

Amphibian chytridiomycosis in Luxembourg

Laura R. Wood¹, Richard A. Griffiths¹ & Laurent Schley²

¹ Durrell Institute of Conservation & Ecology, University of Kent, Marlowe Building, Canterbury, CT2 7NR, UK (laura_rw@yahoo.com; R.A.Griffiths@kent.ac.uk)

² Administration de la nature et des forêts, 16 rue Eugène Ruppert, L-2453 Luxembourg (laurent.schley@ef.etat.lu)

Wood, L.R., R.A. Griffiths & L. Schley, 2009. Amphibian chytridiomycosis in Luxembourg. *Bulletin de la Société des naturalistes luxembourgeois* 110: 109-114.

Abstract. Aquatic-phase amphibians at eight sites in Luxembourg were tested for the fungus *Batrachochytrium dendrobatidis*, which causes a disease responsible for population declines among many amphibian species worldwide. Infected amphibians were found at two of the sites tested; two further sites also showed marginally positive results. The ecology of *B. dendrobatidis* and the necessity for biosecurity protocol implementation by fieldworkers are discussed.

Key words. *Batrachochytrium dendrobatidis*, biosecurity, conservation, fieldwork, mass mortality, population decline.

1. Introduction

In recent years much attention has been focused on the apparent global decline of amphibians. According to the IUCN Red List many more amphibian species are threatened than birds or mammals, with 32.5% amphibians, 23% mammals and 12% birds assessed as 'globally threatened' (Stuart et al. 2004). The threats facing amphibians are complex and mostly caused by humans, including non-native species introductions, exploitation, habitat loss, climate change, pollution and disease (Collins & Storfer 2003).

Following its relatively recent discovery almost simultaneously in wild and captive populations (Berger et al. 1998, Pessier et al. 1999), the chytridiomycete fungus *Batrachochytrium dendrobatidis* (hereafter *Bd*) has been implicated in amphibian declines and extinction events worldwide. The reason for the rapid global emergence of *Bd* is the subject of much debate, but likely due to a suite of factors including introduction by human agency into wild populations, for example release of non-native species, especially the North American bullfrog (*Rana catesbeiana*) (Garner et al. 2006) and the cumulative effect of environmental changes

modifying an already present host-parasite relationship (Pounds et al. 1999).

Prior to the identification of *Bd*, chytridiomycete fungi had not been described in parasitic relationships with vertebrates, they were thought to be either free-living or parasitic on other fungi, algae, plants or invertebrates (Pessier et al. 1999). Therefore *Bd* is the first member of the phylum known to parasitise vertebrates and in a very short space of time has been associated with amphibian declines on every continent they inhabit (see www.spatial-epidemiology.net/bd/). *Bd* infection has been observed to travel very rapidly, described by Lips et al. (2006) as an 'epidemic wave' and its interactions with amphibians and the environment appear to vary geographically (Morehouse et al. 2003).

Infection with *Bd* causes a disease called chytridiomycosis. The fungus only occurs in keratinised tissue and may cause death by interfering with the respiration and osmoregulation properties of amphibian skin (Daszak et al. 1999). However not all amphibian species are susceptible. Out of 28 European species tested by Garner et al. (2005) 20 were found to be infected in some area or at some stage of their life history. According to the latest data available on the

website dedicated to sharing information about *Bd*, 29 species have tested positive in nine European countries (<http://www.spatialepidemiology.net/bd/>).

Bd has been detected in both France and Germany and at very high prevalence in Switzerland. However this does not mean that it is not present in other nearby countries as sampling effort has been patchily distributed and not all data are in the online database. All but two of the amphibian species present in Luxembourg have been found to be susceptible to the disease elsewhere in Europe: only *Triturus cristatus* and *Bombina variegata* have not tested positive so far, but the extent of survey effort has not been quantified.

Increasing knowledge of the global distribution of *Bd* infections is vital. Accordingly, we tested amphibians in Luxembourg for the disease in a simple presence/absence study. The resulting data are useful from both local and global perspectives, informing management decisions on site and contributing to wider knowledge of the disease.

2. Materials and methods

Amphibians from 14 ponds in eight different locations in Luxembourg were tested for *Bd* infection (see Table 1). Test sites were selected mainly (but not exclusively) for having an historical record of the Common

midwife toad, *Alytes obstetricans*, a threatened species known to be particularly susceptible to the disease. Up to 34 individuals were tested at each site between May and July 2008.

During all fieldwork, appropriate biosecurity measures were applied. Footwear and survey equipment were disinfected between sites and clean disposable vinyl gloves were worn for handling amphibians.

Amphibians were caught in bottle traps, by hand or by net. Only species known to be susceptible to *Bd* were tested; see Table 1. Keratinised skin parts were targeted with a cotton swab that was swept three times across the drink patch, feet and thighs of adult animals or rotated on the mouthparts of anuran larvae.

Swabs were stored in individual airtight tubes in a cool, dry environment. The real-time polymerase chain reaction (PCR) Taqman procedure of Boyle et al. (2004) was used to extract and amplify *Bd* DNA from the swabs. Samples were compared to amplification standards of 100, 10, 1 and 0.1 *Bd* zoospore equivalents. This method is widely used and is specific to *Bd*.

Each sample was replicated twice. Samples that amplified ≥ 1.0 *Bd* zoospore equivalent in both replications were taken to be true positives, single amplification samples were retested, and samples that did not

Table 1. Sites of *Bd* testing and the species tested at each site. *A.o* = *Alytes obstetricans*; *B.c* = *Bufo calamita*; *R.k.l.e* = *Rana klepton. esculenta* (green frog complex); *M.a* = *Mesotriton alpestris*; *L.h* = *Lissotriton helveticus*; *L.v* = *L. vulgaris*.

Nearest town or village	Pond name	Grid reference	Number of swabs by species					Total swabs	
			A.o	B.c	R.k.l.e	M.a	L.h		L.v
Putscheid	Alersang	LA 78152 14160	17		6		2		25
Bissen	Laaschtert 2	LC 72266 93271			1		1	2	4
	Laaschtert 3	LC 72520 93396			1	1			2
	Laaschtert 4&5	LC 72679 93499			1	1		2	4
Useldange	Weiden 3&4	LC 64855 92458			30				30
Schoos	Stuppicht 1&2	LC 80024 89074				3	27		30
Hunnebour	Mersch	LC 74102 88067			25				25
Blaschette	Flakewiss2	LC 80068 87043				1	1		2
	Biergwiss	LC 79823 86966	1			4	14		19
Steinfort	Steinfort Carrières	LC 61072 81361	4	6		5	6	13	34
Niedercorn	Giele Botter	LC 59207 67483					19		19

amplify in either replicate were taken to be negative. Due to the very small quantities of DNA involved, and the potential for contamination between samples, those that amplified only once and at very low levels were ignored. The quantity of *Bd* zoospores detected on positive animals is taken from the mean value of *Bd* zoospore equivalents detected in the replicates of each sample.

This study aimed only to establish whether *Bd* is present in Luxembourg, therefore inferences about the prevalence of disease can not be made.

3. Results

Amphibians tested positive for *Bd* zoospores at two of the eight sites surveyed. Two further sites may also be positive, however the number of zoospores detected was very low – if greater confidence in results was required these samples would need further testing. The sites tested and the results are shown in Fig. 1.

No infection was found at Alersang, Laaschtert 2 or 3, Weiden 3&4, Mersch or Biergewiss and Flakewiss2.

A single green frog (*R. kl. esculenta*) tested positive at Laaschtert 4&5; this is a clear positive because *Bd* DNA amplified in both sample replicates on the first PCR run (mean 8.3 zoospores). A clear positive result was also found in one Alpine newt (*Mesotriton alpestris*) at the forest ponds Stuppicht 1&2 (mean 2.5 zoospores).

At Giele Botter pond a palmate newt (*Lissotriton helveticus*) returned a marginal positive result on both replicates, only one of which was ≥ 0.5 zoospore equivalents (0.07 and 0.6). Screening was unable to give conclusive results for two newts swabbed at Steinfort Carrières: a smooth newt (*L. vulgaris*) sample that was tested twice (4 replicates) amplified only a single replicate each time (0.4 and 2.2 zoospores); one palmate newt (*L. helveticus*) gave a marginally positive test on both replicates (mean 0.7 zoospores).

The amplification standards, with a known quantity of *Bd* zoospores, all amplified as expected, indicating that the tests had run successfully.

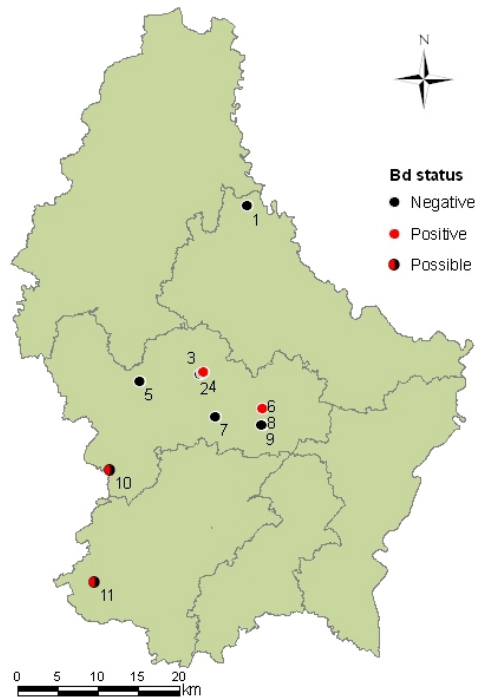


Fig. 1. The locations of ponds tested for *Bd* infection and test results (1 = Alersang; 2 = Laaschtert 2; 3 = Laaschtert 3; 4 = Laaschtert 4+5; 5 = Weiden 3+4; 6 = Stuppicht 1+2; 7 = Mersch; 8 = Flakewiss; 9 = Biergewiss; 10 = Steinfort Carrières; 11 = Giele Botter).

4. Discussion

4.1. *Bd* in Luxembourg

Bd was found at half of the sites tested, although two of the positive results are not clear. The highest infection rate among the animals randomly sampled was 10% at the Laaschtert ponds near Bissen (n = 10); however finding true prevalence rates would require more extensive testing.

The sites tested were selected for their amphibian fauna, with at least four of the eight sites presently hosting the Common midwife toad (*A. obstetricans*) and two other sites from where it has recently become extinct. According to the Luxembourg Musée national d'histoire naturelle (MNHN) records *A. obstetricans* was present at the Laaschtert pond complex until at least 1997 (F. Schoos, 'Amphibia Data' record 466, MNHN, 2007) but it was not detected

during two seasons of intensive survey effort by LRW in 2007 and 2008.

4.2. Biology of *Bd*

Bd was only discovered recently (Berger et al. 1998, Pessier et al. 1999), so relatively little is known about its ecology, and its modes of transmission are still not fully understood. Long-distance transmission seems likely to have occurred principally via infected animals in the global amphibian trade (Garner et al. 2006) for the pet, science and food industries (Kriger & Hero 2007), and then subsequent releases or escapes into the wild. Besides its own motility in water, on a local level there are many possible vectors of *Bd*; for example animals travelling between sites (e.g. on bird feathers) and human vectors such as vehicles and hiking boots (Daszak et al. 2003, Johnson & Speare 2005). It can survive, and remain viable, away from a host amphibian for up to 7 weeks in lake water (Johnson & Speare 2003) and can survive up to three hours out of the water (Johnson & Speare 2005). Therefore it could easily be transported locally.

The effects of *Bd* infection vary widely. In some places mass-mortality events have been observed, for example the large numbers of dead and dying *A. obstetricans* found around ponds in Peñalara National Park in central Spain (Bosch et al. 2001). Yet sometimes there may be a very high prevalence of *Bd*, but no pathological symptoms or any apparent population decline (Kriger & Hero 2007). Furthermore, many of the so-called 'enigmatic declines' (Stuart et al. 2004) could have been caused by undetected disease events. To the authors' knowledge no mass-mortality events of amphibians have been reported in Luxembourg. However, because of the patchy nature of amphibian recording, it is possible that they may have occurred without being witnessed. Alternatively *Bd* may simply be present but not causing animals to die.

Although several treatments or cures for *Bd* infection have been found (Woodhams et al. 2003, Bishop et al. 2008) these are impracticable in the field, therefore *Bd* has not yet been mitigated for in the wild (Garner 2008).

Ascertaining the presence of *Bd* in Luxembourg, and neighbouring countries (Garner et al. 2005), is a key step to understanding the pressures faced by declining amphibian species in the region. The actual rate of infection, whilst regulating the impact of the disease, does not alter the biosecurity protocol that should be implemented by fieldworkers. The disease is present, and care should be taken to prevent further spread by human agency.

4.3. Biosecurity issues

Simple disinfection procedures, such as washing boots in commercially available disinfectants, can be used by fieldworkers to lessen the risk of carrying *Bd* between study sites. At first this is time-consuming, however it rapidly becomes part of the survey routine. It can be argued that such biosecurity measures are 'too little, too late' and even futile because wild animals move freely and frequently between ponds. Despite the counter-opinions, it is generally considered that precautionary principles should be applied because of the potentially severe consequences of spreading *Bd*.

A guideline leaflet produced by UK herpetological conservation organisations provides up-to-date, simple advice on how to minimise the risk of transferring *Bd* between sites and the recommended disinfectants. The leaflet 'Amphibian disease precautions: a guide for UK fieldworkers' (Version 1, February 2008) is free to download from www.arg-uk.org.uk/Publications.htm. Anybody working in or near to amphibian aquatic habitat is strongly recommended to follow the guidelines, to report unusual die-off events and to be aware of the symptoms of disease.

A great deal of research is being conducted on *Bd* around the world. The data from Luxembourg contribute to knowledge of the extent of the disease. Further work could include retesting the samples that gave borderline positive results and making detailed local investigations to determine more precisely where *Bd* infection occurs and its prevalence.

Acknowledgements

The disease testing was funded by the Administration de la nature et des forêts, during research work under LRW's student bursary from the Ministère de la culture, de l'enseignement supérieur et de la recherche (Luxembourg, BFR 06/056). Many thanks to fieldwork assistants Liza Glesener and Véronique Ludwig who sped up the swabbing process greatly, and to Edmée Engel (Musée national d'histoire naturelle) for her collaboration and support throughout the project. Trent Garner and Frankie Clare from the Zoological Society of London (ZSL) kindly instructed LRW in sample collection and processing and provided laboratory facilities. We also thank Jennifer Sears, of ZSL and the Durrell Institute of Conservation and Ecology, whose knowledge of Bd was very helpful in writing this paper.

Literature

Bd database: www.spatialepidemiology.net/bd/ Global surveillance data for *Batrachochytrium dendrobatidis*. Imperial College London. Accessed on the World Wide Web on 26/11/2008.

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, H. B. Hines, K. R. Lips, G. Marantelli & H. Parkes, 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: 9031-9036.

Bishop, P. J., R. T. M. Poulter, J. N. Busby, P. J. Bishop, M. I. Butler, P. Harlow & R. Speare, 2008. *Chloramphenicol is active against chytridiomycosis in frogs*. Paper presented at the 6th World Congress of Herpetology, Manaus, Brazil. 17-22 August 2008.

Bosch, J., I. Martínez-Solano & M. García-Paris, 2001. Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain. *Biological Conservation* 97: 331-337.

Boyle, D. G., D. B. Boyle, V. Olsen, J. A. Morgan & A. D. 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.

Collins, J. P. & A. Storfer, 2003. Global amphibian declines: Sorting the hypotheses. *Diversity and Distributions* 9: 89-98.

Daszak, P., L. Berger, A. A. Cunningham, A. D. Hyatt, D. E. Green & R. Speare, 1999. Emerging infectious diseases and amphibian population declines. *Emerging Infectious Diseases* 5: 735-748.

Daszak, P., A. A. Cunningham, A. D. Hyatt, 2003. Infectious disease and amphibian population declines. *Diversity and Distributions* 9: 141-150.

Garner, T. W. J., M. W. Perkins, P. Govindarajulu, D. Seglie, S. Walker, A. A. Cunningham & M. C. Fisher, 2006. The emerging amphibian pathogen *Batrachochytrium dendrobatidis* globally infects introduced populations of the North American bullfrog, *Rana catesbeiana*. *Biology Letters* 2: 455-459.

Garner, T. W. J., S. Walker, J. Bosch, A. D. Hyatt, A. A. Cunningham & M. C. Fisher, 2005. Chytrid fungus in Europe. *Emerging Infectious Diseases* 11: 1639-1641.

Garner, T. W. J., 2008. *Mitigating Batrachochytrium dendrobatidis in the wild: where we are now and where are we going?* Paper presented at the Zoological Society of London symposium: Halting the global decline in amphibians: Research and practice. London, UK. 20-21 November 2008.

Johnson, M. & R. Speare, 2003. Survival of *Batrachochytrium dendrobatidis* in water: quarantine and disease control implications. *Emerging Infectious Diseases* 9: 922-925.

Johnson, M. & R. Speare, 2005. Possible modes of dissemination of the amphibian chytrid *Batrachochytrium dendrobatidis* in the environment. *Diseases of Aquatic Organisms* 65: 181-186.

Kruger, K. M. & J.-M. Hero, 2007. Large-scale seasonal variation in the prevalence and severity of chytridiomycosis. *Journal of Zoology* 271: 352-359.

Lips, K. R., F. Brem, R. Brenes, J. D. Reeve, R. A. Alford, J. Voyles, C. Carey, L. Livo, A. P. Pessier & J. P. Collins, 2006. Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proceedings of the National Academy of Sciences USA* 103: 3165-3170.

Morehouse, E. A., T. Y. James, A. R. D. Ganley, R. Vilgaly, L. Berger, P. J. Murphy & J. E. Longcore, 2003. Multilocus sequence typing suggests the chytrid pathogen of amphibians is a recently emerged clone. *Molecular Ecology* 12: 395-403.

Pessier, A. P., D. K. Nichols, J. E. Longcore & M. S. Fuller, 1999. Cutaneous chytridiomycosis in poison dart frogs (*Dendrobates* spp.) and

- White's tree frogs (*Litoria caerulea*). *Journal of Veterinary Diagnostic Investigation* 11: 194-199.
- Pounds, J. A., M. P. L. Fogden & J. H. Campbell, 1999. Biological response to climate change on a tropical mountain. *Nature* 398: 611-615.
- Stuart, S. N., J. S. Chanson, N. A. Cox, B. E. Young, A. S. Rodrigues, L. Fischman & R. W. Waller, 2004. Status and trends of amphibian declines and extinctions worldwide. *Science* 306: 1783-1786.
- Woodhams, D. C., R. A. Alford & G. Marantelli, 2003. Emerging disease of amphibians cured by elevated body temperature. *Diseases of Aquatic Organisms* 55: 65-67.