Basic and Applied Herpetology 35 (2021) 5-17

First record of amphibian mortality associated with the fungus *Batrachochytrium dendrobatidis* in Catalonia (NE Spain)

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Received: 08 January 2021; returned for review: 25 March 2021; accepted: 29 May 2021.

We report the first cases of mortality in anurans associated with the presence of the fungus *Batrachochytrium dendrobatidis* at four different localities from Catalonia (NE Iberian Peninsula, Spain). All cases were confirmed by both molecular techniques and histology. The infected individuals were two Mediterranean painted frogs (*Discoglossus pictus*) from Girona province found in 2018 and 2020, one Iberian waterfrog (*Pelophylax perezi*) from Tarragona province found in 2018, and one European common *frog* (*Rana temporaria*) from Barcelona province found in 2019. This is the first time that mortality associated with this pathogen has been confirmed in *D. pictus* and *P. perezi*. The role of the fungus as an agent possibly leading to death, in association with other external environmental factors, is discussed. These findings could suggest a recent increase of the incidence of this disease in the region. Some of these cases are particularly worrying because of their occurrence close to sites where some endemic amphibian species with extremely reduced distributions inhabit.

Key words: Catalonia; chytridiomycosis; *Discoglossus pictus*; oubreak; *Pelophylax perezi*; *Rana temporaria*.

Batrachochytrium dendrobatidis (hereafter *Bd*) is currently found in all continents where amphibians occur (Skerratt *et al.*, 2007) and is considered a major threat to amphibian biodiversity worldwide (Olson *et al*., 2013). However, this pathogen can be present in the environment and on the skin of amphibians without causing fatal disease (Lips, 2016). In this case, infected amphibians are considered asymptomatic

DOI: https://dx.doi.org/10.11160/bah.210

carriers able to spread the pathogen to other more vulnerable species (e.g. Hanselmann *et al.*, 2004). The presence of asymptomatic *Bd* carriers is widely reported in the literature, also within the region of Catalonia in NE Iberian Peninsula (e.g. Bargalló *et al.*, 2016; Martínez-Silvestre *et al.*, 2019, 2020). The first documented *Bd* infection in wild amphibian populations in Europe dates from 1997 (Bosch *et al.*, 2001), when mass mortality episodes of common midwife toads (*Alytes obstetricans*) occurred in the Peñalara protected area in central Spain. Additional mass mortalities in the 2000's were also reported in Spain, including outbreaks in fire salamanders (*Salamandra salamandra*) and spiny toads (*Bufo spinosus*) from the same location (Bosch *et al.*, 2001; Bosch & Martínez-Solano, 2006). Apart from Peñalara, mortalities have also been detected in other montane areas of Spain, including the Pyrenees, the Cantabrian Range (Walker *et al.*, 2010), and the Tramuntana Range in Majorca island (Walker *et al.*, 2008). Interestingly, Walker *et al.* (2010) found an increased *Bd* prevalence along an east-west gradient in northern Iberia, with the eastern Pyrenees and the entire region of Catalonia remaining free of mass mortalities associated with *Bd*.

Between 2016 and 2019, following an active health surveillance plan, numerous routine samplings were carried out in several areas of Catalonia. These studies led to detection of numerous *Bd* infections distributed throughout this territory, although none of them was associated with any mortality episode (Bargalló *et al.*, 2016; Martínez-Silvestre *et al.*, 2017, 2019, 2020; Montori *et al.*, 2019). As a result of this initial surveillance, an action protocol was established, according to which, if a dead amphibian was found during any sampling, it was collected and sent for analysis as quickly as possible to the Catalonian Reptiles and Amphibians Rehabilitation Centre (CRARC).

Here we report three cases of amphibians truly affected by *Bd*, confirmed by both molecular and histological methods. Although *Bd* presence in Catalonia had already been confirmed, this work presents the first evidence of *Bd*-related mortalities detected in this region.

Materials and Methods

Field description, post-mortem *study and sample collection*

Case 1. Mediterranean painted frog (*Discoglossus pictus*). Two male specimens with nuptial calluses were found dead in the Massif de Les Gavarres (Girona; Fig. 1) during an amphibian health surveillance study in the area (Pérez-Novo, 2018). The first animal (1a) was found by Environment Rangers (*Cos d'Agents Rurals*) of the Catalonian government (*Generalitat de Catalunya*) on 9 March 2018 at Riera de Rissec (Madremanya; 41.973449°N, 2.932524°W). The second animal (1b) was found by members of the Catalonian Herpetological Society (*Societat Catalana d'Herpetologia*, SCH) on 12 April 2018 at the Bassa de la Capçana (Cassà de la Selva; 41.869532°N, 2.895320°W). On 1 February 2020, another five Mediterranean painted frogs were found dead, in different stages of decomposition, at the same location as individual 1b (Bassa de la Capçana). Due their decomposition status, only one animal was

Figure 1: Location of the reported cases of *Batrachohytrium dendrobatidis* in amphibians from Catalonia. Orange areas show the protected National (bright orange) or Natural (pale orange) Parks, including Montseny Natural Park (point 3). For a complete interpretation of the figure, the reader is referred to the online, coloured version of the article.

sampled. Necropsy and skin swabbing for PCR diagnosis were performed on the two animals found dead in 2018, and tissue samples (skin, liver, kidney and intestine) were taken for histological studies from one of them. Due to the advanced stage of decomposition of the individuals found in 2020, only skin swabbing was possible.

Case 2. Iberian waterfrog (*Pelophyax perezi*). Five carcasses were found on 20 February 2018 at the bottom of a pond in La Mussara (Tarragona; 41.257584°N, 1.026546°W; Fig. 1). During the same sampling, a live adult waterfrog floating with extended limbs was observed and immediately captured for study. The animal died on the way to CRARC and a *post-mortem* study of this animal was carried out, including skin swabs for PCR and tissue sample collection (skin, liver, kidney, stomach and intestine) for histology.

Case 3. European common *frog* (*Rana temporaria*). A recently deceased adult animal was found on 29 April 2019 in the Santa Fe del Montseny reservoir (Montseny Natural Park, Barcelona; 41.768309°N, 2.470195°W; Fig. 1) by SCH members. The animal was immediately fixed in 70% ethanol and sent to CRARC. The animal was necropsied (despite being fixed), and skin swabs and tissue samples for histology (skin, lung, gastrointestinal tract, liver, spleen, gonads and kidneys) were taken.

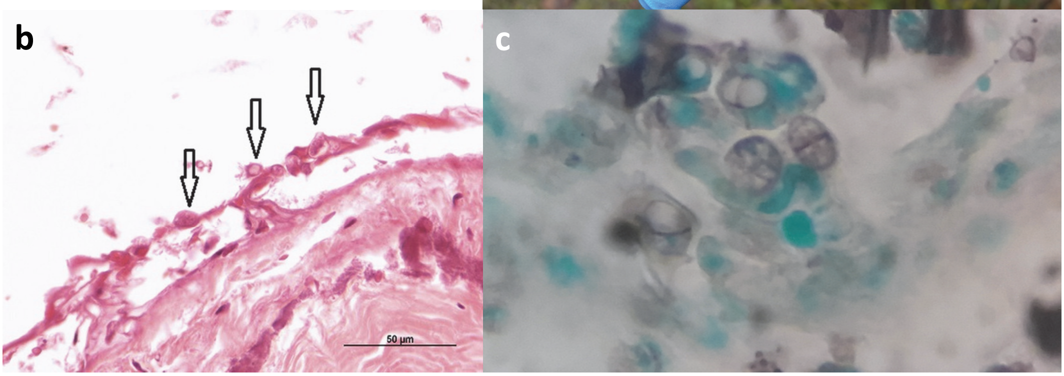
To prevent pathogen transmission and cross-contamination among samples, or even pathogen transmission among sampling sites, sampling was carried out following strict biosecurity guidelines: all animals were handled with nitrile gloves, which were changed between animals in cases of multiple mortality, and all field equipment, including shoes, were disinfected with 1% Virkon™ between sites.

Sampling and molecular methods (end-point PCR and qPCR)

Sampling was performed by swabbing the entire skin of carcasses with sterile dry swabs. Swab samples were stored below

4°C until they were processed in the laboratory, and individuals found dead were stored in 70% ethanol. For *Ranavirus* (hereafter RV) detection, liver samples were used. End-point PCR analyses were performed on skin swabs and liver samples to assess the presence of *Bd*, *Batrachochytrium salamandrivorans* (hereafter *Bsal*) or RV, followed by qPCRs on positive samples performed to quantify infection loads. DNA was extracted using PrepMan™ Ultra reagent and extractions were diluted 1:10 in DNase-free water before PCR or qPCR amplification. PCR products were visualized by electrophoresis on 1.5% agarose gels using SYBR™ Save DNA gel stain and blue light transilluminator. qPCR analyses were performed following BOYLE *et al*. (2004) for *Bd*, a modified singleplex from BLOOI *et al*. (2013) for *Bsal*, and LEUNG *et al*. (2017) for RV. Negative controls and standards with known concentrations of the corresponding pathogen were used in each plate. Infection load of each sample was assessed directly by the machine software according to the reference function obtained with the standards. A sample was assigned as positive when the infection load was equal to or higher than 0.1 genomic equivalents (GE) of zoospores for *Bd* and *Bsal*, or 3 GEs of virions for RV, and when the amplification curve presented a robust sigmoidal shape. Since qPCR was used to quantify infection loads of previously positive samples confirmed by end-point PCR, we did not consider necessary to run the qPCR analyses in duplicate.

Figure 2: Images taken from case 1, corresponding to *Discoglossus pictus*. (a) General view of the animal's ventral side. (b) Skin section (x400) stained with haematoxylin and eosin. Arrows indicate (from left to right) a full *Bd* thallus (zoosporangium), an empty zoospore, and a zoosporangium with discharge tube. (c) Skin section (x1000) stained with Gomori Methenamine Silver.



Histology

Tissue samples were fixed in 4% neutral buffered formalin before being processed for histologic examination. One section per tissue (liver, bone, intestine, muscle and skin from three different locations, including interdigital, ventral and dorsal integument) was examined, resulting in seven sections per individual. Four-µm sections were stained with haematoxylin and eosin (H/E) and Grocott's stain (Gomori Methenamine Silver (GMS)) and examined by light microscopy.

Results

Bd was the only considered pathogen for which PCR analyses tested positive, and none of the samples analysed with molecular methods or histology showed evidence of the presence of either *Bsal* or RV.

Case 1. *Discoglossus pictus*. The necropsy of the animals revealed a mild redness of the ventral skin (Fig. 2a). The qPCR analyses showed an infection load of 1099 GE for animal 1a and 855 GE for animal 1b. The individual found dead in the same pond in 2020 presented a *Bd* load of 62 GE. Histologically, and despite early autolysis, we confirmed the presence of zoospores in the basal layer consistent with a transmural epidermal invasion of the fungus (Fig. 2b). The GMS stain was also positive, revealing numerous fungi with black-stained wall. Active fungal reproduction with numerous thalli and zoosporangia at different stages of maturation were identified, including empty, septated zoosporangia and flask-shaped discharge tubes (Fig. 2c).

Case 2. *Pelophylax perezi*. A reddening of the skin was observed in the ventral area (Fig. 3a), as in case 1. End-point PCR analysis showed the presence of *Bd*, and qPCR yielded an infection load of 1629 GE. Histology showed extensive mild hyperplasia and hyperkeratosis with invasion of the *stratum corneum*, and keratin layers with numerous zoosporangia (Fig.3b) in

Figure 3: Images taken from case 2, corresponding to *Pelophylax perezi*. (a) General view of the animal's ventral side. (b) Skin section (x400) stained with haematoxylin and eosin, where multiple *Bd* zoosporangia can be seen without inflammatory cells.

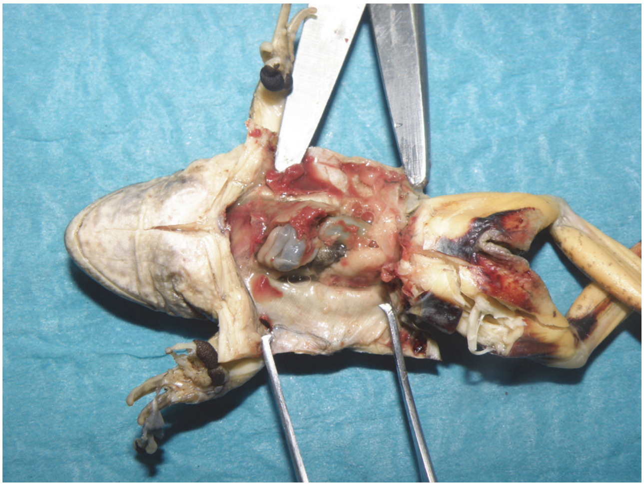


Figure 4: Dissected *Rana temporaria* specimen, corresponding to case 3, showing internal haemorrhages.

several sections of the skin (more than 2 cm^2 evaluated). No inflammatory cells were seen in the dermis. Due to the clear identification of fungal structures by H/E stain, a GMS stain was not necessary.

Case 3. *Rana temporaria*. The necropsy revealed moderate skin redness in the groin area and proximal part of the hind limbs. Internal haematomas were also detected, corresponding to haemorrhages affecting the coelomic cavity (Fig. 4). The PCR was positive for *Bd*, although the presence of some waste material leading to partial inhibition of the qPCR reaction prevented calculation of the infection load. Histology was not congruent with a mortal fungal cutaneous infection because only a single isolated zoospore was detected on the *stratum corneum*, with secondary bacteria detected after evaluation of several sections. In addition, an acute extensive subcutaneous haemorrhage was the only lesion seen in the section from the groin area. As histological changes could not confirm the death of this animal due to chytridiomycosis, we concluded that death, as well as the observed haemorrhages and internal bleeding, were most likely caused by a traumatic incident.

DISCUSSION

We describe the first cases of mortality related to the presence of *Bd* in Catalonia, and also the first cases of mortality due to chytridiomycosis in *D. pictus* and *P. perezi*. Unfortunately, we have not information about the strains involved in these cases. However, according to our previous results published in BYRNE *et al.* (2019), just the GPL strain is present in the Iberian Peninsula. Prior to this report, *Bd*-related mortality had been reported in the Iberian Peninsula only in fire salamanders, spiny toads, and common midwife toads (BOSCH *et al.*, 2001; BOSCH & MARTÍNEZ-SOLANO, 2006; GARNER *et al.*, 2009; WALKER *et al.*, 2010). Although mortality due to chytridiomycosis in the genus *Discoglossus* was described only for the Tyrrhenian painted frog (*Discoglossus sardus*) (BIELBY *et al.*, 2009), most members of the family Alytidae are highly susceptible to chytridiomycosis, including the above mentioned *A. obstetricans* (Bosch *et al*., 2001; Walker *et al*., 2010), Balearic midwife toads (*Alytes muletensis;* Walker *et al*., 2008; Doddington *et al*., 2013), Betic midwife toads (*A. dickhilleni;* Bosch *et al*., 2013; Thumsová *et al*., 2021), Iberian midwife toads (*Alytes cisternasii;* Bosch *et al*., unpublished data), and Moroccan midwife *toads (Alytes maurus;* Thumsová *et al*., unpublished data). *Bd* presence had been already described in *D. pictus* from different localities of Catalonia, but always with highly variable infection loads and not associated with mortality (Saura *et al*., 2016; Montori *et al*., 2019). Other species of the genus, including the Moroccan painted frog *(D. scovazzi)*, have also been found to be asymptomatic carriers of *Bd* (El Mouden *et al*., 2011).

Green frogs are frequently infected with *Bd* in Europe, exhibiting *Bd* prevalence values at background levels (e.g. Italy: Simoncelli *et al*., 2005; Adams *et al*., 2008; Federici *et al*., 2008; Denmark: Scalera *et al*., 2008; Luxembourg: Wood *et al*., 2009; Spain: Walker *et al*., 2010; Fernández-Beaskoetxea *et al*., 2016, Oficialdegui *et al*., 2019; Switzerland: Tobler *et al*., 2012; Germany: Ohst *et al*., 2013; Hungary: Vörös *et al*., 2018). To the best of our knowledge, mortalities in European green frogs associated with *Bd* have not been recorded so far, and although two unwell individuals from Poland tested positive for *Bd*, effects were not associated with chytridiomycosis (Kolenda *et al*., 2017). In Catalonia, *P. perezi* is frequently an asymptomatic carrier of *Bd* (e.g. Bargalló *et al*., 2016; Miras *et al*., 2017). Therefore, it cannot be ruled out that the cause of death of the individual described here as case 2 was related to the stress of transportation to the laboratory. Actually, sudden death of *Bd*-infected individuals due to the stress of handling is relatively common, both in the laboratory and in the field (J. Bosch, personal observations).

Although the cause of death of the individual reported here as case 3 could not be confirmed, it should be noted that this infected animal was within the distribution area of the critically endangered Montseny brook newt (*Calotriton arnoldi*). A comprehensive study conducted between 2007 and 2011 in the whole area of the Montseny Massif revealed an apparent absence of *Bd* (Obón *et al*., 2013). However, more recent surveys performed in 2017 and 2018 confirmed the presence of the pathogen in this area in introduced Iberian *newts* (*Lissotriton boscai*; Martínez-Silvestre *et al*., unpublished data). *Rana temporaria* is normally very resistant to the disease, being frequently infected but without exhibiting associated mortality (Bosch *et al*., unpublished data). In fact, individuals of *R. temporaria* have only been found dead in association with *Bd* with the concurrence of severe episodes of mass mortalities of highly sensitive species (Clare *et al*., 2016). In those cases, only newly emerged metamorphs, and not adults, were found to be affected. Even though the internal bleeding detected in our case did not correspond to the typical casuistry of the disease, the infection could have weakened the animal, turning it more vulnerable to other pathogens, or more prone to be road-killed or predated. Changes in habits (e.g. spending more time out of the water) or behaviour (e.g. lethargy, loss of reflexes) due to *Bd* infection have been described, especially in the terminal phase of disease, making infected animals more vulnerable to other causes of mortality (CAREY *et al*., 2006; VOYLES *et al*., 2007; VIAFARA *et al*., 2020). In any case, the proximity of the area where the specimen was located to populations of *C. arnoldi* is worrying and an important forewarning about the imminent contact of the pathogen with this endemic and endangered species. Even though the sister species *Calotriton asper* has been found to be an asymptomatic carrier of *Bd* (MARTÍNEZ-SILVESTRE *et al*., 2020), contact of Bd with *C. arnoldi* should be prevented because specific thermal or environmental conditions can turn basal infections into mortalities, as described here for *D. pictus* and *P. perezi*.

Regarding diagnosis, even when end-point PCR tests allow a rapid detection of the studied pathogens, a positive PCR does not necessarily imply that the animal is unwell. On the contrary, quantitative PCR is generally much more sensitive than conventional PCR and provides an estimation of the infectious load. Unfortunately, information on the burden of infection alone is not sufficient for determining the cause of death without knowing the reference values that are lethal for the species. In our three study cases, histology using H/E or GMS stains from skin samples were necessary tools to confirm that the animals suffered from the disease, ruling out the possibility that they were merely carriers (see PESSIER, 2007). In addition, the selection of the area where skin samples are collected is particularly important for histological detection. The loss of the epidermis due to processing, decomposition following death, or skin weakening caused by the disease, can make difficult the detection of characteristic lesions and, consequently, the diagnosis. Furthermore, in mild infections, hyperkeratosis can be confined to foci and cause false negatives if these are not detected and sampled during necropsy (BORTEIRO *et al*., 2019), while in fatal infections it is widely distributed (BERGER *et al*., 2005). Finally, macroscopic symptoms such as mild skin redness were common in almost all individuals described here, although these symptoms are highly nonspecific and commonly shared between chytridiomycosis and other diseases like ranavirosis (MARSCHANG, 2019).

In mortality cases as those described here, the following rules could be used to confirm chytridiomycosis as the possible cause of death and to differentiate simple carriers from sick individuals ruling out other causes of mortality: (1) qPCR analyses have to confirm that sick individuals harbour much higher infection loads than asymptomatic co-occurring conspecifics; (2) a histopathological analysis has to confirm that skin lesions are associated with the presence of the fungus; and (3) the necropsy and the histological analysis of the rest of the tissues and viscera have to support the absence of other causes of illness or death.

The presence of *Batrachochytrium* in northeast Iberian Peninsula, including *Bsal* (MARTÍNEZ SILVESTRE *et al*., 2019), is highly concerning. In general, human global travel and commerce is strongly associated with the spill-over of pathogens via introduced alien species (DASZAK *et al*., 2000), and human activities are involved as the cause of the very recent introduction of

Bsal in Catalonia (Martínez-Silvestre *et al*., 2019; Martel *et al*., 2020). In the case of *Bd*, an extensive study has documented its spatiotemporal origins, dating the emergence of this pathogen to the early 20th century (O'Hanlon *et al*., 2018). Since *Bd* was already well distributed throughout Europe (Garner *et al*., 2009) when its emergence was reported in central Spain, it is reasonable to suppose that the introduction of *Bd* to the Iberian Peninsula occurred a long time ago.

Despite increased surveillance of amphibian populations in Spain during the last few years, no well-established protocols to detect disease outbreaks have yet been implemented in the country. As chytridiomycosis due to *Bd* and *Bsal* are listed by the World Organisation for Animal Health (OIE) as diseases of international concern, it is recommended to carry out, at least, routine controls by qualitative PCRs in selected amphibian populations, and histological analyses and quantitative PCRs when mortality cases are found. Rapid control, detection and action on amphibians found dead are critical action tools for reliable diagnosis. Only the adequate inclusion of these tools in field collection protocols will enable greater rates of detection of pathogens, and the possibility of better understanding the ecological repercussions of these diseases.

Acknowledgement

Daniel Guinart (Parc Natural del Montseny); Felix Amat (Museu de Granollers); Francesc Carbonell, Elena Obon, Joan Mayné (Centre de Fauna de Torreferrussa), Francisco Martínez (Forestal Catalana), Joaquim Soler, Zuleika Alonso and Martina Ugrinović (CRARC and Ajuntament de Masquefa), Eudald Pujol, Gerard Carbonell, Joan Maluquer, Oriol Baena, Fernando Loras, Joan Ferrer and Alejandro Garcia (Societat Catalana d'Herpetologia), Javier Burgos and Rubén Sánchez (Associació Herpetològica Timon); Aida Tarrago, Diego Martínez, Vanessa Cadenas, Ricard Casanovas, Francesc Mañas, Albert Sanz (Generalitat de Catalunya) and Cos d'Agents Rurals de la Generalitat de Catalunya. Part of this study is included in the Life Tritó del Montseny (LIFE15 / NAT/ES/000757).

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