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Chytrid fungus infection related to unusual mortalities of *Salamandra salamandra* and *Bufo bufo* in the Peñalara Natural Park, Spain

Jaime Bosch and Iñigo Martínez-Solano

Abstract Chytridiomycosis is a fatal disease associated with amphibian population declines and extinctions worldwide. In a protected area in central Spain, the Peñalara Natural Park, the disease almost extirpated the population of *Alytes obstetricans* over only a few years, but did not apparently affect other amphibians. We present new observations documenting the occurrence of the disease in other species. In 2001–2003 we collected over 400 larvae or recently metamorphosed individuals of *Salamandra salamandra* and also several dead individuals of *Bufo bufo*. The analysis of the skin of post-metamorphic specimens revealed the presence of chytrid sporangia and discharge tubes in both species. According to measures of larval abundances in 1999 and 2003 the population of *S. salamandra* has suffered a marked decline but no significant trend was observed for *B. bufo*. We discuss the possible role of chytridiomycosis in the decline of *S. salamandra* and comment on the differential susceptibility exhibited by various species in the amphibian community at Peñalara.

Keywords Amphibian declines, *Bufo bufo*, chytridiomycosis, Peñalara Natural Park, *Salamandra salamandra*, Spain.

Introduction

Chytridiomycosis is an emerging disease responsible for amphibian declines worldwide (Berger *et al*., 1998; Daszak *et al*., 1999, 2000, 2003). The etiological agent of cutaneous chytridiomycosis in amphibians is the recently discovered and described fungus *Batrachochytrium dendrobatidis* (Order Chytridiales, Longcore *et al*., 1999). Amphibian populations from North, Central and South America, Australia, New Zealand and Europe have suffered sudden and unusually high mortality rates, resulting in some species becoming extinct and many others undergoing sharp population reductions (Richards *et al*., 1993; Laurance *et al*., 1996; Lips, 1999; Bosch *et al*., 2001; Fellers *et al*., 2001; Muths *et al*., 2003).

Two different hypotheses have been proposed to explain the recent emergence of chytridiomycosis in amphibians: the fungus could have been recently introduced into native populations (Berger *et al*., 1999a; Daszak *et al*., 1999, 2003), or environmental changes may have altered a pre-existing host-parasite relationship (Daszak *et al*., 2000; Blaustein & Kiesecker, 2002). Genetic evidence supports the first hypothesis (Morehouse *et al*., 2003). Furthermore, *B. dendrobatidis* displays characteristics typical of a virulent pathogen introduced into a native population, such as low host specificity and high host mortality (Daszak *et al*., 1999).

Despite its broad host range in amphibians, the effects of *B. dendrobatidis* on amphibian populations range from sporadic deaths to sharp declines and complete extinctions (Speare & Berger, 2005), and species-specific differences in life history traits have been invoked to explain these differences in susceptibility (Williams & Hero, 1998).

Although *B. dendrobatidis* apparently lacks resistant stages (Longcore *et al*., 1999), infected larvae can be healthy carriers (Berger *et al*., 1998), providing a mechanism for chytrids to be present even when adult amphibians have disappeared. In addition, *B. dendrobatidis* is able to survive in water bodies without the presence of hosts (Johnson & Speare, 2003), and this capability may also be enhanced in the cooler montane regions (Daszak *et al*., 1999).

The decline of the common midwife toad *Alytes obstetricans* in a montane protected area in central Spain is a well-documented amphibian decline related to chytridiomycosis (Bosch *et al*., 2001). Thousands of dead midwife toads were observed from 1997 to 1999, although other species remained apparently unaffected (Bosch *et al*., 2001; Martínez-Solano *et al*., 2003). Populations of *Salamandra salamandra* and *Bufo calamita* declined at Peñalara during 1980–1999, although we failed to relate these declines to chytridiomycosis (Martínez-Solano *et al*., 2003).

Jaime Bosch (Corresponding author) and **Iñigo Martínez-Solano***
Museo Nacional de Ciencias Naturales, CSIC, José Gutiérrez Abascal 2, 28006 Madrid, Spain. E-mail bosch@mncn.csic.es

*Current address: Museum of Vertebrate Zoology, University of California, Berkeley, 3188 Valley Life Sciences Building, 94720 Berkeley, California, USA.

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The effect of chytridiomycosis on urodeles is poorly understood, and only two cases of infection in the wild have been documented (Davidson *et al*., 2000, 2003; Cleary, 2004). After 1999, when massive die-offs of midwife toads had ceased in Peñalara, we observed several dead larvae, adults and post-metamorphic *S. salamandra* as well as some dead post-metamorphic *B. bufo*. In this paper we present new evidence that suggests that chytridiomycosis is also related to the observed mortalities of *S. salamandra* and *B. bufo*, and also that the former, at least, has experienced a recent and significant decline in the area.

Study area and species

The Peñalara Natural Park is a protected area (768 ha) at 1,800–2,430 m altitude in the Sierra del Guadarrama, central Spain. The area consists mainly of granite outcrops with scattered bogs and alpine grasslands. The amphibian community at Peñalara comprises 10 species (Bosch & Martínez-Solano, 2003). As a result of the harsh climatic conditions at these altitudes amphibian activity is restricted to spring and summer. *S. salamandra* exhibits great developmental plasticity, breeding in a range of water bodies from streams to temporary ponds (Bosch & Martínez-Solano, 2003). Similarly to *A. obstetricans*, *S. salamandra* is mainly terrestrial in habit. Courtship takes place on land, and just one sex (females in *S. salamandra*, males in *A. obstetricans*) releases the larvae into ponds. Both species have overwintering larvae, although those of *S. salamandra* are also able to complete their development within a few months in ephemeral ponds. In contrast, *B. bufo* breeds in the early spring. Adults are also predominantly terrestrial, but mating usually occurs in the largest, permanent ponds (Bosch & Martínez-Solano, 2003) and larvae complete their metamorphosis during the summer.

Methods

Surveys for dead animals and diagnosis of chytridiomycosis

During June-September of 2000–2003 we extensively sampled (daily or at least weekly) the study area in search of dead animals. Dead specimens and living animals in terminal stages were counted and collected, and preserved in 70% ethanol for subsequent scanning electron microscopy (SEM) and histological analyses (moribund animals were euthanized by an overdose of the anaesthetic MS222 -tricaine methyl sulfonate). Following Berger *et al*. (1998) we used SEM techniques to search for evidence of fungal infection in the skin of preserved individuals. To confirm the diagnosis we also performed histological analyses on additional samples following Berger *et al*. (1999b).

Salamander larvae surveys

In 1999 we carried out comprehensive sampling of breeding sites to quantify the abundances of all amphibian species at Peñalara (Martínez-Solano *et al*., 2003). To test if abundance of larval *S. salamandra* had decreased over 1999–2003 we resampled (with the same methodology used in 1999) every group of ponds where dead animals were observed in 2002 (128 ponds in total). These ponds were surveyed at least six times each from June to August 2003, and the number of larvae recorded. We assume abundances represent an estimation of the absolute number of larvae per pond after the peak of the reproductive period was reached. Finally, the observed frequencies of maximum larval abundance in 1999 were compared with the corresponding frequencies observed in 2003 using a χ^2 analysis.

To prevent spreading the disease, disposable plastic gloves were used to handle animals. Other field equipment used during the surveys (including hiking boots) was exclusively used in the study area, and was periodically submerged in bleach for a few minutes and then dried out in the sun for several hours.

Results

Surveys for dead animals

During 2001–2003 a total of 386 dead *S. salamandra* were found, with a slight increase in each year overall (20% of the dead individuals were found in 2001, 38% in 2002, and 42% in 2003; Fig. 1). More than half were larvae (52%), 24% were juveniles and 24% were adults. No dispersal patterns between years could be derived from the spatial location of the carcasses (Fig. 1). Mortalities occurred over the whole area, although 29% of the observations were in the Laguna de los Pájaros. This permanent pond is one of the largest in the Park (4,866 m^2), and in the past supported the highest densities of larval *A. obstetricans*. Overwintering larvae of both *A. obstetricans* and *S. salamandra* occur in this pond.

Adult and post-metamorphic dead animals were mostly found around the ponds and occasionally in the water. Most of the bodies presented abnormal postures when found, usually with a kinked body. *B. bufo* were only found dead at Laguna de los Pájaros, where every year <50 post-metamorphic toads were found dead or ill. These individuals shared refuges with hundreds of healthy animals. Although the most important concentrations of larvae of *B. bufo* occur in the Laguna Grande de Peñalara (Fig. 1), no carcasses were found there.

Examination of the epidermis of seven dead and ill-looking *S. salamandra* and two *B. bufo* with SEM revealed evidence of chytridiomycosis in all cases. Small crater-like structures produced by fungal discharge tubes poking through the epidermis were observed in the

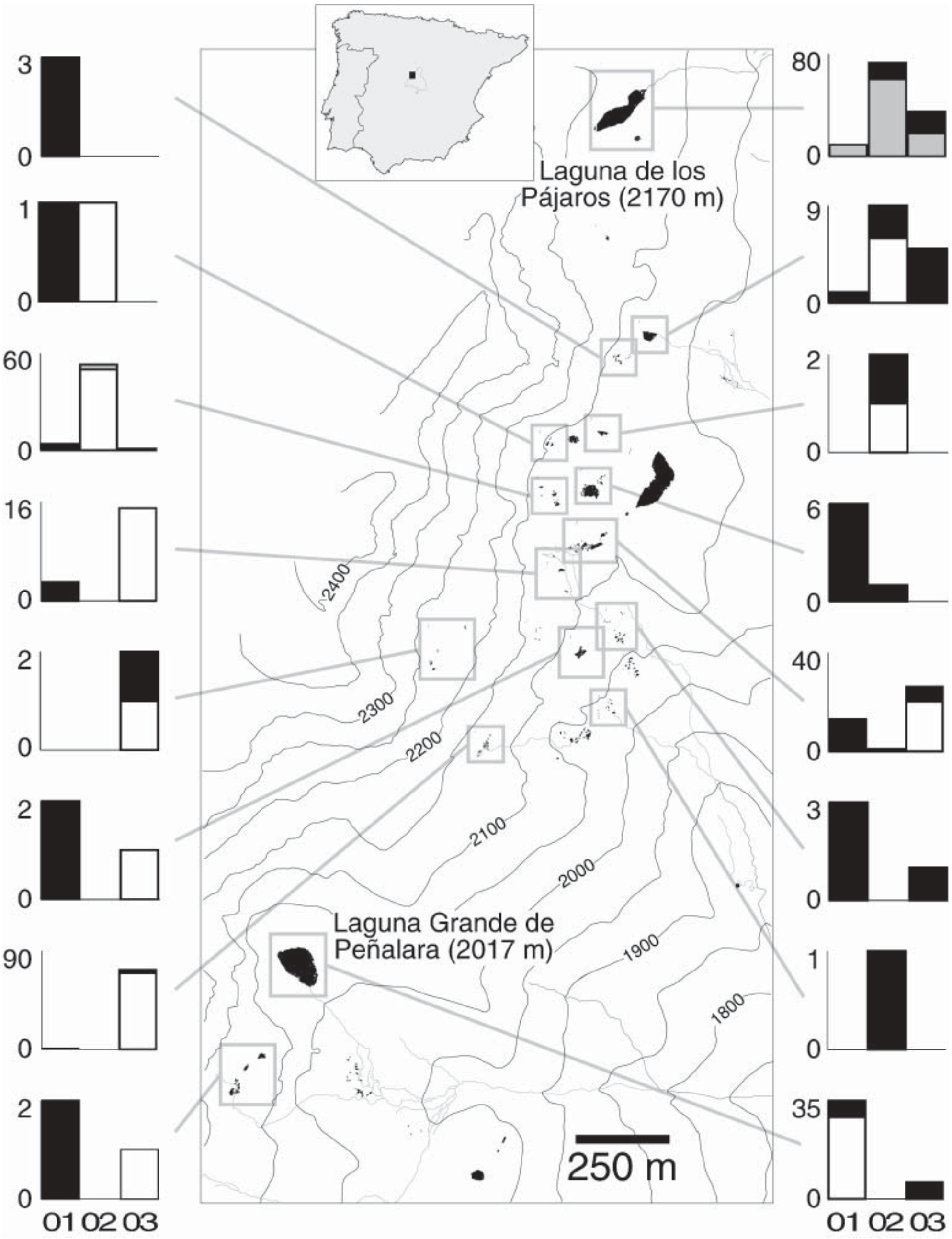

Fig. 1 Peñalara Natural Park (the inset indicates its position in central Spain) showing the location of groups of ponds were dead *S. salamandra* were found. Bars represent the number of carcasses found in 2001, 2002 and 2003 respectively. White bars represent larvae, grey bars juveniles and black bars adult salamanders. Note the differences in the vertical scale across sites.

skin (Plate 1). In addition, histological examination of the epidermis confirmed the diagnosis of chytridiomycosis in three *S. salamandra* and three *B. bufo*. The preparations showed large concentrations of empty sporangia, with some of them showing septae and zoospores (Plate 2). The sporangia presented the size, shape and location characteristic of those of *B. dendrobatidis*. L. Berger (James Cook University, Australia) and A. Cunningham (Institute of Zoology, Zoological Society of London, UK) confirmed our identifications. The presence of chytrids in two carcasses of *S. salamandra* was further confirmed by molecular methods. Genomic DNA was extracted from skin samples and the chytrids' DNA was successfully amplified using a *B. dendrobatidis* – specific nested polymerase chain reaction (M.C. Fisher & J. Bosch, unpubl. data).

Salamander larvae surveys

Maximum abundances of larvae of *S. salamandra* in the 128 ponds resurveyed in 2003 were significantly lower than abundances recorded in 1999 ($\chi^2 = 35.65$, df = 3, $P < 0.0001$). In 1999 only 30% of ponds were unoccupied and 25% of ponds contained more than 50 larvae,

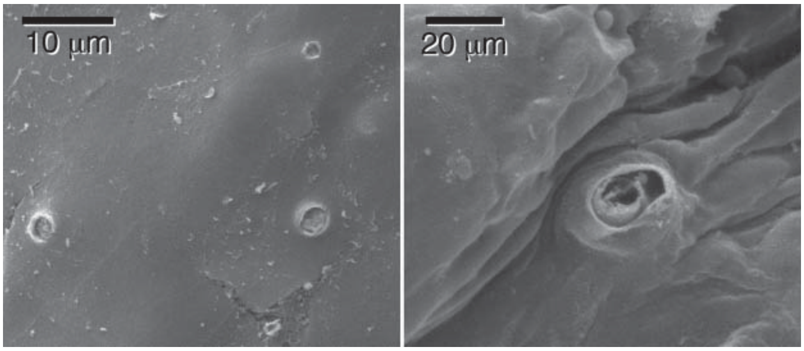

Plate 1 Scanning electron micrograph of the surface of the epidermis of a juvenile *S. salamandra* (left) and a post-metamorphic *B. bufo* (right) from Peñalara Natural Park infected by chytrid fungi, showing discharge tubes of zoosporangia of *B. dendrobatidis*.

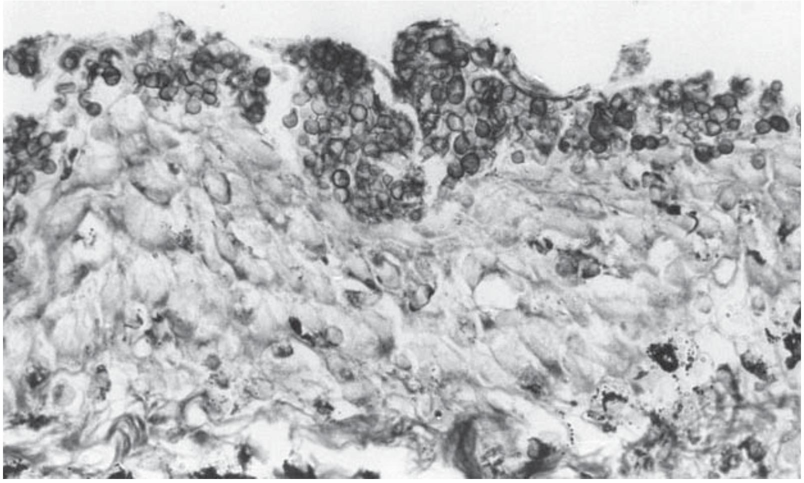

Plate 2 Histological cross-section of the skin of a post-metamorphic *B. bufo* from Peñalara Natural Park with chytridiomycosis. The empty zoosporangia are extremely abundant and visible as dark circles in the superficial keratinized epidermis.

whereas in 2003 42% of ponds were unoccupied and only 9% of ponds contained more than 50 larvae.

B. bufo, however, has apparently healthy population foci at several locations within the Park, with no apparent changes in abundance between years. We also found a new breeding site that had not been used for reproduction in previous years (Laguna de la Mariposa, close to Laguna de los Pájaros).

Discussion

The mortalities of common toads and salamanders reported here cannot be considered a common phenomenon. Before 1997, prior to the occurrence of massive mortalities of midwife toads in the area, no carcasses of *B. bufo* or *S. salamandra* were found, even though the Park was visited almost every day during the breeding seasons of 1993–1996 by JB. Only occasionally were adult salamanders, usually females, found dead in some ponds, probably drowned when releasing their larvae (*S. salamandra* is a poor swimmer). Dead animals were usually found at the beginning of the breeding season (May–June) and not at the end of the summer, the period when most carcasses were found in 2001–2003.

The presence of *B. dendrobatidis* in the samples collected, confirmed by three diagnostic methods, and the heavy infections found in most specimens, suggest that chytridiomycosis is related to mortalities, although other possibilities should not be discarded. In other cases of mass mortalities of salamanders, both chytrids and iridoviruses were present (E.W. Davidson, pers. comm.), a possibility we have not yet confirmed. Other possible factors (e.g. habitat loss or introduced predators) do not seem to have played a significant role in the observed decline. Clinical signs in the individuals in terminal phases were consistent with chytridiomycosis. They were in good body condition, and some dead salamanders were pregnant females with larvae in advanced stages of development, suggesting that they were probably not immunologically depressed. The massive die offs of *S. salamandra* have significantly reduced this population, as indicated by the marked decrease in the abundance of larvae, and the number of dead specimens collected probably represents only a small proportion of the actual number of dead animals.

If the role of chytrids in the decline of *S. salamandra* is confirmed, it will constitute the first report of a population decline of a urodele species caused by chytridiomycosis. Several factors may explain the low incidence of chytridiomycosis in urodeles, including the lack of keratinized tooth rows or beaks in their larvae. However, some larval urodeles, and specifically *Salamandra*, have keratinized skin on their feet (Whitear, 1977), and consequently are susceptible to chytrid infection. Also, the global distribution pattern of chytridiomycosis could explain the lack of data for urodeles because it mainly includes areas where urodeles are historically absent (Oceania, South America), or inhabited by diversified families characterized by direct development in terrestrial habitats (Central and North America). Not surprisingly, the only reports of chytrid infection in wild urodeles involve representatives of families characterized by aquatic habits, such as Ambystomatidae (Davidson *et al*., 2003) and Dicamptodontidae (Cleary, 2004).

Our results contrast with those of Davidson *et al*. (2003) in the observed symptoms of affected post-metamorphic animals and the lethality of the infection. Infected tiger salamanders *Ambystoma tigrinum* presented small black spots on abdomen and head, but infected *S. salamandra* did not have these lesions. Whereas chytridiomycosis appears to have fatal consequences for *S. salamandra* in Peñalara, experimental infection of five *A. tigrinum* did not cause mortality (Davidson *et al*., 2003). We have not, however, carried out laboratory studies involving experimental infections to confirm that the *Batrachochytrium* strain at Peñalara can cause salamander mortality, a key point in demonstrating a causal relationship between chytrids and declines.

Of the 10 amphibian species breeding at Peñalara we have compiled evidence, including the work reported here, of chytrid fungus infection in four (*S. salamandra, A. obstetricans, B. bufo* and *B. calamita*; Bosch *et al*., 2001; Martínez-Solano *et al*., 2003). The species more severely affected, *A. obstetricans* and *S. salamandra*, are similar in certain aspects of their biology. Both are predominantly terrestrial, have low fecundities (two orders of magnitude lower than those of *B. bufo*) and have overwintering larvae. This latter characteristic could explain the differences in susceptibility, because an extended larval period would increase the probability of contact with the waterborne zoospores of *B. dendrobatidis*. In addition, other factors, such as low temperatures, which are favourable for the development of *B. dendrobatidis*, may also play a role. Most first year larvae of *A. obstetricans* and *S. salamandra* will remain in the water after the summer in permanent ponds, thus undergoing low temperatures during the winter. *B. dendrobatidis* develops most rapidly at temperatures as low as 23°C in culture (Longcore *et al*., 1999), and Woodhams *et al*. (2003) found that an environmental temperature of 37°C for less than 16 hours can clear chytrids from *Litoria chloris*. Lower temperatures may decrease the rate of synthesis and secretion of antimicrobial peptides by the skin of amphibians (Rollins-Smith *et al*., 2002). In contrast, *B. calamita* usually breeds in ephemeral ponds (Bosch & Martínez-Solano, 2003), which are shallow and sun-exposed and therefore dry up in the early summer killing the chytrids (the sporangia and zoospores are not resistant to desiccation; Johnson *et al*., 2003). *B. bufo*, however, breeds in permanent and relatively deep ponds (Bosch & Martínez-Solano, 2003) and larval development takes place over a few months. These permanent ponds are also inhabited by *A. obstetricans* and *S. salamandra* larvae, and therefore *B. bufo* larvae must presumably be in contact with infected larvae during their whole development period.

The populations of both *S. salamandra* and *A. obstetricans* are genetically differentiated at Peñalara (Martínez-Solano *et al*., 2003). Molecular evidence indicates that the mountains of the Iberian Sistema Central were colonized by several species of amphibians as recently as the last interglacial period, and these amphibians therefore have low levels of genetic variability (Martínez-Solano, unpubl. data). According to mitochondrial DNA data, haplotype diversity is low among populations of *S. salamandra almanzoris*, a well-differentiated lineage that occupies Peñalara (Martínez-Solano *et al*., 2005). Populations that are genetically impoverished may be more susceptible to infection because of reduced genetic variability in genes involved in the immune response to the chytrid. Thus, both ecological and historical demographic factors may interact to shape patterns of differential host susceptibility to chytrid fungus infection.

Populations of both *S. salamandra* and *B. bufo* have undergone marked declines in the Iberian Peninsula over the last few decades. This process, primarily related to habitat loss, has occurred at lower elevations, largely confining the surviving populations to montane areas. If chytridriomycosis becomes a serious problem for amphibians in such montane refuges, the survival of these species will be seriously jeopardized.

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Biographical sketches

Jaime Bosch has a particular interest in sexual selection in anurans but in the last few years his research has focused on the problem of emerging diseases in amphibians. He is examining this in montane areas in Europe and tropical areas of South America, and is currently carrying out intensive surveys to understand the impact of chytridriomycosis on amphibian populations.

Iñigo Martínez-Solano is interested in the evolutionary biology and conservation of amphibians, with a special interest in their phylogeography and conservation genetics.