



Spread of the pathogen *Batrachochytrium salamandrivorans* and large-scale absence of larvae suggest unnoticed declines of the European fire salamander in the southern Eifel Mountains

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Abstract. Emerging infectious diseases are one of the main suggested reasons for global amphibian decline. Fungal agents play a key role. Since its introduction, the Asian pathogen *Batrachochytrium salamandrivorans* has driven the European fire salamander, *Salamandra salamandra*, to the edge of extinction in the Netherlands and caused severe population declines in Belgium and Germany. We screened 1,526 amphibians (1,431 urodelans and 95 anurans) from 50 sites in a 1,500 km² large area in Germany, south of the next known infected populations. Furthermore, we conducted a presence-absence mapping of larval salamanders in 88 randomly selected creeks and creeks where salamanders had been reported in the past using a standardized removal sampling approach. Our results revealed an expanded distribution of the pathogen in Western Germany and we could detect seven infected urodelan populations including the southernmost locality of the fungus in its exotic range. Larval salamanders were found in 54 out of 63 creeks south, but only in seven out of 25 creeks north of a highway that divides the study area. *Bsal* infection could mainly be detected in newts (*Ichthyosaura alpestris* and *Lissotriton helveticus*) because many previously known European fire salamander populations most likely disappeared in the affected forest regions, however, silently and without observed mass mortalities. The only detectable salamander population north of the highway was found to be *Bsal*-infected at high prevalence in 2019, but not from 2016 to 2018, suggesting a recent infection event. Overall, prevalence at the individual (2.6%) and population level (14%) was very low. Moreover, modelling habitat suitability in seemingly unaffected areas suggests that most reproduction creeks and surrounding land habitats in the seemingly affected area are still suitable for *S. salamandra*, supporting our suspicion that the absence of the species is disease-related rather than habitat related.

Key words. *Bsal*, chytridiomycosis, Germany, *Ichthyosaura alpestris*, *Lissotriton helveticus*, *Salamandra salamandra*.

Introduction

Emerging infectious diseases pose an increasing threat to global biodiversity (DASZAK et al. 2000, 2003). They are often driven by fungal agents (FISHER et al. 2012). One of the most blatant such diseases for amphibians is chytridiomycosis (FISHER et al. 2009, VAN ROOIJ et al. 2015). The chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) has been suggested to play a key role in the sudden and rapid declines that we have started witnessing more than 30 years ago (BERGER et al. 1998, LA MARCA et al. 2005, SKERRATT et al. 2007). This pathogen is known from all continents where amphibians occur, and it has been recorded

from all three amphibian orders (FISHER et al. 2009, VAN ROOIJ et al. 2015). The global pet trade has been suggested to have significantly disseminated *Bd* (FISHER et al. 2009, VAN ROOIJ et al. 2015, O'HANLON et al. 2018). More recently, a second amphibian chytrid fungus (*Batrachochytrium salamandrivorans*, *Bsal*) has been identified as the cause of a mass dying event in a Dutch population of the European fire salamander, *Salamandra salamandra* (SPITZEN-VAN DER SLUIJS et al. 2013, MARTEL et al. 2013).

Bsal is suggested to be native in Asia from where it has apparently been introduced into Western Europe via asymptomatic vectors, most probably via the pet trade (MARTEL et al. 2014, LAKING et al. 2017). After its discovery

in the Netherlands (MARTEL et al. 2013, 2014) and Belgium (SPITZEN-VAN DER SLUIJS et al. 2016, STEGEN et al. 2017), *Bsal* was detected in wild urodelan populations in Germany in 2015 (SPITZEN-VAN DER SLUIJS et al. 2016, DALBECK et al. 2018). Its presence in Germany can be traced back to at least 2004 (LÖTTTERS et al. 2020a, b in this issue), and by now it is known from about 50 sites in the wild in Belgium, Germany, the Netherlands and Spain (for an overview see LÖTTTERS et al. 2020a in this issue). In addition, records of *Bsal* in captive amphibians add Sweden and the UK to the *Bsal*-positive countries outside its native range (SABINO-PINTO et al. 2015, 2018, FITZPATRICK et al. 2018). Different to *Bd*, *Bsal* affects urodelan amphibians only, while anurans can be infected without signs of disease; no data exist on gymnophiones (MARTEL et al. 2014, STEGEN et al. 2017, NGUYEN et al. 2017). So far, one case of *Bd* and *Bsal* coinfection in European fire salamanders, one case in great crested newt (*Triturus cristatus*) and two cases in alpine newts (*Ichthyosaura alpestris*) are known (LÖTTTERS et al. 2018, 2020a in this issue).

From the core area of *Bsal* in Europe (Belgium, Germany, the Netherlands), in addition to the European fire salamander, the following *Bsal* hosts have been confirmed in the wild: alpine newt, palmate newt (*Lissotriton helveticus*), smooth newt (*L. vulgaris*), great crested newt (SPITZEN-VAN DER SLUIJS et al. 2016, DALBECK et al. 2018). In addition, two more *Triturus* species have recently been confirmed as *Bsal* hosts in Spain (MARTEL et al. 2020). While some newt species such as the alpine newt can carry *Bsal* (at low infection levels) without showing clinical symptoms and therefore serve as potential *Bsal* reservoirs and vectors, a *Bsal* infection is lethal for European fire salamanders (STEGEN et al. 2017). As a result, all known Central European outbreaks in wild European fire salamanders led to massive population declines (SPITZEN-VAN DER SLUIJS et al. 2013, 2016, MARTEL et al. 2014, STEGEN et al. 2017, DALBECK et al. 2018, SCHULZ et al. 2018, 2020 in this issue).

Approaches to model the suitable ranges for *Bsal* outside the current invasion range suggest that large areas of Western Europe might be suitable (FELDMEIER et al. 2016, BEUKEMA et al. 2018, LÖTTTERS et al. 2020a in this issue). Accordingly, both future and previously overlooked population collapses of *S. salamandra* are to be expected. WAGNER et al. (2017) hypothesized that the apparent absence of the species in large portions of the northernmost Mountains ('Schneifel') in the Southern Eifel, an area close to known *Bsal* infection sites (cf. SPITZEN-VAN DER SLUIJS et al. 2016, STEGEN et al. 2017, DALBECK et al. 2018) and where formerly European fire salamanders were widespread (BITZ et al. 1996), might be explained by overlooked *Bsal* outbreaks.

Apart from occasional detection of carcasses or living animals showing symptoms of salamander chytridiomycosis, also known as the 'salamander plague', or of *Bsal*-positive skin swabs taken from seemingly healthy specimens (SPITZEN-VAN DER SLUIJS et al. 2016, DALBECK et al. 2018, LÖTTTERS et al. 2018, SCHULZ et al. 2018), indication of *Bsal*-

related population declines or even extinctions can only be gained from a monitoring of European fire salamander populations. However, searching for terrestrial juvenile and adult salamanders is arduous and heavily depends on appropriate weather conditions (THIESMEIER 2004, SCHMIDT et al. 2015). Therefore, monitoring of larval populations has been suggested to control for potentially *Bsal*-induced population declines in European fire salamanders (SCHMIDT et al. 2015).

In Central Europe, female European fire salamanders deposit their larvae in spring, mainly in fish-free first and second order streams (THIESMEIER 2004), but occasionally also in stagnant water bodies (WEITERE et al. 2004, STEINFARTZ et al. 2007). They develop on average within 120 days until metamorphosis (THIESMEIER 2004, WEITERE et al. 2004). In principle, the purely aquatic larvae can easily be detected during the day by active searching, though detection is much easier during the night (THIESMEIER 2004, SCHMIDT et al. 2015). They show almost no weather-related activities, which makes standardised monitoring easier (THIESMEIER 2004). If during the larval season no larva is found in a creek, there are two explanations: either the creek may be unsuitable for reproduction, or no salamanders live in the respective terrestrial habitat. The latter may be due to unsuitability of the terrestrial habitat (or non-colonization of suitable habitats) or, especially if European fire salamanders have been present before, a severe population decline had occurred, e.g. following a *Bsal* infection.

Detecting and documenting population declines is one of the most critical tasks in population ecology and conservation biology, but it is also one of the most difficult (FOURNIER et al. 2019). Usually, post-infection studies will lack solid information about the pre-infection status of populations, and a site-selection bias may mask real population trends in a focal study area (FOURNIER et al. 2019). On the other hand, identifying the ultimate cause of a decline once the decline has occurred is another important but difficult task, as this is the only way to find potential solutions to prevent similar declines in other regions. Therefore, post-decline evidence of disease-related decline can be obtained indirectly by applying a correlative rather than by a causative approach.

To test the assumption of WAGNER et al. (2017) that the absence of European fire salamanders in the northernmost mountains of the Southern Eifel could be due to *Bsal* infection, we hypothesize that salamander-free habitats (especially where salamanders have been present before) in this region are nevertheless suitable for the species. We therefore built a habitat suitability model based on presence-absence of European fire salamander larvae from *Bsal*-unaffected regions (reference area) and projected this model to the potentially *Bsal*-affected parts of the Southern Eifel Mountains to test if these creeks are equally suitable for salamander larvae, plus their surrounding terrestrial habitat for juveniles/subadults and adults, as are those from the reference areas. If in fact *Bsal* had driven European fire salamanders almost to extinction in our focal area, we may still find the pathogen inhabiting few remaining specimens

or other hosts which are less susceptible. We therefore conducted a *Bsal* screening of European fire salamanders and further amphibian species (mainly alpine and palmate newts) using skin swabs to get independent evidence for *Bsal* occurrence in the focal area.

Materials and methods

Study area

The study area is located in the Southern Eifel Mountains (Germany) and encompasses ca. 1,500 km² (Fig. 1). It extends from the south-western German border with Luxembourg and Belgium to the border between the German Federal States of Rhineland-Palatinate and North Rhine-Westphalia. Major parts of it are forested, mainly along river valleys and higher mountainous areas, while on plateaus with calcareous soils, agricultural land use dominates. European fire salamanders and alpine and palmate newts were reported to be quite common in the study area in the 1980s and 1990s (BITZ et al. 1996).



Figure 1. The study area in the Southern Eifel (grey area) is situated in south-western Germany. Red crosses indicate *Bsal*-infected urodelan populations in the Netherlands, Belgium and Germany (cf. SPITZEN-VAN DER SLUIJS et al. 2016, DALBECK et al. 2018, LÖTTERS et al. 2020a in this issue, results of the present study).

The A60 highway divides the study area into a northern and a southern part (Figs 1–3). Highways are well-known barriers for amphibians (LESBARRÈRES et al. 2006, EMEL et al. 2012, VAN BUSKIRK et al. 2012) and thus should hamper *Bsal* transmission through the most important transition pathway, direct animal-to-animal contact (STEGEN et al. 2017, SCHMIDT et al. 2017). This does not mean that highways are strict barriers for *Bsal* (especially due to anthropogenic vectors). Rather, we here use the highway as an unprejudiced hypothetical separator between seemingly non-affected areas and areas where *Bsal* has potentially entered amphibian populations (Fig. 1).

Presence-absence mapping of salamander larvae

From May to July 2016, presence-absence mapping of European fire salamander larvae took place in 88 randomly selected forest creeks as well as creeks where salamanders were recorded in the 1980s/1990s across the study area following a modified removal-sampling of SCHMIDT et al. (2015) (Fig. 2). Close to the source of each creek, a 75 m section was divided into three 25 m subsections. Each subsection was simultaneously examined for 15 min for presence of larvae by three persons using dip nets. As soon as a larva was found, the search was stopped and the creek was classified as presence locality. It was considered an absence locality when this procedure was replicated in another 75 m section of the creek and still no larva was found. For each subsection, environmental variables of the creek and the surrounding terrestrial habitat (potential habitat of juveniles and adults) were recorded (Table 1).

Modelling habitat suitability

Environmental variables used in the habitat model (Table 1) were: the number of pools with slow-running water within the creek (important microhabitat for European fire salamander larvae: BAUMGARTNER et al. 1999, WERNER et al. 2014, SCHMIDT et al. 2015) and the amount of wooden debris (as shelters: THIESMEIER & SCHUHMACHER 1990). Further parameters were the substrate (ordered according to increasing grain size, from mainly muddy over sandy to rocky substrate) and water turbidity (potential effect on detection probability of larvae). Capture occasion (date) was included since mortality rate, drift rate and detection probability of salamander larvae may vary over time (THIESMEIER & SCHUHMACHER 1990, TANADINI et al. 2012, SCHMIDT et al. 2015).

Female European fire salamanders prefer larval deposition in headwater creeks close to the spring (THIESMEIER 2004). As an indicator for the proximity to the spring, creek width was recorded. Presence of predatory fish, especially salmonids, was recorded since they may influence larval abundance (SOUND & VEITH 1994, HECNAR & M'CLOSKEY 1997, FICETOLA & BERNARDI 2004). Creek inclination (which indirectly affects the occurrence of larvae

via water flow/drift (THIESMEIER & SCHUHMACHER 1990, BAUMGARTNER et al. 1999, WERNER et al. 2014) and perpendicular slope inclination (which affects the accessibility of a creek for adult females, which prefer gentle slopes for larvae deposition: MANENTI et al. 2011) are included as relevant topographical features.

Suitable terrestrial habitat is as important for European fire salamander populations as is suitable aquatic habitat (THIESMEIER 2004). Therefore, various terrestrial habitat parameters were included in the model. European fire salamanders are preferentially found in deciduous forests and we therefore quantified the following land cover types within a radius of 100 m and 500 m around a subsection, respectively creek: deciduous, mixed and coniferous forest, settlements, grassland and arable land (BLAUSTEIN & KIESECKER 2002, THIESMEIER 2004). We used CORINE Land Cover Data (<https://land.copernicus.eu/pan-european/corine->

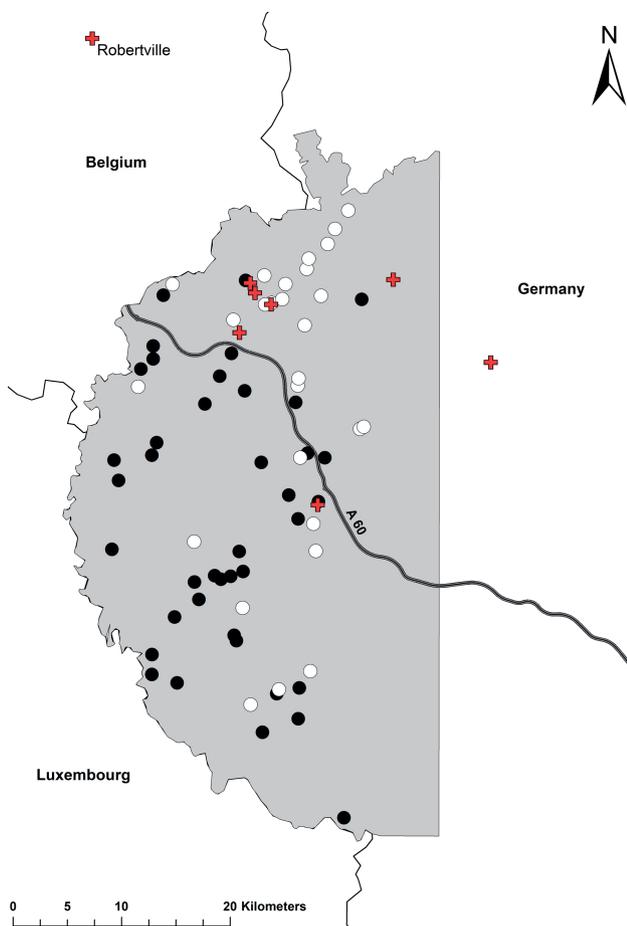


Figure 2. Results of the 2016 presence-absence mapping of fire salamander larvae in the study area (grey area): solid circles = presence, open circles = absence. While 85% of the studied creeks ($N = 63$) were used for reproduction by European fire salamanders south of the A60 highway, larvae were only present in 28% of the studied creeks ($N = 25$) north of the A60 highway. Red crosses indicate *Bsal*-infected urodelan populations found in the present study and the infected fire salamander population in Roberville, Belgium (cf. SPITZEN-VAN DER SLUIJS et al. 2016).

Table 1. Variables used for model building.

Considered variables	Categories
Aquatic habitat	
Water turbidity	Ordinal data (clear – slight turbidity – strong turbidity)
Pools	Count data
Wooden debris in the creek	Ordinal data (few – intermediate – many)
Average creek width	Measured data (cm)
Predatory fish	Binary (presence / absence)
Average creek inclination	Measured data [°]
Average creek perpendicular inclination	Measured data [°]
Substrate	Ordinal data (muddy – sandy – rocky)
Terrestrial habitat	
Land use types	Measured data (%)
Hiding places	Ordinal data (few – intermediate – many)

land-cover) and calculated the proportions of land cover types within buffers using ArcGIS (version 10.5.1). The 100 m radius should cover the core terrestrial habitat of a population, while the 500 m radius should reflect the maximum habitat (REBELO & LECLAIR 2003, SCHULTE et al. 2007). Equally important is a sufficient number of terrestrial hiding places such as rocks and dead wood (THIESMEIER 2004).

We built a habitat suitability model using only creeks south of the A60 highway ($N = 63$), since European fire salamanders are abundant there, and neither the analysis of salamander skin swabs nor population monitoring of larvae indicates any *Bsal* outbreak in this area (except for one newt population; see results). Eventually, habitat suitability projections were compiled for the remaining 25 creeks north of the highway using the best fitting logistic regression model. We used the program R (R Core Team 2020) for statistical calculations. Four different candidate generalized linear models (GLMs) (all variables [global], all terrestrial habitat variables, all aquatic habitat variables, and an individual variable selection: Supplementary document 1) with binomial distribution, i.e. logistic regression models, were built. In this method, non-normal distribution is assumed (DORMANN & KÜHN 2009). Furthermore, all models were automatically simplified to the most important explanatory variables using an information theoretical approach (stepwise AIC) (R-package ‘MASS’: <https://cran.r-project.org/web/packages/MASS/index.html>). An area under the (receiver operation) curve (AUC) value was calculated for each model (R-package ‘verification’: <https://cran.r-project.org/web/packages/verification/index.html>). Eventually, all eight models were compared to each other by their AICc values (R-package ‘MuMIn’: <https://cran.r-project.org/web/packages/MuMIn/index.html>), and the respective best fitting models were selected with a $\Delta AICc < 2$ (BURNHAM & ANDERSON 2002).

Presence and absence predictions were tested individually for each model using a Mann-Whitney U-test because the data were not normally distributed (Shapiro-Wilks-test). To validate the models prior to projection, the dataset was divided into ten random groups (R-package 'dismo'). One group was removed from calculations (test dataset), and with the remaining data (training dataset) a new GLM was calculated. For both the test and the training dataset, an AUC value was calculated. The AUC value of the test data set indicates the quality of the model; only models with an AUC value ≥ 0.7 were considered as plausible (SWETS 1988). In addition to the validation with AUC, the true skill statistics (TSS) quality measurement was used. It was developed specifically for presence-absence data and, unlike other measurements, it is independent of the prevalence. It is the proportion of validation sites where the species was detected. This avoids calculation errors for an unequal number of presence and absence data (ALLOUCHE et al. 2006, 2008). GLM creation and the quality measure calculation were repeated once per group for ten times each. Individual AUC and TSS values were averaged and included in the study as a validation gauge.

Bsal screening

Between 2015 and 2019, we screened European fire salamanders, alpine, palmate and smooth newts as well as syntopic anurans (Supplementary document 2) at 50 sites north and south of the A60 highway for *Bsal* (Fig. 3) using sterile cotton swabs (Medical Wire MW-100). One pair of nitril gloves (CASHINS et al. 2008) was used for each specimen to prevent pathogen transmission and false-positive results. For swabbing, we followed the protocols of HYATT et al. (2007) and VAN ROOIJ et al. (2011) and furthermore collected simultaneously two samples (A and B samples) per specimen for quality insurance. Only individuals where *Bsal* was detected in the A and B sample by two independent laboratories (Trier and Braunschweig Universities, respectively) were considered as confirmed *Bsal*-positive. To prevent for *Bsal* transmission between populations, all materials including field workers' boots were disinfected after each site visit using Virkon S (VAN ROOIJ et al. 2017).

Laboratory analyses of swabs followed BLOOI et al. (2013, 2016), with the modification that only *Bsal* (versus *Bd* and *Bsal* combined) was analyzed in a 'simplex' quantitative real-time PCR (qPCR). DNA was extracted using Prepman (Trier University) or the Quiagen Blood and Tissue Kit (TU Braunschweig). *Bsal*-DNA was amplified and quantified (expressed in GE) using specific primers and fluorescence markers for qPCR as described in BLOOI et al. (2013, 2016). In accordance with THOMAS et al. (2018), only individuals with a *Bsal* load of ≥ 1 GE were considered as *Bsal*-infected. For each locality, Bayesian 95%-credibility intervals for prevalence were calculated using the software R, the R-package R2WinBUGS (<http://cran.r-project.org/web/packages/R2WinBUGS/>) and the software WinBUGS (LUNN et al. 2000) following the method described in LÖTTERS et al. (2012).

Results

Eighty-eight potential and previously known reproduction creeks were investigated for the presence of larval European fire salamanders. Larvae were found in 54 out of 63 creeks south of the A60 highway (= 85%), but only in seven out of 25 creeks (= 28%, Table 2) north of the A60 highway, also not in most of the previously known salamander localities (Fig. 2).

Habitat suitability models and projections

A few variables had to be excluded from further analysis due to no or low values (agricultural land and mixed forest within 100 m, agricultural land, settlement and pasture within 500 m radius). The model selection based on the

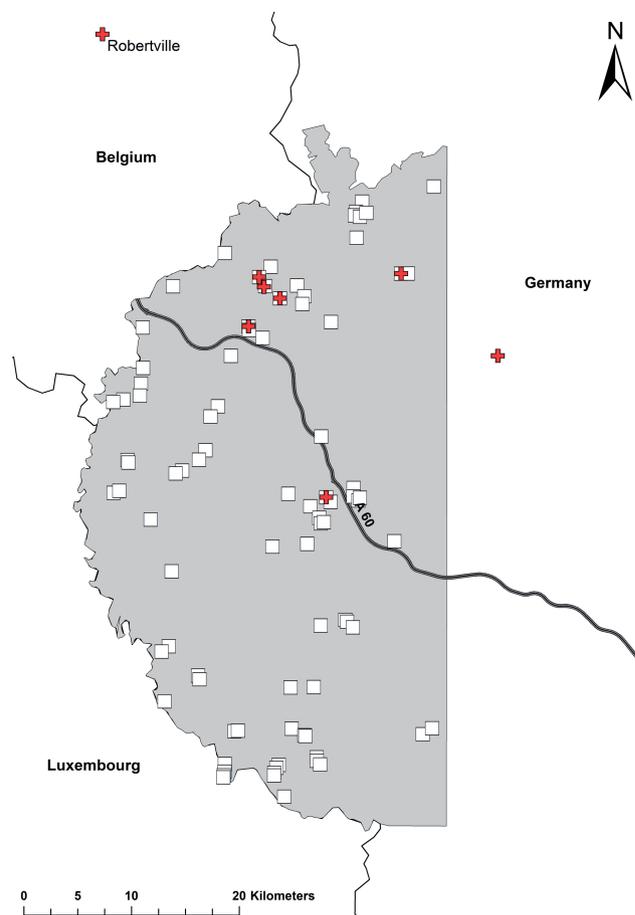


Figure 3. The analysis of 1,526 skin swabs from various amphibian species revealed seven *Bsal*-positive sites confirmed in the A and B samples (red crosses), one confirmed in the A sample only (question mark) and 42 *Bsal*-negative sites (white squares) within and next to the study area (grey area). The northernmost *Bsal*-site in the study area is situated at 26 km from the most proximate *Bsal*-site 'Robertville' (SPITZEN-VAN DER SLUIJS et al. 2016). Note that more white squares are visible; we merged the coordinates of the swabbed amphibians (white squares) to 42 *Bsal*-negative populations in Supplementary data 2.

Table 2. Mapped creek sections north of the A60 highway with the indication whether larvae were found (1) or not (0), as well as the prognosis of the two best models. Only sites marked with * are considered by a model as unsuitable for European fire salamander larvae, delimited by the respective model-specific threshold.

Creek	Larvae	Model 1	Model 2
Alfbach 1	1	99%	87%
Alfbach 2	0	92%	88%
Dreibach	0	97%	89%
Ettelbach	0	99%	98%
Grimmelsbach	0	100%	98%
Hennebach	0	91%	96%
Hennebach Seitenarm	0	57% *	89%
Litzenmehlenbach	0	91%	94%
Lünebach	1	93%	98%
Mattelbusch	0	95%	97%
Mausbach	1	61% *	89%
Mehlenbach	0	99%	97%
Mönbach	0	99%	90%
Pittersbach	0	99%	99%
Prüm	0	99%	89%
Scharrenborn	1	100%	99%
Sellerich	0	97%	90%
Steingert	0	79%	88%
Steinrausch	0	82%	86%
Thierbach 1	0	66% *	87%
Thierbach 2	0	99%	99%
Üchenbach	0	81%	92%
Watzbachzufluss	1	100%	100%
Watzbach	1	100%	100%
Willwerath	1	82%	79% *

corrected Akaike information criterion (AICc) showed that two models with a $\Delta AICc$ value of 0 and 0.51 could be considered as plausible (BURNHAM & ANDERSON 2002). The best fitting model was the automatically simplified global model (Model 1), which took into account the following variables: capture occasion, water turbidity, creek width, creek inclination, substrate, deciduous and mixed forest within 500 m (Supplementary document 3). The two significant predictors were water turbidity and substrate. The turbidity of the water had a significantly negative effect ($Z = -2.25$, $P < 0.05$), and the more rocky the substrate was, the more likely was the presence of larvae ($Z = 2.08$, $P < 0.05$). The AUC value of this model was 0.88, suggesting a 'good' model performance according to SWETS (1988).

The second-best fitting model was the automatically simplified model that considered only the aquatic habitat variables (Model 2). It was limited to the variables: capture occasion, water turbidity, creek inclination and substrate (Supplementary document 4). In this model, only the positive influence of rocky substrate on presence of larvae remained ($Z = 2.10$, $P < 0.05$). With 0.83, this model also had a 'good' performance in terms of AUC (SWETS 1988). The

determined quality measures of the two models, AUC test (Model 1: 0.86 ± 0.25 / Model 2: 0.88 ± 0.13) and TSS (Model 1: 0.85 ± 0.27 / Model 2: 0.82 ± 0.02) of the model validation are in a range of more than 0.80. Model 2 performs better in the evaluation than Model 1 regarding both TSS and AUC.

Based on the thresholds (Model 1: 72%; Model 2: 84%), Model 1 predicts 22 out of the 25 creeks north of the A60 highway as potentially suitable, while Model 2 even predicts 24 creeks as potentially suitable (Table 2). All but one presence creek north of the highway were also predicted as potentially suitable by both models (Table 2).

Regarding the habitat suitability predictions of the investigated creeks north of the A60 highway, divided into the locations where larvae were found during mapping (presence locations, $N = 7$) and those where no larvae were found (absence locations, $N = 18$), the box plots of all sections are in the range of 80–100% probability of presence (Fig. 4). Mean values of the presence (Model 1: 91%; Model 2: 93%) and absence locations (Model 1: 90%; Model 2: 92%) are above the threshold values determined. The Mann-Whitney U-test showed no significant differences between the habitat suitability predictions (Model 1: $P = 0.31$; Model 2: $P = 0.48$) of presence and absence sites.

Bsal screening

Swabbing of 1,526 amphibians (1,431 urodelans and 95 anurans) revealed seven confirmed *Bsal*-positive sites. Eleven out of 355 European fire salamanders were infected (3%). Only seven out of 536 alpine newts (1.3%) and two out of 537 palmate newts (0.4%) were infected with *Bsal* (Supplementary document 2). In on site, one alpine newt was

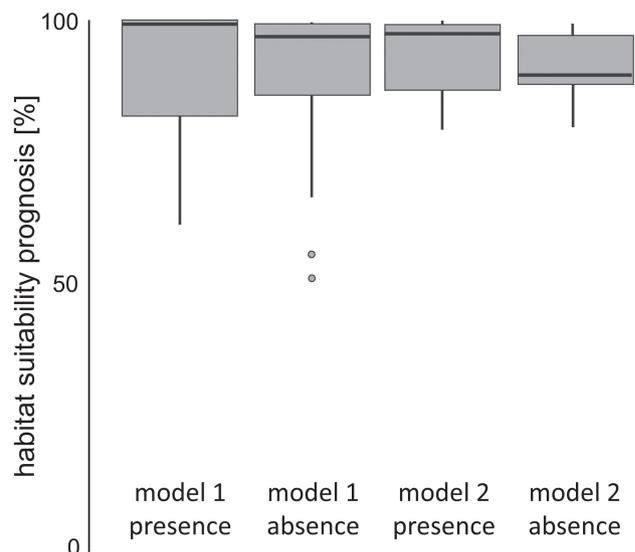


Figure 4. Habitat suitability prognoses for presence and absence creeks north of the A60 highway based on the two best models, respectively.

only positive in the A sample (Supplementary document 2, question mark in Fig. 3). Both swabbed smooth newts and all 43 anurans were *Bsal*-negative (Supplementary document 2). Prevalence was relatively low (7 out of 50 = 14%) and low over all swabbed urodelans (37 out of 1,431 swabs = 2.6%). Bayesian credibility intervals were high for several localities with small sample sizes, while at some localities, sample sizes were reliable (i.e. > 80–90% probability of *Bsal* absence based on Bayesian credibility intervals) to state that *Bsal* was most likely absent (Supplementary document 2).

Six *Bsal*-positive sites were located north of the A60 highway within continuous forest areas where nearly no European fire salamanders or their larvae could be found anymore (cf. Fig. 2). Hence, mainly newts and anurans could be swabbed here, and *Bsal* was detected in seven alpine newts and two palmate newts in the A and B sample (all without any obvious symptoms of chytridiomycosis; Supplementary document 2). In the only swabbed European fire salamander population north of the highway (at the 'Watzbach'), all eleven *Bsal*-positive fire salamanders could be found, while all swabbed fire salamander populations south of the highway tested *Bsal*-negative (Fig. 3, Supplementary document 2). While all specimens swabbed at the 'Watzbach' from 2016 to 2018 were negative for *Bsal*, eleven out of 64 salamanders (17%) were found to be infected in spring and autumn 2019 (Supplementary document 2). We recognized *Bsal*-typical skin lesions in infected individuals (Fig. 5).

One of the *Bsal* sites north of the highway ('Gerolstein') is located east of our study area (Fig. 3) because a suspected case of *Bsal*-caused mortality of several salamanders was reported in spring 2019 via a citizen science project (<https://snu.rlp.de/de/projekte/feuersalamander/>). We could not find dead (or living) salamanders anymore, but eleven alpine and palmate newts; one alpine newt was positive for *Bsal* (Supplementary document 2).

However, south of the highway, we also found at least one *Bsal*-positive site within a continuous forest area with European fire salamanders (Fig. 3). We did not encounter juvenile or adult fire salamanders at night within this forest, but larvae in creeks. Here, *Bsal* was detected on one alpine newt in the A and B sample (again without obvious signs of disease, but with high GE loads of 853 in the A and 671 in the B sample).

Discussion

Presence-absence mapping in the Southern Eifel Mountains showed an increased absence of European fire salamander larvae north of the A60 highway, while nearly all studied creeks south of the highway yielded records of larvae (Fig. 2). When projecting the best fitting habitat suitability models based on data from creeks south of the A60 highway onto the northern area, nearly all absence creeks there appear to be highly suitable for salamander larvae (Table 2). Both best fitting habitat suitability models are

based on reasonable predictors. The capture occasion and water turbidity can be considered as indicators for the detection probability rather than for presence-absence of larvae. Inclusion of these variables in both models could be related to the heavy rainfalls in spring 2016, which most likely decreased detection probability, but also real presence due to an increased flood-induced larval drift (THIESMEIER & SCHUHMACHER 1990). The significant effect of water turbidity on the absence of salamanders in Model 1 argues for low detection probabilities. Hence, final conclusions on real absence in all absence-creeks cannot be made for 2016, but another presence-absence study in 2019 confirmed the absence of salamander larvae in this area (see WAGNER et al. 2020a in this issue). The models also suggest that higher inclination of the studied creek sections is more suitable for salamander larvae in this study area. Inclination also influenced both aquatic and terrestrial habitat parameters in other studies (BAUMGARTNER et al. 1999, WERNER et al. 2014), but we hypothesized earlier (in the Material and methods section) that higher inclination automatically enhances drift and should negatively affect presence, which was not statistically supported at least for this study region. Furthermore, in low mountain regions, such as the Eifel, a higher creek inclination is expected close to the spring where female salamanders preferably deposit their larvae (THIESMEIER 2004). Both models suggest that in creeks with a rocky substrate, larvae are more likely to be found. This may be due to an increased number of hiding places and pools suitable for the larvae, but here they can also be detected more easily during fieldwork. Mod-



Figure 5. *Bsal*-infected fire salamander with typical skin lesions, found in autumn 2019 at the Watzbach near Brandscheid (Eifelkreis Bitburg-Prüm). Photograph taken by S. FELDMIEIER.

el 1 also included creek width and the proportions of deciduous and mixed forest within a 500 m radius (although without significant explanation). Females prefer narrower headwater creeks in proximity to the spring to deposit larvae (THIESMEIER 2004). The positive, but not significant, relationship between proportions of deciduous and mixed forest in the terrestrial habitat is also plausible because juvenile and subadult/adult European fire salamanders are predominantly found there (THIESMEIER 2004, FICETOLA et al. 2009, MANENTI et al. 2009). In addition to the plausible predictor variables in both models, the quality measures AUC and TSS were used to verify them. Both values are above 0.80 for each model so that they can be considered validated (SWETS 1988).

Of course, also other factors such as land-use changes, chemical pollutants or competitive interaction with other species are known drivers of amphibian extinction events (BLAUSTEIN & KIESECKER 2002). None of them was included in our habitat models. However, they are not likely to have simultaneously eradicated all salamander populations in one part of the study area while not having a similar effect in the adjacent part. In additions, all study sites are inside sustainably managed forests, which are known to provide comparatively stable conditions for biodiversity over long periods (PAILLET et al. 2010). In conclusion, the models are suitable to test creeks for their suitability but also detection probability of larvae of the European fire salamander. Based on the creeks south of the A60 highway, the models predict 22 and 24, respectively, out of 25 creeks north of the highway to be potentially suitable for European fire salamanders. Given the previously documented presence of European fire salamanders in large parts of this area (BITZ et al. 1996) our results lead us to hypothesize that absence in most creeks in the northern study area may be *Bsal*-related.

In fact, the presence of *Bsal* north of the A60 highway was confirmed by our analyses of skin swabs. *Bsal*-infected European fire salamanders, alpine and palmate newts were found at six sites north of the highway (Fig. 3, Supplementary document 2); however, *Bsal* prevalence and infection loads were mainly low. From an epidemiological perspective, such patterns could arise because the peak of the epidemic has passed and/or because the primary host has already been driven to extinction. Infected newts had usually low GE loads (only the infected alpine newt south of the highway had a high infection load). STEGEN et al. (2017) found that *Bsal* is lethal for alpine newts only at high infection loads, but they can act as reservoirs and vectors at low infection loads because they are able to clean themselves within about two weeks in the laboratory. North of the highway, only at the ‘Watzbach’ and ‘Watzbachzufluss’, juvenile and subadult/adult European fire salamanders could be detected at surveys between 2015 and 2019. This site is only about 1 km from the next known *Bsal*-infected newt population as well as sites of apparent absence of European fire salamanders. An isolated but still *Bsal*-free salamander population also exists next to the type locality of *Bsal* at Bunderbos in the Netherlands (SPITZEN-VAN DER SLUIJS et

al. 2018). This gave hope that *Bsal* does not spread into all populations in an infected area (SPITZEN-VAN DER SLUIJS et al. 2018). Sadly, the ‘Watzbach’ can no longer be regarded as a *Bsal*-free refugium as apparently *Bsal* was introduced into the population between the swabbing events 2018 and 2019. Since all salamanders swabbed until 2019 were found to be *Bsal*-negative (Supplementary document 2) and also large larvae populations were found (see WAGNER et al. 2020b in this issue), we conclude that this population was infected recently.

It is difficult to detect disease-driven population declines relying on presence data of previously known amphibian populations only. SPITZEN-VAN DER SLUIJS et al. (2013) needed monitoring data of about one decade to realize that a population was sharply declining in the Netherlands, an observation that ultimately led to the discovery of *Bsal*. This was only possible because the European fire salamander is a rarity in the Netherlands, and the population was permanently monitored by volunteers and scientists. The same accounts for an infected salamander population in Belgium, which was monitored before and after the *Bsal* outbreak (STEGEN et al. 2017). However, in overviews on *Bsal* distribution in the Netherlands, Belgium and Germany, for most infected populations no statement on decline could be made due to missing monitoring data (SPITZEN-VAN DER SLUIJS et al. 2016, DALBECK et al. 2018), in several infected sites not even after five years of standardized larvae monitoring (WAGNER et al. 2020b in this issue). Hence, long-term monitoring of at least selected salamander and newt populations within the invasive range of *Bsal* should be one major task for further amphibian conservation.

Conclusions

Most creeks of the northern part of our study area provide suitable habitat for larvae of the European fire salamander. However, the significant predictors water turbidity and substrate may also relate to detection probability. Nevertheless, given the similar or even identical results of presence-absence mapping in 2015 (unpub. data) and 2019 (see WAGNER et al. 2020a in this issue), the extensive absence of the species in the northern part of our study area does not appear to be habitat-related. Certainly, the absence of larvae of the European fire salamander does not necessarily have to be the result of a *Bsal* outbreak. Post-infection studies of a susceptible host species usually lack information from the pre-infection period, and therefore conclusions about a potential pathogen-driven extinction scenario must remain speculative. Nevertheless, due to the detection of *Bsal*-infected newts and the recent introduction of *Bsal* into the, up to 2018, *Bsal*-free Watzbach population (Figs 3, 5, Supplementary document 2), it is very likely that a previous *Bsal* outbreak rather than unsuitable habitats account for the lack of European fire salamanders in the northern part of our study area.

The potential large-scale extinction of European fire salamanders in our northern study area is astounding.

We are aware of the problems associated with negative records, but our larvae and adult monitoring efforts since 2015 clearly suggest absence of the species. Conversely, in other *Bsal*-infected areas, such as the Netherlands or the Eifel Mountains in North Rhine-Westphalia (Germany), infected populations declined sharply, but they did not get fully extinct (SPITZEN-VAN DER SLUIJS et al. 2013, 2016, STEGEN et al. 2017, DALBECK et al. 2018, SCHULZ et al. 2018). Also, in the *Bsal* area south of the highway A60, European fire salamanders are still present in all creeks, which are situated in the continuous forest area where *Bsal* was detected (cf. Fig. 2 and results from larvae monitoring in WAGNER et al. 2020a,b in this issue). The same accounts for *Bsal*-infected fire salamander populations in the northern Eifel (see results from larvae monitoring in WAGNER et al. 2020b). It therefore remains unclear why European fire salamanders are nearly completely extinct (or at best are only present in such low numbers that we could neither detect terrestrial nor aquatic life-stages in all seemingly negative sites) in the northern part of our study area. Perhaps these populations were already small, but unfortunately, there is no historical data on abundance (see BITZ et al. 1996). Thus, our study illustrates how difficult it can be to identify a presumably disease-driven decline once the decline has happened.

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Supplementary data

The following data are available online:

Supplementary document 1. Candidate models, which were compared using information-theoretical approaches.

Supplementary document 2. Overview on amphibians, which have been swabbed in the whole study area from spring 2015 to autumn 2019.