Epidermal galactose spurs chytrid virulence and predicts amphibian colonization

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The chytrid fungal pathogens Batrachochytrium dendrobatidis and Batrachochytrium salamandrivorus 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. salamandrivorus 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.

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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 the extinctions or declines of hundreds of amphibian species worldwide. 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, 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* and *B. salamandrivorans*. The latter was recently discovered from a collapsing fire salamander (*Salamandra salamandra*) population in the Netherlands and is causing mass mortality events in wild salamander populations across Europe. In susceptible animals, *B. salamandrivorans* causes epidermal necrosis, resulting in loss of the epidermal barrier and subsequent overgrowth and invasion by opportunistic bacteria, bacterial septicemia, and death.

Infection of the amphibian skin by the motile fungal spores is a complex and poorly understood process that requires recognition, 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. 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. 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. Following attachment, the *B. salamandrivorans* arsenal of proteases is thought to play a key role in subsequent cell invasion.

The extent of epidermal infection correlates with the severity of the disease, which varies strongly between, and even within, amphibian host species. The outcome of infection depends on complex host, pathogen, and environmental interactions and can vary from the absence of clinical signs to rapid death. Predicting host susceptibility is crucial for risk assessments and the development of mitigation action plans and is currently only possible using invasive infection experiments.

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-galactoside 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 4 anuran species and of different life stages of fire salamanders (*Salamandra salamandra*) demonstrates a 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 and 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 mannnose, but not to N-Acetylgalactosamine (GlcNAc) (Fig. 1b and Supplementary Tables 3, 4), identifying galactose and mannnose 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 and Supplementary Table 5). This is in contrast with *B. dendrobatidis*, which is attracted by the tested carbohydrates (mannose, galactose, fucose, N-acetylgalactosamine, N-acetylgalactosamine, and N-acetylenuraminic acid) without specific preference. 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 beta-1,4-linked galactose residues.

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-galactoside 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 a 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.
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. In choanoflagellates, protein tyrosine kinases have been found to regulate cell proliferation and react to the environmental nutrient
availability.27. Protein tyrosine kinases have also been shown to play a crucial role during fungal infections, including attachment to host cells.28,29. BSLG_08965, also specifically linked to galactose treatment, was annotated as belonging to the alpha/beta hydrolase fold family of proteins. Alpha/beta hydrolase fold proteins have been reported to regulate the intercellular transport of cellular components and particularly proteins (e.g., secretory exocyst component, kinesin motor domain, transporters, nexin, clathrin, and 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 H2O-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.3,30,32,34. M36 metallopeptidase candidates BSLG_08963 (Log2 fold change vs H2O = 2.25) and BSLG_09557 (Log2 fold change vs H2O = 2.55), together with the peptidase S41 candidate BSLG_06886 (Log2 fold change vs H2O = 3.66), showed an upregulation in galactose-treated zoospores, compared to H2O-treated zoospores, but not in glucose- or mannose-treated zoospores (Fig. 2c). As a general response to carbohydrates, a significant upregulation (Log2 fold change vs H2O ≥ 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), H2O- (p = 0.036) or protease inhibitor (PI)-treated (p < 0.001) (Fig. 3 and Supplementary Table 7). Increased protease activity corroborates the initiation of a virulent response of B. salamandrivorans upon contact with galactose.

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,30 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,31,32 larvae are not considered susceptible to chytridiosis.12 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 and 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(transformed) = 6, Fig. 4a). Disease signs (skin ulcers) 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 (β = 2.81, p = 0.014, n = 43, regression F-statistic (2,40) = 5.40 and p-value = 0.008; Fig. 4c–e). Infection load and disease course are thus correlated with ontogenetic galactose patterns in salamander skin.

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.
We con explain 87.2% of the variance in using dummy variables for RCA scores and found that RCA scores peak loads (in lethality. Rather, innate and acquired defence mechanisms and the three biological replicates in total. In the box-and-whisker plots, the median based on Tukey's multiple comparisons test comparing two-sided linear mixed models. 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 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 h. The skin washes were subsequently examined for the concentration of oligosaccharides.

The proportion of galactose in the total carbohydrate fraction of the skin washed 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 and 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 the infection intensity of B. salamandrivorans infection, we do not recommend using skin washes for predictions at species level if more invasive sampling is allowed.

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. wallis (n = 11), I. alpestris (n = 12) and S. salamandra (n = 10) were 18.56, 13.74 and 0.00%, respectively. Mortality rates after experimental exposure for these species are 61.56, 19.75, 19.39 and 100%6,19,39,41. 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

RCA histochemistry revealed a clear positive correlation between B. salamandrivorans infection peak loads and RCA staining intensity across the fourteen species included (Fig. 5 and 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 and Supplementary Table 10). Indeed, extensive colonization needs not necessarily result in lethality. Rather, innate and acquired defence mechanisms and the environmental context are likely to determine the disease outcome. Thus, RCA staining of the tail or toeclips can be useful as a predictor of susceptibility for a species 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. 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), H2O 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, the median is shown as a 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.
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.

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...
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 1.5 × 10⁵ spores/ml for 24 h. 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. The line indicates linear regression, implemented with RCA score as an ordered categorical variable. Source data are provided as a Source Data file.

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**Methods**

*Batrachochytrium salamandrivorans* (*B. salamandrivorans*) culture conditions and zoospore isolation. *B. salamandrivorans* type strain (AMFP 13/01)* 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 motility were 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 lystate binding assay is shown in Supplementary Fig. 3.

To collect the sloughed skin, ten captive-bred adult fire salamanders were housed at 13 ± 1 °C on moist tissue. The sloughed skin samples were ground with liquid nitrogen into a fine powder and then homogenized, using 3 ml Radiolmmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich) per gram of tissue. Samples were incubated for 1 h at 4 °C, centrifuged at 27.000 × g for 10 min and the supernatant was subsequently collected. Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). The obtained skin lystate 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 h. 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 min to separate the precipitated proteins from the supernatant. The precipitated proteins were resuspended in 300 µl of 0.05 M carbonate bicarbonate coating buffer (3.7 g NaHCO₃, 0.64 g Na₂CO₃, 1 L distilled water, pH 9.6). Each skin lysate solution was coated in each well of 96-well poly styrene 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 for 20 min at 13 °C and washed five times with distilled water to remove the unbound zoospores. Digital photographs were taken through an inverted light microscope at 100 × magnification. Five pictures were taken for each well and each species 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-acetylgalactosamine (GlcNAc), N-acetylgalactosamine (GalNAc), mannose, and lactose was tested. The three mono saccharides and the disaccharide (Siga ma-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 20 h. Plates were rinsed three times with washing buffer and blocked with 1% BSA overnight at 4°C. % intercepted; 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*, LHY = *Lyciasalamandra helversoni*, SPS = *Speleomantes strinatii*, PH = *Paramesotriton hongkongensis*, PG = *Plethodon glutinosus*, CL = *Chioglossa lusitanica*, PS = *Pachyphonus 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), LHY = (n = 3), SPS = (n = 2), PH = (n = 2), CL = (n = 3), PS = (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), LHY = (n = 3), SPS = (n = 3), PH = (n = 2), CL = (n = 6), PS = (n = 3), SA = (n = 5), AO = (n = 10), 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.
Hundred µl of B. salamandrivorans zoospore suspension (1 × 10⁷ zoospores per ml) was added in each well and incubated for 20 min 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 × 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). The highest level of B. salamandrivorans spores binding to lactose was observed. Lactose is a disaccharide consisting of glucose and galactose. Therefore, in the following experiments galactose, glucose and their derivatives will be tested separately.

Carbohydrate chemotaxis test. Chemotaxis of B. salamandrivorans toward free carbohydrates was tested as previously explained (Supplemental Fig. 4)⁴. The sugars 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 signalling activity of the amides. Sugars were dissolved in distilled water, filter sterilized, and tested at a 0.1 M concentration. Hemocytometric capillaries (75 mm length; Hirschmann laborgärtner, 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 laborgärtner, Eberstadt, Germany) at one side. Each capillary was swept on the outside with lens paper (Kimtech Science, Kimberly Clark, Roswell, GA, USA) to remove possible attractant spillover. Capillaries were incubated in 400 µl incoim brain containing 10⁹ B. salamandrivorans zoospores in water and placed in a holder inclined about 65° upwards. The assay was incubated for 2 min at 15 °C and the supernatant was removed as much as possible. The assay was repeated in 100 µl Prepmann 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 quantitative real-time PCR (qPCR)⁴, and data were analyzed 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 h after induction of spore release by adding TGhL broth with distilled water, which was filtered using a sterile mesh filter with pore size 10 µm (PluriSelect, PluriSelect)). Six biological replicates containing 4 × 10⁷ zoospores were obtained. Each biological replicate consisted of a pool of spores harvested from three cell culture flasks by replacing the TGhL broth with distilled water, which was filtered using a sterile mesh filter with pore size 10 µm (PluriSelect). The spores were treated with different carbohydrates. Therefore, zoospores were obtained. 200 µl of the spore suspension (10⁷ spores) was added to eppendorfs (107 zoospores eppendorf) which were treated for 1 h at 15 °C and the supernatant was collected. Protease activity in the supernatant was assessed. Therefore, zoospores were harvested from TGhL broth with distilled water, which was filtered using a sterile mesh filter with pore size 10 µm (PluriSelect) and centrifuged for 2 min at 15,000 × g. The supernatant was removed as much as possible. The remaining supernatant was removed as much as possible. The assay was repeated in 100 µl Prepmann 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 quantitative real-time PCR (qPCR)⁴, and data were analyzed 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).

Differential expression analysis was performed using the DESeq2 R package⁴. 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 were defined 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 package⁴. GO enrichment analysis was used the FDR for correcting P-values. Genes with corrected P-value < 0.05 were considered significantly enriched by differentially expressed genes. ClusterProfiler R package⁴ was also used to test the statistical enrichment of differentially expressed genes in KEGG pathways.

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 1 March 2018) using the stringent e-value cutoff of 1e−50 to avoid spurious hits⁴,⁶. 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 settings⁴. Expression of two of these genes (BSLG_00833 and BSLG_02674) was analyzed using the Primer Fluorescent Protease Assay Kit (Thermo Fisher Scientific), according to the manufacturer’s instructions. Three independent repeats of the experiment were performed (biological replicates).

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 were used in a B. salamandra density interaction trial for lectin–histochemical staining, skin samples were collected from amphibian species Salamandra salamandra (n = 10), Lithobates alpestris (n = 12), Lissotriton helveticus (n = 13), Pleurodeles waltli (n = 11), Lissotriton boscai (n = 3), Alytes obstetricans (n = 10), Cynops pyrrhogaster (n = 3), Triturus alpestris (n = 3), Triturus marmoratus (n = 3), Calotriton asper (n = 10), Bombina variegata (n = 5), Rana temporaria (n = 10), Epidelma calamina (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 sodium pentobarbital 20% (KELA). The collected samples were immediately fixed in Bouin solution, adapted with paraffin wax, and cut into 4 µm thick slices. Microwave samples were collected by bathing animals in HPLC-grade water for 1 h from 21 amphibian species (different animals as the ones used for the
tissue clips), namely *Lissotriton helveticus* (n = 3), *Pleurodeles waltli* (n = 5), *Lissotriton boscai* (n = 3), *Triturus anatolicus* (n = 3), *Triturus marmoratus* (n = 3), *Cynops pyrrhogaster* (n = 4), *Echinophora alpestris* (n = 3), *Salamandra salamandra* (n = 3), *Lysiasalamandra helvensis* (n = 2), *Scoloeplanis stratiotes* (n = 2), *Parasemotriton hongkongensis* (n = 2), *Plethodon glutinosus* (n = 2), *Ochloglossa lusitanica* (n = 5), *Pachychelys shangchengensis* (n = 3), *Calotriton asper* (n = 3), *Salamandra algira* (n = 3), *Salamandra lanzai* (n = 2), *Alytes obstetricans* (n = 2), *Bufo viridis* (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 were inoculated with 1.5 × 10^5 *B. salamandrivorans* spores per ml water during 24 h. Ten days after the inoculation all the early-stage and sixteen late-stage larvae were euthanized. The two hind legs were analyzed by qPCR to detect the *B. salamandrivorans* GE load. A tail clip was stained with fluorescein-labelled RCA 1 (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 × 10^5 spores for 24 h. The animals were euthanized 10 days after inoculation. The hind legs were analyzed by qPCR to detect the *B. salamandrivorans* GE load. A tail clip was stained with fluorescein labelled RCA 1 (see below).

**Lectin-histochemical staining.** Fluorescein labelled RCA 1 (*Ricinus communis* agglutinin I) (Vector Laboratories) and Con A (Concanavalin A) has been used to detect the expression of galactose and mannosse or glucose in the epidermis of amphibians. After fixation in Bouin’s medium (Sigma-Aldrich), samples were washed first with tap water until the water ran colourless, then washed for 24 h 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 min plus 450 W for 10 min). The slides were rinsed with PBS (0.01 M, pH 7.4) and immersed in 1% BSA (Sigma-Aldrich) for 15 min, to prevent non-specific lectin binding. Subsequently, the sections were incubated with either lectin RCA 1 (15 µg/ml) or lectin Con A (5 µg/ml) for 30 min. 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 with 200 mM glucose or mannose, to detect the expression of galactose and mannose or glucose in the epidermis of amphibians.

Fluorescent intensities were measured using the AlexA 700 nm diode laser for Hoechst staining. Staining pictures were observed using a Leica TCS SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany). Staining results were assessed using Image J software (National Institutes of Health, USA). Correlations of RCA scores with percentage of free galactose were calculated by using a two-tailed Pearson Correlation Coefficient test (correlation significant at p < 0.05), and regression analyses were assessed by a quadratic regression model.

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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 the statistical analysis and experiments; M.K. delivered genetic data; Y.W., E.V., M.K., L.M., and D.S. performed the statistical analysis and experiments; A.M., F.P., Y.W., and E.V. designed the study; Y.W., E.V., and A.M. conducted the 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 paper. All authors reviewed the paper.

Competing interests

The authors declare no competing interests.
