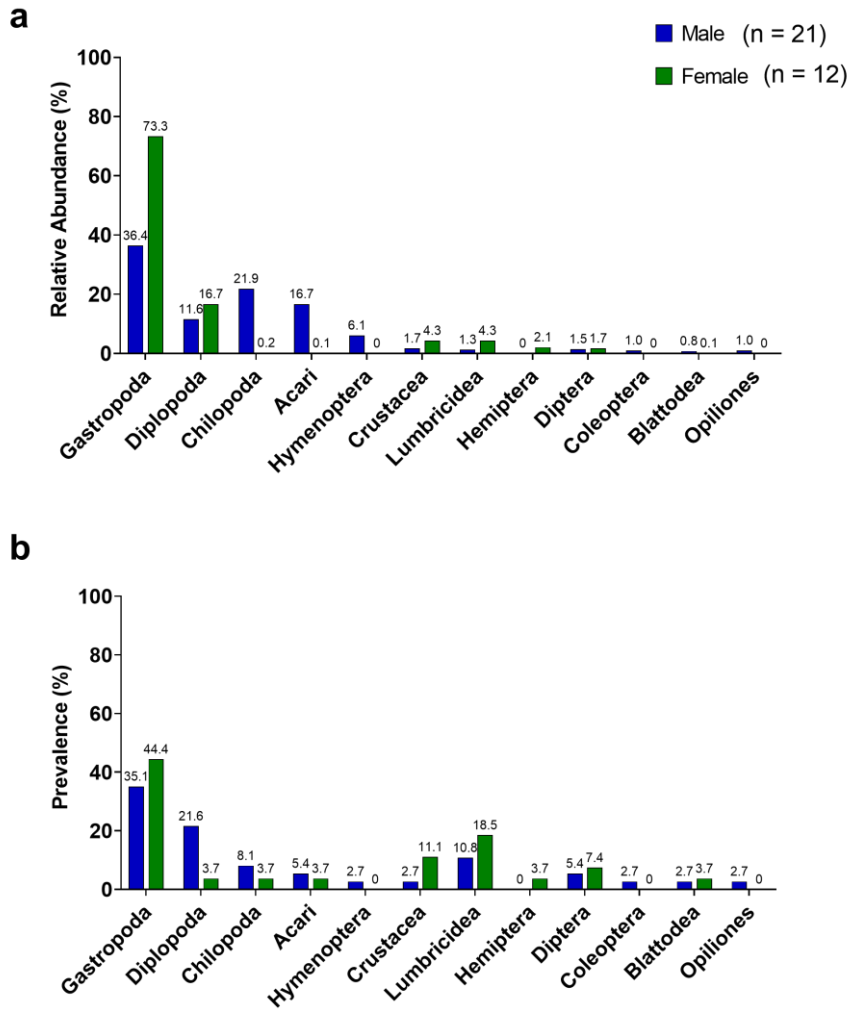

Figure 1. Diet composition between sexes. a) Relative abundance (relative number of sub-OTU reads for each prey) of fire salamander diet in male and female animals. b) Prevalence (presence/absence) of prey taxa in male and female animals.

Fire salamander diets were largely made up of the same general taxa, and the relative abundances of prey reads did not differ between the forests of HG (Heilig Geestgoed) (total n = 11, male n = 5, female n = 5, juvenile n = 1), M (Makegem) (total n = 17, male n = 9, female n = 5, juvenile n = 3)

and S (Smetledebos) (total n = 9, male n = 7, female n = 2, juvenile n = 0) (RDA F = 0.50, df = 2.28, p = 0.738). Gastropods and earthworms were found to have a similar prevalence in salamander diets between forests (Supplementary Figure S1). Differences were seen in millipedes, where values fluctuated between locations, although not significantly ($\chi^2_{2,12}$ = 4.35, p = 0.113). Many of the Insecta species were not part of the salamander diet in all three forests (Supplementary Figure S1).

Alpha diversity of the salamander diet did not differ between locations (Chao2 H_2 = 0.013, p = 0.994; sub-OTU richness H_2 = 0.016, p = 0.992) or sexes (Chao2 W = 99, p = 0.246; OTU Richness W = 108, p = 0.407). Beta diversity of the salamander diet showed a large overlap between sexes, as well as forests (Figure 2a, 2b) and there was no significant difference in diet composition between sexes (PERMANOVA Pseudo-F =1.20, df = 2. 30, p = 0.26), as well as locations (PERMANOVA Pseudo-F = 1.03, df = 2. 30, p = 0.41). Moreover, we found that the salamander body condition (SMI) did not correlate to the diversity of diet (OTU richness r_s = 0.074, p = 0.640; Chao2 r_s = 0.067, p = 0.694).

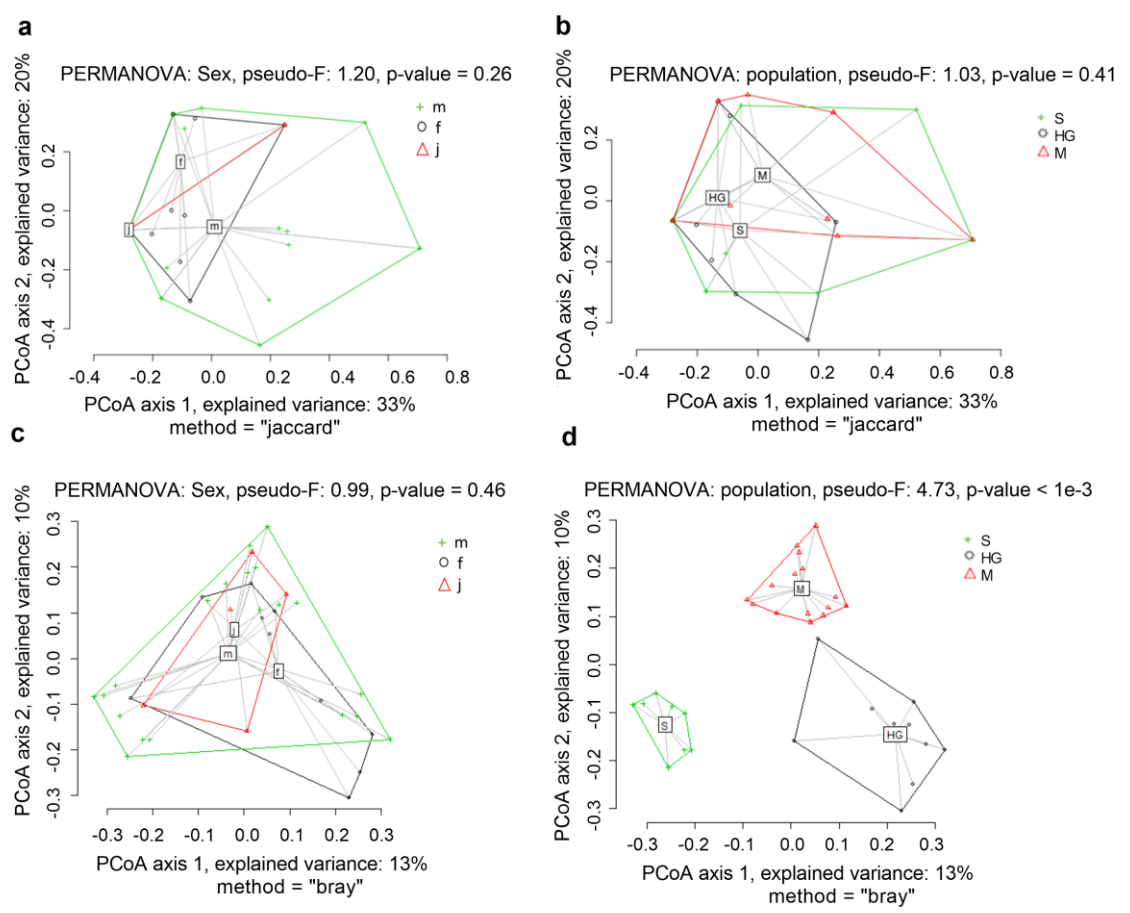

Figure 2. Principal coordinates analysis of Jaccard beta diversity matrices for diet of sexes (a) and locations (b). Principal coordinates analysis based on Bray-Curtis dissimilarity distance for microbiome composition of sexes (c) and locations (d). m = male, f = female, j = juvenile, HG = forest Heilig Geestgoed, M = forest Makegem and S = forest Smetledebos.

Gut microbiome analysis and pathogen load based on fecal DNA metabarcoding

Within all fecal samples, we found 15 different bacterial phyla. Bacteroidetes made up the largest portion of bacteria at 47.8%, followed by Firmicutes at 32.1% and Proteobacteria at 15.3%. These three phyla made up 95.2% of all bacteria found in fecal samples. The remaining bacteria were identified as Verrucomicrobia (3.7%), Desulfobacterota (0.6%), Cyanobacteria (0.2%), Actinobacteriota (0.1%), Elusimicrobiota (0.1%), Deferribacterota (0.009%), Bdellovibrionota (0.006%), Patescibacteria (0.003%), Myxococcota (0.001%), Planctomycetota (0.001%), Fusobacteriota (0.001%), and others (0.001%). When comparing the fecal microbiome from

salamanders captured in different forests, significantly more Proteobacteria and Elusimicrobia were observed in forest HG, as compared to forest M or S (Supplementary Table S1).

Looking at the family level, Bacteroidetes was represented by four main families: *Bacteroidaceae* (HG 24.0% / M 28.2% / S 25.4%), *Tannerellaceae* (HG 7.2% / M 6.3% / S 10.5%), *Rikenellaceae* (HG 5.7% / M 8.5% / S 6.7%) and *Marinifilaceae* (HG 5.7% / M 4.4% / S 4.4%). For Firmicutes the four main families were: *Lachnospiraceae* (HG 8.6% / M 10.7% / S 7.9%), *Ruminococcaceae* (HG 3.6% / M 7.3% / S 6.4%), *Oscillospiraceae* (HG 3.2% / M 3.1% / S 3.5%) and *Oscillospirales_fa* (HG 1.7% / M 2.9% / S 2.7%). Proteobacteria was represented by: *Yersiniaceae* (HG 9.3% / M 0.2% / S 0.1%), *Enterobacteriaceae* (HG 2.8% / M 6.8% / S 3.4%), *Diplorickettsiaceae* (HG 5.7% / M 1.1% / S 0.4%) and *Pseudomonadaceae* (HG 1.4% / M 0.5% / S 1.8%). Most of the remaining families within these phyla contain less than 1% of the relative abundance (Figure 3). In total, 138 families were found to be present in at least one location. Of those, only four families were identified to be significantly different in abundance between locations (Supplementary Table S1).

Based on redundancy analysis (RDA), the gut microbiome composition was significantly affected by location (RDA $F_{2,36} = 4.07$, $p = 0.001$) but not by sex (RDA $F_{2,36} = 0.85$, $p = 0.720$). Alpha diversity of the gut microbiome varies between locations (Figure 3b), with significant differences for ASV richness ($\chi^2 = 7.52$, df = 2, $p = 0.023$), Chao1 ($\chi^2 = 9.18$, df = 2, $p = 0.010$) and Shannon Index ($\chi^2 = 11.09$, df = 2, $p = 0.004$). We found that forest HG has significant lower alpha diversity than the other two forests (Figure 3b). When comparing between sexes, alpha diversity of the gut microbiome is significantly lower for females than males: ASV richness (W = 43, $p = 0.013$), Chao1 (W = 48, $p = 0.021$), Shannon Index (W = 38, $p = 0.005$). The dissimilarity in microbiome composition was analyzed with PERMANOVA on Bray-Curtis distance, where no difference could be seen comparing between sexes (Figure 2c, Pseudo-$F_{2,29} = 0.9$, $p = 0.556$), but when comparing between locations, the three forests were significantly different from each other (Figure 2d, Pseudo-$F_{2,29} = 4.73$, $p < 0.001$). Moreover, SMI (RDA $F_{1,19} = 1.80$, $p = 0.340$) was not correlated with gut microbiome composition.

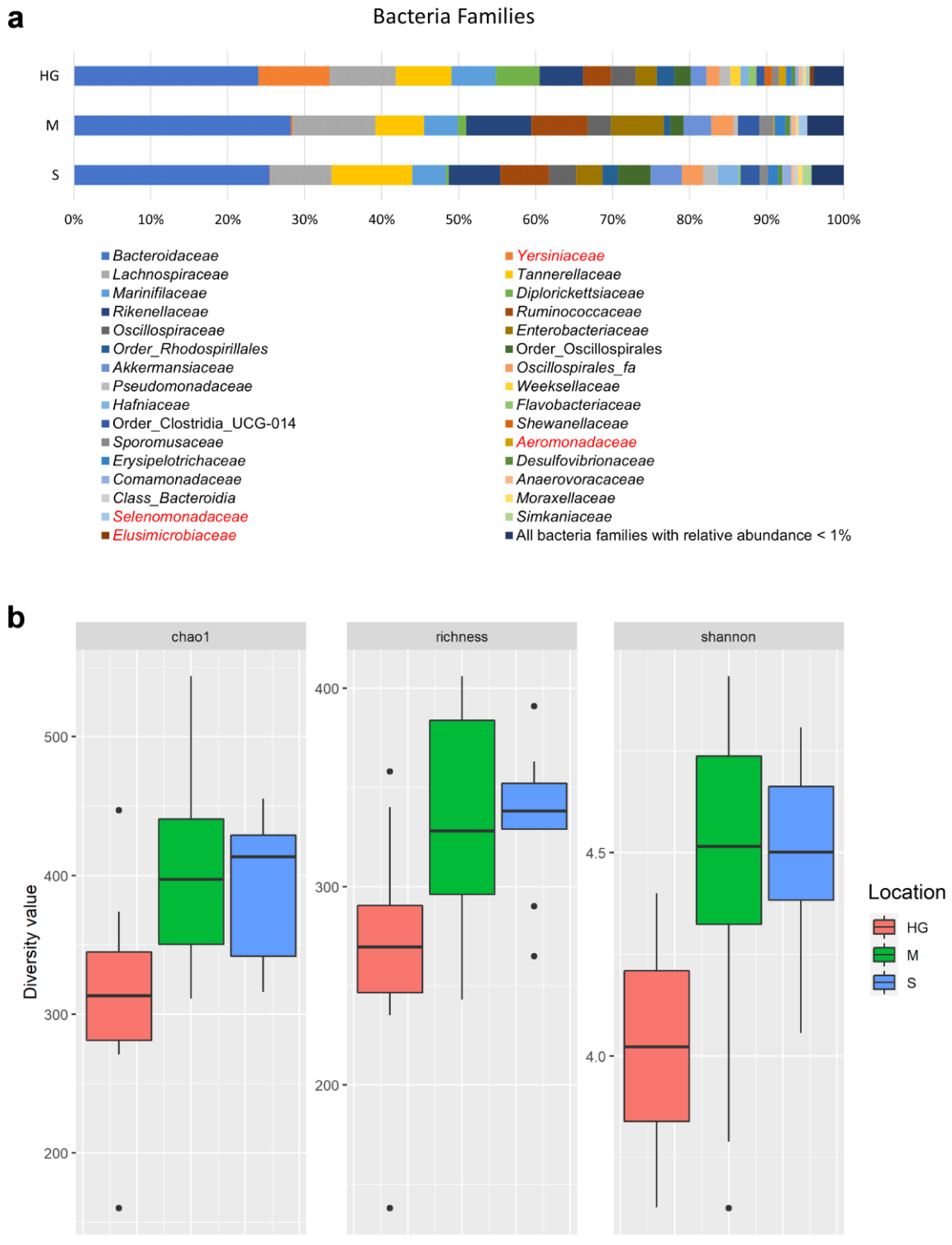

Figure 3. a) Bacteria families found in the fecal samples of fire salamanders from forests HG (Heilig Geestgoed), M (Makegem), and S (Smetledebos). Bacteria families that are significantly

different between locations are highlighted in red. b) Box plots showing alpha diversity (Chao1, ASV richness and Shannon Index) of gut microbiome between locations HG (Heilig Geestgoed), M (Makegem), and S (Smetledebos). Boxes are extended from the 25th to 75th percentiles, and the horizontal line inside the boxes defines the median. Whiskers indicate variability outside the upper and lower quartiles. Black circle indicates outliers.

We included pathogens of the genera *Flavobacterium*, *Chryseobacterium*, *Sphingobacterium*, *Aeromonas*, *Citrobacter*, *Yersinia*, *Acinetobacter* and *Stenotrophomonas* as a proxy for pathogen load, given their known involvement in amphibian pathology (Botzler, Wetzler and Cowan, 1968; Cooper, Needham and Griffin, 1978; Hird *et al.*, 1984; Olson *et al.*, 1992; Pearson, 1998; Green *et al.*, 1999; Bernardet *et al.*, 2005; Pasteris *et al.*, 2011; Kirk *et al.*, 2013; Suzina *et al.*, 2018; Hallinger, Taubert and Hermosilla, 2020). The presence of potential pathogenic amphibian bacteria in the fire salamander gut microbiome was analysed at genus level (Supplementary Figure S2). We focused on eight genera well known to contain pathogenic bacterial taxa (Table 1). The total relative abundance of the eight pathogenic bacterial genera makes up less than two percent of the fire salamander gut microbiomal communities in forest M (0.5%) and S (1.4%), but more than 13% in forest HG. The relative abundance of the pathogen load is significantly higher in forest HG, compared to forest M ($p < 0.0001$). Similarly, a tendency towards increased pathogen load was observed when comparing forests HG and S ($p = 0.8649$). Comparison of beta diversity of the pathogen load between forests was analysed with PERMANOVA on Bray-Curtis distance and revealed a significant difference between locations (Supplementary Figure S3, Pseudo-F =4.69, df = 2. 80, $p = 0.0001$).

Genus	**Mean relative abundance**			***p*-value**		
	HG	**M**	**S**	**HG-M**	**HG-S**	**M-S**
Flavobacterium	1.0%	0.02%	0.3%	0.976	0.550	0.898
Chryseobacterium	1.3%	0.01%	0.1%	0.669	1.000	1.000
Sphingobacterium	0.1%	0.002%	0.004%	1.000	1.000	1.000
Aeromonas	0.9%	0.1%	0.0%	0.614	< 0.001	< 0.001
Citrobacter	0.2%	0.1%	0.3%	0.573	0.882	1.000
Yersinia	9.2%	0.02%	0.05%	< 0.001	< 0.001	1.000
Acinetobacter	0.3%	0.2%	0.6%	0.809	0.967	1.000
Stenotrophomonas	0.1%	0.01%	0.04%	0.951	1.000	1.000

Table 1. Mean relative abundance and level of significance of potentially pathogenic bacterial genera in the fire salamander gut microbiome between different forests (HG = forest Heilig Geestgoed, M = forest Makegem and S = forest Smetledebos).

Diet and gut microbiome

A Mantel test revealed that dietary differences between individual salamanders are not similar to the microbiome differences (r = -0.05, p = 0.747). Exploring the structure between diet and microbiome we found a co-inertia coefficient of 0.42, indicating that the two categories varied independently (p = 0.721). Furthermore, no correlation was observed between the alpha diversity (Chao 1 & 2 index) of diet and gut microbiome (Supplementary Figure S4, Spearman's r_s = -0.05, p = 0.748).

Discussion

To estimate the accuracy of the DNA barcoding technique, validation studies have been conducted using feeding experiments of captive animals, where exact diet inputs were compared to fecal DNA sequencing outputs (Deagle *et al.*, 2010, 2013; Nakahara *et al.*, 2015). Whilst the species of prey could be successfully identified in these studies, the proportion of detected DNA varied and was not completely on par with the dietary proportions. Amounts of DNA in the fecal matter need to be exactly proportionate to ingested prey biomass, which is not always the case, due to biological and technical biases, such as different speeds of digestion, size of ingested prey, possible presence of multicopy genes, DNA degradation in fecal samples and availability of DNA reference sequences of potential prey in public databases (Deagle, Kirkwood and Jarman, 2009; Pompanon *et al.*, 2012; Thomas *et al.*, 2014). Nonetheless, DNA sequencing of fecal matter is a more viable technique to identify soft-tissued, easily digestible prey (Deagle, Kirkwood and Jarman, 2009), and able to provide higher resolution than conventional stomach content analysis (Deagle and Tollit, 2007; Ando *et al.*, 2020). This technique has been applied successfully in unravelling diets for many species, including birds (Deagle *et al.*, 2007, 2010), mammals (Deagle *et al.*, 2005; Parsons *et al.*, 2005; Deagle, Kirkwood and Jarman, 2009), fish (Meekan *et al.*, 2009; Guillerault *et al.*, 2017) and reptiles (Brown, Jarman and Symondson, 2012), but so far has not been used for amphibians.

In this study, we characterise the diet of fire salamanders by means of DNA metabarcoding of fecal samples. Our data identifies gastropods as the most prevalent prey of fire salamanders, and reveals the diet differences between sexes. As reported previously, fire salamanders are opportunistic and generalist predators that focus on slow-moving and soft prey items (Ferenti *et al.*, 2008; Ferenti, David and Nagy, 2010; Lezău *et al.*, 2010; Balogová *et al.*, 2015). Our findings are in line with previous publications of fire salamander diet compositions, where Gastropoda was also found to be the most important prey (Lezău *et al.*, 2010). Other amphibian species have significantly different diet compositions, Monte Albo cave salamander (*Speleomantes flavus*), for example, mostly consume Hymenoptera, Ambrosi's cave salamander (*Speleomantes ambrosii*) feed mostly on Arachnida and Strinati's cave salamander (*Speleomantes strinatii*) mostly consume Diplopoda (Sebastiano *et al.*, 2012; Lunghi, Cianferoni, Ceccolini, Mulargia, *et al.*, 2018; Lunghi, Cianferoni, Ceccolini, Veith, *et al.*, 2018). This likely explains, why our results are in line only with previous studies on diet conducted specifically on fire salamanders. The sex-dependent change in diet has been thoroughly investigated in a number of amphibian species (Measey, 1998; Sebastiano *et al.*, 2012; Pamintuan and Starr, 2016; Lunghi, Cianferoni, Ceccolini, Veith, *et al.*, 2018; Le *et al.*, 2020), but no difference of diet composition has been found. Our study observed for the first time diet differences between sexes in amphibians, with female fire salamanders consuming more gastropods and millipeds. While in other vertebrates, such as reptiles, this is often linked to sexual dimorphism (Plummer and Farrar, 1981; Shetty and Shine, 2002; Vincent, Herrel and Irschick, 2004) and/or the utilisation of different microhabitats (Manenti, Conti and Pennati, 2017), we attribute the observed difference to different activity patterns. In North American plethodontid salamanders it has been found that females are less active than males (Keen, 1979; Forester, 1981; Harris, 2008), resulting in them eating more slow-moving organisms, such as millipedes and gastropods (Anderson and Mathis, 1999). Similarly, during the breeding season in autumn, female fire salamanders are less active outside the shelters than males (Manenti, Conti and Pennati, 2017), which could explain the diet difference between sexes.

Interestingly, the alpha diversity of the gut microbiome was also found to be significantly different between sexes, with diversity of species being lower in female than in male animals. Looking at this lower diversity of the gut microbiome in female animals, especially with regards to the sex-dependent diet, these findings suggest that decreased prey variation results in lower gut microbiome diversity. Previous studies on gut microbiome compositions between sexes in

amphibians have yielded mixed results. One study on Chinese concave-eared frogs (*Odorrana tormota*) observed a significant difference in gut microbiome composition at family and genus levels between sexes (Shu *et al.*, 2019). In cane toads (*Rhinella marina*) a study elucidated that the difference in variation of gut microbiome communities is mostly due to the factor of sex, but did not statistically quantify the effect on microbiome diversity (Zhou *et al.*, 2020). In rice field frogs (*Fejervarya limnocharis*), however, no statistical difference of gut microbiome composition could be found between sexes (Huang and Liao, 2021). Therefore, differences of gut microbiome diversity between sexes depends on the respective species, and is most likely subject to individual behaviour, prey selection, biology and environmental factors.

The alpha diversity of the fecal micriobiome was also found to be significantly different between locations, with a lower diversity of species found at forest HG, which is located adjacent to farmland, unlike the other two forests in this study. Forest HG also stood out, in that it had a remarkably higher pathogen load, compared to the other forests. We assume that the higher pathogen load coincided with the close vicinity to farmland. This hypothesis is supported by the relatively high amount of Proteobacteria, and the presence of members of the phylum Elusimicrobia in these salamanders, which has been associated with exposure to fertilizers and pesticides in farmland frogs (Chang *et al.*, 2016). Generally, a substantial proportion of Proteobacteria is typical for amphibian gut microbiomes, but more so in larvae than adults (Kohl *et al.*, 2013; Colombo *et al.*, 2015; Vences, Lyra, Kueneman, *et al.*, 2016). An additional explanation is that, in contrast to the other two sites, the salamanders from this forest are exposed to raw sewage from the neighbouring houses, which may also explain increased numbers of *Yersiniaceae*, notably of the genus *Yersinia*, which has been previously reported as an indicator of fecal pollution in fish (Novoslavskij *et al.*, 2015). *Yersinia* has also been reported in amphibian species, such as common mudpuppy (*Necturus maculosus*) (Standish *et al.*, 2019), Northern leopard frog (*Rana pipiens*) (Hird *et al.*, 1983) and green frog (*Rana clamitans*) (Botzler, Wetzler and Cowan, 1968). This, however, is the first time *Yersinia* has been found in gut microbiome of amphibians from a farmland adjacent habitat, which may possibly be linked to sewage and fecal pollution. Therefore, the salamander gut microbiome is likely associated with land use and/or pollution, which may have consequences for salamander physiology and health.

Furthermore, we observed no correlation between diet alpha diversity and gut microbiome alpha diversity in fire salamanders. However, previous studies on the correlation between microbiome richness and dietary richness did not yield consistent results. A positive correlation between diet diversity and gut microbome diversity was found in humans (Heiman and Greenway, 2016), black howler monkeys (Amato and Righini, 2015), kudus (Kartzinel *et al.*, 2019) and predatory insects (Tiede *et al.*, 2017). In contrast, a negative correlation was observed in fish, where a study showed that feeding on mixed diets resulted in a lower gut microbiome diversity, compared to pure diets (Bolnick *et al.*, 2014). Moreover, no correlation between diet diversity and gut microbiome diversity was observed in numerous mammalian herbivore species, such as pika, elephant and camel (Kartzinel *et al.*, 2019). Diet-microbiome correlation is thus difficult to distill into a general rule across the animal kingdom. A previous study looked specifically at the diet and gut microbiome correlation, where a positive correlation was found in tadpoles of Malagasy frogs (Vences, Lyra, Kueneman, *et al.*, 2016). We were unable to confirm this in our study. So for now it appears that variety of diet does not always correlate with the variety and biodiversity of the gut microbiome in amphibians.

Similar studies of the gut microbiome have been conducted in fire salamander larvae (Bletz *et al.*, 2016), where they found Proteobacteria, Firmicutes and Bacteroidetes to be the most abundant phyla. This is in line with our findings, where we have found Bacteroidetes, Firmicutes and Proteobacteria to be the most abundant phyla. Exception is the phylum Actinobacteriota, which was found to be more abundant in both the pond larvae (3.9%) and stream larvae (6.6%), than in our adult fire salamanders (0.1%). Nonetheless, these findings could indicate that the most abundant phyla in the gut microbiome composition do not undergo major changes throughout the different life stages of fire salamanders. The aforementioned publication also found that gut microbiome composition changes depending on habitat-specific diet in ponds versus streams. We were unable to see such stark contrasts of habitat-dependent diet in our adult animals, since prey was similar across different forest habitats.

To conclude, this is the first study to investigate the correlation between the diversity of diet and gut microbiome of adult fire salamanders in Belgian forests, using high-throughput DNA metabarcoding techniques. We show that diet composition is driven by sex, and influences

microbiome composition in the fire salamander intestine. However, no correlation was observed between diet diversity and gut microbiome diversity.

Methods

All methods were carried out in accordance with relevant guidelines and regulations.

Field sampling

All samples were collected from October to November 2016 at three forest locations in East Flanders, Belgium. Heilig Geestgoed (hereafter HG), a 29.77 ha public forest in the municipality of Merelbeke with 9 ponds (latitude 50.946881, longitude 3.726310). Makegem (hereafter M), a 54.24 ha private forest in Harentbeekbos with 10 ponds (latitude 50.945331, longitude 3.714886) approximately 1 km from HG and finally Smetledebos (hereafter S) with 45.75 ha, a public forest with 4 ponds (50.976308, longitude 3.906562), approximately 22 km from all other forests. All locations from which the salamanders were collected consisted predominantly of beech trees (*Fagus sylvatica*) with minimal undergrowth. Permits for sampling and experimental protocols of fire salamanders were granted by Agentschap voor Natuur en Bos of East Flanders in Belgium, license number ANB/BL/FF-V15-00015. According to the Belgian and EU legislation (EU directive 2010/63/EU), the collection of faeces obtained after spontaneous defecation is not considered an animal experiment and therefore does not require the approval of an ethical committee. Permission for collecting the samples in the forests (public and private) were obtained from the owners in the framework of the UGent GOA project Scaling up Functional Biodiversity Research: from Individuals to Landscapes and Back (TREEWEB).

Sample collection

Fire salamanders were collected from each of these three forests. They were kept individually without food in sterile boxes (16 × 11 × 5 cm) with moist towel, air holes and hiding places for 1 to 3 days at 15 ^{o}C at the Faculty of Veterinary Medicine, University of Ghent in Merelbeke and then returned to their exact same locality in the forest. All animals were weighed (to 0.1 g) and measured (to 0.1 cm). Scaled mass index (SMI) of body condition ($\widehat{M}_i$) was calculated for all

salamanders: $\widehat{M}_i = M_i[\frac{L_0}{L_i}]^{b_{SMA}}$ (Peig and Green, 2009). Animals were checked and the boxes were cleaned each day and any fecal samples were collected in Eppendorf tubes and frozen at -20 ºC until DNA extraction. In total, 60 fecal samples were collected from 49 individual fire salamanders. 13 individual fire salamanders were collected from forest HG (7 males, 5 females and 1 juvenile), 21 individuals from forest M (11 males, 6 females and 4 juveniles) and 15 individuals from forest S (9 males, 4 females and 2 juveniles).

Fecal sample DNA metabarcoding

DNA was extracted from all fecal samples with the MoBio PowerSoil DNA Isolation Kit (MoBio, Carlsbad, CA, USA) following the manufacturer's protocol. Extracted DNA was stored at -20 ºC until further processing.

Diet

Sequencing methods were used as previously described (Vences, Lyra, Perl, *et al.*, 2016). The V9 regions of nuclear 18S rRNA gene were amplified using modified primers 1391F (5´-GTACACACCGCCCGTC-3´) and EukBr (5´-TGATCCTTCTGCAGGTTCACCTAC-3´) (Amaral-Zettler *et al.*, 2009). We performed index PCR with unique combinations of indexed forward and reverse primers for each sample using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific). For quantification of amplicon DNA concentration, we roughly assessed intensity of their signal on agarose gels and then added 2 to 6 µl to a pooled library. This library was gel-purified by cutting out the band of the correct amplicon size, and subsequently cleaned with a Qiagen MinElute Kit. DNA concentration of the purified library was determined with a Qubit 2.0 fluorometer. The library was sequenced on the Illumina MiSeq platform using MiSeq Reagent Kit v3 for 300 cycles in both directions, including 10% phiX. Sequences were processed with MacQIIME v1.9.1 (Caporaso *et al.*, 2010), filtering the forward reads as previously described (Vences, Lyra, Perl, *et al.*, 2016). Quality filtered sequences were clustered into sub-operational taxonomic units (sub-OTUs) using the deblur workflow (Caporaso *et al.*, 2010; Amir *et al.*, 2017); (https://github.com/biocore/deblur). Within this workflow, all sequences were trimmed to 150 bp and subsampled to 13000 reads per sample. Sub-OTUs clusters with less

than 10 reads were removed. Sequences of sub-OTUs were taxonomically identified through BLAST searches. Only potential salamander prey items were selected from the 18S data, discarding sub-OTU reads from taxa such as Bacteria, Fungi and Protists. Prey taxa were identified into the main taxa: Gastropoda, Diplopoda, Chilopoda, Arachnida (Acari and Opiliones), Crustacea, Annelida (Lumbricidae), and six insect clades: Hymenoptera, Hemiptera, Dipera, Coleoptera, Blattodea and Collembola (Supplementary Table S2).

Gut microbiome

The V3-V4 hypervariable region of the 16S rRNA gene was amplified using gene-specific primers S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21 (Klindworth *et al.*, 2013). The PCR amplification procedures were performed according to a previous study (Aguirre *et al.*, 2019). The final barcoded libraries were combined to an equimolar 10 nM pool and sequenced with 30% PhiX spike-in using the Illumina MiSeq v3 technology (2 x 300bp, paired-end) at the Oklahoma Medical Research Centre (Oklahoma City, USA). Demultiplexing of the amplicon dataset and deletion of the barcodes was done by the sequencing provider. Quality of the raw sequence data was checked with the FastQC quality-control tool (Babraham Bioinformatics, Cambridge, United Kingdom; http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), after which the sequences were trimmed, quality-filtered and dereplicated using the DADA2 algorithm (v1.14) (Callahan *et al.*, 2016). An initial amplicon sequence variant (ASV) table was constructed before chimaeras were identified using the *removeBimeraDenovo* function. Finally, taxonomy was assigned using DADA2's native naïve Bayesian classifier against the Silva database (v138) (Quast *et al.*, 2012).

To select the appropriate subsampling depth, alpha rarefaction curves were generated (Supplementary figure S5). One sample (sample MF12) was unsufficiently sequenced, and was therefore excluded from the final ASV table. Amplicon sequence variants were subsampled (rarefied) by random sampling to a depth of 5047 ASVs per samples (depth of the least sequenced sample).

As our sequencing samples are high bacterial biomass faecal samples, negative controls (buffer control) were included in our sequencing runs, to guard against reagent contamination, as this is only a problem in low bacterial biomass samples. We rarefied to 1300 reads per sample for 18S

and 5047 ASVs for 16S and eliminated all sub-OTUs with fewer than 10 reads overall. 43 fecal samples from 37 individuals were used for subsequent diet statistic analysis and 41 fecal samples from 35 individuals were used for gut microbiome analysis. The mean abundance of 16S and 18S data of multiple samples from same individuals was calculated.

Statistical analysis

Sequence reads per fecal DNA sub-OTU found in the samples of each salamander were used as approximate proxy for ingested biomass. We calculated the prevalence of each prey taxon in the salamander diet, determined by the number of samples in which a prey category was found present or absent.

To evaluate alpha diversity (in-samples diversity) of salamander diets, OTU richness and Chao2 were calculated from prey prevalence data, and for bacteria ASV richness, Chao 1 (estimated ASV richness) and Shannon indexes (estimated community diversity) were calculated from relative abundance data using the fossil package v 3.2.5(Vavrek, 2011). To compare alpha diversity between location and sexes, a Kruskal Wallis One-Way Analysis of Variance was performed between locations and a Wilcoxon rank sum test was performed between sexes (due to non-normal distribution in both cases). To evaluate the differences of the prey prevalence data between different locations and sexes, Pearson's Chi squared was calculated. Differences in microbial relative abundance between locations were assessed at the phylum, family and genus level, and differences between sexes were assessed at the phylum and family level, using the DESeq2 algorithm from the phyloseq package (v 1.30) (McMurdie and Holmes, 2013). Differentiallly abundant taxa were identified through applying the negative binomial Wald test with p-values corrected for multiple hypothesis testing using the Benjamini-Hochberg method (Love, Huber and Anders, 2014). The fire salamander gut microbiomes contained at least eight potentially pathogenic genera (*Flavobacterium*, *Chryseobacterium*, *Sphingobacterium*, *Aeromonas*, *Citrobacter*, *Yersinia*, *Acinetobacter*, *Stenotrophomonas*). The differences in relative abundances of pathogen load between locations were assessed using SPSS (IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY, USA), with Kruskal-Wallis analysis followed by Dunn's multiple comparison tests (significance values adjusted by the Bonferroni correction for multiple tests).

Redundancy analyses (RDA) were performed to examine the effect of location and sex on the relative abundance of ingested biomass, as well as to further quantify the effect of location, sex, SMI and diet on gut bacteria. Spearman's Rank correlation was used to test whether the alpha diversity of individual diet and gut bacteria was significantly related to salamander SMI. Additionally, correlations were used to examine the relationship of alpha diversity of diet on gut bacteria.

To explore the beta diversity, principal coordinates analysis was performed, based on the Jaccard distance (Jaccard, 1912), to compare community dissimilarities with presence or absence of OTUs for diet. We performed the principal coordinates analysis based on Bray-Curtis distance, to compare the community dissimilarities of relative abundance of ASVs, for both gut microbiome and pathogen load. We used PERMANOVA to test if the divergent species composition differed significantly between locations and sexes. Analyses were done with the package vegan (v 2.4) (Oksanen *et al.*, 2020) and the functions vegdist and adonis 2 in R (v 3.4).

To measure the correlation between individual salamander diet and the fecal microbiome, Mantel tests were performed on the Jaccard and Bray-Curtis distance matrices using R. To further explore the inter-relationship between the diet and the bacterial community matrices, a co-inertia analysis was run using the ade4 package (Dray and Dufour, 2007) in R.

Data Availability

The demultiplexed raw amplicon reads were submitted to the Sequence Read Archive (NCBI-SRA) under Bioproject accession PRJNA768724 (18S data) and PRJNA767645 (16S data).

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Author contributions

A.M., F.P. and M.V. conceived and designed the study. HK.S. conducted the experiments. E.G., HK.S., Y.W., MC.B. and L.H. performed statistical analysis and figure generation. Y.W., HK.S., F.P., and A.M. prepared the original manuscript. D.B., K.V. and L.L. contributed key study material. All authors reviewed the manuscript.

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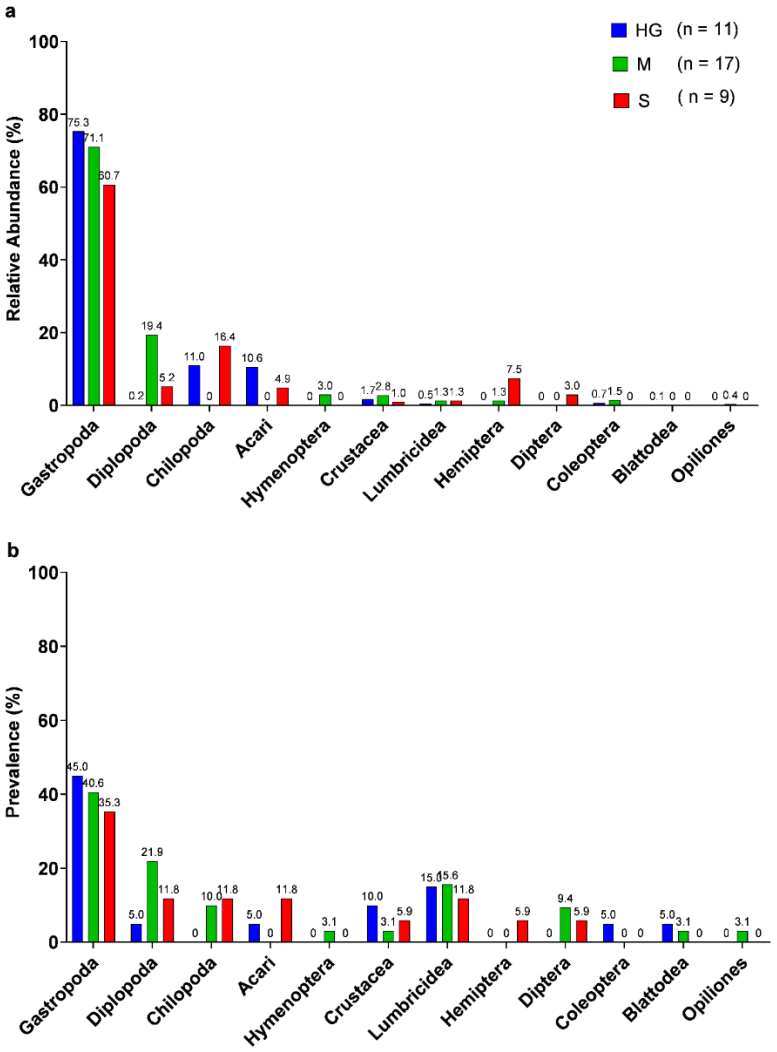
Additional information

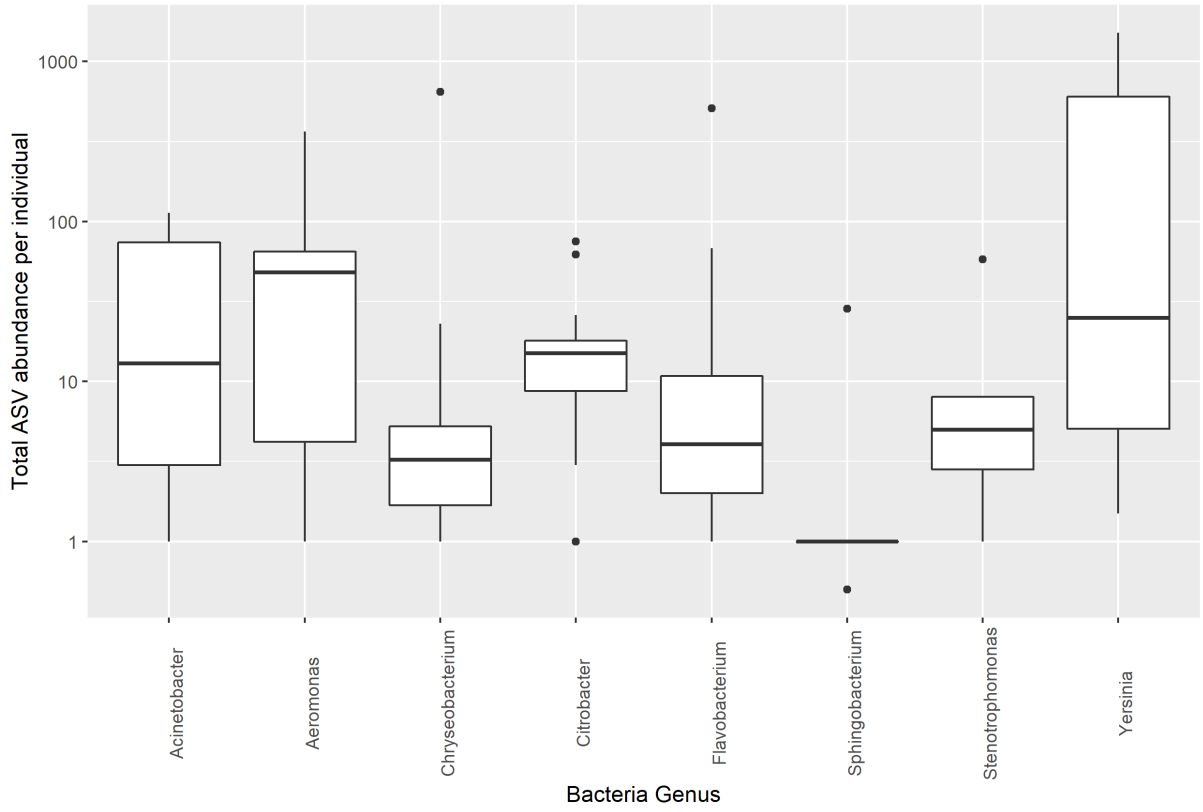
Competing interests statement

The authors declare no competing interests.

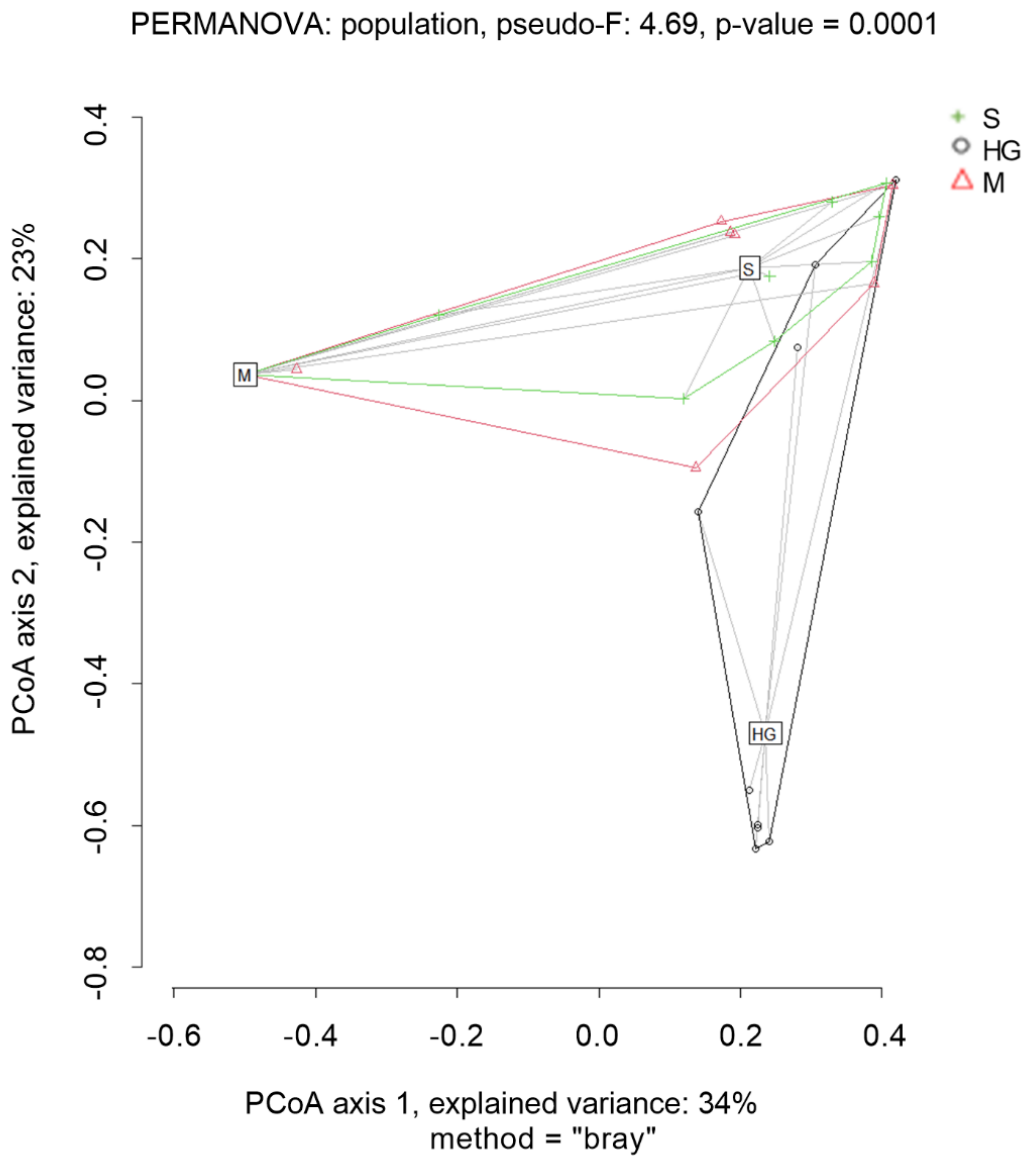
Supplementary information



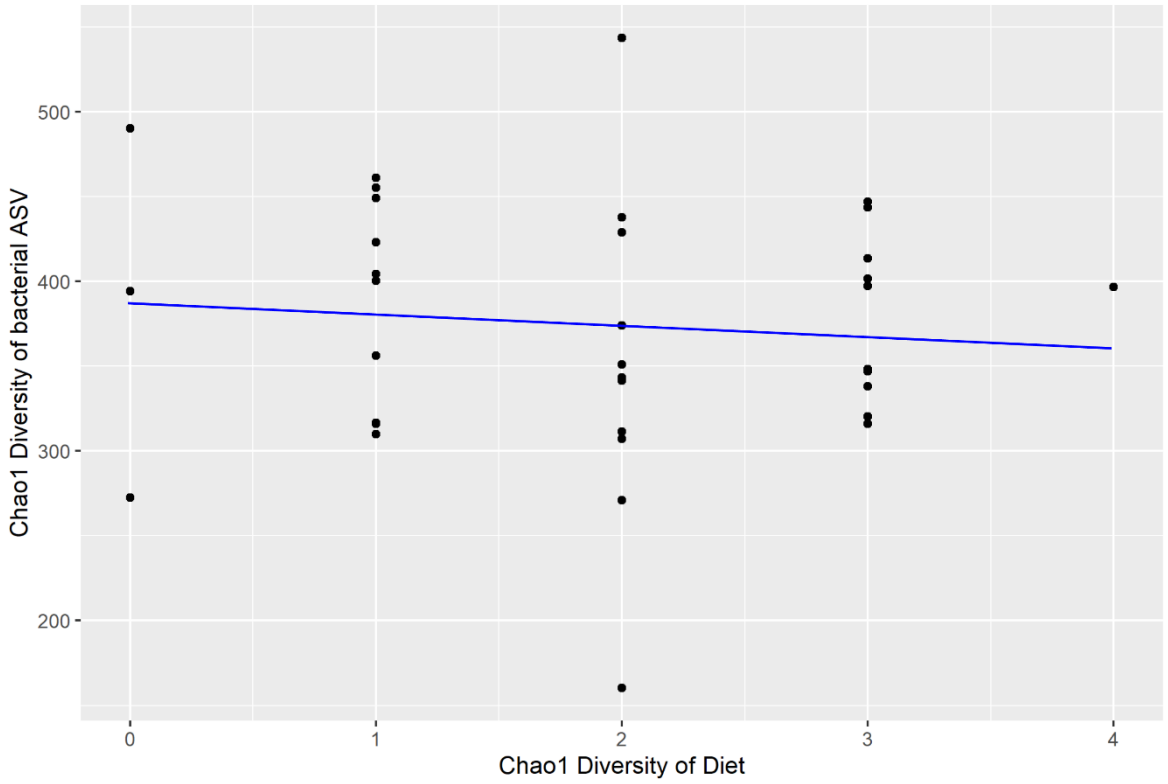
Supplementary Figure S1. Diet composition between forests. a). Relative abundance (relative number of sub-OTU reads for each prey) of fire salamander diet in forests HG, M, S. b). Prevalence of prey taxa (presence /absence) in fire salamander diet between forests HG, M, S. HG = Heilig Geestgoed, M = Makegem and S = Smetledebos.



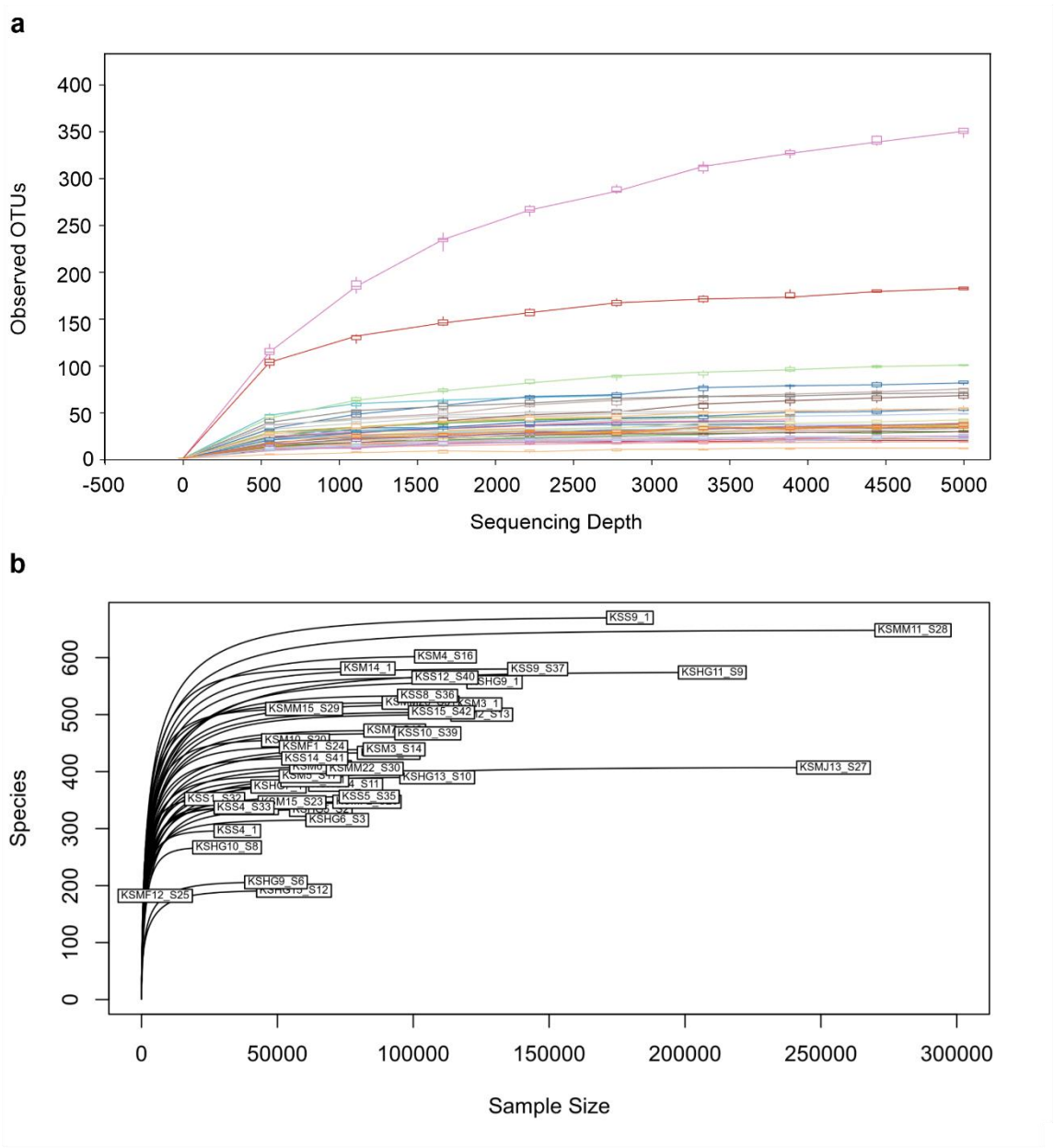
Supplementary Figure S2. Total ASV abundance per individual of each potential pathogenic bacteria in the fire salamander gut microbiome. Boxes are extended from the 25th to 75th percentiles, and the horizontal line inside the boxes defines the median. Whiskers indicate variability outside the upper and lower quartiles. Black circles indicate outliers.



Supplementary Figure S3. Principal coordinates analysis of Bray-Curtis dissimilarity distance for total pathogen load of locations. HG = forest Heilig Geestgoed, M = forest Makegem and S = forest Smetledebos.



Supplementary Figure S4. Linear regression of the correlation between alpha diversity of fire salamander diet and gut microbiome.



Supplementary Figure S5. Rarefaction curves of a) observed sub-OTU counts for 18S rRNA sequences, and b) the number of ASVs for 16S rRNA sequences in each sample. Each line represents one sample.

Phylum	Family	Mean relative abundance			*p*-value		
		HG	M	S	HG-M	HG-S	M-S
Phylum level							
Proteobacteria		25.6%	10.4%	12.6%	0.008	0.028	1
Elusimicrobia		0.5%	0.0%	0.0%	0.008	0.007	1
Family level							
Proteobacteria	*Yersiniaceae*	9.3%	0.2%	0.1%	< 0.001	< 0.001	1
Proteobacteria	*Aeromonadaceae*	0.9%	0.1%	0.0%	1	< 0.001	< 0.001
Elusimicrobiota	*Elusimicrobiaceae*	0.5%	0.01%	0.0%	0.137	0.045	1
Firmicutes	*Selenomonadaceae*	0.3%	1.1%	0.1%	1	0.922	0.025

Supplementary Table S1. Mean relative abundance and level of significance of bacterial phyla and families in fire salamander gut microbiome between different forests. HG = forest Heilig Geestgoed, M = forest Makegem and S = forest Smetledebos.

Phylum (sub)	Class (sub)	Order	Family	Species	F	BLAST
Mollusca	Gastropoda			*Alinda biplicata* (Two lipped door snail)	29	99.3
				Helicoidea sp. (land snail)	1	99.3
Annelida	Oligochatea		Lumbricidea	*Dendrobaena clujensis*	10	100
Anthropoda	Diplopoda			*Cylindroiulus punctatus (*Blunt-tailed snake millipede)	14	98
				Polydesmus sp.	6	98.6
				Proteroiulus fuscus	1	98.6
	Chilopoda			*Himantarium mediterraneum* (centipede)	3	97.3
	Arachnida	Opiliones		*Nelima sylvatica* (harvestman)	1	99.3
		Acari		*Acari* sp. (tick/mite)	5	96.6
				Atropacarus striculus (mite)	2	100
	Insecta	Hymenoptera		*Neodiprion* sp. (sawfly)	1	99.3
				Microplitis sp. (solitary endroparasitic wasp)	1	99.3

		Hemiptera	*Dactylopius coccus* (Cochineal scale insect)	1	94.7
		Diptera	*Haematobia irritans* (hornfly)	1	99.3
			Drosophila melanogaster (fruitfly)	1	99.3
			Bradysia hygida	1	97.3
			Ochlerotatus caspius	1	97.2
		Coleoptera	*Trochoideus goudoti* (fungus beetle)	1	99.3
		Blattodea	*Blattella germanica* (German cockroach)	2*	85.4
	Collembola		*Sminthurinus bimaculatus* (springtail)	8	97
Crustacea			*Peracarida* sp. (pillbugs/sowbugs)	1	95.3

Supplementary Table S2. Taxa found in fecal samples in functional taxonomic groups. Frequency of occurrence of each taxon. Species as given by the closest BLAST hit.

CHAPTER 3

CHAPTER 3

Epidermal galactose spurs chytrid virulence and predicts amphibian colonization

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Abstract

The chytrid fungal pathogens *Batrachochytrium dendrobatidis* and *B. salamandrivorans* cause the skin disease chytridiomycosis in amphibians, which is driving a substantial proportion of an entire vertebrate class to extinction. Mitigation of its impact is largely unsuccessful and requires a thorough understanding of the mechanisms underpinning the disease ecology. By identifying skin factors that mediate key events during the early interaction with *B. salamandrivorans* zoospores, we discovered a marker for host colonization. Amphibian skin associated beta-galactose mediated fungal chemotaxis and adhesion to the skin and initiated a virulent fungal response. Fungal colonization correlated with the skin glycosylation pattern, with cutaneous galactose content effectively predicting variation in host susceptibility to fungal colonization between amphibian species. Ontogenetic galactose patterns correlated with low level and asymptomatic infections in salamander larvae that were carried over through metamorphosis, resulting in juvenile mortality. Pronounced variation of galactose content within some, but not all species, may promote the selection for more colonization resistant host lineages, opening new avenues for disease mitigation.

Keywords: Chytridiomycosis – amphibian – skin carbohydrates – *Batrachochytrium salamandrivorans*

Introduction

Mitigation of infectious diseases has become a key challenge in curbing biodiversity loss. One of the wildlife diseases contributing to Earth's sixth mass extinction is the lethal skin disease chytridiomycosis. This fungal disease is linked to extinctions or declines of hundreds amphibian species worldwide (Scheele, Pasmans, Skerratt, Berger, Martel, Beukema, Acevedo, Burrowes, Carvalho, Catenazzi, De la Riva, *et al.*, 2019; Lambert *et al.*, 2020). Efforts to contain the impact of the disease, including host removal, the use of chemical disinfectants, probiotics and habitat alteration, have thus far had limited success (Bosch *et al.*, 2015; Garner *et al.*, 2016; McKenzie, Kueneman and Harris, 2018; Martel *et al.*, 2020), and such actions can themselves be controversial, given the dire state of amphibian populations and their often vulnerable habitats. Designing more targeted and sustainable mitigation strategies, for example vaccination or selective breeding of resistant host lineages, requires a thorough understanding of host – pathogen – environment interactions.

Chytridiomycosis is caused by the chytrid fungi *Batrachochytrium dendrobatidis* (Berger *et al.*, 1998) and *B. salamandrivorans* (Martel *et al.*, 2013). The latter was recently discovered from a collapsing fire salamander (*Salamandra salamandra*) population in the Netherlands (Martel *et al.*, 2013) and is causing mass mortality events in wild salamander populations across Europe (Spitzen-van der Sluijs *et al.*, 2016; Lötters *et al.*, 2020; Martel *et al.*, 2020). In susceptible animals, *B. salamandrivorans* causes epidermal necrosis (Martel *et al.*, 2013), resulting in loss of the epidermal barrier and subsequent overgrowth and invasion by opportunistic bacteria, bacterial septicemia and death (Bletz *et al.*, 2018).

Infection of the amphibian skin by the motile fungal spores (Van Rooij *et al.*, 2015; Fisher and Garner, 2020) is a complex and poorly understood process that requires recognition of, attraction to, and subsequent attachment to the outer layers of the skin, in order to be able to invade the skin surface. To successfully invade the skin the fungus needs to overcome the physical (mucus and stratum corneum), chemical (antimicrobial peptides and toxins), cellular (immune cells), and microbiological barriers of the epidermal layer (Varga, Bui-Marinos and Katzenback, 2019). Little is known about the molecules that participate in the early interactions between pathogen and host, but they likely include polypeptides (proteins) or polycarbohydrates (carbohydrates), since fungal ligand-host receptor binding is mediated mainly by either protein-protein or protein-carbohydrate

interactions (Dwek, 1996; De Las Rivas and Fontanillo, 2010). The first matrix encountered is the epidermal mucosome, comprising host mucus and other host secreted and microbiome derived compounds. This saccharide-rich environment contains oligosaccharides that are known to attract *B. dendrobatidis* zoospores (Moss *et al.*, 2008; Van Rooij *et al.*, 2015). Following attachment, the *B. salamandrivorans* arsenal of proteases is thought to play a key role in subsequent cell invasion (Farrer *et al.*, 2017).

The extent of epidermal infection correlates with the severity of the disease, which varies strongly between, and even within, amphibian host species (Martel *et al.*, 2014, 2020; Van Rooij *et al.*, 2015; Carter *et al.*, 2019). The outcome of infection depends on complex host, pathogen and environmental interactions and can vary from absence of clinical signs to rapid death (Martel *et al.*, 2014). Predicting host susceptibility is crucial for risk assessments and the development of mitigation action plans and is currently only possible using invasive infection experiments (Martel *et al.*, 2020).

In this work we unravelled the early interactions of *B. salamandrivorans* with its amphibian host and investigate the potential use of skin galactose as a biomarker for fungal colonization. Using *in vitro* assays we show that beta-galactose mediates fungal attraction and adhesion to the amphibian skin. Beta galactose selectively upregulates virulence associated fungal genes and increases protease activity in zoospores, suggesting the initiation of a virulent fungal response. Histochemistry of the skin of 9 urodele and 5 anuran species and of different life stages of fire salamanders (*Salamandra salamandra*) demonstrates marked variation of the cutaneous glycosylation pattern of amphibian skin between species and life stages, notably of galactose, which correlates with susceptibility to chytrid colonization. A similar correlation between the proportion of galactose in the total carbohydrate fraction of skin washes of 17 urodele and 4 anuran species and chytrid colonization corroborates the use of galactose as a biomarker for susceptibility to *B. salamandrivorans* colonization. While *B. salamandrivorans* infections in salamander larvae are asymptomatic, infections may be carried over to metamorphosis, resulting in lethal infections of juveniles. Significant variation of cutaneous galactose content in two of three urodele species examined, further suggests the possibility of selection for increased colonization resistant lineages in some urodele species.

Results and discussion

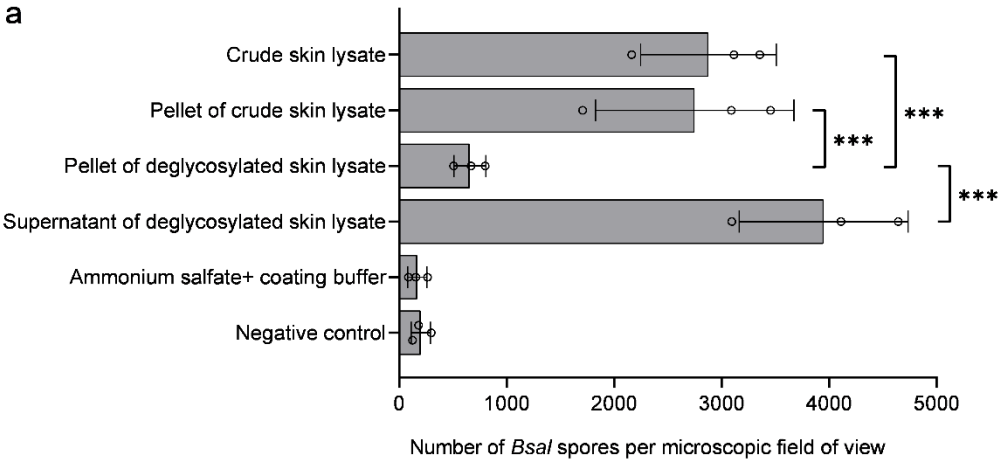
Galactose mediates *B. salamandrivorans* attraction and adhesion to salamander skin

We first identified key factors in the attraction and attachment of *B. salamandrivorans* to the amphibian skin, representing the first steps in the pathogenesis of *B. salamandrivorans* infection. We investigated if *B. salamandrivorans* spores bind to the carbohydrate or protein fraction of the amphibian skin. A water soluble lysate was prepared from sloughed skin of fire salamanders (*Salamandra salamandra*) and this crude preparation was used to compare the binding ability of *B. salamandrivorans* spores to precipitated skin proteins, precipitated skin proteins lacking carbohydrates (through enzymatic deglycosylation), and supernatant of the latter, containing the enzymatically removed carbohydrate fraction. *B. salamandrivorans* zoospore binding was quantified by counting the attached zoospores in a binding assay. While binding of spores was comparable between the crude preparation, the precipitated protein fraction and the carbohydrate fraction, binding to the deglycosylated proteins was significantly reduced, compared to the crude preparation ($p = 0.0002$), precipitated protein fraction ($p = 0.0018$) and carbohydrate fraction ($p < 0.0001$) (Fig. 1a, Supplementary Tables 1–2). *B. salamandrivorans* zoospores thus predominantly bind to carbohydrates of salamander skin.

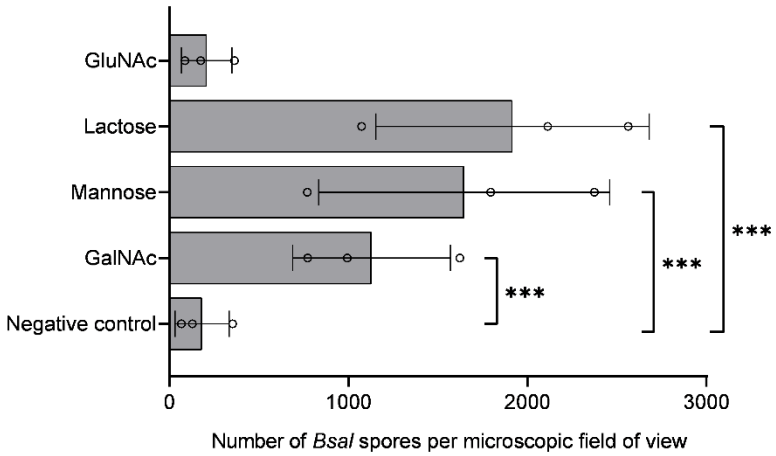
To identify the carbohydrates involved in *B. salamandrivorans* binding, we then coated a series of carbohydrates on microtiter plates and quantified the number of attached zoospores. The fungal spores predominantly bound to lactose, N-Acetylgalactosamine (GalNAc) and mannose, but not to N-Acetylglucosamine (GlcNAc) (Fig. 1b, Supplementary Tables 3–4), identifying galactose and mannose as *B. salamandrivorans* binding sites on salamander skin. In addition, a capillary tube chemotaxis assay demonstrated movement of *B. salamandrivorans* zoospores towards carbohydrates, with a high affinity towards galactose (Fig. 1c, Supplementary Table 5). This is in contrast with *B. dendrobatidis*, which is attracted by the tested carbohydrates (mannose, galactose, fucose, N-acetylglucosamine, N-acetylgalactosamine and N-acetylneuraminic acid) without specific preference (Van Rooij *et al.*, 2015). To corroborate these findings, we searched for evidence of carbohydrate binding proteins in the *B. salamandrivorans* genome (AMFP13/01, NCBI database, Bioproject PRJNA311566), which yielded two ricin B lectins, two legume-like lectins and one concanavalin A (Con A)-like lectin (Supplementary Table 6). Ricin B lectin binds oligosaccharides containing either terminal beta-GalNAc or β-1,4-linked galactose residues

(Wales *et al.,* 1991). Both legume-like lectins were identified as mannose-specific lectins (Itin *et al.*, 1996; Kamiya *et al.,* 2005), and the Con A-like lectin binds specifically to α-D-mannosyl and α-D-glucosyl residues(Sumner, Gralën and Eriksson-Quensel, 1938). The expression of a ricin B lectin gene (BSLG_00833) and the Con A-like lectin gene (BSLG_02674) upon *B. salamandrivorans* exposure to salamander skin was demonstrated previously (Farrer *et al.*, 2017). These results suggest that *B. salamandrivorans* expresses lectins to bind to galactose and mannose in the salamander skin.

a


Crude skin lysate
Pellet of crude skin lysate
Pellet of deglycosylated skin lysate
Supernatant of deglycosylated skin lysate
Ammonium salfate+ coating buffer
Negative control
***
***
***
0
1000
2000
3000
4000
5000
Number of *Bsal* spores per microscopic field of view

b


GluNAc
Lactose
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GalNAc
Negative control
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0
1000
2000
3000
Number of *Bsal* spores per microscopic field of view

c

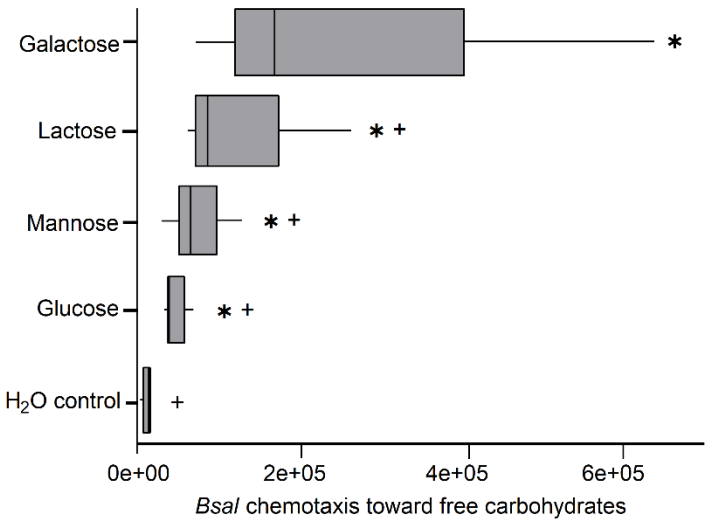
Galactose
Lactose
Mannose
Glucose
H_2O control
0e+00
2e+05
4e+05
6e+05
Bsal chemotaxis toward free carbohydrates

Fig. 1: Galactose mediates *B. salamandrivorans* attraction and adhesion. a *B. salamandrivorans* zoospores bound on microtiter plates coated with crude and enzymatically treated skin lysate of sloughed fire salamander skin. Pellet and supernatant fractions were obtained by protein precipitation. **b** *B. salamandrivorans* zoospores bound on microtiter plates coated with different carbohydrates. GlcNAc = N-Acetylglucosamine, GalNAc = N-Acetylgalactosamine.a-b Negative control = wells coated with coating buffer only. Three technical replicates were performed per biological replicate, with three biological replicates in total. Figures represent the mean and variation (standard deviation) of the biological replicates. Black circles represent biological replicates. The significance of difference between the distributions is shown by *** $p < 0.001$, based on Tukey's multiple comparisons with the glht function in R package multcomp, comparing (two-sided) negative binomial models. **c** Chemotaxis of *B. salamandrivorans* toward free carbohydrates. The carbohydrates D-glucose, D-mannose, lactose and D-galactose were tested as attractants at a 0.1 M concentration, using a traditional capillary tube test. Water was used as vehicle and control attractant. Genomic equivalents (GE) of *B. salamandrivorans* zoospores in the capillaries were quantified after 90 minutes using quantitative realtime PCR. At least three technical replicates were performed per biological replicate, with three biological replicates in total. Box-and-whisker plot present the mean and variation in chemotaxis of the biological replicates; median is shown as vertical line inside the box, the first and third quartiles shown as the lower and upper edges of the box, respectively, and the minimum and maximum values shown as whiskers. An * indicates a significant difference compared to the water control, whereas + designates a significant difference compared to galactose; with adjusted *p*-values obtained via Tukey's multiple comparisons corrections. **a-c** Individual P-values are shown in Supplementary Tables 2, 4 and 5. Source data are provided as a Source Data file.

Contact with galactose spurs fungal virulence

To better understand how these carbohydrates mediate the early stages of *B. salamandrivorans* infection, we performed RNA-seq analysis and found a selective upregulation of putative *B. salamandrivorans* virulence factors during initial contact with carbohydrates and galactose in particular. RNA-seq analysis detected 25 uniquely expressed genes in galactose-treated zoospores (Fig. 2a). Among those, BSLG_05880 and BSLG_09248 were annotated as protein tyrosine kinase candidates, which are known to participate in signalling transduction pathways and regulate a series of essential cellular processes, such as cell growth, differentiation and death (Wang and Cole, 2014). In choanoflagellates, protein tyrosine kinases have been found to regulate cell proliferation (Ruiz-Trillo *et al.*, 2007) and react to the environmental nutrient availability (King, Hittinger and Carroll, 2003). Protein tyrosine kinases have also been shown to play a crucial role during fungal infections, including attachment to host cells (Limongi, De Souza and Rozental, 2003; Mendes Giannini *et al.*, 2005). BSLG_08965, also specifically linked to galactose treatment,

was annotated as belonging to the alpha/beta hydrolase family (Fig. 2a), which is one of the biggest groups of structurally related hydrolytic enzymes with diverse catalytic functions, such as hydrolases, lyases or transporters of other proteins (Nardini and Dijkstra, 1999; Holmquist, 2000). Alpha/beta hydrolase fold proteins have been reported to regulate the interactions between pathogenic bacteria, and were previously associated with the efficient infection and adaptation of hosts by *B. dendrobatidis* (Mei *et al.*, 2010; Zhu *et al.*, 2012; Sun *et al.*, 2016). Gene groups uniquely expressed in mannose- and glucose-treated spores contained repair enzymes, proteins linked to transport, but also possible virulence candidates including protein kinase (mannose BSLG_00482 and glucose BSLG_08672) and copper/zinc superoxide dismutase (mannose BSLG_09793), which is known as a superoxide radical scavenger linked to fungal virulence (Thirach *et al.*, 2007). Genes uniquely expressed in mannose were mainly associated with mitochondria, whereas the majority of the genes uniquely expressed in glucose seemed to be associated with the transport of cellular components and particularly proteins (e.g. secretory exocyst component, kinesin motor domain, transporters, nexin, clathrin, exportin) (Supplementary Data 1).

Differential gene expression analysis showed a significant upregulation/downregulation of 1079/610, 1017/418 or 925/746 genes in, respectively, galactose, glucose or mannose-treated zoospores, compared to the H_2O-treated zoospores (Fig. 2b). The fungalysin metallopeptidase (M36) family and the serine-type peptidase (peptidase S41) family are highly expanded in both *B. dendrobatidis* and *B. salamandrivorans*, compared to non-pathogenic chytrid fungi and are considered virulence factors, involved in the initial stages of zoospore colonization of amphibian skin and entry into host cells (Rosenblum *et al.*, 2008; Farrer *et al.*, 2013, 2017). M36 metalloprotease candidates BSLG_08963 (Log_2 fold change vs H_2O = 2.25) and BSLG_09557 (Log_2 fold change vs H_2O = 2.55), together with the peptidase S41 candidate BSLG_06886 (Log_2 fold change vs H_2O = 3.66), showed an upregulation in galactose-treated zoospores, compared to H_2O-treated zoospores, but not in glucose- or mannose-treated zoospores (Fig. 2c). As a general response to carbohydrates, a significant upregulation (Log_2 fold change vs H_2O $\geqslant$ 2.0) was observed in possible virulence genes including multiple protein kinase candidates (BSLG_01979, BSLG_09370, BSLG_07973, BSLG_05106, BSLG_09982, BSLG_01828, BSLG_02473 and BSLG_08449) and the peptidase family S41 candidate (BSLG_07398). Two-tailed Fisher's exact test for Gene Ontology (GO) terms in the genes significantly upregulated in response to

carbohydrate exposure against the remaining gene set showed enrichment of kinase activity (GO:0016301) and transferase activity (GO:0016772) (FDR *p*-value < 0.05). Exposure to carbohydrates, and specifically galactose, initiates a cascade of protein changes, including expression and upregulation of a number of virulence candidates.

This was translated to increased protease activity of zoospores. When exposed to galactose, protease activity is significantly higher than when mannose- ($p = 0.008$), H_2O- ($p = 0.036$) or protease inhibitor (PI)-treated ($p < 0.001$) (Fig. 3, Supplementary Table 7). Increased protease activity corroborates the initiation of a virulent response of *B. salamandrivorans* upon contact with galactose.

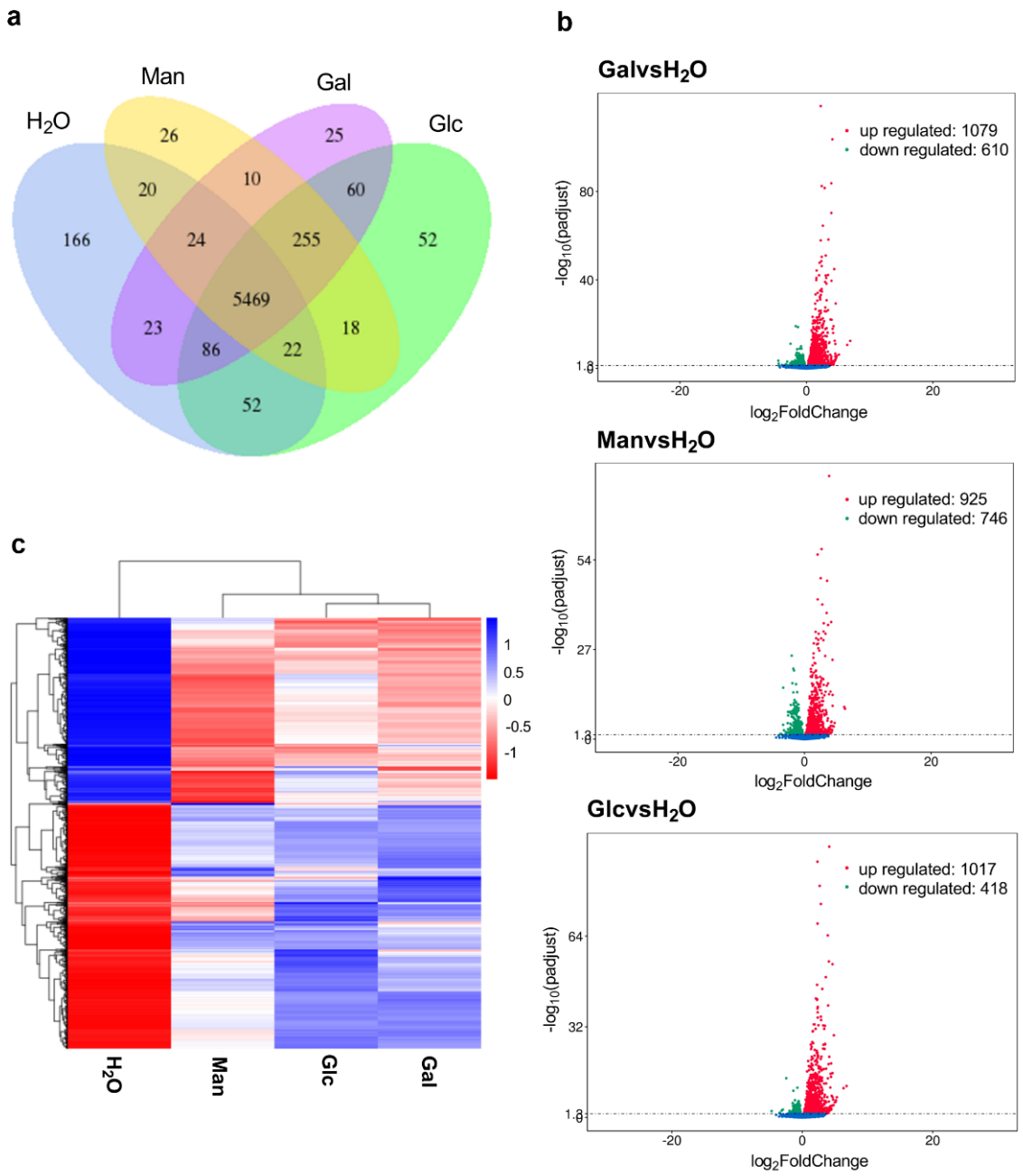

Fig. 2: Gene expression in *B. salamandrivorans.* **a** Co-expression Venn diagram presents the number of genes that are uniquely expressed within each group per sample, with the overlapping regions showing the number of genes that are co-expressed in two or more groups per samples. **b** Volcano plots of differentially expressed genes identified between the galactose, glucose or mannose group and control group. The green dots denote down-regulated gene expression, the red dots denote up-regulated gene expression, and the blue dots denote the gene expression without marked differences. **c** FPKM cluster analysis, clustered using the $\log_{10}$ (FPKM+1) value. Blue

denotes genes with high expression levels, and red denotes genes with low expression levels. The color ranging from blue to red indicates $\log_{10}$ (FPKM+1) value from large to small. Trees indicate hierarchical clustering between data sets (above) and genes (left of heatmap). Gal = galactose; Glc = glucose; Man = mannose. Source data are provided as a Source Data file.

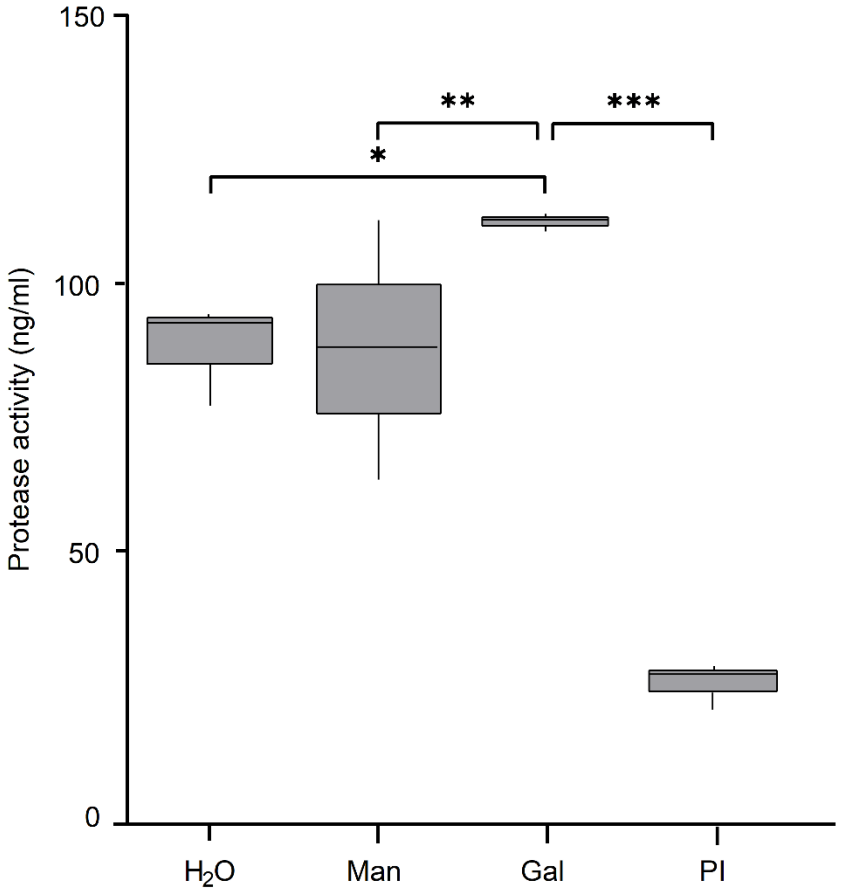

Fig. 3: Protease activity detected in supernatants of *B. salamandrivorans* zoospores. Supernatants were collected by centrifuging the *B. salamandrivorans* zoospore suspensions treated with 50 mM D-galactose (Gal), 50 mM D-mannose (Man), H_2O and protease inhibitor mix (PI). Three technical replicates were performed per biological replicate, with three biological replicates in total. In the box-and-whisker plots, median is shown as line inside the box, the first and third quartiles shown as the lower and upper edges of the box, respectively, and the minimum and maximum values shown as whiskers. The significance of difference between the means is shown by *p = 0.036, ** p = 0.008, *** $p < 0.001$; based on Tukey's multiple comparisons test comparing two-sided linear mixed models. Source data are provided as a Source Data file.

Life stage dependent susceptibility to *B. salamandrivorans* colonization correlates with ontogenetic galactose patterns in the amphibian skin

Marked differences in lectin binding patterns have been observed in amphibians (Zaccone *et al.*, 1999), suggesting profound differences in carbohydrate patterns. Since *B. salamandrivorans* is attracted by and attaches to carbohydrates, the salamander skin carbohydrate content may predict the magnitude of host skin colonization. Ontogenetic carbohydrate patterns of different life stages of fire salamanders were compared to explain intraspecific differences of host susceptibility. While *B. salamandrivorans* infection in fire salamanders post metamorphosis is consistently lethal (Martel *et al.*, 2013, 2014), larvae are not considered susceptible to chytridiomycosis (Van Rooij *et al.*, 2015). *Ricinus communis* agglutinin (RCA) and concavalin A (Con A) histochemistry was used to score the presence of galactose and mannose/glucose in the skin, respectively. The presence of galactose but not mannose or glucose in the skin of fire salamander larvae markedly increases with age and climaxes towards metamorphosis (Fig. 4, Supplementary Fig. 1). Post metamorphosis, these high galactose levels are maintained.

The correlation between galactose levels and susceptibility to *B. salamandrivorans* infection was studied by experimental exposure of different life stages of fire salamanders (early and late stage larvae and metamorphs) to *B. salamandrivorans.* The infection prevalence and load was much higher in metamorphs than in larvae (Wilcoxon rank sum test of *B. salamandrivorans* load in genomic equivalents $p = 0.0001$, z-score= -3.885 n(larvae) = 37, n(metamorphs) = 6; Fig. 4a). Disease signs (skin ulcerations) were noted in metamorphs only and not in the pre-metamorphic stages (Fig. 4b). Five out of ten inoculated late stage larvae carried over the infection through metamorphosis, resulting in lethal disease of juveniles. The infection load in the larvae correlated with the intensity of the galactose staining ($\beta = 2.81$, $p = 0.014$, n = 43, regression F-statistic (2,40) = 5.40 and p-value= 0.008; Fig. 4 c, d, e). Infection load and disease course are thus correlated with ontogenetic galactose patterns in salamander skin.

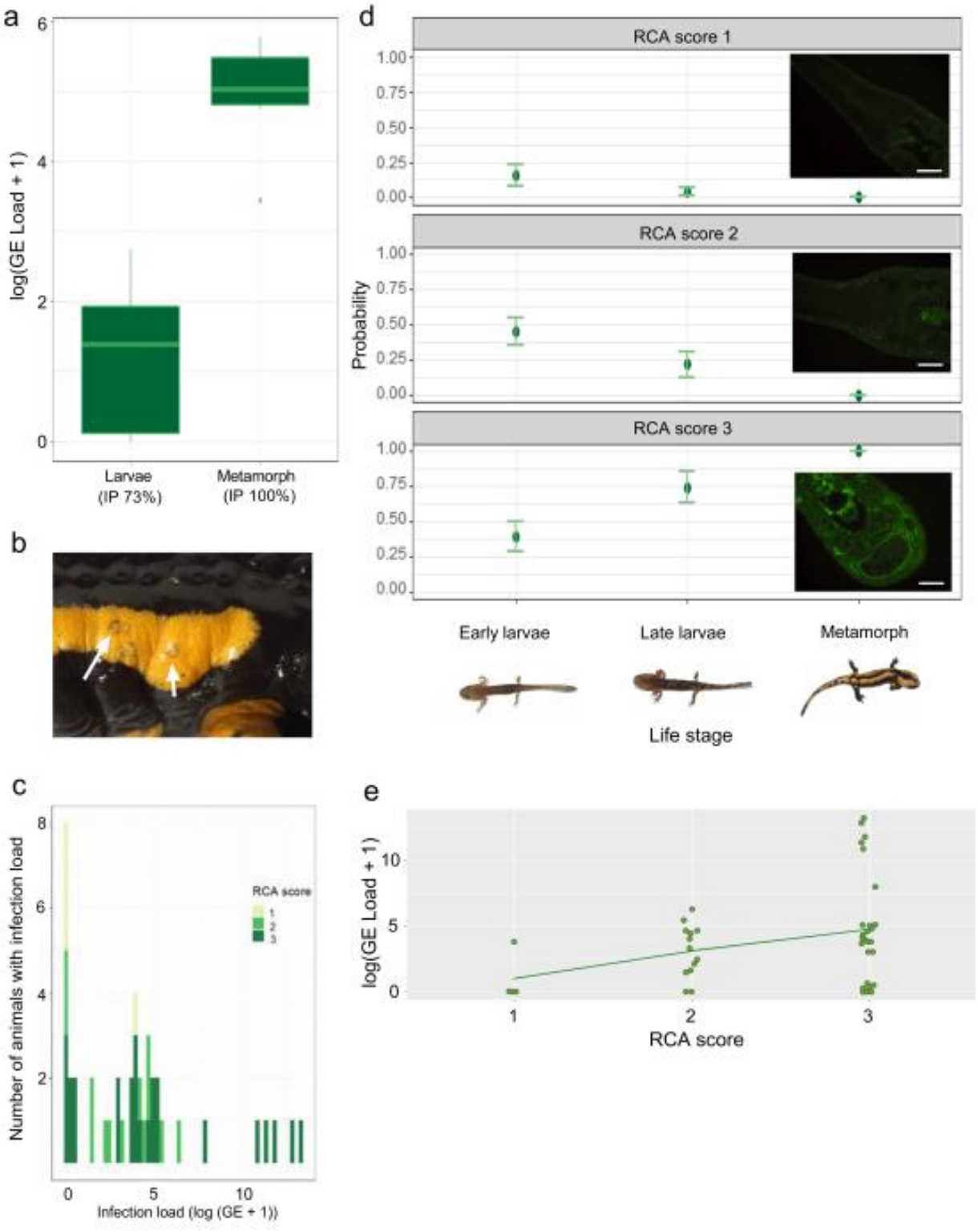

Fig. 4: Susceptibility to *B. salamandrivorans* infection correlates with life stage dependent skin galactose presence. a *B. salamandrivorans* infection loads (expressed as genomic equivalents (GE)) on fire salamander larvae (*n* = 37 biologically independent animals) and

metamorphs (n = 6 biologically independent animals) 10 days after exposure to $1.5x10^5$ spores/ml for 24 hours. In the boxplots, horizontal lines represent median and interquartile ranges, with the vertical line representing min/max. Dots represent outliers, whiskers indicate highest/lowest value within 1.5*IQR from hinge. *B. salamandrivorans* infection prevalence (IP) of fire salamander larvae and metamorphs are shown. **b** Macroscopic picture of infected fire salamander metamorphs, arrow indicates skin ulcerations. **c** Histogram of *B. salamandrivorans* infection log (GE load + 1) load and equivalent RCA scores of fire salamander larvae and metamorphs. **d** Probability of RCA scores in early larvae stage (n = 21 biologically independent animals), late larvae stage (n = 16 biologically independent animals), and fire salamander metamorphs (n = 6 biologically independent animals) predicted from ordinal logistical regression fit with polr(), error bars indicate RCA score probability +/- one standard error. Photomicrographs represent different RCA staining scores. RCA score: 1 = weak staining, 2 = strong staining, 3 = intense staining. RCA score of 0 is not shown because no slides were scored 0. Scale bars = 100 µm. Morphological characters of fire salamander larvae and metamorphs are shown. **e** *B. salamandrivorans* infection log (GE load + 1) load and respective RCA scores of individual fire salamander larvae and metamorphs. Line indicates linear regression, implemented with RCA score as an ordered categorical variable. Source data are provided as a Source Data file.

Skin galactose staining predicts *B. salamandrivorans* infection intensity and survival probability in amphibians

We then compared the presence of epidermal galactose and susceptibility to *B. salamandrivorans* infection across fourteen amphibian species (nine urodelan species, five anuran species; Fig. 5). To exclude anatomical topology differences in carbohydrate pattern, we first compared the presence of galactose between different body sites (ventral and dorsal skin, toeclips and tailclips) of the same animal in three species (alpine newts, fire salamanders and palmate newts; Supplementary Table 8). The RCA staining was consistent in the skin of the different body parts. Hence, tailclips and toeclips were used for urodelan and anurans, respectively. We used average peak loads as a proxy for susceptibility.

RCA histochemistry revealed a clear positive correlation between *B. salamandrivorans* infection peak loads and RCA staining intensity across the fourteen species included (r_{pb} = 0.687, p = 0.007; Fig. 5a, Supplementary Table 9). We confirmed this with a linear model using dummy variables for RCA scores and found that RCA scores can explain 87.2% of the variance in *B. salamandrivorans* infection peak loads (R^2 = 0.872; Supplementary Table 10). All seven species (five anuran species, two urodelan species) reported to be less susceptible to *B. salamandrivorans*

infection showed a negative to weak RCA signal (Fig. 5, Supplementary Table 9). These species were previously shown to develop low level and asymptomatic infections (Stegen *et al.*, 2017; Martel *et al.*, 2020). The eight susceptible species that are extensively colonized by *B. salamandrivorans* upon exposure (Martel *et al.*, 2014, 2020) showed marked galactose presence in their skin, represented by an intense RCA signal (Fig. 5, Supplementary Table 9). We found that galactose content in the skin is a poor predictor of disease course after infection (r_{pb} = 0.193, p = 0.509, R^2 = 0.564; Fig. 5b, Supplementary Table 10). Indeed, extensive colonization needs not necessarily result in lethality. Rather, innate and acquired defense mechanisms and the environmental context are likely to determine the disease outcome (Fisher and Garner, 2020). Thus, RCA staining of the tail or toeclips can be useful as a predictor of susceptibility of a species for *B. salamandrivorans* colonization and, less reliably, for severity of disease progression. This information can inform mitigation strategies and action plans. We applied this to tailclips of the endangered Lanzai salamander (*Salamandra lanzai*), which showed intense RCA staining. Based on the dummy variable regression models between RCA scores with infection peak loads and mortality rates (Supplementary Table 10), we predict that infection of *Salamandra lanzai* would result in high infection intensities and a high probability of lethal infections. This estimate is supported by its phylogenetic vicinity to closely related, and known susceptible species. We thus propose the galactose staining pattern in salamander skin to be a valuable predictor of infection intensity and, to a lesser extent, survival probability.

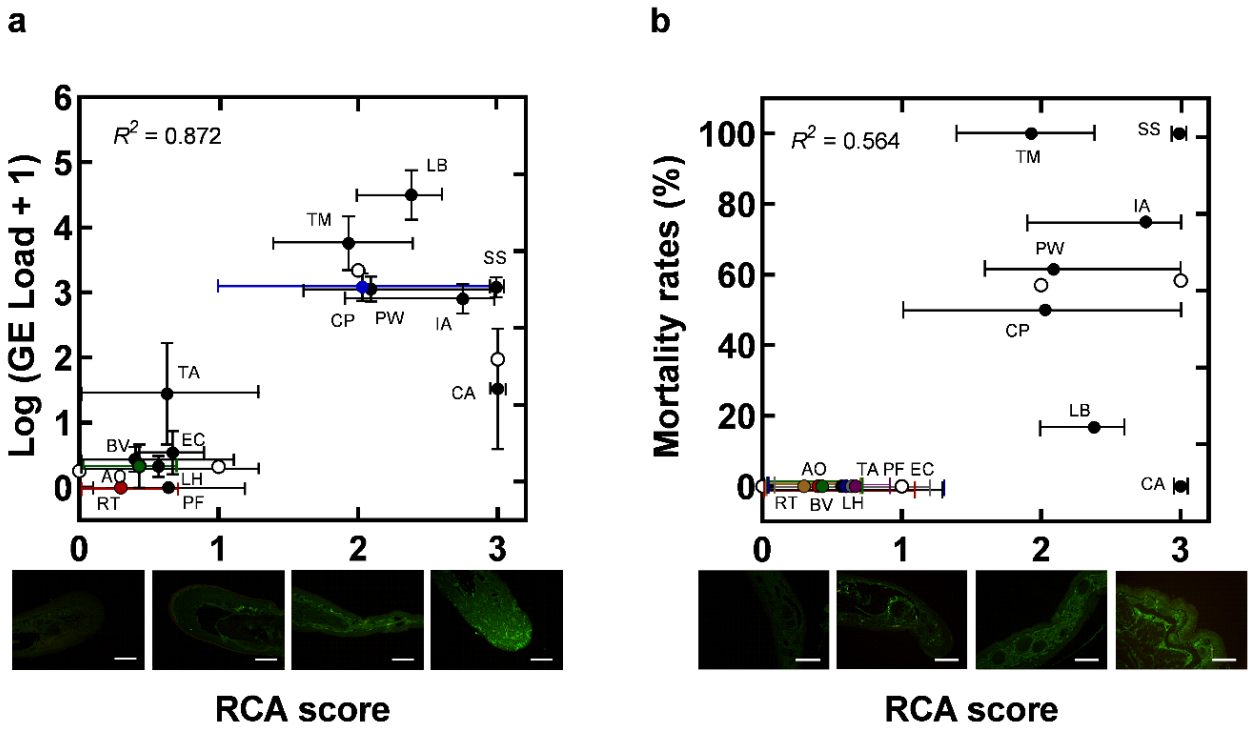

Fig. 5: Skin galactose staining predicts *B. salamandrivorans* infection intensity and survival probability. a Intensity of RCA staining in relation to *B. salamandrivorans* infection peak loads of different amphibian species. *B. salamandrivorans* infection peak loads are expressed in log (genomic equivalents (GE) load +1). Vertical error bars indicate standard error of mean. **b** Intensity of RCA staining in relation to mortality rates of different amphibian species. **a-b** Horizontal error bars indicate minimum and maximum RCA scores, black and coloured dots represent the mean value overall. Linear regressions were performed using dummy variables for RCA scores. White dots represent the predicted values of infection peak loads and mortality rates for each RCA score generated by the regression model. R^2 indicates the coefficient of multiple determination in the regression model. RCA score: 0 = negative staining, 1 = weak staining, 2 = strong staining, 3 = intense staining. Photomicrographs represent different RCA staining scores. Scale bars = 100 µm. Urodele species: LH = *Lissotriton helveticus*, PW = *Pleurodeles waltl*, LB = *Lissotriton boscai*, TA = *Triturus anatolicus*, TM = *Triturus marmoratus*, CP = *Cynops pyrrhogaster*, IA = *Ichthyosaura alpestris*, SS = *Salamandra Salamandra* and CA = *Calotriton asper*. Anuran species: AO = *Alytes obstetricans*, RT = *Rana temporaria*, BV = *Bombina variegata*, PF = *Pelobates fuscus* and EC = *Epidalea calamita*. Number of biologically independent animals used for RCA staining: LH (n = 13), PW (n = 11), LB (n = 3), TA (n = 3), TM (n = 3), CP (n = 3), IA (n = 12), SS (n = 10), CA (n = 10), AO (n = 10), RT (n = 10), BV (n = 5), PF (n = 5) and EC (n = 5). Number of biologically independent animals in infection trials: LH (n = 23), PW (n = 13), LB (n = 6), TA (n = 6), TM (n = 6), CP (n = 8), IA (n = 20), SS (n = 26), CA (n = 5), AO (n = 13), RT (n = 5), BV (n = 4), PF (n = 5) and EC (n = 5). Source data are provided as a Source Data file.

Noninvasive sampling of amphibians for galactose levels as a biomarker for infection intensity

While quantifying galactose in the skin may be a promising tool to predict amphibian susceptibility to *B. salamandrivorans* infection, noninvasive sampling is preferable over the collection of tissue samples. We therefore studied whether testing galactose levels in the amphibian skin mucosome (Woodhams *et al.*, 2014) could be a viable alternative. Amphibian skin washes were collected by bathing animals from seventeen species of urodeles and four species of anurans in water for 1 hour. The skin washes were subsequently examined for the concentration of oligosaccharides.

The proportion of galactose in the total carbohydrate fraction of the skin washes yielded results in line with those obtained from the RCA staining of tissues. The four anuran species, reported to be *B. salamandrivorans* resistant or tolerant, showed a low percentage of free galactose in the total carbohydrate fraction (Fig. 6, Supplementary Fig. 2, Supplementary Table 9). Moderate correlations were observed between the percentage of free galactose with *B. salamandrivorans* infection peak loads (Pearson r = 0.641, p = 0.003; Fig. 6a) and with mortality rates (Pearson r = 0.523, p = 0.026; Fig. 6b), though the sensitivity of free galactose likely varies across species, as suggested by *Lyciasalamandra helverseni*, *Salamandra salamandra* and *Calotriton asper* (Fig. 6a, b), which seem better able to tolerate higher free galactose levels than expected based on the observed linear correlations (i.e. observed infection peak loads and mortality rates outside the 95% CI for both Pearson's correlations). Quadratic regression models show that 70.4% of the variance in infection peak loads (R^2 = 0.704; Fig. 6a) and 54.3% of the variance in mortality rates (R^2 = 0.543; Fig. 6b) can be explained by the percentage of free galactose from the mucosome washes. Meanwhile, a clear correlation was also found between the galactose levels in mucosome washes and the RCA staining intensity, when comparing samples within the same species (r_{pb} = 0.565, p = 0.035; Fig. 6c). However, RCA scores account for a larger variation in the infection peak loads of *B. salamandrivorans* (R^2 = 0.872) than the method of measuring galactose in the mucosome washes (R^2 = 0.704). Therefore, although the galactose concentrations in skin washes can predict infection intensity of *B. salamandrivorans* infection, we do not recommend using skin washes for predictions at species level if more invasive sampling is allowed.

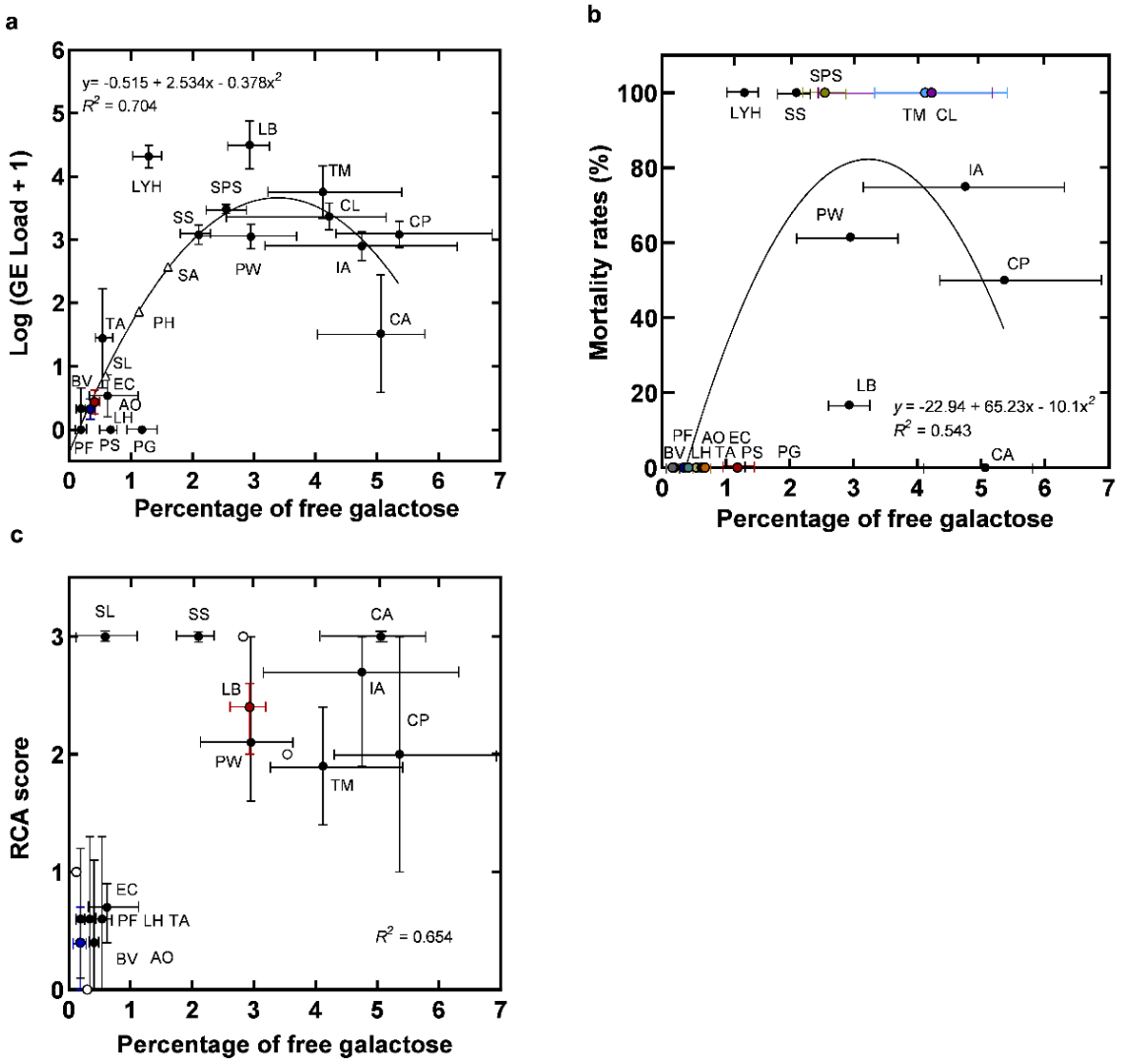

Fig. 6: Free galactose levels as a biomarker for infection intensity. a Percentage of free galactose per total carbohydrate in mucosome washes in relation to *B. salamandrivorans* infection peak loads of different amphibian species. *B. salamandrivorans* infection peak loads are expressed in log (genomic equivalents (GE) load +1). Vertical error bars indicate standard error of mean. **b** Percentage of free galactose per total carbohydrate in mucosome washes in relation to mortality rates of different amphibian species. **c** Percentage of free galactose per total carbohydrate in mucosome washes in relation to intensity of RCA staining. Vertical error bars indicate minimum and maximum RCA scores. **a-c** Horizontal error bars indicate minimum and maximum of percentage of free galactose. Black and coloured dots represent the mean value overall. R^2 indicates the coefficient of multiple determination. **a-b** The regression analyses were performed by quadratic regression. White triangles represent the predicted infection peak load values calculated

by the equation of quadratic regression model. Line indicates quadratic regression. **c** Linear regression was performed using dummy variables for RCA scores. White dots represent the predicted values of percentage of free galactose for each RCA score generated by the regression model. RCA score: 0 = negative staining, 1 = weak staining, 2 = strong staining, 3 = intense staining. **a-c** Urodele species: LH = *Lissotriton helveticus*, PW = *Pleurodeles waltl*, LB = *Lissotriton boscai*, TA = *Triturus anatolicus*, TM = *Triturus marmoratus*, CP = *Cynops pyrrhogaster*, IA = *Ichthyosaura alpestris*, SS = *Salamandra salamandra*, LYH = *Lyciasalamandera helverseni*, SPS = *Speleomantes strinatii*, PH = *Paramesotriton hongkongensis*, PG = *Plethodon glutinosus*, CL = *Chioglossa lusitanica*, PS = *Pachyhynobius shangchengensis*, CA = *Calotriton asper*, SA = *Salamandra algira* and SL = *Salamandra lanzai*. Anuran species: AO = *Alytes obstetricans*, BV = *Bombina variegata*, EC = *Epidalea calamita* and PF = *Pelobates fuscus*. Number of biologically independent animals used for measuring the percentage of free galactose in mucosome washes: LH (n = 3), PW (n = 3), LB (n = 3), TA (n = 3), TM (n = 3), CP (n = 3), IA (n = 3), SS (n = 3), LYH (n = 3), SPS (n = 2), PH (n = 2), PG (n = 2), CL (n = 3), PS (n = 3), CA (n = 3), SA (n = 3), SL (n = 2), AO (n = 3), BV (n = 2), EC (n = 3) and PF (n = 3). Number of biologically independent animals in infection trials: LH (n = 23), PW (n = 13), LB (n = 6), TA (n = 6), TM (n = 6), CP (n = 8), IA (n = 20), SS (n = 26), LYH (n = 3), SPS (n = 3), PG (n = 5), CL (n = 6), PS (n = 3), CA (n = 5), AO (n = 13), BV (n = 4), EC (n = 5) and PF (n = 5). Number of biologically independent animals used for RCA staining: LH (n = 13), PW (n = 11), LB (n = 3), TA (n = 3), TM (n = 3), CP (n = 3), IA (n = 12), SS (n = 10), CA (n = 10), AO (n = 10), RT (n = 10), BV (n = 5), PF (n = 5) and EC (n = 5). Source data are provided as a Source Data file.

Intraspecies variation in carbohydrate pattern could promote selection towards increased resistance

RCA scores were shown to vary significantly between individuals of some, but not all species examined. The coefficient of variation (CV%) for RCA scores of species *P. waltl* (n = 11), *I. alpestris* (n = 12) and *S. salamandra* (n = 10) were 18.56%, 13.74% and 0.00%, respectively (Supplementary Table 14). Mortality rates after experimental exposure for these species are 61.5% (Martel *et al.*, 2014, 2020), 75% (Martel *et al.*, 2014; Stegen *et al.*, 2017) and 100% (Blooi *et al.*, 2013; Martel *et al.*, 2014, 2020; Stegen *et al.*, 2017). This variation in glycosylation patterns within species may be exploited to produce lineages with increased resistance against infection and disease, provided such characteristics are hereditary and do not incur harmful side effects such as decreasing the defensive capacity of the skin microbiome. In naturally infected populations, such variation may result in the selection of increased resistance and thus may predict resilience

against disease. Lack of variation in the galactose pattern observed in fire salamanders coincides with sharp population declines and the apparent lack of developing increased resistance observed in this species (Stegen *et al.*, 2017).

In this study, we were able to demonstrate that the interaction between host galactose-containing oligosaccharides and *B. salamandrivorans* ricin B-like lectin plays a vital role in the early pathogenesis of *B. salamandrivorans*-induced chytridiomycosis by mediating chemotaxis, adhesion and the initiation of a virulent fungal response. Host cutaneous galactose content effectively predicted susceptibility to *B. salamandrivorans* colonization. Intraspecific variation of galactose patterns may thus provide an opportunity for selection towards increased colonization resistance.

Methods

Batrachochytrium salamandrivorans (*B. salamandrivorans*) culture conditions and zoospore isolation

B. salamandrivorans type strain (AMFP 13/01) (Martel *et al.*, 2013) was grown in tryptone-gelatin hydrolysate-lactose (TGhL) broth and incubated for 5-7 days at 15 °C. Zoospores were harvested by replacing the TGhL broth with distilled water. The collected water was filtered through a sterile mesh filter with pore size 10 µm (Pluristrainer, PluriSelect) to remove sporangia. Zoospore viability and mobility was confirmed using light microscopy.

Salamander skin lysate binding assay

Binding of *B. salamandrivorans* spores to the protein or carbohydrate fractions from fire salamander (*Salamandra salamandra*) skin was tested by treating fire salamander sloughed skin lysates enzymatically with glycoside hydrolases, followed by protein precipitation. An overview of the skin lysate binding assay is shown in Supplementary Fig. 3.

To collect the sloughed skin, ten captive bred adult fire salamanders were housed at 15 ± 1 °C on moist tissue. The sloughed skin samples were ground with liquid nitrogen into a fine powder and then homogenized, using 3 ml RadioImmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich)

per gram of tissue. Samples were incubated for 1 hour at 4 °C, centrifuged at 27.000 g for 10 minutes and supernatant was subsequently collected. Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). The obtained skin lysate was equally divided, one part was treated with Protein Deglycosylation Mix II and two parts were kept as crude skin lysates. Protein Deglycosylation Mix II (New England BioLabs) was used to remove N-linked and O-linked glycans from glycoproteins. According to the manufacturer's instructions, 5 µl 10x Deglycosylation Mix Buffer I and 5 µl Protein Deglycosylation Mix II were added to 40 µl skin lysate. The mixture was incubated at 37 °C for 16 hours. Protein precipitation was conducted on the redundant Protein Deglycosylation Mix II treated and crude skin lysates. The precipitation was performed by slowly adding saturated ammonium sulfate solution to the skin lysates to achieve a final concentration of 75%. Samples were then centrifuged at 21.130 × g for 30 minutes to separate the precipitated proteins from supernatant. The precipitated protein pellets were resuspended in 300 µl of 0.05 M carbonate-bicarbonate coating buffer (3.7 g $NaHCO_3$, 0.64 g Na_2CO_3, 1 L distilled water, pH 9.6). Each skin lysate solution was adjusted to the volume of 300 µl by adding coating buffer. One hundred µl of each skin lysate solution were coated in each well of 96-well polystyrene microtiter plates (MaxiSorp™ plate, Thermo Fisher Scientific) in three technical replicates. As controls, coating buffer (negative control) and 75% ammonium sulfate solution were also coated on the 96-well plates. After incubation at 4 °C for 24 hours the coated plates were washed three times with washing buffer (0.01 M PBS-Tween 20, pH 7.4) and blocked with 1% BSA overnight at 4 °C. Plates were then again washed three times with washing buffer and three times with distilled water. One hundred µl of *B. salamandrivorans* zoospore suspension (1 x 10^7 zoospores per ml) were added in each well. Plates were incubated for 20 minutes at 15 °C and washed five times with distilled water to remove the unbound zoospores. Digital photographs were taken through a via an inverted light microscope at 100 x magnification. Five pictures were taken for each well and zoospores in each photograph were counted in a blind fashion. Three independent repeats of the experiment were conducted (biological replicates).

Carbohydrate binding assay

To further determine which carbohydrates expressed on fire salamander sloughed skin can mediate the binding of *B. salamandrivorans* zoospores, *B. salamandrivorans* binding against four

carbohydrates; N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), mannose and lactose was tested. The three monosaccharides and the disaccharide (Sigma-Aldrich) were dissolved and thereafter diluted in coating buffer to achieve a concentration of 5% (w/v). Then they were coated in triplicate wells by incubating at 4 °C for 24 hours (Paul *et al.*, 2009). Plates were rinsed three times with washing buffer and blocked with 1% BSA overnight at 4 °C.

Hundred µl of *B. salamandrivorans* zoospore suspension (1 x 10^7 zoospores per ml) was added in each well and incubated for 20 minutes at 15 °C. After washing the wells five times with distilled water to remove unbound zoospores, the plates were evaluated using a light microscope. Digital photographs were taken at 100 x magnification. Five pictures were taken for each well and zoospores in each photograph were counted in a blind fashion. Three independent repeats of the experiment were conducted (biological replicates).

In this experiment the highest level of *B. salamandrivorans* spores binding to lactose was observed. Lactose is a dissacharide consisting of glucose and galactose. Therefore, in the following expriments galactose, glucose and their derivatives will be tested separately.

Carbohydrate chemotaxis test

Chemotaxis of *B. salamandrivorans* toward free carbohydrates was tested as previously explained (Supplementary Fig. 4) (Van Rooij *et al.*, 2015). The oligocarbohydrates D-Glucose (Sigma-Aldrich), D-mannose (Sigma-Aldrich), Lactose (Sigma-Aldrich) and D-galactose (Sigma-Aldrich) were tested as attractant for *B. salamandrivorans*. The monosaccharides instead of the amide derivatives were used in this experiment to exclude any chemotactic signaling activity of the amides. Carbohydrates were dissolved in distilled water, filter sterilized and tested at a 0.1 M concentration. Hematocrit capillaries (75 mm length; Hirschmann laborgeräte, Eberstadt, Germany) were filled with 60 µl carbohydrate solution, vehicle control capillaries with 60 µl sterile distilled water. To prevent leakage, the capillaries were sealed with wax plugs (Hirschmann laborgeräte, Eberstadt, Germany) at one side. Each capillary was swiped on the outside with lens paper (Kimtech Science, Kimberley Clark, Roswell, GA, USA) to remove possible attractant spillover. Capillaries were incubated in 400 µl inoculum containing 10^6 *B. salamandrivorans* zoospores in water and placed in a holder inclined about 65 degrees upwards. The assay was incubated for 90 minutes at 15 °C, after which the capillaries were removed and swiped again at

the outside to remove *B. salamandrivorans* zoospores possibly adhering on the outside. Inocula were checked for motility of the zoospores using an inverted microscope (Olympus CKX 41, Hamburg, Germany). Contents of the capillaries were collected and centrifuged for 2 minutes at 16.000 x g . The supernatant was removed as much as possible. The pellet was suspended in 100 µl Prepman Ultra Sample Preparation reagent (Applied Biosystems, Life Technologies Europe, Ghent, Belgium) and DNA was extracted according to the manufacturer's guidelines. For each sample, the number of *B. salamandrivorans* zoospores was quantified using a quantitative real-time PCR (qPCR) (Blooi *et al.*, 2013) and data was analysed using the Bio-Rad CFX manager 3.1. The primers and probe can be found in Supplementary Table 11. Within each assay, all carbohydrates and negative controls were tested at least in triplicate (technical replicates) and three independent repeats of the assay were performed (biological replicates).

Carbohydrate transcriptome test

RNA preparation: Total RNA was isolated from *B. salamandrivorans* zoospores treated with different carbohydrates. Therefore, newly released zoospores (less than 2 hours after induction of spore release by adding water) were harvested from 175 cm^2 cell culture flasks by replacing the TGhL broth with distilled water, which was filtered using a sterile mesh filter with pore size 10 µm (Pluristrainer, PluriSelect). Six-biological replicates containing 4 x 10^7 zoospores were obtained. Each biological replicate consisted of a pool a spores harvested from three cell culture flasks. Per biological replicate, the spores were divided in 4 eppendorfs (10^7 zoospores/eppendorf) which were treated for 1 hour at 15°C with H_2O (control), 50 mM (D-galactose), 50 mM (D-glucose) or 50 mM (D-mannose) (Supplementary Fig. 5). After 1 hour, the zoospores were centrifuged for 5 minutes at 4.000 x g at 15°C to remove the supernatant, after which RNA was extracted using the RNeasy mini kit (Qiagen) (Farrer *et al.*, 2017). The RNA was treated with Turbo™ DNase (Ambion), following the manufacturer's instructions. RNA degradation and contamination was monitored on 1% agarose gels. The RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). Finally, the RNA integrity and quantitation were assessed using the RNA Nano 6000 assay kit of the Bioanalyzer 2100 system (Agilent Technologie, CA, USA).

Library preparation for transcriptome sequencing: Whole-transcriptome sequencing libraries were constructed and sequenced on the Illumina HiSeq platform (Novogen, China). A total amount of 1 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3' ends of DNA fragments, NEBNext Adaptor with hairpin loop structure were ligated to prepare for hybridization. In order to select cDNA fragments of preferentially 150~200 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). Then 3 μl USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37 °C for 15 minutes followed by 5 minutes at 95 °C before PCR. Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. At last, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system.

Clustering and sequencing: The clustering of the index-coded samples was performed on a cBot Cluster Generation System using PE Cluster Kit cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina platform and paired-end reads were generated.

Quality analysis, mapping and assembly: Raw data (raw reads) of FASTQ format were first processed through fastp (version 0.20.0). In this step, clean data (clean reads) were obtained by removing reads containing adapter and poly-N sequences and reads with low quality from raw data. At the same time, Q20, Q30 and GC content of the clean data were calculated (Supplementary Table 12). All the downstream analyses were based on the clean data with high quality. Reference genome and gene model annotation files were downloaded from genome website browser (NCBI/UCSC/Ensembl) directly. Paired-end clean reads were mapped to the *B.*

salamandrivorans reference genome using HISAT2 (version 2.0.5) software (Farrer *et al.*, 2017). Featurecounts (version 1.5.0-p3) was used to count the read numbers mapped to each gene, including known and novel genes (Supplementary Table 13). And then RPKM (reads per kilobase per million) of each gene was calculated based on the length of the gene and reads count mapped to this gene.

Gene expression, differential expression, enrichment and coexpression- analysis: Differential expression analysis was performed using the DESeq2 R package (Anders and Huber, 2010). The resulting *P*-values were adjusted using the Benjamini and Hochberg's approach for controlling the False Discovery Rate (FDR). Genes with an adjusted *P*-value < 0.05 found by DESeq2 were assigned as differentially expressed. Protein domains were annotated with PFAM version 27 and 33 and KEGG domains, Gene Ontology (GO) enrichment analysis of differentially expressed genes was implemented by the clusterProfiler R package (Yu *et al.*, 2012) and dcGOR R package (Fang, 2014). GO terms with corrected *P*-value less than 0.05 were considered significantly enriched by differential expressed genes. ClusterProfiler R package (Yu *et al.*, 2012) was also used to test the statistical enrichment of differentially expressed genes in KEGG pathways.

Detection of protease activity

The influence of carboydrate exposure on protease activity of *B. salamandrivorans* zoospores was assessed. Therefore, zoospores were harvested from 175 cm^2 cell culture flasks by replacing the TGhL broth with distilled water, which was filtered using a sterile mesh filter with pore size 10 µm (Pluristrainer, PluriSelect). A pool containing approximately 5 x 10^7 zoospores/ ml was obtained. 200 µl of the spore suspension (10^7 spores) was added to eppendorfs containing 200 µl H_2O (H_2O; n = 3), 200 µl 100 mM D-Glucose (Glc; n = 3), 200 µl 100 mM D-mannose (Man; n = 3), 200 µl 100 mM D-galactose (Gal; n = 3), or as a control, 200 µl H_2O containing protease inhibitor mix (P8215, Sigma-Aldrich) (PI; n = 3). After 1.5 hours at 15°C, the zoospores were centrifuged for 5 minutes at 4.000 x g at 15°C and the supernatant was collected. Protease activity in the supernatant was analysed using the Pierce Fluorescent Protease Assay Kit (Thermo Fisher Scientific), according to the manufacturer's instructions. Three independent repeats of the experiment were performed (biological replicates).

Identification of *B. salamandrivorans* lectin genes

Potential candidates of carbohydrate binding molecules (CBMs) were identified in the *B. salamandrivorans* (AMFP) genome listed in the NCBI database (Bioproject PRJNA311566).

B. salamandrivorans (AMFP 13/01) coding regions from the single annotated genome present on NCBI database (Bioproject PRJNA311566) were used to single out potential lectin genes of interest that could serve as genes of carbohydrate binding proteins. The lectin candidates were identified with BLASTp (BLAST+2.9.0) over the FungiDB database (constituting 199 candidates, database accessed 1st March 2018) using the stringent e-value cutoff of 1e-50 to avoid spurious hits (Altschul *et al.*, 1997; Basenko *et al.*, 2018).

From these, five candidates that referred to lectins and carbohydrate binding were manually selected using the NCBI CDD (v3.16) conserved domain software with default settings (Marchler-Bauer *et al.*, 2017).

Expression of two of these genes (BSLG_00833 and BSLG_02674) was confirmed by a previous mRNA expression analysis (Bioproject PRJNA311566) (Farrer *et al.*, 2017).

Animals

The animal experiments were performed following the European law and with the approval of the ethical committee of the Faculty of Veterinary Medicine (Ghent University EC) (EC2015/86). Only captive bred animals were used. Fire salamander larvae belonging to different life stages (Steinfartz, Weitere and Tautz, 2007) were used in a *B. salamandrivorans* infection trial.

For lectin-histochemical staining, skin samples were collected from amphibian species *Salamandra salamandra* (*n* = 10), *Ichthyosaura alpestris* (*n* = 12), *Lissotriton helveticus* (*n* = 13), *Pleurodeles waltl* (*n* = 11), *Lissotriton boscai* (*n* = 3), *Alytes obstetricans* (*n* = 10), *Cynops pyrrhogaster* (*n* = 3), *Triturus anatolicus* (*n* = 3), *Triturus marmoratus* (*n* = 3), *Calotriton asper* (*n* = 10), *Bombina variegata* (*n* = 5), *Rana temporaria* (*n* = 10), *Epidalea calamita* (*n* = 5), *Pelobates fuscus* (*n* = 5) and *Salamandra lanzai* (*n* = 3). Tail or toe clips, ventral and dorsal skin samples were collected from animals that were euthanized with natrium pentobarbital 20% (KELA). The

collected samples were immediately fixed in Bouin's solution (5% acetic acid, 9% formaldehyde and 0.9% picric acid) for 24 hours.

Mucosome samples were collected by bathing animals in HPLC-grade water for 1 hour from 21 amphibian species (different animals as the ones used for the tissueclips), namely *Lissotriton helveticus* (*n* = 3), *Pleurodeles waltl* (*n* = 3), *Lissotriton boscai* (*n* = 3), *Triturus anatolicus* (*n* = 3), *Triturus marmoratus* (*n* = 3), *Cynops pyrrhogaster* (*n* = 3), *Ichthyosaura alpestris* (*n* = 3), *Salamandra salamandra* (*n* = 3), *Lyciasalamandera helverseni* (*n* = 3), *Speleomantes strinatii* (*n* = 2), *Paramesotriton hongkongensis* (*n* = 2), *Plethodon glutinosus* (*n* = 2), *Chioglossa lusitanica* (*n* = 3), *Pachyhynobius shangchengensis* (*n* = 3), *Calotriton asper* (*n* = 3), *Salamandra algira* (*n* = 3), *Salamandra lanzai* (*n* = 2), *Alytes obstetricans* (*n* = 3), *Bombina variegata* (*n* = 2), *Epidalea calamita* (*n* = 3) *and Pelobates fuscus* (*n* = 3).

Exposure of fire salamander larvae and metamorphs to *B. salamandrivorans*

Twenty-two early stage and 26 late stage larvae (Steinfartz, Weitere and Tautz, 2007; Sanchez *et al.*, 2018) were inoculated with 1.5 x 10^5 *B. salamandrivorans* spores per ml water during 24 hours. Ten days after the inoculation all the early stage and sixteen late stage larvae were euthanized. The two hind legs were analysed by qPCR to detect the *B. salamandrivorans* GE load. A tail clip was stained with fluorescein labeled RCA I (see below). Ten late stage larvae were further kept until five weeks after metamorphosis.

Six one week old fire salamander metamorphs were inoculated with 1 ml of water containing 1.5 x 10^5 spores for 24 hours. The animals were euthanized 10 days after inoculation. The two hind legs were analysed by qPCR to detect the *B. salamandrivorans* GE load. A tail clip was stained with fluorescein labeled RCA I (see below).

Lectin-histochemical staining

Fluorescein labeled RCA I (*Ricinus communis* agglutinin I) (Vector Laboratories) and Con A (Concanavalin A) has been used to detect the expression of galactose and mannose or glucose in the epidermis of amphibians (Zaccone *et al.*, 1999).

After 24 hours fixation in Bouin's medium (Sigma-Aldrich), samples were washed first with tap water until the water ran colorless, then washed for 24 hours in 70% ethanol saturated with lithium carbonate (Sigma-Aldrich) to remove picric acid. Tissues were then dehydrated in a graded ethanol series, cleared in xylene, embedded in paraffin, and sectioned in 4-6 µm slices. Before lectin staining, the sections were deparaffinized in xylene and hydrated in a series of ethyl alcohols. For better presenting the carbohydrate antigens, we performed antigen retrieval by submerging slides in citrate buffer (10 mM citric acid, pH 6.0) and heat treating in microwave (850 W for 3.5 minutes plus 450 W for 10 minutes). The slides were rinsed with PBS (0.01 M, pH 7.4) and immersed in 1% BSA (Sigma-Aldrich) for 15 minutes, to prevent non-specific lectin binding. Subsequently, the sections were incubated with either lectin RCA I (15 µg/ml) or lectin Con A (5 µg/ml) for 30 minutes. Lectins were diluted with lectin binding buffer (10 mM Hepes, 0.15 M NaCl, pH 7.5). As a negative control, lectin RCA I was mixed with 200 mM galactose and lectin Con A was mixed with 200 mM mannose + 200 mM glucose, before incubating with skin sections to inhibit lectin binding. For positive control, a slide of fire salamander ventral skin sample for RCA staining, and midewife toad ventral skin sample for Con A staining, was included in each experiment. The slides were then washed in PBS, and cell nuclei were stained with 10 µg/ml Hoechst 33342 Solution (Invitrogen). Coverslips were mounted with Prolong™ Gold Antifade Reagent (Invitrogen). Staining results were observed using a Leica fluorescence microscope under 10x magnification, with a 450-490 nm BP excitation filter for lectin staining and a 355-425 nm BP excitation filter for Hoechst staining. Staining pictures were taken using Leica Application Suite (LAS) X software. The lectin staining intensities were classified as intense (3), strong (2), weak (1) or negative (0) staining (Supplementary Fig. 6). Experimental positive and negative controls were defined as intense (3) and negative (0) stained, respectively, and other slides were then evaluated in comparison to the set parameters. Hoechst staining results were paired with corresponding lectin staining results, making it easier to discern the tissue structure from dark background. The fluorescent intensities were scored by three reviewers, respectively scoring the same dataset of pictures blinded three separate times, and the mean value was taken as final result.

Free galactose, mannose and total carbohydrates in amphibian mucosome

Mucus was collected from 21 amphibian species (see above). The animal body surface and volume of bathing water was calculated according as follows: surface area of anuran species in cm^2 = 9.9* (mass in g)^(0.56), surface area of urodelan species in cm^2 = 8.42* (mass in g)^(0.694), and the quantity of HPLC-grade water to add to both anuran and urodelan species was determined by dividing surface area by 4), and animals were bathed in respective amounts of HPLC-grade water for 1 hour (Spight, 1967; Woodhams *et al.*, 2014). Animal washes were collected and concentrated by SpeedVac Vacuum Concentrators (Thermo Fisher Scientific) to 100 µl. The quantities of free galactose, mannose and total carbohydrates in 100 µl of concentrated animal wash were measured using the Galactose Assay Kit (Abcam), Mannose ELISA Kit (Aviva Systems Biology) and Total Carbohydrates Assay Kit (Abcam), as per instructions. Concentrations of free galactose, mannose and total carbohydrates in animal washes were divided by animal body surface to get the final results of carbohydrate concentrations per square centimeter of body surface.

Statistical analysis

Statistical analyses of fire salamander skin lysate binding assay, carbohydrate binding assay, chemotaxis assay and protease activity assay were performed using R version 4.0.3. To account for the experimental design, Generalized Linear Mixed Models (GLMM, R library lme4 (Bates *et al.*, 2014)) were used, specifying a nested random effect whereby technical replicates are nested within biological replicates. Count data were modelled first using a Poisson distribution, but as significant overdispersion was present in the data, a negative binomial error structure was implemented. For the protease activity assay, data do not represent counts and a log transformation on the raw values was used to ensure normality of model residuals (Shapiro-Wilk $W > 0.95$) allowing a Gaussian error structure (i.e. a Linear Mixed Model (LMM). To test for differences between categories, the (G)LMMs were directly fed to the glht function of the R library multcomp (Hothorn, Bretz and Westfall, 2008), setting up contrasts for Tukey's all-pair comparisons, resulting in Bonferroni-corrected *p*-values adjusted for multiple testing. Statistical analyses of the larvae infection trial were performed in R version 4.0.0, with tidyverse (Wickham *et al.*, 2019) version 1.3.0, MASS (Venables and Ripley, 2002) version 7.3-51.6, VGAM (Yee, 2015) version 1.1-3, DHARMa (Hartig, 2020) version 0.3.1 and glmmTMB (Brooks *et al.*, 2017)

version 1.0.2.1. Infection loads of larvae and metamorphs were compared, using the Wilcoxon rank sum test, formula Chytrid GE load ~ larvae vs metamorph status, from the stats package. The correlation between larvae *Ricinus communis* agglutinin (RCA) scoring (1 = weak staining, 2 = strong staining, 3 = intense staining) and infection load was performed using the glm() function on log transformed genomic equivalents with formula $\log_{10}$(*B. salamandrivorans* load in Genomic equivalents)~ RCA score, treating RCA score as an ordered factor with guassian distribution. As non-transformed chytrid loads showed zero-inflation and overdispersion, we also fit a generalized linear model with negative binomial distribution (GE load ~ RCA score) with RCA score as an ordered factor, using glmmTMB with a zero-inflation model (~ RCA score), which showed a comparable positive correlation between RCA score and GE load (conditional model coefficient = 5.67, $p = 0.003$, zero-inflation model coefficient = -2.21, $p = 0.016$). Residuals and chi square test indicated the negative binomial model was not a significant improvement and so the simpler generalized linear model on transformed data was included. RCA scoring and larval stage prediction probabilities in Fig. 4c were generated by polr(RCA score ~ life stage) from MASS. Model fit and appropriateness was tested using Chisq test ($p = 0.003$), the model fit compared favorably to a more complex multinomial logit model and a model fit based on 70% of the data predicted 70-75% of remaining data (when data repeatedly sampled with different seeds, with the final model fit to all data).

The regression and correlation analyses of different amphibian species were perfomed in SPSS (IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY, USA). Correlations of RCA scores with *B. salamandrivorans* infection peak loads, mortality rates and percentage of free galactose were calculated by two-tailed Point-Biserial Correlation ($p < 0.05$ considered as significant), and the regression analyses were assessed by linear regression model with dummy variables. Dummy variables were generated from RCA scores, rounded to the nearest whole number. Correlations of percentages of free galactose with *B. salamandrivorans* infection peak loads and mortality rates were calculated using a two-tailed Pearson Correlation Coefficient test (correlation significant at $p < 0.05$), and regression analyses were assessed by quadratic regression model.

Data availability

All data reported in this study are provided in the Supplementary Information file and Supplementary Data files. Source data are provided with this paper. Carbohydrate binding genes were searched in the NCBI database, under Bioproject PRJNA311566. Reference genome and gene model annotation files used for RNA-seq annotation were downloaded from genome website browser NCBI (https://www.ncbi.nlm.nih.gov/), UCSC (http://genome.ucsc.edu/) and Ensembl (https://www.ensembl.org/index.html). RNA-seq data are available on the GEO website (http://www.ncbi.nlm.nih.gov/geo/) with Accession number GSE161129 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE161129).

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Author contributions

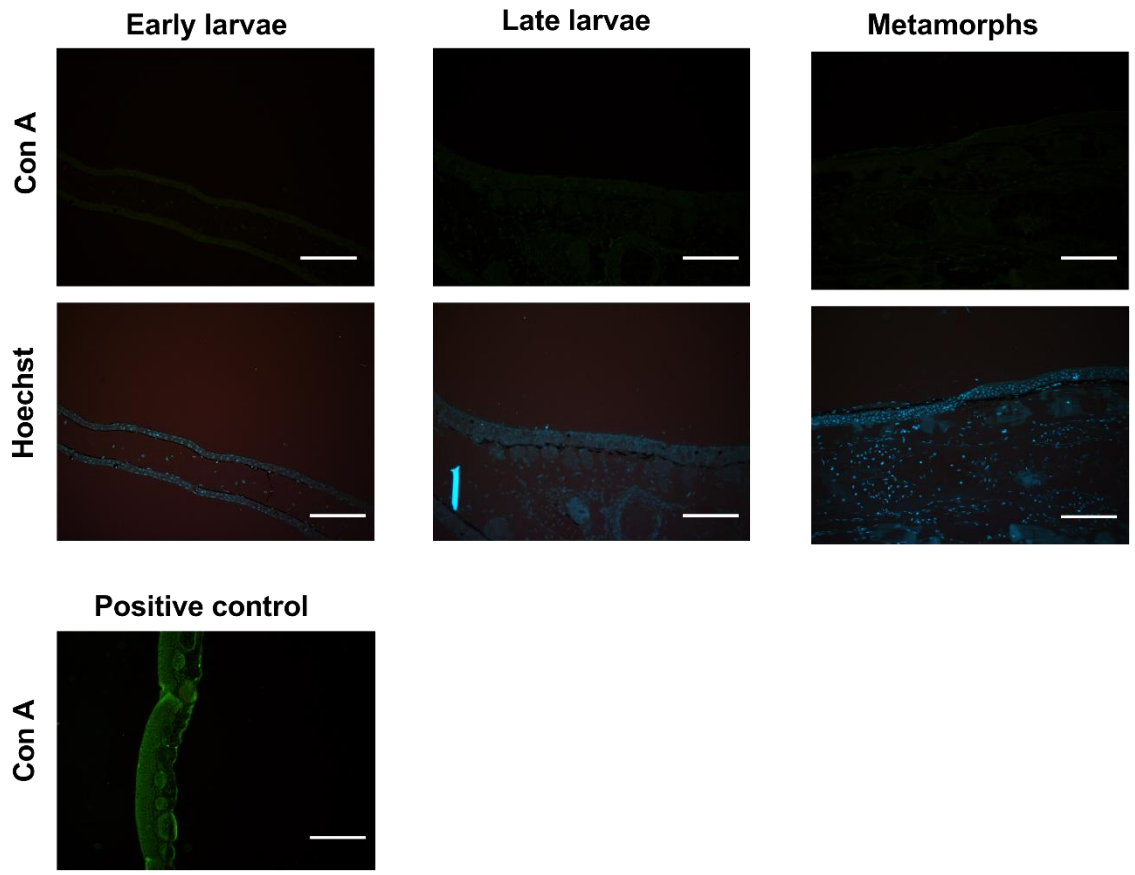
A.M., F.P., Y.W. and E.V. designed the study; Y.W., E.V. and A.M. conducted the experiments; M.K. delivered genetic data; Y.W., E.V., M.K., L.M. and D.S. performed statistical analysis and figure generation; K.C. and N.C. contributed key study material; Y.W., E.V., A.M. and F.P. prepared the original manuscript. All authors reviewed the manuscript.

Competing interests statement

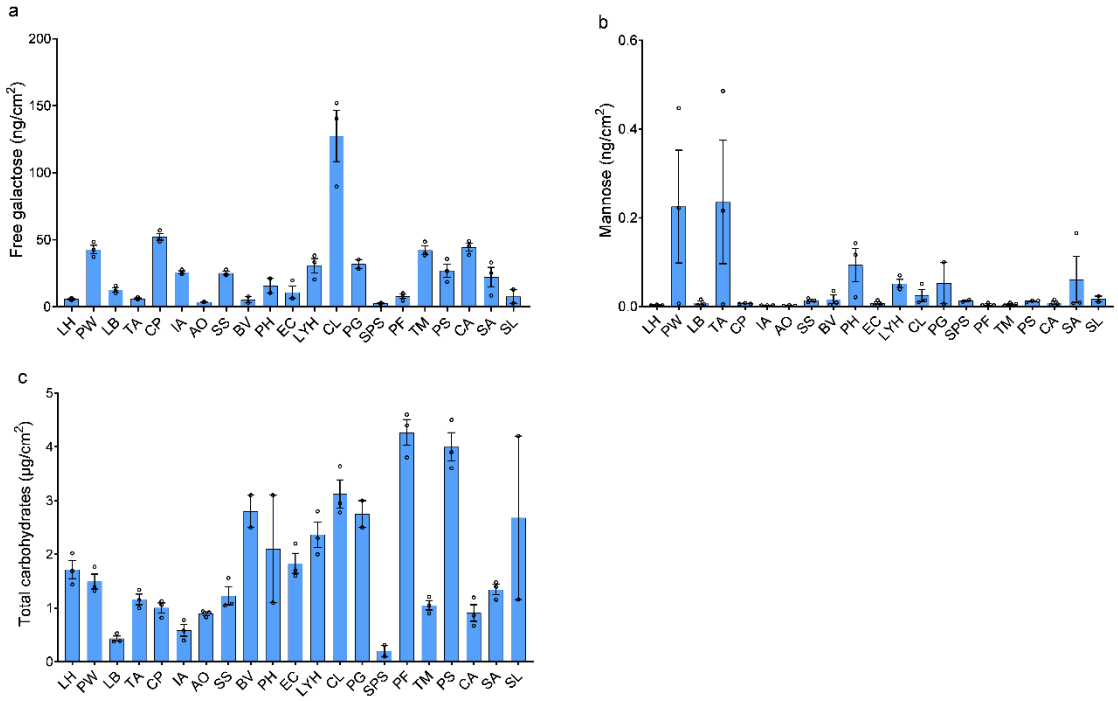
The authors declare no competing interests.

Supplementary information

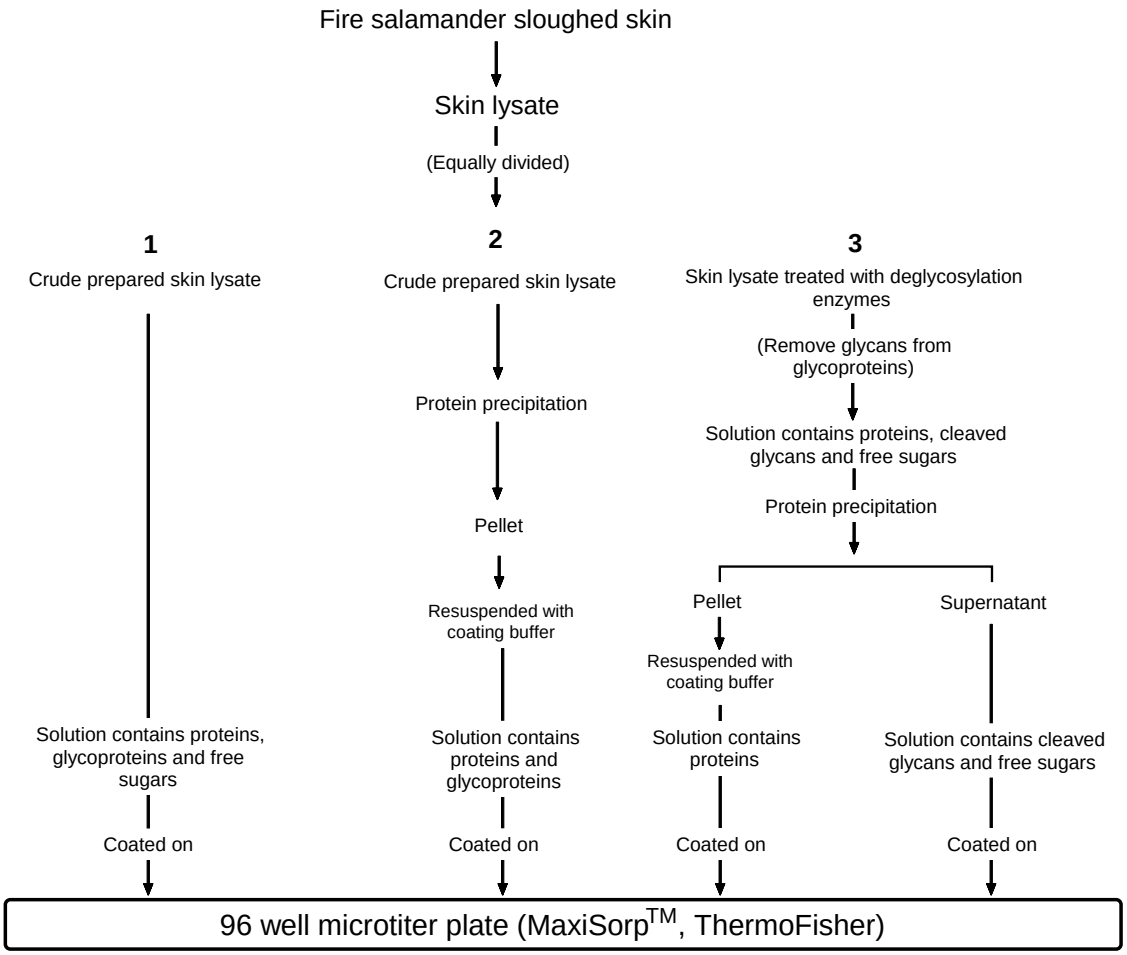
Figures



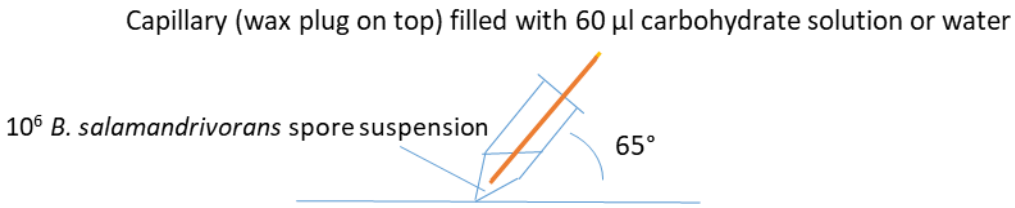
Supplementary Figure 1: Con A and Hoechst staining of different life stages of fire salamander larvae and metamorphs. Skin section of *Alytes obstetricans* was used as a Con A positive control. Scale bar = 100 µm.



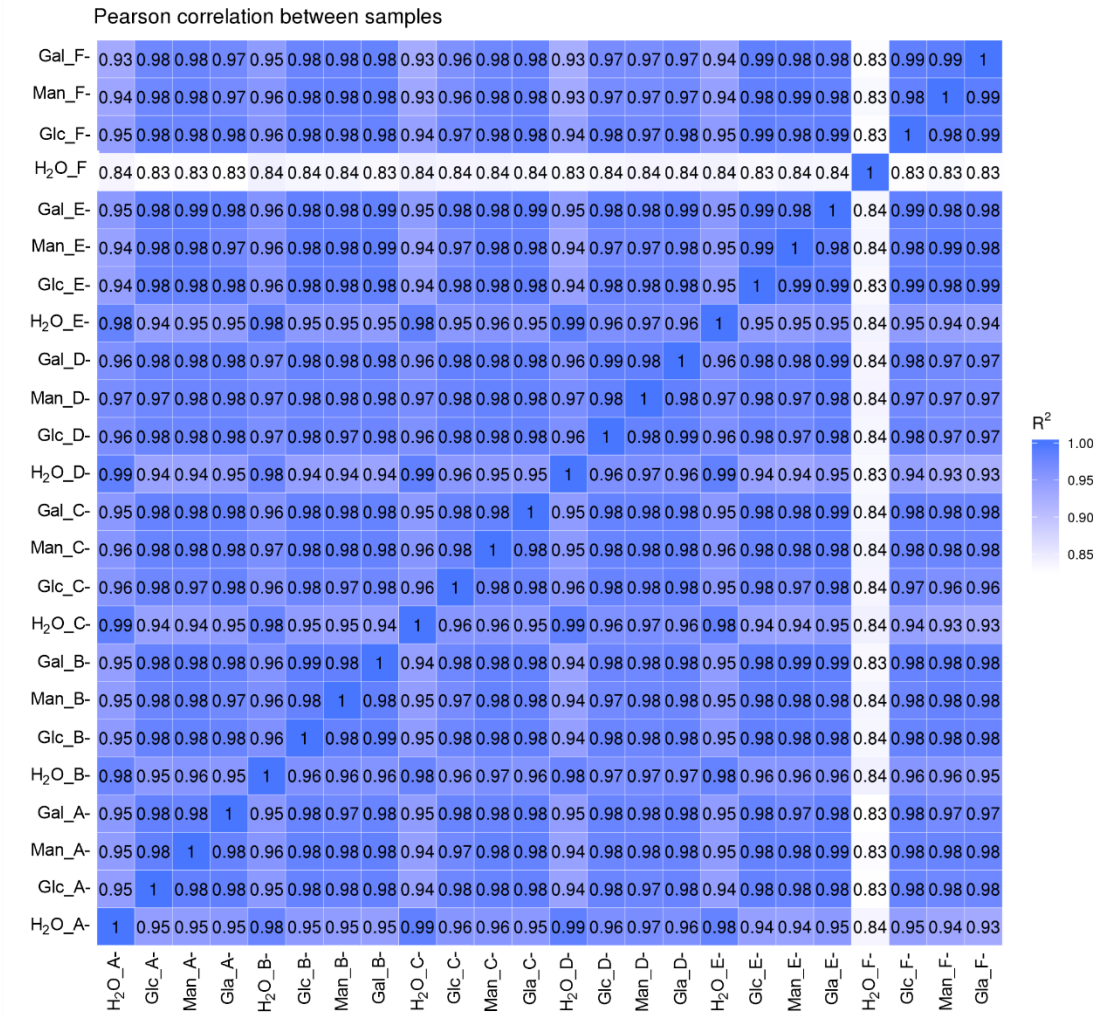
Supplementary Figure 2: Concentration of (a) free galactose and (b) mannose and (c) total carbohydrate per square centimeter of body surface in animal washes. Amphibian species: LH = *Lissotriton helveticus* (*n* = 3), PW = *Pleurodeles waltl* (*n* = 3), LB = *Lissotriton boscai* (*n* = 3), TA = *Triturus anatolicus* (*n* = 3), CP = *Cynops pyrrhogaster* (*n* = 3), IA = *Ichthyosaura alpestris* (*n* = 3), AO = *Alytes obstetricans* (*n* = 3), SS = *Salamandra salamandra* (*n* = 3), BV = *Bombina variegata* (*n* = 2), PH = *Paramesotriton hongkongensis* (*n* = 2), EC = *Epidalea calamita* (*n* = 3), LYH = *Lyciasalamandra helverseni* (*n* = 3), CL = *Chioglossa lusitanica* (*n* = 3), PG = *Plethodon glutinosus* (*n* = 2), SPS = *Speleomantes strinatii* (*n* = 2), PF = *Pelobates fuscus* (*n* = 3), TM = *Triturus marmoratus* (*n* = 3), PS = *Pachyhynobius shangchengensis* (*n* = 3). CA = *Calotriton asper* (*n* = 3), SA = *Salamandra algira* (*n* = 3) and SL = *Salamandra lanzai* (*n* = 2). Data are presented as mean ± SEM; dots represent values of individual animals. Source data are provided as a Source Data file.



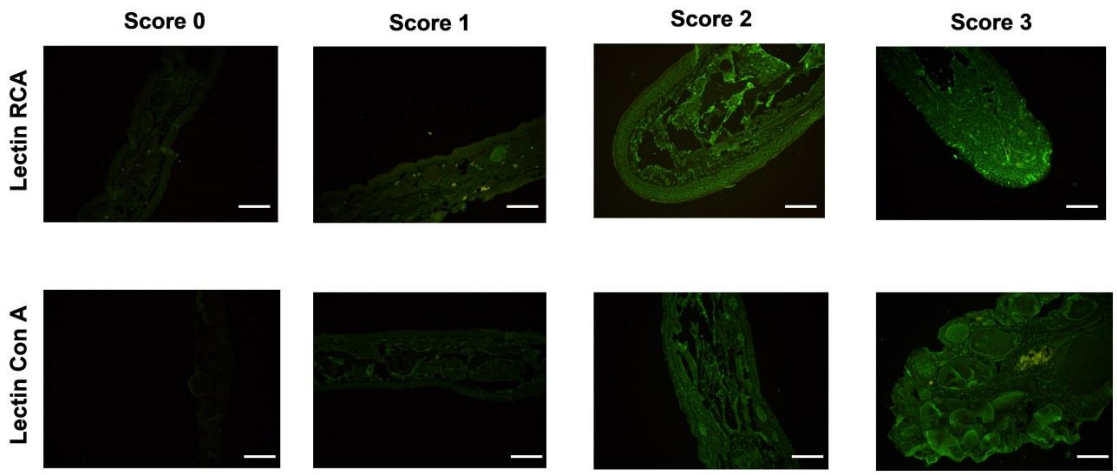
Supplementary Figure 3: Overview of skin lysate binding assay.



Supplementary Figure 4: Schematic overview of the chemotaxis assay.



Supplementary Figure 5: Pearson's correlation of transcriptome sequencing between different samples.



Supplementary Figure 6: Representative pictures for lectin staining evaluation scores. 0 = negative staining, 1 = weak staining, 2 = strong staining, 3 = intense staining. Scale bar = 100 µm.

Supplementary Tables

Supplementary Table 1: Descriptive statistics accompanying Fig. 1a. Number of *B. salamandrivorans* zoospores bound on microtiter plates coated with different treated skin lysate samples. N = three independent experiments. Mean, standard deviation, Standard Error of the mean, 95% Confidence Interval, minimum and maximum of three independent experiments are given. Negative control: wells coated with only coating buffer.

	N	Mean	Std. Deviation	Std. Error of Mean	95% CI for Mean		Minimum	Maximum
					Lower	Upper		
Crude skin lysate	3	2878.38	630.65	364.10	1311.77	4444.99	2163.40	3355.53
Pellet of deglycosylated skin lysate	3	658.89	148.26	85.60	290.60	1027.18	505.87	801.87
Supernatant of deglycosylated skin lysate	3	3950.60	786.81	454.26	1996.06	5905.14	3095.47	4643.93
Pellet of crude skin lysate	3	2751.69	922.44	532.57	460.23	5043.15	1707.60	3456.20
Ammonium sulfate + coating buffer	3	169.07	89.29	51.55	-52.73	390.86	87.20	264.27
Negative control	3	202.09	88.89	51.32	-18.72	422.90	125.87	299.73

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Supplementary Table 2: Multiple comparisons based on a (negative binomial) GLMM linking the number of zoospores attached to the different treatments (see Fig. 1a.) Results present Bonferroni-corrected multiple comparisons test, showing significance of differences in binding of *B. salamandrivorans* zoospores on different treated skin lysate samples. Adjusted *p*-value of $p < 0.05$ is considered as significant.

multiple comparisons test	estimate	se	z-value	*P*-Value
Crude skin lysate vs. Pellet of deglycosylated skin lysate	-1.48	0.03	-43.31	<0.001
Crude skin lysate vs. Supernatant of deglycosylated skin lysate	0.32	0.03	9.54	<0.001
Crude skin lysate vs. Pellet of crude skin lysate	-0.07	0.03	-2.13	0.271
Crude skin lysate vs. Ammonium sulfate + coating buffer	2.89	0.04	81.05	<0.001
Crude skin lysate vs. Negative control	-2.70	0.04	-76.32	<0.001
Pellet of deglycosylated skin lysate vs. Supernatant of deglycosylated skin lysate	1.80	0.03	52.78	<0.001
Pellet of deglycosylated skin lysate vs. Pellet of crude skin lysate	0.39	0.03	11.66	<0.001
Pellet of deglycosylated skin lysate vs. Ammonium sulfate + coating buffer	1.42	0.04	39.26	<0.001
Pellet of deglycosylated skin lysate vs. Negative control	1.22	0.04	34.16	<0.001
Supernatant of deglycosylated skin lysate vs. Pellet of crude skin lysate	-1.40	0.03	-41.18	<0.001
Supernatant of deglycosylated skin lysate vs. Ammonium sulfate + coating buffer	3.21	0.04	90.10	<0.001
Supernatant of deglycosylated skin lysate vs. Negative control	3.02	0.04	85.48	<0.001
Pellet of crude skin lysate vs. Ammonium sulfate + coating buffer	2.82	0.04	79.15	<0.001
Pellet of crude skin lysate vs. Negative control	2.62	0.04	74.34	<0.001
Ammonium sulfate + coating buffer vs. Negative control	0.20	0.04	5.29	<0.001

Supplementary Table 3: Descriptive statistics accompanying Fig. 1b. *B. salamandrivorans* zoospore binding to different oligocarbohydrates. N = three independent experiments. Mean, standard deviation, Standard Error of the mean, 95% Confidence Interval, minimum and maximum of three independent experiments are given. Binding normalized to the negative control. GlcNAc = N-Acetylglucosamine, GalNAc = N-Acetylgalactosamine, negative control = wells coated with only coating buffer.

	N	Mean	Std. Deviation	Std. Error of Mean	95% CI for Mean		Minimum	Maximum
					Lower	Upper		
GlcNAc	3	208.31	141.24	81.54	-142.54	559.16	86.93	363.33
GalNAc	3	1129.67	441.06	254.65	34.01	2225.33	722.67	1622.73
Mannose	3	1646.87	812.57	469.14	-371.67	3665.41	770.47	2375.27
Lactose	3	1916.98	763.96	441.07	19.20	3814.75	1074.13	2563.87
Negative control	3	182.73	150.19	86.71	-190.36	555.83	66.80	352.40

Supplementary Table 4: Multiple comparisons based on a (negative binomial) GLMM linking the number of zoospores attached to different oligocarbohydrates (see Fig. 1b.) Results present Bonferroni-corrected multiple comparisons test, showing significance of differences in binding of *B. salamandrivorans* zoospores on different oligocarbohydrates. Adjusted *p*-value of $p < 0.05$ is considered as significant.

multiple comparisons test	**estimate**	**se**	**z-value**	***P*-Value**
GluNAc - GalNAc	-1.81	0.067	-26.94	<0.001
Lactose - GalNAc	0.51	0.066	7.65	<0.001
Mannose - GalNAc	0.31	0.066	4.72	<0.001
Negative control - GalNAc	-1.99	0.068	-29.37	<0.001
Lactose - GluNAc	2.32	0.067	34.55	<0.001
Mannose - GluNAc	2.13	0.067	31.70	<0.001
Negative control - GluNAc	-0.18	0.068	-2.60	0.071
Mannose - Lactose	-0.19	0.066	-2.93	0.028
Negative control - Lactose	-2.50	0.068	-36.94	<0.001
Negative control - Mannose	-2.30	0.068	-34.12	<0.001

Supplementary Table 5: Multiple comparisons based on a (negative binomial) GLMM assessing chemotaxis of *B. salamandrivorans* toward free carbohydrates (see Fig. 1c). Results present Bonferroni-corrected multiple comparisons test, showing significance of differences in chemotaxis among different carbohydrats and a water control attractant. Adjusted *p*-value of $p < 0.05$ is considered as significant.

multiple comparisons test	estimate	se	z-value	*P*-Value
Glucose - Galactose	-1.67	0.270	-6.174	< 0.001
H20 control - Galactose	-3.57	0.265	-13.462	< 0.001
Lactose - Galactose	-0.73	0.265	-2.751	0.0466
Mannose - Galactose	-1.23	0.266	-4.614	< 0.001
H20 control - Glucose	-1.90	0.286	-6.652	< 0.001
Lactose - Glucose	0.94	0.278	3.375	0.00659
Mannose - Glucose	0.44	0.280	1.560	0.523
Lactose - H20 control	2.84	0.283	10.036	< 0.001
Mannose - H20 control	2.34	0.282	8.308	< 0.001
Mannose - Lactose	-0.50	0.277	-1.802	0.372

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Supplementary Table 6: Lectin candidates selected over PFAM annotation pipeline and protein blasting against the FungalDB database. Conserved domains and e-values are listed.

Gene	Domain	e-value
BSLG_00643	Lectin legume-like	1.08E-77
BSLG_00833	Ricin B lectin	6.73E-09
BSLG_03242	Ricin B lectin	1.33E-14
BSLG_05191	Lectin legume-like	6.55E-39
BSLG_02674	Laminin G3 concanavalin A-like lectin	1.34E-05

Supplementary Table 7: Multiple comparisons based on a Linear Mixed Model assessing protease activity detected in supernatants of *B. salamandrivorans* zoospores (see Fig. 3). Results present Bonferroni-corrected multiple comparisons test, showing significance of differences in protease activity. Adjusted *p*-value of $p < 0.05$ is considered as significant.

multiple comparisons test	estimate	se	z-value	*P*-Value
Glucose - Galactose	-0.02	0.08	-0.31	0.998
H20 - Galactose	-0.23	0.08	-2.85	0.036
Mannose - Galactose	-0.27	0.08	-3.33	0.008
Protease Inhibitor - Galactose	-1.55	0.08	-19.14	< 0.001
H20 - Glucose	-0.21	0.08	-2.54	0.082
Mannose - Glucose	-0.24	0.08	-3.02	0.021
Protease Inhibitor - Glucose	-1.52	0.08	-18.83	< 0.001
Mannose - H20	-0.04	0.08	-0.48	0.989
Protease Inhibitor - H20	-1.32	0.08	-16.29	< 0.001
Protease Inhibitor - Mannose	-1.28	0.08	-15.81	< 0.001

Supplementary Table 8: Lectin RCA staining patterns in epidermis of ventral skin, dorsal skin, toeclips and tailclips of fire salamanders, alpine newts and palmate newts. Three individuals for each species. SEM = Standard Error of the Mean. Staining scores: 0 = negative; 1 = weak; 2 = strong; 3 = intense.

	Fire salamander				**Alpine newt**				**Palmate newt**			
	Animal 1	Animal 2	Animal 3	Mean	Animal 1	Animal 2	Animal 3	Mean	Animal 1	Animal 2	Animal 3	Mean
	Score (SEM)	Score (SEM)	Score (SEM)	Score (SEM)	Score (SEM)	Score (SEM)	Score (SEM)	Score (SEM)	Score (SEM)	Score (SEM)	Score (SEM)	Score (SEM)
Ventral skin	3.0 (0.0)	3.0 (0.0)	3.0 (0)	3.0 (0.0)	2.2 (0.1)	2.9 (0.1)	3.0 (0.0)	2.7 (0.3)	0.9 (0.1)	0.3 (0.2)	0.4 (0.1)	0.5 (0.2)
Dorsal skin	3.0 (0.0)	3.0 (0.0)	3.0 (0)	3.0 (0.0)	1.9 (0.1)	2.9 (0.1)	3.0 (0.0)	2.6 (0.3)	0.9 (0.1)	0.4 (0.1)	0.0 (0.0)	0.4 (0.3)
Toeclips	3.0 (0.0)	1.9 (0.1)	2.0 (0)	2.3 (0.3)	3.0 (0.0)	3.0 (0.0)	2.8 (0.1)	2.9 (0.1)	0.9 (0.1)	0.7 (0.2)	0.0 (0.0)	0.5 (0.3)
Tailclips	3.0 (0.0)	3.0 (0.0)	3.0 (0)	3.0 (0.0)	2.4 (0.1)	3.0 (0.0)	3.0 (0.0)	2.8 (0.3)	0.9 (0.1)	0.2 (0.1)	0.4 (0.1)	0.5 (0.3)

Supplementary Table 9: Summary of infection trials, lectin histochemical studies and carbohydrate measurements from animal washes. Order of species, *B. salamandrivorans* susceptibility, infection peak loads, mortality rates, lectin staining scores and percentage of free galactose and mannose are listed. SEM = Standard Error of the Mean. Staining scores: 0 = negative; 1 = weak; 2 = strong; 3 = intense. NA = data not available.

Species		***B. salamandrivorans* susceptibility**	**Infection peak loads (log GE load +1) (SEM)**	**Mortality rates (%)**	**References**	**RCA lectin staining**	**Con A lectin staining**	**Percentage of Free galactose**	**Percentage of mannose**
Name	Order					Score (SEM)	Score (SEM)		
Salamandra salamandra	Urodela	Susceptible	3.08 (0.2)	100.00	(Blooi *et al.*, 2013; Martel *et al.*, 2014, 2020; Stegen *et al.*, 2017)	3.0 (0.0)	1.2 (0.2)	2.10	0.0011
Ichthyosaura alpestris	Urodela	Susceptible	2.90 (0.2)	75.00	(Martel *et al.*, 2014; Stegen *et al.*, 2017)	2.7 (0.1)	1.7 (0.1)	4.75	0.0005
Lissotriton helveticus	Urodela	Resistant	0.32 (0.2)	0.00	(Martel *et al.*, 2014)	0.6 (0.1)	2.5 (0.4)	0.34	0.0002
Pleurodeles waltl	Urodela	Susceptible	3.05 (0.2)	61.50	(Martel *et al.*, 2014, 2020)	2.1 (0.1)	2.7 (0.3)	2.95	0.0167
Lissotriton boscai	Urodela	Susceptible	4.50 (0.4)	16.67	(Bosch *et al.,* 2021)	2.4 (0.2)	2.4 (0.2)	2.93	0.0020
Alytes obstetricans	Anura	Resistant	0.44 (0.2)	0.00	(Martel *et al.*, 2014; Stegen *et al.*, 2017)	0.4 (0.2)	2.7 (0.3)	0.41	0.0003
Cynops pyrrhogaster	Urodela	Susceptible	3.08 (0.2)	50.00	(Martel *et al.*, 2014)	2.0 (0.6)	1.4 (0.4)	5.36	0.0007
Triturus anatolicus	Urodela	Resistant	1.44 (0.8)	0.00	(Martel *et al.*, 2020)	0.6 (0.4)	0.8 (0.3)	0.54	0.0196
Triturus marmoratus	Urodela	Susceptible	3.75 (0.4)	100.00	(Martel *et al.*, 2020)	1.9 (0.3)	2.2 (0.2)	4.12	0.0006
Calotriton asper	Urodela	Resistant	1.52 (0.9)	0.00	(Bosch *et al.,* 2021)	3.0 (0.0)	1.9 (0.6)	5.06	0.0009
Salamandra lanzai	Urodela	Not yet determined	NA	NA	NA	3.0 (0.0)	NA	0.59	0.0011
Rana temporaria	Anura	Resistant	0.00 (0.0)	0.00	(Martel *et al.*, 2014)	0.3 (0.1)	NA	NA	NA
Bombina	Anura	Tolerant	0.33 (0.3)	0.00	(Martel *et al.*,	0.4 (0.1)	NA	0.19	0.0007

variegata					2014)				
Epidalea calamita	Anura	Resistant	0.54 (0.3)	0.00	(Martel *et al.*, 2014)	0.7 (0.1)	NA	0.62	0.0004
Pelobates fuscus	Anura	Resistant	0.00 (0.0)	0.00	(Martel *et al.*, 2014)	0.6 (0.2)	NA	0.19	0.0001
Plethodon glutinosus	Urodela	Resistant	0.00 (0.0)	0.00	(Martel *et al.*, 2014)	NA	NA	1.18	0.0021
Pachyhynobius shangchengensis	Urodela	Tolerant	0.00 (0.0)	0.00	(Martel *et al.*, 2014)	NA	NA	0.67	0.0003
Salamandra algira	Urodela	Not yet determined	NA	NA	NA	NA	NA	1.60	0.0044
Lyciasalamander a helverseni	Urodela	Susceptible	4.32 (0.2)	100.00	(Bosch *et al.*, 2021)	NA	NA	1.29	0.0023
Chioglossa lusitanica	Urodela	Susceptible	3.37 (0.2)	100.00	(Bosch *et al.*, 2021)	NA	NA	4.22	0.0008
Speleomantes strinatii	Urodela	Susceptible	3.49 (0.1)	100.00	(Martel *et al.*, 2014)	NA	NA	2.55	0.0125
Paramesotriton honkongensis	Urodela	Reservoir	NA	NA	(Yuan *et al.*, 2018)	NA	NA	1.13	0.0068

Supplementary Table 10: Regression results of RCA scores with *B. salamandrivorans* infection peak loads, mortality rates and percentage of free galactose, using RCA score 0 as the reference group.

Independent variables	Regression Coefficients (β)	t value	*p*-value	95% Confidence interval		R^2	Adjusted R^2
				Lower Bound	Upper Bound		
Dependent variable: infection log (GE load + 1)							
RCA=0 (Constant)	0.257	0.712	0.493	-0.547	1.060	0.872	0.833
RCA=1	0.318	0.667	0.520	-0.745	1.381		
RCA=2	3.338	6.998	0.000	2.275	4.401		
RCA=3	1.973	3.869	0.003	0.837	3.110		
Dependent variable: mortality rates							
RCA=0 (Constant)	5.80E-15	0.000	1.000	-38.510	38.510	0.564	0.434
RCA=1	-7.85E-15	0.000	1.000	-50.944	50.944		
RCA=2	57.043	2.495	0.032	6.099	107.986		
RCA=3	58.333	2.387	0.038	3.872	112.794		
Dependent variable: percentage of free galactose							
RCA=0 (Constant)	0.300	0.316	0.758	-1.814	2.414	0.654	0.550
RCA=1	0.122	0.105	0.918	-2.467	2.712		
RCA=2	3.540	3.046	0.012	0.951	6.129		
RCA=3	2.825	2.431	0.035	0.236	5.414		

Supplementary Table 11: Sequences of the primers and probe used in quantitative real-time PCR (qPCR) for quantifying the number of *B. salamandrivorans* zoospores.

Forward primer (STerF)	5´-TGCTCCATCTCCCCCTCTTCA-3´
Reverse primer (STerR)	5´-TGAACGCACATTGCACTCTAC-3´
Cy5-Probe (SterC)	5´-/Cy5/ACAAGAAAATACTATTGATTCTCAAAC AGG CA/IAbRQSp/-3´
Reference	(Blooi *et al.*, 2013)

Supplementary Table 12: Data quality summary of sequencing results. Glc = glucose; Gal = galactose; Man = mannose.

Sample name	Raw reads	Clean reads	Raw bases	Clean bases	Error rate (%)	Q20 (%)	Q30 (%)	GC content (%)
Glc_B	13345700	12962299	4.0	3.9	0.03	97.83	94.11	48.10
Glc_C	12005909	11740794	3.6	3.5	0.03	97.82	94.05	48.12
Glc_A	12146267	11844741	3.6	3.6	0.02	97.93	94.34	47.94
Glc_F	16948742	16603588	5.1	5.0	0.03	97.46	93.22	48.11
Glc_D	12223887	11955721	3.7	3.6	0.03	97.90	94.18	47.94
Glc_E	17762535	17353477	5.3	5.2	0.03	97.41	93.15	48.31
H_2O_A	14696959	14311212	4.4	4.3	0.02	97.93	94.33	48.05
H_2O_C	14443191	14061285	4.3	4.2	0.03	97.61	93.57	48.24
H_2O_B	11632267	11398588	3.5	3.4	0.03	97.66	93.73	48.12
H_2O_E	14792764	14475716	4.4	4.3	0.03	97.61	93.58	47.97
H_2O_D	16341281	15939571	4.9	4.8	0.03	97.86	94.18	48.13
H_2O_F	15907874	15378637	4.8	4.6	0.03	96.89	92.32	48.61
Gal_B	14343277	14023042	4.3	4.2	0.03	97.82	94.13	48.11
Gal_C	13767498	13304744	4.1	4.0	0.03	97.86	94.10	48.13
Gal_A	13574960	13335481	4.1	4.0	0.03	97.80	94.05	48.02
Gal_F	19623272	19208633	5.9	5.8	0.03	97.63	93.60	48.10
Gal_D	14462516	14218086	4.3	4.3	0.03	97.83	94.08	48.07
Gal_E	18458636	18042658	5.5	5.4	0.03	97.42	93.18	48.30
Man_D	11317621	11056172	3.4	3.3	0.03	97.80	94.01	48.08
Man_E	16480735	16087958	4.9	4.8	0.03	97.39	93.04	48.35
Man_F	17060602	16695412	5.1	5.0	0.03	97.67	93.64	48.25
Man_A	13005267	12741066	3.9	3.8	0.03	97.85	94.13	48.04
Man_B	11788470	11533701	3.5	3.5	0.03	97.48	93.30	48.09
Man_C	13274399	12917376	4.0	3.9	0.02	97.90	94.29	48.15

Supplementary Table 13: Summary of mapping results. Glc = glucose; Gal = galactose; Man = mannose.

Sample name	Total reads	Total mapped reads	Uniquely mapped reads	Multiple mapped reads	Total mapping rate	Uniquely mapping rate	Multiple mapping rate
Glc_B	25924598	21216351	20794533	421818	81.84%	80.21%	1.63%
Glc_C	23481588	19022142	18642392	379750	81.01%	79.39%	1.62%
Glc_A	23689482	19367025	19005977	361048	81.75%	80.23%	1.52%
Glc_F	33207176	28136919	27571043	565876	84.73%	83.03%	1.70%
Glc_D	23911442	19448680	19067074	381606	81.34%	79.74%	1.60%
Glc_E	34706954	29894540	29325493	569047	86.13%	84.49%	1.64%
H_2O_A	28622424	23420602	22948075	472527	81.83%	80.18%	1.65%
H_2O_C	28122570	22791750	22316057	475693	81.04%	79.35%	1.69%
H_2O_B	22797176	18634376	18297507	336869	81.74%	80.26%	1.48%
H_2O_E	28951432	23161442	22721885	439557	80.00%	78.48%	1.52%
H_2O_D	31879142	26315059	25776268	538791	82.55%	80.86%	1.69%
H_2O_F	30757274	23376903	23030764	346139	76.00%	74.88%	1.13%
Gal_B	28046084	23184760	22732566	452194	82.67%	81.05%	1.61%
Gal_C	26609488	21837846	21408651	429195	82.07%	80.45%	1.61%
Gal_A	26670962	22117078	21710534	406544	82.93%	81.40%	1.52%
Gal_F	38417266	32990195	32336144	654051	85.87%	84.17%	1.70%
Gal_D	28436172	23530609	23093569	437040	82.75%	81.21%	1.54%
Gal_E	36085316	30505584	29936126	569458	84.54%	82.96%	1.58%
Man_D	22112344	18201476	17883228	318248	82.31%	80.87%	1.44%
Man_E	32175916	27507455	26972151	535304	85.49%	83.83%	1.66%
Man_F	33390824	28675319	28105689	569630	85.88%	84.17%	1.71%
Man_A	25482132	21089309	20701958	387351	82.76%	81.24%	1.52%
Man_B	23067402	18789259	18445798	343461	81.45%	79.96%	1.49%
Man_C	25834752	20818999	20422249	396750	80.59%	79.05%	1.54%

Supplementary Table 14: The coefficient of variations for RCA staining between different individuals of species *S. Salamandra, I. alpestris* and *P. waltl*. Mean RCA score of each animal is provided.

S.salamandra		
	Mean RCA score	The coefficient of variation (%)
Animal 1	2.9	0.00
Animal 2	3.0	
Animal 3	3.0	
Animal 4	3.0	
Animal 5	3.0	
Animal 6	3.0	
Animal 7	3.0	
Animal 8	3.0	
Animal 9	3.0	
Animal 10	3.0	
I. alpestris		
	Mean RCA score	The coefficient of variation (%)
Animal 1	1.9	13.74
Animal 2	2.8	
Animal 3	3.0	
Animal 4	2.4	
Animal 5	3.0	
Animal 6	3.0	
Animal 7	2.8	
Animal 8	3.0	
Animal 9	2.8	
Animal 10	3.0	
Animal 11	2.7	
Animal 12	2.7	
P. waltl		
	Mean RCA score	The coefficient of variation (%)
Animal 1	1.7	18.56
Animal 2	1.7	
Animal 3	3.0	
Animal 4	1.8	
Animal 5	2.1	
Animal 6	1.6	
Animal 7	2.0	
Animal 8	2.4	
Animal 9	1.9	
Animal 10	2.2	
Animal 11	2.6	

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GENERAL DISCUSSION

Amphibian population declines have been observed for decades, with a plethora of species having already gone extinct, and nearly one third of all amphibian species now being classified as endangered (Wake and Vredenburg, 2008; Alroy, 2015). Chytridiomycosis is one of the major drivers of this mass extinction, and one of the most significant and urgent threats to amphibian diversity (Farrer *et al.*, 2011; Martel *et al.*, 2014; Alroy, 2015; Scheele, Pasmans, Skerratt, Berger, Martel, Beukema, Acevedo, Burrowes, Carvalho, Catenazzi, Riva, *et al.*, 2019). The chytrid fungus *Bsal* has been known to be responsible for significant salamander population declines in Europe (Martel *et al.*, 2014; Spitzen-van der Sluijs *et al.*, 2016), and appropriate mitigation methods will be necessary to control further spread and subsequently limit the adverse impact of *Bsal* on amphibian diversity. Here, we will discuss current amphibian conservation methods. These will be set in context with findings from dietary intake and the gut microbiome of fire salamanders, as well as findings of the initial host-pathogen interactions in *Bsal* pathogenesis. These novel findings could provide tools, that will potentially aid in improving amphibian conservation in the future.

1. The limitation of Chapter 2

In Chapter 2, we found that gut microbiome composition in fire salamanders is different, depending on the forest. However, another, more detailed study might be necessary to cement our findings, as our study included only three forests. The forest HG is a private forests, where contamination could affect forest biodiversity, which could subsequently have an impact on the salamander gut microbiome. Forest M is further away from the other two forest, and biodiversity could be altered. Therefore, it would be prudent to repeat this study with a higher number of forest habitats sampled, and check the biodiversity and potential contamination beforehand, in order to exclude other potential factors affecting fire salamander gut microbiome. This would lend credence to our findings of diet and gut microbiome difference between locations and sexes, as well as increase the statistical significance.

2. Conservation methods for saving fire salamanders in Belgium

In Chapter 1 we have summarised potential *Bsal* mitigation methods. However, so far, no long-term, sustainable and effective method has been introduced for mitigating *Bsal*. Therefore,

tailoring appropriate conservation methods to fire salamander populations could save the population from extinction. For fire salamander populations näive to *Bsal* it would be sensible to set up active disease surveillance programs, and actively screen for *Bsal* emergence once or twice a month, by checking skin health of individuals for skin lesions, as well as taking skin swaps for PCR. This early warning system would certainly increase the chances of fire salamander population survival, if, at emergence of *Bsal* immediate action is taken, healthy animals are separated and infected animals are treated. This, however, would require sufficient funding and human resources. Forests that already have an active *Bsal* outbreak, should prohibit unauthorized or public visits, in order to decrease the spread of *Bsal*. Furthermore, affected forests should be separated as best as possible from healthy habitats, possibly by plastic quarantine cordons, no more than one meter in height. This would prevent the species which function as *Bsal* reservoir to aid in the spread of the outbreak.

3. Effects of breeding season on fire salamander diet and gut microbiome

Fire salamander diet has also been studied in spring (Ferenti, David and Nagy, 2010; Lezău *et al.*, 2010) and winter (Balogová *et al.*, 2015). Therein Gastropoda was found to be the most important prey in those studies, with the diet composition of fire salamander in other seasons also being similar with the results in Chapter 2. Therefore, the breeding season (autumn) of fire salamander show no effects on the diet composition. We are the first study to profile the gut microbiome of fire salamander. Therefore, we have no previous data for comparison. However, there is a previous study on gut microbiome of fire salamander larvae (Bletz *et al.*, 2016), this study was conducted during spring, where they found Proteobacteria, Firmicutes and Bacteroidetes to be the most abundant phyla. This is in line with our findings. Therefore, we assume that gut microbiome will not be significantly different in composition in different seasons. However, to know it for sure, more studies of fire salamander gut microbiome need to be conducted in other seasons. Understanding the diet and gut microbiome throughout the year would be a benefit for conservation strategies, so we have an idea what the health composition of diet and gut microbiome should be. It would make it easier to identify animals with abnormal diet or gut microbiome as potentially unhealthy animals.

4. The role of diet in captive breeding conservation

As described in Chapter 1, captive breeding and reintroduction are important ex-situ conservation methods for preventing endangered amphibian population extirpation. Numerous studies on mammals (Linklater *et al.*, 2010), birds (Dufty Jr and Wingfield, 1986; Lombardo and Thorpe, 2009), reptiles (Moore, Thompson and Marler, 1991; Morales and Sánchez, 1996; Aldridge and Arackal, 2005) and amphibians (Zerani *et al.*, 1991; Gobbetti and Zerani, 1996; Titon *et al.*, 2018) have reported a decreased reproduction rate for animals in captivity. This negative impact does not only affect first-generation animals, but also subsequent generations. In species-comprehensive studies it has been found that captive-born animals have a 42% decreased likelihood of reproductive success in captivity, compared to wild-born counterparts (Farquharson, Hogg and Grueber, 2018), and the offspring survival rate changes over generations of captive breeding (Farquharson, Hogg and Grueber, 2021). This also goes for amphibians in captivity, where it has been reported that females frequently fail to produce and retain mature eggs and males, in turn, often fail to elicit breeding behaviour or fail to produce sperm (Browne *et al.*, 2006; Kouba, Vance and Willis, 2009; Bronson *et al.*, 2021). Nutrition has been previously reported to be a modifying factor for reproduction in amphibians (Ferrie *et al.*, 2014). Especially for female amphibians, inadequate nutrition has been found to decrease ovarian function and egg development, which could result in decreasing of fecundity and offspring survival rates (Alexander and Bellerby, 1935; Girish and Saidapur, 2000; Wright and Whitaker, 2001; Dziminski, Vercoe and Roberts, 2009). Therefore, during breeding season, adjusting diet composition to the needs of female amphibians in captivity may lead to the improvement of reproduction rates. However, this has so far not been tried. In chapter 4 we report for the first time dietary differences between sexes of amphibians in the wild, which indicates that modifying the diet according to sex-dependent needs could have a beneficial effect. We show that during breeding seasons, male fire salamanders consume a diverse spectrum of prey species, with Gastropoda and Chilopoda are the top two prey taxa, making up to 58.3% of the total prey. In contrast, female fire salamanders consume mostly slow-moving prey, with Gastropoda and Diplopoda making up to 90% of the total prey. Therefore, we assume that providing a higher quantity of slow-moving prey for female fire salamanders during breeding season may help them to fall back to natural feeding behaviour by hunting slow moving prey, and, therefore, saving energy from hunting and improving the reproductive success in captivity. It has been previously reported that female amphibians with higher energy stores are more likely to

successfully reproduce (Girish and Saidapur, 2000; Wells, 2010). However, excess energy intake can cause obesity-related effects such as fatty liver, which can have a negative impact on reproduction (Wright and Whitaker, 2001; Regnault *et al.*, 2018). Therefore, providing a more balanced diet or reducing feeding rates after breeding season would be beneficial, to prevent obesity and subsequently improve reproductive rates.

Here, we suggest an experiment will need to be performed, in order to test whether dietary optimisation can lead to higher reproductive success in captivity. Fire salamander population could be divided into two groups with the same ratio of female and male animals. The breeding season for fire salamanders occurs in autumn, which is why the experiment will be conducted from September until December, when the offspring hatches. One group of animals will be fed twice a week with prey animals, in which the bodymass corresponds to 90% of Gastropoda and 10% of Chilopoda. The second group of animals will be fed in the same interval with bodymass, corresponding to 10% of Gastropoda and 90% of Chilopoda. At the end of the experiment, the reproduction rate can be compared and a conclusion can be felled, whether or not the optimisation of the diet has a beneficial effect on the animal reproduction program in captivity.

5. Agricultural land use may affect animal health in neighbouring forests

Agricultural land expansion has become one of the major anthropogenic changes in the environment, driving massive habitat loss for wild animals (Cardinale *et al.*, 2012). The toxic chemicals and pesticides released in agriculture can contaminate the soil and water, further affecting already declining populations and the ecosystem as a whole (National Research Council, 1993; Seiler and Berendonk, 2012; Kanianska, 2016). For amphibians, it has been shown that pesticides alter the immune system (Christin *et al.*, 2004; Brodkin *et al.*, 2007), increasing animal susceptibility to diseases (Hua *et al.*, 2017; McCoy and Peralta, 2018), as well as affecting the microbial biodiversity on the skin (Krynak, Burke and Benard, 2017; McCoy and Peralta, 2018; Jiménez *et al.*, 2020) and in the gastrointestinal tract (Chang *et al.*, 2016). So far, almost all studies of the agricultural impact on animal health focused only on the animals that are living on the farmland itself or locations which were directly contaminated by chemicals. Very little attention has been paid to animals that reside in neighbouring forests. The potential impact of agricultural

land use on neighbouring animal habitats, however, should also be considered, in order to select the healthiest and most promising individuals for conservation programs.

In chapter 2, we observed that the fire salamanders from a farmland-adjacent habitat showed a significantly lower diversity in their gut microbiome, compared to animals from other habitats. These findings are somewhat contrary to a previous publication (Chang *et al.*, 2016), where it has been reported that frogs living on farmland show higher gut microbiome diversity than frogs from natural habitats. They also reported a lower diversity of diet in farmland frogs than frogs from natural habitats. The authors argued that the monotonous diet can weaken the immune system of farmland frogs, leading to a more diverse gut microbiome. However, we observed no significant difference of diet composition between the farmland adjacent forest and other forests. We therefore concluded that the agricultural site has no detectable impact on food webs of salamanders in adjacent forests. Furthermore, we can conclude that the decreased gut microbiome diversity we have observed in fire salamanders of this forest is not caused by diet. It is more likely that the soil of pastoral agriculture land has a higher bacteria richness than the adjacent forest (Wu *et al.*, 2021), which would explain the difference in gut microbiomes between animals from farmland adjacent and non-adjacent forests, as a result of altered environmental microbiomes and microbial agents that are ingested with the food. Our results in chapter 2 may provide the evidence that the agriculture land use could have effects on the immune system of adjacent wild animals. We found higher pathogen loads in the gut microbiome of fire salamanders from farmland adjacent forest, which is in line with the high pathogen loads observed in farmland frogs (Chang *et al.*, 2016). A healthy gut microbial community can train the host immune system to prevent the growth of pathogenic bacteria (Round and Mazmanian, 2009; Ahern, Faith and Gordon, 2014). Therefore, the higher pathogenic loads suggest that the animals may suffer from a weakened immune system. It is possible, but unproven, that the weakened immune system can be caused by exposure to pesticides and fertilizers used in adjacent farmland. The results of our study do seem to suggest that contamination from pesticides and fertilizers from the adjacent farmland could account for an increased pathogenic load on fire salamanders. But since the study design was aimed at determining microbial variability between different habitats, a more thorough follow-up study should be conducted, potentially with multiple sampled locations adjacent and not adjacent to farmland, with the aim of determining whether the animals from the habitats adjacent to farmland contain higher pathogenic bacterial loads in their gut microbiome, and whether the soil

from the farmland adjacent habitats also contains higher pathogenic bacteria. It could also be possible, that chemical contaminants negatively affect the fire salamander immune system, making them more susceptible to pathogenic bacteria. Would our observation hold true, that animals adjacent to farmland have higher pathogen loads, this would have repercussions on animal selection for captive breeding programs, as animals adjacent to farmland would be not as healthy as their counterparts from wildlife habitats that are less subjected to anthropological contaminations.

6. Selective breeding

With the end goal of overcoming the pathogenic threat of chytridiomycosis and preserving ecological biodiversity, selective breeding could be a long-term sustainable method to increase resistance against *Bsal* infections in certain species. This would permanently avert the risk of *Bsal* induced population decline and achieve host–pathogen co-existence in the environment (Garner *et al.*, 2016). So far, selective breeding for increasing host resistance to *Bsal* hasn't been implemented in amphibians. As shown in chapter 5, skin galactose intensities vary significantly between individual animals of some, but not all species. This variation is negatively correlated with species mortality. For example, fire salamanders show intense RCA staining in all tested individuals (0% variation of RCA staining scores) and exhibit a 100% mortality rate. Spanish newts (*Pleurodeles waltl*) have 18.6% of variation in RCA staining scores and exhibit a 61.5% mortality rate. RCA staining and mortality rates were done on different individuals in our experiments, but the variation of skin galactose presence between individual animals of susceptible species could also be determined in this manner. Furthermore, this method could subsequently be used for selecting more resistant individuals, in order to produce lineages with higher resistance to *Bsal* infection. To test this hypothesis, firstly we have to confirm that the skin galactose presence is inheritable. Secondly, we have to test whether subsequent generations that have a lower skin galactose presence also correlate with lower infection loads and a higher chance of survival in infection trials. An experiment with Spanish newts could be conducted, where individual animals would be selected and bred, based on lower skin galactose presence, compared to a control group without selection. The galactose content of all offspring would have to be measured, in order to selectively breed for multiple generations. These measured galactose levels

could then be compared to infection load levels in subsequent infection trials. The outcome of selective breeding could also be achieved by genetic engineering, which could particularly useful for species with 100% mortality rates, as is the casein fire salamanders. So far we only know that galactose presence on skin glycoproteins is crucial for initial colonization of *Bsal*. However, we have no idea which genes are responsible for expressing these glycoproteins. Therefore, identifying the specific genes behind the galactose-containing glycoproteins would provide a genetic marker to modify encoding genes and decreasing disease susceptibility.

7. Future outlook

The widespread use of disinfectants and antifungals, as a means of decontaminating the environment, has previously been proposed as a *Bsal* mitigation method. However, this method raises concerns of adverse effects on the environment and wildlife. In chapter 5, we discovered for the first time that galactose plays a crucial role in *Bsal* adhesion to the host epidermis. Therefore, designing a galactose-based inhibitor could reduce the success of *Bsal* for initial colonization after contact with host skin, which may provide a more sustainable and environmentally friendly method of mitigating *Bsal* in affected areas. The development of glycoconjugates, which are drug-like compounds and mimic the glycans displayed on host cell surfaces (Sattin and Bernardi, 2016), is already an established technique to inhibit the adhesion of bacteria (Mitchell *et al.*, 2002; Wellens *et al.*, 2008), viruses (Reuter *et al.*, 1999; Sun, 2007) and fungi (Martin *et al.*, 2021) to their respective targets. Synthetic carbohydrates, such as the divalent galactoside glycomimetics 1 and 6, have already shown potential as a new treatment against *Candida albicans* infection, by inhibiting binding of *C. albicans* towards human buccal epithelial cells (Martin *et al.*, 2020, 2021). Direct potential uses of the galactose-inhibitor for mitigating *Bsal* could be found in preventing disease infections in captive populations of lethally susceptible species, and also in more broad uses in *Bsal* affected population habitats, decreasing the likelihood of infections in *Bsal* hotspots and protecting already vulnerable population. That is, of course, if the glucomimetic compound is cheap enough to produce, is deemed non-toxic and safe for dispersal in the environment, and, lastly, does not have strong taste properties. This is very difficult to predict with certainty, but as most glucomimetic compounds are much larger than simple sugars, and as it is reported that even small modifications of simple sugars can change the taste (Birch, 1987), it is unlikely that

glucomimetics will have a low molar basis of sweetness. Should a low molar basis of sweetness or sourness occur, this glucomimetic would be unfit for use. This would need to be tested beforehand, in order to not unleash a taste environmental pollutant like other sweeteners (Kokotou, Asimakopoulos and Thomaidis, 2012). In order to design and synthesize glycomimetics, it is important to have crystal structures of the cell surface glycans, that are to be mimicked. So far, we only know that terminal galactose residues mediate *Bsal* zoospore adhesion to the amphibian host epidermis. Further studies are necessary to reveal the detailed structure of galactose-containing oligosaccharides that are expressed on amphibian skin, in order to subsequently design and synthesize glycomimetics, that are able to mitigate *Bsal* in an environmentally friendly and sustainable manner.

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SUMMARY

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Western Palaearctic amphibian biodiversity is under serious threat of *Batrachochytrium salamandrivorans* (*Bsal*), which was first described in 2013, after a population collapse of fire salamanders in the Netherlands. Subsequently, *Bsal* has also been found in the wild in Spain, Belgium and Germany and has brought multiple native European salamander populations to the brink of extinction. Therefore, increased efforts are necessary in setting up conservation strategies to protect endangered salamander populations from extirpation.

Chapter 1 provides an overview of the potential conservation strategies for amphibian populations threatened by *Bsal*. However, no long-term, sustainable, and effective conservation methods have been introduced thus far. In order to protect and safeguard fire salamander (*Salamandra salamandra*) populations, behavioural, molecular biological and microbiological aspects of these animals and host pathogen interactions will need to be thoroughly understood and characterized.

In chapter 2, we used high-throughput DNA metabarcoding techniques to characterize both diet and gut microbiome of fire salamander populations from Belgian forests. Differences of diet composition between males and females were observed for the first time in amphibians in this study, with female fire salamanders consuming more slow-moving prey. Differences between sexes were also found in fire salamander gut microbiome communities, with diversity of species being lower in female than in male animals. We found no correlation between fire salamander diet and gut microbiome composition. In addition, a higher pathogen load was detected in the forest located adjacent to farmland. These findings deepen the understanding of the diet and gut microbiome of fire salamander populations, and provide the necessary insight to improve conservation strategies.

Understanding the mechanisms of disease ecology is crucial for developing effective conservation strategies against *Bsal*. However, the knowledge of *Bsal* pathogenesis is still in its infancy. In chapter 3, we successfully identify host factors that mediate early interactions between *Bsal* zoospores and host skin. We found that beta-galactose plays a crucial role in the initial step of *Bsal* pathogenesis, mediating zoospore chemotaxis, adhesion to host skin, and initiating a virulent fungal response. We observed a clear positive correlation between lectin staining intensity and *Bsal* infection loads of different amphibian species, by assessing the skin galactose content using RCA lectin staining on animal tail or toe clips. We conclude that skin galactose can be used as a biomarker to predict *Bsal* species susceptibility. At species level, these markers could be used to

classify species according to their *Bsal* susceptibility, which would make a new methodology possible for assessing the risk *Bsal* poses for global urodelan diversity and subsequent species prioritization in conservation efforts.

In conclusion, the findings produced in this PhD thesis could have far-reaching implications, improving not only present animal conservation strategies, by characterizing diet and gut microbiome dynamics in fire salamanders, but also establishing novel methods for classifying *Bsal* species susceptibilities, which in turn could be used for more targeted, effective and long-term conservation strategies. The finding of beta-galactose residues as a binding target for the pathogenic *Bsal*, gives a foundational knowledge that can be build on.

SAMENVATTING

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De biodiversiteit van amfibieën in de West-Palearctische regio staat zwaar onder druk ten gevolge van *Batrachochytrium salamandrivorans* (*Bsal*). Deze schimmel werd voor het eerst beschreven in 2013 als de oorzaak voor de dramatische reductie van de Nederlandse populatie vuursalamanders. Ondertussen is *Bsal* in het wild sterk verspreid binnen de EU (o.a. Spanje, België en Duitsland) en heeft het al verschillende inheemse Europese salamander populaties op de rand van uitsterven gebracht. Het is daarom van groot belang om in te zetten op strategieën voor het behoud en de bescherming van bedreigde salamander populaties.

In hoofdstuk 1 wordt er een overzicht gegeven van mogelijke conservatie strategieën om populaties van amfibieën te beschermen tegen *Bsal*. Jammer genoeg zijn er momenteel geen effectieve en duurzame oplossingen beschikbaar die op lange termijn bescherming bieden in het wild. Om in de toekomst populaties van vuursalamanders (*Salamandra salamandra*) te vrijwaren van *Bsal* zullen de fundamenten blootgelegd moeten worden van zowel gedrags-, moleculaire, biologische en microbiologische aspecten van deze dieren en de interacties tussen gastheer en pathogenen.

In hoofdstuk 2 wordt er dieper ingegaan op het microbioom van de vuursalamander. Via DNA metabarcoding werd zowel het ‘dieet microbioom’ als het ‘darmmicrobioom’ bepaald van vuursalamanders afkomstig uit Belgische bossen. Afhankelijk van het geslacht werden verschillen in eetpatroon waargenomen, waarbij vrouwelijke salamanders meer traag bewegende prooien consumeren. Daarenboven had het geslacht ook een impact op de samenstelling van het darmmicrobioom. Vrouwelijke salamanders vertoonden een lagere diversiteit in species ten opzichte van mannelijke dieren. Er werd echter geen correlatie gevonden tussen het dieet en het darmmicrobioom. Vuursalamanders afkomstig uit bossen gelegen naast landbouwgebied, bleken een hogere pathogeen load te hebben. Deze data verwerven meer inzicht in het dieet en de microbioom samenstelling van vuursalamanders en hoe deze elkaar beïnvloeden, wat belangrijk is voor conservatie van deze diersoort.

Het verwerven van inzichten in de ziekte-ecologie van *Bsal* is cruciaal om effectieve conservatie strategieën te ontwikkelen tegen deze verwoestende schimmel. Momenteel staat de kennis over *Bsal* pathogenese nog in de kinderschoenen. In hoofdstuk 3 werden de gastheerfactoren geïdentificeerd die de vroege interactie tussen *Bsal* zoosporen en de huid van de gastheer mediëren. We hebben aangetoond dat beta-galactose een cruciale rol speelt in de initiële stappen

van de *Bsal* pathogenese, namelijk chemotaxis van de zoosporen richting de huid, adhesie van de zoosporen aan de huid en de opregulatie van virulentiegenen. Er werd een positieve correlatie tussen de *Bsal* infectie load en de intensiteit van een RCA-lectine kleuring aangetoond bij verschillende amfibiesoorten. De lectine kleuring is een indicator voor beta-galactose en deze werd uitgevoerd op een huidbiopt van zowel de staart als de teen. Niet alle amfibiesoorten zijn even gevoelig voor deze schimmel en onze resultaten tonen aan dat de aanwezigheid van galactose in de huid gebruikt kan worden als een biomerker om de gevoeligheid van verschillende amfibiesoorten voor *Bsal* te bepalen. Dit onderzoek verschaft niet alleen cruciale informatie over de eerste stappen in het infectieproces, maar het beschrijft ook een niet-invasieve techniek om het risico van *Bsal* te bepalen voor de wereldwijde urodelan diversiteit wat essentieel is om prioriteiten voor beschermingsmaatregelen te stellen.

We kunnen concluderen dat deze PhD heel wat fundamentele kennis verschaft die de huidige beschermingsmaatregelen voor amfibieën verbetert. Door zowel de dynamiek tussen het dieet- als het darmmicrobioom van vuursalamanders te bepalen, alsook door de ontwikkeling van nieuwe niet-invasieve methoden om *Bsal* gevoeligheid bij verschillende amfibiesoorten te bepalen, kunnen meer gerichte, effectieve en lange termijn conservatie strategieën ontwikkeld worden. Daarnaast zullen de nieuwe inzichten tussen beta-galactose en *Bsal* gebruikt worden om verder onderzoek aan te sturen.

CURRICULUM VITAE

CURRICULUM VITAE

Yu Wang was born on June 7, 1990 in Hohhot, Inner Mongolia, China. She received her Bachelor of Science degree in Veterinary Medicine from the Shanxi University, China in 2013. In the fall of the same year, she continued her studies at the University of Liverpool, UK, School of Veterinary Medicine. In the fall of the following year, she finished her studies with the thesis "Can prophage WO of an insect *Wolbachia* symbiont be reactivated?" with merit. In 2016, she finished her second Master of Science at the department of Infection and Immunity at the University of Edinburgh with distinction, with the research project titled "Investigating the clonality and formation of memory populations of non-conventional NKp46+ CD3+ T-cells".

In January 2017, she started her doctoral training in the Wildlife Health Ghent research group, located within the Department of Pathology, Bacteriology and Poultry Diseases, at the Faculty of Veterinary Medicine of Ghent University. Under the supervision of Prof. Dr. An Martel and Prof. Dr. Frank Pasmans. The research was funded by the Ghent University Special Research Fund and led to various scientific publications in international journals.

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