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Key questions about the impact of the salamander plague on the Northern Crested Newt, *Triturus cristatus* – a German perspective

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Abstract. For at least two decades, European amphibians have been affected by the 'salamander plague', an emerging infectious disease caused by the invasive chytrid skin fungus *Batrachochytrium salamandrivorans* (*Bsal*) that originated from Asia. Resulting dramatic declines are well known from the European Fire Salamander, while the impact of *Bsal* on other European amphibians in the wild is less clear. We here focus on the Northern Crested Newt (*Triturus cristatus*) in Germany. This species is susceptible to *Bsal* and strictly protected under Annex II of the EC Habitats Directive. While *T. cristatus* is declining in the *Bsal*-hotspot Germany, it remains to be answered if the salamander plague is one of the leading drivers. By asking five key questions we review the available information with the goal of providing a baseline for further research. In two investigated newt communities, *Bsal*-prevalence was found to be considerably high, with 75–89% of newts being infected, reaching the highest peaks in spring. Later in the year, some *T. cristatus* were apparently able to clear their *Bsal* infection, even when individual infection load was previously high. Although we observed mortality of infected specimens and declines in populations with *Bsal*, declines are also seen outside known *Bsal* regions. Thus, it appears that the *Bsal* epidemic is not exclusively responsible for the species' current status. With this, *Bsal* poses a yet poorly-understood threat to Northern Crested Newt survival, making further research on pathogen/host interactions and long-term survival indispensable to fulfil our legal responsibility of conserving this emblematic species.

Key words. Caudata, Salamandridae, amphibian crisis, *Batrachochytrium salamandrivorans, Bsal,* chytrid fungus, conservation, decline, emerging infectious disease, population monitoring.

Introduction

Invasive emerging diseases are a significant threat to the global biodiversity (Daszak et al. 2000). Amphibians suffer particularly from the spread of pathogens. The chytrid skin fungi *Batrachochytrium dendrobatidis* (*Bd*) and *B. salamandrivorans* (*Bsal*), both of Asian origin, are associated with many instances of amphibian decline and extinction worldwide (van Rooij et al. 2015, O'Hanlon et al. 2018, Scheele et al. 2019, Fisher & Garner 2020). *Bsal* mainly affects caudates and has been invasive in Europe for at least two decades (Lötters et al. 2020a). It poses a serious threat to the entire Western Palaearctic amphibian diversity. Therefore, national and international conservation measures are urgently needed (Martel et al. 2014, 2020, Spitzen-van der Sluijs et al. 2016, Thomas et al. 2019). Among the four European countries from which *Bsal* is known in the wild (BE, DE, ES, NL), Germany is the 'hotspot' with more than four-fifths (94) of all European records published to date (105), and with the most outbreaks in recent years (FOD 2017, Lötters et al. 2020b, Schulz et al. 2020, authors' unpubl. data).

The European Fire Salamander (*Salamandra salamandra*) has been particularly hard hit by *Bsal*-induced chytridiomycosis, responding to infections with local mass mortality (Spitzen-van der Sluijs et al. 2013, 2016, Stegen et al. 2017), which was the reason why Dalbeck et al. (2018) introduced the catch phrase 'salamander plague' for it. Infection experiments and observations in the wild (*Bsal* outbreaks) suggest that European newts may be less affected by the salamander plague (Martel et al. 2014, Spitzen-van der Sluijs et al. 2016, Dalbeck et al. 2018, Gilbert et al. 2020, Lötters et al. 2020b, Bosch et al. 2021). However, little is known about the dynamics of *Bsal* infection and its threat to wild newt populations. Palmate and Smooth Newts, *Lissotriton helveticus* and *L. vulgaris*, are probably resistant or tolerant (Martel et al. 2014, Dalbeck et al. 2018, Lötters et al. 2020b). Nevertheless, mortality caused by *Bsal* has been observed in the latter species (Spitzen-van der Sluijs et al. 2016, Bates et al. 2019). The Alpine Newt (*Ichthyosaura alpestris*) is known to be susceptible and may act as a reservoir, but disease dynamics are dose-dependent (Stegen et al. 2017). The species may even promote *Bsal* dispersal (Beninde et al. 2021).

The picture is even less conclusive regarding the Northern Crested Newt (*Triturus cristatus*), which is strictly protected under Annex II of the EC Habitats Directive (Council Directive 92/43/EEC). Martel et al. (2014) showed that high pathogen doses were lethal to *T. cristatus*. However, infected specimens survived these expositions for at least 30 days, suggesting more complex response mechanisms. Bosch et al. (2021) suggested that levels of *Bsal* susceptibility might be transferable among phylogenetically related taxa. Since the Marbled Newt (*T. marmoratus*) is highly susceptible to *Bsal* (Greener et al. 2020, Martel et al. 2020), it can be assumed that *T. pygmaeus* and the entire *T. cristatus* superspecies are likewise highly susceptible. In contrast, however, *T. anatolicus* seems to be tolerant to this pathogen (Martel et al. 2020).

So far, Fitzpatrick et al. (2018) reported one dead Northern Crested Newt in a captive collection in the United Kingdom that tested positive for *Bsal*. In addition, Lötters et al. (2020b) noted a dramatic decline of *T. cristatus* in the wild in the German Eifel region, which could be related to the presence of *Bsal,* as no other threats were obvious. In some cases, *Bsal* infection loads of Northern Crested Newts were markedly high. On the other hand, declines of *T. cristatus* were also reported from regions in Germany (Fromhage 2001, Kupfer & Von Bülow 2011, Geiger et al. 2020) where *Bsal* apparently is absent (cf. Lötters et al. 2020b). The same applies to populations in France, Switzerland, Scotland, and other parts of the United Kingdom (e.g., Jenny & Arlettaz 2008, Denoël 2012, Lewis et al. 2017, Grillas et al. 2018, Harper et al. 2018), i.e., countries where the pathogen has not yet been reported from the wild (Thomas et al. 2019, Gilbert et al. 2020).

Here, we discuss key questions regarding the potential impact of *Bsal* on the Northern Crested Newt based on observations in Germany. We outline preliminary results of case studies examining the individual infection dynamics of *Bsal* in two local newt communities in North Rhine-Westphalia with *T. cristatus* as the focal species. Finally, we aim to identify research needs with regard to the salamander plague that can contribute to the conservation of this species.

Methods

Two populations (site 1 = Winkelenberg, Eifel National Park; site 2 = Bochum-Günnigfeld) were studied across three breeding seasons (March 2019 to August 2021). We generated time series for two years (2020–2021) of both infected and healthy Northern Crested Newts, combining capture-recapture analyses with skin swabbing to monitor individual infection histories at both sites. *Triturus cristatus* individuals were primarily captured with newt traps or dip nets during their aquatic phase (e.g., Schlüpmann & Kupfer 2009, Schlüpmann 2014, Bodingbauer & Schlüpmann 2020). In addition, syntopic newt species were surveyed during spring migration and during the aquatic phase.

On some occasions, we adopted an eDNA approach that targeted both *T. cristatus* and *Bsal* (Biggs et al. 2015, Harper et al. 2018, Spitzen-van der Sluijs et al. 2020). For DNA extraction from skin swabs, we used the DNeasy Blood and Tissue Kit (QIAGEN®) following the manufacturer's guidelines with slight modifications (see Schulz et al. 2020); eDNA samples were processed using the Power Water Kit (QIAGEN®) according to the protocol provided by the manufacturer. For *Bsal* detection, skin swabs and/or eDNA samples were analyzed using qPCR (sensu Blooi et al. 2013, 2016, Rees et al. 2014, Standish et al. 2018, Spitzen-van der Sluijs et al. 2020). All positive qPCR signals at the labs of Trier University and Bochum University were calculated in ITS copies and then converted into genomic equivalents (GE) following Schulz et al. (2020) for comparability with earlier studies.

All captured individuals were photographed ventrally for individual identification and recapture analysis with the software AmphIdent (Matthé et al. 2008) or Wild-ID (Bolger et al. 2012).

We used information on the Northern Crested Newt distribution from the online atlas of amphibians and reptiles in Germany (DGHT 2018). *Bsal* localities, except the new ones reported here (see below), were adopted from Lötters et al. (2020b). All other data were obtained from the references quoted throughout the text and from our own field studies (Supplementary Table S1). Maps were created in ArcMap (v. 10.7.1, ESRI). We calculated prevalence and 95% Bayesian credible intervals (CI) following Lötters et al. (2012) by using the 'R2Winbugs' package in R (v. 4.0.3).

Results and discussion
How do Northern Crested Newt populations with known *Bsal* histories develop?

At Study Site 1, *Bsal* has been known since 2019 (Lötters et al. 2020b), at Study Site 2 since 2020 (Fig. 2, Supplementary Table S1). At Study Site 1, 28 *T. cristatus* (18 females, 10 males) were caught, of which 21 (75%) tested *Bsal*-pos-

itive. Time series for 11 recaptured individuals (5 females, 6 males) are available. At Study Site 2, 18 *T. cristatus* (7 females, 11 males) were caught, of which 16 (89%) tested *Bsal*-positive. Time series for 9 recaptured males are available. Syntopic newts tested *Bsal*-positive in both years at both sites (Site 1: *L. vulgaris,* N = 6*; L. helveticus*, N = 15; *I. alpestris*, N = 24; Site 2: *L. vulgaris,* N = 32; *I. alpestris*, N = 37).

Overall, the numbers of captured *T. cristatus* were low at both sites, despite extensive monitoring over 16–26 weeks. Fewer Northern Crested Newts were caught at Study Site 1 in 2021 than in 2020. At the same time, infection prevalence decreased from 69% (14 out of 21; CI: 49–85%) to 50% (5 out of 10; CI: 23–75%). Out of the 14 infected specimens in 2020, 1 male was found dead at the margin of the pond and 2 were recaptured *Bsal*-free in 2021. One *Bsal*-negative specimen caught in 2020 was recaptured *Bsal*-free again in 2021. At the same time, 4 other *Bsal*-negative individuals from 2020 were not recaptured in 2021. In contrast, at Study Site 2, the number of caught individuals increased from 4 (2020) to 17 (2021), while infection prevalence increased from 75% (3 out of 4; CI: 27–95%) to 88% (15 out of 17; CI: 64–96%). Of the 3 infected specimens in 2020, 2 were recaptured with *Bsal* loads in 2021. The *Bsal*-negative individual from 2020 was recaptured *Bsal*-positive in 2021.
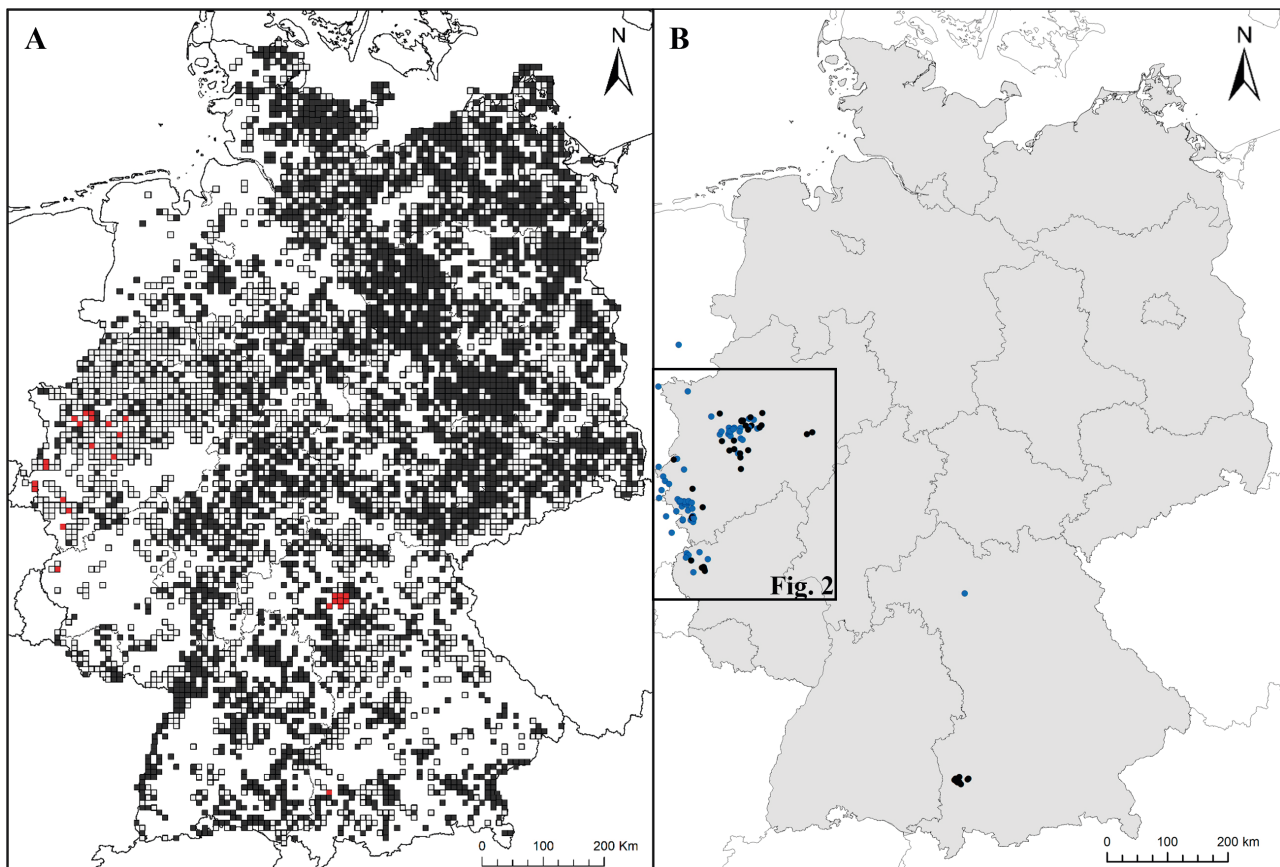

Figure 1. (A) Northern Crested Newt (*Triturus cristatus*) occurrence in 10 km × 10 km grid cells in Germany: black = presence in 2000–2018; light grey = 1900–1999; red = cells with present populations potentially affected by *Bsal* (modified after DGHT 2018). (B) Presence of *Bsal* in Germany: blue = sensu Lötters et al. (2020b) and authors' unpubl. data; black = new records.

We cannot exclude the possibility that the differences between the two study sites represent distinct stages of rampant *Bsal* infections. Both populations persist for now with a low number of individuals, suggesting that each site is already in a late invasion (epidemic) or established (endemic) phase (sensu GILBERT et al. 2020). Hence, we do not rule out mass mortality events in these populations after first *Bsal* contact.

Moreover, we cannot exclude the possibility of potential effects of increased sampling effort at both study sites (i.e., number of traps, sampling events and netting surveys at least doubled in 2021) on capture numbers/recapture rates. However, increased effort did not result in higher numbers of individuals in the second year (Fig. 3). Study Site 1 even yielded a reduction of *T. cristatus* observations from 2020 to 2021 despite an identical sampling scheme.
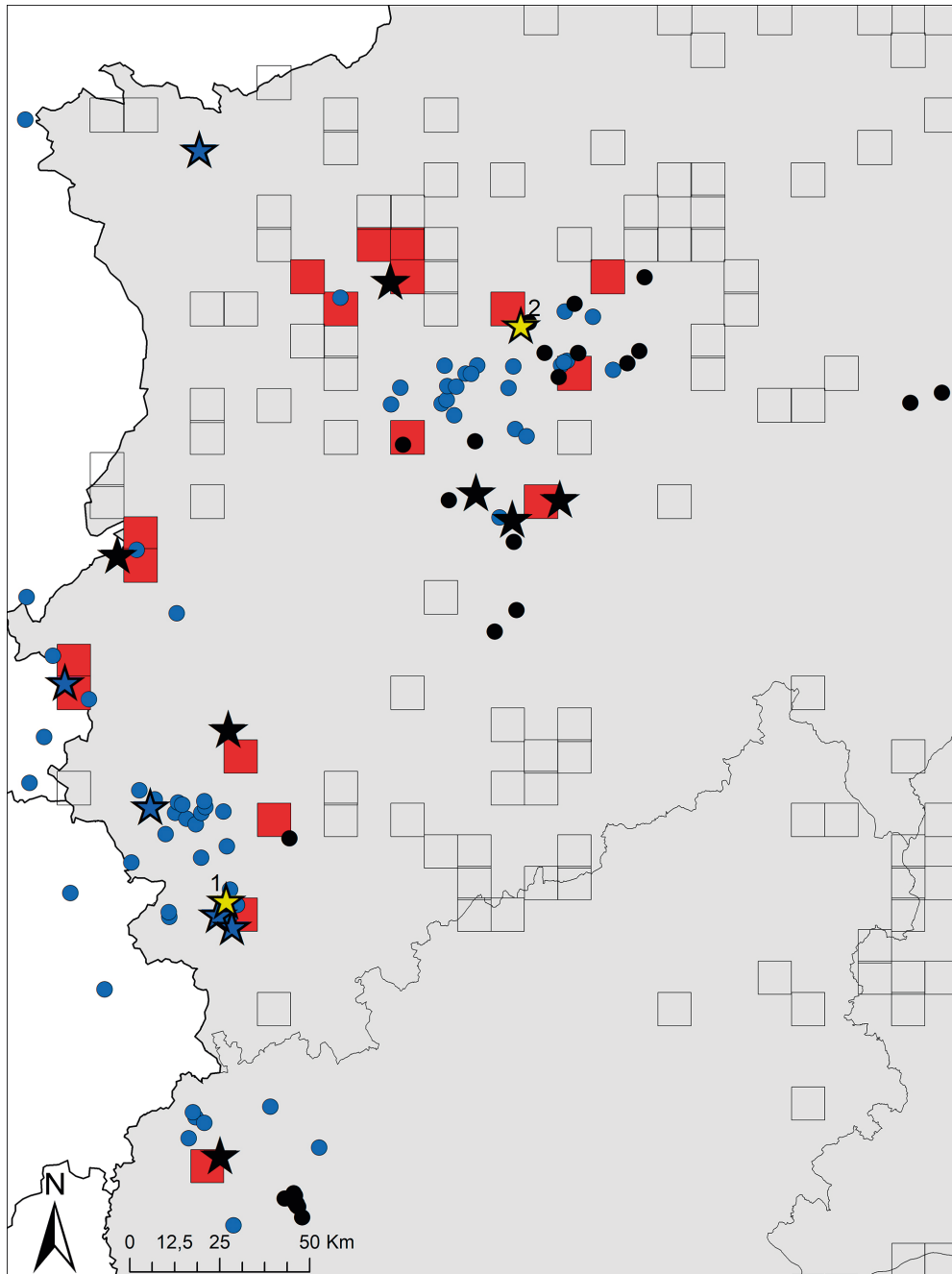

Figure 2. Detailed view of *Bsal* records (dots, as in Fig. 1B) in North Rhine-Westphalia and Rhineland Palatinate and Northern Crested Newt (*Triturus cristatus*) presence 2000–2018 (squares), including populations potentially affected by *Bsal* (filled in red). Stars indicate populations with confirmed *Bsal* presence (blue = sensu LÖTTERS et al. (2020b); black = new records; yellow = Study Sites 1 and 2).

Furthermore, as we framed only a two-year dataset, our observations potentially lie within normal fluctuations (e.g., due to individual reproduction breaks) of Northern Crested Newt populations (Thiesmeier et al. 2009, Von Bülow & Kupfer 2019). Nevertheless, it should be emphasized that a large proportion of the infected animals from 2020 were not traced in the subsequent year, and multiple recaptures of infected individuals in 2021 were rare at both sites. A more profound knowledge about mortality as well as resistance rates of newts in natural populations is required to comprehensively interpret our findings. Furthermore, the role of abiotic factors needs to be further investigated, as higher temperatures may temper infections later in spring and may reduce mortality both by decreasing fungal growth and by increasing newt defenses.

How does a *Bsal* infection manifest in *Triturus cristatus*?

Based on our data at Study Sites 1 and 2, no Northern Crested Newts with external skin lesions were detected during our monitoring, suggesting that visual diagnosis of *Bsal* infection in this species is challenging in the field (if possible at all) despite high detected zoospore loads (i.e., > 800 GE, sensu Martel et al. 2014, Stegen et al. 2017, Beninde et al. 2021). Hence, inferring disease progression from detected zoospore loads appears unfeasible.

Subadult and adult Northern Crested Newts carried *Bsal* zoospores in varying amounts (Supplementary Table S1). Our highest detected GE amount on a live *T. cristatus* was 477,900 GE. Moreover, there is notable GE variation within specimens (Study Site 2: in 2021, 174–
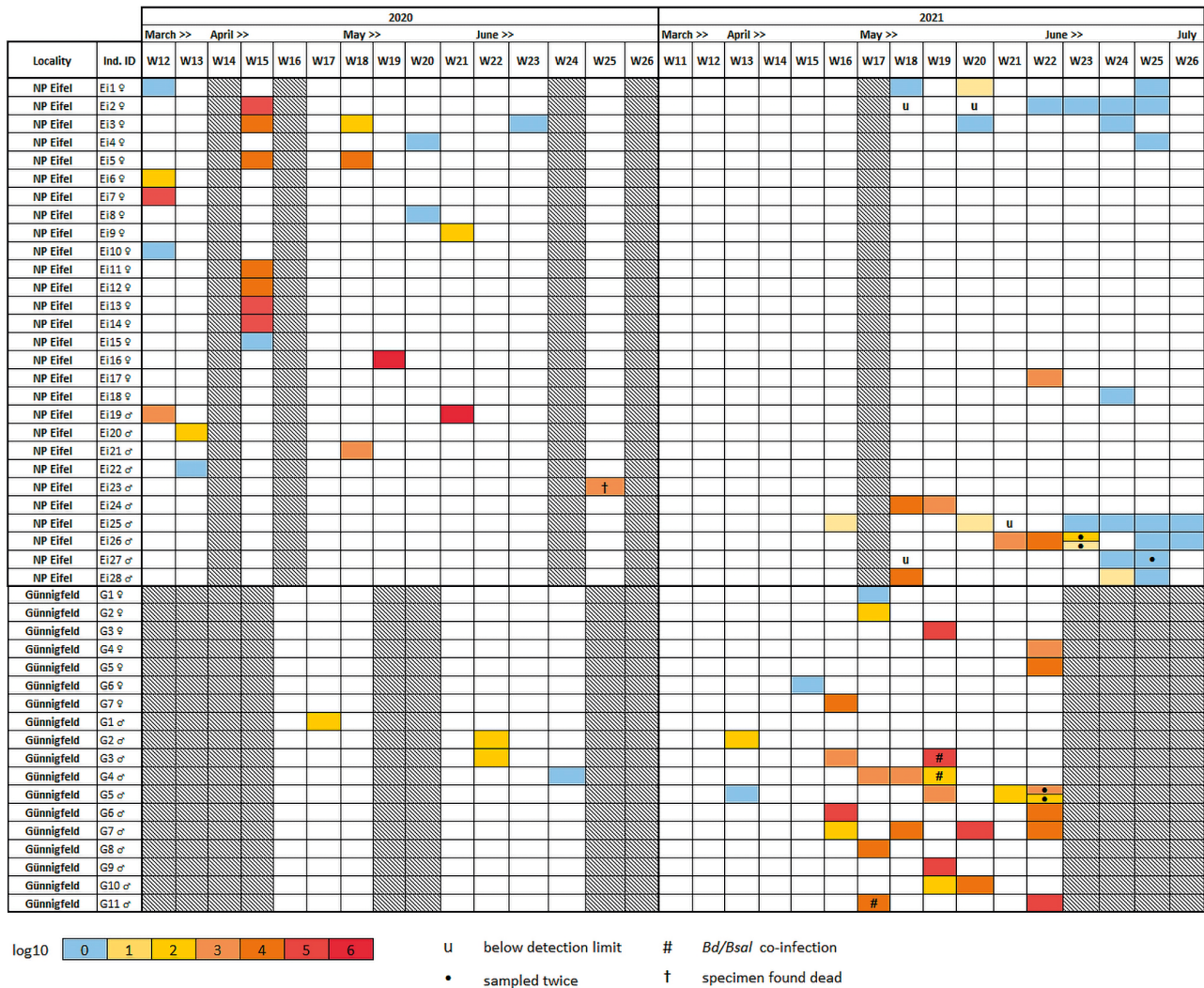

Figure 3. Individual *Bsal* infection histories of 46 Northern Crested Newts (*Triturus cristatus*) in 2020 and 2021 at two localities in Germany (Weeks 12 to 26 of the year; for details of study sites see text). *Bsal* infection loads (GE) are shown in colours, with warmer colours indicating higher infection loads, given in steps of the common logarithm (log$_{10}$). Open cells indicate that only newt species other than the Northern Crested Newt were sampled; shaded cells indicate that no newts were sampled.

477,900 GE), between years (e.g., Study Site 1: in 2020, 2–40,406 GE; in 2021, 1–524 GE), as well as across populations (Fig. 3, Supplementary Table S1). Notably, three *Bsal*-positive individuals repeatedly tested negative at later stages of the study. These individuals may (tentatively?) have cleared *Bsal* infections both within one year and after two years (Fig. 3). This observed variation might be considered in further studies, as both methodological issues (e.g., the 'swabber effect', Simkins et al. 2014) and biological factors such as sloughing may play a role (Bletz et al. 2018, Meyer et al. 2020). However, findings from infection trials seem different to our observations in the field, since in the laboratory, infected *T. cristatus* individuals die on average after 34 days with infection loads > 1,000 GE (Martel et al. 2014).

Since data is sparse (more so even in larvae and metamorphs), it remains difficult to draw a comprehensive picture on how *Bsal* infections manifest in *T. cristatus* over time. Moreover, there is no information on how a *Bsal* infection affects life history, including reproductive behaviour. Anecdotally, we observed that *Bsal*-positive females laid eggs and that *Bsal*-positive males developed similar breeding costumes to those that tested *Bsal*-negative.

Where, when, and how do Northern Crested Newts become *Bsal*-infected?

Based on what is known from the pertinent literature, we assume that both motile and encysted spores may play a role in direct and indirect transmission, both on land and in the water (cf. Martel et al. 2013, Stegen et al. 2017). We captured an infected Northern Crested Newt as early as in March (Week 12 at Site 1 in 2020; Week 13 at Site 2 in 2021), while the latest infected individuals appeared in June (at both sites in both years; Fig. 3). The detection of infected individuals during the early aquatic phase may either indicate that such individuals returned already infected from their respective winter habitats, or that they may have become infected when they entered the ponds and came into contact with infected individuals of other newt species. As a result, the zoospore load of breeding ponds may be initially low in spring and increase during the year (cf. Spitzen-van der Sluijs et al. 2020). However, as other parameters (e.g., pond size, exposition, eutrophic level, annual climate, host density, zooplankton density) may enhance or hamper annual zoospore density, our suggestion needs to be further elucidated by eDNA analyses. Only in a single case we detected an initial infection during the aquatic phase (M5 at Site 2 between Weeks 13 and 19 in 2021; Fig. 3). It may be worth mentioning that syntopic *I. alpestris*, *L. helveticus* (at Study Site 1 only) and *L. vulgaris* also tested *Bsal*-positive during the entire sampling season, both during the terrestrial and the aquatic phase (authors' unpubl. data). This detail is important, because other newt species have been suggested to be less susceptible to *Bsal* and may act as vectors and reservoirs (Stegen et al. 2017, Beninde et al. 2021).

Can *Triturus cristatus* recover from a *Bsal* infection

Infection experiments suggest that Northern Crested Newts can die from the salamander plague (Martel et al. 2014, Bates et al. 2019). However, mortality rates in natural environments and whether this newt species can survive a *Bsal* infection are not yet understood. Our field data demonstrate that infections can last over short (several days) to long periods (several weeks to months; 2 ± 1 weeks at Site 1, 5 ± 1 weeks at Site 2; Fig. 3). Thus, animals in the wild can apparently have a high infection load (> 800 GE, see above) for several weeks to months without noticeable external impairments. In line with this, our data indicate that at least some individuals lowered or even cleared a previously detectable infection (Fig. 3; at Site 1, in 3 *T. cristatus* with low initial zoospore loads the *Bsal* infection was no longer detectable after one week).

Mechanisms of *Bsal* clearing remain mostly unknown; this holds also for the Northern Crested Newt. However, based on *Bd* studies we assume that, besides other aspects (abundance of conspecifics and syntopic carriers of other taxa, temperature, habitat structure, etc. sensu Malagon et al. 2020), skin sloughing could play a role when entering the aquatic phase, during the aquatic phase, and when entering the terrestrial phase (for *Bd* see Ohmer et al 2015, 2017, Russo et al. 2018). Schulz et al. (2020) already suggested that higher water temperatures may help to lower infection rates, as *Bsal* growth is hampered by higher temperatures *in vitro* (Martel et al. 2013). Beyond this, several authors have discussed the possibility that a combination of sloughing and changes in the individual skin microbiome may play a role in clearing a *Bsal* infection (Sabino-Pinto et al. 2017).

Where in its German range is *Triturus cristatus* declining, and where has *Bsal* been detected?

Germany lies in the centre of the Northern Crested Newt's distribution range, and this species occurs in all federal states (Fig. 1A). However, Geiger et al. (2020) stated that there is a strongly declining trend in the long term in Germany, whereas this decline is 'moderate' from a short-term perspective. Reasons for this decline are primarily linked to habitat loss and fish stocking. Geiger et al. (2020) also added *Bsal* to the list of potential threats, though. To date, the salamander plague has been reported from 94 sites in three German federal states (Lötters et al. 2020b and new data herein; Fig. 1B): North Rhine-Westphalia, Rhineland Palatinate, and Bavaria. Among them are 16 records where *Bsal* was detected on *T. cristatus*, including six previously undocumented populations (Fig. 2; Supplementary Table S2). Moreover, assuming a 4-km maximum dispersal ability of syntopic Central European newt species (e.g., Kupfer 1998, Schäfer 1993, Jehle & Sinsch 2007) that hypothetically can act as *Bsal* reservoirs (Beninde et al. 2021), this amounts to 27 additional grid cells of current (2000–2018) *T. cristatus* occurrences that potentially

are infested with *Bsal* (Fig. 1A). Despite intensive *Bsal* sampling effort in recent years, this data is still sparse, and it is not yet possible to confirm the pathogen as a major causal factor for *T. cristatus* decline in Germany.

Reasons for this are mainly incomplete sampling of Northern Crested Newt populations for this pathogen as well as varying survey methods in the past (cf. GEIGER et al. 2020). However, the information obtained thus far suggest that *Bsal* could act as a causal cofactor in *T. cristatus* population declines, at least on a local scale. This is supported by the results of the surveys at the *Bsal*-positive sites Helingsbach, Winkelenberg and Günnigfeld (Supplementary Table S1). Here, the number of captured individuals has decreased since *Bsal* was first detected, while simultaneously the number of *Bsal*-infected individuals has increased. At the locality Hiesfelder Wald in Oberhausen, we observed a drastic decline in the number of individuals, although *Bsal* had only been discovered there in 2021, after *Bsal* screening did not detect the pathogen two years earlier (Supplementary Table S1). That means that – assuming the pathogen as the main reason for this observation – a *Bsal*-caused mass mortality event must have happened within a timespan of only two years. We may see a similar pattern at the localities Stiepel, Bochum and Walsum-HG, Duisburg. In these populations, the numbers of captured Northern Crested Newts likewise dropped within two years from 17 to zero and 18 to zero, respectively (Supplementary Table S1). Both localities are within the above assumed contact zone of the pathogen. In contrast, also droughts, especially in the years 2017 and 2020, may have led to these observed declines. However, the pathogen may increase its negative impact in synergy with other threats identified by GEIGER et al. (2020) even though the mentioned field observations show that if *Bsal* were regarded "only" as a cofactor, the pathogen constitutes still an enormous threat to *T. cristatus* populations within its distribution range.

Recommendations and outlook

The overall decline of the Northern Crested Newt in Germany obviously is the result of several, probably synergistic factors, of which *Bsal* might be one (cf. GEIGER et al. 2020). Both the frequent spatial overlap of *Bsal* infections in other caudates, in particular the European Fire Salamander (cf. Fig. 1B), and the temporal overlap between infection events and *T. cristatus* declines suggest causality (cf. DALBECK et al. 2018, LÖTTERS et al. 2020b, SCHULZ et al. 2020). It cannot be ruled out that *Bsal* infections add additional stress to local populations (GEIGER et al. 2020). Such tandem effects between 'old' (e.g., habitat change) and 'new' (e.g., emerging infectious diseases) threats have already been proposed by COLLINS & STORFER (2003) as playing a role in the global amphibian crisis.

Despite an increasing interest in the little-understood *Bsal* life history and disease dynamics, we have only begun to understand how *Bsal* affects individuals and populations (GILBERT et al. 2020). Studies investigating differ-

ent immediate outcomes of infections (death vs. recovery), long-time effects (e.g., "Long *Bsal*" or loss in reproductive fitness), *Bd*/*Bsal* co-infections (LONGO et al. 2019 and as observed at Site 2 in 2021; see also LÖTTERS et al. 2020b, GREENER et al. 2020 for *T. marmoratus*), and the proximate causes underlying apparent recovery (e.g., individual health status or abiotic factors that inhibit *Bsal* growth or spread) need to be conducted in detail. Although they undoubtedly add valuable insight into individual host's or host species' responses to *Bsal*, lab trials including infection experiments do not suffice to replace long-term field studies to understand the mechanisms underlying infection and disease dynamics as well as the life history and dynamics of the pathogen itself in the environment.

Novel information should be made rapidly available to both stakeholders and the public. This will ensure that current knowledge is integrated into decision-making processes and applied fieldwork. Due to the inefficiency of macroscopic diagnostics in newts, there is an enormous risk of misinterpreting a population's health status and thus of pathogen spread (i.e., *Bsal*). The latter is especially important in *T. cristatus* as a species that is protected by the EC Habitats Directive (Annex II+IV).

Both histology and molecular genetic methods (skin swabbing and qPCR) are the most widely used and most reliable tools for *Bsal* detection. Despite known limitations (e.g., SIMKINS et al. 2014), the latter should be applied in a standardized manner during monitoring. Handling specimens and equipment during monitoring and fieldwork should strictly follow the established hygiene protocols (see LANUV 2021). Furthermore, direct animal-to-animal contact should be restricted to a minimum during fieldwork to avoid infection. Here we see a promising potential in the application of non-invasive, highly informative methods, such as the use of eDNA for presence/absence screening of the pathogen in the wild (cf. BRANNELLY et al. 2020, SPITZEN-VAN DER SLUIJS et al. 2020). However, when using this new and promising technique, potential problems, such as false positive and/or false negative signals, should be taken into account (BOSCH et al. 2021). An inconsistent pattern of negative eDNA samples despite finding infected animals in the traps was also noted in our results.

It is evident that the dynamics of *Bsal* outbreaks and linked population declines, along with the Northern Crested Newt potentially acting as a reservoir, need to be investigated in more detail. In addition, synergistic climate change-associated stressors and their interaction with *Bsal* epidemics need to be better understood. This is the only way to design sensible measures for the protection of the Northern Crested Newt that will prevent further reductions of its European populations.

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Supplementary data

The following data are available online:

Supplementary Table S1. Localities in Germany with *Batrachochytrium salamandrivorans* sampling in Northern Crested Newt populations (*Triturus cristatus*).

Supplementary Table S2. Known sites with *Batrachochytrium salamandrivorans* occurrence in Germany until July 2022.