Connectivity of Alpine newt populations \((I\text{chthyosaura } a\text{lpestris})\) exacerbates the risk of \(B\text{atrachochytrium sala}\text{mandrivorans}\) outbreaks in European fire salamanders \((S\text{alamandra sala}\text{mandra})\)

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Abstract
The pathogenic chytrid fungus \(B\text{atrachochytrium sala}\text{mandrivorans (Bsal)}\) was introduced from Asia to Europe, most likely via the pet trade. It is currently causing a severe local decline in populations of the European fire salamanders \((S\text{alamandra sala}\text{mandra})\). Laboratory tests confirmed that all infected individuals succumbed to the disease within two weeks. Furthermore, mass mortality events in the wild were observed in Germany, Belgium and the Netherlands. Some newt species, including the Alpine newt \((I\text{chthyosaura alpestris})\), can also become infected with \(Bsal\). However, they seem to be ‘tolerant’ and can often survive the disease, but maintain an infectious load, which they could spread among connected populations. It is, therefore, hypothesized that this species functions as a vector of the disease and threatens syntopic populations of susceptible species such as the European fire salamander. To assess the risk of Alpine newts as vectors for \(Bsal\), we sampled 233 individuals from 50 sites in 2017 and performed a genetic population connectivity analysis using microsatellites. The results showed that populations of Alpine newts are not spatially structured, suggesting high gene flow across the study area. Tests for \(Bsal\) of all Alpine newts and 150 individuals of two other newt species confirmed five infected individuals. Infected newts can thus function as reservoirs and vectors of \(Bsal\) and spread it through dispersal. As a result, the risk of European fire salamander declines in this region is further exacerbated by the connectivity of syntopic Alpine newt populations.

Keywords Isolation · Landscape genetics · Herpetofauna · Risk management · Human-mediated dispersal

Introduction

The necessity to maintain population connectivity is a central paradigm of conservation biology with the aim of ensuring the long-term survival of a species. At the same time, dispersal among connected populations poses a risk of spreading pathogens, such as \(B\text{atrachochytrium sala}\text{mandrivorans (Bsal)}\), a chytrid fungus that is lethal to many urodelan species, especially the European fire salamander \((S\text{alamandra sala}\text{mandra})\) (Martel et al. 2013, 2014; Stegen et al. 2017; Dalbeck et al. 2018; Wagner et al. 2019; Lötters et al. 2020a). \(Bsal\) has only recently been described from salamanders in the Netherlands (Martel et al. 2013), but has been shown to be present in Central Europe (Germany) at least since 2004 (Lötters et al. 2020b). It was most likely introduced to Europe via the pet trade from Asia (Martel et al. 2014), and so far the exotic range of this invasive fungus includes parts of Germany, Belgium, the Netherlands and Spain (Spitzen-van der Sluijs et al. 2016; Dalbeck et al. 2018; Wagner et al. 2019; Martel et al. 2020; Lötters et al. 2020a).

In highly susceptible species, such as \(S. sala\text{mandra}\), infection with this pathogen leads to the disease chytridiomycosis, which sometimes results in a strongly increased mortality (Martel et al. 2014; Stegen et al. 2017). As the European fire salamander appears to be the most susceptible species in the wild, the disease has also been termed ‘salamander plague’ (Dalbeck et al. 2018). In European fire salamanders it has been linked to severe population declines and local or even regional extinctions (Martel et al. 2013; Spitzen-van der Sluijs et al. 2016; Wagner et al. 2019;
Fig. 1  A The study area in Northern Rhineland-Palatinate with sample sites of Alpine newts, CORINE forest cover and OSM highways. B Expanding on Germany, distribution of Alpine newts and records of European fire salamanders (data based on confirmed records from national monitoring efforts between 2000–2018; newt occurrences buffered by 10 km for clarity). C Expanding on Europe, distribution of Alpine newts and European fire salamanders is shown (based on IUCN data)
Lötters et al. 2020a; Sandvoß et al. 2020). In addition, models predict that outbreaks can occur at low densities, lower than those known from the field, and have the potential to spread rapidly across the landscape (Schmidt et al. 2017).

Other urodelans, such as the Alpine newt (Ichthyosaura alpestris), seem to be ‘tolerant’, i.e. they could be parasitized, but often survive infections as they show a dose-dependent course of disease: Low doses most often result in individuals with limited or no infection, while high doses can also lead to death (Stegen et al. 2017). Recent observations suggest that populations of Alpine newts in areas affected by BsAl may remain stable or decrease only slightly; however, long-term monitoring has yet to confirm this (Spitzen-van der Sluijs et al. 2016; Dalbeck et al. 2018; Wagner et al. 2019; Lötters et al. 2020a). Importantly, Alpine newts can be re-infected (Stegen et al. 2017), and experiments have shown that infected newts can transmit the disease to previously uninfected individuals (Spitzen-van der Sluijs et al. 2018).

BsAl in forest soil, in addition to zoospores in the water, was detectable via qPCR for up to 200 days after contamination, while transmission from soil or water is likely limited to 48 h and < 7 days after contamination, respectively (Stegen et al. 2017). Alpine newts may thus pose a serious threat to syntopically occurring salamanders as they are a reservoir for the pathogen, and host infection is assumed to occur both through direct contact among animals and via environmental pathways such as soil and water (Stegen et al. 2017). Therefore, knowledge on population connectivity for Alpine newts, which are common and widely distributed in Germany (DGHT 2018), is essential to better understand the epidemiology of BsAl and to assess and manage its impacts.

The tri-border region of Germany, Belgium, and the Netherlands is at the ‘center of BsAl outbreaks’ in Europe (Spitzen-van der Sluijs et al. 2016; Dalbeck et al. 2018; Wagner et al. 2019; Lötters et al. 2020a). Almost all amphibians positive for BsAl in Germany originate from the federal states of North Rhine-Westphalia and Rhineland-Palatinate, which cover this region. Spatially, these findings coincide with declining populations of European fire salamanders (Dalbeck et al. 2018; Wagner et al. 2019; Lötters et al. 2020a; Sandvoß et al. 2020). To assess the capability of Alpine newts to spread BsAl in northern Rhine-Palatinate, Germany, we sampled 233 individuals for genetic connectivity analysis using microsatellites. The study region is mountainous (Eifel Mts.) and divided by a highway, which may impede movement of newts. If the landscape structured the regional population of Ichthyosaura alpestris into geographically distinct clusters, with no or only minimal exchange of individuals, the spread of BsAl by Alpine newts could be contained within such species-specific barriers for gene flow. In addition, we tested all newts for BsAl to quantify the prevalence of BsAl infections and to assess the capacity of Alpine newts to function as a reservoir for this pathogen.

Material and methods

The Alpine newt is widespread in Central Europe, up to 2500 m a.s.l. (Thiesmeier and Schulte 2010). It is terrestrial for most of the year, changing to an aquatic lifestyle in spring to breed in permanent and ephemeral waters (Thiesmeier and Schulte 2010). Our study area covers 1200 km² in northern Rhineland-Palatinate, Germany (Fig. 1), and is located within the ‘center of BsAl outbreaks’ in Europe (Spitzen-van der Sluijs et al. 2016; Dalbeck et al. 2018; Wagner et al. 2019; Lötters et al. 2020a). It is within a 30 km radius of the BsAl-positive sites Robertville, Belgium, and Belgenbachtal, Germany, the closest known outbreaks at the time of starting field work for this study in early 2017 (Spitzen-van der Sluijs et al. 2016).

Sampling was conducted from March to June 2017, the time of the year when newts can be caught in their aquatic breeding habit (Thiesmeier and Schulte 2010). While Alpine newts are considered to be distributed continuously throughout the study region, official records documented occurrences of both Alpine newts and European fire salamanders only until the 2000s (DGHT 2018). To adequately sample Alpine newts throughout our study area we employed two different strategies. Landscape coverage (i.e. number of sampling sites) and genetic coverage per locality (i.e. number of individuals sampled per site) represent a trade-off between population genetic inferences and fine-scale inferences of landscape effects on gene flow (Prunier et al. 2013). For an increased robustness of genetic results, and as recommended by Prunier et al. (2013), we made use of both sampling schemes: We aimed to sample a minimum of 10 populations evenly distributed throughout the study area, with at least 10 individuals each, and, in addition, we filled the area between population samples with samples of single or a few individuals from as many additional locations as feasible. In order to minimize spatial bias, we divided the study area into ten grid cells (120 km²), which represent the minimum number of populations to be sampled, and distributed the sampling intensity evenly among these cells. Overall, we sampled Alpine newts at 50 sites (Table 1). At the majority of these sites, we obtained one or two samples (in 13 and 23 cases, respectively), while sample numbers at the remaining 14 sites ranged from 3 to 28 samples (mean N = 10). While the focus of this study and the population genetic results presented here concern the Alpine newt, we also sampled 147 syntopic palmate newts (Lissotriton helveticus), at 50 sites, and three smooth newts (Lissotriton vulgaris), at one site, to test for presence of BsAl. We took three different dermal swabs of each individual, one for genetic
analyses (A-sample; following Prunier et al. 2012) and two for testing of Bsal (B- and C-samples). The results for Bsal have already been published elsewhere (Wagner et al. 2019; Sandvoß et al. 2020), so we here refer to the results of these studies (Table S1). All equipment used for sampling, such as dip nets, traps and rubber boots, was disinfected after use at any location by submerging it in a solution of Virkon S for 10 min, to prevent spread of Bsal (van Rooij et al. 2017; More et al. 2018).

DNA was extracted using the DNeasy Blood & Tissue Kit by QIAGEN. Population genetic analyses were based on 14 microsatellite loci designed for Alpine newts (Prunier et al. 2012; Garner et al. 2003), which were amplified in Multiplex-PCRs (Table S2) and run on an Applied Biosystems 3500 sequencer. Genotypes were checked for null alleles (Micro-Checker v2.2.3; van Oosterhout et al. 2004), deviation from Hardy-Weinberg-equilibrium (R-package PopGenReport v3.0.4; Adamack & Gruber 2014) and linkage disequilibria (FSTAT v2.9.3.2; Goudet 1995). To determine population structure, we performed a PCA (R-package adegenet v2.1.0; Jombart 2008) and ran both STRUCTURE, vers. 2.3.4 (Pritchard et al. 2000) and GENELAND, vers. 4.0.7 (Guillot et al. 2008) with 10⁶ simulations, a burn-in of 2 × 10⁵ and from K = 1–20. Since both spatial scale and the number of individuals can affect measures of genetic similarity and inferences of landscape barriers, we performed all analyses on three different sets of individuals (Table 1) in order to ensure robustness of results: spatial scales used to pool samples varied between 100 and 1500 m, and two thresholds, 3 and 10 individuals, were used as the minimum number of samples per population.

Comparisons of the landscape were based on 2018 CORINE Land Cover data (https://land.copernicus.eu/) and OpenStreetMap (OSM) data (http://download.geofabrik.de/europe.html). We summarized CORINE land cover classes into urban land use, agricultural land use, broad-leaved/ mixed forest or coniferous forest cover (Table S3), and used OSM data to quantify linear features at a finer scale, i.e. road and water cover (Table S4; section LAND COVER DATA in online resources). Within a radius of 100 m (OSM data only), 500 m, 1000 m, 1500 m, 2000 m, 3000 m and 4000 m around sites, covering known dispersal distances of I. alpestris (Schmidt et al. 2006; Jehle and Sinsch 2007), we compared landscape characteristics at sites where Bsal was detected to Bsal-free sites using t-tests or Wilcoxon tests, depending on the result of a Shapiro–Wilk test for normality of data, and using Holm-corrected p-values for multiple testing. In addition, using the same methodology, we quantified landscape characteristics at the type locality of Bsal in the Netherlands (Bunderbos), where Bsal was detected both in European fire salamander and Alpine newt.

Results and discussion

Two loci (CopTa6 and CopTa14) had to be excluded from analyses due to the presence of null alleles, deviations from Hardy-Weinberg equilibrium and linkage equilibrium that were consistent across sample locations (Tables S5–S7; section POPULATION GENETIC SUMMARY in online resources). The remaining twelve loci were retained. Population structure was identified as K = 1 by GENELAND (Fig. S1) and K = 3 by STRUCTURE (based on Delta K values, Evanno et al. (2005); Fig. S2). Noteworthy, STRUCTURE cannot identify K = 1 as the best fitting model, and the resulting three clusters at K = 3 showed no specific spatial pattern: based on Q-values of cluster membership, individuals from multiple clusters were found at all sites containing more than two samples. Similarly, PCA showed a high overlap of all populations (Fig. S3). These results were consistent across the three sets of individuals used for analyses (Tables 1 and S8; section POPULATION STRUCTURE in online resources), corroborating a lack of spatial genetic structure of Alpine newts in this region.

Alternatively, lack of spatial genetic structure could be explained by large effective population sizes or insufficient genetic resolution to detect barriers (McCarty-Melstad et al. 2018). However, despite large effective population sizes, population genetic studies on Alpine newts conducted with similar sets of microsatellite markers revealed significant genetic structure at spatial scales even below that of our study (Prunier et al. 2014, Luqman et al. 2018). Similarly, and using only seven microsatellite loci at a finer spatial scale made possible to detect reduced migration rates due to urban land use, roads and highways (Van Buskirk 2012). Furthermore, simulations by Prunier et al. (2014) demonstrated that strong barrier effects can be detected with our set of microsatellites loci. Therefore, we are confident that
Alpine newts in this region in fact show no significant spatial genetic structure. Suitable habitat cover, such as broad-leaved and mixed forests (Thiesmeier and Schulte 2010), covering > 24% of the studied landscape, may facilitate connectivity throughout the study region and allow for dispersal despite the presence of large-scale agricultural land uses in the study area, characterized predominantly by pastures used for grazing or mowing (> 30%) and non-irrigated arable land (> 17%). Our findings of connected Alpine newt populations resemble results of other studies, which showed that gene flow in Alpine newts can persist (Emaresi et al. 2011) despite highways (Prunier et al. 2014; Luqman et al. 2018) or rivers (Luqman et al. 2018) as putative barriers.

Tests for Bsal detected only one highly infected Alpine newt (> 800 genomic equivalents, GE) among the individuals sampled by us in 2017 (Wagner et al. 2019; Sandvoß et al. 2020). Additional screening of 150 newts in the study region in 2018 and 2019 identified six further Bsal-sites based on seven Alpine and two palmate newts, all with a low infection rate (< 100 GE) (Wagner et al. 2019; Sandvoß et al. 2020). These findings are concerning for two reasons. Firstly, the lack of spatial genetic structure in Alpine newts means that these populations are likely to be interconnected, and migration may potentially spread Bsal throughout the region. Secondly, the presence of infected newts means that Bsal infected hosts that can survive infections persist in the ecosystem where they can function as pathogen reservoirs (Stegen et al. 2017). Therefore, Alpine newts can facilitate both the spread and the persistence of Bsal in the study area. Yet, the significance and specific role of Alpine newts in the local spread of Bsal remains controversial. The case of the Bsal type locality in Bunderbos might even indicate a low risk of Bsal spreading by Alpine newts. While infected European fire salamanders and Alpine newts were found here in 2010, salamanders at a nearby site—only 800 m away—remained uninfected until 2017, despite the presence of Alpine newts at both sites (Spitzen-van der Sluijs et al. 2018). On the contrary, a recent finding of eleven infected European fire salamanders (high Bsal loads with > 100,000 GE) in a population previously tested Bsal-free (20–30 salamanders tested per year between 2016 and 2018; Lotters et al. 2020a; Sandvoß et al. 2020) suggests a recent introduction of Bsal. Coincidentally, the nearest confirmed Bsal site is at a distance of 1 km and traces back to an infected Alpine newt (Wagner et al. 2019; Sandvoß et al. 2020). This is within reach of the regular dispersal distances of Alpine newts, as mark-recapture studies of this species have shown individuals covering distances of 1.8 km are no exception (Schmidt et al. 2006) and distances up to 4 km have been recorded (Jehle and Sinsch 2007). However, individuals infected with Bsal may have reduced dispersal ability, as hypothesized for the European fire salamander (Canessa et al. 2018). In addition to animal-animal contact, Bsal may also be spread by other vectors, such as humans (Spitzen-van der Sluijs et al. 2016).

We did not detect significant differences in landscape characteristics between sites where Bsal has been detected and those that are Bsal-free, (Table S9), casting doubt on the landscape as a relevant factor for the spread of Bsal. At the same time, considerable landscape differences between our study area and the Bsal type locality in Bunderbos exist (Table S10). Within 5000 m, the dominating land use types around Bunderbos are agricultural (> 60%) and urban (> 24%), while habitat most suitable for Alpine newts, such as broad-leaved and mixed forests (Thiesmeier and Schulte 2010), make up less than 5%. Compared to our study extent, urban land use and road cover are ~ 10 times and ~ 4 times higher, respectively, at Bunderbos compared to any site within our study area, a pattern which held up across all scales. Urban land use cover and roads were shown to reduce migration rates in this species elsewhere (Van Buskirk 2012) and it may contribute to lower connectivity of Alpine newt populations in the vicinity of Bunderbos than in our study area.

Our study highlights that, based on the demonstrated genetic connectivity, Alpine newts have the potential to act as a vector for and to promote the spread of Bsal. The wide distribution of palmate newts in our study region adds further concern, since two Bsal infected individuals were found. While laboratory data have so far only confirmed transmission among individuals of Alpine newts, palmate newts may also act as an interspecific vector. Conclusions drawn from epidemiological models considering only Alpine newts and European fire salamanders as Bsal hosts highlight the need to locally remove at least 80% of both species to prevent a disease outbreak (Canessa et al. 2019). Our results strongly suggest that mitigation efforts for Bsal are complex (Thomas et al. 2019) and emphasize that this pathogen is likely to spread via multispecies host communities.

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Data availability Data is located in the supplementary material.
Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Research did not involve experiments or invasive sampling of animals.

Consent to participate Research did not involve human participants.

Consent for publication All authors approved the final version of the manuscript.

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