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SHORT NOTE

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Chytridiomycosis surveillance in the critically endangered Montseny brook newt, *Calotriton arnoldi*, northeastern Spain

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Chytridiomycosis, a disease caused by the pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*) has caused significant declines of amphibian populations in different areas of Spain. The critically endangered Montseny brook newt (*Calotriton arnoldi*) is endemic to the mountain region of Montseny in Catalonia, northeast Spain. As part of its conservation plan, special attention was needed to evaluate the population health status, which remained uncertain. From 2007 to 2011, we conducted a survey in Montseny Natural Park and examined 158 Montseny brook newts, 14 fire salamanders and 2 common toads for the presence of *Bd* using quantitative real-time Taqman PCR (qPCR) assay. All samples were negative to this pathogen suggesting that *Bd* is absent in the region or present in such a low level that it was undetected. The implementation of disease surveillance in wildlife, especially in endangered species, is of crucial importance for the detection of subclinical infection and prompt adoption of counter measures.

Key words: amphibian, *Batrachochytrium dendrobatidis*, *Calotriton arnoldi*, chytridiomycosis, emergent diseases

The Montseny brook newt (*Calotriton arnoldi*) is an endemic amphibian only found in seven mountain streams of Montseny Natural Park (Catalonia, northeast Spain). Based on its extremely small distribution range (40km^2) and population size (1500 adults; Amat, 2004; Amat & Carranza, 2005; Amat & Carranza, 2007), it is considered critically endangered by IUCN (Carranza & Martínez-Solano, 2009).

Chytridiomycosis, a disease caused by the waterborne chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), has been associated with amphibian mortality and population declines on at least four continents (Berger et al., 1998; Bosch et al., 2001; Fisher et al., 2009). In Spain *Bd* has been vastly studied in certain areas, especially in Peñalara National Park (Bosch et al., 2001; Bosch & Martínez-Solano, 2006) and Ibon Acherito (Walker et al., 2010) where it caused severe declines in common midwife toads (*Alytes obstetricans*). Moreover it has been detected in reintroduced captive bred populations of the vulnerable Mallorcan midwife toad (*A. muletensis*) (Walker et al., 2008) and in many amphibian species in Doñana National Park (Hidalgo-Vila et al., 2012). Thus *Bd* could be considered a potential risk for the conservation of the Montseny brook newt, and still this endemic species has not been screened for infection. The aim of this study was to assess for the presence and prevalence of *Bd* in this critically endangered species.

Fieldwork was conducted between October 2007 and November 2011 and we obtained samples from 158 animals. The population is split in two differentiated and non-connected sectors in the Tordera river basin, eastern and western (Montori & Campeny, 1991; Carranza & Amat, 2005; Valbuena-Ureña, 2010). Fifty four samples came from the three populations of the eastern sector (refereed to in Table 1 as A1, A2, A3) and 104 samples came from the four populations of the western sector (referred in the table as B1, B2, B3, B4). Sample size was calculated using Win Episcope v.2. 0 (Clive, Edinburgh, UK), and with a hypothetic infection prevalence of 2% and a population of 1500 animals we aimed for at least 142 samples to achieve a 95% confidence level. We considered that an ideal ecosystem approach to disease monitoring would also have to include other amphibian species with wider ranges. Thus we decided to include samples from 14 adult fire salamanders (*Salamandra salamandra*) and two adult common toads (*Bufo bufo*) which were captured in the mountain streams of Montseny. We sampled each animal by firmly running a cotton tipped swab (MW100 swabs, Medical Wire & Equipment) over the skin of ventral surface including undersides of thighs and toes. All animals were handled with gloves and all equipment was disinfected using 1% Virkon between sites. All swab-samples were stored dry and refrigerated until analyzed.

DNA swab extraction was made using PrepMan Ultra (Applied Biosytems; Hyatt et al., 2007). The amount of

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DNA present in each sample was calculated using real time PCR, with a *Bd*-specific Taqman Assay (Boyle et al., 2004) in a CFX96 Real Time PCR Detection System (Bio-Rad) and using standards containing known genome-equivalents. Each sample was run in duplicate and infection load was measured as the number of zoospore genome equivalents per swab. Individuals were considered *Bd* positives when the results of the two replicates were consistent and obtained zoospore equivalents that were >0.1. We used an internal positive control (IPC) to measure PCR inhibition in randomly selected samples that tested negative for *Bd* infection. Following the methodology of Hyatt et al. (2007), a VICTM labelled synthetic amplicon was used as the IPC (VICTM dye, Applied Biosystems). The IPC was included in one of each duplicate well as 1 µl 10x Exo IPC mix and 0.5 µl 50x Exo IPC DNA.

None of the samples collected from wild amphibians showed evidence of amplification by the *Bd*-diagnostic primers. IPCs showed that there was no evidence for PCR inhibition in any sample. All results are summarized in Table 1 and data has been deposited in the EU *Bd*-surveillance archive at http://www.bd-maps.eu.

This study demonstrates for the first time that *Bd* is not present in Montseny brook newts, or in other amphibian species of Montseny Natural Park. Thus our results indicate that this fungus is not a risk for the Montseny brook newt population at the present time. Some aspects of the biology of the species may be protective against invasion and spread of an introduced pathogen. The population density is low, and very limited dispersal has been recorded by a capture-recapture study (Amat, unpublished data). Moreover, all developmental life stages are fully aquatic, making movements between streams impossible by natural means. On the other hand, aquatic amphibians with limited distribution are in general recognized as the most endangered by extinction due to chytridiomycosis (Bielby et al., 2008), therefore the risk should not be underestimated.

Surveillance for novel infectious disease surveillance is of paramount importance in the management of amphibian populations (Pessier & Mendelson, 2010) given the disease-related aspects of global amphibian declines. Despite the secretive behaviour of the Montseny brook newts we were able to collect samples from approximately 10% of the expected wild adult

Table 1. Field testing for *Batrachochytrium dendrobatidis* in Montseny Natural Park between 2007 and 2011 with negative PCR results (populations A1, A2, A3 belong to the eastern sector and B1, B2, B3 and B4 belong to the western sector of Montseny brook newts).

Year-Month	Population Code	*C. arnoldi*	*S. salamandra*	*B. bufo*
2007 October	A1	10		
	B1	4		
	B2	4		
2009- June	A1	13		
	B2	5		
2009- October	B1	2		
	B2	12		
	B3 & B4	7		
2009- November	B4	9		
2010- April	A1	2		
	A3	4	1	
	B1	6	2	
	B3	14	4	
	B4	4		
2010- May	A1	1		
	A2	2		
	A3	1		
	B1	8		1
	B3	6		
2010- June	A2	3		
2011- March	B1	3		
	B2	8		
2011- May	A1	5		
	A3	3		
	B1	5		
	B2	4		1
	B3	2		
2011- October	B2		4	
	B3	1	3	
2011- November	A1	10		
TOTAL		158	14	2

population. Our surveillance did not detect the presence of *Bd* so this could mean that the pathogen is absent in the region or that it is present in such low levels that it was undetected. Assuming a disease low prevalence of 2% might have existed, there was a 96.57 % probability that we would have detected at least one positive individual by sampling 158 animals as calculated using Win Episcope v.2. 0 (Clive, Edinburgh, UK). It is also remarkable because we obtained samples in different months to minimize the possibility of seasonal variation in detection, as it is known that chytridiomycosis prevalence can vary dramatically depending on air temperature (Kriger & Hero, 2007).

The impact of chytridiomycosis on representatives of the order Caudata is still poorly understood and variable symptomatology has been reported. This ranges from the loss of digits and skin discolouration in Sardinian newts (Bovero et al., 2008) to massive die-offs in salamanders (Bosch & Martínez- Solano, 2006). Ohst et al. (2011) reported that the alpine newt (*Ichthyosaura alpestris*) had one of the highest *Bd* prevalences (14.9%) after conducting an extensive survey in Germany that included many endemic amphibian species (urodele and caudate). Similarly, both in Austria and Spain (Doñana National Park) several species of caudate amphibians tested positive to *Bd* with variable prevalences (Hidalgo-Vila et al., 2012; Sztatecsny & Glaser, 2011). All these data support the importance of including caudate amphibians for *Bd* surveillance. In future, surveillance for *Bd* will continue in Montseny Natural Park aiming for an early detection of infection in the Montseny brook newt and other more common amphibians. Management strategies are being implemented to avoid the introduction and dissemination of the pathogen by rangers and other occasional visitors, with specific information about working routines and disinfection of materials.

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