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Observations on the intensity and prevalence of *Batrachochytridium dendrobatidis* in sympatric and allopatric *Epidalea calamita* (native) and *Discoglossus pictus* (invasive) populations

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We analyse if the presence of the invasive *Discoglossus pictus* and the native *Epidalea calamita* in the same geographic area can shape the dynamics of infection by *Batrachochytridium dendrobatidis* (Bd). Both amphibian species share breeding habitat preferences in the area (i.e. ephemeral and temporary ponds) and are common syntopic competitors. We sampled adults of either species in a total of nine breeding localities in Catalonia (NE Iberian Peninsula): six localities within sympatric geographic areas, two localities outside the current invasion range of *D. pictus*, and one locality comprising solely *D. pictus* due to its recent introduction and the ancient extinction of *E. calamita*. We analysed the prevalence and intensity of infection by Bd in 183 individuals (81 *D. pictus* and 101 *E. calamita*). The presence of Bd was detected in eight of the localities, all of which had not been yet confirmed as positive for the fungus. Only in one locality, with presence of *D. pictus*, Bd presence was not detected. Mean prevalence was slightly but non-significantly higher in *E. calamita* (46.5%) than in *D. pictus* (36.6%). Allopatric populations of *E. calamita* showed significantly lower prevalence of Bd infection than sympatric ones, but similar differences were not found for infection intensity. Likewise, no significant differences were found in infection intensity between allopatric and sympatric *D. pictus* populations. The presence of abundant Bd outside the invasion range of *D. pictus* supports the assertion that Bd has not been introduced in the studied area because of *D. pictus* invasion. However, the presence of the two species in sympatry may enhance the infection rates and therefore the prevalence of Bd in the native species.

Key words: Chytridiomycosis; *Discoglossus pictus*; *Epidalea calamita*; invasive species.

Infectious diseases are a well-known driver of animal declines worldwide (Fisher *et al*., 2012). Population declines caused by fungal diseases have been reported in bats, corals, bees, snakes and amphibians (Fisher *et al*., 2012). Particular-

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ly, amphibians have been exceptionally impacted by emerging infectious diseases (EID, DASZAK *et al.*, 1999; SKERRAT *et al.*, 2007), with observations of massive pathogen-associated amphibian die-offs dating back to the 1970s and 80s (BARRINAGA, 1990). Chytridiomycosis is one of those EID (DASZAK *et al.*, 2000), caused by the fungal pathogen *Batrachochytrium dendrobatidis* (Bd). Although globally the number of species affected by habitat loss and degradation is higher than species affected by EID, Bd is considered to be one of the major causes of amphibian declines (LIPS *et al.*, 2006; SKERRATT *et al.*, 2007). Nowadays, chytridiomycosis is a global threat, related to the mass decline and extinction of amphibian populations worldwide (BERGER *et al.*, 1998; BOSCH *et al.*, 2001; RACHOWICZ *et al.*, 2006). This EID is considered as the most devastating ongoing vertebrate disease (BRANNELLY *et al.*, 2018).

The pathogen infects over 500 species of amphibians (GLOBAL BD MAPPING GROUP, 2018) and is found in all continents except Antarctica. In Europe, Bd was first recorded causing mass amphibian die-offs during the summer of 1997 (BOSCH *et al.*, 2001), and is widely distributed nowadays. It has been found in Spain, Portugal, Italy, Germany, Belgium, Netherlands, Great Britain, France, Switzerland, Austria, Hungary, Denmark and Sweden (e.g. GARNER *et al.*, 2005; SPITZEN-VAN DER SLUIJS & ZOLLINGER, 2010). Spain is the European country where Bd distribution is best known (Table 1), with a total of 345 sites sampled so far (GLOBAL BD MAPPING GROUP, 2018).To date, Bd has been found infecting 22 amphibian species in Spain (Table 1), causing mass mortality to at least three of them: the common midwife toad (*Alytes obstetricans*), the fire salamander (*Salamandra salamandra*) and the common toad (*Bufo spinosus*) (BOSCH *et al.*, 2001; BOSCH & MARTINEZ-SOLANO, 2006).

The composition and the dynamics of the host community can affect host-pathogen dynamics. For instance, it has been noted that a high diversity of amphibians can dilute the effects of Bd (SEARLE *et al.*, 2011). The dilution effect occurs when biodiversity and disease risk are inversely related, which can occur through a reduction in the encounter rates between susceptible and infectious individuals. By contrast, the near extinction of *A. obstetricans* by Bd in the Peñalara mountains (central Spain) increased the presence of *B. spinosus*, promoting the spread and maintenance of the fungus to new areas (BOSCH et al 2014). According to these possible scenarios, invasive species may help to dilute the effects of Bd due to the increase in diversity, assuming that those invasive species do not reduce the native species richness. By contrast, when there is a positive relationship between biodiversity and disease risk, the invasive species may help to expand and enhance the presence of the fungus in the invaded areas (MIAUD *et al.*, 2016). In that case, the presence of the invasive species has an amplification effect. For example, the invasive North American bullfrog (*Lithobates catesbeianus)* is known to act as a host reservoir of Bd (MAZZONI *et al.*, 2003; DASZAK *et al.*, 2004; MIAUD *et al.*, 2016; BORZÉE *et al.*, 2017), while CUNNINGHAM & MINTING (2008) found a strong association between the presence of non-native amphibian species and Bd infection in the UK.

Species can act differently relative to

Table 1: Prevalence of the amphibian chytrid fungus *Batrachochytridium dendrobatidis* in Spanish amphibians estimated from published studies compiled by GLOBAL BD MAPPING GROUP (2018). (1) Each entry from the database was considered as a different site. (2) Data from BOSCH *et al.* (2013). (3) Data from the present study.

Species	N samples	N positive samples	Prevalence	N sites (1)	N positive sites	Occurrence
Salamandra salamandra	139	47	33.81	43	14	32.56
Pleurodeles waltl	9	9	100.00	5	5	100.00
Calotriton asper	21	4	19.05	7	4	57.14
Triturus marmoratus	21	2	9.52	8	2	25.00
Triturus pygmaeus	22	13	59.09	11	5	45.45
Mesotriton alpestris	83	23	27.71	29	14	48.28
Lissotriton helveticus	19	3	15.79	7	3	42.86
Discoglossus jeanneae	2	1	50.00	2	1	50.00
Alytes dickhilleni	36	0	0.00	10	0	0.00
Alytes dickhilleni(2)	461	43	9.33	30	3	10.00
Alytes muletensis	589	78	13.24	48	10	20.83
Alytes obstetricans	1456	468	32.14	174	57	32.76
Bufo spinosus	246	74	30.08	45	15	33.33
Epidalea calamita	93	4	4.30	31	3	9.68
Bufotes balearicus	54	0	0.00	5	0	0.00
Hyla molleri	24	3	12.50	6	2	33.33
Hyla meridionalis	19	17	89.47	11	10	90.91
Pelobates cultripes	12	7	58.33	8	5	62.50
Pelodytes punctatus	10	0	0.00	4	0	0.00
Rana iberica	9	2	22.22	5	2	40.00
Rana pyrenaica	9	4	44.44	3	2	66.67
Rana temporaria	72	1	1.39	27	1	3.70
Pelophylax perezi	67	26	38.81	31	19	61.29
Total	3012	786	26.10	520	174	33.46
Discoglossus pictus(3)	82	30	36.58	8	6	75.00
Epidalea calamita(3)	101	47	46.53	6	6	100.00

the risk of infection, either as reservoir hosts or as resistant species. Reservoir hosts are species that can harbour a pathogen by transmitting it among themselves and to other taxa, thus maintaining the pathogen within the ecosystem in the absence or with a low density of susceptible hosts. Resistant species, in contrast, are refractory to infection or quickly clear infection, and thereby have a limited potential to maintaining a disease within the system (BRANNELLY *et al.*, 2018). In this way, those invasive amphibian species that act as reservoir hosts could multiply their negative impact on native amphibian species by adding increased infection risk to the well-known competition effect.

The Mediterranean Painted Frog (*Discoglossus pictus)* is an invasive species that has spread in the northeast of the Iberian Peninsula. This species is a north African amphibian introduced to southern France in the early 20th century that became invasive and spread up to 170 km southwards and northwards along the Mediterranean coastal plains (MONTORI *et al.*, 2007; LLORENTE *et al.*, 2015). The species usually co-occurs with native anurans that breed in ephemeral and temporary ponds, sharing these breeding sites mostly with natterjack toads (*Epidalea calamita*) and common parsley frogs (*Pelodytes punctatus*) (ESCORIZA & BOIX, 2012; RICHTER-BOIX *et al.*, 2013). Because the invasive species represents an addition, in terms of diversity and biomass, to the community of many ephemeral and temporary ponds from the area, the aims of this study were (i) to investigate Bd presence in the invasive species *D. pictus* and the native species *E. calamita* from the same geographic area, and (ii) to analyse if the presence of *D. pictus* in sympatry modifies the prevalence or intensity of Bd infection in *E. calamita*. Given that, during the many years we have been prospecting the study area, we have not observed any *D. pictus* dead because of Bd infection, we assume that the invasive species is a tolerant host. According to previous literature on tolerant host invasive species, like *L. catesbeianus* and the results obtained by CUNNINGHAM & MINTING (2008) in the UK, we hypothesized that Bd infection prevalence in the native species should increase in the presence of the invasive *D. pictus*, acting the invasive species as a reservoir host of the fungal pathogen.

MATERIALS AND METHODS

Sample collection and processing

From February 2010 to May 2013, we carried out a survey on selected aquatic environments previously identified as suitable breeding sites of *D. pictus* and / or *E. calamita* in the north-eastern region of the Iberian Peninsula. We sampled adults of either species from a total of nine ephemeral and temporary ponds (Fig. 1). Six of these sites were located in geographic areas that comprised populations of both species; we obtained samples from both species at one of the sites (05), samples from *D. pictus* only at three sites (01, 02 and 03), and samples from *E. calamita* only at two sites (06 and 09) (Table 2). Two additional localities were outside the invasion range of *D. pictus* and had the regular presence of *E. calamita* only, and another one had only *D. pictus* due to the extinction of *E. calamita* from the area during the third quarter of the last century (MONTORI *et al.*, 2009) (Fig. 1, Table 2). MINTING (2012) found that detection of Bd in adult amphibians is increased when animals are in the aquatic phase, and so all individuals sampled in the present study were captured from aquatic habitats during the reproductive period. In total, we captured 198 individuals, 84 *D. pictus* and 114 *E. calamita*.

We collected a sample of skin tissue by rubbing a sterile cotton swab (MW100-100; Medical Wire & Equipment Co, Cors-

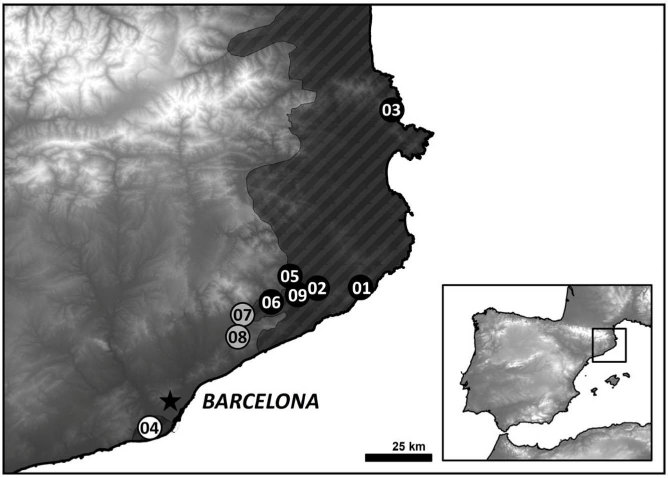

Figure 1: Location of sampling sites and current distribution of *Discoglossus pictus* in the Iberian Peninsula (shadowed area). Black circle represent sites where both species occur in sympatry, grey circles represent sites where only *E. calamita* allopatric populations occur and the white circle represents the site where only the *D. pictus* allopatric population occurs. For details on names and coordinates of the sites, see Table 2.

Table 2: Detail on sampling sites and Bd prevalence (in %) and intensity (mean and range, excluding zeros, of zoospore genome equivalents) in *Discoglossus pictus* (DP) and *Epidalea calamita* (EP) individuals sampled in Catalonia. S/A: sympatric (S) or allopatric (A) population with the other species. (*) Population introduced in the Llobregat River delta from an unknown northernmost population .

Sp	Code	Locality	UTM x	UTM y	S/A	N	Prev	GE
DP	01	Solius	497511	4630028	S	21	0	0
DP	02	Vidreres	480427	4626272	S	19	89.5	80.4 (4-420)
DP	03	Portbou	510841	4697190	S	4	75.0	83.3 (10-200)
DP	04	El Prat de Llobregat	424754	4571162	A*	11	54.5	66.51 (1-370)
DP	05	Riudarenes	476581	4630824	S	25	12.0	1323,5 (0,5-3930)
EC	06	St. Feliu de Buixalleu	466582	4620440	S	20	75.0	60.13 (3.7-500)
EC	07	Sant Çeloni	459557	4616686	A	21	4.8	0.4 (0.4-0.4)
EC	08	Llinars del Vallès	453001	4612353	A	14	28.6	1.5 (0.2-3.2)
EC	09	Sils	476935	4629315	S	18	77.8	20.8 (3.5-110)
EC	05	Riudarenes	476581	4630824	S	28	46.4	3653,3 (0,5-18280)

ham, UK), over the ventral side of the body and thighs (20 strokes) and the webbing of the hind feet (10 strokes), consistent with standard swabbing protocols. Samples were stored at 4ºC until processing and DNA was extracted with PrepMan® Ultra (Thermo Fisher, Waltham, Massachusetts, USA) following BOYLE *et al.* (2004). Extractions were diluted 1:10 in Milli-Q-grade water before real-time PCR amplification, performed in duplicate with a CFX96 thermocycler (Bio-Rad, Hercules, California, USA) following BOYLE *et al.* (2004). Each 96-well assay plate included samples, a negative control and standards of 100, 10, 1, and 0.1 Bd zoospore genome equivalents (GE) in duplicate. Samples were considered positive when both replicates were ≥ 0.1 GE and the amplification curves had the typical sigmoidal shape. When only one of the replicates of a sample amplified, we ran this sample a third time. If the third run did not result in an amplification profile, we considered the sample as negative for infection. Samples that showed signs of inhibition (non-sigmoidal amplification) were further diluted to 1:100 and re-analysed. If signs of inhibition remained, the samples were excluded from the analyses.

Statistical analysis

Prevalence of Bd-infected animals was calculated by dividing the number of individuals positive for Bd by the corresponding number of individuals that we swabbed in the population. We fitted binomial models for the number of infected and uninfected individuals per population (1: infected individual, 0: uninfected individual) to detect differences in prevalence between species and among areas. Using the package *nlme* (PINHEIRO *et al.*, 2018), we fitted a global model including species and geographic status (sympatric vs. allopatric), and all possible nested simpler models, including a null model. We ordered all models according to the corrected Akaike Information Criterion (AICc) and tested the relevant pairs of models against each other using likelihood ratio tests (LRTs). Since interactions between species and distribution status were significant (see Results), we explored the differences between distribution statuses separately for each species by fitting a binomial model that included distribution status as explanatory variable. This model was tested using a LRT against a null model. Prevalence analyses were conducted using R (R CORE TEAM, 2018).

Intensity analyses were performed using QP 3.0 software (Quantitative Parasitology 3.0, Budapest; RÓZSA *et al.*, 2000; REICZIGEL & RÓZSA, 2005), comparing the following pairs of groups: (i) *E. calamita* total vs. *D. pictus* total, (ii) *E. calamita* allopatric vs. sympatric and (iii) *D. pictus* allopatric vs. sympatric. We use de Mood's median test to compare median intensities and the test of two-sample comparison of mean crowding of 97.5% of confidence limits with 2000 bootstrap replications.

RESULTS

Of the 198 adults captured, 183 yielded conclusive analyses (81 *D. pictus* and 101 *E. calamita*). Fifteen samples continued showing signs of inhibition after having been diluted 1:100, and were therefore eliminated from the analyses. Only one of the sampled populations, that of *D. pictus* from Solius (site 01, Table 2), did not give any positive result for Bd presence, whereas all the analysed *E. calamita* populations in both sympatry and allopatry tested positive for Bd (Table 2).

The best model explaining the prevalence of infection in a sampled population included the species, distribution status (allopatric or sympatric), and their interaction, signalling that populations of each species react differently to their distribution status (Table 3). This model was the best according to AICc and was significantly better than any other tested option, as corroborated by LRTs (Table 3). Mean prevalence of Bd infection was higher in *E. calamita* than in *D. pictus* (46.5% vs. 36.6%), although this difference cannot be considered as statistically significant because the model including only the species was worse, according to AICc, than the null model, and its LRT against the null model was above the significance threshold (P = 0.163). Differences between allopatric and sympatric populations of a given species were found for *E. calamita* ($P < 0.001$) but not for *D.* pictus ($P = 0.182$).

Table 3: Selection of the best-fit model, according to the corrected Akaike Information Criterion (AICc), to explain the prevalence of Bd infection in sampled populations as a function of the species and distribution status. (*) Refers to interaction between terms and (+) refers to additive effects.

Model	**df**	**AICc**	**Δ(AICc)**
prevalence ~ distribution status * species	4	103.3	0
prevalence ~ distribution status + species	3	111.8	8.55
prevalence ~ distribution status	2	112.3	9.06
null model	1	117.8	14.52
prevalence ~ species	2	119.1	15.79

Infection intensity was not significantly different between the two species (mean ± SD GE: *E. calamita* = 482.1 ± 2178.3, *D. pictus* = 77.9 ± 438.2; P = 0.147). The same results were obtained by comparing allopatric and sympatric populations of *E. calamita*. De Mood's median test comparing median intensities was not significant (P = 0.060) despite mean GE in allopatric *E. calamita* populations (1.40 ± 0.89) being much lower than in sympatric ones (1159.26 ± 3281.69). By contrast, the two-sample comparison of mean crowding of distributions between *E. calamita* allopatric and sym-

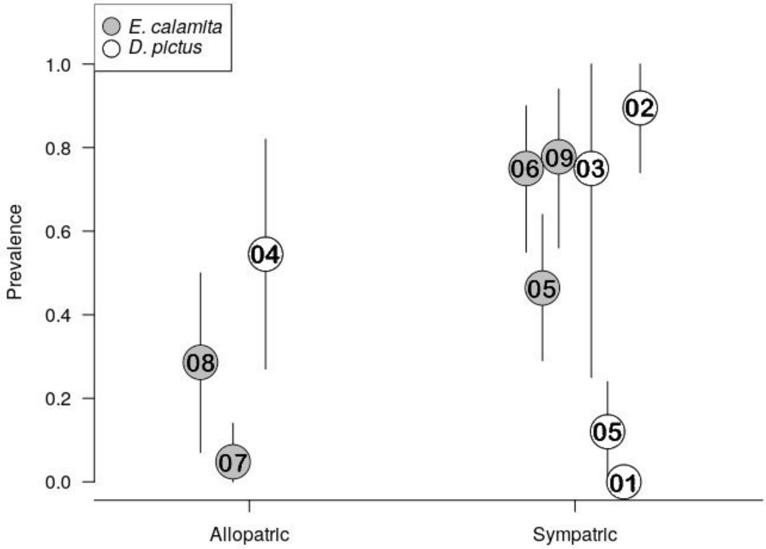

Figure 2: Prevalence (proportion of infected individuals) of Bd in *Epidalea calamita* and *Discoglossus pictus* populations sampled in the present study. Vertical lines indicate the 95 % confidence intervals. For details on site characteristics and location, see Figure 1 and Table 2.

patric populations indicated that the 97.5 % confidence intervals did not overlap, and therefore the difference between the two samples is significant (*P* < 0.05) (Fig. 2).

No significant differences were found in intensities between both allopatric and sympatric *D. pictus* populations according to either the de Mood's median test (mean ± SD GE: allopatric = 66.7 ± 148.7, sympatric = 7212.9 ± 711.8; *P* = 0.182) or the mean crowding 97.5 % confidence intervals (*P* > 0.05).

DISCUSSION

The results of our sampling show eight new localities with confirmed presence of Bd in northeast Iberian Peninsula. Hitherto, only a positive detection of Bd in *D. pictus* in Aiguamolls de l'Empordà (SAURA-MAS *et al.*, 2016) and eight in Iberian green frog (*Pelophylax perezi*) had been reported (FERNÁNDEZ-GUIBERTEAU *et al.*, 2016; MIRAS *et al.*, 2017) in Catalonia. These new localizations indicate that the Bd presence in Catalonia could be more spread than currently assumed.

The anthropogenic spread of pathogens has been responsible for the emergence of the known agents of amphibian chytridiomycosis, *B. dendrobatidis* and *B. salamandrivorans* (CUNNINGHAM *et al.*, 2017). Species invasions and commercial trade of amphibians have been demonstrated to provide a vehicle for the global movement of amphibian hosts and their associated pathogens (PICCO & COLLINS, 2008). It has even been hypothesized that the invasive bullfrog is responsible for the dispersal of the hypervirulent Bd-GPL lineage globally (SCHLOEGEL *et al.*, 2012). The alien nature and captive origins of the *D. pictus* populations in southern Europe could suggest a certain role of the species as a reservoir or enhancer of the presence of Bd in the invaded areas. Despite the numerous cases of the so-called "pathogen pollution" via invasive species, our data indicate that Bd was present in the area before contact between the two analysed species because both allopatric and sympatric populations of *E. calamita* are infected. This result is consistent with various positive detections of Bd in other species and populations from the area (FERNÁNDEZ-GUIBERTEAU *et al.*, 2016), and the detection of Bd in many other parts of the Iberian Peninsula where the introduced species *D. pictus* is not present (GARNER *et al.*, 2005; WALKER et al. 2010). Unfortunately, while there is a consensus in the Algerian origin of the *D. pictus* populations introduced in Europe (ZANGARI *et al.*, 2006; MONTORI *et al.*, 2007; LLORENTE *et al.*, 2015), there are no existing data of Bd in Algeria. Data on a nearby and similar species (*Discoglossus scovazzi*, in Morocco), reflect a level of infection prevalence (8.3%) clearly lower than that found in our study (36.6%), but with similar values of intensity (mean: 77.9 GE, range: 10-100 GE) (EL MOUDEN *et al.*, 2011), suggesting that *Discoglossus* from northern Africa are not a reservoir of Bd. In the same genus, unusual mortalities of the Tyrrhenian painted frog (*Discoglossus sardus*) were found at three sites in the Limbara Mountains of northern Sardinia (BIELBY *et al.*, 2009). These authors indicated that infection and mortality occurred at locations with characteristics that are not consistent with the published habitat requirements of the pathogen (Bd), and that *D. sardus* would be a primary host for Bd, unlike *D. pictus* in NE Iberian Peninsula.

In our study, we observed lower Bd prevalence in areas where there is only one of the two analysed species (Fig. 2) and a trend towards lower intensities in allopatric populations of both species compared with sympatric ones. These results could point to cross infection when both species coexist in the same geographic area. This would agree with the results of FERNÁNDEZ-BEASKOETXEA *et al.* (2016), who experimentally found that *B. spinosus* larvae had a significantly higher infection burden when raised in the presence of *A. obstetricans* larvae than when raised in the absence of *A. obstetricans*. On the other hand, our results also concur with those of CUNNINGHAM & MINTING (2008), who found a strong association between Bd infection and presence of allochthonous amphibians in aquatic habitats. This finding supports the hypothesis that some non -native species, like *D. pictus*, might be linked to the spread of Bd and could increase the infection level of autochthonous species.

Discoglossus pictus coincides with *E. calamita* in the use of temporary and ephemeral aquatic environments as breeding habitats (BOSCH & MARTÍNEZ-SOLANO, 2003). These habitats are generally shallow and sun-exposed, and are therefore prone to drying up in the early summer, which may help to kill or remove chytrid fungi because the sporangia and zoospores are not resistant to desiccation (JOHNSON *et al.*, 2003). These observations agree with our data, as the only studied population negative for Bd presence was sampled in autumn, after pond desiccation. These results are in line with those of MINTING (2012), who demonstrated that adults of *E. calamita* showed higher infection rate in wet conditions during the breeding season than when they had a completely terrestrial activity. The same results have also been found in *B. spinosus* and the Alpine newt (*Ichthyosaura alpestris*), where infections of terrestrial individuals are rare, suggesting that terrestrial habitats can be less suitable for Bd than aquatic ones (DAVERSA, *et al.*, 2018a,b). As Bd detection was performed from swabs on the surface of the skin, the lack of autumnal detections of Bd might not represent a real absence of infection but a change in the activity of the pathogen. In the current study, detection was based on the presence of the infectious stage of Bd zoospores, which may not continuously emerge from infected animals. Bd zoospores are aquatic and killed by drying (JOHNSON *et al.*, 2003), so their emergence in dry conditions is probably maladaptive (MINTING, 2012). In this sense, some studies have evidenced the existence of cryptic but persistent infection in terrestrial hosts (MINTING, 2012).

Up to date, no symptomatology or mortality due to Bd infection has been detected in the surveyed area. However, parasite infection differs dramatically across years and habitats, and severe epidemics occur infrequently and unpredictably in a relatively small subset of habitats (HITE *et al.*, 2016). The study conducted by HITE *et al.* (2016) found that the structure of habitat and the existence of permanent waterbodies were linked to the density of Bd. Our results show great differences among populations, possibly caused by important differences in structure among ephemeral or temporary ponds. All the individuals analysed in our study were adults and use different terrestrial habitats, so it is probably during the larval period when cross infection can happen.

Discoglossus pictus is a dominant species in ephemeral ponds within their distribution area. Our findings indicate that their occupancy of these aquatic environments can be an important contribution as a reservoir host of Bd to the maintenance of the fungal pathogen in sympatric species using ephemeral ponds as breeding habitats.

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