In-situ severe breeding habitat intervention only achieves temporary success in reducing *Batrachochytrium dendrobatidis* infection

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type of manuscript: short-note

total number of words in the whole manuscript: 3311

total number of words in the abstract: 150

Abstract

Chytridiomycosis, an emerging infectious disease caused by the fungal *Batrachochytrium dendrobatidis* (*Bd*), is causing sharp declines in amphibian populations around the globe. A substantial research effort has been made to study the disease, including treatments against *Bd*, but most treatments have been applied to captive amphibians only. We report a study aimed at clearing wild populations of the Common Midwife toad *Alytes obstetricans*. We removed all larvae from natural breeding sites (cattle troughs) and conducted two types of severe breeding habitat manipulation (complete drying and fencing for the whole breeding season). While larval removal followed by drying was a successful method of *Bd* elimination, the effect was only temporary. Since terrestrial habits of adult *A. obstetricans* prevent them from infection, our findings suggest that, even in simple breeding habitats where all aquatic amphibian stages can be handled and extreme habitat intervention is possible, *Bd* cannot be eliminated without controlling other potential *Bd* reservoirs in the surroundings of breeding sites.

Keywords: Environmental manipulation, *Alytes obstetricans*, drying natural breeding points, chytridiomycosis.

Emerging infectious diseases represent a major threat to global biodiversity, as they can cause severe declines or extinctions of local populations (Daszak et al., 1999; Kilpatrick et al., 2009). Some well-known examples in animals are the White Nose Syndrome in bats, caused by the fungus *Geomyces destructans;* the highly deleterious crayfish plague driven by the fungus *Aphanomyces astaci*; and the Chytridiomycosis, caused by the pathogenic chytridiomycete fungus *Batrachochytrium dendrobatidis* (hereafter *Bd*), which has been linked to extinctions and dramatic declines of hundreds of amphibian species worldwide (Scheele et al., 2019). *Batrachochytrium dendrobatidis* is listed as one of the top 100 world's worst invasive alien species and has been identified as the main culprit of the greatest loss of vertebrate biodiversity attributable to disease in recorded history (Skerratt et al., 2007).

Disease ecology depends on interactions between hosts, pathogens and their environment, the three elements being key determinants of infection transmission and persistence, host susceptibility and pathological effects. Thus, besides direct interventions towards the pathogen, the vector or the host, environmental management could also be potentially used to control disease, even if results may take longer to be measurable (Delahay et al., 2009). For example, habitat manipulation has been used to decrease infection rates and influence of water-borne disease development (Wobeser, 1994). Some studies suggest that air temperature, water temperature, soil moisture, and even the action of microscopic aquatic predators could be important extrinsic risk factors driving *Bd* infection dynamics (Raffel et al., 2010; Forrest and Schlaepfer, 2011; Heard et al., 2014; Schmeller et al., 2014; Fernández-Beaskoetxea et al., 2015), and others advocate for habitat manipulation as a possible strategy to minimize the impact of the disease on amphibian populations (Raffel et al., 2010; Daskin et al., 2011; Geiger et al., 2011; Puschendorf et al., 2011; Becker et al., 2012; Heard et al., 2014; Scheele et al., 2014). Given that *Bd* growth, reproduction and infection prevalence and intensity are negatively affected by high air and water temperature (Piotrowski et al., 2004; Stevenson et al., 2013), some studies propose reducing canopy cover to increase solar insolation and thus warm patches where temperature would be above *Bd* upper optimal threshold, acting as amphibian thermal refuges in the water (Raffel et al., 2010; Geiger et al., 2011; Becker et al., 2012; Heard et al., 2014) or ground (Daskin et al., 2011; Puschendorf et al., 2011).

There has been great investment in studying the biology and dynamics of chytridiomycosis and implementing treatments to clear amphibian populations from the pathogen. However, the vast majority

63 of treatments have consisted of applying high temperatures (Woodhams et al., 2003; Chatfield and
64 Richards-Zawacki, 2011; Geiger et al., 2011) or antifungal products, such as itraconazole (Garner et al.,
65 2009; Tobler and Schmidt, 2010; Brannelly et al., 2012; Jones et al., 2012), chloramphenicol (Bishop et
66 al., 2009), thiophanate-methyl (Hanlon et al., 2012) or voriconazole (Martel et al., 2011), to captive
67 amphibian collections under monitored conditions. While the treatment of captive amphibians has been
68 successful, translating this knowledge into safe, reliable, transferable, cost-effective and long-term
69 solutions to manage chytridiomycosis in the wild remains a challenge.

70 Four different strategies have been used to try mitigate the impacts of the disease in natural
71 habitats (Garner et al., 2016), with no or partial success: (1) translocations or reintroductions; (2)
72 bioaugmentation of the host microbiome; (3) treatment of individuals with antifungals; and (4) the
73 combination of antifungal treatments with environmental disinfection using chemicals. Other potential
74 mitigation strategies, such as obtaining resistant amphibians through genetic manipulation and selective
75 breeding, remain untested. Here we report a management study aimed at mitigating the effects of
76 chytridiomycosis on a wild population of the Common Midwife toad (*Alytes obstetricans*). A distinctive
77 natural history trait of this species is its extremely long larval stage (up to several years), which is
78 associated with the persistence and transmission of the disease (Bosch et al., 2001). We used a
79 combination of two methods, avoiding the use of chemicals in the environment: (1) the removal of
80 overwintering larvae, which are responsible for maintaining and producing large amounts of fungal
81 infective zoospores (Fernández-Beaskoetxea et al., 2016); and (2) habitat intervention, which consisted of
82 either drying or fencing the breeding sites to avoid the entrance of metamorphosed individuals.

83 We conducted the study in twelve cattle troughs located in a forested mountain area (mean
84 altitude of 1550 m.a.s.l.) in the Teruel province (Aragón, Spain). These cattle troughs, which are used as
85 natural breeding sites by *A. obstetricans*, were selected following three years of sample collection (2010-
86 2012) from a wide range of breeding sites, which showed high variation in *Bd* loads (S1). Each site was
87 randomly assigned to one of the following experimental groups, comprised of three sites each: control
88 (Blanca, Juan and Milano sites); removal only (Jorcas, Reguero and Torreta sites); removal and fencing
89 (Blandina, Cuerda and Molino sites); and removal and drying (Abrevador, Cebo and Gil sites). All larvae
90 were removed from the sites in all cases; in the control group, larvae were immediately returned to their

original sites; in the fencing group, larvae were removed and then the site was isolated using a fence that was left there during the whole breeding season (45 days), to avoid the entrance of metamorphosed individuals; in the drying group, larvae were removed and then the site was completely dried out and kept it dry for at least 20 days, during which no rain occurred and no other persisting sources of moisture were present. Habitat interventions were conducted in May 2012, immediately after the collection of larvae, which were taken to a close captive facility and used in other studies, except for larvae from the control group.

We collected twenty samples per site before habitat intervention and ten samples per site annually for three consecutive years after intervention. We took samples from the mouthparts of overwintering (OW) larvae using cotton swabs (MW100 rayon tipped dry swabs from MWE Medical Wire), extracted the DNA with PrepMan Ultra, and amplified the DNA using a BIO-RAD CFX96 Real-Time PCR Detection System following Boyle et al., (2004). Each 96-well assay plate included a negative control and four different standards containing DNA from 100, 10, 1 and 0.1 *Bd* genome equivalents (GE). We tested all the samples, as well as the negative control and the standards, in duplicate. We considered samples with greater than 0.1 GE in both replicates, and the expected sigmoidal shaped curve, positive for *Bd*. We transformed *Bd* loads to the $\log_{10}(x+1)$ to reach normality and used a general linear model to analyse variation before and after intervention. We considered treatment and year as fixed factors and site as fixed factor nested within treatment, and used post-hoc Tukey's honest significance tests to compare pairs of *Bd* load means for the treatment by year interaction (S2).

The *Bd* loads varied across treatments, sites within treatments and years (Fig. 1, S3), and the treatment by year interaction was also significant ($p<0.05$ in all cases), explaining the model 54% of the observed variation. While complete clearance of *Bd* was not achieved in any of the experimental groups (Fig. 1, S3), the drying group remained *Bd*-clean for two years. In the control group, infection load levels remained constant at Blanca and decreased at Milano in 2013 and 2104; we found no larvae at Juan in 2013 and 2014, or in any control site in 2015. In the removal group, we found <10 larvae in 2013 and 2014, and no larvae in 2015 at Jorcas (which prevented comparisons), and *Bd* loads remained similar or increased after habitat intervention at Reguero and Torreta. Similar results were found in the fencing group, with increased *Bd* loads at Blandina in 2014 and 2015, and no larvae in 2013 and 2015 at Cuerda

and in 2014 at Molino. In contrast, *Bd* loads were null at all sites in the drying group for two years (2013 and 2014), although infected larvae were collected again in 2015 at Cebo (with extremely low *Bd* loads) and Gil; Abrevador did not present overwintering larvae in 2015, but ten non-overwintering larvae resulted *Bd*-negative (data not shown).

This study shows how some types of habitat intervention, but not others, are able to affect the dynamics of chytridiomycosis in natural habitats by reducing *Bd* loads. In particular, we achieved the elimination of *Bd* after removing larvae of *A. obstetricans* from their natural breeding sites, and then completely drying out the sites and keeping them dry for at least 20 days. As a result of this intervention, *Bd* remained absent for at least two years (three in one site), which contrasted with other interventions (i.e., larval removal only, or larval removal plus site fencing), which rendered no significant or consistent effects on *Bd* loads. Other authors had suggested that drying out the natural habitat could be a viable way of suppressing *Bd* in the wild, given the high sensitivity of *Bd* zoospores to desiccation as shown in laboratory conditions (Woodhams et al., 2011). However, Bosch et al. (2015) failed to eradicate *Bd* in wild populations of *Alytes muletensis* populations after completely drying out the aquatic habitat until a chemical was applied. Our study supports those results and emphasizes the need of using chemicals to completely and permanently eradicate *Bd* from the environment.

Mitigation strategies (including direct mitigation actions) have proven ineffective in order to obtain long-term control of chytridiomycosis in the wild. The only exception is Bosch et al. (2015), who successfully eradicated *Bd* in 2013, with no reappearance to date (JB, unpublished results). For example, the use of antifungals for in-situ treatment of amphibian adults (Hudson et al., 2016) or larvae, using a capture-treat-release approach (Geiger et al., 2017), has only obtained short-term success, similar to the one described here. Still, in-situ antifungal treatments have been shown to reduce disease prevalence, infection loads and mortality of post-metamorphic juveniles, which is helpful when coping with an epidemic wave of chytridiomycosis (Garner et al., 2016; Hudson et al., 2016; Geiger et al., 2017) but not for critically endangered species with reduced distributions. Reducing *Bd* loads and preventing critical infection thresholds can avoid mass mortality episodes and extinctions (Vredenburg et al., 2010), allowing some individuals to persist and populations to overcome the epidemic (Briggs et al., 2010). Drastic population reductions are never desirable for amphibians, which are critically affected by many

147 other anthropogenic factors. Even when some studies have shown that *Bd* has no negative effects on
148 populations of vulnerable species when it reaches an enzootic stage (Tobler et al., 2012; Spitzen-van der
149 Sluij et al., 2014; Kieran et al., 2018), long surveying programs have indicated that other populations are
150 not able to recover even decades after the outbreak disease (Bosch et al., 2018). Within this context, our
151 results demonstrate that even drastic habitat interventions (i.e., drying out natural breeding sites) can
152 reduce *Bd* infection loads but cannot be considered an eradication method.

153 There are two possible ways of reinfection of dried habitats that may have occurred in our study,
154 namely the arrival of infected amphibians and the existence of potential reservoirs in the surroundings of
155 breeding sites. Data are insufficient to completely discard the arrival of infected individuals, even when
156 *A. obstetricans* is the only amphibian species present in the area. The persistence of infection in
157 postmetamorphic *A. obstetricans* could explain why water bodies were reinfected after two complete
158 years being utterly cleared of *Bd*. However, this explanation could also be deemed unlikely, since *A.*
159 *obstetricans* adults are fully terrestrial and generally live far away from the water, being rarely infected by
160 the chytrid fungus (Allain and Goodman 2018; JB, unpublished results; but see Spitzen-van der Sluij et
161 al., 2014). Thus, the existence of terrestrial *Bd* reservoirs in the surroundings of breeding sites could be
162 considered as a more plausible explanation for habitat reinfection. If such environmental reservoirs of *Bd*
163 were confirmed by field studies, then the use of chemicals in the surroundings of water bodies could be
164 one of the best methods, already proved as efficient, in order to remove the chytrid fungus from the
165 environment (Bosch et al., 2015). Obviously, environmental disinfectant application would need to be
166 coupled with appropriate management of adult recolonization for those species with aquatic or
167 semiaquatic adults. The use of chemicals in natural habitats has been criticized for its potential damage on
168 aquatic organisms and ecosystems, but this mostly applies to the massive use of pesticides in agriculture
169 (Köhler and Triebskorn, 2013). In contrast, using biocides for specific conservation purposes often has
170 more advantages than disadvantages (Martín-Sánchez et al., 2012, Peay et al., 2019), and should be
171 considered as a viable option to eradicate the chytrid fungus from natural habitats.

172

Acknowledgements

Animal handling procedures were approved by the Instituto Aragonés de Gestión Ambiental (Gobierno de Aragón, Spain). We thank all the workers of Servício Provincial de Medio Ambiente de Teruel, and specially Miguel Ángel Martín, Fran Osses, Óscar Bailón, Antonio Fortea and Jaime Lahoz for their crucial collaboration on this study helping through field work and habitat manipulation interventions. We thank also people of Fortanete (Teruel) for logistic support during this study. An anonymous reviewer provided helpful comments and suggestions. This work was funded by Fundación General CSIC and Banco Santander (Zero projects, PI: J. Bosch). LB was supported by Basque Government funds (IT951-16) to the Stream Ecology Group at UPV/EHU led by J. Pozo.

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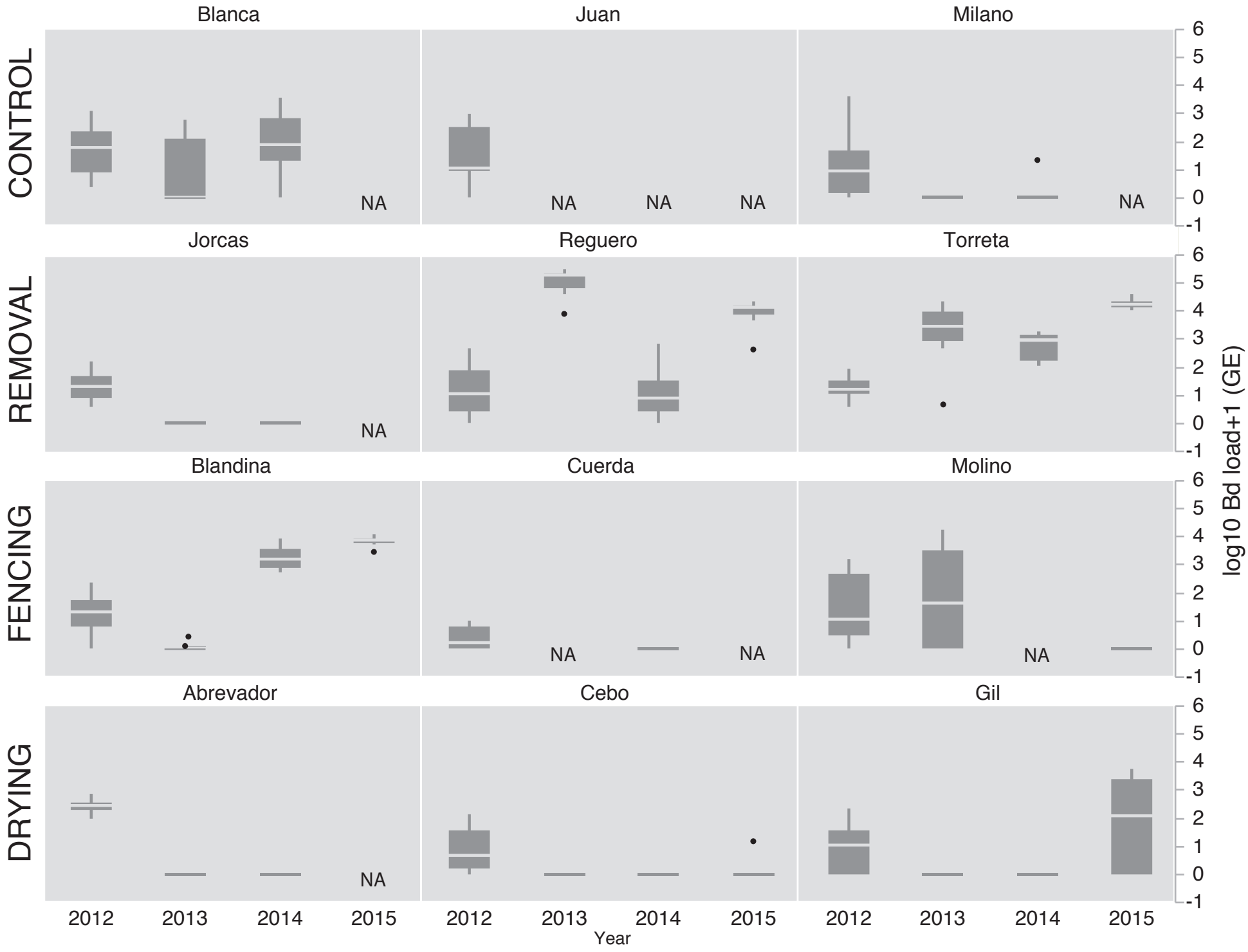
307 Fig. 1. Box plots of *Bd* load (log_{10} x+1 transformed) of overwintering *Alytes obstetricans* larvae at the
308 twelve study sites immediately before (2012) and after habitat intervention (2013-2015). NA: no
309 overwintering larvae present.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Amphibia-Reptilia

In-situ habitat intervention without chemicals only achieves temporary success in reducing *Batrachochytrium dendrobatidis* infection

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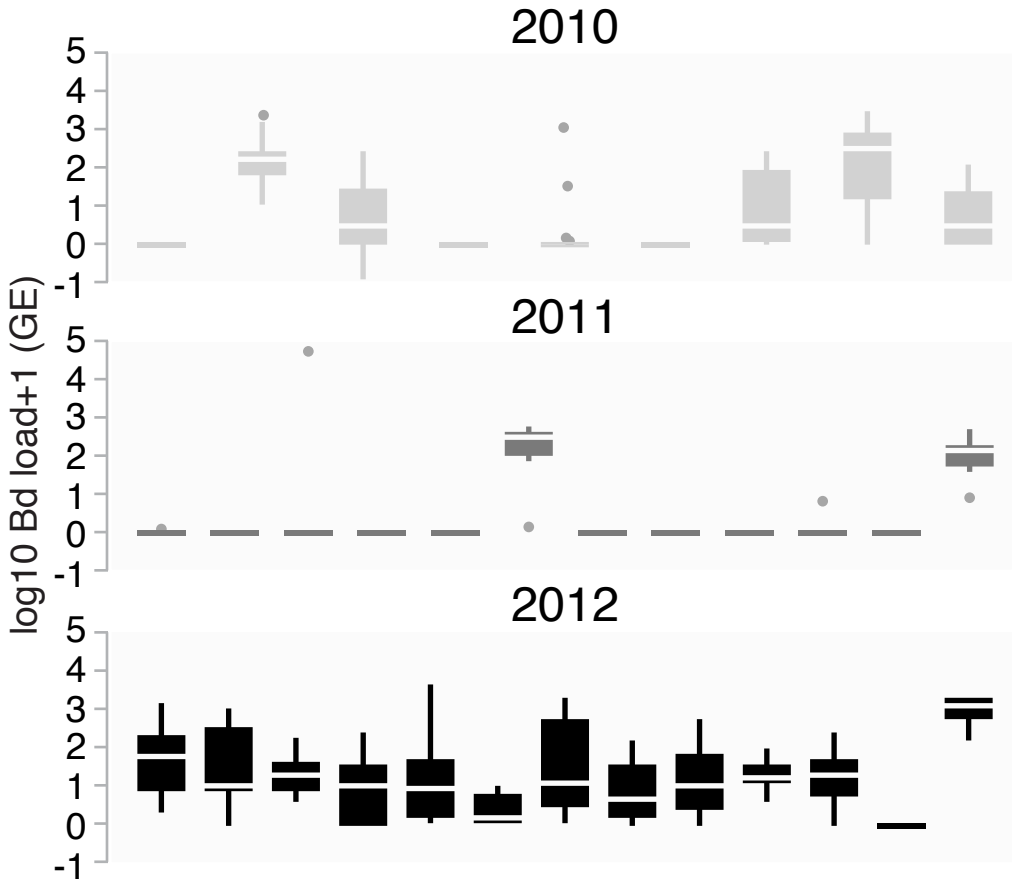
Supplementary material

S1. Box plots of *Bd* load (log_{10} x+1 transformed) of overwintering *Alytes obstetricans* larvae at different breeding sites sampled in 2010, 2011 and 2012 before habitat intervention.



Amphibia-Reptilia

In-situ habitat intervention without chemicals only achieves temporary success in reducing *Batrachochytrium dendrobatidis* infection

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Supplementary material

S2. Tukey's post-hoc tests comparing pairs of means of *Bd* loads for the treatment by year interaction. Letters not connected by the same letter are significantly different.

Level		Least Sq Mean
REMOVAL, 2015	A	3.764
REMOVAL, 2013	A B	3.314
FENCING, 2015	B C	2.309
FENCING, 2014	C D	1.905
DRYING, 2012	C D E	1.523
CONTROL, 2012	C D E	1.395
REMOVAL, 2012	C D E F	1.296
CONTROL, 2014	C D E F G	1.161
CONTROL, 2015	A B C D E F G H	
DRYING, 2015	D E F G	1.116
REMOVAL, 2014	C D E F G	1.107
FENCING, 2012	E F G	0.915
CONTROL, 2013	F G H	0.429
FENCING, 2013	G H	0.2876
DRYING, 2014	H	3.442e-15
DRYING, 2013	H	-5.898e-16

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Supplementary material

S3. *Bd* load (logarithmic scale) and prevalence of infection (mean +/- 95% CI) across the four experimental groups (control, before (2012) and after habitat intervention (2013-2015). There is no data from the control group in 2015 because no overwintering larvae were found.