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Detection of Chytridiomycosis Caused by *Batrachochytrium dendrobatidis* in the Endangered Sardinian Newt (*Euproctus platycephalus*) in Southern Sardinia, Italy

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ABSTRACT: The chytridiomycete fungus *Batrachochytrium dendrobatidis* is known to be focally distributed across Europe, but has only been linked to chytridiomycosis at a few locations in Spain. Here we report the second occurrence of chytridiomycosis in European amphibians. We found a population of endangered Sardinian newts (*Euproctus platycephalus*) exhibiting clinical signs of disease including loss of digits and patchy, discolored skin. Molecular examination of skin samples tested positive for *B. dendrobatidis*. The population of *E. platycephalus* has been in decline on a timescale consistent with the global emergence of chytridiomycosis, and the ecology of this salamander suggests that the disease in this species warrants concern.

Key words: *Batrachochytrium dendrobatidis*, chytridiomycosis, *Euproctus platycephalus*, Sardinia.

The chytridiomycete fungus *Batrachochytrium dendrobatidis* has been identified as a major cause of global amphibian declines (Berger et al., 1998; Stuart et al., 2004). Although recently emerged (Berger et al., 1998; Morehouse et al., 2003), it has been implicated in mass mortality events, and even in species extinctions, on various continents (Stuart et al, 2004; La Marca et al., 2005). In Europe, the most well-studied system is the series of mass die-offs of common midwife (*Alytes obstetricans*), fire salamander (*Salamandra salamandra*), and common toad (*Bufo bufo*) in Spain (Bosch et al., 2001, 2005); however, the fungus is known to be broadly, but focally, distributed across this continent (Garner et al., 2005; 2006). In Italy, *B. dendrobatidis*-related declines or mortality events have not been reported, but infections have been described in introduced North American bullfrogs (*Rana catesbeiana*; Garner et al. 2006), native Apennine yellow-bellied toads (*Bombina pachypus*; Stagni et al., 2002), pool frogs (*Rana lessonae*; Simoncelli et al., 2006) and the Italian agile, or Lataste's, frog (*Rana latastei*; Garner et al., 2004). Until now, the presence of *B. dendrobatidis*-infected amphibians on Sardinia has not been reported.

The Sardinian newt (*Euproctus platycephalus*) is endemic to the island of Sardinia in Italy and is found primarily on the east side of the island in the Limbara, Gennargentu, and Sette Fratelli Mountains (Lecis and Norris, 2003; Bovero et al., 2005). It is considered, by some, to be the rarest and most threatened salamander in Europe (Andreone and Luiselli, 2000). It is listed as endangered by the IUCN (IUCN, 2006) and has special conservation status at both the regional and national levels in Italy (Lecis and Norris, 2003). *Euproctus platycephalus* prefers cooler waters and is most often found in streams, small lakes, pools (Puddu et al., 1988), and even artificial canals (Bovero et al., 2005). Declines have been reported since the early 1980s (Puddu et al., 1988; Van Rooy and Stumpel, 1995), and possible causes include the reduction and fragmentation of habitats arising from water redirection for agricultural purposes (Van Rooy and Stumpel, 1995), agricultural water pollution, illegal fishing methods (Schenk et al., 1995), and the introduction of allochtonous fishes (Beebee, 1996). Disease has not been considered a cause of the observed population declines. In this paper, we report the occurrence of disease

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in adult Sardinian newts and present data relating to two important pathogens, *B. dendrobatidis* and ranavirus, both associated with mass die-offs of amphibians.

In a survey of the Sette Fratelli Mountains (Southern Sardinia, Italy) from 1–3 August 2005, we observed a population of *E. platycephalus* at 525 m above sea level (a.s.l.) in a pool along the Maidopis stream. We observed several individuals with extensive damage to the tips of the digits on both hindlimb and forelimb feet. In some cases, tissue was so degraded that bone was clearly exposed, and tissue loss was associated with the concurrent loss of either the terminal phalanx or the entire toe. Although dead newts were not found at this location at that time, both skin and muscle samples were collected from the digit tips from five of these newts prior to release. Tissue samples were sent to the Institute of Zoology of London for *B. dendrobatidis* and ranavirus testing. DNA was extracted from the skin and muscle of diseased animals using a bead beating protocol, and samples were tested by PCR for *B. dendrobatidis* (Boyle et al., 2004). All samples were tested in replicate, with four concentrations of standard *B. dendrobatidis* and negative controls. Positive PCR results were detected from three of five skin samples. Genomic equivalents (GE), or the quantitative estimate of the number of *B. dendrobatidis* genomes present in the three PCRs, were 0.79, 8.44 and 17.94 GE.

We further tested extractions for evidence of ranavirus DNA by using a standard PCR targeting the major capsid protein locus of the ranavirus genome, following the protocol of Pearman et al. (2004). Amplification was again performed in replicate, using both positive and negative controls. While positive controls were consistently amplified, none of the samples tested positive for ranavirus.

Although ulcerative syndrome reported by Cunningham et al. (1996) is caused by ranavirus infection, and is often associated with necrosis of one or more distal digits, all samples taken from the diseased Sardinian newts tested negative for this pathogen. In contrast, the majority of samples tested positive for *B. dendrobatidis*. Due to the lack of keratinized mouthparts in caudates, Fellers et al. (2001) recommended toe tips of larval salamanders as targets for detecting *B. dendrobatidis* infection. Typical signs of amphibians with chytridiomycosis include lethargy, anorexia, lack of a righting reflex, extensive skin sloughing, and patches of discolored skin (Pessier et al., 1999). Signs in salamanders may also include black spots on both the dorsal and ventral surfaces of the head, as reported for *Ambystoma tigrinum* (Davidson et al., 2003). We observed extensive skin sloughing and patches of discoloured skin on *E. platycephalus* specimens collected along Maidopis stream, and we previously observed black spots on the dorsal surface of newts from another location in Central Sardinia; however, attempts to detect *B. dendrobatidis* from individuals with these lesions have not been made. Such gross lesions are not commonly associated with chytridiomycosis (Berger et al.,1998), although lesions consisting of acanthotic and hyperkeratotic foci have been reported by Pessier et al. (1999) and Lamarinde and Nichols (2002). When present, such lesions are small but may be located on the feet as well as on the ventral surface of the hind limbs and the drink patch (Lamarinde and Nichols, 2002). However, we believe this is the first report of digit loss associated with chytridiomycosis.

The presence of *B. dendrobatidis* infection may be of major significance for the conservation of *E. platycephalus*. Amphibian species, in decline or extinct due to chytridiomycosis, are often associated with higher elevations. Populations of *E. platycephalus* can occur at elevations up to 1800 m a.s.l. (Lecis and Norris, 2003), but the majority of known populations occur between 500–900 m a.s.l. The detection of *B. dendrobatidis* in *E. platycephalus* collected as low as 525 m a.s.l., suggests that the species may be affected over much of its current range. Research on the

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physiology of *B. dendrobatidis* also has shown that optimal growth occurs at 17 C–25 C; this optimum range overlaps with the water temperatures of habitats utilized by *E. platycephalus* (12.4 C–24.5 C). Recent population losses of this species have been recorded; over half of the sites previously known to be occupied by *E. platycephalus*, and surveyed by Lecis and Norris (2003) in 2001, lacked any evidence of current occupation.

Population declines and extirpations related to chytridiomycosis have occurred repeatedly in amphibian species globally. Many more of these events are probably unrecognized or are not confirmed by diagnostic testing. In Northern Sardinia, for example, unusual mass die-offs of Sardinian painted frogs (*Discoglossus sardus*) were recorded at three different locations ("Zirichiltaggi" S.W.C., unpubl. data) in June 2004 and April 2006; the cause of these declines is unknown. For effective conservation, it is imperative to determine the distribution of *B. dendrobatidis* among populations of critically endangered Sardinian newts, as well as among other amphibians which may be affected or involved in the epidemiology of this disease.

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