Presence of low virulence chytrid fungi could protect European amphibians from more deadly strains

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Wildlife diseases are contributing to the current Earth’s sixth mass extinction; one disease, chytridiomycosis, has caused mass amphibian die-offs. While global spread of a hypervirulent lineage of the fungus Batrachochytrium dendrobatidis (BdGPL) causes unprecedented loss of vertebrate diversity by decimating amphibian populations, its impact on amphibian communities is highly variable across regions. Here, we combine field data with in vitro and in vivo trials that demonstrate the presence of a markedly diverse variety of low virulence isolates of BdGPL in northern European amphibian communities. Pre-exposure to some of these low virulence isolates protects against disease following subsequent exposure to highly virulent BdGPL in midwife toads (Alytes obstetricans) and alters infection dynamics of its sister species B. salamandrivorus in newts (Triturus marmoratus), but not in salamanders (Salamandra salamandra). The key role of pathogen virulence in the complex host-pathogen-environment interaction supports efforts to limit pathogen pollution in a globalized world.
Global invasion by highly virulent chytrid fungi has resulted in the most extensive disease-driven loss of biodiversity ever recorded, with the decline of 500 amphibian species worldwide. The skin disease chytridiomycosis is caused by two fungal species, Batrachochytrium dendrobatidis (Bd) and B. salamandrivorans (Bsal). The vast majority of chytridiomycosis-driven declines have been attributed to the hypervirulent and panzootic lineage of Bd (BdGPL). Despite its presumed high virulence and global distribution, amphibian community declines caused by BdGPL are predominantly limited to disease hotspots in North, Central and South America, Australia, Europe and Africa. Since BdGPL is also widely distributed in regions not affected by declines, its mere presence is a poor predictor of disease dynamics. In Europe, BdGPL impacts vary from regional mortality events and declines in a limited number of species to widespread endemism characterized by host–pathogen co-existence in a broad range of host species. Current knowledge of the host–pathogen–environment interaction, notably the role of local BdGPL virulence types, offers an incomplete explanation of these different infections and disease dynamics.

Here we predict that BdGPL co-existence with amphibian communities is mediated by low virulence of the present BdGPL isolates and explore the extent to which low-virulence isolates confer protection against hypervirulent Bd or Bsal. We determined the occurrence of BdGPL in native amphibian populations in Belgium. We then compared pathogen virulence traits of northern European BdGPL isolates to those of a known hyper-virulent isolate. Finally, we estimated the impact of pre-existing infections with less virulent BdGPL isolates on virulent BdGPL and Bsal infections.

Results

Populations persist in the presence of BdGPL. Opportunistic sampling of 1483 amphibians for Bd in 2015–2016 revealed the presence of BdGPL in 17 out of 63 locations and 5 out of 7 species sampled in Flanders, Belgium (Supplementary Fig. 1). Prevalence ranged between 0 and 35% per species (Supplementary Table 1). Besides, 25 cases of amphibian mortality, comprising 47 animals belonging to seven species (1 Rana temporaria, 20 Bufo bufo, 2 Triturus cristatus, 11 Ichthyosaura alpestris, 7 Lissotriton vulgaris, 3 Salamandra salamandra, 3 Lissotriton helveticus) were reported to the regional hotline (passive surveillance system) and submitted for postmortem analysis during a 4-year period (2015–2018). Chytridiomycosis was not diagnosed in any of these reported cases.

The impact of Bd on native amphibians was estimated both in remnant populations of a threatened chytridiomycosis susceptible species (midwife toads, Alytes obstetricans) and in populations of a common newt considered to be a Bd supershedder (alpine newt, Ichthyosaura alpestris). During the 4-year study, the midwife toad populations persisted in the absence of Bd infections (Supplementary Table 2). Bd occurrence was observed in 10 of 26 study populations of alpine newts and did neither result in reduction of newt abundance ($F(2,76.2) = 0.04$, $p = 0.96$) nor in reduction of body condition (i.e. scaled mass index, $F(1,1728.13) = 1.66$, $p = 0.20$) (Supplementary Table 3, Supplementary Fig. 2).

High fecundity in hypervirulent BdGPL compared to local BdGPL isolates. Ten BdGPL isolates were cultured from midwife toads (A. obstetricans), alpine newts (I. alpestris) or invasive bullfrogs (Lithobates catesbeianus) across Flanders, Belgium (Supplementary Table 4). These isolates were confirmed to belong to the GPL clade using a lineage-specific PCR and this was corroborated by whole-genome sequencing of 6 isolates. Their culture phenotype (growth characteristics and morphological traits) was compared to that of the hypervirulent BdJEL423, isolated from an episode of amphibian mortality in the neotropics. After 7 days of culturing, the hypervirulent BdJEL423 had significantly larger sporangia (linear model $\beta = 0.28 \pm 0.02$, $t(10,416.8) = 14.9$, $P < 0.001$; pairwise Tukey’s HSD contrasts $P < 0.01$; Fig. 1) and produced significantly more zoospores (linear model $\beta = 335.4 \pm 26.4$, $t(10,32) = 12.72$, $P < 0.001$; pairwise Tukey’s HSD contrasts $P < 0.01$; Fig. 1) than any of the local Bd isolates (Fig. 1), corroborating previous reports that zoospore production and sporangium size are linked to pathogen virulence. Although significant variation in phenotypic traits was also noticed among the local isolates, multivariate ordination of phenotypic traits grouped BdJEL423 apart from all other isolates (Fig. 1).

Midwife toads are a European sentinel species for Bd-induced chytridiomycosis. To assess the potential impact of invasion of hypervirulent BdGPL in the broader amphibian community, we compared susceptibility of seven indigenous amphibian species to exposure to the hypervirulent BdGPL isolate BdJEL423 and the local isolate BdBE1. In contrast to the midwife toads that died after exposure to BdJEL423, European tree frogs (Hyla arborea), fire salamanders (Salamandra salamandra), spadefoot toads (Pelobates fuscus), alpine newts (I. alpestris) and natterjack toads (Epidalea calamita) became infected with both isolates (Supplementary Fig. 3) but did not develop lethal chytridiomycosis within the 13 weeks of the experiment. Throughout the trial, great crested newts (Triturus cristatus) and common toads (Bufo bufo) never tested positive for Bd (Supplementary Fig. 3). The marked susceptibility of midwife toads and the low susceptibility of fire salamanders and common toads correlate with marked fungal growth in mucosomes collected from midwife toads and with fungal growth inhibition by mucosomes collected from fire salamanders (Fig. 2a, Supplementary Fig. 4) and reflects the European field situation (endemic and widespread presence of Bd, with local outbreaks). Based on these results we classify the midwife toad as a Bd sentinel (canary in the coal mine for virulent Bd infections in Europe) species in Europe and decided to use this species in further experiments to test the pathogenicity of local isolates.

Midwife toads tolerate infection with local BdGPL isolates but are highly susceptible to the hypervirulent BdGPL isolate JEL423. Compared to the hypervirulent BdGPL isolate BdJEL423, all four local BdGPL isolates included were low pathogenic in an infection trial using recently metamorphosed midwife toads (Alytes obstetricans) (Fig. 3). BdJEL423 caused 100% mortality (9 out of 9 animals, housed individually) with a mean time to death after exposure of 27 days (range 16–64). Of the 29 toads infected with the local BdGPL isolates, only one died due to infection. The probability of death was not significantly different from the uninfected control group (for penalized GLM coefficients, all $z$ (5.43) $< 0.7$, $p > 0.5$; Fig. 3, Supplementary Table 5).

BdGPL pathogenicity correlates with Crinkler and Necrosis (CRN) gene expression pattern and host cell invasion. Searching for pathogenicity determinants, the isolates were further compared by analysing the expression of a selection of Bd virulence genes in spores and spores that were first exposed to midwife toad skin. Regarding virulence gene expression, marked inter-isolate variation was noticed. Distinctive features mainly pertained to the expression of CRN and metalloprotease genes (Supplementary Tables 6–7). Expression of CRN genes has been linked to the early infection stage of BdJEL423, and was low in spores of local BdGPL isolates (BdBE1, BdBE3, BdBE4 and BdBE5).
**Fig. 1 Experimental results for phenotypic traits of B. dendrobatidis isolates.** a-d Boxes indicate 25th and 75th percentiles, central lines the mean, bars the 95th percentiles, and points indicate individual samples. N of sporangia = number of sporangia in the central 1000 × 1000 pixels, n = 4 independent experiments; area of sporangia = area of the largest 10 sporangia, n = 10 technical replicates per independent experiment; N of zoospores = number of sporangia in the central 1000 × 1000 pixels of the well/image, n = 4 independent experiments; fecundity was calculated following the formula (average \( N_{ZOOSPORE} / \text{average} N_{SPOR} ) / \text{average} N_{SPOR}, n = 4 \) independent experiments. e Non-metric multidimensional scaling across the four variables in (a–d); labels indicate one point per group as a colour legend. BdBE1–10 are local isolates isolated in this study. JEL423 is a BdGPL isolate isolated from an episode of amphibian mortality in the neotropics\(^5\). Source data are provided as a Source data file.

**BdBE5** compared to the hypervirulent BdJEL423. Incubation with midwife toad tissue resulted in increased CRN gene expression for isolates BdJEL423, BdBE1 and BdBE3 and decreased expression for BdBE4 and BdBE5 (Fig. 2b, Supplementary Fig. 5, Supplementary Tables 6–7). In vitro, this pattern of CRN gene expression was correlated with invasiveness and intracellular growth in amphibian A6 cells. Intracellular growth, characterized by the formation of intracellular daughter thalli, was only observed for BdJEL423, BdBE1 and BdBE3. Growth of BdBE4 and BdBE5 was dominated by an epibiotic growth, limited to Bd development outside the host cells (Fig. 2d, e, Supplementary Figs. 6–8). Relative CRN gene expression followed the same pattern as colonisation capacity in the above mentioned in vivo infection experiment, with significant correlation between CRN gene expression of fresh spores of local BdGPL isolates relative to BdJEL423 and skin colonisation capacity (CRN_22492 \( r_s = 0.88, p \leq 0.05, n = 38 \) (CRN_25085 \( r_s = 0.73, p \leq 0.05, n = 38 \)) (CRN_23176 \( r_s = 0.88, p \leq 0.05, n = 38 \))\(^3\).

**Genome-wide variance associated with BdGPL virulence and host cell invasion.** Whole-genome sequence data were mapped against the reference genome for BdJEL423 to identify genome-wide association with the low virulence (BdBE1, BdBE3, BdBE4 and BdBE5) and cell invasion (BdBE1, BdBE3 and BdJEL423) phenotypes. Annotation of variants predicted to be highly deleterious or disruptive to gene function predicted 101 genes presenting differentiating variants between local isolates and the hypervirulent BdJEL423 isolate and 64 genes differentially affected between isolates displaying the invasive versus the epibiotic phenotype. Many of these genes were annotated as candidate Crinkler genes (12 genes), or contained common effector protein domains such as SigP4 secretory signals (28 genes), transmembrane domains (10 genes), or metalloprotease (4 genes), aspartate proteases (12 genes) and CBM18 chitin-binding PFAM domains (4 genes, Supplementary Datasheet 1). Fisher’s exact test indicated gene enrichment of five PFAM domains in both sets of affected genes, including Aspartate proteases, the chitin-binding CBM18 domain and the M36 metalloprotease-associated FTP domain (Supplementary Datasheet 1). Three of the genes affected by deleterious mutations in all local isolates show upregulated expression in BdJEL423 in vivo\(^5\), including BDEG_22285, a secreted M36 metallopeptidase. The established role of Crinkler proteins\(^30\), M36 metallopeptidases\(^31,32\) and aspartate proteases\(^33–35\) in fungal pathogenesis suggest these may be Bd effector proteins that are differentially associated with function-altering mutations in virulent and invasive phenotypes, and highlights targets for future investigation. As previous studies have found pathogen virulence attenuation in BdGPL to be associated with an overall decrease in copy number\(^34\), this was compared in the local and BdJEL423 isolates. While the local isolates showed extensive copy number variation of large sequence regions up to entire supercontigs, no such overall decrease in copy number was observed in the local isolates compared with BdJEL423 (Supplementary Fig. 9).

**Local BdGPL protects midwife toads against hypervirulent BdGPL.** To assess the level of protection conferred by the local BdGPL isolates against invasion by hypervirulent BdGPL, we exposed the animals that were previously infected with one of four local BdGPL isolates to the hypervirulent BdJEL423 and...
compared infection dynamics and disease with that of age-matched, naive toads exposed to *Bd*JEL423 only. Animals that had been previously infected with the *Bd*BE1 and *Bd*BE3 isolate showed significantly higher survival than those infected directly with *Bd*JEL423 (GLM $\beta = -2.59 \pm 1.29$, $z(2,32) = -2.01$, $p = 0.044$; Fig. 3, Supplementary Table 8).

In a final proof of concept study, infection dynamics of the hypervirulent *Bd*JEL423 were compared between midwife toadlets that were either pre-exposed or not pre-exposed (naive) to low-virulence *Bd*BE3 ($n = 20$ per treatment, all animals kept individually) 4 weeks before *Bd*JEL423 challenge. Exposure to *Bd*BE3 resulted in infection in all animals in the absence of mortality and a median peak load of 140 GE (range 14–1164 GE). Subsequent challenge of these pre-exposed toads with *Bd*JEL423 resulted in detection of low *Bd* levels in only two toadlets (3 and 23 GE) and only on the first sampling occasion. In contrast, all naive animals developed significant infection loads during the 4-week follow-up period after challenge with *Bd*JEL423 (median peak load 1802 GE, range 58–19,100 GE) (Fig. 3). Prior infection with the low-virulence *Bd*BE3 thus resulted in less infections upon challenge with the highly virulent *Bd*JEL423 (Fisher’s exact test, two-tailed $P$ value < 0.0001).

*Bd*GPL alters virulent *Bsal* infection in marbled newts, but not fire salamanders. Local *Bd*GPL temporarily colonized fire salamanders (*S. salamandra*), marbled newts (*Triturus marmoratus*) and ribbed newts (*Pleurodeles waltl*) without causing clinical signs or mortality (Fig. 4). The effect of pre-exposure to local *Bd*GPL on the course of a subsequent *Bsal* infection depended on the infected species (Fig. 4, Supplementary Table 9). In fire salamanders, exposure to local *Bd*GPL had no effect on *Bsal*-induced mortality (penalized GLM $\beta = 0.00 \pm 2.17$, $z(2,26) = 0.00$, $p = 1$) or infection course (parametric survival regression $\beta = -0.07 \pm 1.27$).
Fig. 2 BdGPL virulence coincides with growth ability in mucosomes, Crinkler (CRN) gene expression and adhesion and invasion capacities. a Growth (expressed in corrected GE values) of BdBE1 and BdJEL423 in mucosomes collected from different amphibian species. PC: positive control; Ao: Alytes obstetricans; Ss: Salamandra salamandra; NC: negative control. Significant differences are shown with an asterisk and were assessed by a Kruskal–Wallis analysis, followed by pairwise Mann–Whitney U-test (two-tailed) with a Bonferroni-corrected P value of 0.017 (Ao vs Ss: P value = 0.001 (BdJEL423) and 0.009 (BdBE1); Ao vs PC: P value < 0.001 (BdJEL423) and 0.003 (BdBE1)). The experiment was carried out in tenfold and individual data points are shown (blue = BdJEL423; red = BdBE1), with the mean ± s.e.m. depicted by the horizontal bars. b Box plots of mean fold changes in mRNA expression of CRN genes (CRN_22492, CRN_25085 and CRN_23176). The data in the left panel show the normalized target gene amount in spores of each isolate (n = 4) relative to spores of BdJEL423 (n = 4) which is considered 1. The data in the right panel show the normalized target gene amount in spores that were incubated with skin tissue of A. obstetricans for 2 h (n = 4) relative to spores of the respective isolate (n = 4) which is considered 1. Boxxy indicate 25th and 75th percentiles, central lines the median, bars the minima and maxima, and points indicate individual samples. Target genes were based on Farner et al.5. Left panel: an asterisk indicates a significant difference compared to BdJEL423 spores (Kruskal–Wallis analysis, followed by pairwise Mann–Whitney U-tests (two-tailed) with a Benjamini–Hochberg-adjusted P value < 0.05). Right panel: an asterisk indicates a significant difference compared to spores of the respective isolate (Kruskal–Wallis analysis, followed by pairwise Mann–Whitney U-tests (two-tailed)). Individual P values are shown in Supplementary Tables 6–7. c Representative image showing germ tube formation of BdJEL423. Fluorescent signals of Bd (Calcofluor White (blue)) and A6 cells (green cell tracker) were merged to assess the ability of germ tube formation, 4 h after contact with the cells. d, e Representative images demonstrating endobiotic growth (d: BdJEL423) and epibiotic growth (e: BdBE4) of Bd. The cell wall of extracellular Bd was coloured using Calcofluor White (blue). Bd was visualized using Alexa Fluor 568 targeting a polyclonal antibody against Bd (Thomas et al.62), resulting in red fluorescence of both intracellular and extracellular Bd. The Bd-exposed A6 cells were stained with a cell tracker (green). d New intracellular chytrid thalli are formed and the cell content of the mother thallus is transferred into the new daughter thallus (DT). e Epibiotic growth is limited to Bd development outside the host cells. GT germ tube, EX GT extracellular germ tube, IN GT intracellular germ tube, MT mother thallus, DT daughter thallus. Scale bar = 20 μm. Three independent in vitro experiments were conducted with every condition tested in triplicate, with similar results. Source data are provided as a Source data file.

Fig. 3 Local BdGPL isolates protect midwife toads against infection with virulent BdGPL. a (Left) Toads exposed to local (BdBE) isolates had survival comparable to the control (non-exposed) group; toads exposed to hypervirulent BdJEL423 had 100% mortality. (Right) When all survivors were re-infected with BdJEL423, toads previously exposed to BdBE1 and BdBE3 had higher survival than naive toads (control) or toads previously exposed to BdBE4–5. For each group, curves indicate infection load (log$_{10}$[GE+1]), crosses indicate times of individual deaths (crosses offset vertically for readability, dead individuals shaded). The asterisk * indicates one animal that died of non-treatment-related causes between the two experiments. b Tukey-style box plots of Bd peak loads in midwife toads either pre-exposed (BE: green) or not pre-exposed (N: red) to the low-virulence BdBE3 isolate and 4 weeks later challenged with the highly virulent BdJEL423 (BE/JEL and N/JEL). n = 20 biologically independent animals per condition. Source data are provided as a Source data file.
0.15, z(1,18) = −0.43, p = 0.67); Bsal exposure always resulted in lethal infection at an average of 35 days for both treatments. In ribbed newts, BdGPL exposure did not significantly change mortality (penalized GLM $\beta = 0.00 \pm 2.18$, z(2,23) = 0.00, p = 1) or the duration of the infection period (model could not be fitted; Fig. 4b), yet, mortality was observed in the non-Bd pre-exposed newts only. All marbled newts that were inoculated with Bsal only died within an average of 37 days after exposure, whereas four out of seven newts survived when pre-exposed to BdGPL (penalized GLM $\beta = 2.95 \pm 1.74$, z(2,19) = 1.71, p = 0.088, one-sided t-test, $p = 0.037$). However, BdGPL pre-exposure increased the duration of the infectious period (parametric survival regression $\beta = 1.49 \pm 0.21$, z(1,14) = 7.14, $P < 0.001$) and it took on average 107 days before three of the newts died.

Discussion

Although BdGPL has caused death and species extinction on a global scale, its impact in Europe is currently limited to specific regions. Our results suggest that low-virulence BdGPL isolates co-occur with amphibian communities in the absence of population declines and that only a limited number of European amphibian species may be susceptible to the highly virulent isolates associated with massive declines elsewhere. Results of our laboratory trials with seven indigenous amphibian species largely match the in situ condition in northern Europe1,13,15,27–29. None of the tested species was susceptible to Bd-induced chytridiomycosis after exposure to the local BdGPL isolates but several showed infection patterns that corroborate their potential as a chytrid reservoir. Midwife toads persisted in small and isolated populations during at least 4 years in the presence of Bd and Bd infection could not be linked to decreased body condition or host abundance in alpine newts. This finding corroborates the current coexistence of low-virulence BdGPL with northern European amphibian assemblages, despite the occurrence of erratic mortality13,36.

In contrast, the hypervirulent BdGPL isolate did cause lethal infections in a single amphibian species, the midwife toad, while several other species showed relatively high-level long-term infections. These experimental results agree with Bd epizootics.
and population declines observed in midwife toads in Southern Europe.10–12,37.

Our results suggest that if a hypervirulent Bd/GPL similar to the one used in this study invaded our study area, two scenarios might occur. If the invaded host population is entirely Bd-naïve, susceptible hosts (e.g. midwife toads) are likely to be lethally affected. Co-occurring, tolerant hosts (e.g. alpine newts) may sustain high-level infections and act as pathogen reservoirs. If spatio-temporal factors shape a conducive environment, these disease dynamics might result in mass die-offs, population declines and even extirpation of the susceptible species.18. However, pre-existing low-virulence Bd isolates may at least initially affect disease dynamics of an invading hypervirulent Bd. Their protective effect against disease is associated with several traits that correlate with their colonisation ability and invasiveness: expression and function of Bd crinkler genes, metalloproteases and other effector proteins. However, the hypervirulent isolate’s high fecundity, marked invasiveness in amphibian cells and more pronounced colonisation capacity in vivo suggest a distinct competitive advantage over the local isolates. Moreover, co-infections may result in super-infection and/or hybridisation, with highly unpredictable outcomes. Reduced mortality and delayed time to death demonstrate that prior exposure to Bd reduces pathogenicity of Bsal in marbled newts. This may equally pertain to the ribbed newts, in which Bsal mortality occurred only in newts that were not previously exposed to Bd. However, low mortality rate and relatively small sample size hamper meaningful statistics for this species. In contrast, all fire salamanders succumbed to Bsal infection within a similar timeframe, regardless of prior Bd infection. The host species-dependent protective effect of a pre-existing Bd infection against Bsal may present as a double-edged sword for the amphibian community. The reduced pathogenicity in the marbled newts coincides with a longer infectious period and high Bsal infection loads, which is likely to facilitate Bsal transmission to susceptible animals. Increased transmission opportunities may offer an additional explanation for the local extirpations observed in Bsal-infected fire salamander populations in parts of Europe where Bd is widespread.42. In amphibian communities, simultaneous introduction of both pathogens may be subject to different disease dynamics. Simultaneous co-infections with Bd and Bsal in American newts (Notophthalmus viridescens) resulted in disease exacerbation.43.

In conclusion, our results show a complex disease landscape with multiple implications for biodiversity conservation. First, the mere demonstration of Bd/GPL presence in amphibian communities is not sufficient to predict disease impacts on susceptible species. Second, co-existence of amphibian communities with low-virulence Bd/GPL may alter the outcome of an invasion by a hypervirulent isolate.44 Protection may increase the probability of host populations surviving a hypervirulent chytrid incursion, but also amplify reservoir dynamics and increase the risk to highly susceptible species. European mitigation programmes could be fine-tuned to focus on the highly virulent isolates, but this would require the availability of a rapid diagnostic pathogen virulence assay.

Methods

Impact of Bd on amphibian communities in Flanders. In a first study, Bd prevalence was determined across our study area (Flanders, Belgium). We sampled 1483 amphibians belonging to 62 populations in 2015–2016 (Supplementary Fig. 1). To detect the presence of Bd, we collected swabs from the superficial skin surface of metamorphosed animals or the mouthparts of larval anurans. Before use in an experiment, isolates were passaged one time in TGhL broth at 20 °C. Belonging to three clades. Full information on each isolate can be found in (Supplementary Table 3), weighed to the nearest 0.1 g and the snout-vent length was 42.7 mm. We thus calculated the SMI as (body mass × (42.7/snout-vent length)2.87). The average number of individuals caught per fyke per pond was used as proxy for newt density. To test whether trends in newt density (i.e. average number of newts per fyke) differed between Bd positive and Bd negative ponds, a generalised linear mixed model (GLMM) was used specifying newt density as the dependent variable and the interaction between time (month) and Bd status (positive versus negative) as independent variables. Trends in newt density were better approximated by a quadratic relationship compared to a linear trend (delta AIC = 5.73). To test whether trends in Bd differed between Bd positive versus Bd negative ponds, a GLMM was implemented using SMI as the dependent variable and time (month), Bd status (positive versus negative), newt sex (male versus female) and newt density (see above) as independent variables. The initial model contained all two-way interactions between the independent variables. For GLMMs, pond was included as a random factor, and a random error structure was specified (model residuals were normally distributed, Shapiro–Wilks W = 0.90). A frequentist approach was adopted whereby initial models were reduced in a stepwise manner, by excluding the variable with the highest P value until only P < 0.05 predictors remained.

This field study was performed with approval of the Flemish government (derogation number ANB/BL/F-F15-00015). Animals were handled while wearing a fresh pair of non-powdered, disposable gloves. Equipment and field clothing were cleaned and disinfected before visits to sampling locations.

Detection of chytrid associated disease in the study region was done by postmortem examination of field cases of amphibian disease or mortality over a period of 4 years. Wildlife Health Ghent hosts an amphibian disease hotline where suspect cases of infectious disease are submitted (cases with obvious traumatic causes such as predation or traffic are not withheld). The dead amphibians are routinely examined for the presence of Bd, Bsal and Ranavirus using (q)PCR.46–48

Isolate collection. Isolates were obtained from wild amphibians collected July 2015–September 2016 in the Flanders region of Belgium. Isolation was carried out using the protocol described in Fisher et al.49. Using a sterile needle, small skin sections 1–2 mm were cleaned of surface-contaminating bacteria and fungi by dragging it through agar-medium. Subsequently, the tissue sample was transferred into a sterile TGhL medium with antibiotics (200 mg/L penicillin-G and 400 mg/L streptomycin sulphate) and incubated at 20 °C. Six isolates were genotyped and their phylogeny depicted in the global phylogeny in O’Holland et al. These isolates belong to three clades. Full information on each isolate can be found in (Supplementary Table 4). All isolates were preserved in liquid nitrogen at passage 10. Before use in an experiment, isolates were passaged one time in TGhL broth at 20 °C.

Hygiene and biosafety protocols. The animal experiments were performed under strict BSL2 conditions. During the fieldwork, each individual was handled with a new pair of nitrile gloves. At the end of each field visit, boots, dipnets, funnel traps and other equipments were disinfected with a 1% Virkon® solution for at least 5 min.38

Phenotypic characterisation of local Bd isolates. All cultures (Bd/BE1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and Bd/E423) were grown on TGlh agar at 20 °C, for 3 days. Plates were replicated with sterile distilled water, and the water containing the zygospores was passed over a sterile mesh filter with pore size 10 mm (Pluristrainer, Plur-iSelect). Using a haemocytometer and inverted microscope (Nikon Eclipse TS100, Nikon) the spores were counted. Bd spores (1 × 10 spores per well) were seeded in TGlh medium (1.6% tryptone, 0.4% gelatin hydrolysate and 0.2% lactose in H2O) supplemented with 50% H2O into 24-well tissue culture plates. Wells were photographed using an Olympus CKX41 with an attached camera (Olympus SC50, Olympus) with a (2560 × 1920) pixel field, –72, 96, 120, 144 and 168 h after inoculation. The experiment was performed in quadruplicate. Images were analysed using ImageJ 1.52d software, with the following measurements taken: (1) NnewSpores = number of spores in the central 1000 × 1000 pixels of the well per image, (2) NSpores = number of sporangia in the central 1000 × 1000 pixels, (3) ASpores = area of the largest 10 sporangia, (4) AZonospore = area of 10 random
speros. To measure fecundity, at day 7, the formulation was used (average $N_{ZOOPORE}$/average $N_{SPOR}$/average $N_{ASPOR}$) as described in Fisher et al.26. The number and the number of sporangia was modelled using generalized linear models (GLM) with Poisson error distributions, while the area of sporangia and the calculated fecundity were modelled using linear models, all with isolate as the response variable. For the area of sporangia, a random effect at the replicate level was included to account for pseudo-replication. The significance of pairwise differences among isolates was assessed using Tukey’s HSD test. In addition to differences among isolates for individual characteristics, their overall similarity was assessed using two-dimensional non-metric multidimensional scaling (NMS). NMDS maps the relationship between the dissimilarity matrix (Bray-Curtis index) to locate each sample along two coordinates in the ordination space26. All analyses were performed in R25.

**Infection trials.** The animal experiments were performed with the approval of the ethical committee of the Faculty of Veterinary Medicine (Ghent University EC2016/20, EC2015/86). Only captive-bred animals were used in standardized experiments, using identical environmental conditions that allow comparison of isolate virulence and host susceptibility. All animals were housed individually in terraria at 15°C on filter substrate with a hiding place and water dish. All animals/ females) were clinically healthy and derived from breeding colonies that are free of Bd, Bsul and Ranavirus as assessed by sampling the skin using cotton-tipped swabs and subsequent performing qPCR or PCR46,47,53. Individuals were randomly assigned to treatments. All animals were clinically inspected daily. Skin sampling was done weekly and the swabs were analysed for the presence of Bd using qPCR described by Hyatt et al.53 with the respective isolate used as standard. Sample analysis was blinded. Animals were euthanized by an overdose of pentobarbital. Humane endpoints were set at the loss of self-righting ability and/or change in posture.

**Susceptibility of native amphibians to BdGpL isolates under standardized laboratory conditions.** Five captive-bred individuals of seven species (fire salamander (Salamandra salamandra), common spadefoot (Pelobates fuscus), natterjack toad (Epidalea calamita), common toad (Bufo bufo), alpine newt (Lissotriton alpestris), great crested newt (Triturus cristatus) and European tree frog (Hyla arborea)) were exposed to the local BdGpL isolate BdBE1 or the hypervirulent isolate BdJEL423. In addition, two midwife toads (A. obstetricans) were included as BdJEL423-infected positive control group (the limited number of midwife toads in this experiment was modelled using a binomial regression, with the respective isolate used as standard). In this species were conducted separately). For inoculum, isolates was modelled using a binomial GLM. Penalized regression (package splines46,47,53). Individuals were randomly assigned to groups of six for each species, with 3 replicates for each isolate. After 3 weeks, the volume of water was calculated according to animal surface56,57. The number of animals of each species becoming infected from either isolate using a two-dimensional non-metric multidimensional scaling (NMS). NMDS maps the relationship between the dissimilarity matrix (Bray-Curtis index) to locate each sample along two coordinates in the ordination space26. All analyses were performed in R25.

**Bd growth in amphibian mucosones.** Mucosones were collected from healthy midwife toads (Alytes obstetricans) and fire salamander (Salamandra salamandra) using the bathing method described by Woodhams et al.55. For each species, mucosones were collected from 15 individuals that were exposed to 106 spores of BdJEL423 (prepared as previously) for 24 h. Animals were then housed individually in plastic containers containing tissue, a hiding place and water dish in a climate-controlled (~20 °C & ~80% relative humidity) room and examined as previously described. Four weeks later, all animals were exposed to 1 ml of 1 × 106 of fresh spores of BdJEL423 (prepared as previously) for 24 h. Animals were then transferred to individual plastic containers containing tissue, a hiding place and water dish. Small crickets were given ad libitum, providing a constant food supply. Clinical examination of the animals was carried out daily, and tissue replacement and swabbing weekly. Sample analysis was blinded. The readout for this experiment was the proportion of Bd infected animals after challenge with the highly virulent BdJEL423 in each of the two treatment groups. Significance of protection was calculated using two-tailed Fisher’s exact test.

**BdGpL gene expression.** We compared gene expression of a selection of virulence genes as identified by Farrer et al.7. Using the RNeasy mini kit (Qiagen), total RNA was isolated from fresh Bd spores (2.5 × 106 spores per condition) and Bd spores (2.5 × 105 spores per condition) that were incubated for 2 h at 20 °C with chytrid filtrate (1 × 106 sporangia of A. obstetricans) and with a sterile water control. RNA was isolated from BdJEL423-infected animals as described by Hyatt et al.53, with the respective isolate used as standard. Sample analysis was blinded. The readout for this experiment was the proportion of Bd infected animals after challenge with the highly virulent BdJEL423 in each of the two treatment groups. Significance of protection was calculated using two-tailed Fisher’s exact test.

**Protective capacity of low-virulence BdGpL against hypervirulent BdGpL challenge in midwife toads.** After completion of the multi-isolate A. obstetricans infection experiment, all remaining individuals were exposed to 105 fresh spores of BdJEL423 (prepared as previously) for 24 h. Animals were then transferred to individual plastic containers containing damp tissue and a hiding place in a climate-controlled (~15°C & ~80% relative humidity) room. Small crickets were given ad libitum, providing a constant food supply. Clinical examination of the animals was carried out daily, and tissue replacement and swabbing weekly. Sample analysis was blinded. As for the initial infection trials, penalized binomial GLMs were used to estimate whether the probability of death following infection with BdJEL423 depended on (1) the isolate individuals were exposed to in the initial experiment and (2) the mean maximum/total infection load sustained during the initial experiment. Parametric survival regression was also used to assess whether the survival rates of BdJEL423-re-infected individuals depended on (1) the isolate they had been exposed to in the initial trial and (2) on the mean load of infection in the initial trial. In both cases, the response variable (death) was modelled using a Weibull survival function while being censored as soon as the animals were exposed to a local isolate and before any signs of infection were observed. The relationship between probability of death and the infection load sustained was modelled using a binomial penalized GLM (as above). Infection load was always modelled as the log10 of the genomic equivalent. Mean, maximum and total load over the course of infection were all assessed as predictors.
In vitro infection of A6 cells. Here, we compared the invasive capacity between hypervirulent and low-virulence local BdGGL isolates using a cell culture model. The Xenopus laevis kidney epithelial cell line A6 (ATCC–CCL 102) was grown in 75 cm² cell culture flasks and maintained in complete growth medium (74% NCTC 109 media, 10% foetal bovine serum (FBS) and 1% of a 0.1% penicillin-streptomycin solution (P/S)) and the cells were incubated at 26 °C and 5% CO₂ until they reached confluence. Upon trypsinisation, the cells were detached, washed with 70% Hank’s Balanced Salt Solution without Ca²⁺, Mg²⁺ (HBSS−) and centrifuged for 5 min at 1500 rpm and resuspended in the appropriate cell culture medium for invasion assays, which were performed as described in Verbrugghe et al.11. To assess the germ tube formation, A6 cells were stained with 3 µM CellTrackerTM Green CMFDA, seeded (10⁵ cells per well) in 24-well tissue culture plates containing collagen-coated glass coverslips and they were allowed to attach for 2 h at 20 °C and 5% CO₂. After washing three times with 70% HBSS−, the cells were inoculated with 750 µl of Bd zoospores in invasion medium at a MOI of 1:10. Two hours p.i., the cells were washed three times with 70% HBSS+ and the invasion medium was replaced by staining medium. Four hours p.i., the infected cells were washed three times with HBSS+ and they were incubated with Calcofluor White stain (1 µg ml⁻¹ in 70% HBSS+) for 10 min. After washing three times with 70% HBSS+, the cells were fixed, mounted and analysed using fluorescence microscopy. To assess the invasive growth, A6 cells were seeded and inoculated with Bd zoospores as described above. Two days p.i., the infected cells were stained with 3 µM CellTrackerTM Green CMFDA, washed three times with 70% HBSS+, and they were incubated with Calcofluor White stain (10 µg ml⁻¹ in 70% HBSS+) for 10 min. After washing three times with 70% HBSS+, the cells were fixed, mounted and analysed using fluorescence microscopy. To assess the infection involved in the decline of the common midwife toad (Alytes obstetricans) in protected areas of central Spain. Biol. Conserv. 97, 331–337 (2001). 9. Briggs, C. I., Knapp, R. A. & Vredenburg, V. T. Enzootic and epizootic dynamics of the chytrid fungal pathogen of amphibians. Proc. Natl Acad. Sci. USA 107, 9695–9700 (2010). 10. Weldon, C., Channing, A., Misinzo, G. & Cunningham, A. A. Disease driven extinction in the wild of the Kihansi spray toad (Nectophryne asperginsi). bioRxiv https://doi.org/10.1101/67791 (2019).
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Author contributions
A.M. and F.P. designed the research. A.M., M.S.G., E.V., M.K., M.B., W.B., N.D.T., Z.L., G.S., R.V.L. and S.V.P. carried out the research. A.M., M.S.G., E.V., M.K., W.B., S. Canessa, L.L., Z.L., D.S., R.V.L. and F.P. analysed the data. M.S.G., E.V., W.B., S. Canessa, S. Carranza, S.C., D.F.G., P.G., L.L., Z.L., D.S., R.V.L., M.V.E., M.V., F.P. and A.M. interpreted the data. A.M., F.P., M.S.G., E.V., M.K., W.B. and S. Canessa wrote the paper with input from all other authors.

Competing interests
The authors declare no competing interests.

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