Evidence of a Chytrid Fungus Infection Involved in the Decline of the Common Midwife Toad (Alytes obstetricans) in Protected Areas of Central Spain

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Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain

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Abstract

During the summers of 1997, 1998 and 1999 mass mortality episodes of post-metamorphic common midwife toads (*Alytes obstetricans*) occurred in a protected area in central Spain. The population suffered a sharp decline, disappearing from 86% of the ponds where they were known to reproduce some years ago. Scanning electron microscopy and histological techniques revealed the presence of a chytridiomycosis infection in the skin of the toads. This evidence supports chytridiomycosis as the most plausible cause of the decline of the species in the area. This is the first report of an apparent chytridium-caused amphibian decline in Europe.

Keywords: Chytridiomycosis; Decline; Midwife toad; Spain

1. Introduction

Declines of amphibian populations in their natural habitats, with no identifiable direct causes, have become a subject of increasing concern for the scientific community in recent years (Blaustein and Wake, 1990; Wake, 1991; Blaustein, 1994; McCoy, 1994). It is a complex problem with multiple potential causes whose relative importance in local extinction processes has yet to be established, as well as the extent to which these factors affect normal population dynamics of amphibians (Pechmann et al., 1991; Alford and Richards, 1999). Several studies have provided solid evidence of disappearances of populations that were common only a few decades ago, particularly in montane, well conserved habitats (Bradford, 1991; Carey, 1993; Richards et al., 1993; Lips, 1998). Some of these studies have implicated abiotic agents in the declines, mostly physical changes in the environment which act as stressing factors affecting normal physiological functions of the amphibians (Bradford et al., 1992; Blaustein et al., 1994). Biotic factors have also been implicated in some cases, as in the recent description of a pathogenic fungus which has been causally related to amphibian population declines in Australia and Northern, Central and South America (Berger et al., 1998; Daszak et al., 1999; Pessier et al., 1999). This fungus belongs to the order Chytridiales, and has been included in a new genus, *Batrachochytrium* (Longcore et al., 1999). It is waterborne and pathogenic to adult amphibians, although it does not kill tadpoles; it prefers cooler temperatures, and is not solely dependent upon the highly susceptible host species for its continued existence (Berger et al., 1999a). Chytrids were not known to parasitise vertebrates prior to these studies.

Chytridiomycosis is a fatal disease which has been identified in an increasing number of amphibian species, as preserved material from populations affected some years ago is being re-studied. The disease can be carried by healthy tadpoles and has also been found in a small proportion of apparently healthy frogs and tadpoles (Berger et al., 1999a). In post-metamorphic amphibians it causes a widespread infection of the skin, resulting in hyperkeratosis, sloughing and erosions of the epidermis, and occasional ulcerations (Berger et al., 1999a).

No fungal infections causing amphibian declines have been reported so far in Europe, and such declines have mainly occurred as a result of conventional reasons

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associated with habitat loss and disturbance (e.g. Cooke, 1972; Beebee, 1977; Kuzmin, 1994; Cunningham et al., 1996). Unlike other parts of the world, no species in Europe has gone extinct recently, and the cases of apparently inexplicable declines are very unusual (e.g. Semb-Johansson, 1992).

In Spain, a recent case was observed in July 1992 and 1994, when two episodes of mass mortality of larval and metamorphic common midwife toads (*Alytes obstetricans*) were recorded in a lake in the Pyrenean Mountains (Márquez et al., 1995). In that case, the bacterium *Aeromonas hydrophila*, which produces a disease commonly known as "red leg", was shown to be responsible for the mortality.

In the summers of 1997 and 1998, thousands of dead, post-metamorphic *A. obstetricans* were found around the ponds in the Peñalara Natural Park in central Spain. They had an apparently healthy general aspect, showing no traces of red-leg infection. Dead tadpoles or adults were not found. A limnological study was performed during the summer of 1998 in which 25 water chemistry variables were measured in selected ponds, and a rare increase in pH (up to 9) in some of the ponds was reported (Toro and Granados, personal communication). In the summer of 1999 we carried out a study of the *A. obstetricans* population to detect the potential causes of the mass mortality in order to act as soon as possible to ensure the conservation of the populations, which are isolated from other populations in central Spain (García-París, 1995).

2. Study area and biology of *A. obstetricans*

The Peñalara Natural Park is an alpine area in central Spain, very close to Madrid. The area has been protected for 70 years and, in spite of the high number of visitors (>100 000 per year), conservation and restoration practices maintain its preservation status and ecological health in good condition. The area is located at about 2000 m of elevation, and mainly consists of bogs and alpine grasslands with granitic outcrops. The Natural Park holds >250 ponds from 0.3 to 6463 m^2 in area (average 102 m^2 Fig. 1). More than half (55%) of the ponds are permanent, with water all year round, whereas the rest are ephemeral ponds, which are dry for some months. As a typical boggy area, the ponds are slightly acid (pH about 6, Toro and Granados, 1999).

The Natural Park has 10 species of amphibians among which *A. obstetricans* was one of the most abundant. During the spring, *A. obstetricans* males normally formed large choruses in several locations, and reproduction was known in at least 35 ponds (García-París and Martín, 1987; Bosch, 1997). This species has a very small clutch size, and a remarkable reproductive behaviour, for males carry the eggs twined around their hind legs on land for about a month, from fertilization to hatching (Barbadillo, 1987). Usually, more than one season is needed to complete the larval development (sometimes up to three seasons, Angelier and Angelier, 1964), so tadpoles from previous years coexist in the ponds together with recently released tadpoles, forming relatively high density groups.

3. Methods

During the summer of 1999 (June–September) we extensively sampled every pond of the Peñalara Natural Park at least six times, in search of amphibian larvae. In addition, we searched for adults in the surroundings of the ponds by day (when they hide under rocks) or at dawn (when they can be detected by listening for male choruses). We compared the data with previous results obtained during the summers of 1981–1986 by the third author (García-París and Martín, 1987, and unpublished data).

Dead specimens and living animals in terminal stages were counted and collected, and then preserved in refrigerators located at El Ventorrillo, a biological station 10 km away from the Park. In addition, we collected a few tadpoles and live post-metamorphic toads and preserved them in 10% formalin or 70% ethanol for ulterior scanning electron microscopy (SEM) and histological analysis. Following the procedures cited in Berger et al. (1998), we used SEM techniques searching for evidence of fungus infection in the skin of preserved post-metamorphic individuals. To confirm the diagnosis of chytridiomycosis we also performed histological analyses of the tadpoles and post-metamorphic individuals preserved in formalin (procedures cited in Berger et al., 1999b).

In order to know if some environmental process could be involved in the decline, we focussed on a limnological survey conducted in 1998. Specifically, we tried to identify differences in water chemistry between ponds that still supported *A. obstetricans* tadpoles and those that no longer did so. Some of the water chemistry variables analyzed in the limnological study were pH, conductivity, alkalinity, levels of Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, SO_4^{2-}, Cl^{-}, H^{+}, and NH_4^{+}. We used a stepwise discriminant analysis of these eight ponds.

4. Results

In the past decade *A. obstetricans* tadpoles were present in at least 35 ponds, which constitute 44% of the ponds sampled before 1999. In 1999 they were present in only five ponds across the Natural Park; i.e. they had disappeared from 86% of the ponds that were occupied a few years ago (Fig. 1). Although we do not have precise quantitative density data from the past, the estimated

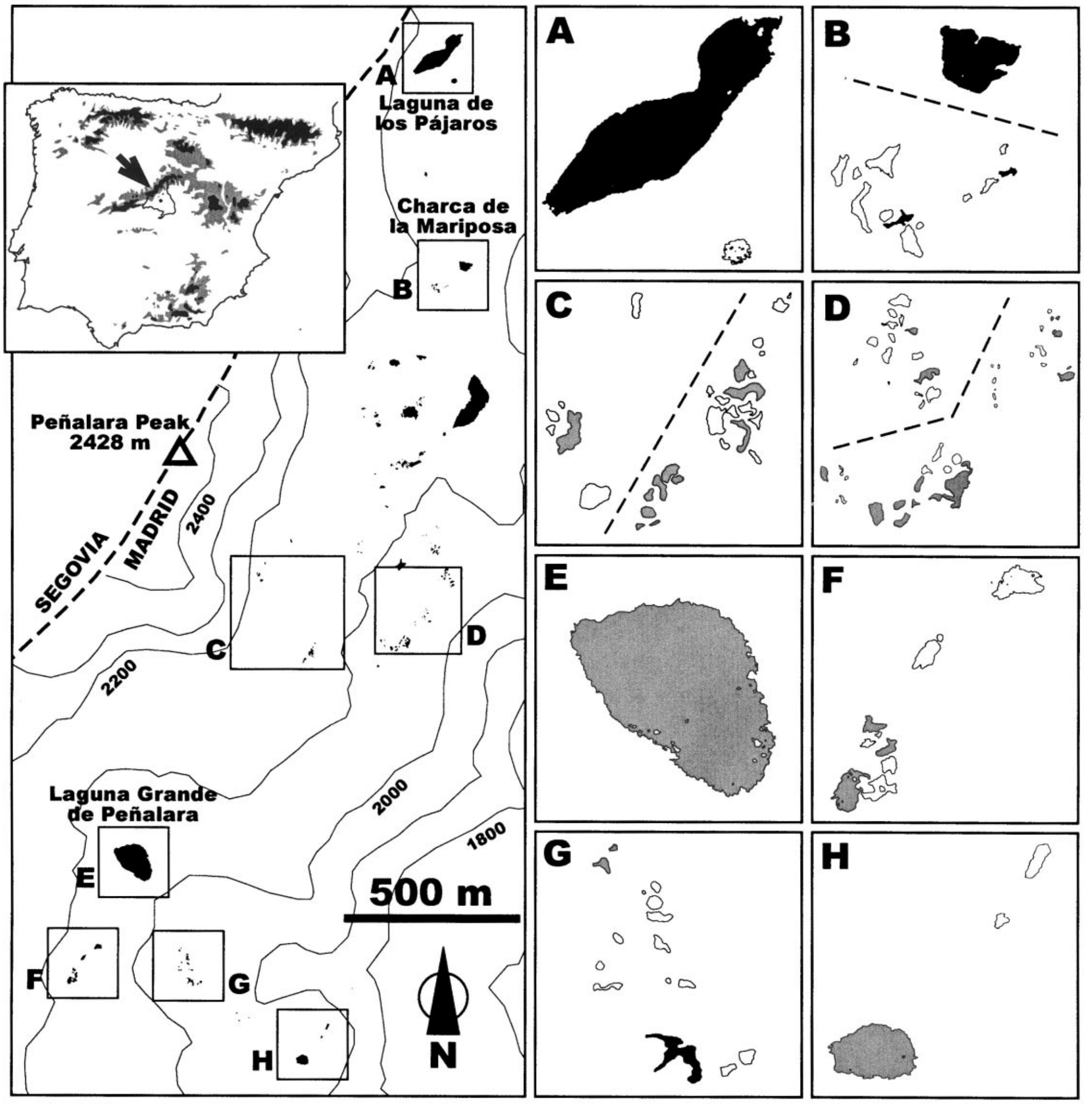

Fig. 1. Some ponds of the Peñalara Natural Park and the location of Madrid Province in the Iberian Peninsula (altitude levels above 1000 and 1500 m appear in gray and black respectively). (A–H) Ponds that were known to have held *Alytes obstetricans* tadpoles before 1997 appear filled, either in gray (ponds that did not hold tadpoles in 1999) or in black (ponds that still held tadpoles in 1999). The ponds that never supported tadpoles or for which no data were available from previous studies appear empty. Dashed lines in B–D indicate that the groups of ponds are not represented at the same scale.

tadpole density has decreased sharply in three of the five ponds that still maintained tadpoles in 1999, when compared with qualitative estimations from the past.

At the beginning of the summer, the Laguna de los Pájaros (area = 4866 m^2; Fig. 1A) still supported a high density of *A. obstetricans* tadpoles, ca. 5000, but during the summer we collected about 700 dead post-metamorphic individuals there. We found evidence of reproduction in 1999 at this site (i.e. tadpoles in early stages of development), but we found no more than 10 tadpoles. We also found about 50 dead post-metamorphic common toads (*Bufo bufo*). Dead material was also preserved and analyses are now in progress.

The Charca de la Mariposa (area = 483 m^2; Fig. 1B) is the only other pond that still supported high density of *A. obstetricans* tadpoles at the beginning of the summer. We estimated the presence there of at least 300 tadpoles. During the summer, we again collected dead post-metamorphic individuals.

Two other smaller ponds (Fig. 1B) near the Charca de la Mariposa, supported a few *A. obstetricans* tadpoles at the beginning of the summer, but a similar count of dead post-metamorphic toads at the end of the summer suggested that no tadpoles were able to survive the season there. Finally, we found a few larvae in September at another small pond (Fig. 1G) in the south of the Park.

Summarizing, we found tadpoles in early stages of development, presumably from clutches released in 1999, in just two of the five ponds. This, combined with the lack of direct or indirect observations of adult toads (e.g. male choruses), lead us to suppose that probably just a few pairs managed to reproduce in the Park during 1999.

At all of the five pools containing tadpoles at the beginning of the summer, the tadpoles seemed healthy. Some of them displayed light spots on their skin, but their behaviour was not different from usual. Most of the dead post-metamorphic individuals showed no evident signs of disease. Some living post-metamorphic toads displayed metallic green spots, and their vitality was reduced, while other living post-metamorphic toads were apparently healthy and with normal vitality, showing no unusual-coloration spots. Both categories of toads were observed sharing the same refuges.

Examination of epidermis of dead specimens with SEM showed evidence of fungal infection. Small crater-like structures were observed all along the skin of the specimens (Fig. 2A). These tubular structures are probably produced by fungal sporangia on their way out of the epidermis, including the small openings by which the zoospores would emerge from the skin to complete dispersal (Figs. 2B and C). Histological examination of the epidermis both of specimens found dead and those killed and preserved, showed empty sporangia and some others still containing zoospores (Fig. 3). Dr. L. Berger (James Cook University, Australia; personal communication) supported our identification.

Out of the 25 water chemistry variables measured, only four contributed significantly to the discriminant analysis (Table 1). Levels of Ca^{2+}, H^{+}, Mg^{2+} and NO_3^- were combined in a mathematical function that correctly classified all eight ponds according to the presence or absence of *A. obstetricans* tadpoles. Relatively low levels of Ca^{2+} and $Mg^{2+}$and high levels of H^{+} (i.e. more typical pH values) characterized the ponds that still maintained *A. obstetricans* tadpoles compared to those where *Alytes* populations disappeared in recent years.

5. Discussion

Sudden episodes of massive death of post-metamorphic individuals suggest that the observed decline has been caused by an epidemic disease, and clearly exclude the possibility of natural population fluctuations. Other causes, as for example human impacts, were not examined but, do not seem probable in this case. *A. obstetricans* was one of the most abundant amphibian species in the Park in the past, not only in

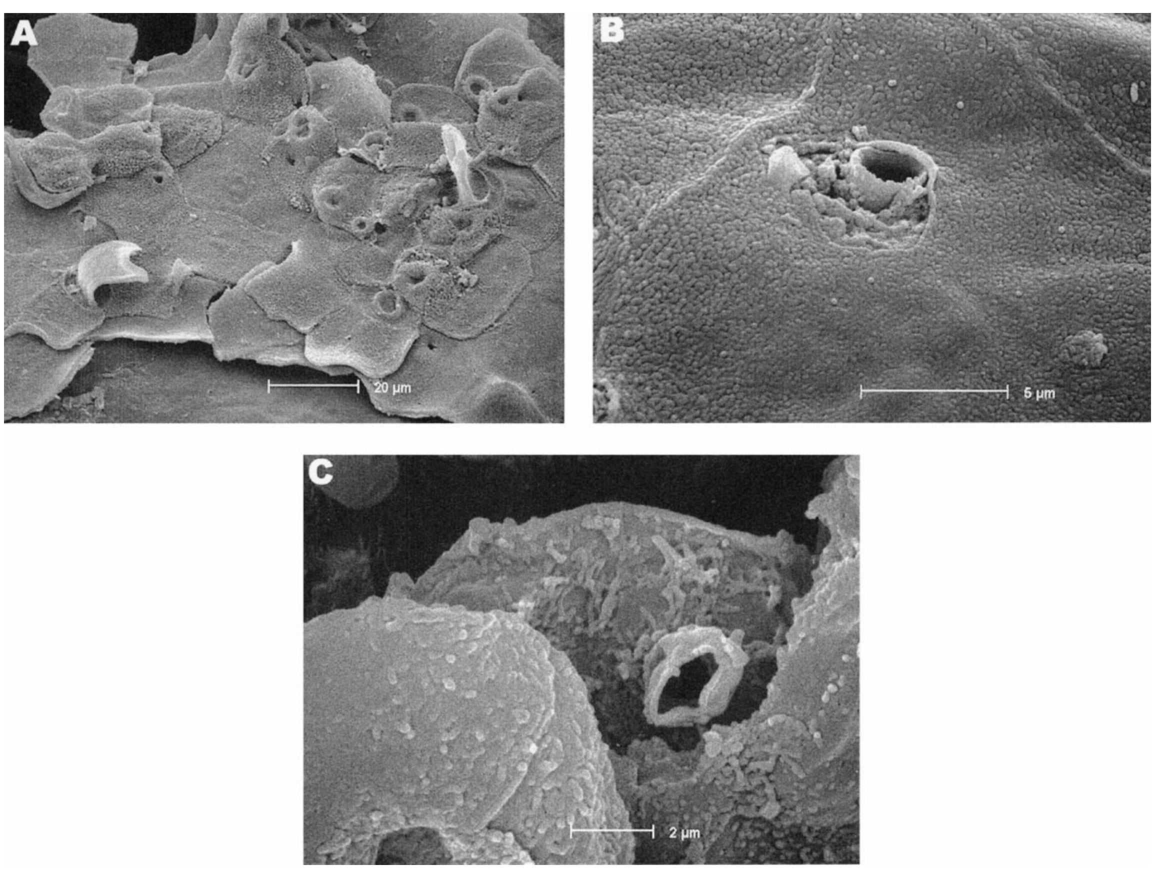

Fig. 2. (A) Scanning electron micrograph of skin from an infected post-metamorphic *Alytes obstetricans* from the Peñalara Natural Park showing fungal infection evidence. (B) Discharge tubes of the sporangia emerging through the surface of epidermal cells. (C) Discharge tubes open after zoospores are released.

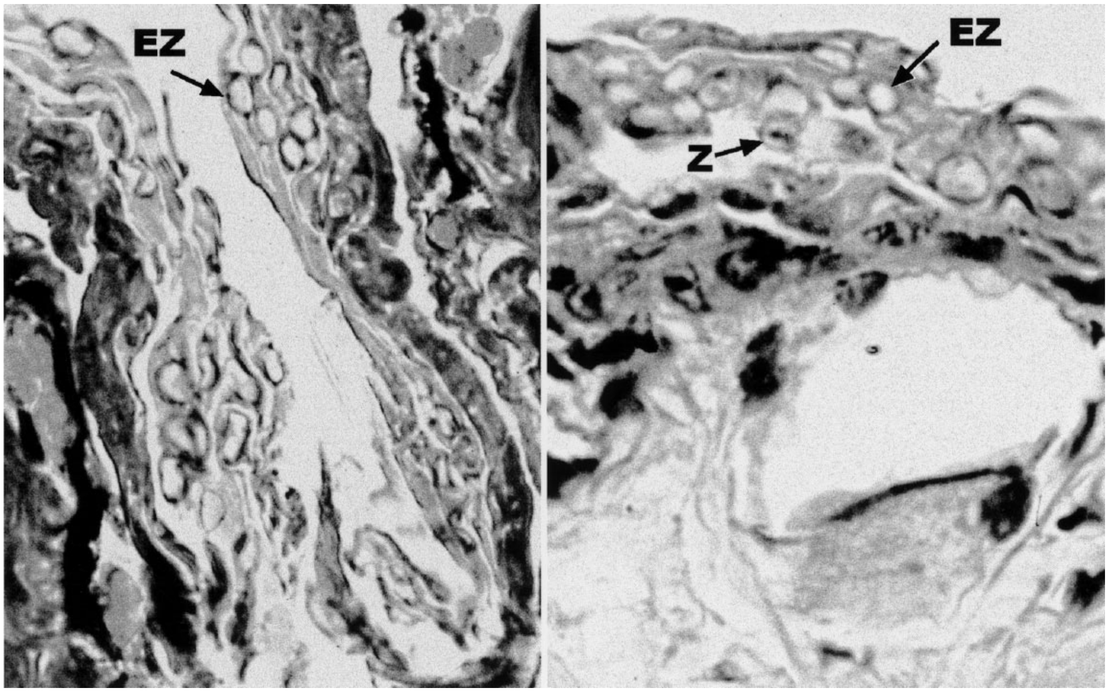

Fig. 3. Two sections of skin from a infected post-metamorphic *Alytes obstetricans* from the Peñalara Natural showing empty zoosporangium (EZ) formed after zoospores have been discharged and zoosporangium (Z) containing zoospores. The empty zoosporangium appear as small, clear circular spaces in the superficial keratinised epidermis.

Table 1
Results of the stepwise discriminant analysis of the ponds in which *Alytes obstetricans* was known to reproduce in recent years, showing the four significant variables included at each step[a]

Variable	Mean and S.D. values (meq./litre) in ponds		Wilks lambda	*P*
	With tadpoles	Without tadpoles		
Ca^{2+}	35 (1.6)	71(14.47)	0.28477	0.0082
H^{+}	0.62 (0.29)	0.35 (0.3)	0.04743	0.0005
Mg^{2+}	14.5 (5.32)	24.75 (7.45)	0.00854	0.0001
NO_3^-	6.42 (11.05)	3.4 (5)	0.00059	<0.001

[a] Mean and S.D. (in parentheses) values of the four variables for the two groups of ponds are given for ponds that still hold *Alytes* tadpoles in 1999 vs. ponds where they have disappeared in recent years.

the relatively high number of ponds used for reproduction, but also with respect to the larval densities observed.

This is the first report of an amphibian population decline caused by a fungal infection in Europe. The circumstances are quite similar to those described by Berger et al. (1998): rapid declines in populations living at high altitudes in well preserved habitats. The presence of other aquatic zoosporic fungi (Saprolegniaceae) is correlated with altitude and unpolluted areas (Sparrow, 1968). The extended larval period of *A. obstetricans* involves high probability of contact with the waterborne zoospores, and therefore the tadpoles can carry the fungus. This, and the relatively small clutch size that characterizes this species, are factors that could partly explain why *A. obstetricans* has been the only species largely affected in Peñalara Natural Park. Common midwife toads appear to be a highly sensitive species, considering other reported recent mortalities in high altitude populations within its distribution range (Márquez et al., 1995; L. J. Barbadillo, personal communication). These factors could also explain the lower impact detected on the Peñalara population of *B. bufo*, whose clutches usually contain >5000 eggs, and whose larvae develop in a shorter time span and never have overwintering larvae. However, more studies are needed to test the relative sensitivity of other species to the pathogen and the identification of other possible factors involved in the decline.

The pH change detected in 1998 in some ponds of the Park could have acted as a stress factor that enhanced the susceptibility of the host to the pathogenic infection. On the other hand, it is known that water pH affects the epidemiology of chytrid blooms (Sparrow, 1968); Berger et al. (1999a) pointed out that this may be a relevant consideration when investigating the causes of outbreaks of chytridiomycosis.

It should also be noted that our findings, although described for a high altitude area, concern a different type of ecosystem and amphibian populations, not confined to rain forests but to high altitude small lakes surrounded by grasslands above the treeline in a temperate area. The main factor related to amphibian declines cited in these open areas is UV-B radiation

(Lizana and Pedraza, 1998, but see Langhelle et al., 1999). This possibility has not been tested here, because *A. obstetricans* embryos are protected from UV-B damage since males carry the eggs on land until fully formed tadpoles are ready to hatch. The characterization of the pathogen as responsible for the species decline therefore seems well founded, although other approaches and new insights to the problem would be very helpful.

Explaining the disease in recent years is not possible without additional data. What has changed in the Natural Park in the last 5 years that could account for this severe decline? Several alternatives have been proposed for similar cases by Berger et al. (1998), of which two might explain the observed decline.

The first hypothesis is a recent introduction of the pathogen to the area. This could have happened through uncontrolled introduction of infected non-native species or maybe as a human-borne disease. We found evidence of amphibian introductions in both relatively old and recent dates. *Triturus alpestris* was found to be introduced in the Park ca. 1980 (Arano et al., 1991), and we found a single female specimen of the pygmy marbled newt (*Triturus marmoratus pygmaeus*) during the sampling period in 1999.

The second possible explanation relates the origin of the disease to an increased sensitivity of the host to parasites due to environmental changes that reduce the toad's normal immune response capabilities. There is evidence of changes in pH in some of the ponds at Peñalara. Unfortunately, there is still no explanation for these variations in water quality, and their role in enhanced pathogen infection has also to be proved in further studies.

Although these explanations are not mutually exclusive and together could explain the decline, both need further evidence.

The current status of *A. obstetricans* in the Park is now critical and survival of the species is unlikely under present circumstances. Most amphibian species in central and southern Spain are patchily distributed, and populations are largely isolated from each other, rendering recolonization unlikely. Further studies on the disease and on preservation measures are urgently needed to prevent the midwife toad being exterminated in Spain by further episodes of infection.

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