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Batrachochytrium salamandrivorans in the Ruhr District, Germany: history, distribution, decline dynamics and disease symptoms of the salamander plague

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Abstract. The chytrid fungus *Batrachochytrium salamandrivorans* (*Bsal*), recently introduced from Asia to Europe, causes mortality in numerous species of salamanders and newts and has led to catastrophic declines and local extinctions of the European fire salamander (*Salamandra salamandra*) in the Netherlands, Belgium, and Germany. Due to the continuous spread of the pathogen, Germany can be considered as the current 'hotspot' of *Bsal*-driven salamander declines. The pathogen was detected in 2015 in the Eifel Mountains where it probably has been present at least since 2004. Moreover, *Bsal* was found in 2017 in the Ruhr District where it also might occur since 2004. The Ruhr District is a heavily urbanized and industrialized region in western Germany, which offers an unprecedented opportunity to monitor range expansion and infection dynamics of *Bsal* in an area affected by intense human activities. We here review the current knowledge on *Bsal* in the Ruhr District where the pathogen by now has been recorded based on qPCR data from 18 sites distributed over eight cities. Transect counts (adult salamanders) and larval removal-sampling at two sites where *Bsal* was recorded in 2017 and 2018, confirm that fire salamander populations at the affected sites have declined dramatically. However, single negative-tested individuals were still observed and reproduction could be ascertained. Moreover, we successfully detected *Bsal* by analysing environmental DNA (eDNA) from samples obtained from a standing water body as well as a stream. Detailed monitoring of a site in Essen (Kruppwald) from January to May 2019 provided data on infection and disease dynamics during an acute *Bsal*-outbreak in a population of European fire salamanders. After initial observation of single dead infected salamanders in January and February 2019, the maximum *Bsal* loads in the population ranged from 7.90E+03 ITS copies in early March to 2.29E+09 ITS copies at the end of March. Prevalence of infection ranged from 4% to 50% and significantly increased over time; prevalence of externally visible disease symptoms peaked on May 2 and May 8. Single dead salamanders were encountered throughout the monitoring period. Recaptures of two infected salamanders indicated an increase of *Bsal* load by about one order of magnitude within one week. Infected salamanders showed small-sized regular round ulcerations usually of 0.25–1 mm but sometimes up to 2.5 mm in diameter, which gave the impression of outward growth from the centre of each ulceration. Among salamander individuals monitored in the Kruppwald, such ulcerations were only found in infected salamanders, but we found no significant correlation between the intensity of the ulcerations and *Bsal* load. Heat treatment proved effective to cure even deep ulcerations when salamanders were kept for 10 days at 25–27°C or 14 days at 25°C, but infection persisted and ulcerations reappeared six weeks after the end of the treatment; only heat treatment at 25°C for 21 days proved effective to reliably clear the infection in three tested salamanders.

Key words. Amphibia, Caudata, *Salamandra salamandra*, European fire salamander, *Bsal*, chytridiomycosis, heat treatment, emerging infectious disease, amphibian disease, eDNA.

Introduction

Globalization, through increased mobility of people and goods, has opened the gates for invasive species, including pathogenic bacteria, viruses and fungi, to spread into new areas harbouring organisms vulnerable to these pathogens (Marano et al. 2007, Perrings et al. 2018). Especially areas with strong human footprint and activities, e.g. extended urbanization and dense networks of roads, are characterized by a high invasion intensity (e.g. Ehrenfeld 2008, Wichmann et al. 2009, Gallardo et al. 2015), whereas protected areas can offer refuge from invasive species (Gallardo et al. 2017).

In central Europe, one of the most urbanized areas is the Ruhr District ('Ruhrgebiet') in western Germany. Named after the river Ruhr, this large polycentric urban area in the federal state of North Rhine-Westphalia is characterized by a high human population density (~5 million residents) and numerous industrial agglomerations. The Ruhr District is geologically a 'Börde', i.e. a highly fertile lowland with loess substrate, located in a transition area from a low mountain range (Bergisches Land and Sauerland) to lowlands (Lower Rhineland and Münsterland) (Feldmann & Schlüpmann 2011a). As such, it has been largely deforested and was historically first used for agriculture, then industrialized with numerous heavy industries and associated urban areas built around intensive black coal mining. In this landscape mosaic, natural and agricultural elements were strongly reduced and fragmented, but remained locally preserved. For instance, numerous deciduous (beech) forest patches remain scattered among urban and industrial areas, often used as local recreation areas.

The Ruhr District is one of currently two areas in Germany where amphibian mass mortality caused by the invasive pathogenic fungus *Batrachochytrium salamandrivorans* (*Bsal*) have been observed in the wild. The outbreaks caused by this pathogen (also dubbed as 'salamander plague'; Dalbeck et al. 2018) have triggered a fast response by regional and national stakeholders who engaged in numerous monitoring and research activities, and our purpose is to herein provide an overview and summary of the data obtained so far. While the dense infrastructure in the Ruhr District presumably facilitates the spread of invasive species and pathogens, it also makes this area ideal for close monitoring of pathogen dispersal, prevalence, and host survival in a natural environment, as most sites are effortlessly accessible. Moreover, since the 1970s, the amphibian fauna of the Ruhr District has been intensively studied (Sell & Sell 1977, Feldmann 1981, Geiger & Niekisch 1983, Klewen 1988, Kordges et al. 1989, Hamann & Uthoff 1994, Kordges & Schlüpmann 2011), which provides an excellent baseline to estimate the impact of this pathogen.

Amphibians are among the vertebrates being most affected by infectious diseases caused by invasive pathogens (Blaustein et al. 2018). The chytrid fungi *B. dendrobatidis* (*Bd*) and *B. salamandrivorans* are native to Asia (Martel et al. 2014, Laking et al. 2017, Nguyen et al. 2017, Yuan et al. 2018, O'Hanlon et al. 2018) and have the potential to push species from all amphibian orders to the brink of extinction. While *Bd* infects and causes mortality in anurans, caecilians as well as caudates, *Bsal* appears to affect only caudates, with some anurans functioning as potential vectors and reservoirs (Martel et al. 2014, Stegen et al. 2017). Presumably, the global movement of goods and materials, including the pet trade, facilitated the spread of *Bd* into every continent inhabited by amphibians, causing massive die-offs in anurans, caudates and caecilians e.g. in the Neotropics (Scheele et al. 2019). The origin of *Bsal* in Europe is considered to be a recent introduction (Martel et al. 2014) with subsequent invasive range expansion (Spitzen-van der Sluijs et al. 2016, Thomas et al. 2019).

The most susceptible species to *Bsal* infection within the current range of the pathogen appears to be the European fire salamander, *Salamandra salamandra* (Stegen et al. 2017), a species with a substantial portion of its global population in Germany (Thiesmeier 2004); for simplicity hereafter mostly named "fire salamander". Within days upon infection, fire salamanders develop superficial skin lesions and deep epidermal ulcerations. After complete disruption of the important skin barrier, secondary bacterial infections will lead to septicaemia and death within less than a month (Martel et al. 2013, 2014, Stegen et al. 2017). Newt species can also develop and die from *Bsal*-driven chytridiomycosis as recently reported in marbled newts in Spain (Martel et al. 2020). However, large-scale population collapses have not been reported in other European newt species so far, suggesting they could also act as unpredictable reservoirs of *Bsal* (Martel et al. 2014, Wagner et al. 2019). The dispersal vectors are theoretically manifold (conspecifics, resistant amphibians, wildlife, humans, etc.), but practically unknown (Stegen et al. 2017). The only cure for chytridiomycosis to date is a temperature treatment at 25°C for 10 days and/or a fungicide (Blooi et al. 2015a, b), although intensive research is conducted regarding microbiome manipulation and vaccination/immunisation (McMahon et al. 2014, Bletz et al. 2018). The invasive distribution of *Bsal* currently encompasses the Netherlands, Belgium, Germany (Spitzen-van der Sluijs et al. 2016, Dalbeck et al. 2018, Lötters et al. 2020a in this issue), and Spain (Martel et al. 2020). The first discovery of dead fire salamanders in Europe dates back to 2008 in the Netherlands (Spitzen-van der Sluijs et al. 2013), but it was not until 2013 that the mortality causing pathogen was identified and scientifically named (Martel et al. 2013). Subsequently, more infected populations were discovered in nearby areas in Belgium and Germany (Eifel) due to intensive monitoring (Spitzen-van der Sluijs et al. 2016, Dalbeck et al. 2018), and a record from 2004 (Dalbeck et al. 2018, Lötters 2020b in this issue) suggests that the pathogen may have been present in the Eifel for much longer than originally assumed.

In the Ruhr District, sick or dead fire salamanders infected with *Bsal* were first recorded and confirmed by quantitative PCR (qPCR) in 2017 (Lötters et al. 2018, Schulz et al. 2018, Wagner et al. 2019). The species in this area inhabits a variety of habitats, from isolated forest remains to more extensive patches of beech forest, with abundances dropping rapidly towards the northern and western lowland areas. In the most recent Red List of endangered reptiles and amphibians of North Rhine-Westphalia (Schlüpmann et al. 2011b), the fire salamander was assessed as Least Concern for the entire state, but as Critically Endangered for the strongly urbanized part of the Ruhr District ('Ballungsraum Ruhrgebiet'; see map in Thimm & Weiss 2011) where only a few populations occur and are threatened by ongoing urban development (Schlüpmann et al. 2011b). The Ruhr District is part of a potentially wide contact zone between two subspecies that reached the area by postglacial recolonization, *S. s. salamandra* and *S. s. terrestris* (Weitere et al. 2004). Fire salamanders in the region usually show the striped pattern typical for the western subspecies *S. s. terrestris,* but carry some alleles typical for the eastern subspecies and may have evolved unique genetic characteristics through hybridization and adaptation (Weitere et al. 2004).

So far, 18 positive *Bsal* sites associated with eight distinct cities/counties were recorded in the Ruhr District (Wagner et al. 2019, Lötters et al. 2020a in this issue, V. Schulz unpubl. data) and the number is steadily increasing. As we report herein, the earliest unconfirmed indication of the possible presence of *Bsal* within this urban area, documented by a photo, dates back to 2004. The close collaboration between universities, biological stations, nature conservation authorities and volunteers facilitates an unprecedented opportunity to monitor infection dynamics in real-time. During 2017–2019, we repeatedly sampled fire salamanders and newts in infected as well as non-infected sites across the Ruhr District for *Bsal* infection by skin swabbing. Molecular analysis was conducted using qPCR. Every individual was photographed for recapture analysis which enabled us to compare infection loads over time and to search for possible resistant individuals that would be tested positive at first and negative at a later time. Furthermore, we conducted a removal sampling in streams to determine the abundance of fire salamander larvae as a proxy for the abundance of adults. Additionally, we conducted the first trials to detect *Bsal* by using environmental DNA. In this review, we aim to give an overview of the discovery and spread of *Bsal* in the Ruhr District, the observed population declines, and the observed disease symptoms. Moreover, we provide a report on our experiences in the treatment of infected fire salamanders and discuss future perspectives for their conservation management in the Ruhr District.

Materials and methods

Study area and distribution of European fire salamanders in the Ruhr District

From an administrative and political perspective, the Ruhr District (Fig. 1) is defined since 1900 as a conglomerate of many cities and counties, including Bergkamen, Bochum, Bottrop, Castrop-Rauxel, Dinslaken, Dortmund, Duisburg, Essen, Gelsenkirchen, Gladbeck, Hamm, Hattingen, Herdecke, Herne, Herten, Kamen, Lünen, Mülheim an der Ruhr, Oberhausen, Recklinghausen, Wetter and Witten. Also the city of Hagen, the entire Ennepe-Ruhr District (in the west Sauerland), the district of Wesel (predominantly Niederrhein), the district of Recklinghausen (in the north Münsterland) and the district of Unna (in the north Münsterland, in the south Sauerland) are politically linked to the region by the 'Regionalverband Ruhr'.

Geographically, the southern border of the current Ruhr area is bordered by the Ruhr valley and its ridges immediately to the south. The zone south of the river Ruhr is part of the natural area of the 'Süderbergland', bordering the 'Bergisches Land' in the west and the 'Sauerland' in the east. The Süderbergland is densely populated by the fire salamander (Schlüpmann et al. 2006, 2011a) which occurs in almost all of the countless V-shaped valleys, with spring streams in deciduous forest, as is well documented in Hagen (see Schlüpmann 2004, 2008).

North of the river Ruhr lies the ridge of the Ardey. It extends from Mülheim an der Ruhr in the west and the district of Unna in the east, with an overall mountainous and forested character in the south, on the mountains sloping towards the Ruhr, with many areas of beech forests with small streams flowing into the Ruhr, being characteristic habitats populated by fire salamanders (Kordges & Schlüpmann 2011). Towards the north, the terrain gradually slopes down to the Emscher valley which can be regarded as the core zone of the Ruhr District with the highest density of human settlements. The small and medium-sized brooks to the river Emscher had been used here since the beginning of the 20th century as open sewers, and only here some patches of forest can be found with clean source streams, such as in the mountains of Ardey near Witten and Dortmund. Fire salamanders were and are only found in a few places (Kordges & Schlüpmann 2011).

North of the Emscher, the metropolitan area gradually merges with the agricultural landscape, with some important extensions of beech forest that often host large fire salamander populations, such as the Sterkrader Wald, Hiesfelder Wald, Köllnischer Wald and Kirchheller Heide.

The fire salamander reaches the limit of its continuous distribution in North Rhine-Westphalia in the Ruhr area on the heights of the Ardey in the southern Ruhr District (Schlüpmann et al. 2006). To the north, only few isolated fire salamander populations inhabit the lowlands (e.g., the Hiesfelder Wald) and the Münsterland (e.g. Wollbecker Tiergarten or in the woodlands near Selm and Cappenberg). Throughout, these populations dwell in so-called old forest areas that have survived the medieval deforestation unscathed (Schlüpmann et al. 2006, Feldmann & Schlüpmann 2011b). West of the Rhine and in the northern lowlands the fire salamander is completely missing.

Besides the fire salamander, the Ruhr District is also inhabited by several newt species that are susceptible to *Bsal* (Martel et al. 2014). Alpine newt (*Ichthyosaura alpestris*) and smooth newt (*Lissotriton vulgaris*) are widespread, whereas the great crested newt (*Triturus cristatus*) is only locally represented (Kordges et al. 1989, Kordges & Schlüpmann 2011). The palmate newt (*L. helveticus*) reaches its northern distribution border in the Ruhr District; only at a few known sites does this species occur further north. To the west, in a locally limited area of the Duisburg-Mülheimer forest, it reaches the edge of the northern Rhine lowland. However, to the north there is an isolated occurrence of the palmate newt in the forests north of Oberhausen (Schlüpmann 2006) where it co-occurs with the fire salamander.

Bsal screening and detection

For the detection of *Bsal*, fire salamanders were caught by hand. Each individual was handled with clean nitrile gloves and its ventral surface was gently rubbed 10 times, simultaneously with two sterile rayon swabs representing A and B sample (MW113; Medical Wire & Equipment, Corsham, UK). Each swab was kept separately in a sterile 1.5 ml centrifuge tube and stored at -20°C until DNA extraction. One swab per sampled individual was analysed in the laboratory of the Technische Universität Braunschweig (Braunschweig, Germany). In case of a positive result, the B-sample was analysed in the laboratory of Trier University (Trier, Germany) for validation.

First, genomic DNA was extracted from the swabs using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's Animal Tissues protocol with pretreatment for gram-positive bacteria. Incubation for the initial enzymatic lysis was extended to 1 h, and the temperature of the proteinase K lysis was increased to 70°C to increase DNA yield. For the detection of the chytrid fungus *Bsal*, the region of the ITS (internal transcribed spacer) rRNA (120 bp) was amplified by quantitative PCR, following a standard protocol by Blooi et al. (2013) with the alteration of using a *Bsal*-specific FAM-labeled probe (Biomers, Ulm, Germany) and KlearKall Master Mix (LGC genomics, Middlesex, UK).

Quantitative polymerase chain reactions (qPCRs) were performed on a CFX96 Real-Time System (Bio-Rad Laboratories Inc., Hercules, USA) in Braunschweig, and on a StepOnePlus (Applied Biosystems, Foster City, USA) in Trier. Each sample was run in duplicate in Braunschweig. If contradictory results of the replicates occurred, a third

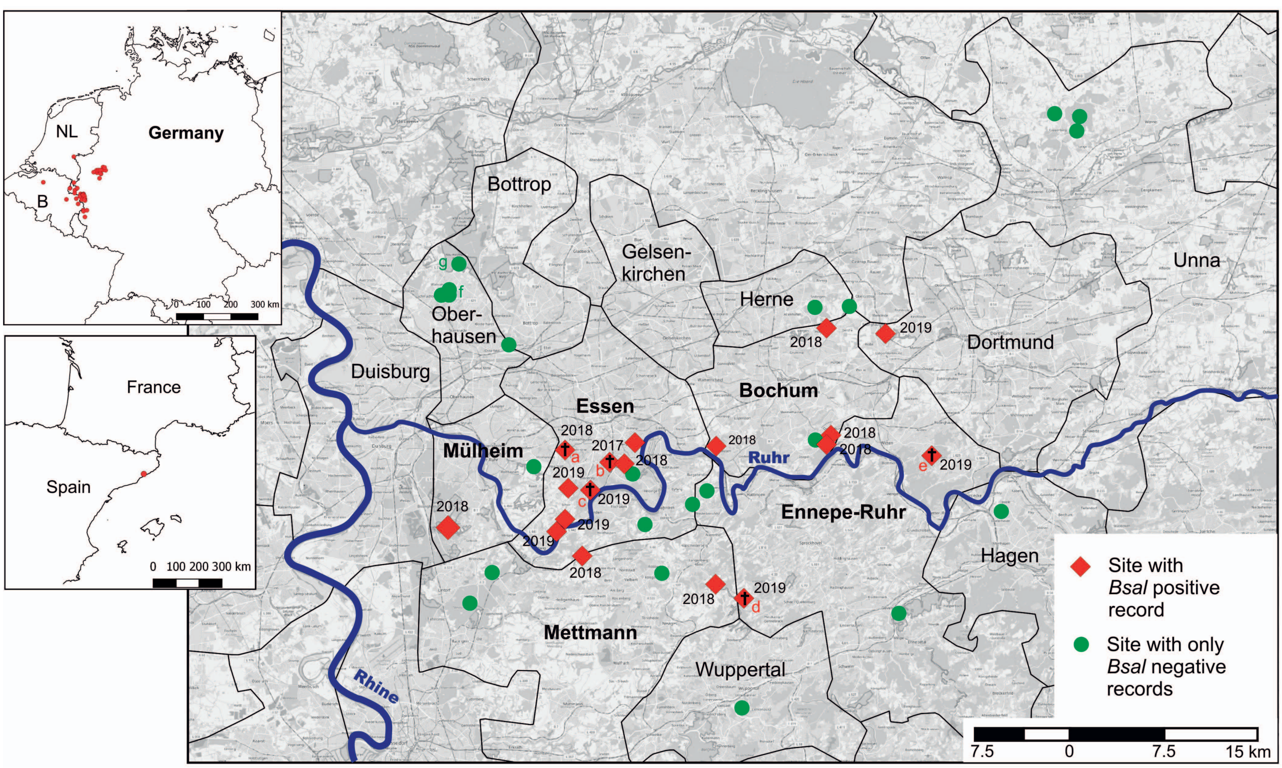

Figure 1. Map showing counties in the Ruhr District (Ruhrgebiet) in western Germany with populations surveyed for *Bsal*. For each positive site, the year of first *Bsal* detection is given. Counties with positive records are in boldface. Black crosses mark sites where mass mortality (> 5 dead salamanders) or drastic population crashes were detected: (a) Südwestfriedhof Essen, (b) Stadtwald Essen, (c) Kruppwald Essen, (d) Hattingen, (e) Witten-Annen. The two *Bsal*-negative sites used as reference monitoring sites in 2019 are (f) Sterkrader Wald and (g) Hiesfelder Wald. The map is based on Bundesamt für Kartographie und Geodäsie, Frankfurt am Main, 2011; note that county borders are only approximate. The main rivers in the area, Rhine and Ruhr, are indicated in blue. Small inset maps give overview of all *Bsal*-positive sites (red dots) in Europe known until 2019 (upper left: Germany, B: Belgium and NL: The Netherlands; below: Spain with one confirmed positive site).

replicate was run. Samples analysed at Trier University were run in triplicate. Each qPCR plate in Braunschweig contained two replicates of *Bsal* standards (10-fold dilution scale from 0.1–1,000 zoospores and 100–10,000,000 ITS copies) and two negative controls, while in Trier each plate had the *Bsal* standard (either zoospores or ITS copies) and the negative controls in triplicate. The threshold for the ITS copies was set to 100 ITS based on a comparison of Ct values between both standards (1 GE = 33 cycles/ 100 ITS copies = 35 cycles). Amplification signals were only considered as positive when the signal was between the highest and the lowest standard and when the amplification curve was logarithmic. Based on the extraction volume used, the estimated zoospore equivalents were converted to numbers of zoospores per swab (hereafter z/s) and ITS copies per swab (hereafter ITS/s).

The presence of *Bsal* at the site Kruppwald was verified for several individuals by independent qPCR at Trier University and at Ghent University (A. Martel, Ghent, Belgium), and furthermore by histology at Ghent University. However, analyses of *Bsal* prevalence and load over time, and of correlation with the presence of external ulcerations, are based on data from Braunschweig only.

Analysis of *Bsal* eDNA from water samples

This part of the study aimed to test the usability of DNA from environmental samples, so-called environmental DNA (eDNA), for *Bsal* detection. Eight streams and forty-five ponds were sampled between February and August 2019, comprising streams in the immediate vicinity of infected as well as non-infected fire salamander populations in Bochum (Botanical Garden of the Ruhr University Bochum/Kalwes, Dahlhausen, Lottental, Stiepel, Tippelsberg) and Essen (Fulerum, Kruppwald, Stadtwald). All samples were collected with clean nitrile gloves. In streams, four water samples of 500 ml each were taken from slow-moving areas of the main stream or side puddles. In ponds, four water samples were taken in a clockwise orientation using sterile 500 ml bottles that were either directly filtered at the same day or stored at 4°C overnight. Water samples were filtered using sterile 0.2 µm nitrocellulose membrane filter funnels (Thermo Fisher, Waltham, USA) and a water-jet vacuum pump. Each filter was kept separately in a sterile 1.5 ml Eppendorf tube and stored at -20°C until DNA extraction. Strict spatial separation of all eDNA related work was ensured during water filtering, DNA extraction and qPCR in different (sterile) laboratories to exclude DNA contamination. Filters were halved, shredded into small pieces and genomic DNA was extracted as already described for the swab extraction. For the detection of the chytrid fungus *Bsal*, a region of the ITS rRNA (120 bp) was amplified by quantitative PCR, following a standard protocol with the alteration of using SYBR Green GoTaq® qPCR Master (Promega, Fitchburg, USA). qPCRs were performed on Light Cycler® 96 System (Roche, Mannheim, Germany) and plates consisted of eight (stream) and four (pond) technical replicates for each sample, two replicates of synthetic *Bsal* standard (10-fold dilution scale from 100–10,000,000 ITS copies) and two negative controls. Amplification signals were only considered as positive when the signal was between the highest and the lowest standard and when the amplification curve was logarithmic. Positive samples were Sanger sequenced at the Faculty of Chemistry and Biochemistry at the Ruhr University Bochum to verify that the amplified DNA fragments corresponded to *Bsal*.

Visualization of *Bsal*-related ulcerations

High-resolution images of living salamanders and external symptoms of *Bsal* infection (ulcerations) were made with a Nikon D850 camera and a Sigma 105 mm macro lens. Amplified figures of ulcerations in a euthanized individual (in terminal stage of disease) were made with a Keyence VHX 900F digital microscope. For scanning electron microscopy, skin fragments were fixed with 5% formaldehyde and 2% glutaraldehyde overnight, washed twice with TE buffer and dehydrated in a graded series of acetone (10, 30, 50, 70 and 90% on ice, and twice in 100% acetone at room temperature) for 20 minutes each. Critical-point drying with liquid CO_2 was done with a Balzers CPD 30 and gold-palladium sputter coating with a Bal-Tec SCD500. The samples were examined with the field emission scanning electron microscope Merlin from Zeiss using both, the HESE2 Everhart Thornley SE detector and the in-lens SE detector and an acceleration voltage of 5 kV.

Heat treatment of *Bsal*-infected salamanders

A subset of fire salamanders with *Bsal*-related lesions and positive qPCR signal were subjected to heat treatment either (i) in a room with normal heaters and an additional electric heater, or (ii) in a climate chamber. As these treatments were primarily carried out to cure infected animals, they did not follow a rigorous experimental protocol (e.g. there were no controls). The animals were housed individually in plastic boxes on a moist tissue, with access to a hiding place as described in Blooi et al. (2015a). The tissues were humidified every day and renewed every second day. Fresh nitrile gloves were used for each box and animal. Earthworms and crickets powdered with vitamin supplement were provided as food. For minimizing the risk of spreading *Bsal* spores, only one person had access to the room / climate chamber, which was equipped with disinfection spray (1% Virkon S solution) in a bottle as well as filled in a disinfection mat placed in front of the door. After the treatment, the temperature was lowered slowly down to 15°C and animals were transferred into new plastic boxes which contained clay, wood and moss. All materials used in the rooms, including any substrate, plastic boxes, tissues or gloves were disinfected in a large tray filled with 1% Virkon S solution before disposal.

Capture-mark-recapture study

We used the natural individual dorsal colour pattern of fire salamanders for non-invasive individual recognition as implemented in the software AmphIdent, developed for automated amphibian photo analysis (http://www.amphident.de, Matthe et al. 2008, Drechsler et al. 2015). This software is based on a pattern recognition algorithm for the analysis of spotted dorsal patterns. We used photographs of the dorsal sides of each fire salamander, standardized in AmphIdent by adjusting the automatically generated body contour points during the process of uploading.

Larval monitoring

Larval monitoring was carried out following a removal sampling protocol suggested by Schmidt et al. (2015). A total of 75 m of a stream was divided into three transects of 25 m each. Each transect was searched for salamander larvae by one researcher during 15 min, using visual encounter and dip-netting, and temporarily removing all encountered larvae. Subsequently, researchers rotated and examined another 25 m transect, until all three transects were examined three times. The total number of larvae removed over the total of 75 m stream were counted and all larvae were released at the place of capture. The larval monitoring was always conducted at daytime to avoid a potential bias based on larval activity. Only at the *Bsal*-positive sites Stadtwald and Südwestfriedhof, larval monitoring was also conducted at night to confirm the decline.

Results and discussion

Bsal records in the Ruhr District

The first alert of suspected *Bsal*-infected fire salamanders in the Ruhr District was received in July 2017. A citizen found a fire salamander in the Stadtwald in Essen sitting exposed during daytime, obviously not in good shape as the body was covered by lesions and ulcerations characteristic for *Bsal*-infected animals. The animal was caught for treatment but died one day later. Analysis by qPCR in Braunschweig, Ghent and Trier confirmed that the animal was infected with *Bsal* and thus the pathogen's presence for the first time detected outside of the Eifel area in Germany. Swabs of 97 fire salamanders taken during two nights (August/September 2017) at this site yielded eight additional *Bsal*-positives by qPCR. Shortly afterwards, we received a report of several dead fire salamanders found on the graveyard in Essen-Fulerum (Südwestfriedhof) (Figs 2b, c), around 3.5 km from the Stadtwald. In late December 2017 and in January 2018, more than 60 dead fire salamanders (some already almost decomposed, several with *Bsal*-related ulcerations) had been observed on the paths (Fig. 2a), constituting the first documented mass mortality event in the Ruhr District. Our subsequent survey in the same period yielded eight fire salamanders, four of which were dead and all of which tested positive (qPCR results confirmed by duplicate analyses at Braunschweig and Trier Universities).

Subsequent surveillance was carried out in close collaboration with the local Nature Conservation authorities, the Ruhr University Bochum and the Biological Station of Western Ruhr (hereafter BSWR). In 2018, we screened a fire salamander population in Bochum-Querenburg and Stiepel; three out of 40 swabbed salamanders tested *Bsal*-positive, and one of these had a few of the typical *Bsal*-related skin ulcerations. Subsequent work led to the discovery of one dead *Bsal*-infected fire salamander each in Bochum-Hiltrop and Bochum-Dahlhausen, respectively.

A further *Bsal*-positive site in Essen was recorded in April 2018 close to the Stadtwald. In this case, only alpine newts were affected, with several dead individuals being observed. One living newt showed skin ulcerations characteristic for *Bsal* (Figure 3). In Essen-Bergerhausen, we also detected *Bsal* in alpine, smooth and palmate newts screened at a temporary amphibian drift fence consisting of pitfall traps to prevent road mortality events. At the same time, one dead, *Bsal*-positive, fire salamander was found in Velbert (Stumpsberg), approximately 5 km south of Essen. In June 2018, a citizen living at the border between Essen and Heiligenhaus discovered two dead fire salamanders showing obvious skin damage which we confirmed positive for *Bsal*. Some months later, we also located a *Bsal*-infected fire salamander in Mülheim an der Ruhr, west from Essen, simultaneously with the report of a citizen of a fire salamander with skin lesions and deep ulcerations in a garden, found during the day; in a previous screening in 2017, this population (N = 30) did not yield any *Bsal*-positives.

In January 2019, we received records from citizens in Essen of two dead fire salamanders indicating characteristic *Bsal*-lesions at Ruthertal (Essen-Kettwig and Essen-Werden), both of which we confirmed *Bsal*-positive. Just some days later, the first dead salamander with visual *Bsal*-symptoms was found at the site Essen-Kruppwald, where previous surveys in spring 2018 (N = 8) and autumn 2018 (N = 22) indicated no sign of *Bsal*. A second mass mortality event was then recorded in Witten, Borbach, by a citizen in February 2019. Here, 18 dead fire salamanders were lying close to a house next to a dry stone wall; in 13 we could still see ulcerations, and these were collected and tested *Bsal*-positive. Two months later, eight dead fire salamanders were observed in Hattingen (Deilbach); three in good condition for analysis and with signs of *Bsal*-ulcerations were collected and tested positive. Most recently, in December 2019 two dead fire salamanders were also found to the west of Dortmund, at the Dellwiger Bachtal. Both individuals tested *Bsal*-positive and had damaged skin parts with several ulcerations.

Although qPCR is currently the most reliable and widely used method for *Bsal* detection, a final confirmation of the pathogen's presence requires combination of qPCR with a second diagnostic technique (Thomas et al. 2018). In 2019, tissue samples of a dead infected fire salamander from the Kruppwald site were sent to the Central Diagnostic Laboratory for *Bsal* in Europe at the University of Ghent, Belgium (http://bsaleurope.com/laboratories/); here, qPCR from freshly taken swabs of this sample, as well as histology provided an official confirmation of *Bsal* in the Ruhr District (A. Martel & F. Pasmans pers. comm. May 2019).

In summary, at present the distribution of *Bsal* in the Ruhr District is covering an area of 800 km² including the cities Bochum, Dortmund, Essen, Hattingen, Heiligenhaus, Mülheim an der Ruhr, Velbert and Witten (Table 1, Fig. 1).

Earliest indication of *Bsal* presence in the Ruhr District

Bsal is known to be present in the Ruhr District since 2017 (Essen Stadtwald) proved by qPCR. By involving local media and other networks, we received several additional reports of fire salamanders with suspected *Bsal* infection. One of these indicated that in 2016 several dead fire sala-

Figure 2. Habitats of European fire salamanders (*Salamandra salamandra*) in the Ruhr District: (a) Dead salamander encountered next to a trail in the Südwestfriedhof graveyard area of Essen-Fulerum during a *Bsal*-related mass mortality event in early 2018; (b, c) stream valley adjacent to the Südwestfriedhof used for reproduction by the almost extinct population at this site; (d, e) stream and pond in the Sterkrader Wald, sites of larval development of fire salamanders; (f) fire salamander in the deciduous Kruppwald forest in Essen; (g, h) stream in the Kruppwald.

Table 1. Sites/districts (with decimal geographic coordinates) within the Ruhr District (Ruhrgebiet) in western Germany where different amphibians were screened for *Bsal* in the years 2017–2019. *Bsal*-positive sites are underlined. The amount of *Bsal*-positive individuals (in boldface) in relation to the amount of individuals sampled. *S.s.* = *Salamandra salamandra; I.a.* = *Ichthyosaura alpestris; L.h.* = *Lissotriton helveticus; L.v.* = *L. vulgaris; T.c.* = *Triturus cristatus; R.t.* = *Rana temporaria; B.b.* = *Bufo bufo*; † = dead individuals.

City / County	Site *(Coordinates given in decimal degrees)*	2017	2018	2019
Essen	Stadtwald (Forest) (51.423354, 7.020767)	*S.s.*: **8**/97	*I.a.*: **1**/2	*S.s.*: 0/1 *I.a.*: **1**/4 *B.b.*: 0/4 *R.t.*: 0/1
	Stadtwald (Garden) (51.423306, 7.033296)	No sampling	*I.a.*: **1**/25	*I.a.*: **2**/50
	Fulerum, Südwestfriedhof (51.431025, 6.968945)		*S.s.*: **8**/68 (64 †)	*I.a.*: 0/22*L.h.*: 0/5 *L.v.*: 0/10 *B.b.*: 0/32*S .s.*: **1**/5*R.t.*: 0/3
	Ruthertal (51.382679, 6.972078/ 51.376889, 6.959599)		*S.s.*: **1**/23	*S.s.*: **3**/3 (3 †)
	Kruppwald (51.403281, 6.995988)		*S.s.*: 0/30	*S.s.*: **73**/333 (8 †)
	Wolfsbachtal (51.404382, 6.973143)		No sampling	*S.s.*: **6**/15 *I.a.*: **1**/1
	Bergerhausen (amphibian fence) (51.436599, 7.048501)		No sampling	*I.a.*: **2**/5 *L.h.*: 0/2 *L.v.*: **6**/17
	Fischlaken (51.378457, 7.057662)	*S.s.* 0/1	No sampling	No sampling
	Byfang (51.392093, 7.112520)	No sampling	*R.t.* 0/2 *L.h.* 0/1 *I.a.* 0/3	No sampling
	Schellenberger Forest (51.413459, 7.043988)	*S.s.* 0/12	No sampling	No sampling
Bochum	Querenburg (several spots) (51.440654 7.254040/ 51.442381, 7.270643 / 51.448365, 7.280179)	No sampling	*S.s.*: **2**/25	*S.s.*: 0/5
	Stiepel (several spots) (51.418149, 7.251696/ 51.422972, 7.21072)		*S.s.*: **1**/15	*S.s.*: **1**/26 *I.a.*: 0/2 *L.v.*: 0/4
	Dahlhausen (51.435223, 7.139897)		*S.s.*: **1**/1 †	*S.s.*: 0/3 *I.a.*: 0/2 *L.v.*: 0/2
	Hiltroper Volkspark (51.52021, 7.26652)		*S.s.*: **1**/1 †	*L.v.*: 0/1 *B.b.*: 0/2
	NSG Tippelsberg/Berger Mühle (51.512009, 7.235154)		No sampling	*S.s.*: 0/43
Dortmund	Dellwiger Bachtal (51.511440, 7.336399)	No sampling	No sampling	*S.s:* **2**/2 (2 †)
Herne	Langeloh (51.532323, 7.289932)	No sampling	No sampling	*S.s.*: 0/18 *B.b.*: 0/1
	Gysenberg (51.532070, 7.250724)	No sampling	No sampling	*S.s.*: 0/1
Selm	Cappenberger Wald (51.669187, 7.525273/ 51.666870, 7.551980/51.656975, 7.550057)	No sampling	*S.s.*: 0/49 *I.a.*: 0/1	No sampling
Mülheim an der Ruhr	Rottbachtal (Forest/Garden) (51.377275, 6.839299)	*S.s.*: 0/30	*S.s.*: **1**/3 *I.a.*: **1**/1	*S.s.*: **3**/13 *R.t.*: **1**/1 *B.b.*: 0/1 *I.a.*: **4**/36 (0/3 †) *L.v.*: 0/18
	Rumbachtal (51.416847, 6.936570)	*S.s.*: 0/7	*S.s.*: 0/2	*S.s.*: 0/1 *I.a.*: 0/3 *R.t.*: 0/1 *B.b.*: 0/1
Wuppertal	Barmen, Hesselnberg (51.247764, 7.168684)	No sampling	*S.s.*: 0/11 *I.a.*: 0/3 *R.t.*: 0/1	No sampling
Kreis Mettmann	Ratingen (51.343460, 6.884168/51.321740, 6.858843)	*S.s.*: 0/22	*S.s.*: 0/10	*S.s.*: 0/24 *L.v.*: 0/1 *R.t.*: 0/2
	Heiligenhaus (51.358604, 6.990914)	No sampling	*S.s.*: **2**/2 †	No sampling
	Velbert (Bleibergquelle/Deilbach) (51.343183, 7.077412/ 51.337388, 7.142683)	No sampling	*S.s.*: **2**/3 (2†)	No sampling
Ennepe-Ruhr Kreis	Witten-Annen (51.429405, 7.387315)	No sampling	No sampling	*S.s.*: **13**/18 (18 †) R.t.: 0/2
	Hattingen - Deilbach (51.326008, 7.171701)	No sampling	No sampling	*S.s.*: **2**/8 (8 †)
	Hattingen – Niederwenigern (51.401187, 7.126893)	*S.s.* 0/1	No sampling	No sampling
	Ennepetal–Gevelsberg (51.315666, 7.346786)	No sampling	No sampling	*S.s.* 0/1
Hagen	Hagen-Nord (51.387469, 7.463797)	No sampling	*I.a.*: 0/2	No sampling
Oberhausen	Sterkrader Wald (51.542313, 6.833602)	No sampling	No sampling	*S.s.*: 0/109 *I.a.*: 0/2 *B.b.*: 0/1
	Hiesfelder Wald (51.562273, 6.846767/ 51.567875, 6.832103)	No sampling	No sampling	*S.s.*: 0/58 *I.a.*: 0/2 *L.h.*: 0/1 *T.c.*: 0/24
	Vonderort (51.505806, 6.902312)	No sampling	*S.s.*: 0/7	No sampling

manders had been observed in Essen-Ruthertal; qPCR of the preserved specimens did not yield *Bsal*-positive results, but this could be due to suboptimal conditions of preservation. Two other citizens reported having seen several dead fire salamanders in the winter of 2016 in their gardens or in the surroundings of their houses (Hattingen-Niederwenigern and Essen-Byfang, both sites at just 1.2 km from each other), and an absence or near-absence of fire salamanders at these sites since that time. Since no samples had been preserved, we could not confirm that the mortality at these two sites was due to chytridiomycosis. However, it seems possible that the pathogen was present already pre-2017 in the Ruhr District, especially when combining those reports with a photograph of a fire salamander in Bochum made in 2004 that shows typical *Bsal*-related lesions and ulcerations on the head (M. Maschka, pers. comm., March 2020; Fig. 3). No samples were taken of this salamander in 2004, and the population where it was photographed does not exist anymore; a confirmation that this salamander was indeed infected by *Bsal* therefore cannot be obtained. However, it is striking that the first evidence of *Bsal* in Germany, and in all of Europe, is also from 2004, referring to a preserved fire salamander from the Eifel Mountains where the pathogen was confirmed by histological examination at Ghent University (Dalbeck et al. 2018, Lötters et al. 2020b in this issue).

Quantifying *Bsal*-related declines

As reported above, for two sites in the Essen area, indications for fire salamander population crashes became available in 2017–2018 (Fig. 1a, b): In the Stadtwald, 97 individuals had been swabbed and analysed by us in 2017, eight of which turned out to be *Bsal*-positive and three had *Bsal*-characteristic external ulcerations; surveys in 2018 yielded no observations of infected fire salamanders. In the Südwestfriedhof graveyard, large numbers of dead individuals were observed at the end of 2017 and beginning of 2018, of which eight could be tested and resulted to be positive (Schulz et al. 2019, Wagner et al. 2019); afterwards, only two observations of a single fire salamander each were recorded by citizens during 2018 (Supplementary document 1). The Südwestfriedhof is highly frequented by promenaders, and many of these confirmed to us that in previous years fire salamanders were a common sight. However, given the secretive habits and not fully predictable activity pattern of fire salamanders, it is problematic to confidently assess the decline or disappearance of a population – a lack of encounters may simply be due to climate-induced inactivity or to a low overall activity in the respective night.

Figure 3. Photograph of a European fire salamander individual (*Salamandra salamandra*) taken in April 2004 in Bochum, Dahlhausen, by Martin Maschka. The animal shows possibly *Bsal*-related lesions and ulcerations especially on the head.

To obtain more reliable information on the status of declines/extinctions of the fire salamander populations at Südwestfriedhof and Stadtwald, we conducted an additional monitoring in spring 2019, along with standardized comparisons with two reference populations. As references, we chose two dense fire salamander populations without known *Bsal*-positives in the vicinity, Sterkrader Wald (Figs 1f, 2d, e) and Hiesfelder Wald in Oberhausen (Fig. 1g). At all four sites, transects of ca. 2 km were defined within suitable habitat, i.e. deciduous forest with mainly beech trees and close to small streams used by fire salamanders for reproduction. Transect walks took place during the night by a minimum of two observers, slowly walking along the transect and scanning the area left and right of the transect with electric torches for active salamanders and other amphibians. Because all encountered salamanders were photographed and swabbed, time per transect was not standardized.

At the two *Bsal*-positive decline sites, on five survey nights a total of only three salamanders could be recorded (Table 2): On April 2 and April 24, one individual each at the Südwestfriedhof site, on April 2, one individual at the Stadtwald site. In contrast, the two reference sites yielded 8–232 (Sterkrader Wald) and 14–28 (Hiesfelder Wald) salamander records per night (Table 2). On the two nights with highest numbers of active salamanders at the two reference sites (April 24 and May 2), surveys at all four sites were conducted simultaneously. Data suggest a heavy impact of the disease outbreak at the *Bsal*-positive sites, and considering the anecdotal observations from 2017 and 2018, it appears that the populations have drastically declined almost to complete extinction within only one or two years.

The single fire salamander found in 2019 at the Stadtwald was tested negative for *Bsal*; of other amphibians sampled at this site, two out of four alpine newts tested *Bsal*-positive, confirming the presence of the pathogen at this site. At the Südwestfriedhof, two fire salamanders were found during our five monitoring nights, and two additional individuals at other nights in 2019; one of these was found dead and tested *Bsal*-positive. Additionally, one living fire salamander without any visible sign of infection was recorded by a citizen during that time (Supplementary document 1). All of the 21 alpine newts, ten smooth newts

Table 2. Results of fire salamander surveys in the spring of 2019 at two *Bsal*-positive sites in Essen (Ruhr District) where declines were detected or assumed in 2017/2018 and at two *Bsal*-negative reference sites. During the first two surveys, not all sites could be assessed at the same day (* assessed on March 16; # assessed on April 3). NA = not assessed.

Site	March 14, 16	April 2, 3	April 24	May 2	May 21
Bsal-positive					
Südwestfriedhof	0	1	1	0	0
Stadtwald	0	1	0	0	0
Bsal-negative (Reference)					
Sterkrader Wald	8*	45#	232	118	NA
Hiesfelder Wald	NA	14	19	28	NA

Table 3. Results of monitoring of larvae of the European fire salamander (removal sampling) in streams in 2019, at two *Bsal*-positive sites in Essen (Ruhr District) where declines had been detected or assumed in 2017/2018, and at two *Bsal*-negative reference sites. Dates of surveys are listed with regard to the order of the sites. Numbers marked with (N) refer to nocturnal surveys.

Site	March 29/29/ 20/26	April 1	April 24/24/ 18/23	April 29	May 21/20/ 17/22	May 23/20
Bsal-positive						
Stadtwald	0	2 (N)	1	5 (N)	0	1 (N)
Südwestfriedhof	0	29 (N)	0	4 (N)	0	1 (N)
Bsal-negative (Reference)						
Sterkrader Wald	43	–	78	–	177	–
Hiesfelder Wald	237	–	463	–	364	–

and five palmate newts swabbed at the Südwestfriedhof in 2019 tested negative.

To obtain further quantitative data on population sizes in the *Bsal*-positive vs. reference sites, we performed monitoring of salamander larvae via removal sampling in all four sites. During diurnal sampling, we estimated numbers of 43–177 larvae (Sterkrader Wald) and 237–463 larvae (Hiesfelder Wald) per 75 m of stream in the reference sites, but only up to a maximum of one larva (Stadtwald) or no larvae (Südwestfriedhof) could be found at the *Bsal* positive sites (Table 3). To ascertain whether indeed at the decline sites larvae were absent, we sampled these sites three times at night, considering that fire salamander larvae are often more exposed and thus easier to spot in the streams during the night. At each sampling event, we obtained at least one larva at the *Bsal*-positive sites, with a maximum of up to 29 larvae on April 1 at Südwestfriedhof (Table 3).

Additionally, monitoring of the two *Bsal*-sites Stadtwald and Südwestfriedhof was conducted in October 2019 which resulted in no fire salamander observations over a period of five nights at the Stadtwald and one negatively tested individual over a period of seven nights at the Südwestfriedhof. In total, three negative and one positive tested fire salamanders were observed between March and October; only one per night out of 17 nights surveyed while only one non-infected fire salamander was found in one of 11 nights at the Stadtwald (Supplementary document 1).

Overall, these data confirm that the populations at the *Bsal*-positive sites have undergone severe declines, with only few females having deposited larvae in the respective streams in 2019. However, taking into account the results of further opportunistic searches for conservation genetic sampling, a total of 42 larvae from Stadtwald and 38 larvae from Südwestfriedhof were observed and sampled in 2019 (Schulz et al. 2019), and therefore we cannot consider the populations as being completely extinct yet.

Real-time history of a population crash

In late 2018, a large population of fire salamanders occurring in the Kruppwald (Figs 2f–h), northwest of the Baldeneysee, Essen-Bredeney, was selected for monitoring. Initially, this population was flagged as a *Bsal*-negative control site, based on the negative swabbing results of 30 fire salamanders (April 11, 2018: 8 individuals; September 21, 2018: 22 individuals). A transect was defined along a small stream serving salamanders for breeding, approximately between the geographical coordinates 51.4012N, 6.9973E and 51.4033N, 6.9957E.

In early 2019, two salamanders were found dead in this forest and reported to us by a citizen (one salamander each on January 3 and February 3). The site therefore offered the unique opportunity to observe the progress of a *Bsal* outbreak in real time. Nocturnal transect walks in the Kruppwald (transect length ca. 2 km) were carried out from February 28, 2019 to May 27, 2019 (plus three diurnal visits on March 21, 26 and 28), and altogether 278 salamanders were recorded during this time (including at least 20 individuals recaptured once or several times).

Table 4. Summary of individuals of European fire salamanders (*Salamandra salamandra*) from Kruppwald (Essen, Ruhr District) sampled in 2019 which were recaptured on at least one occasion during the study period. N and P refer to negative or positive (in boldface) results of qPCR tests of *Bsal* infection. Individual numbering as used in the AmphIdent project. * Infected salamander subsequently taken into the lab and cured by heat treatment. † Fire salamander found dead at the site.

Individual	Initial capture	First recapture	Second recapture	Third recapture
SA21	April 2 (N)	May 2 (N)	**September 23 (P)***	
SA61	April 2 (N)	May 21 (N)		
SA115	April 24 (N)	May 21 (N)		
SA118	April 24 (N)	May 8 (N)		
SA120	April 24 (N)	May 8 (N)		
SA122	April 24 (N)	May 21 (N)		
SA128	April 24 (N)	May 2 (N)	May 8 (N)	May 21 (N)
SA186	April 27 (N)	May 21 (N)		
SA217	May 2 (N)	May 21 (N)		
SA231	May 2 (N)	May 8 (N)		
SA238	May 2 (N)	May 8 (N)		
SA243	May 2 (N)	May 21 (N)		
SA246	May 2 (N)	May 21 (N)	**September 23 (P)**	
SA247	May 2 (N)	**May 8 (P)**		
SA265	**May 8 (P)**	**May 21 (P)**		
SA266	May 8 (N)	May 21 (N)	September 23 (N)	
SA272	May 8 (N)	September 23 (N)	October 4 (N)	
SA278	May 8 (N)	May 27 (N)		
SA289	May 8 (N)	May 17 (N)		
SA297	May 8 (N)	May 17 (N)		
SA323	May 21 (N)	May 27 (N)		
SA340	September 29 (N)	October 6 (N)		
SA343	October 4 (N)	October 6 (N)		
SA348	October 4 (N)	October 6 (N)		
SA352	September 23 (N)	September 29 (N)		
SA359	September 29 (N)	October 4 (N)		
SA466	March 1 (N)	April 2 (N)		
SA517	April 2 (N)	September 21 (N)		
SA534	**May 2 (P)**	**May 8 (P) †**		

Data are summarized in Table 4, with trends of prevalence and *Bsal* load over time given in Figure 4 / Table 5. Counting only the nights on which a regular transect walk of several hours was carried out, between 8 and 47 salamanders were observed, with maximum activities usually coinciding with rainfall. Although a general decrease in numbers of observed salamanders was observed during the last three survey days in May 2019, this cannot necessarily be interpreted as an indication of a major population decline because during these days the activity of salamanders decreased in general due to drier conditions, as also observed in *Bsal*-negative control populations (see below).

Maximum *Bsal* loads (in ITS/s) were relatively low until the beginning of March, (7.90E+03 to 5.66E+05), reached higher values at most sampling visits between the end of March to the end of April, with a maximum value of 2.29E+09 recorded on March 28, and reached moderate values in the last three visits in May, not exceeding a maximum value of 7.66E+06. The trend of mean *Bsal* loads of infected individuals followed a similar trend, as did prevalences which reached a maximum of 50% on the last sampling day, May 27, 2019 (Fig. 4). Both, *Bsal* loads and prevalences dropped towards the end of April 2019, with *Bsal* loads reaching its lowest post-March value on April 24 and prevalences dropping to 20% on April 24 and to 4% on April 27. Individuals with visible ulcerations scored from photographs peaked on May 2 and 8, 2019. Dead salamander individuals were observed on six occasions from January to March 2019, and one additional individual in May.

Using data from Winter 2018 and Spring 2019, and ordering all sampling days chronologically, a non-parametric Spearman correlation revealed a significant correlation between sampling day and prevalence (Spearman's R = 0.721; P = 0.019), and the relation between mean *Bsal* load and time was close to significance (Spearman's R = 0.486; P = 0.066). This suggests a probable overall trend of increasing prevalence of *Bsal* infection, and possibly an increasing *Bsal* load in infected individuals, in this population over

Table 5. Number of European fire salamander individuals (*Salamandra salamandra*) encountered during nocturnal transect walks at the Kruppwald (Essen, Ruhr District) in April and May 2019 (plus three salamanders encountered during diurnal visits in March 2019), and their *Bsal* load. Mean air temperature (Temp; °C) measured during the respective night or day. Note that the first two records in 2019 refer to dead salamanders encountered by citizen science observers. Prevalence values were only calculated for nights with > 2 individuals. *Bsal* loads were determined as numbers of ITS copies per swab, except in 2018 and early 2019 (zoospore genomic equivalents, abbreviated GE, per swab – transformed into ITS copies per swab by x 100). NA, not applicable/data not taken.

Date	Temp	Negatives	Positives (dead)	With lesions	Prevalence (%)	*Bsal* load mean	*Bsal* load mean positives	*Bsal* load maximum
April 4, 18	NA	8	0	NA	0	0	NA	NA
September 21, 18	NA	22	0	NA	0	0	NA	NA
January 3, 2019	NA	0	1 (1)	NA	NA	7.23E+04(723 GE)	7.23E+04 (723 GE)	7.23E+04(723 GE)
February 3, 2019	NA	0	1 (1)	NA	NA	3.00E+03(30 GE)	3.00E+03(30 GE)	3.00E+03(30 GE)
February 28, 2019	NA	19	1 (1)	NA	5.0	2.83E+04(283 GE)	5.66E+05(5660 GE)	5.66E+05(5660 GE)
March 1, 2019	NA	30	6 (1)	NA	16.7	7.79E+03	6.62E+04	1.37E+05
March 3, 2019	NA	0	1 (1)	NA	NA	7.90E+03	7.90E+03	7.90E+03
March 21, 2019	15	1	0	NA	NA	NA	NA	NA
March 26, 2019	9	0	1 (1)	NA	NA	1.58E+07	1.58E+07	1.58E+07
March 28, 2019	12	0	1	NA	NA	2.29E+09	2.29E+09	2.29E+09
April 2, 2019	12	27	15	0	35.7	3.12E+07	8.73E+07	1.16E+09
April 8, 2019	11.5	1	1	1	NA	1.10E+06	2.20E+06	2.20E+06
April 24, 2019	10	16	4	0	20.0	8.53E+03	4.27E+04	6.29E+04
April 27, 2019	8	22	1	1	4.3	NA	NA	NA
May 2, 2019	10.5	29	17	8	37.0	3.58E+07	9.68E+07	1.05E+09
May 8, 2019	10.5	27	20 (1)	12	42.6	1.13E+07	3.05E+07	3.80E+08
May 17, 2019	11	4	3	0	42.9	3.53E+05	8.24E+05	1.83E+06
May 21, 2019	11.5	16	7	2	30.4	2.03E+05	6.65E+05	1.28E+06
May 27, 2019	11	4	4	2	50.0	1.38E+07	2.75E+07	7.66E+06

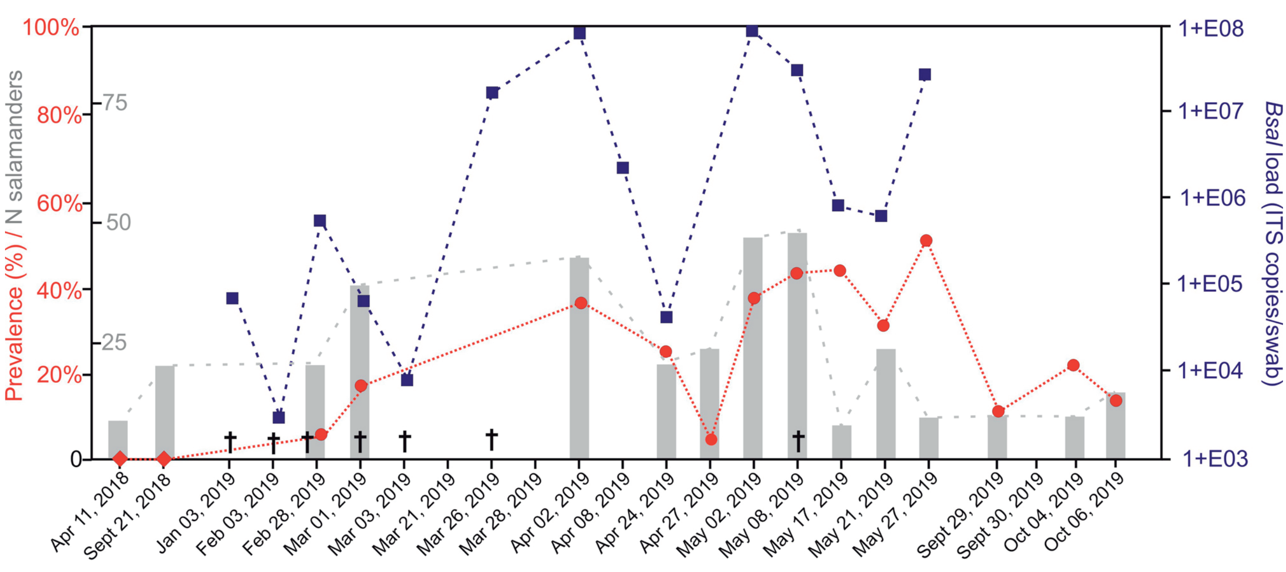

Figure 4. Graph showing number of European fire salamanders (*Salamandra salamandra*) encountered, *Bsal* infection prevalences (in percent of *Bsal*-positive individuals), average *Bsal* loads (ITS copies per swab) of positive individuals, and records of dead salamanders (crosses; on each marked day, a single dead individual was found) at various survey days in 2018 and 2019 at the Kruppwald site, along a ca. 2 km transect. Prevalences were calculated only for days with > 2 salamanders recorded; numbers of salamanders are only reported for nocturnal transect surveys with > 5 individuals. *Bsal* loads for some survey days were not calculated due to the use of different qPCR standards (see text).

the study period, despite the temporary drop of both variables at the end of April.

Additional surveys carried out in September 2019 revealed a comparatively low number of salamanders persisting in the population, many of which were subadults with relatively low infection prevalence (18%). A number of individual salamanders were recaptured on at least one occasion, as revealed by analysis of colour patterns in AmphIdent. In 24 out of 30 cases, recaptured individuals were tested *Bsal*-negative in the initial capture and all recaptures. Of the six recaptured individuals that were tested *Bsal*-positive on at least one occasion, three were already positive at first capture. In two of these, the data allowed comparing *Bsal* loads at two sampling dates: SA247 had a load of 1.37E+04 ITS/s on May 2 and 8.39E+05 on May 8; and SA265 had a load of 3.39E+04 ITS/s on 8 May and 2.12E+06 on 21 May. In SA247, *Bsal* load thus increased by one order of magnitude within a week; the individual had no visible external ulcerations at first capture but a few recognizable ulcerations at recapture. In SA265, the load increase was almost two orders of magnitude within 2 weeks; from the photos, a few ulcerations were recognizable at first capture but not at recapture, despite the higher infection loads. The other individual that was positive on the first sampling occasion (SA534) was observed on May 2 with a load of 1.05E+09 ITS/s, and was found dead on May 8.

Bsal loads and external symptoms

Bsal was first discovered in a declining population of fire salamanders in the Netherlands (Martel et al. 2013); external symptoms described consist of multifocal epidermal erosions and deep ulcerations on the entire body (Martel et al. 2013; see also van Rooij et al. 2015). Histologically, the periphery of these erosions is characterized by keratinocytes each containing one centrally located thallus, and the area of the lesions is often superficially colonized by bacteria (Martel et al. 2013). The increase in relative abundance of these opportunistic bacteria due to the destruction of the epidermis can contribute to septicaemic events, these bacteria thus acting as pathogens (Bletz et al. 2018).

At the Kruppwald site, we observed 84 *Bsal*-positive salamanders, of which 78 were alive. Many of these presented the typical ulcerations described previously for *Bsal*-infected fire salamanders (Figs 5–7), allowing for an

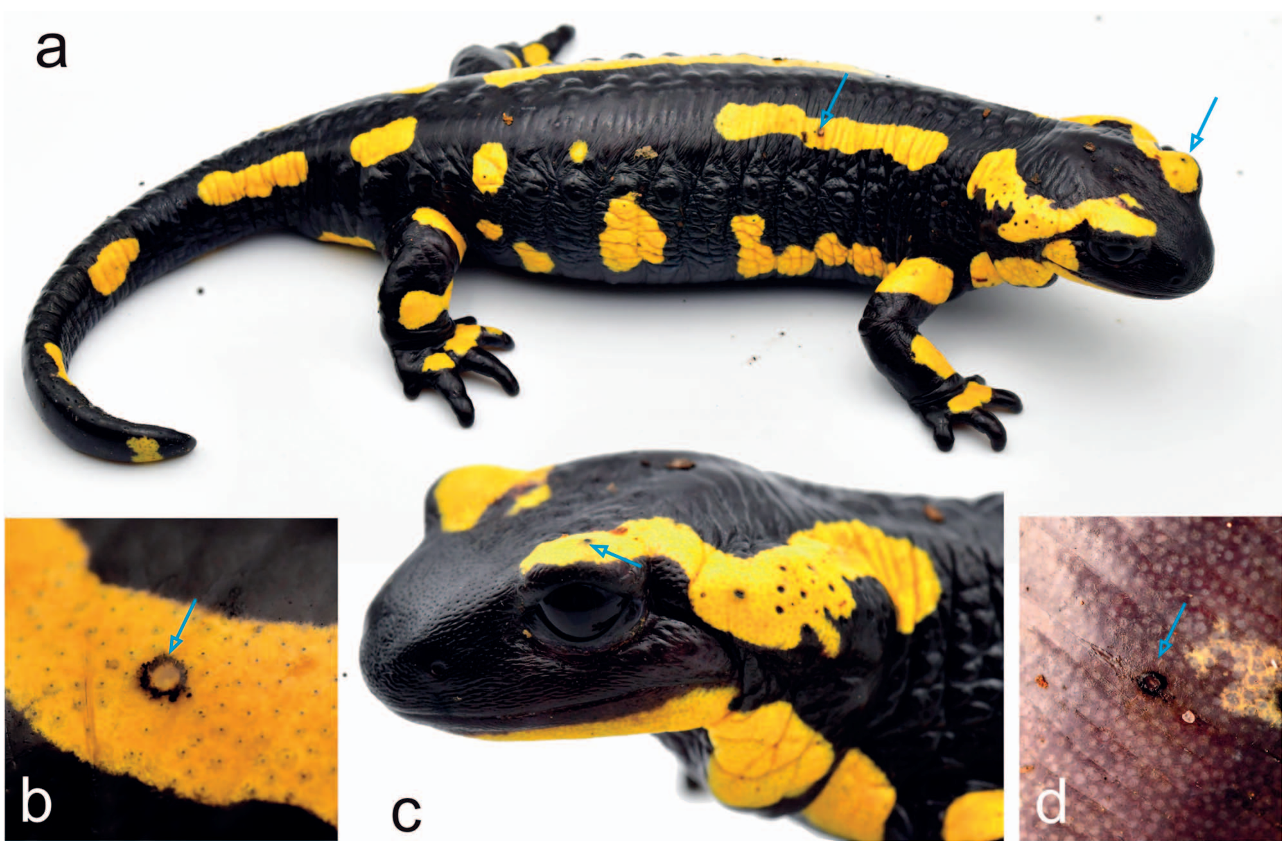

Figure 5. A *Bsal*-positive European fire salamander (*Salamandra salamandra*) female with a few external ulcerations on the dorsum (a, b), head (a, c) and venter (d) (indicated by blue arrows) detected at the Kruppwald site on April 2, 2019 (individual K0). This was the first salamander with external symptoms discovered at this site, and it survived for 39 days at the Technische Universität Braunschweig at a low temperature regime (ca. 15°C), with some of the ventral ulcerations and lesions healing, before it eventually died.

extended (macroscopic) description of these symptoms which may prove important for future initial detection of the disease, e.g. in the course of citizen science projects. By far, most salamanders with visible signs of disease had small ulcerations which were best visible on the yellow skin patches but also exist on the black-coloured parts of the

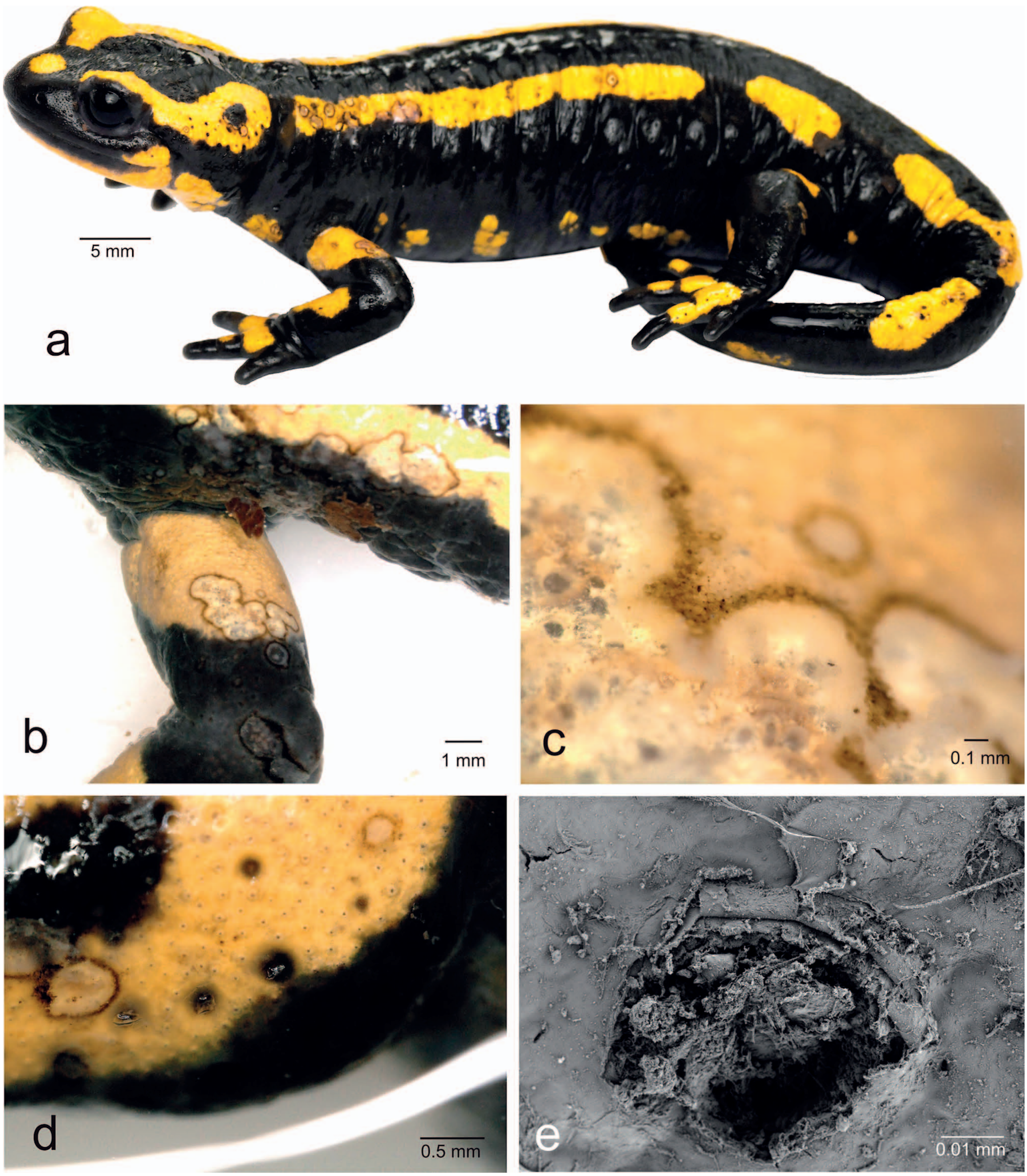

Figure 6. A European fire salamander (*Salamandra salamandra*) male from Kruppwald with a large number of partly large-sized ulcerations. (a) Dorsolateral view in life (snout–vent length 98.8 mm; voucher preserved under field number ZCMV 15514). This individual was sacrificed for close examination of the ulcerations under a light microscope. (b–d) Lesions visible in the living animal on the parotoid gland and forelimb. (e) Scanning electron microscope showing one of the crater-like ulcerations. The individual tested *Bsal*-positive.

Figure 7. Different individuals of European fire salamanders (*Salamandra salamandra*) from Kruppwald with externally visible *Bsal*-related ulcerations. All these individuals tested *Bsal*-positive.

body. Because in the Kruppwald population, salamanders have only rarely yellow colour on the ventral side, the ventral ulcerations are less readily detectable. In some individuals only single ulcerations were present, and sometimes it was not easy in such cases to macroscopically distinguish tiny dorsal ulcerations from poison gland duct openings (especially on the parotoid glands), small abnormal coloration phenomena (such as reddish pigment elements or small black dots on yellow patches), or small ventral injuries. Other individuals had multiple small ulcerations over the whole body (Figs 5, 6), and often these were present on the head, both in the region of snout and throat, and in some individuals also in high density on the parotoid glands (Figs 6d, 7g). In most cases, salamanders had a few or many small-sized regular round ulcerations of 0.25–1 mm in diameter, but in several individuals (Fig. 6) some ulcerations were larger (up to ca. 2.5 mm) and confluent with each other, giving the impression of outward growth from the centre of each ulceration, similar to a 'fairy circle'. The edge of the growth zone is brownish and in magnified view consists of bulbous structures probably corresponding to keratinocytes and maybe fungal thalli (Figs 6c, d). Examined with a scanning electron microscope, the ulcerations are revealed as deep crater-like formations in which the original skin layer is completely destroyed (Fig. 6e). In advanced stages of disease, these ulcerations become so deep that they form bleeding lesions, often in the region of the snout (Fig. 7e).

Because all recorded salamanders from April 2019 were photographed for individual recognition, it was possible to score the presence of these externally visible ulcerations and lesions for all individuals and relate the presence and intensity of these symptoms to *Bsal* presence and loads determined by qPCR. We classified individuals into four categories: (1) no recognizable ulcerations, (2) ulcerations visible according to field notes only, not visible in photos, (3) few/weak ulcerations, (4) moderate number of ulcerations, (5) many ulcerations. These data revealed that ulcerations were never found in *Bsal*-negative salamanders (Fig. 8a), suggesting that in regions with qPCR-confirmed *Bsal* outbreaks the presence of the typical lesions described here can be seen as a suitable indicator for individuals likely to be *Bsal*-infected. However, the majority of infected salamanders showed no visible ulcerations (47 of 74 scorable, *Bsal*-positive individuals), and only three and four individuals were observed in the highest categories of a moderate and high number of ulcerations, respectively (Fig. 8b). The median *Bsal* load for *Bsal*-positive individuals was lowest for individuals without lesions and highest for individuals with many ulcerations (Fig. 8c), but the differences among categories were not statistically significant (Kruskal-Wallis-ANOVA, $P > 0.5$), which could be either due to low sample sizes in the categories of moderate number and many ulcerations, or to differences in individuals regarding e.g. microbiome composition (Bataille et al. 2016), genetic variation (Horner et al. 2017), individual thermal preference (Sauer et al. 2018) or immunogenetic variation (Savage & Zamudio 2011).

Clearing *Bsal* infection with heat treatment

Disease dynamics are linked to the pathogen virulence, host factors and environmental determinants, and temperature seems to be one key environmental factor for both

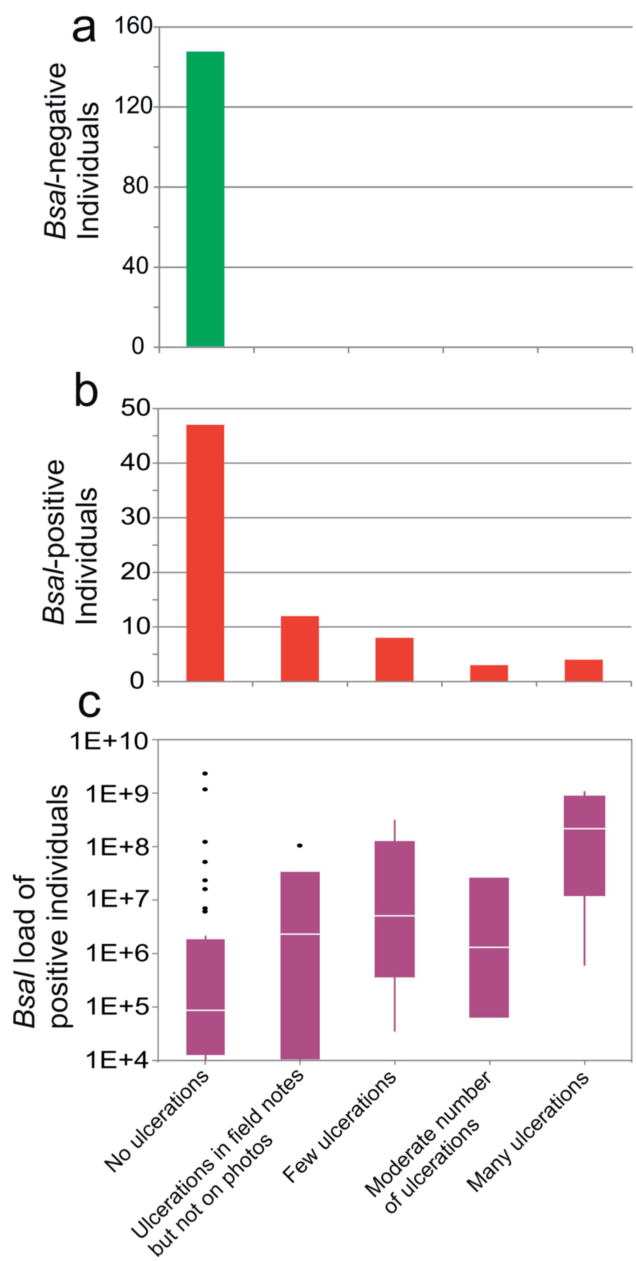

Figure 8. Occurrence of externally visible lesions and ulcerations in European fire salamanders (*Salamandra salamandra*) from Kruppwald, based on photographs and field notes of living individuals recorded in April and May 2019. Panels (a) and (b) show the distribution of categories of ulceration occurrence in salamanders that tested positive and negative for *Bsal* in qPCR assays, respectively (no ulcerations were visible in any *Bsal*-negative individual). Panel (c) shows boxplot of *Bsal* load (in ITS copy number per swab) for the four categories, based on data from *Bsal*-positive individuals only.

fungi, *Bd* and *Bsal* (Berger et al. 2004, Kriger & Hero 2007, Martel et al. 2013, Blooi et al. 2015a, b). Consequently, heat treatment protocols were published to clear infection in animals. However, since both fungi show different thermal growth characteristics most probably due to differences in their host spectrum (Olson et al. 2013, Martel et al. 2014), different protocols need to be followed for treating chytridiomycosis induced either by *Bd* or *Bsal* (Martel et al. 2011, Woodhams et al. 2012, Blooi et al. 2015a, b). Here, we used the heat treatment protocol published by Blooi et al. (2015a) to cure *Bsal*-infected fire salamanders.

First treatment in an irregularly heated room: Six *Bsal*-infected fire salamanders with few (Box C), several (Box K1) or many lesions (Box A, B) and two non-infected fire salamander (Box G, E) were transferred from the field to the lab at Braunschweig. They were kept individually in plastic boxes on a moist tissue in a room with normal heaters and an additional electric heater. When the animals arrived, the room temperature was about 15°C for two days. After two days, the heaters were turned up slowly until the temperature in the room reached 25°C after four days, fluctuating between 25–27°C. At these temperatures, all animals were intensively shedding their skin and ulcera-

Figure 9. Healing process during the heat treatment period 10–20 May 2019 at 25–27°C of the fire salamander that was kept in Box A (see Fig. 12) and had a high amount of small ulcerations at the dorsal and ventral sides, as well as deep wounds at the head. Skin recovered very fast during the treatment due to intensive skin shedding.

tions became smaller and darker every day, and had almost completely disappeared after seven days (Figs 9, 10). During the whole treatment period, the animals rejected food. After the suggested 10-day treatment (Blooi et al. 2015a) they were transferred to new plastic boxes with clay, wood and moss, first at 20°C, and three days later at 15°C. After the treatment, qPCR results revealed still very low amplification values for *Bsal* regarding the animals in Box A, B and C (below 100 ITS/s, mean Cq value 36.24 ± 0.51), i.e. below the suggested threshold of 1 GE (Thomas et al. 2018) which would correspond to an approximate Cq value around 33 (33.32 ± 0.62 with BSA; 32.99 ± 0.19 without BSA) (Blooi et al. 2013). These animals tested negative by the second laboratory. Approximately six weeks (40 days) after the end of the treatment (animals kept at 15°C during this time), the non-infected animals in Box G and E were still *Bsal*-negative. However, new skin ulcerations were observed in the other four animals (Boxes A, B, C and K1), and all of them

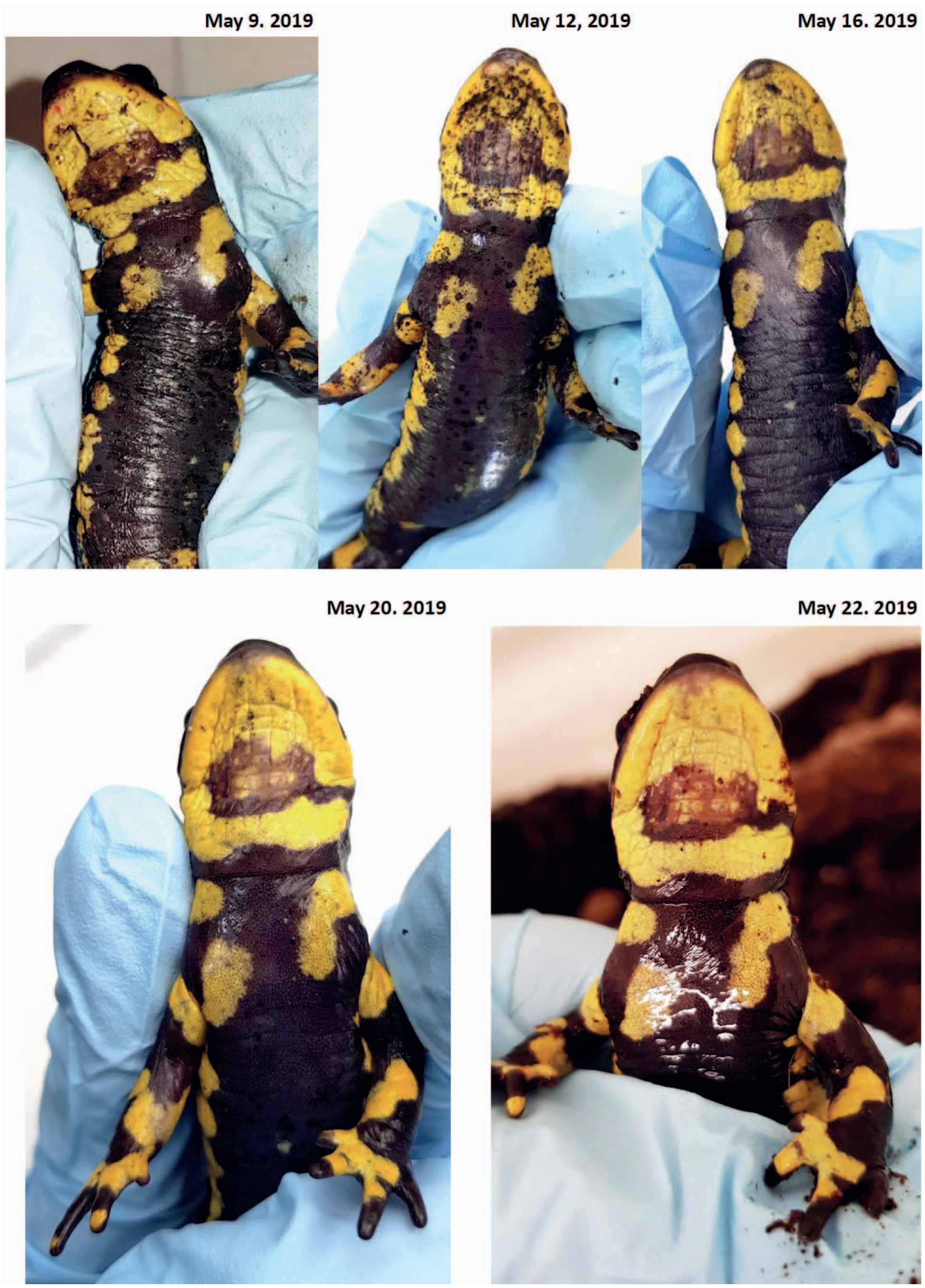

Figure 10. Healing process during the heat treatment period 10–20 May 2019 at 25–27°C in the fire salamander kept in Box B (see Fig. 12) which had some small ulcerations at the dorsal and a high amount at the ventral side, including a deep wound at the chin.

tested *Bsal*-positive again in qPCR. Although loads were similar for all four (mean load 2.16E + 07 ITS/s), the form and amount of ulcerations differed, suggesting an irregular expression of symptoms. The infected fire salamanders were transferred into new plastic boxes and placed into a climate chamber for a second heat treatment, one day at 20°C, one day at 23°C, and 21 days at 25°C (Fig. 11a). All three animals tested *Bsal*-negative directly after the end of the treatment, and again 19 days later (Fig. 12). It is of relevance to note that initially *Bsal*-negative fire salamanders in Box G and E had no indication of external symptoms of chytridiomycosis, and consistently tested *Bsal*-negative in all qPCRs. This confirms that transmission of *Bsal* spores among salamanders kept in separate boxes in the same room can be avoided if working under strict hygiene protocol.

Second treatment in a climate chamber under fully controlled conditions: An additional fire salamander collected in September 2019 that showed *Bsal*-lesions followed by a positive qPCR-result was directly transferred into a climate chamber where the temperature could be kept constant at 25°C after the third day of acclimation. Considering our previous experience we chose to treat the salamander for 14 days before lowering the temperature. Directly after the treatment, the animal tested negative, and was then transferred to a box with clay, moss and wood and kept at 15°C and below. Surprisingly, also this fire salamander again showed the first clinical signs of a *Bsal* infection after approximately 5 weeks (34 days) (Fig. 11b), confirmed by qPCR. After the animal was taken back into treatment (this time at 26°C), no immediate improvement could be

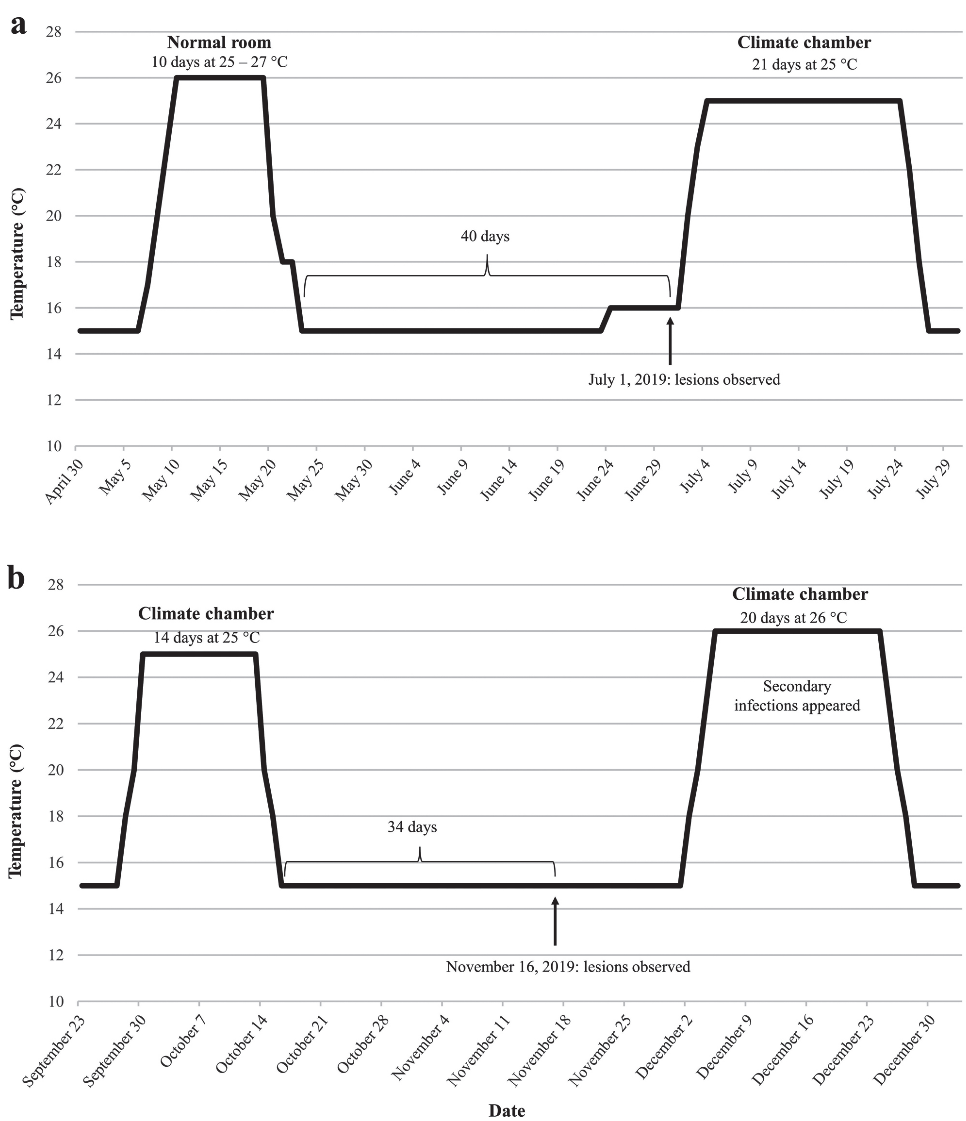

Figure 11. Graphs showing heat treatment time frames used to cure the infected European fire salamanders (*S. salamandra*) found in the wild in 2019. Panel a shows the ad-hoc treatment chosen for salamanders in boxes A, B, K1, C and E. Panel b shows the treatment chosen for box Z. Each box was used to house a single salamander during this time. See Fig. 12 for qPCR loads of salamanders subjected to these treatments.

noticed after 3 days as seen in previous treatments. In contrast, extended wounds emerged on the head where lesions were previously visible, almost certainly in concert with secondary infections caused by bacteria and other fungi. After 14 days of heat treatment (disinfecting wounds by dabbing them with a tissue dipped in Bactine every two days), *Bsal* could still be detected via qPCR (Fig. 12). The detected loads (mean value around 50 ITS/s) remained below the threshold, but positive curves were visible for more than five weeks. Eight weeks after the treatment the qPCR detection was again positive above the threshold, with recurrence of lesions and ulcerations especially at the head and back. This demonstrates the presence of viable *Bsal* when signals below the threshold are detected rather than just residual DNA from dead fungal remains in this individual. Since the wounds were still not healed and the animal obviously still infected by *Bsal*, we eventually decided to euthanize this salamander (by MS222 overdose).

In conclusion, our experiences with the treatment of *Bsal*-infected fire salamanders in principle agree with those of Blooi et al. (2015a): heat treatment can be an effective and low-cost option to heal *Bsal*-infected fire salamanders if well monitored. However, we stress that (i) in some fire salamander individuals infection may not be completely cleared after a 10 days / 25°C treatment, highlighting the importance of intensive monitoring during and after treatment, and the danger of missing post-treatment weak-intensity infections by qPCR (i.e. signals below threshold or even negative); and (ii) the possibility (as with the salamander in our second treatment) that in heavily infected animals with many external symptoms, the stressful heat treatment even for 14 days may be insufficient, and may even favour secondary infections.

This information might help private amphibian keepers conducting the treatment at home where a continuous monitoring of the infection status is not feasible. Animals might not feed properly or not at all during the treatment which needs to be considered. However, once the heat treatment is started we do not recommend to interrupt it (except if secondary infections occur that need specific treatment by a veterinary), to prevent reinfection and further stress for the fire salamander. Cured animals should be fed properly after treatment in combination with mineral and vitamin supplement to facilitate full recovery. We also highlight, once more that any material that came into contact with infected animals must be either immersed in disinfectant (e.g. 1% Virkon S solution or 80% ethanol), autoclaved or burnt.

Last but not least, we appeal to all amphibian keepers to test their individuals for detecting asymptomatic infections to prevent further passive spread of spores in captivity and into the wild. A few studies have already shown that the pathogen occurs in captive collections (Fitzpatrick et

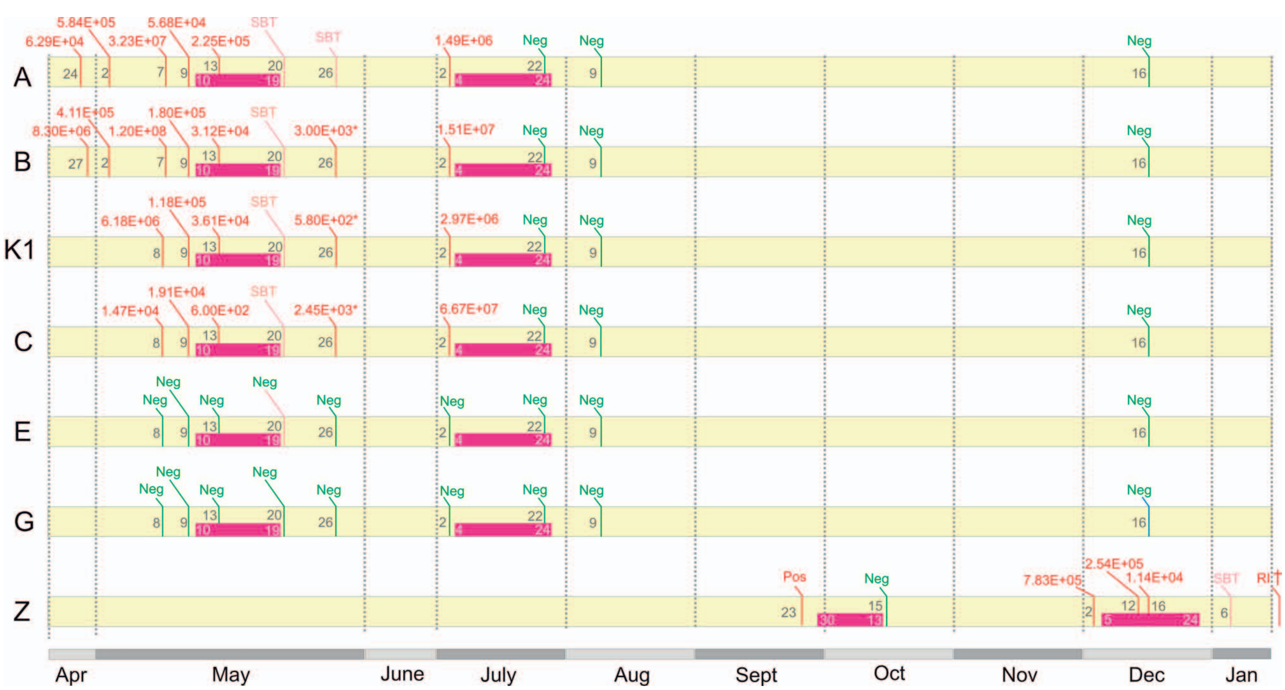

Figure 12. Chronology of results of qPCR testing for *Bsal* infection in European fire salamanders (*Salamandra salamandra*) collected from the wild in the Ruhr District and subjected to heat treatment to cure chytridiomycosis between April 2019 and January 2020. Each light yellow bar represents one salamander individual. Numbers within light yellow bars are dates of the respective month; heat treatment is indicated by magenta bars (see Fig. 11 for temperature regimes applied). Values above light yellow bars are qPCR results; red values give *Bsal* load for samples that tested positive; Neg in green font marks *Bsal*-negative test results, and pink SBT marks possibly positive samples with qPCR curves below the threshold. In order to graphically fit all values, May occupies a disproportionally wide part and June a disproportionally narrow part of the yellow bars. Salamander Z after January 2020 turned out to be reinfected (RI) and was euthanized.

al. 2018, Sabino-Pinto et al. 2018) although in other encouraging cases captive collections turned out to be apparently free of *Bsal* despite intensive testing (Jung et al. 2020 in this issue). Especially, reports about asymptomatic infections in several, non-native species are of concern (Sabino-Pinto et al. 2018), as are the recently documented chronic, non-lethal *Bsal* infections in Anatolian crested newts (*Triturus anatolicus*) invasive in Spain that may indicate latency periods of undetectable infection and subsequent flare-ups leading to spillover of infection to native species (Martel et al. 2020).

The role of newts as potential victims, reservoirs and vectors in the Ruhr District

Beside the fire salamander, at least three of the four native German newt species are also affected by *Bsal*: alpine newts, smooth newts and great crested newts died due to artificially induced *Bsal* infection (Martel et al. 2014); the fourth species, the palmate newt, could not be artificially infected, but *Bsal*-infected wild palmate newts in the field have been observed (Dalbeck et al. 2018, Lötters et al. 2020a in this issue). While in fire salamanders, *Bsal* infection is typically lethal, experimental infection of alpine newts at low zoospore doses resulted in frequent shedding for several months with fungal clearing and clinical cure (Stegen et al. 2017), suggesting newts may function as a pathogen reservoir and vectors for *Bsal*.

Only a few of the *Bsal*-positive sites in the Ruhr District are populated solely by newts. One affected alpine newt population (Stadtwald, garden) occurs at a garden pond located at a linear distance of only ca. 900 m from a *Bsal* site (Stadtwald, forest) populated by fire salamanders. In this pond, used by at least 50 alpine newts for reproduction, a citizen observed in April 2018 several dead newts and others with ulcerations (Fig. 13). In the same month, 20 individuals were swabbed of which one newt was unambiguously *Bsal*-positive while several others indicated very low loads (< 1 GE) in one of two samples. In September 2018, three tested alpine newts were *Bsal*-negative, and in May 2019, two out of 50 newts were *Bsal*-positive (mean load 5.00E + 03 ITS/s), and no decline of the newt population was apparent since the number of animals was still as high as in the previous year.

In Mülheim an der Ruhr, another garden pond has moved into focus after a *Bsal*-infected fire salamander was discovered in the garden in September 2018. The pond is used by both alpine and smooth newts for reproduction, and in April 2019, six out of 33 alpine newts and five out of 17 smooth newts tested *Bsal*-positive, while three dead alpine newts tested negative. A month before, in the forest surrounding this garden, we also detected three infected out of 13 tested fire salamanders, one infected alpine newt and, interestingly, one possibly *Bsal*-positive common frog (*Rana temporaria*); however, given that the latter observation refers to a single individual only, we consider the possibility of *Bsal* infection in this anuran species to be in need of confirmation by an independent method (e.g. histologically; see Thomas et al. 2018). However, it is worth noting that the possibility of a sample confusion can be excluded as from the same swab used for *Bsal* detection, we also sequenced a fragment of the mitochondrial 16S rRNA gene of the host which was confirmed as *R. temporaria* (Genbank accession number MT408024). In the Hiesfelder Wald in Oberhausen, a great crested newt population was also screened in spring 2019, but all animals were *Bsal*-negative.

The current data are insufficient to flag newts as unaffected reservoirs and vectors of *Bsal* in the Ruhr District, and we cannot exclude that they also undergo long-term population declines. Although newts are at high risk of infection when entering water bodies potentially infested by *Bsal* zoospores, they may in many cases also have an advantage over the almost purely terrestrial adult fire salamanders due

Figure 13. Garden pond in Essen-Stadtwald. The size of the pond (left) is 1 × 1.5 m and a depth of 30 cm. It represents the spawning water for an alpine newt population (*Ichthyosaura alpestris*) of > 50 individuals. Five out of 75 newts were tested *Bsal*-positive (April and September 2018 / May 2019). One individual showed ulcerations similar to those observed in infected fire salamanders (right).

to their semiaquatic life cycle, alternating between aquatic and terrestrial phases. In particular, in many populations living in stagnant water bodies, aquatic-phase newts may experience at least temporarily warm temperatures, potentially implying a periodic, passive heat treatment that may help them to clear *Bsal* infection. Additional factors may include the drastic changes in skin structure and cutaneous microbiome that newts undergo in their transition between aquatic and terrestrial phases (Perrotta et al. 2012, Sabino-Pinto et al. 2017). Clearly, a further close monitoring of the known newt sites, also including one pond in the Südwestfriedhof site, is warranted to understand the long-term impact of *Bsal* on newt populations. This in particular applies to the great crested newt, where in the Eifel Mountains two monitored populations disappeared within two years (Lötters et al. 2020a in this issue).

In areas of dense human population such as the Ruhr District, habitat fragmentation and road mortality (Fahrig et al. 1995, Hels & Buchwald 2001, Andrews et al. 2008) can impact amphibian populations, and temporary drift fences with pitfall traps are widely used to maintain connectivity between breeding sites and terrestrial habitats and to monitor population trends and breeding phenology (e.g. Houlahan et al. 2000, Bonari et al. 2011). As volunteers are often involved, an educational effect is often an added benefit (Schmidt & Zumbach 2008). However, the pitfall traps might also increase the risk of pathogen transmission among amphibians, and volunteers handling the animals may also contribute to the spread of the disease. Of 24 randomly screened newts caught at a temporary drift fence in Essen-Bergerhausen, about 2 km from the *Bsal*-positive site Essen Stadtwald (forest) in March 2019, two out of five alpine newts and six out of 17 smooth newts tested *Bsal*-positive. Despite this evidence for *Bsal*-positive newts at drift fences, we do not advocate abandoning the drift fence activities to rescue migrating amphibians given that (i) *Bsal* already seems to be widespread in the Ruhr District, (ii) it is likely that the pathogen is further spreading by multiple mechanisms, and (iii) anurans, which are predominantly caught in pitfall traps, are not affected by *Bsal*. However, next to the routine disinfection measures suggested for shoes, dip nets or newt traps, at least in areas with known *Bsal* occurrence it may be recommendable to disinfect pitfall buckets each time they are controlled (disinfection solution for effective exposure time: see van Rooij et al. 2017; then rinsing with water before next use); furthermore, gloves may be used, and changed for each bucket, to minimize the risk of transferring the pathogen. Ideally, the drift fence equipment should be used in only one area, and volunteers should not be in charge of multiple, geographically distant drift fences.

Use of water-borne environmental DNA for *Bsal* detection

The isolation and detection of amphibian pathogens via DNA from environmental samples, so-called environmental DNA (eDNA), is a promising tool for their early detection (e.g. Pierson & Horner 2016, Hall et al. 2016, Kamoroff & Goldberg 2017). In a pilot study, we here tested the potential of eDNA for the detection of *Bsal* in 52 water bodies located in the vicinity of either infected or non-infected fire salamander populations. *Bsal* was confirmed by eDNA analysis in two out of the 52 sample sites: from a stream at the site Essen-Kruppwald with a *Bsal* load of 2.23E + 03 to 4.48E + 03 ITS copies per 500 ml of water; and from a pond near the botanical Garden RUB/Kalwes, with amplification products in all four 500 ml water samples. The qPCR products of both sites were verified as *Bsal* by means of Sanger sequencing. Also, at both sites, several animals were confirmed as *Bsal*-positive by qPCR analysis from swabs, and many salamanders were observed along the stream at the Essen-Kruppwald site during water sampling. Therefore, it is possible that the detected *Bsal*-DNA originated directly from infected fire salamanders, potentially from females releasing larvae into the water.

Although only two of the water bodies showed positive results, our study points to the potential of detecting the pathogen *Bsal* based on DNA from environmental samples. More data on the reliability of this eDNA approach (rates of false positives/false negatives; sensitivity to low concentrations of *Bsal* DNA) are needed to understand which role it could play for studying and tracking the spread of the disease in the environment, and identifying potential reservoirs. Potentially, it could be used alongside simultaneous amphibian detection via eDNA in a landscape-wide approach (Thomas et al. 2019). However, little is known about the persistence, mobility, position and infection potential of the different stages of *Bsal* in water (Rosenblum et al. 2010, Gray et al. 2015, Stegen et al. 2017). Also, different sources of origin of the detected DNA have to be considered, as it can originate from infectious spores as well as from degenerated and non-infectious DNA. Therefore, further studies are needed to evaluate both the limitations and the benefits of using eDNA as a tool for *Bsal*-detection.

Future perspectives for salamander conservation management in the Ruhr District

This review is a testimony of the calamity that fire salamanders are experiencing in forests across the Ruhr District. It allows us a glance into a future with this once so abundant amphibian gone from this area if we do not take action. The fire salamander is a very charismatic species that engages the sympathy of citizens regardless of their understanding of nature. To halt the ongoing loss of salamanders due to *Bsal* infection, we aim to develop a broad conservation action plan.

The unpredictability of *Bsal* in terms of dispersal and reservoirs renders in situ conservation difficult (Stegen et al. 2017, Thomas et al. 2019), and the persistence of this highly infectious pathogen in a habitat is still uncertain. It is obvious, however, that with the decline and disappearance of every population the species can lose valuable genetic adaptations. Our data illustrate once more how fast *Bsal*-induced declines of salamander populations can proceed, thus demonstrating the urgency for conservation actions. At the short time, ex situ conservation (i.e, keeping animals outside their natural environment) appears to be the only viable solution to preserve the genetic diversity of the fire salamander from areas with *Bsal* outbreaks. This option is a last resort to save a species, subspecies or an important genetic lineage from extinction (Pritchard et al. 2012, McGowan et al 2017). Several amphibian species that can no longer survive in the wild due to habitat destruction, pollution or disease are currently bred in captivity to preserve them until the natural conditions improve (Valbuena-Ureña et al. 2017, Lewis et al. 2019).

During the massive *Bsal*-related population declines in the Netherlands, the remaining fire salamanders were rescued and are since then sheltered in a zoo (Spitzen-van der Sluijs et al. 2013). But an infectious pathogen like *Bsal* ignores political borders. Saving the fire salamander by means of ex situ breeding must be a joint effort of all affected countries which is currently represented by the 'Ex situ Salamandra Group' (ESG; Spitzen-van der Sluijs et al. 2018a). The ESG develops breeding guidelines, engages zoos and private keepers, and holds a consultative role, whereas the translation of ex situ conservation into practice should be a regional responsibility. By keeping fire salamanders, zoos in the Ruhr District can conserve an imperilled local species while simultaneously engage the public and conduct environmental education. As a further aspect, especially in a densely populated area like the Ruhr District, where citizens are directly witnessing the mass die-offs, it is crucial to also consider animal welfare aspects. Concepts for a temporary rescue centre for infected fire salamanders may therefore be necessary to be able to perform heat treatments of infected salamanders encountered by citizens.

As a long-term perspective, animals from zoos or rescue centres may be reintroduced to their natural habitats in a stepwise procedure, first to outdoor enclosures that allow a close monitoring of individuals and for periodically testing for infection, and eventually – hopefully – into the wild if the pathogen has disappeared. Only time can tell whether this strategy can be successful – but without immediate action we are at risk of losing the Ruhr District populations of the charismatic fire salamander without any option for return.

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Supplementary data

The following data are available online:

Supplementary document 1. Overview of all reports and site visits with documented and tested fire salamanders from 2017–2019 at two *Bsal*-positive sites in Essen.