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Absence of infection with the amphibian chytrid fungus in the terrestrial Alpine salamander, *Salamandra atra*

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Amphibian declines and species extinctions are worrying conservationists around the globe, and the emerging infectious disease chytridiomycosis is suggested to play a key role in these processes (Fisher et al. 2009). The disease's etiological agent, the chytridiomycete fungus *Batrachochytrium dendrobatidis* (*Bd*), has been reported to be present on all continents inhabited by amphibians (Fisher et al. 2009). Amphibian mass mortalities, however, seem to be geographically restricted, and it has recently been suggested that one particular currently emerging, globalised and highly virulent strain of *Bd* is responsible for the most dramatic consequences of the disease (Farrer et al. 2011). Besides the strain, the specific susceptibility of host species or populations as well as host-environment interactions might play a role in the outcome of an infection (e.g., Woodhams et al. 2007a, Tobler & Schmidt 2010, Savage & Zamudio 2011, Searle et al. 2011).

Bd is known to infect more than 400 amphibian species of both anurans and salamanders, and the most dramatic mass mortalities have occurred in mountainous areas of the Americas, Australia, and southern Europe (Berger et al. 1998, Bosch & Martinez-Solano 2006). The pathogen spreads through motile infectious zoospores released from zoosporangia growing on keratinised parts of the amphibian skin. Despite this aquatic transmission stage, *Bd* is known also to infect purely terrestrial amphibian species (Weinstein 2009). If an individual is infected and develops symptoms of chytridiomycosis, it may eventually die from a breakdown of neurological functions (Voyles et al. 2009).

To better understand possible consequences of *Bd* spread, it is important to know which species are susceptible to *Bd* infection and chytridiomycosis. Bielby et al. (2008) provided evidence that, at least in anuran amphibians, *Bd* susceptibility is related to life history. They found that species from high altitudes within a geographically 'restricted' range and having an aquatic life stage accompanied by low fecundity suffer from higher risk of *Bd*-related decline. Woodhams et al. (2007a, b) provided evidence that susceptibility can also depend on species-specific skin peptides or skin bacteria. *Bd* susceptibility may furthermore be related to the environment in which amphibians live. Based on the pathogen's temperature sensitivity, Rödder et al. (2009) identified worldwide regions in which climatic conditions are most suitable to *Bd* and concluded that at lower latitudes higher elevations and at higher latitudes lower elevations would provide the best environment for the survival of *Bd*.

Bd infection is widespread among European amphibians including those occurring in the Alps (Garner et al. 2005, Sztatecsny & Glaser 2011). While it has caused mortality and population extinctions in some mountain ranges (Bosch & Martinez-Solano 2006, Bielby et al. 2009, Walker et al. 2010), *Bd* apparently leaves many Eu-

Figure 1. Alpine salamander from the Hinterstein Valley, Bavarian Alps, Germany (not collected). Photo: U. Schulte

ropean species and populations unaffected. Here, we report the results of a study on *Bd* infection in the viviparous and entirely terrestrial Alpine salamander, *Salamandra atra* Laurenti, 1768 (Fig. 1). This caudate is endemic to the Alps and the Dinaric Alps (Griffiths 1996).

We suggest that there is reason for concern that this species may be at risk of *Bd* infection because (i) it has a low fecundity (Bielby et al. 2008, by implication), (ii) it occurs under climatic conditions where outbreaks of chytridiomycosis may occur (see Walker et al. 2010), (iii) it inhabits mountain ranges climatically suitable to *Bd* (Rödder et al. 2009) and where this fungus occurs (Sztatecsny & Glaser 2011), and (iv) *Bd*-associated mass mortality has been observed in the congeneric *Salamandra salamandra* (Bosch & Martinez-Solano 2006).

We tested for *Bd* infection 310 Alpine salamanders living at different altitudes in nine separated populations well spaced over the species' geographic range (Table 1, Fig. 2). For sampling, we used sterile cotton swabs (Copan Italia S.p.A., Brescia, Italy; Medical Wire & Equipment, Wiltshire, England) to swab ventral surfaces of body, hands and feet of salamanders. To avoid that the same individuals would be tested twice, one site within a population was only sampled once and specimens were released only after all specimens had been swabbed. Afterwards, swabs were frozen as quickly as possible upon return from the field trip (Hyatt et al. 2007). For *Bd* screening, we used quantitative real-time PCR (polymerase chain reaction) of the ITS-1/5.8S ribosomal DNA region of *Bd* (Boyle et al. 2004) with internal positive control (Hyatt et al. 2007). *Bd* data has been made available to the global *Bd* mapping project at http://www.bd-maps.net/maps/.

Bd was detected in none of our samples (Table 1), indicating that none of the *S. atra* specimens sampled were infected. To obtain a Bayesian 95% credible interval for prevalence, we used WinBUGS to estimate the posterior distribution of prevalence (Kéry 2010, see Appendix). Posterior distributions were left-skewed towards zero and all 95% credible intervals included a prevalence of zero.

Table 1. Details of Alpine salamander and accompanying amphibian species sampling (see Fig. 2). Altitude in metres above sea level.

Country	State, locality, altitudinal range	Approximate coordinates	Number of individuals	Observed prevalence (Bayesian 95% Credible Interval)	Date	Additional species sampled (n)
Austria	Salzburg, Hagengebirge (Schlumsee), 490–1,200 m	13.1 E, 47.5 N	35	0% (0.00, 0.10)	7 July 2009	
Austria	Salzburg, Krimmler Achental (NP Hohe Tauern), 1,622 m	12.19 E, 47.14 N	20	0% (0.00, 0.16)	14 June 2009	
Austria	Steiermark, Wörschach (Totes Gebirge), 1,715 m	14.13 E, 47.60 N	8	0% (0.00, 0.35)	11-12 June 2009	
Austria	Tirol, Imst (Lechtaler Alpen), 1,700–1,800 m	10.6 E, 47.26 N	10	0% (0.00, 0.28)	30 July 2009	
Austria	Vorarlberg: Schoppenau (Bregenzer Wald), 915–1,000 m	10.03 E, 47.31 N	8	0% (0.00, 0.35)	31 July 2009	
Germany	Bayern, Hintersteiner Tal (Allgäu), 850–1,825 m	10.4 E, 47.4 N	120	0% (0.00, 0.03)	9-12 July 2009	*Bufo bufo* (13), *Ichthyosaura alpestris* (59)
Switzerland	Nidwalden, near Wolfenschiessen, 550–1,705 m	8.38 E, 46.9 N	53	0% (0.00, 0.07)	24-25 July, 10 August 2009	*Bufo bufo* (2), *Ichthyosaura alpestris* (3), *Salamandra salamandra* (2)
Switzerland	St. Gallen, Murgtal, 1,160–1,604 m	9.11 E, 47.03 N	41	0% (0.00, 0.09)	2 August 2009	*Bufo bufo* (1), *Ichthyosaura alpestris* (4)
Switzerland	Glarus, near Braunwald, 1,500 m	8.98 E, 46.93 N	15	0% (0.00, 0.20)	8 August 2008	

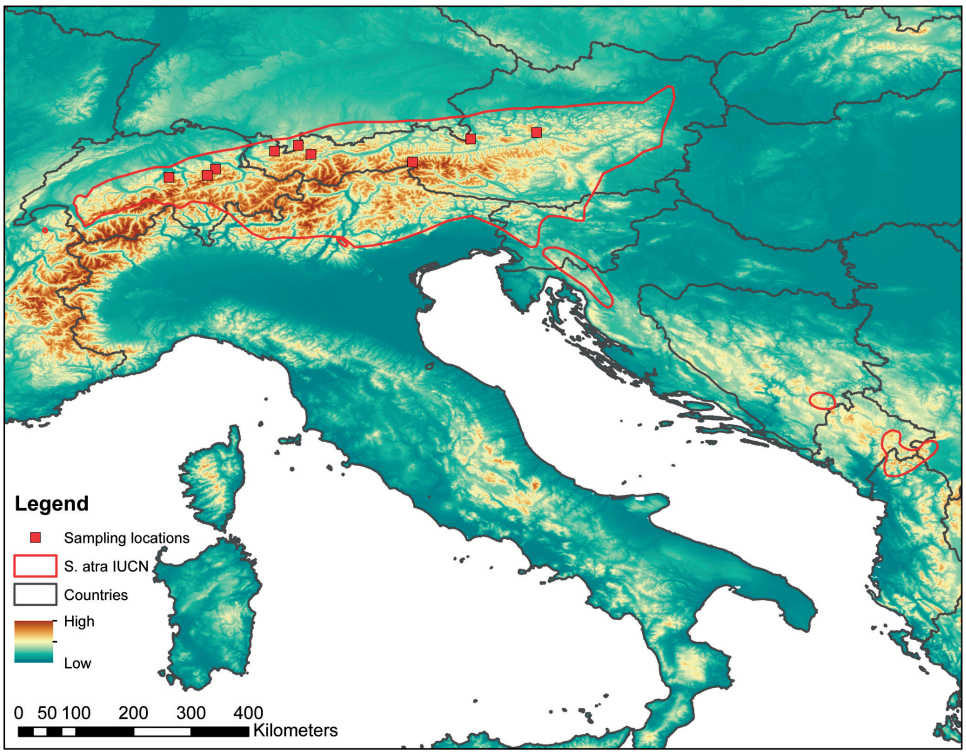

Figure 2. Distribution of the Alpine salamander (solid red line, taken from www.iucn.org) and populations sampled (red squares, Table 1).

How can we explain the apparent absence of *Bd* infection in the Alpine salamander? We here discuss four possible explanations.

(1) The simplest explanation would be that we failed to detect *Bd* when it was in fact present. Given our sample sizes, we may have missed *Bd* in some localities (DiGiacomo & Koepsell 1986, Marti & Koella 1993). The range of possible prevalences is given by the 95% credible intervals (Table 1). However, because all 95% credible intervals included zero and because total sample size was 310 (Table 1), our results suggest an absence or at least a very low prevalence of *Bd* in the populations studied (DiGiacomo & Koepsell 1986, Marti & Koella 1993). Peyer (2010) tested 52 museum specimens of *S. atra* for *Bd* and none tested positive (one specimen collected in 1972 gave an equivocal result, but this also occurred in other species that were tested by Peyer [2010]). Although it is clear that *Bd* may occur in very low prevalence in nature, our data support that *Bd* was most likely truly absent rather than not detected.

(2) One might also argue that *Bd* was simply not present in the general area of our tested salamander populations. This, however, seems unlikely, as *Bd* is known to occur at high elevations and in cold climates (Seimon et al. 2007, Knapp et al. 2001, Muths et al. 2008) including the Swiss and Austrian Alps (Peyer 2010, Sztatecsny & Glaser 2011). We note, however, that at some localities we tested syntopic amphibian species (Table 1) for *Bd* and they all tested negative either.

(3) Another explanation could be that the risk of *Bd* infection is minimized in this species as a result of its strictly terrestrial life cycle. Under this assumption, the Alpine salamander might be susceptible to *Bd,* but in practice does not, or rarely becomes, infected and/or has a low intraspecific transmission rate. Several studies of life history traits and *Bd* susceptibility suggest that it is more likely to affect species linked to permanent water bodies (Bielby et al. 2008, Bancroft et al. 2011). However, experimental infection trials conducted on strictly terrestrial salamanders clearly demonstrated susceptibility to both *Bd* infection and clinical chytridiomycosis (Chinnadurai et al. 2009, Vasquez et al. 2009, Weinstein 2009). Moreover, a wealth of studies have provided field records of *Bd*-infected terrestrial salamanders and anurans both in temperate and tropical zones (Bell et al. 2004, Cummer et al. 2005, Kolby et al. 2009, Weinstein 2009, Becker & Harris 2010, Longo & Burrowes 2010). Thus, a strictly terrestrial life history does not *per se* exclude or reduce the likelihood of infection by *Bd*.

(4) We favour an alternative explanation. We suggest that it is plausible that *S. atra* is resistant to *Bd* because of innate immunity caused by skin peptides or skin microbiota in the manner observed in a number of other amphibians (Woodhams et al. 2007a, b). This hypothesis

should be tested through experimental infection trials on *S. atra* involving infection of salamanders under natural environmental conditions with and without suppressed immune function. In principle, a species that is immune because of skin peptides or microbiota should become susceptible by a combination of disinfection using antimicrobials and mechanical or chemical depletion of skin peptide reservoirs. Additionally, it could be studied *in vitro* whether the salamander's skin peptides and bacteria inhibit the growth of *Bd*. If such anti-*Bd* properties were found, they might be used as part of a strategy to mitigate the effects of *Bd* on wild amphibians (Woodhams et al. 2011).

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Appendix

WinBUGS code to compute Bayesian 95% credible intervals for prevalence

We assume that the reader will access WinBUGS from the statistical software R (see Kéry 2010 for an introduction to both R and WinBUGS). In R, if there are, say, 20 individuals that tested negative for *Bd*, data could be entered using the command

```
data <- c(rep(0,20))
```

The code for the WinBUGS model is

```
prevalence ~ dunif(0,1) # uniform, non-informative prior
for (i in 1:n.ind) {# n.ind is the number of individuals in the data set
data[i] ~ dbern(prevalence)}
```

We ran three parallel Markov chains with 2,000 iterations each and discarded the first 1,000 iterations as burn-in; we did not thin the chains.