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FIRST RECORD OF *BATRACHOCHYTRIUM DENDROBATIDIS* IN THE NORTHERN NETHERLANDS

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ABSTRACT

Batrachochytrium dendrobatidis (*Bd*) infects amphibians and has been linked to the decline of hundreds of anuran amphibians all over the world. In the province of Groningen in the Netherlands, this fungal pathogen was not detected before this study. To determine whether Groningen was *Bd*-free, we surveyed 12 locations in this province in 2020 and 2021. Samples were then used to quantify the presence of *Bd* with a qPCR assay. In total, 2 out of 110 (~0.02%) collected in 2020 and 11 out of 249 samples collected in 2021 tested positive for *Bd*. Infected amphibians were found in 4 out of the 12 sites, and the prevalence of *Bd* was estimated at 4% for both years combined. Our study provides the first record of *Bd* in Groningen, and we hypothesize that *Bd* is present throughout the Netherlands in regions currently considered "*Bd*-free." Furthermore, we warn scientists and policymakers to be apprehensive when calling a site free from *Bd* when sampling is limited or not recent.

Amphibian populations worldwide are declining because of the chytridiomycosis pandemic, caused by the fungal pathogens *Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrivorans* (Berger et al., 1998; Fisher and Garner, 2007; Spitzen-van der Sluijs et al., 2013, 2014; Scheele et al., 2019). *Batrachochytrium dendrobatidis* was first discovered in the 1990s, after dramatic population declines of anuran amphibians in Queensland (Australia) and Panama (Berger et al., 1998). In 2013, *B. salamandrivorans* was described, following the discovery of massive crashes of salamander populations in northwestern Europe (Martel et al., 2013). Due to its global widespread, the fungus *Bd* has since received much research attention. Ongoing research aims to identify its global distribution pattern, its impact on the species it infects, how to protect biodiversity, and how to mitigate the disease (Fisher et al., 2009; Olson et al., 2021). Because *Bd* is capable of hybridizing across lineages and hybrid lineages can be more virulent, it is unknown how amphibian host populations will react to the introduction of a new or hybrid *Bd* lineage (Voyles et al., 2018). Additionally, small amphibian host communities that are recovering from *Bd* outbreaks are especially vulnerable to new disease outbreaks (Voyles et al., 2018). Screening of populations that are facing *Bd*-related die-offs and declines, as well as less affected populations that are not or seem not to be as threatened by *Bd*-related outcomes, is therefore important for mitigation planning (Byrne et al., 2019).

Bd infects a broad range of species, including 520 anuran amphibians (frogs and toads), urodeles (salamanders and newts), and caecilians (Gower et al., 2013; Olson et al., 2013; Scheele et al., 2019). Most anurans are susceptible to *Bd* infection during all life stages (excluding eggs), although morbidity and mortality vary between species, life stages, and environmental conditions (Gower et al., 2013). The microhabitat and environment that a host species inhabits are key determinants for infection and disease outcome, as, for instance, virulence is reduced at warmer temperatures (>28 C) (Daskin et al., 2011; Van Rooij et al., 2015). Some tolerant amphibian species maintain infection below lethal levels and function as carrier species (Reeder et al., 2012); this makes them potential pathogen vectors as well as environmental reservoirs (Reeder et al., 2012; Kolby et al., 2014).

In Europe, *Bd* is considered to be widely distributed, infecting a broad range of amphibian species (Garner et al., 2005). Although in northern, southern, and central Europe *Bd* is known to be widespread (Martel et al., 2012; Allain and Duffus, 2019; Meurling et al., 2020), limited data exist on its distribution in western Europe. *Batrachochytrium dendrobatidis* has been present in western Europe (including Belgium and the Netherlands) since the late 1990s, although mass mortalities or steep declines in host populations have not been observed (Spitzen-van der Sluijs et al., 2014). In the northern part of the Netherlands, *Bd* has been reported at very low incidence and prevalence and was not found in all provinces (Spitzen-van der Sluijs et al., 2014); however, large-scale

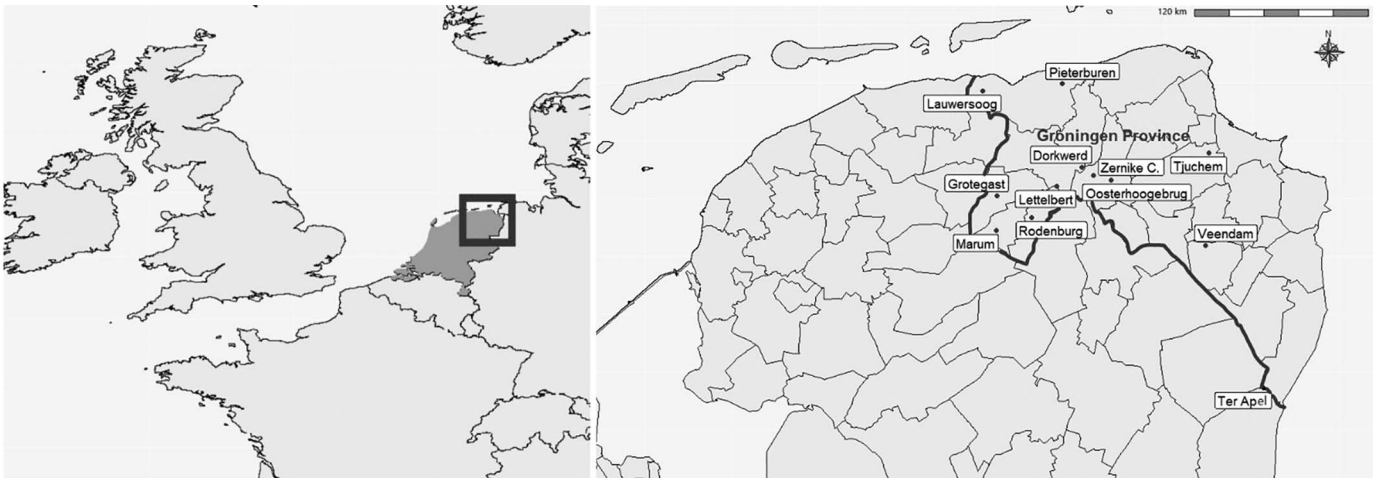

Figure 1. Map of the province of Groningen in the northern Netherlands and its position in relation to the rest of Europe (left) and detail showing the locations of the 12 sampling sites included in this study (right).

surveillance has not been conducted. The Netherlands hosts 16 native amphibian species: 5 urodeles and 11 anurans. Additionally, there are some non-native species such as the Italian crested newt (*Triturus carnifex*). Several anuran and urodele species have tested positive for *Bd* in the Netherlands and can act as *Bd* carriers (Spitzen-van der Sluijs et al., 2014). However, at the present time research efforts in the Netherlands mostly focus on *B. salamandrivorans* (*Bd*'s sister species), which is responsible for massive die-offs of the fire salamander (*Salamandra salamandra*) in northwestern Europe (Spitzen-van der Sluijs et al., 2013).

We aimed to screen the province of Groningen for *Bd* to verify the status of *Bd* presence and to broaden the existing data on *Bd* distribution in the Netherlands. We sampled 12 locations across the region and performed molecular diagnostic tests to identify the presence of *Bd*. Our study expands our knowledge of *Bd* distribution and supports the value of larger sample sizes in pathogen-monitoring studies.

MATERIALS AND METHODS

Sampling

The province of Groningen is situated in the northeast part of the Netherlands (Fig. 1). The landscape is flat, and a large part of the province is below sea level. About 60.9% of the land is used for agriculture, 9% is built-up or semi-built-up area, 6% is natural vegetation, and 9.7% is inland water (Statistics Netherlands, 2018). The province has an oceanic climate, with a year-round daily mean temperature of 10 C (Royal Netherlands Meteorological Institute, 2019–2021).

We surveyed 12 locations around the province of Groningen in July 2020 and May–June 2021, aiming to sample during the amphibian reproductive period to ensure sufficient sample size (Fig. 1). To achieve that, in 2021 sites in Groningen were checked for active amphibian presence (e.g., active calling, newts in aquatic-reproductive form) twice a week since the beginning of spring (March) to determine when sampling could start. Amphibians were found to aggregate only in late May–June, which is later in the year than what was observed the previous year (observations by authors). Sampling sites included different types of inland water (i.e., canals, streams, natural ponds, and garden ponds). Sampling locations were selected based on the observed amphibian species richness and abundance according to RAVON's (Reptile, Amphibian and Fish Conservation Netherlands, Nijmegen, the Netherlands) distribution data and public access to the sites. Each site was screened for 40 min by 2 persons, and amphibians were captured with a dip net or by hand (amphibians were always handled with sterile nitrile gloves). Our goal was to sample at least 20 amphibian individuals per site, based on the prevalence estimates for *Bd* in the Netherlands, as that was 4.8% (Spitzen-Van Der Sluijs et al., 2014) and at least 20 individuals need to be sampled for obtaining an objective subset of the population to estimate *Bd* prevalence. Captured adults and juveniles were kept in individual bags, and tadpoles were kept in water-filled buckets until *Bd* sampling. All collected amphibians were handled according to safety protocols to prevent the spreading of *Bd*, and all equipment was disinfected with 70% ethanol between sites (Van Rooij et al., 2017). Each specimen was handled by 1 person, while another person collected skin samples by rubbing the abdomen (adults and juveniles) or the oral disc (tadpoles) with rayon swabs (Hyatt et al., 2007; Image DELTALAB, Barcelona, Spain). Two swab replicates were collected simultaneously (for tadpoles, samples were collected sequentially because of the impossibility of placing 2 swabs in a tadpole disc at the same time) per specimen and were placed in aseptic 1.5 ml microcentrifuge tubes. Pictures of all individuals were taken, and identification of species, sex, and life stage was performed on-site. Individuals were categorized as tadpoles, juveniles, or adults. Individuals that had not reached sexual maturity (<4.5 cm snout-vent length and no clear nuptial pads) were classified as juveniles, and all sexually mature individuals were classified as adults and sexed afterward. If metamorphosis was not complete, individuals were grouped into tadpoles. Individuals were released immediately after sampling. One duplicate of each swab was kept at −20 C until DNA extraction (University of Groningen); the other was kept at −80 C until all extractions, and qPCRs of the first duplicate were performed, to be sent to an independent external laboratory (University of Leipzig) for validation.

DNA extraction of collected samples

At the University of Groningen, DNA extraction of skin swabs was performed with the Biokè NucleoSpin tissue kit (Cat. No./ID

740952.250, Biokè, Leiden, the Netherlands) with an additional step of enzymatic lysis to enhance the lysis of the fungal cell (Belden et al., 2015). DNA extraction protocols followed the single-swab method described in Mantzana-Oikonomaki et al. (2021). During each round of extractions, a blank sample was extracted (no swab) as an extraction negative control (extraction negative controls were all negative). Eluted DNA was stored at −20 C until qPCR runs.

At the University of Leipzig, DNA extraction of skin swabs was performed with the Qiagen Blood and Tissue kit (Qiagen, Hilden, Germany) with the same enzymatic lysis step described above (Belden et al., 2015). Eluted DNA was stored at −20 C until qPCR runs.

qPCR analysis

After DNA extractions, qPCR was performed using established protocols and *Bd*-specific primers (*Bd* [ITS] 5.8S region) (Boyle et al., 2004; Hyatt et al., 2007). In Groningen qPCRs were performed on a CFX96 Real-Time System (Bio-Rad Laboratories Inc., Hercules, California); in Leipzig, qPCRs were performed on a qTower^3 (Analytik, Jena, Germany). Each qPCR plate included a series of 5 plasmid-based *Bd* (Standish et al., 2018) standard dilutions (10, 100, 1,000, 10,000, and 100,000 ITS copies in University of Groningen and 100, 1,000, 10,000, 100,000, and 1,000,000 ITS copies in University of Leipzig) and a negative control containing deionized water. In each qPCR run, the samples, the negative control, and standard dilutions were run in duplicates. In case the replicates showed contradictory results, a third replicate was run.

For both laboratories, a sample was considered *Bd* positive when 2 qPCR replicates provided a Cq (quantification cycle) value lying between the amplification signals estimated for the lowest and highest standard. In addition, for a sample to be considered positive, the amplification curve had to be sigmoidal, and the standard error had to be smaller than the mean of the 2 replicates. The quantification of zoospore equivalents (i.e., 1 zoospore equivalent [ze] = 10 ITS copies) was calculated as the average of the replicates for that sample. An individual was considered *Bd* infected if both sample duplicates (one processed in Groningen, the other in Leipzig) were positive.

RESULTS

A total of 359 individuals (adult, juveniles, and tadpoles or larvae) from 8 different amphibian species were sampled in years 2020 and 2021 (110 collected in 2020 and 249 in 2021): *Pelophylax lessonae* ($n_{2020} = 17$, $n_{2021} = 31$), *Pelophylax esculentus* ($n_{2020} = 1$, $n_{2021} = 4$), *Pelophylax ridibundus* ($n_{2020} = 4$), unidentified juvenile/tadpole *Pelophylax* sp. ($n_{2020} = 62$, $n_{2021} = 82$), *Rana temporaria* ($n_{2020} = 11$, $n_{2021} = 2$), *Bufo bufo* ($n_{2020} = 2$, $n_{2021} = 57$), *Epidalea calamita* ($n_{2020} = 1$), *Lissotriton vulgaris* ($n_{2021} = 73$), and *Triturus cristatus* ($n_{2020} = 12$) (Suppl. Data, Table S1).

In total, 11 out of the 249 samples collected in 2021 and 2 out of the 110 samples collected in 2020 tested positive for *Bd*. Prevalence of *Bd* was estimated at 4.4% for 2021 and 0.02% for 2020. For both sampling periods, positive individuals were found in 4 out of 12 sites (~33%) with 2 sites being infected in 2020 and 2 new sites being infected in 2021. Overall, for both years pooled together, across *Bd*-positive sites, prevalence was between 1% and 15%. There was an 18% prevalence of *Bd* in all anuran juvenile individuals captured (no Caudata juveniles were captured) and a 4% prevalence of *Bd* in all captured adults (Fig. 2; Tables I, S2). No *Bd*-infected tadpoles or newts were found. Average *Bd* load for positive samples ranged from 11.07 to 681.07 ze (Table II). Of the 13 *Bd*-positive samples, 8 samples came from unidentified *Pelophylax* juveniles.

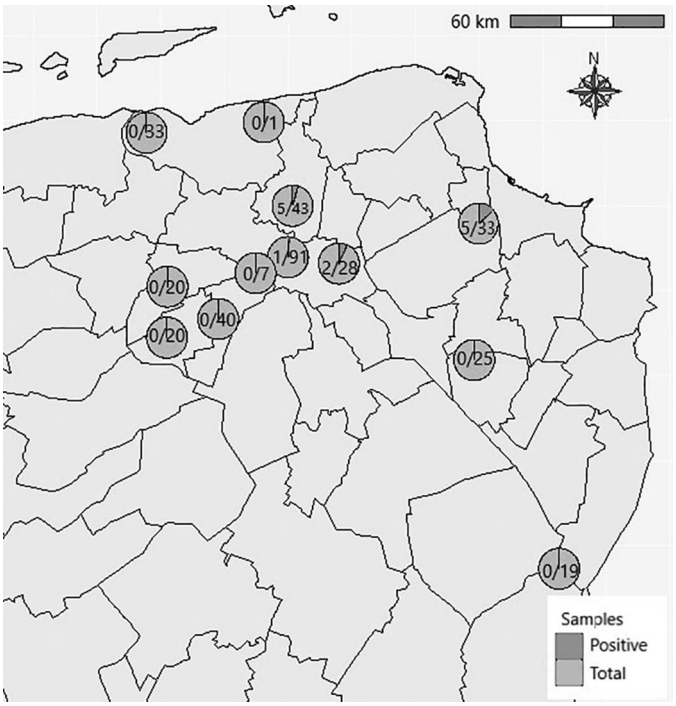

Figure 2. Number of *Batrachochytrium dendrobatidis* positive samples detected in each site compared to the total sample size for each site. Values inside pie charts represent the number of positive samples (dark) over the number negative samples collected in each site (light).

DISCUSSION

This study provides the first documentation of the chytrid pathogen *Bd* in the province of Groningen in the Netherlands (Fig. 2). A previous survey, between March and September 2009, in this province, did not detect *Bd* (Spitzen-van der Sluijs et al., 2010, 2014). However, the small sample size of total individuals sampled in Groningen in that study (12 individuals across 3 sites; Spitzen-van der Sluijs et al., 2010) does not allow us to conclude that the pathogen was only recently introduced into the province of Groningen. Instead, and in line with what was suggested by Spitzen-van der Sluijs et al. (2010), it is more likely that the pathogen was already present, but not detected, due to very low prevalence and small sample size. In the present study, a large enough proportion of the population was sampled to detect *Bd* and conclude that *Bd* is present in the province of Groningen.

We encountered *Bd* infected individuals of the species *P. lessonae*, *P. esculentus*, and unidentified *Pelophylax* sp. juveniles. In contrast, sampled *B. bufo*, *R. temporaria*, *P. ridibundus*, *E. calamita*, *L. vulgaris*, and *T. cristatus* were not found to be infected in any of the sampling sites. The fact that no Caudata species (0 infected individuals out of 85 total Caudata individuals sampled) were found infected agrees with the overall trend for a higher tendency of anuran species getting infected with *Bd* rather than Caudata, as shown also in earlier surveys (Spitzen-van der Sluijs et al., 2014). In each of the sampling sites, only a very small percentage of the samples tested positive for *Bd* (1–15%), which is in

Table I. Number of infected individuals detected and total sample size (positive/total) in each sampling site and total number of infected individuals per species detected. In infected species columns: *Tc: Triturus cristatus*, *Lv: Lissotriton vulgaris*, *Psp: Pelophylax* sp., *Pl: Pelophylax lessonae*, *Pe: Pelophylax esculentus*, *Pr: Pelophylax ridibundus*, *Rt: Rana temporaria*, *Bb.: Bufo bufo*, *Ec: Epidalea calamita*.

Sampling site	Sampling year(s)	Sample size 2020 (positive/total)	Sample size 2021 (positive/total)	Infected species								
				Tc	*Lv*	*Psp*	*Pl*	*Pe*	*Rt*	*Bb*	*Ec*	*Pr*
Groningen (Zernike Campus)	2020–2021	1/5	0/86	0	0	0	1	0	0	0	0	0
Groningen (Dorkwerd)	2020–2021	0/19	2/24	0	0	***3***	***2***	0	0	0	0	0
Groningen (Oosterhoogebrug)	2020–2021	1/7	1/21	0	0	0	***1***	1	0	0	0	0
Veendam	2020–2021	0/23	0/2	0	0	0	0	0	0	0	0	0
Ter Apel	2020–2021	0/11	0/8	0	0	0	0	0	0	0	0	0
Rodenburg	2020–2021	0/20	0/20	0	0	0	0	0	0	0	0	0
Lauwersoog	2020–2021	0/12	0/21	0	0	0	0	0	0	0	0	0
Tjuchem	2020–2021	0/13	5/20	0	***0***	***5***	0	0	0	0	0	0
Grotegast	2021	0/1	0/19	0	0	0	0	0	0	0	0	0
Marum	2021	—	0/20	0	0	0	0	0	0	0	0	0
Pieterburen	2021	—	0/1	0	0	0	0	0	0	0	0	0
Lettelbert	2021	—	0/7	0	0	0	0	0	0	0	0	0

line with the prevalence of *Bd* across the Netherlands and Belgium (4.8%; Spitzen-van der Sluijs et al., 2014). A large majority of the total infected individuals were juveniles (61.5%), showing a higher proportion of infected individuals in this life stage. This is not surprising as juvenile anuran amphibians have higher *Bd*-related mortality rates than adults (Russell et al., 2010), and in Europe chytridiomycosis has the tendency to be expressed in newly metamorphosed animals (Bakar et al., 2016). Interestingly, the average number of zoospores found in infected individuals was much higher in the province of Groningen (this study), in comparison to other areas in the Netherlands and Belgium (Martel et al., 2012; Spitzen-van der Sluijs et al., 2014). This pattern could be related to the fact that during our sampling period (17 C, May 2020 and 17–20 C, May–June 2021), temperatures were slightly lower than average temperatures during the sampling periods in Spitzen-van der Sluijs et al. (2014) (18–20 C, May–June 2009) (Royal Netherlands Meteorological Institute, 2019–2021), and therefore conducive to higher *Bd* zoospore production (Woodhams et al., 2011).

Previous reports indicate that the species found in this study, like *P. esculentus* and *L. vulgaris*, have a high tolerance against *Bd* and are disease-resistant (Cheatsazan et al., 2013; Ujszegi et al., 2021). Tolerant species, however, can act as reservoirs of the disease, both to sympatric species and to their habitat, and can further spread the pathogen or lead to new disease variants emerging. While increasing the sample size of disease screenings leads to higher confidence when asserting that a site is uninfected, the required sample size is only rarely met (DiGiacomo and Koepsell, 1986). Absolute certainty that a site is *Bd*-negative is impossible to achieve, and therefore it is safest to assume that all sites are potentially *Bd*-positive. We therefore recommend that all protection measures (e.g., disinfecting equipment, using gloves) are put into place. Additionally, strict disease mitigation practices are needed in infected areas, independently of the potential hosts' susceptibility to the disease, to protect other areas, populations, and species, and to limit the possibility of new variants emerging.

CONCLUSION

In this study we report the presence of the *Bd* pathogen in Groningen for the first time. We observe differences in infection prevalence between species and between sampling years, highlighting the importance of regular screenings and continuous efforts to maintain basic hygiene procedures. Finally, since the *Bd* pathogen is now known to have spread over almost all of the Netherlands apart from Zuid-Holland and Zeeland (Spitzen-van der Sluijs et al., 2010), conservation efforts should focus on preventing introductions of new and more highly virulent disease strains that can hybridize and could shift the disease outcome and eventually lead to an increase in mortality rate or to a new variant with higher virulence emerging.

Table II. Average *Batrachochytrium dendrobatidis* (*Bd*) load (in zoospore equivalents: ze) estimated for each *Bd* positive sample. M = male, NA = undetermined sex.

Area	Species	Life stage	Sex	Average *Bd* load (ze)
Groningen (Dorkwerd)	*P. lessonae*	Adult	M	399.58
Groningen (Dorkwerd)	*P. lessonae*	Adult	M	222.45
Groningen (Dorkwerd)	*Pelophylax* sp.	Juvenile	NA	637.92
Groningen (Dorkwerd)	*Pelophylax* sp.	Juvenile	NA	17.60
Groningen (Dorkwerd)	*Pelophylax* sp.	Juvenile	NA	41.91
Groningen (Oosterhoogebrug)	*P. lessonae*	Adult	M	16.87
Groningen (Oosterhoogebrug)	*Pelophylax esculentus*	Adult	NA	26.04
Groningen (Zernike Campus)	*P. lessonae*	Adult	NA	30.72
Tjuchem	*Pelophylax* sp.	Juvenile	NA	25.36
Tjuchem	*Pelophylax* sp.	Juvenile	NA	347.61
Tjuchem	*Pelophylax* sp.	Juvenile	NA	15.17
Tjuchem	*Pelophylax* sp.	Juvenile	NA	73.55
Tjuchem	*Pelophylax* sp.	Juvenile	NA	93.65

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LITERATURE CITED

Allain, S., and A. Duffus. 2019. Emerging infectious disease threats to European herpetofauna. Herpetological Journal 29: 189–206. doi:10.33256/hj29.4.189-206.

Bakar, A. A., D. S. Bower, M. P. Stockwell, S. Clulow, and M. J. Mahony. 2016. Susceptibility to disease varies with ontogeny and immunocompetence in a threatened amphibian. Oecology 181: 997–1009. doi:10.1007/s00442-016-3607-4.

Belden, L. K., M. C. Hughey, E. A. Rebollar, T. P. Umile, S. C. Loftus, E. A. Burzynski, and R. N. Harris. 2015. Panamanian frog species host unique skin bacterial communities. Frontiers in Cellular and Infection Microbiology 6: 1171. doi:10.3389/fmicb.2015.01171.

Berger, L., R. Speare, P. Daszak, D. E. Green, A. A. Cunningham, C. L. Goggin, R. Slocombe, M. A. Ragan, A. D. Hyatt, K. R. McDonald, et al. 1998. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and central America. Proceedings of the National Academy of Sciences USA 95: 9031. doi:10.1073/pnas.95.15.9031.

Boyle, G. D., B. D. Boyle, V. Olsen, J. Morgan, and A. Hyatt. 2004. Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Diseases of Aquatic Organisms 60: 141–148. doi:10.3354/dao060141.

Byrne, A. Q., V. T. Vredenburg, A. Martel, F. Pasmans, R. C. Bell, D. C. Blackburn, M. C. Bletz, J. Bosch, C. J. Briggs, R. M. Brown, et al. 2019. Cryptic diversity of a widespread global pathogen reveals expanded threats to amphibian conservation. Proceedings of the National Academy of Sciences USA 116: 20382–20387. doi:10.1073/pnas.1908289116.

Cheatsazan, H., A. P. de Almedia, G. Lugon, A. F. Russell, and C. Bonneaud. 2013. Experimental evidence for a cost of resistance to the fungal pathogen, *Batrachochytrium dendrobatidis*, for the palmate newt, *Lissotriton helveticus*. BMC Ecology and Evolution 13: 27. doi:10.1186/1472-6785-13-27.

Daskin, J. H., R. A. Alford, and R. Puschendorf. 2011. Short-term exposure to warm microhabitats could explain amphibian persistence with *Batrachochytrium dendrobatidis*. PLoS One 6: e26215. doi:10.1371/journal.pone.0026215.

DiGiacomo, R. F., and T. D. Koepsell. 1986. Sampling for detection of infection or disease in animal populations. Journal of Veterinary Medical Education 189: 22–23.

Fisher, M. C., and T. W. J. Garner. 2007. The relationship between the emergence of *Batrachochytrium dendrobatidis*, the international trade in amphibians and introduced amphibian species. Fungal Biology Reviews 21: 2–9. doi:10.1016/j.fbr.2007.02.002.

Fisher, M. C., T. W. J. Garner, and S. F. Walker. 2009. Global emergence of *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis in space, time, and host. Annual Review of Microbiology 63: 291–310. doi:10.1146/annurev.micro.091208.073435.

Garner, T. W. J., S. Walker, J. Bosch, A. Hyatt, A. Cunningham, and M. Fisher. 2005. Chytrid fungus in Europe. Emerging Infectious Diseases 11: 1639–1641. doi:10.3201/eid1110.050109.

Gower, D. J., T. Doherty-Bone, S. P. Loader, M. Wilkinson, M. T. Kouete, B. Tapley, F. Orton, O. Z. Daniel, F. Wynne, E. Flach, et al. 2013. *Batrachochytrium dendrobatidis* infection and lethal chytridiomycosis in caecilian amphibians (Gymnophiona). EcoHealth 10: 173–183. doi:10.1007/s10393-013-0831-9.

Hyatt, A., D. Boyle, V. Olsen, D. Boyle, L. Berger, D. Obendorf, and A. Coiling. 2007. Diagnostic assay and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. Diseases of Aquatic Organisms 73: 175–192. doi:10.3354/dao073175.

Kolby, J. E., K. M. Smith, L. Berger, W. B. Karesh, A. Preston, A. P. Pessier, and L. F. Skerratt. 2014. First evidence of amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) and ranavirus in Hong Kong amphibian trade. PLoS One 9: e90750. doi:10.1371/journal.pone.0090750.

Mantzana-Oikonomaki, V., M. Maan, and J. Sabino-Pinto. 2021. Wildlife pathogen detection: Evaluation of alternative DNA extraction protocols. Biology Methods and Protocols 6: bpab018. doi:10.1093/biomethods/bpab018.

Martel, A., M. Sharifian, P. Van Rooij, R. Jooris, F. Boone, F. Haesebrouck, D. Rooij, and F. Pasmans. 2012. Road-killed common toads (*Bufo bufo*) in Flanders (Belgium) reveal low prevalence of Ranaviruses and *Batrachochytrium dendrobatidis*. Journal of Wildlife Diseases 48: 835–839. doi:10.7589/0090-3558-48.3.835.

Martel A., A. Spitzen-van der Sluijs, M. Blooi, W. Bert, R. Ducatelle, M. C. Fisher, A. Woeltjes, W. Bosman, K. Chiers, F. Bossuyt, and F. Pasmans. 2013. *Batrachochytrium salamandrivorans sp. nov.* causes lethal chytridiomycosis in amphibians. Proceedings of the National Academy of Sciences USA 110: 15325–15329. doi:10.1073/pnas.1307356110.

Meurling, S., S. Kärvemo, N. Chondrelli, M. C. Chinarro, D. Åhlen, L. Brookes, P. Nyström, M. Stenberg, T. W. J. Garner, J. Höglund, et al. 2020. Occurrence of *Batrachochytrium dendrobatidis* in Sweden: Higher infection prevalence in southern species. Diseases of Aquatic Organisms 140: 209–218. doi:10.3354/dao03502.

Olson, D. H., D. M. Aanensen, K. L. Ronnenberg, C. I. Powell, S. F. Walker, J. Bielby, T. W. J. Garner, G. Weaver, The Bd Mapping Group, and M. C. Fisher. 2013. Mapping the global emergence of *Batrachochytrium dendrobatidis*, the amphibian chytrid fungus. PLoS One 8: e56802. doi:10.1371/journal.pone.0056802.

Olson, D. H., K. L. Ronnenberg, C. K. Glidden, K. R. Christiansen, and A. R. Blaustein. 2021. Global patterns of the fungal pathogen *Batrachochytrium dendrobatidis* support conservation urgency. Frontiers in Veterinary Science 8: 774. doi:10.3389/fvets.2021.685877.

Reeder, N. M. M., A. P. Pessier, and V. T. Vredenburg. 2012. A reservoir species for the emerging amphibian pathogen *Batrachochytrium dendrobatidis* thrives in a landscape decimated by disease. PLoS One 7: e33567. doi:10.1371/journal.pone.0033567.

Royal Netherlands Meteorological Institute (Koninklijk Nederlands Meteorologisch Instituut). 2019–2021.

KNMI (KDB as of 2020) Data Platform. Data set Tg1. Available at: dataplatform.knmi.nl/dataset/tg1-5. Accessed 11 October 2021.

Russell, D. M., S. C. Goldberg, P. L. Waits, and B. E. Rosenblum. 2010. *Batrachochytrium dendrobatidis* infection dynamics in the Columbia spotted frog *Rana luteiventris* in north Idaho, USA. Diseases of Aquatic Organisms 92: 223–230. doi:10.3354/dao02286.

Scheele, B. C., N. C. Foster, A. D. Hunter, B. D. Lindenmayer, R. B. Schmidt, and W. G. Heard. 2019. Living with the enemy: Facilitating amphibian coexistence with disease. Biological Conservation 236: 52–59. doi:10.1016/j.biocon.2019.05.032.

Spitzen-van der Sluijs, A., A. Martel, A. C. Hallmann, W. Bosman, T. W. J. Garner, P. Van Rooij, R. Jooris, F. Haesebrouck, and F. Pasmans. 2014. Environmental determinants of recent endemism of *Batrachochytrium dendrobatidis* infections in amphibian assemblages in the absence of disease outbreaks. Biological Conservation 28: 1302–1311. doi:10.1111/cobi.12281.

Spitzen-van der Sluijs, A., F. Spikmans, W. Bosman, M. De Zeeuw, T. Van der Meij, E. Goverse, M. Kik, F. Pasmans, and A. Martel. 2013. Rapid enigmatic decline drives the fire salamander (*Salamandra salamandra*) to the edge of extinction in the Netherlands. Amphibia-Reptilia 34: 233–239. doi:10.1163/15685381-00002891.

Spitzen-van der Sluijs, A., R. Zollinger, W. Bosman, F. Pasmans, A. Martel, P. Van Rooij, and F. Clare. 2010. *Batrachochytrium dendrobatidis* in amphibians in the Netherlands and Flanders (Belgium). Short report. Stichting RAVON, Nijmegen, the Netherlands, 32 p.

Standish, I., E. Leis, N. Schmitz, J. Credico, S. Erickson, J. Bailey, J. Kerby, K. Phillips, and T. Lewis. 2018. Optimizing, validating, and field testing a multiplex qPCR for the detection of amphibian pathogens. Diseases of Aquatic Organisms 129: 1–3. doi:10.3354/dao03230.

Statistics Netherlands. 2018. Bodemgebruik; uitgebreide gebruiksvorm, per gemeente. Data from 1996 to 2015. Available at: opendata.cbs.nl/#/CBS/nl/dataset/70262ned/table. Accessed 11 October 2021.

Ujszegi, J., K. Ludányi, M. A. Móricz, D. Krüzselyi, L. Drahos, T. Drexler, Z. M. Németh, J. Vörös, T. W. J. Garner, and A. Hettyey. 2021. Exposure to *Batrachochytrium dendrobatidis* affects chemical defences in two anuran amphibians, *Rana dalmatina* and *Bufo bufo*. BMC Ecology and Evolution 21: 135. doi:10.1186/s12862-021-01867-w.

Van Rooij, P., A. Martel, F. Haesebrouck, and F. Pasmans. 2015. Amphibian chytridiomycosis: A review with focus on fungus-host interactions. Veterinary Research 46: 137. doi:10.1186/s13567-015-0266-0.

Van Rooij, P., F. Pasmans, Y. Coen, and A. Martel. 2017. Efficacy of chemical disinfectants for the containment of the salamander chytrid fungus *Batrachochytrium salamandrivorans*. PLoS One 12: e0186269. doi:10.1371/journal.pone.0186269.

Voyles, J., D. C. Woodhams, V. Saenz, A. Q. Byrne, R. Perez, G. Rios-Sotelo, M. J. Ryan, M. C. Bletz, F. A. Sobell, S. McLetchie, et al. 2018. Shifts in disease dynamics in a tropical amphibian assemblage are not due to pathogen attenuation. Science 359: 1517–1519. doi:10.1126/science.aao4806.

Woodhams, D. C., J. Bosch, J. C. Briggs, S. Cashins, R. L. Davis, A. Lauer, E. Muths, R. Puschendorf, R. B. Schmidt, B. Sheafor, et al. 2011. Mitigating amphibian disease: Strategies to maintain wild populations and control chytridiomycosis. Frontiers in Zoology 8: 1–24. doi:10.1186/1742-9994-8-8.