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Presence of the Fungus *Batrachochytrium dendrobatidis*, but not *Batrachochytrium salamandrivorans*, in Wild Pyrenean Brook Newts (*Calotriton asper*) in Spain and France

In the last 20 years, the emergence of chytridiomycosis due to the chytrid fungi *Batrachochytrium dendrobatidis* (*Bd*), and the more recently described *Batrachochytrium salamandrivorans* (*Bsal*), has caused severe amphibian population regressions across the planet (Bosch et al. 2001; Spitzen-van der Sluijs et al. 2016; Scheele et al. 2019). This has generated an increase in scientific interest to decipher the complex interaction between the environment, the fungus and amphibian hosts, and increased surveillance efforts in many localities (Canessa et al. 2020). *Batrachochytrium* spp. affect the vital function of the amphibian skin, leading to lethargy or skin discoloration, hyperkeratosis, erosions (even ulcerations in *Bsal*) of the epidermis, and eventually death (Berger et al. 1998; Weldon et al. 2004; Stuart et al. 2004; Wake and Vredenburg 2010; Martel et al. 2013). *Bd* is currently found on all continents where amphibians are present (Skerratt et al. 2007), affecting more than 700 species within the three orders of amphibians and has been considered a major threat to amphibian biodiversity worldwide (Crawford et al. 2010; Fisher et al. 2012; Olson et al. 2013; Olson and Ronnenberg 2014).

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The first documented chytrid infection of wild amphibian populations in Europe dates from 1997 (Bosch et al. 2001) when mass mortality episodes of Common Midwife Toads (*Alytes obstetricans*) occurred due to *Bd* in a protected area in central Spain. Additional mass mortalities in the 2000s were reported mainly in Spain, including outbreaks in the Fire Salamander (*Salamandra salamandra*) and the Spiny Toad (*Bufo spinosus*; Bosch et al. 2001; Bosch and Martínez-Solano 2006). Some species are known to be potential asymptomatic carriers, tolerant to infections with little or no evidence of mortality, as in the American Bullfrog (*Lithobates catesbeianus*; Daszak et al. 2004). In other species, the emergence of *Bd* is related to high mortality events, as in the Common Midwife Toad (Bosch et al. 2001). Asymptomatic individuals are good candidates for maintaining *Bd* within the *Bd*-amphibian complex system (*Bd* reservoir hosts; Brannelly et al. 2017). Indeed, reservoir hosts can facilitate the global spread of *Bd* and pathogen transmission among other species (Haydon et al. 2002; Reeder et al. 2012). Currently, *Bd* is widely distributed all around Europe (including Mediterranean, central, and insular countries) with an exceptionally high prevalence in Spain (Garner et al. 2005).

More recently, *Bsal* was discovered in Europe, causing massive declines in salamander populations between 2010 and 2013 in the Netherlands (Martel et al. 2013, 2014). The fungus affects mainly salamanders, and anuran species seem to be tolerant of infections and can act as infection reservoirs for *Bsal* (Martel et al. 2014; Stegen et al. 2017). A 2016 study showed a significant range expansion of *Bsal* in native amphibian populations in the Netherlands, Germany, and Belgium (Spitzen-van der Sluijs et al. 2016), generating concerns that *Bsal* might soon colonize other countries, including France and Spain. Recently, a *Bsal* outbreak was recorded affecting *Triturus marmoratus* and *Salamandra salamandra* in Catalonia (Martínez-Silvestre et al. 2019; Martel et al. 2020).

Both *Bd* and *Bsal* scenarios in Europe become a high risk that threaten endemic and fragile European salamander species including, among others, species of the genus *Calotriton*. The Pyrenean Brook Newt, *Calotriton asper* (Dugès 1852), is a large-bodied salamander endemic to the Pyrenean Mountains, where

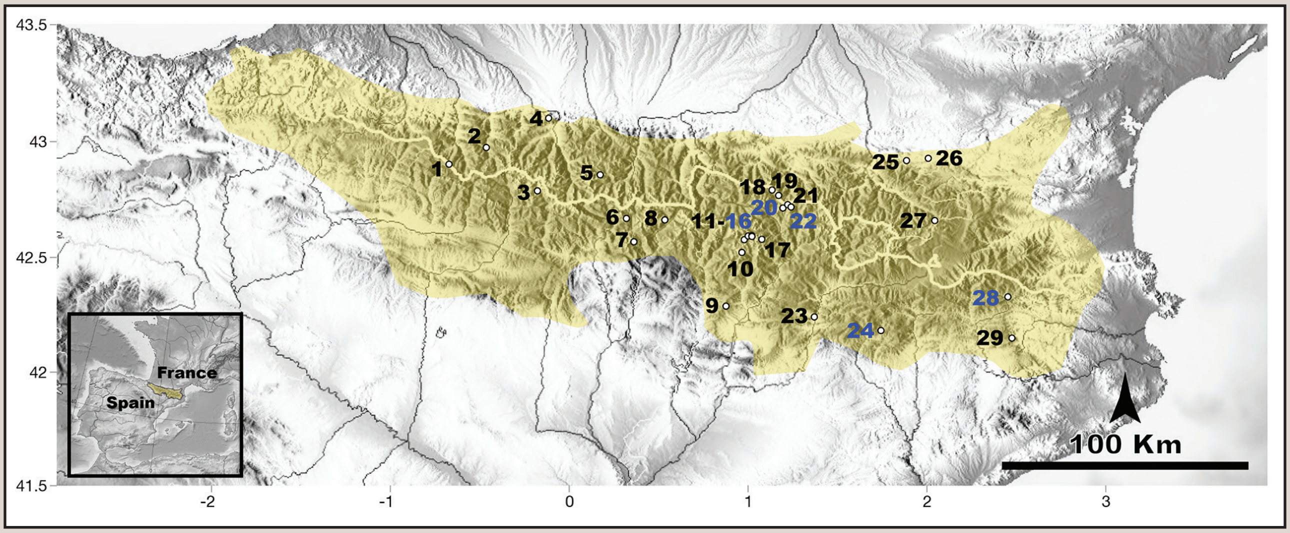

FIG. 1. Location of the 29 study populations of the Pyrenean Brook Newt (*Calotriton asper*) in the Pyrenees Mountains along the border of France and Spain, numbered from west to east (number codes, site names, and coordinates in Table 1). Yellow shading depicts the natural distribution area of the species. Blue numbers are locations with positive *Batrachochytrium dendrobatidis* samples.

FIG. 2. Emaciated Pyrenean Brook Newt (*Calotriton asper*) found in 2007 in Lake Acherito, Spain.

it occurs in brooks, lakes, springs, and aquatic caves, from 250 to 2500 m elev. (Clergue-Gazeau and Martinez-Rica 1978). The species is protected by national legislation, listed in Appendix II of the Bern Convention, Annex IV of the EU Habitats Directive, and classified as "near threatened" by the International Union for the Conservation of Nature (IUCN) Red List because of its vulnerability to climate change. To our knowledge, *Bsal* has not been reported in *C. asper* populations (Martínez Silvestre et al. 2017) and *Bd* infection has been reported in one study (Montori et al. 2019). In addition, *Bd* has been linked to mortality events in the related Sardinian Brook Newt (*Euproctus platycephalus*; Bovero et al. 2008).

Here, in order to advance understanding of the current infection status related to the presence of *Batrachochytrium* spp. in different populations, we tested for the presence/absence (qualitative analysis) of *Bd* and *Bsal* in 13 monitored populations and measured the *Bd* infection loads (quantitative analysis) of 16 populations of the Pyrenean Brook Newt in the French and Spanish Pyrenean Mountains and present details of a population health monitoring program.

Pyrenean Brook Newts from 29 French and Spanish populations were sampled for *Bd* and *Bsal* for this study (Table 1; Fig. 1). Animals were captured by hand during June–August of 2016 and 2017 and samples were collected *in situ* using external sterile skin swabs (Dryswab MW113, Medical Wire & Equipment, Corsham, England). Every animal was stroked 30 times to ensure the greatest amount of DNA was gathered on the swab. We swabbed the middle of the venter, both sides of the venter, and each thigh and foot of each animal. Samples collected in the field were kept dry and under ambient temperature. Upon return to the laboratory samples were stored at 4°C or frozen at -20ºC until processing. Some populations were resampled several times every 2 months during the same year in order to investigate potential variability in infection occurrence over time. In order to avoid spreading the pathogens within and among populations, during field work we followed the disinfection protocols recommended by Pessier and Mendelson (2017).

A subset of field-tested animals were used for a laboratory experimental study. We collected animals from 10 populations (Spain: Rivert, Vall Fosca, Organyà, Camprodon, and Hostalets d'en Bas; France: Fontestorbes, Belesta, Orlu, Néouvielle, and Salau) and housed them in the laboratory separately by population of origin for six months to carry out a parallel study of thermal preferences (Trochet et al. 2018). During this time, data about sloughing frequency, feeding, and performance activity were taken. Captive animals were returned to the wild after confirming PCR-negative results for chytrid fungi and for ranavirus. Ranavirus prerelease PCR analysis was performed only in these captive animals from cutaneous samples and following the international rules to avoid a negative impact on wild amphibian populations and which can inadvertently spread pathogens via released animals (Daszak et al. 1999). To avoid mixing individuals and populations in the study, these animals were identified by a subcutaneous PIT tag and released at the same sampling points. We examined these 189 animals for disease symptoms across the time span of their six-month captivity.

Samples from the Orlu, Rivert, and Organyà populations were analyzed as pools of a maximum of 4–5 swabs/sample following published recommendations (Hyatt et al. 2007), whereas all other samples were analyzed at the level of individual swabs. Laboratory analyses were performed at Museo Nacional de Ciencias Naturales-CSIC (Spain) or LABOKLIN laboratories (Bad Kissingen, Germany). DNA was extracted with the Roche MagNA

Table 1. Characteristics of the Pyrenean Brook Newt, *Calotriton asper*, populations studied for the detection of *Batrachochytrium dendrobatidis* (*Bd*) and *B. salamandrivorans* (*Bsal*). The table shows the code (number in the map) and site name of each population (from west to east), the number of sampled individuals (N), the altitude (meters above sea level), the geographic coordinates (referred to the centroid of the UTM location (10 km × 10 km)), the sampling date, and the number of samples positive for *Bd*. All samples were negative for *Bsal*.

Code	Site Name	Place	N	Altitude	Latitude	Longitude	Side	Sample date: Day/Mo/Yr	% *Bd* -positive samples
1	Lake Acherito	Lake	6	1872	42.88089	-0.70608	Spain	17/08/16	–
2	Eaux-Chaudes	Stream	12	854	42.95300	-0.49761	France	15/07/17	
3	Lake Serrato Alto	Lake	4	2459	42.76581	-0.21160	Spain	19/08/16	–
4	St-Pé-de-Bigorre	Stream	21	582	43.07941	-0.14894	France	20/07/17	
5	Néouvielle	Lake	20	2189	42.83526	0.13947	France	25/07/17	
6	Lake Barleto Alto	Lake	5	2531	42.64654	0.28567	Spain	19/08/16	–
7	Lake de la Mora	Lake	5	1903	42.54526	0.32771	Spain	18/08/16	–
8	Lake Perramó	Lake	5	2254	42.64033	0.50084	Spain	24/08/16	–
9	Rivert	Stream	41	893	42.26569	0.84278	Spain	29/07/17	–
10	Riqüerna	Stream	20	2400	42.49904	0.93161	Spain	13/06/17	–
11	Lake Mig Dellui	Lake	2	2314	42.55451	0.94468	Spain	19/07/16	–
12	Lake Natural Dellui	Lake	38	2316	42.55231	0.94501	Spain	19/07/16	–
								14/07/17	–
								01/07/17	–
13	Coma Peixerani	Stream	20	2205	42.57065	0.96661	Spain	24/08/17	–
14	Subenuix river	Stream	20	2260	42.56892	0.98682	Spain	17/08/17	–
15	Lake Subenuix	Lake	3	2195	42.57199	0.98735	Spain	07/08/17	–
16	Lake Xic Subenuix	Lake	46	2272	42.56845	0.98774	Spain	14/07/16	4.3
								13/07/17	–
								07/08/17	–
17	Vall Fosca	Stream	19	1828	42.55612	1.04297	Spain	30/06/17	–
18	Lake Aula	Lake	5	2128	42.76951	1.10045	Spain	15/07/16	–
19	Salau	Stream	20	1258	42.74596	1.13705	France	22/07/17	–
20	Buixase	Lake	21	2246	42.69165	1.16103	Spain	22/06/17	10
21	Gallina inferior	Lake	5	2268	42.70618	1.18763	Spain	16/07/16	–
22	Lake Vedós	Lake	5	2263	42.69591	1.20659	Spain	17/07/16	20
23	Organyà	Stream	20	773	42.21870	1.33656	Spain	01/07/17	
24	Ensija	Stream	14	1600	42.15943	1.70920	Spain	12/09/16	–
								03/07/17	–
								07/06/18	20
25	Fontestorbes	Stream	22	613	42.89675	1.85220	France	25/07/17	
26	Belesta	Stream	25	795	42.90644	1.97375	Spain	23/07/17	
27	Orlu	Stream	20	1313	42.63730	2.01010	France	19/07/17	
28	Camprodon	Stream	22	979	42.30617	2.41901	Spain	03/07/17	10
								13/12/17	–
29	Hostalets d'en Bas	Stream	20	625	42.12652	2.44156	Spain	02/07/17	–

Pure 96 system using the MagNa Pure 96 DNA and Viral NA small volume kit according to the manufacturer's instructions or using the PrepMan Ultra Reagent Protocol as described by Boyle et al. (2004). The presence of *Bd* and *Bsal* was assessed using quantitative PCR (qPCR) protocols as described by Boyle et al. (2004) and Blooi et al. (2013). Samples were analyzed independently (the same DNA extract but with two different qPCRs).

A total of 486 animals were sampled for *Bd* and *Bsal* from the 29 study populations (Table 1; Fig. 1). *Bsal* was not detected in any of the study populations, whereas *Bd* was found in five Spanish populations (Camprodon, Ensija, Lake Xic de Subenuix, Lake de Vedós, and Buixase). The overall *Bd* prevalence was 3.29% (16 of 486 animals tested *Bd*-positive) with infections occurring at 5 of 29 (17.2%) sites. No specific signs of disease were detected in any animal during collection of field samples, and no mass-mortality episode linked to *Bd* or *Bsal* has been detected in the species so far. Infected animals harbored very small infection loads (ranging from 0.1 genomic equivalents [GE] at Buixase and Lake de Vedos to 39.2 GE at Lake Xic de Subenuix), with no apparent pathological effect.

Of the five *Bd*-positive populations, three (Camprodon, Ensija, and Lake Xic de Subenuix) were resampled to examine disease occurrence over time. These samples were taken every 6–12 months after first sampling. Newts at Camprodon were sampled two times; the first sample was *Bd*-positive and the second was *Bd*-negative. Increased skin-slough frequency was observed in the captive positive Camprodon population during these two resampling occasions. Newts at Ensija were sampled three times; the first sample was *Bd*-negative, the second was *Bd*-positive, and the third sample was *Bd*-negative. Lake Xic de Subenuix was sampled three times, the first resample was *Bd*-positive and the second and third samples were *Bd*-negative. Skin sloughing was not observed at Ensija or Lake Xic de Subenuix over the course of multiple sampling occasions.

For the laboratory experiment, 159 of the 496 field-sampled Pyrenean Brook Newts were retained in captivity for six months. Twenty captive animals came from *Bd*-positive populations. No specific signs of disease or skin sloughing were detected in any of these laboratory animals. As mentioned before, only the positive Camprodon population was observed with extra sloughing behavior.

Our study is the first comprehensive study of *Bd* and *Bsal* infection in wild populations of Pyrenean Brook Newts from both sides of the Pyrenees. Our *Bd*-infection data are lower than the previously described prevalence in Spanish *C. asper* estimated from different unpublished analyses and compiled for the world Bd-maps database by the Global *Bd* Mapping Project (2018; D. H. Olson, pers. comm.) which was estimated to be 19% (4 of 21 sampled animals were *Bd*-positive) with an occurrence of 57% (4 of 7 sites). However, annual fluctuations in the prevalence of infection, and even changes in the infection status of adult individuals have been described, for example in Spiny Toads (*Bufo spinosus*) in central Spain (Daversa et al. 2018). Our infection rates are highly variable, and in three areas sampled repeatedly over the course of the study, positive detection was only achieved once per population. There was no apparent causal mechanism, such as correlated extreme weather conditions, for the alternating appearance of positive and negative test results. Similar variability in detection has also been reported in amphibians of the Sierra Nevada (California, USA; Briggs et al. 2020). Only one previous internal report showed a single individual found in 2007 in one of our study locations (Lake Acherito) that was positive for *Bd* and presented signs not specific for chytridiomycosis, including emaciation, cachexia, and malnutrition (J. Bosch, unpubl. data).

Despite its conservation status, species of *Calotriton* (previously placed in *Euproctus*) have received little scientific attention with respect to the emergence of the chytrid fungi. Bovero et al. (2008) recorded *Bd* in a species related to *C. asper*, the Sardinian Brook Newt (*Euproctus platycephalus)* following a series of mass die-offs, highlighting its vulnerability to chytridiomycosis. On the other hand, a recent study failed to find *Bd*-infected populations either in wild or captive Montseny Brook Newts (*Calotriton arnoldi*) in Spain (Obón et al. 2013).

To date, symptomatic Pyrenean Brook Newts have rarely been observed. Nevertheless, external parasites, thinner individuals, or individuals with physiological abnormalities of unknown origin have been reported on occasion (Dalibard et al. 2020; Trochet A., Calvez O., and Deluen M., unpubl. data) both in wild populations and in experimental conditions. Mass mortalities of other amphibians (Common Frog, *Rana temporaria;* Common Midwife Toad, *Alytes obstetricans*; Fire Salamander, *Salamandra salamandra*) have been reported in the Pyrenees, partly attributable to ranavirus and *Bd* (Walker et al. 2010). Another survey was conducted in the French Alps between 2012 and 2013 and seemed to indicate that Common Frogs were not infected by *Bd* (Miaud et al. 2016). In some cases, fortuitous detection of *Bd* has been reported in specimens that died due to other causes. For example, in Lake Perramó several Pyrenean Brook Newts died from traumatic causes and were also positive for *Bd*. Nonetheless, no direct connection between the fungus and the cause of death could be made (Amat et al. 2018).

The geographic distribution of *Bd* in the Pyrenean Brook Newt does not overlap with the previously reported *Bd* distribution in other amphibian species in the Pyrenees. Indeed, all Pyrenean Brook Newts from Néouvielle (2000 m elev.) tested negative for *Bd* despite being the site of one of the most important outbreaks of mortality associated with *Bd* in the Pyrenees for Common Midwife Toads and Common Frogs in 2010 (Clare et al. 2016) and for Common Midwife Toads in 2011 (Schmeller et al. 2014). Subsequently, in 2019, several Pyrenean Brook Newts in Néouvielle were found with ulcerative eye injuries but were negative for *Bd*, *Bsal*, and ranavirus (A. Martínez-Silvestre, unpubl. data).

In our study, infected Pyrenean Brook Newts did not display any of the typical signs of chytridiomycosis. Only one sick individual mentioned above in one of our study locations (Lake Acherito) was positive for *Bd* in 2007 (Fig. 2). Indeed, it is possible that *Bd* was present as a co-infection with another pathogen such as ranavirus, which is also present in this area (J. Bosch, unpubl. data). Unfortunately, no further sanitary tests were carried out on that specimen. Therefore, given the lack of observed morbidity and mortality, we can assume that the Pyrenean Brook Newts testing positive here were asymptomatic carriers of *Bd*.

The low amount of *Bd* found in the positive samples may have been influenced by a number of factors, including warming water temperatures, immune response, and increased sloughing rates. Consequently, the season of collection of samples (summer) may also have affected the detected load. Variation in water temperatures may affect both the immune status of animals and the level of infection in amphibians (Greenspan et al. 2017), and it could similarly affect Pyrenean Brook Newts. According to our results, we have not observed differences in detection at different altitudes, or between streams and lakes. Temperature has been shown to influence amphibian susceptibility to disease caused by *Bd* (Berger et al. 1998; Lips 1998; Bosch et al. 2001; Young et al. 2001; Stuart et al. 2004; Woodhams and Alford 2005), and high-altitude populations have been found to be more vulnerable to the disease (Walker et al. 2010). Even if sampled individuals did not show clinical signs of the disease, *Bd* virulence could be greater at higher altitudes, affecting the vital functions of the skin of individuals and ultimately endangering the population. Prediction models show that the timing of spring thaw in this region will advance markedly by the 2050s, indicating that climate change may further the severity and impacts of infection (Clare et al. 2016). Since we expect high-altitude populations to be more susceptible to the disease, long-term monitoring along the altitudinal gradient is warranted.

Finally, it is important to consider that sloughing rates vary significantly between phylogenetic groups and presently cannot be related to available evidence of *Bd*-driven declines in anurans (Ohmer et al. 2019). However, variation in skin-sloughing frequency may play a role in the intraspecific variation in susceptibility to the disease (Ohmer et al. 2015). An increase in sloughing rate in amphibians with lower infection loads can reduce *Bd* loads up to 100%, leading to infection clearance (Ohmer et al. 2017). This finding is in accordance with the increased sloughing behavior observed in our captive positive animals.

We found a higher prevalence of *Bd* in the Spanish Pyrenees than in the French Pyrenees, but also in the eastern rather than western part of our study area. Walker et al. (2010) reported opposite findings in Common Midwife Toads with just positive results in the western Pyrenees but in both slopes. Therefore, our results do not support their hypothesis that different climates across the longitudinal gradient could be a determining factor for *Bd* prevalence and pathogenesis, thus leading to specific differences in frequency of symptomatic and asymptomatic individuals.

The recent emergence of *Bsal* in European urodeles (Martel et al. 2013) raises the question of the vulnerability of the Pyrenean Brook Newt. This pathogen causes severe skin infections and was first reported in Fire Salamanders in the Netherlands (Martel et al. 2013; Spitzen-van der Sluijs et al. 2016). Moreover, this pathogen has been introduced in Montnegre Natural Park (Catalonia, Spain; Martel et al. 2020), a region less than 100 km from the Pyrenees and close to the distribution area of *C. asper*. The prevalence observed in infested populations may be affected by the virulence of the disease, since in case of high virulence there would be less chance to collect dead or ill individuals. However, the detection of *Bsal* also occurs in animals with low loads, asymptomatic and carriers (Martel et al. 2020), which makes it possible to ensure that the disease is not yet affecting wild populations of Pyrenean Brook Newt. In order to inform future conservation of this Pyrenean endemic species, we recommend prompt monitoring of the *Bd*-positive populations reported here to: 1) follow the propagation of *Bd* among Pyrenean Brook Newt populations, as well as the likely arrival of *Bsal* in amphibian populations in the Pyrenees; 2) scrutinize populations for potential clinical signs of chytridiomycosis in this species; and 3) confirm whether the populations of *Bd*-positive but asymptomatic Pyrenean Brook Newts act as an infection reservoir for sympatric anurans.

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