From the eastern lowlands to the western mountains: first records of the chytrid fungus *Batrachochytrium dendrobatidis* in wild amphibian populations from Austria

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Chytridiomycosis is a fungal disease that has been made responsible for amphibian declines around the globe. We found the causative agent of the disease, *Batrachochytrium dendrobatidis*, at six amphibian breeding sites in the eastern lowlands of Austria and four in the western parts of the country (30% of all sampled sites), including the highest record for the European Alps to date at 1630 m a.s.l. Nine amphibian species were infected, and metamorphosing *Bombina bombina* had the highest prevalence (40%). No individual showed obvious signs of disease, but our data are insufficient to draw any conclusions on disease-associated effects.

Key words: alpine amphibians, chytridiomycosis, infectious disease

The fungal disease chytridiomycosis has been recognized as a major driver for global amphibian population declines (Berger et al., 1998; Skerratt et al., 2007). The origin of the causative organism of the disease, *Batrachochytrium dendrobatidis* (*Bd*), is not completely clear, but it has spread very quickly to all continents inhabited by amphibians, most probably assisted by international trade (Fisher & Garner, 2007). Whereas *Bd* is widely distributed, fatal outbreaks of chytridiomycosis have been limited to parts of the Americas, Australia and Western Europe (Berger et al., 1998; Bosch et al., 2001; Lips et al., 2006). The variability in virulence observed seems to be mostly a consequence of different strains of *Bd* and varying susceptibility of species and life stages, with climatic conditions playing an additional role (Fisher et al., 2009).

Europe's amphibians have been declining for decades, and habitat fragmentation and destruction are the major causes (Stuart et al., 2004). Small and fragmented populations could be particularly vulnerable to additional stressors such as diseases (Smith et al., 2009). Except for Spain and Switzerland, there have been no reports of chytridimycosis-linked amphibian declines so far (Bosch et al., 2001; Tobler & Schmidt, 2010). The detection of subtle disease effects, however, would require data on the presence of *Bd* linked with population trends. Austrian amphibians, for instance, have not been tested for *Bd* except for a captive tropical species (Richter & Kubber-Heiss, 2010), and samples from two sites that proved negative (Garner et al., 2005; Sztatecsny & Hödl, 2009). The amphibians occurring in Austria all have a central to eastern European distribution (Gasc et al., 1997), and Austria stands out through the conjunction of maritime (western and northern parts of the country), continental (east) and Mediterranean (south) climate regimes, with additional vertical effects caused by the Alps (Auer et al., 2007). Given this high diversity within a small geographic area, we considered it important to know whether or not *Bd* is present.

We collected juvenile and adult amphibians in April and May 2008 in eastern Austria (10 sites) and in May and June 2009 in western Austria (20 sites). To increase the probability of detecting *Bd* we attempted to sample a minimum of 20 individuals per site and focused on species with a long aquatic phase (i.e. several months), which is likely to increase the probability of infection by aquatic zoospores. Our focal species were the smooth newt (*Lissotriton vulgaris*), the alpine newt (*Ichthyosaura alpestris*), crested newts (*Triturus cristatus*, *T. carnifex*, *T. dobrogicus*), fire and yellow bellied toads (*Bombina bombina*, *B. variegata*) and the water frogs (*Pelophylax* lessonae, *P. ridibundus*, *P. kl. esculentus*, pooled because of doubtful species determination for subadults). Other species were tested in small numbers when present (Table 1). We captured all animals by dipnetting and handled them with unused latex gloves to reduce the risk of possible disease transmission. Samples were taken by firmly running sterile cotton swabs (Medical Wire & Equipment, MW 100) over the amphibians' ventral surface, flanks, feet and tail (for newts only) in a standardized manner (constant number of sweeps per animal) as described by Kriger et al. (2006). All animals were released at the point of capture immediately after sampling. Swabs were air dried, replaced in their original containers and stored at room temperature. We disinfected our equipment either with VirkonS (2g l^{-1}, Webb et al., 2007), or by complete drying.

We screened the swabs for the presence of *Bd* using the quantitative real-time polymerase chain reaction (qPCR) as described in Boyle et al. (2004). We applied the changes suggested by Kriger & Hero (2006); however, we ran all samples in duplicate and always added a negative control and four reactions containing DNA from 100, 10, 1 and 0.1 *Bd* genome equivalents (GE) to create a standard curve. We scored samples as positive only if both replicates clearly amplified and estimated infection burden from mean GE as described in Bai et al. (2010). Because we had small numbers of positives from most species, we calculated GE only for *L. vulgaris*, *I. alpestris*, *B. bombina*, *B. variegata* and water frogs and pooled individuals from all sites in case of positives.

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Table 1. Infected species and prevalence of *Bd* at 10 Austrian amphibian breeding sites. Long. = longitude, Lat. = latitude, Elev. = elevation, Prev. = prevalence by site, Lv = *Lissotriton vulgaris*, Ia = *Ichthyosaura alpestris,* Tcr = *Triturus cristatus,* Tc = *T. carnifex*, Td = *T. dobrogicus*, Bb = *Bombina bombina*, Bv = *B. variegata,* Ha = *Hyla arborea,* Ra = *Rana arvalis,* Rt = *R. temporaria*, wf = water frogs.

No.	Site	Long. °E	Lat. °N	Elev. (m)	Species (no. tested/positives)	Prev. (%)
1	Hohenau	16.92320	48.60550	150	Lv(6/1), Td(2/0), Bb(16/1), Ra(2/0), wf(3/0)	6.9
2	Vienna 1	16.48420	48.17660	136	Lv(1/0), Td(1/1), Bb(15/6), wf(3/2)	45.0
3	Vienna 2	16.50150	48.17250	156	Lv(18/3), Bb(3/2), Ha(2/0), wf(4/1)	22.2
4	Vienna 3	16.44540	48.19160	145	Lv(19/6), wf(1/0)	30.0
5	Vienna 4	16.24700	48.14590	311	wf(4/1)	25.0
6	Rohrau	16.86070	48.06040	146	Bb(19/3), wf(1/0)	15.0
7	Ebbs	12.19350	47.59678	480	Bv (19/6), Tcr (2/1), Ia (4/0)	28.0
8	Walchsee	12.28773	47.65628	655	Rt (3/1), wf (20/3)	17.4
9	Stans	11.73701	47.37391	530	Ia(2/0), Tc(14/1), wf(1/0)	5.9
10	Kauns	10.74261	46.96845	1630	Ia(20/2)	10.0

We found 10 amphibian breeding sites and nine amphibian species (the three species of water frogs pooled) in Austria to be infected by *Bd*, with the prevalence at each site ranging from 5.9% to 45% (Table 1; Fig. 1, data on negative sites not shown). *Bd* was present in the low elevation flood plains of eastern Austria (sites 1 and 6), all sites within the city limits of Vienna (sites 2–5) and in the Alpine valleys of western Austria, with the highest recording from an elevation of 1630 m a.s.l. (sites 7–10, Table 1, Fig. 1). The highest prevalence was reached at Vienna site 1 where we tested 15 juvenile *B. bombina* of which six proved positive (Tab. 1). Zoospore load in mean GE±SE across all sites with positives was 119.8±5.0 for *L. vulgaris* (3 sites), 284.2±182.2 for *I. alpestris* (1 site), 18.4 ±1.5 for *B. bombina* (4 sites), 24.1±3.8 for *B. variegata* (1 site) and 90.6±49.2 for water frogs (4 sites).

As we did not use an internal positive control in our qPCR assay (Hyatt et al., 2007), we may have underestimated the number of positive sites as well as prevalence rates. To our knowledge, the two infected alpine newts found at 1630 m a.s.l. represent the highest occurrence of *Bd* in the European Alps to date. None of the animals tested showed obvious signs of disease, and a mass mortality observed earlier at a montane breeding site was not associated with chytridiomycosis (Sztatecsny & Hödl, 2009). Our results are consistent with findings suggest-

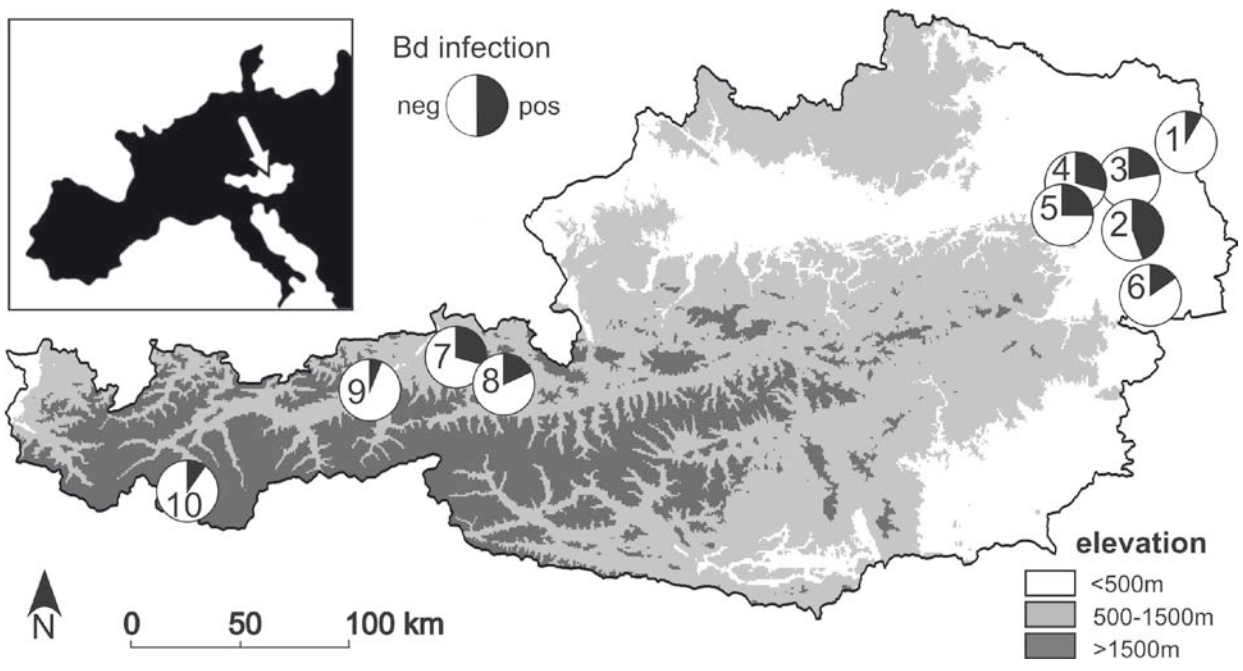

Fig. 1. Location, prevalence, and elevation of ten Austrian amphibian breeding sites infected with *Batrachochytrium dendrobatidis* (*Bd*).

ing that *Bd* is an ecological generalist and can be found in various habitats (Walker et al., 2010). Environmental conditions, however, act in synergy with other factors such as *Bd* strain, infected species and life stages (Fisher et al., 2009a; Garner et al., 2009), influencing the actual risk of a fatal disease outbreak (Garner et al., 2009; Harris et al., 2009; Tobler & Schmidt, 2010; Woodhams et al., 2007). Zoospore load varied considerably between sites, species and individuals, but was generally low in all cases compared to fatally infected captive *A. obstetricans* from Switzerland (mean GE: 970000; Tobler & Schmidt, 2010). To clarify if the observed variation in GE is species-specific or influenced by environmental conditions, larger sample sizes are needed. Metamorphs of *B. bombina* exhibited the highest prevalence in our survey, confirming a higher infection probability at this life stage (Garner et al., 2009). These findings indicate that *Bd* could be a threat to Austrian amphibian populations even in the absence of adult mortality, because juvenile survival can play an important role in population persistence (Garner et al., 2009; Vonesh & De la Cruz, 2002).

The detection of *Bd* in Austria was not surprising, given its occurrence in many of Austria's neighbouring countries (Duffus & Cunningham, 2010; Garner et al., 2005). It was worrying, however, to find infected amphibians at high altitudes, where *Bd* caused fatal disease outbreaks in Spain (Walker et al., 2010).

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