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Divergent population responses following salamander mass mortalities and declines driven by the emerging pathogen *Batrachochytrium salamandrivorans*

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Understanding wildlife responses to novel threats is vital in counteracting biodiversity loss. The emerging pathogen *Batrachochytrium salamandrivorans* (*Bsal*) causes dramatic declines in European salamander populations, and is considered an imminent threat to global amphibian biodiversity. However, real-life disease outcomes remain largely uncharacterized. We performed a multidisciplinary assessment of the longer-term impacts of *Bsal* on highly susceptible fire salamander (*Salamandra salamandra*) populations, by comparing four of the earliest known outbreak sites to uninfected sites. Based on large-scale monitoring efforts, we found population persistence in strongly reduced abundances to over a decade after *Bsal* invasion, but also the extinction of an initially small-sized population. In turn, we found that host responses varied, and *Bsal* detection remained low, within surviving populations. Demographic analyses indicated an ongoing scarcity of large reproductive adults with potential for recruitment failure, while spatial comparisons indicated a population remnant persisting within aberrant habitat. Additionally, we detected no early signs of severe genetic deterioration, yet nor of increased host resistance. Beyond offering additional context to *Bsal*-driven salamander declines, results highlight how the impacts of emerging hypervirulent pathogens can be unpredictable and vary across different levels of biological complexity, and how limited pathogen detectability after population declines may complicate surveillance efforts.

1. Introduction

The current biodiversity extinction crisis takes place through a complex interplay of environmental changes, including habitat loss, climate change, pollution and infectious diseases [1]. Understanding how species respond to these anthropogenic threats presents a central challenge in biology, and a challenge to our ability to anticipate detrimental effects on biodiversity and guide effective conservation action [2,3]. Emerging infectious diseases are an increasingly documented factor causing biodiversity loss [4]. Contingent on other ecological stressors and the vulnerability of naive populations, introduced pathogens have

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the potential to drive once-abundant species to severe decline or local extinction [5] and, consequently, alter community dynamics and ecosystem function [6].

Global amphibian declines are among the most severe in the ongoing biodiversity crisis, with an estimated 41% of species threatened with extinction [7]. Over recent decades, chytridiomycosis, a disease caused by fungal pathogens of the genus *Batrachochytrium*, has been associated with the decline and extirpation of amphibian populations around the world [8,9]. Thus far, these declines are largely caused by the species *B. dendrobatidis* (*Bd*), which has been implicated in the declines of hundreds of amphibian taxa, and especially eroded frog diversity in the Neotropics and Australia following its emergence in the 1970s [8,10]. In the early twenty-first century, a second amphibian-pathogenic chytrid fungus, *B. salamandrivorans* (*Bsal*), emerged in northwestern Europe [11,12]. Like its sister species *Bd*, the pathogen was probably introduced through the international amphibian trade from its centre of origin in East Asia [13]. Being particularly lethal to salamander species, however, *Bsal* was projected to have devastating effects on salamander biodiversity hotspots, most notably in North and Central America as well as southern Europe [14–16]. Its emergence was therefore signalled as a key conservation threat that has the potential to strongly amplify the amphibian extinction crisis [17].

Presently, *Bsal* still has a clustered but rapidly expanding distribution in northwest to central Europe [18,19], and shows a localized outbreak in the northeast of Spain [20]. Within this European invasive range, its effects have mainly been studied in populations of fire salamanders (*Salamandra salamandra*), a large-bodied, terrestrial and nocturnal species, in which it caused mass mortality events with estimated population reductions of 90 to over 96% [21,22]. Other studies noted the large-scale absence of fire salamanders around *Bsal*-infected areas in which mass mortalities went unnoticed [23]. Although integral disease-risk models predict that population extinctions are indeed a highly likely scenario [24], individuals have been encountered in several outbreak sites multiple years after *Bsal* was detected [19,25,26]. Hence, much remains uncertain about *Bsal* biology and around the central question of how susceptible salamander populations respond to *Bsal* invasion.

The extinction risk of wildlife populations following the emergence of a novel pathogen has been strongly linked to host population sizes and pathogen transmission patterns [5,27]. Evaluating host numbers and pathogen prevalence in natural populations will therefore be an essential step in characterizing variation in population-level responses following disease emergence. Given the precipitous *Bsal*-driven salamander declines, the question remains whether predicted population extinctions present the predominant longer-term disease outcome, or to what extent population numbers may stabilize or rebound into a prolonged phase of host–pathogen coexistence (the enzootic state; cf. [28]).

In turn, however, pathogen transmission patterns may be strongly determined by host behaviour and life-history traits [29], and thus show system- or species-specific characteristics. For instance, when impacts vary along a host's life cycle, diseases may also affect the demographic structure of populations (e.g. [30]). In the salamander-*Bsal* system, direct transmission through courtship and territorial behaviour was hypothesized to cause disproportionate mortality in adults, raising the question if *Bsal* causes prolonged demographic shifts that hamper recruitment and population persistence following outbreaks [22]. Local environmental conditions may additionally create spatial variation in infection dynamics, thus possibly influencing host responses on smaller habitat scales [31]. In this context, preceding risk assessments for urodeles predicted that ecological features such as microclimate [32,33] and habitat complexity [34] shape *Bsal* infection dynamics, and could therefore mediate population-level responses to outbreaks in the wild.

Disease-driven population declines can furthermore affect population genetic structure by causing genetic bottlenecks [35,36]. Yet, these effects have been seen to vary substantially across host–pathogen systems, including both strong reduction but also preservation of genetic diversity following steep population declines [35]. Although bottleneck effects take shape over several generations, disease-afflicted salamander populations might show early signatures of genetic diversity loss that signal a weakened recovery potential. However, in turn, disease-driven declines can drive adaptation in host populations by favouring individuals with more adept immune responses [36,37]. While an increase in host resistance was not observed in the immediate aftermath of a *Bsal* outbreak [22], resistance may have increased with prolonged disease exposure following initial declines.

Taking the fire salamander as a highly susceptible study species, we here investigate the persistence of populations more than a decade following *Bsal* emergence in Europe. By comparing some of the earliest known outbreak sites (figure 1*a*) to uninfected reference sites, we (1) quantify post-outbreak population dynamics to assess population survival and relative population declines; (2) quantify post-outbreak pathogen prevalence to assess ongoing *Bsal* infection pressure and transmission; (3) evaluate population demographic structure and recruitment in reference to long-term behavioural data to assess disease-induced demographic change and the role of direct-contact behaviour; (4) perform spatial analyses to infer clustering patterns and habitat associations of salamander distributions; (5) perform microsatellite analyses to scan for potential early detectable effects of *Bsal*-driven declines on population genetic diversity and structure; and (6) perform non-invasive mucosome assays as a preliminary test for increased host resistance. Please refer to the electronic supplementary material for more context on the life history of fire salamanders in our study area.

2. Results

(a) Persistence of *Bsal*-affected salamander populations at low abundance compared to uninfected reference sites, and the apparent extinction of a small-sized population

Three outbreak sites show persistence of fire salamander populations after *Bsal* invasion and subsequent declines (figure 1*b*). Of these, the *Bsal* index site Bunderbos shows population survival more than a decade after the first detection of mortality, while two more populations both harbour persisting populations up to six years after *Bsal* detection in 2014. Population numbers in persisting populations remained low and showed no distinct pattern of recovery, with encounter rates in outbreak sites consistently ranking below the 5th

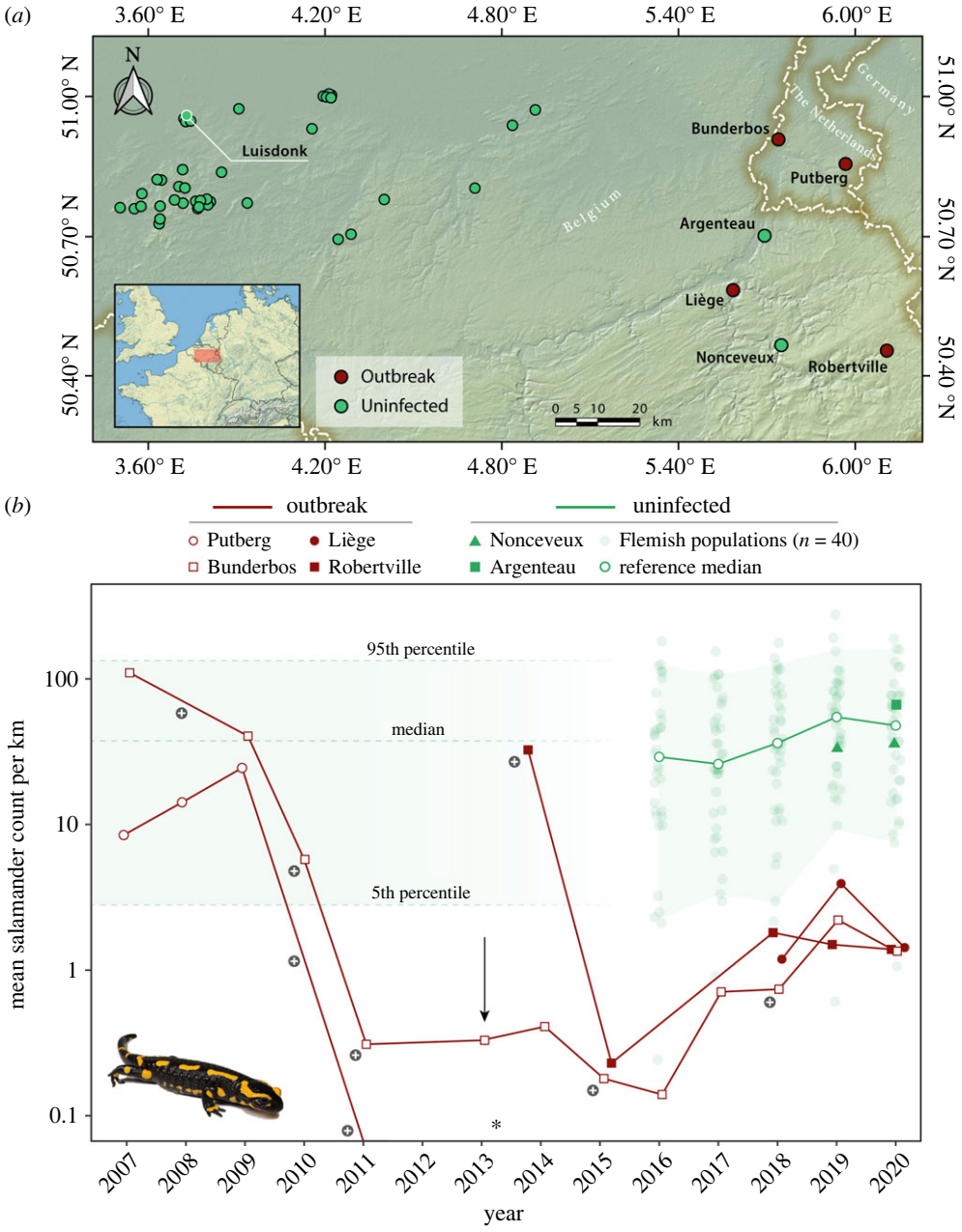

Figure 1. Population dynamics and pathogen detection in fire salamander (*Salamandra salamandra*) populations that experienced a *Batrachochytrium salamandrivorans* (*Bsal*) outbreak compared to uninfected reference sites. (*a*) Overview of focal populations. In addition to two reference populations (Nonceveux and Argenteau) situated in between outbreak populations, encounter rates in uninfected populations were characterized from 40 reference populations situated just outside of the infection front, monitored as part of the Flemish citizen science monitoring network during 2016–2020. Basemap adapted from GMRT (gmrt.org), inset adapted from Natural Earth (naturalearthdata.com). (*b*) Annual encounter rates of fire salamanders in *Bsal* outbreak populations compared to uninfected populations. The green shading highlights the variation within the 95th and 5th percentile of encounter rates in uninfected populations (with the overall variation across uninfected reference populations, as recorded in the period 2016–2020, extrapolated by dashed lines). An arrow denotes the discovery of *Bsal* in Bunderbos in 2013 [11]. Crosses denote the detection of *Bsal* and/or dead individuals, including infections identified retrospectively after the discovery of *Bsal*. An asterisk indicates the last living individual that was found at Putberg (and hence a non-zero encounter rate), after which no more fire salamanders were found in subsequent years up until the end of the study period in 2020. Note that *Bsal* outbreaks in Robertville and Liège were both detected in 2014 (see [38]), but unlike Robertville, no population monitoring was conducted in Liège directly following pathogen detection.

percentile of those recorded across uninfected reference populations. Mean annual counts ranged from a minimum of 0.14 salamanders per km of transect to a maximum of 3.93 salamanders per km following *Bsal* outbreaks. By contrast, in a probably introduced population (Putberg), which showed low pre-outbreak population counts and experienced declines concurrent with Bunderbos, a single living individual was last observed in 2013. Despite continued monitoring efforts, no remaining individuals (including larvae) were encountered up until the end of the study period. Log-linear regression coefficients furthermore indicated no population declines in uninfected reference sites equalling those seen in outbreak sites (electronic supplementary material, figure S1).

(b) Low recurrent *Bsal* detection and estimated prevalence in outbreak populations

Following initial pathogen detection and population decline, *Bsal*-positive and/or fire salamanders with clinical signs were only detected in 2015 and 2018 in Bunderbos (figure 1*b*). Post-outbreak *Bsal* prevalence estimates consequently remained

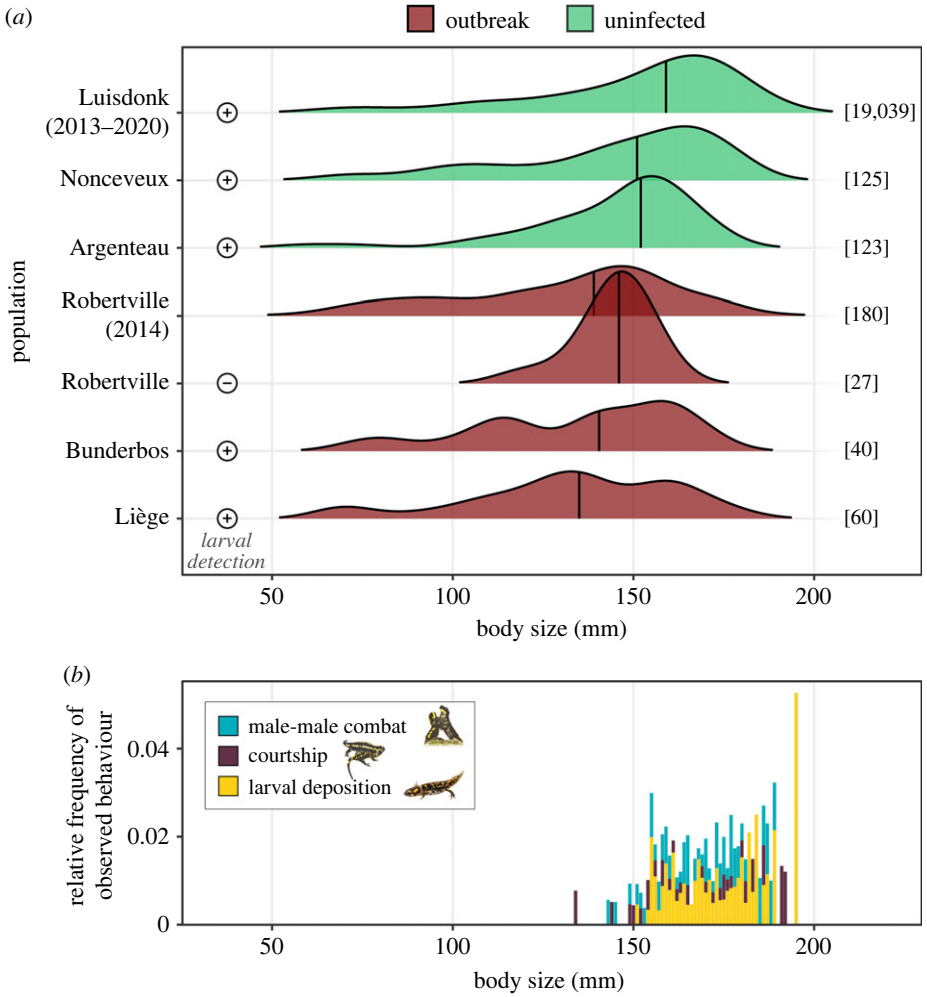

Figure 2. Body size distributions of fire salamanders (*Salamandra salamandra*) in *Batrachochytrium salamandrivorans* (*Bsal*) outbreak sites compared to uninfected reference sites and in reference to long-term behavioural data. (*a*) Recorded body sizes and larval detection across fire salamander populations, with vertical lines denoting population medians and sample sizes indicated between brackets. Body size data were collected around the recent study period 2018–2020, except where noted for: (1) The 2014 dataset of Robertville, which was obtained during the initial outbreak and demographic shift [22], providing a temporal comparison to recent observations; and (2) The 2013–2020 dataset of Luisdonk, providing a high-detailed reference of an uninfected population. The Luisdonk dataset was further used to evaluate the potential for sampling error in outbreak sites through resampling analyses and assessment of annual demographic stability (see electronic supplementary material, table S2 and figure S2). Larval presence (+) indicates detection in all study years, while absence (−) indicates no larval detection throughout the study period. (*b*) Observations of reproductive and territorial direct-contact behaviour ($n = 201$), relative to the total amount of observations per body size class.

low (electronic supplementary material, table S1), with a maximum estimated prevalence of 0.19 ($n = 16$, 95% CI: 0.07–0.45) in 2015. A single infected animal was found and prevalence estimates did not exceed 0.02 ($n = 61$, 95% CI: 0–0.08) in 2018. No recurrent infections or clinical signs were recorded among persisting animals found in the other two surviving populations. Also in 2019, the year showing the highest overall encounter rates across salamander populations, *Bsal* remained undetected across outbreak sites. Yet, upper confidence levels generally remained high as a result of small sample sizes in outbreak sites, thus not excluding *Bsal* occurrence at low prevalence.

(c) Enduring differences in the demographic structure of outbreak populations coincide with direct-contact behaviour

The demographic structures of outbreak populations showed ongoing signs of a size-structure truncation (figure 2*a*), as reflected by lower body sizes recorded in outbreak populations compared to uninfected populations (LMM, ß = 0.24, s.e. = 0.07, $t_{5.51} = 3.50$, $p = 0.01$; coefficient presents inverse relation following data transformation, see methods). Resampling analyses of uninfected sites and a high temporal stability of the long-term Luisdonk dataset (electronic supplementary material, figure S2) furthermore suggested that observed body size differences in outbreak sites unlikely resulted from sampling error due to smaller sample sizes or random temporal fluctuation. Since direct-contact behaviour was hypothesized to cause disproportionate adult mortality in *Bsal*-afflicted populations, demographic patterns were subsequently placed in context of long-term behavioural data.

Reproductive and territorial direct-contact behaviour was largely associated with body sizes above 150 mm (figure 2*b*). Body sizes associated with male–male combat (168.0 ± 10.6 mm, $n = 67$), courtship (166.2 ± 14.1 mm, $n = 30$) and females depositing larvae (169.2 ± 9.7 mm, $n = 104$) thus coincided with the larger size classes more frequently encountered within uninfected populations. However, despite

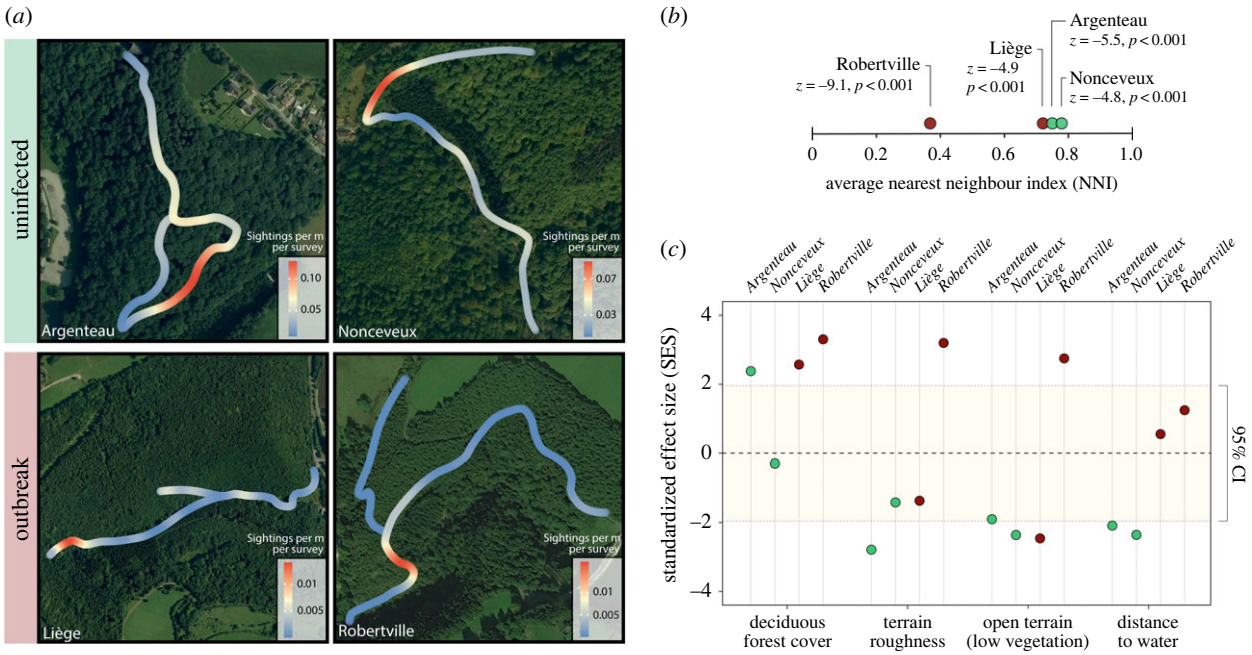

Figure 3. Spatial population patterns and habitat associations of fire salamanders (*Salamandra salamandra*) in *Batrachochytrium salamandrivorans* (*Bsal*) outbreak sites compared to uninfected reference sites. (*a*) Densities of fire salamander populations observed along transects surveys within the study period 2018–2020. Note the difference in absolute densities between outbreak and uninfected sites (see legend). (*b*) The Average Nearest Neighbour Index (NNI) of observed distributions in outbreak sites (magenta) and uninfected sites (green) in relation to the mean expected distance in randomized null models (1000 iterations). (*c*) Standardized effect sizes (SES) of observed distribution patterns in outbreak sites (magenta) and uninfected sites (green) compared to the mean expected values of null models for selected environmental parameters at the scale of ecotopes (see electronic supplementary material, table S3 for details). Satellite imagery copyright: Google.

showing a higher relative proportion of smaller individuals compared to uninfected sites, the outbreak sites Bunderbos and Liège retained a broad range of body sizes, including larger animals in reproductive size classes, and both larvae and juveniles being detected each year (figure 2*a*). Conversely, only a narrow cohort of young adult animals was recorded in the outbreak site of Robertville, and no larvae, juveniles or large adult animals could be detected. This is in contrast to 2014, when these size classes were still found during the early stages of the outbreak and demographic shift.

(d) Variable post-outbreak distribution patterns, including a population remnant in aberrant habitat

Individuals in both outbreak and uninfected study populations displayed a degree of clustering based on transect surveys completed in the period 2018–2020 (figure 3*a*), with all salamander distribution patterns showing an Average Nearest Neighbour Index (NNI) below one, and differing significantly from randomized null models (figure 3*b*). The NNI nevertheless indicated a disparity between the outbreak sites. While individuals in Liège showed clustering comparable to those in reference populations (with NNI ratios ranging between 0.72 and 0.78), persisting individuals in Robertville showed a considerably more clustered occurrence with an NNI of 0.37. The observed salamander distributions were predominantly linked to ecotopes with high deciduous forest cover, low terrain roughness and low open terrain cover in comparison to null distributions (figure 3*c*), in line with known fire salamander habitat preferences. The population cluster in Robertville, however, showed an inverse association with ecotopes containing high terrain roughness and open terrain, with observed mean values above the 95% confidence intervals of expected null distributions. Uninfected populations were furthermore observed in relative proximity to water, while outbreak populations showed no distinct association.

(e) No early indications of a strong loss of genetic diversity, yet nor of increased host resistance

Temporal comparisons in outbreak populations thus far show neither a significant decrease in allelic richness (table 1; permutation test, $p = 0.5$) nor expected heterozygosity ($p = 0.87$) between early outbreak and recent time points. However, population structure inferences provided some indications that the allelic composition of outbreak populations was more uniform in the recent sampling period, hinting at a recent loss of variation (figure 4*a*; electronic supplementary material, figure S3). A significant genetic divergence (F_{ST}) between time points in Bunderbos furthermore indicates a shift in allele frequencies over time (figure 4*b*). This is corroborated by a depletion of low frequency alleles between 2012/2014 and 2018–2020 at outbreak sites (figure 4*c*), even though no mode-shift was detected. Yet, reflecting the thus far limited indications for early stage genetic decline, a significant excess in heterozygosity, i.e. a bottleneck was only indicated for outbreak populations when the analysis assumed merely 20% of the mutations to follow the SMM (table 1). A third outbreak site, Liège, for which no temporal reference was available, furthermore showed comparatively high levels of genetic diversity similar to nearby uninfected reference sites.

Mucosome samples collected in both outbreak and uninfected sites in the period 2018–2020 showed a very limited capacity for *Bsal* zoospore inhibition (electronic

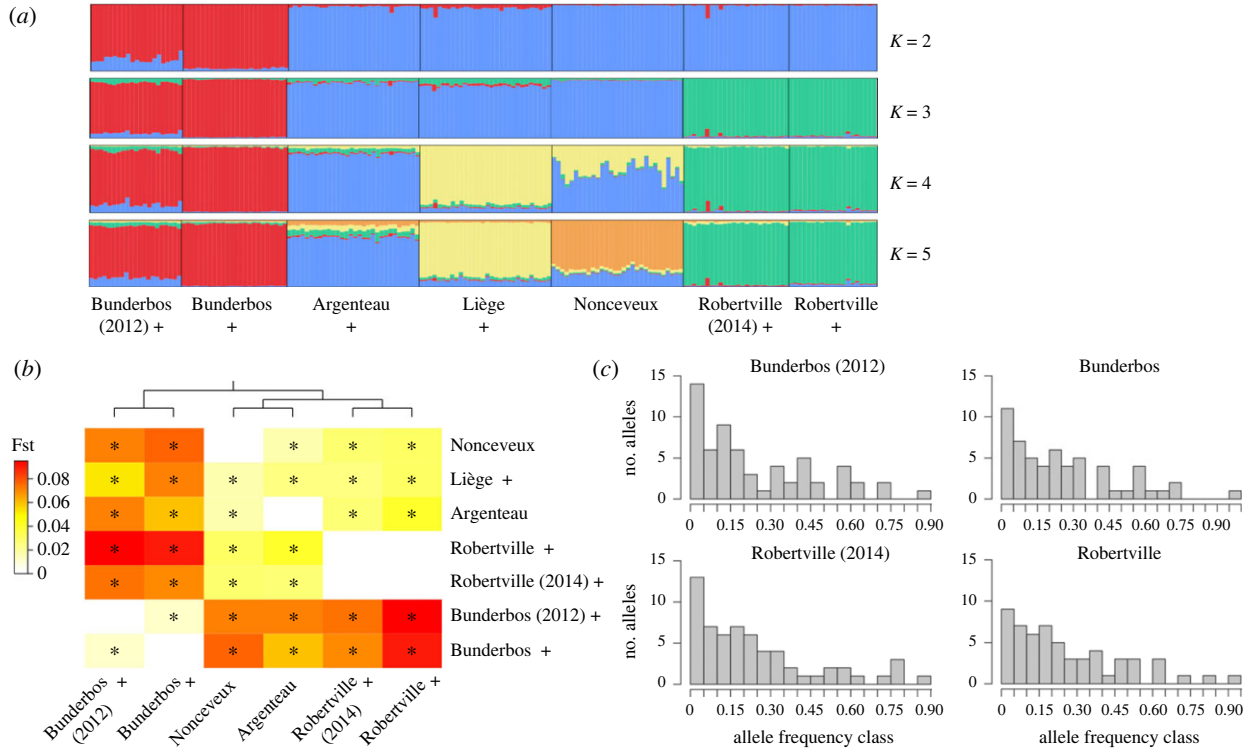

Figure 4. Population genetic comparisons of fire salamanders (*Salamandra salamandra*) in *Batrachochytrium salamandrivorans* (*Bsal*) outbreak sites and uninfected reference sites. (*a*) Genetic population structure inferred using the Bayesian clustering algorithm STRUCTURE at $K = 2$–5. (*b*) Heatmap and dendrogram of pairwise F_{ST} distances. Significant comparisons are indicated by an asterisk. (*c*) Number of alleles per allele frequency class (mode-shift indicator). Genetic samples were collected in the field in the recent study period 2018–2020, except where noted for individuals sampled from *ex-situ* populations, stemming from early-outbreak phases in Bunderbos in 2012 and Robertville in 2014. Outbreak sites are indicated with a cross when compared to uninfected populations.

Table 1. Diversity metrics and bottleneck results for *Bsal* outbreak sites and uninfected reference sites. *n*: sample size A: mean number of alleles per locus, A_R: allelic richness, H_O/H_E: observed/expected heterozygosity, F_{IS}: inbreeding coefficient, SMM: stepwise mutation model (bottleneck), at various increments. Significant probabilities for heterozygosity excess are indicated by an asterisk. Genetic samples were collected in the field in the recent study period 2018–2020, except where noted for individuals sampled from *ex-situ* populations, stemming from early-outbreak phases in Bunderbos in 2012 and Robertville in 2014.

							bottleneck: Wilcoxon test		
site	*n*	*A*	A_R	H_O	H_E	F_{IS} (95% CI)	20% SMM	70% SMM	90% SMM
Bunderbos (2012)	21	4.1	3.65	0.54	0.52	−0.03 (−0.14–0.08)	0.114	0.380	0.848
Bunderbos	24	3.8	3.46	0.49	0.5	0.003 (−0.07–0.12)	0.023*	0.227	0.598
Robertville (2014)	24	4.1	3.7	0.5	0.5	−0.006 (−0.08–0.07)	0.138	0.401	0.789
Robertville	20	3.8	3.54	0.52	0.52	−0.008 (−0.11–0.07)	0.041*	0.165	0.38
Liège	30	4.8	4.31	0.53	0.53	0.01 (−0.06–0.07)	0.467	0.805	0.968
Nonceveux	30	4.3	4.18	0.56	0.54	−0.04 (−0.10–0.02)	0.319	0.719	0.939
Argenteau	30	5.0	3.83	0.51	0.52	0.01 (−0.04–0.06)	0.151	0.532	0.885

supplementary material, figure S4), and did not differ between populations (ANOVA, $F_{4,45} = 0.54$, $p = 0.71$), thus tentatively providing no indications of increased host resistance.

3. Discussion

Gaining a better understanding of the direct and continued impact of environmental threats on amphibian populations is essential in confronting global species and population extinctions. Here, we conducted a multidisciplinary study to the ongoing effects of *Batrachochytrium salamandrivorans* (*Bsal*) invasion on wild populations of the fire salamander (*Salamandra salamandra*), a highly susceptible European species.

In contrast to predictions of epidemiological models [24], our continued monitoring studies further substantiate the possibility for populations to persist following *Bsal*-driven declines [19,22,25], as we highlight prolonged population survival from 6 years in Robertville and Liège up to more than a decade at Bunderbos. Our results nevertheless also underline an important nuance to these patterns, as post-outbreak population numbers remained very low, even across the long study period in Bunderbos. Consequently, persisting populations most likely remain at increased vulnerability to

further environmental stressors [3,5], Allee effects, or demographic and environmental stochasticity. Local extirpation can nonetheless be a realistic disease outcome in the short term after *Bsal* invasion, as evidenced by the complete absence of fire salamanders at Putberg since 2013. Notably however, this single extinction event occurred in an initially small-sized population, which was likely introduced from a small founder base. Hence, in line with theoretical risk factors for disease-induced extinction [5], *Bsal* may especially imperil species and populations with low initial population sizes or those thinned by pre-outbreak perturbations, including endemic and already endangered species [27]. This will particularly hold true given at least partial density-independent transmission patterns (also see further below).

In the persisting, strongly reduced populations, *Bsal* was only sparsely and intermittently re-detected. This yielded low post-outbreak prevalence estimates, although low sample sizes leave considerably wide confidence intervals. Yet, these combined observations indicate there is a limited window of opportunity to detect *Bsal* invasion in natural populations, which mostly only becomes apparent by sudden observations of mass mortality [21,22]. Indeed, as *Bsal* also caused unnoticed declines in fire salamanders [23], a conspicuous and large-bodied species, detecting the pathogen could be especially challenging in more secretive species, or in species that receive less research attention. This emphasizes the importance of active monitoring networks and crowd-sourced reports to detect declines, delineate *Bsal*'s presence, and provide an early warning upon disease emergence.

Inevitably therefore, our insights are thus far based on a limited number of populations, a limited geographical extent, and a still limited time period after *Bsal*'s relatively recent emergence in Europe. Our demographic, spatial and genetic comparisons nevertheless reveal various midterm effects of *Bsal* invasions, thereby giving rise to hypotheses and avenues for future research to assess its ongoing threats to salamander populations.

In addition to low population numbers, our results suggest that—at least in fire salamanders—*Bsal*-afflicted populations might be characterized by enduring demographic signatures. Outbreak sites showed a low relative abundance of large individuals in reproductive age/size classes up to multiple years after outbreak, and apparent recruitment failure in Robertville. These observations could be a lingering effect of a large demographic shift during initial population crashes [22]. However, the possibility that *Bsal* transmission becomes functionally frequency-dependent towards maturity warrants further attention in conservation assessments (see also [39]). Hence, multiyear mark–recapture studies would be needed to assess survival and recruitment rates, but also potential life-history changes, following *Bsal* invasion. As shown in other wildlife disease systems, including frogs impacted by *Bd* [40], but also Tasmanian devils impacted by facial tumour disease [30], earlier maturation and increased recruitment may allow populations to persist by compensating for strong adult mortality.

Another key question remains to what extent local landscape features are conducive to disease spread, or might facilitate population persistence (see also [18]). Following outbreaks in 2014, individuals in Liège showed clustering and habitat associations comparable to uninfected populations, but those in Robertville showed a highly clustered occurrence in ecotopes with relatively rough terrain and a lower, more open vegetation structure. This pattern in Robertville is interesting, as increased ambient temperatures [32,33] (reduced canopy cover increases maximum temperatures at ground level; [41]) as well as habitat complexity [34] have been shown to reduce infection pressure in experimental salamander-*Bsal* studies. Also under natural conditions, such as seen in frogs affected by *Bd* [31] and birds affected by avian malaria [42], local environmental conditions may constrain pathogen impacts. Our observations add to the notion that (partial) environmental refuge from *Bsal* may contribute to population persistence, yet requires further research to establish causal relationships. Our results also show it is generally advisable that post-outbreak searches extend beyond core forest habitats to allow detection of salamanders residing in more atypical habitats [26,31].

Considering post-outbreak pathogen prevalence stayed low, and increased host resistance is unlikely (see also [22]), our overall results lead us to hypothesize that the observed persistence of fire salamander populations is a result of individuals staying free from transmission events. The persistence of small and size-truncated populations indicates this may be partially mediated by life-history characteristics. While transmission might become functionally frequency-dependent towards maturity, density-dependent transmission may still predominate throughout early life. A plausible explanation for this is that juvenile salamanders are known to preemptively avoid conspecifics to escape predation and general competition (e.g. [43]). Still, density-dependent transmission may occur regardless of age or below a certain population density threshold which, following basic disease theory, could also explain the persistence of low-density populations [44]. Indeed, as for example suggested for bats impacted by white-nose syndrome [45], density-dependent effects could modulate population impacts in some cases. However, our results also indicate that local population densities may not necessarily decrease linearly with overall population size, as persisting animals may be locally clustered and clustering patterns can differ between outbreak sites. In this regard, we expect (micro)habitat conditions to play an important role in regulating spatial separation and, with that, transmission between individuals. More studies on host movement across different life stages and in relation to abiotic variation will therefore be needed to better understand what environmental features may effectively facilitate salamander persistence.

Along these lines, the timing of pathogen emergence might be another important yet unaddressed factor in determining population-level effects; in view of the fast mortality of fire salamanders upon infection [11], individuals probably have a higher chance of avoiding transmission when invasion takes place outside of the breeding season. The potential for disease spread during periods of inactivity remains however unknown, as animals may still come into contact in underground refuges. However, as salamander movement is restricted during these periods and climatic conditions are generally above or below *Bsal* thermal optima [32], potential transmission events can be expected to be more localized. A further uncertain aspect is to what extent repeated introductions, either anthropogenic or through animal and non-animal reservoirs, might perpetuate infection pressure in populations. In Bunderbos and Putberg, *Bsal* was also detected in the newt species *Lissotriton vulgaris* and *Ichthyosaura alpestris* [38], which occur in syntopy with fire salamanders. Some *Bsal* strains are furthermore able to sustain themselves in aquatic

habitats as a facultative saprophyte [46], and spores may infect amphibians from contaminated soil [22]. Consequently, the periodic re-detection of *Bsal* in fire salamanders as seen in Bunderbos might have been a result of repeated spillover from external sources. These disease reservoirs moreover suggest that *Bsal* can continue to inflict mortality in (density) reduced populations.

Interestingly, contrary to theoretical models and small-scale experiments, a recent large-scale study across hundreds of frog populations affected by *Bd* revealed that population densities only have a very limited influence on real-life disease dynamics and outcomes [47]. Instead, environmental variables such as climatic factors and disease reservoirs were found to be better predictors of amphibian population responses. Such findings illustrate the challenge of identifying large-scale drivers of amphibian declines based on a limited number of populations in the relatively short term after pathogen emergence. The present study provides much needed insights into the longer-term impact of *Bsal* across multiple levels of complexity. Still, the patterns observed among the four focal outbreak sites might have been influenced by stochastic variation among the various biotic and abiotic factors possibly driving disease dynamics. Thus, follow-up studies in the increasing number of known European outbreak sites are essential to further characterize the variability in population responses to *Bsal* as revealed in our study. Additionally, insights into *Bsal*'s effects on wild populations of other species are only just starting to emerge [20,48], and probably differ significantly among urodeles given their diverse life histories and ecological niches.

Eventually, co-evolutionary interactions between host resistance and pathogen virulence may increase the chances for population persistence [37]. However, fire salamanders have a relatively long generation time, and potential population recovery and acquiring resistance can therefore be a slow process. In addition, demographic models suggest that amphibian population recovery after severe disease-driven declines can take many years in general (e.g. [49]). Indeed, our mucosome assay showed a still low zoospore inhibition potential that is comparable to previous studies [33,50], provisionally indicating that individuals are still highly vulnerable to *Bsal*. Yet, it should be noted that these analyses do not rule out increased resistance as a factor (partially) contributing to the observed population persistence. Also, exogenous protection by microbial symbionts may convey a degree of resistance on shorter timescales than acquired immunity. Our analysis shows no strong loss of neutral genetic diversity thus far, suggesting these populations hold the potential to largely retain their genetic integrity. Complementary sampling and immunogenetic analyses across an extended timespan will nevertheless be required to confirm these trends and establish adaptive processes.

Overall, our findings illustrate how the impacts of emerging hypervirulent pathogens can be unpredictable, even for some of the most susceptible host species. For one, we confirm the possibility of extinction, and point at the role of pre-epidemic population sizes in determining extinction risk [5,27]. Yet, we additionally show how host responses within surviving populations may vary in a demographic, ecological as well as a molecular context, despite showing similar (reduced) host encounter rates after outbreaks. In turn, this emphasizes the importance of continued field assessments in the wake of pathogen emergence, especially since the efficacy of conservation measures will be highly dependent on the specific mechanisms underlying host persistence in the wild [3]. Finally, our findings also demonstrate how wildlife disease studies may become complicated by reduced pathogen detectability after initial population declines. Consequently, when pathogen introductions into naive populations lead to near-extinctions, passive surveillance systems may not be sufficiently effective in detecting emerging pathogens across broad spatial scales.

4. Methods

(a) Study area and population monitoring

Fire salamander populations in Bunderbos, Putberg (the Netherlands), Robertville and Liège (Belgium) were the focal *Bsal* outbreak sites in our study (figure 1*a*). To characterize ongoing host–pathogen dynamics in these disease-afflicted populations, we integrated long-term inventory data from both infected and uninfected populations. First, Bunderbos and Putberg have been part of a long-running Dutch citizen science monitoring programme, which led to the detection of salamander mass mortality around 2010 and the subsequent discovery of *Bsal* in Bunderbos in 2013 (see [11,21]). The population of Putberg was probably introduced from nearby Belgian populations, and showed low historical population counts since its discovery in 1994 [21,51]. We included pre-outbreak and recent monitoring data from these populations to span the period 2007–2020. Second, Robertville and Liège present two of the earliest known outbreak sites in Belgium, in which disease-driven mortality was recorded in 2014 (see [22,38]). As no surveys were performed in intervening years, we conducted new surveys in these populations in the period 2018–2020. Third, to put post-outbreak encounter rates in perspective, outbreak sites were analysed in reference to 40 uninfected populations situated just outside of the infection front, surveyed as part of the Flemish citizen science monitoring network from 2016 to 2020. We additionally selected two uninfected populations in Nonceveux and Argenteau, situated in between the focal outbreak sites (figure 1*a*), to serve as geographically adjacent reference sites in demographic, spatial, mucosome and genetic analyses (see next sections). While it is impossible to completely rule out *Bsal* presence within a given site, fire salamander populations were considered uninfected when they remained free from observed mortality events, clinical signs or pathogen detection during preceding large-scale disease surveys (*sensu* [18]).

In each of the study populations, monitoring activities followed a similar protocol, in which standardized transect-based surveys were performed during salamander activity peaks in spring and/or autumn, and during which all individuals within visual range of the transect path were recorded. All surveys were conducted under the requirement of meeting favourable conditions for fire salamander activity: taking place after dusk, following or during rainfall and with an ambient temperature of ≥ 5°C. Only completed transects were included to ascertain the total sampling effort within a year. The surveys did not account for imperfect detection, which is unlikely to be problematic because we assumed that there was no temporal trend in detectability, as no such trends were indicated by mark–recapture studies [52]. To accommodate variation in transect lengths and the number of completed transects, mean salamander encounter rates were quantified relative to the total distance covered in each population in a given sampling year. Fire salamander populations can however show strong natural fluctuations in abundance [52]. To assess whether population drops as seen in *Bsal*-infected sites equally occurred in uninfected sites, we performed log-linear regression analysis of annual

encounter rates in individual populations, taking all sites with at least 5 years of available data.

(b) *Bsal* screening

We collected ventral skin swabs from persisting animals encountered in the field to assess the continued presence of *Bsal*. Swab samples were analysed using real-time PCR after Blooi *et al*. [53], all performed in duplicate to minimize detection error. Prevalence estimates and associated 95% confidence intervals were obtained using the *epiR::epi.prev* function in R while accounting for test sensitivity and specificity, set at 96.2% and 100%, respectively following qPCR assay validations [54]. See electronic supplementary material, table S1 for details on post-outbreak sampling and detection.

(c) Demographics and recruitment

To investigate the extant demographic structure and recruitment in populations, we performed morphometric comparisons and larval surveys. To this end, we measured the total body size of post-metamorphic salamanders (to the nearest mm) in the three surviving outbreak populations and the nearby reference sites Nonceveux and Argenteau during field surveys around the study period 2018–2020 (includes a visit early 2021 in Bunderbos and two visits late 2017 in Liège, not forming complete transects). For each population, we took measurements at various points within a year (provided weather and logistical constraints) to avoid large biases stemming from seasonal demographic variation. In parallel, populations were surveyed for larvae by visually scanning suitable reproductive water bodies along the established transects, including both perennial streams and ephemeral forest ponds. This was repeated at least once following the larval deposition period in spring to minimize false-absence detection error.

The collected measurements were compared to two other data sources. First, we incorporated morphometric data taken in Robertville in 2014. These were collected at the onset of *Bsal* outbreak (see [22]), thus allowing us to draw comparison to the demographic structure during the initial period of population decline. Second, we included a long-term population dataset from the Flemish population in Luisdonk. This includes an extensive number of individual records ($n = 19\,039$) collected during 2013–2020, thus providing a high-detailed reference of the demographic structure of an uninfected population, as well as elaborate reference data of host behaviour (see next section).

We used a linear mixed-effects model (LMM) to assess differences in body sizes between outbreak and uninfected reference sites, with *Bsal* (infected/uninfected) as fixed effect, while including site as a random factor using the *lme4::lmer* function in R. For the LMM, the 2014 and recent datasets for Robertville were pooled to represent all data at population level. As model residuals were negatively skewed using the unedited response values, data points were reflected and log-transformed to ensure a normal error distribution (Shapiro–Wilk $W > 0.95$). In addition, we sought to evaluate the potential of sampling error leading to recorded body size differences in outbreak sites, either from smaller sample sizes or random demographic fluctuation. This was done through resampling analysis of uninfected population and linear regression of annual body size data of the extended Luisdonk dataset (see electronic supplementary material, S3).

(d) Behavioural data

As demographic shifts are thought to be causally linked to host behaviour [22], we additionally assembled fire salamander behavioural data. Across the eight-year study period in Luisdonk, the behaviour of individuals was recorded at the moment of observation. We collated all observations made of behaviour directly pertaining to reproductive and territorial contact: male–male combat, courtship and larval deposition ($n = 197$). Four additional observations of these behaviours during recent surveys in Nonceveux and Argenteau were also included, amounting to 201 total observations. Behavioural records were then expressed relative to the number of observations per body size class.

(e) Spatial analyses

We further aimed to evaluate whether persisting individuals might occur in confined distributions (i.e. potential refugia) and/or show specific environmental correlates following *Bsal* outbreaks. For this purpose, we focused our analyses on the two most recent outbreak sites, Liège and Robertville. In conjunction with morphometric data collection efforts in the period 2018–2020, we logged the position of measured animals using a handheld GPS (Garmin GPSmap 62s), and compared spatial distribution patterns with those in the nearby reference sites Argenteau and Nonceveux. For this comparison, transects in these four populations were established to cover various distances from aquatic habitat, and leading through various amounts of habitat heterogeneity provided the site characteristics and existing network of paths.

First, we analysed population patterns by means of the average Nearest Neighbour Index (NNI) [55]. For analyses, we only included records taken on completed transects to omit spatially biased field visits. Transects and coordinates were plotted in ArcGIS 10.1, and we projected a 15 m radius around the transects, which was seen to best approximate the visual range from within which the vast majority of animals were observed during surveys. Coordinates falling outside of this radius were removed to correct for inaccurate GPS measurements. These restrictions resulted in 112 coordinates in Argenteau, 102 in Nonceveux, 46 in Liège and 27 in Robertville comprising the observed distribution patterns. For each population, we then generated null models by resampling the observed coordinates within the specified 15 m radius across 1000 randomizations using the *spatstat::rpoint* function in R. The NNI, standard errors and derived *z*-scores and *p*-values were calculated after the original description by Clark and Evans [55].

Subsequently, we assembled a set of environmental parameters from the LifeWatch ecotope database (http://maps.elie.ucl.ac.be/lifewatch/ecotopes.html). This database combines LIDAR-based height models, spectral data and topography to delimit ecotopes, which are considered to be the smallest ecologically distinct features in a landscape. From the LifeWatch polygon maps (2015, v2.15), we selected a range of underlying parameters pertaining to habitat complexity and terrain variability, and extracted values for the salamander distribution patterns across the ecotopes in each site. In addition, we mapped streams within study sites in ArcGIS 10.1 based on maps of the Belgian National Geographic Institute (https://topomapviewer.ngi.be/), and computed the distance of recorded animals to the nearest waterway (streams/brooks) using the *sf::st_distance* function in R. Collinearity between all initial variables of interest was assessed through Pearson correlation coefficients (r). We omitted similar parameters ($r > |0.75|$), and retained four variables for analyses: distance to water, terrain roughness, open terrain and deciduous forest cover. See electronic supplementary material, table S3 for a metadata overview of considered parameters, data sources and collinearity. Subsequently, we generated null distributions for parameters using the same null models as used in NNI analyses. These were assessed for normality using QQ plots and, if necessary, log- or square root-transformed to improve fit. To compare the values of observed distribution patterns against those found in the ecotopes throughout the entirety of the transect, standardized effect sizes were calculated as $SES = (I_{obs} - I_{exp})/\sigma_{exp}$, in which I_{obs} presents the mean parameter value of the observed distribution pattern, and I_{exp} and σ_{exp} respectively present the mean

and standard deviation of the parameter values across the 1,000 randomizations of the null distribution.

(f) Genetic analyses

We collected buccal swabs to evaluate potential early detectable effects of *Bsal*-driven declines on population genetic structure and diversity through microsatellite analyses. A total of 179 individuals ($n = 20$–30 per population; table 1) were genotyped for 19 microsatellite loci (following [56]). Samples were collected in the period 2018–2020 across focal outbreak sites and adjacent reference sites. For temporal comparison, we additionally sampled fire salamanders safeguarded in *ex-situ* populations during and following early outbreak phases in 2012 and 2014 in Bunderbos and Robertville, respectively. For Bunderbos, this includes three F1 descendants from meanwhile deceased founder females and three from already sampled founder females. As these females were gravid when taken into the breeding programme, all six F1 individuals presented unsampled paternal lineages. Please refer to the electronic supplementary material, S5 for a description of the different genetic analyses and associated software packages.

(g) Mucosome assay

We used the mucosome assay developed by Smith *et al*. [50] to gain a preliminary indication whether persisting populations show increased resistance against *Bsal* (strain AMFP13/01). The amphibian mucosome (skin mucosal ecosystem) contains both host immune factors as well as microbial symbionts, and presents the first line of defence against pathogens. By testing the chytrid zoospore inhibition potential of mucosome samples gathered in the field, this method provides a non-invasive yet indirect proxy of host susceptibility. Mucosome samples were collected from salamanders *in situ* in the three surviving outbreak sites and two nearby reference sites ($n = 10$ per population) during 2018–2020 field efforts. Please refer to Smith *et al*. [50] for details on the collection and laboratory protocols specific to mucosomes.

Ethics. Data collection was approved by the Service Public de Wallonie Agriculture, Ressources naturelles et Environnement and carried out under permit 2016/RS/n°34 avenant2 and 2020/RS/n°01 for studies in Wallonia, by Agentschap Natuur en Bos under permit ANB BL FF V21-202358 for studies in Flanders, and by the Netherlands Enterprise Agency under permit FF/75A/2016/015 for studies in the Netherlands. Strict biosecurity protocols were followed to prevent disease spread: handling salamanders with clean nitrile gloves, no visits to outbreak and uninfected sites on the same sampling occasion, rigorous disinfection of all materials after each site visit, and maximum use of different sets of field gear between outbreak and uninfected sites. All data collection steps were designed to minimize disturbance in populations, no animals were translocated, and all animals were returned to their original location directly after collection of the necessary data.

Data accessibility. All data and code are available at the Figshare open access repository: https://doi.org/10.6084/m9.figshare.21076129 [57].

Supplementary material is available online [58].

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. J.E.: conceptualization, formal analysis, investigation, methodology, visualization, writing—original draft, writing—review and editing; K.P.: formal analysis, writing—review and editing; J.S.: data curation; W.B.: methodology; A.S.-v.d.S.: data curation; T.S.: data curation; A.L.: resources; T.K.: resources; B.R.S.: methodology; A.M.: conceptualization, methodology, supervision, writing—review and editing; S.S.: conceptualization, methodology, supervision, writing—review and editing; F.P.: conceptualization, methodology, supervision, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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