SUCCESSFUL ELIMINATION OF A LETHAL WILDLIFE INFECTIOUS DISEASE IN NATURE

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Abstract

Methods to mitigate the impacts of emerging infectious diseases affecting wildlife are urgently needed to combat loss of biodiversity. However, the successful mitigation of wildlife pathogens *in situ* has rarely occurred. Indeed, most strategies for combating wildlife diseases remain theoretical, despite the wealth of information available for combating infections in livestock and crops. Here we report the outcome of a five year effort to eliminate infection with *Batrachochytrium dendrobatidis* affecting an island system with a single amphibian host. Our initial efforts to eliminate infection in the larval reservoir using a direct application of an antifungal were successful *ex situ* but infection returned to previous levels when tadpoles with cleared infections were returned to their natal sites. We subsequently combined antifungal treatment of tadpoles with environmental chemical disinfection. Infection at four of the five pools where infection had previously been recorded was eradicated, and remained so for two years post-application.

Keywords

Chytridiomycosis
Batrachochytrium dendrobatidis
Mitigation
Alytes muletensis
Mallorca

1. Introduction

Emerging infections are on the increase, incurring extraordinary economic and health costs and globally degrading our natural capital. In response, several efforts to eradicate animal pathogens are underway, however with few successes reported [1,2]. Research on livestock pathogens predominates and provides insight as to how pure wildlife pathogens may be combated for host conservation purposes [1,2]. Delivery of an efficient and practical intervention is a cornerstone of any scheme to eliminate infectious diseases, and the direct application of antimicrobials to infected hosts or immunization can be used effectively to control pathogen replication within a host and to reduce the likelihood of transmission to susceptible individuals [3]. However, for these types of interventions to be effective, control of environmental reservoirs of (re)infection must also be achieved. Local control of pathogens through the use of environmental chemical treatments has been effectively used to disinfect areas where environmental transmission of parasites can occur, but the impact of chemical treatment on transmission and maintenance of infection in concert with antimicrobial treatments has rarely been examined [4].

Amphibian chytridiomycosis, a disease predominantly caused by the aquatic chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) has driven population declines, local extirpations and species extinctions across five continents [5]. The pathogen is an extreme generalist, infecting over 700 amphibian species (http://www.bd-maps.net). Strategies developed to ameliorate the impacts of chytridiomycosis are predominantly geared towards disease-free maintenance of captive assurance colonies, and multiple methods have been developed to treat captive amphibians against infection with *Bd* [6-8]; however, most attempts at immunization have failed [9]. The remaining approaches that hold promise for *in situ* control include bioaugmentation with bacteria, direct application of antifungal drugs, and environmental application of anti-*Bd* chemicals. Although not without promise, research on the application of bioaugmentation so far describes complex interactions between host, beneficial bacteria, the broader microbiota and pathogen that are strongly dependent upon environmental context and amphibian community structure [10,11]. For this reason, bioaugmentation strategies are unlikely to converge on an intervention that can be generalized across amphibian communities and ecosystems. The immediacy of the epizootic of chytriomycosis calls for an intervention that can be applied across systems, so we chose to explore direct application of antifungal drugs to infected hosts and environmental application of chemicals as strategies to eliminate *Bd* from a simple, single host system [12].

2. Material and methods

Biannual surveys at the five permanent ponds (3 X Torrent des Ferrerets, 2 X Cocó de sa Bova; Mallorca, Spain) were undertaken from 2008 and are ongoing. We sampled Mallorcan midwife toad (*Alytes muletensis*) tadpoles as terrestrial stages are rarely captured as they take refuge in inaccessible locations. Tadpoles of this and other *Alytes* sp. are recognized as reservoirs of infection [13,14]. To sample we swabbed tadpole mouthparts following established protocols [12,13]. All ponds affected by chytridiomycosis on the island were included in the study and none were left as untreated controls due to conservation requirements. However, chemical disinfection at Torrent de Ferrerets preceded those at Cocó de sa Bova, affording us the opportunity to compare across sites.

Swabs were processed according to standard extraction and quantitative PCR methods [15] in duplicate and run against negative controls and positive controls (0.1, 1, 10 and 100 zoospore genomic equivalents, GE).

For antifungal treatments, tadpoles were collected and transported in plastic bottles containing pond water. We used air pumps and tubes with aeration stones to ensure tadpole survival during the outward hikes. Tadpoles were then transported to the lab and kept in several cooled, glass aquaria. All tadpoles were bathed daily for seven days in aged tapwater containing 1.0 mg/l itraconazole (Sporanox, Janssen-Cilag Inc.) and returned to aquaria after each treatment. Aquaria water was replaced every day during the 7 days treatment. After treatment, tadpoles were returned to the collection sites by helicopter, either immediately if ponds were not drained, or after ponds were refilled by autumn rain. In these cases subsets of 40 tadpoles from each aquarium were swab-sampled 15 days post treatment.

Environmental disinfection was done using Virkon S (DuPont Inc.) at 1% final concentration and a single application applied *ad libitum* to the environment. The disinfectant was liberally applied to all rock, gravel, crevice and vegetated areas that surrounded the immediate environs of each breeding site.

3. Results

We initially attempted mitigation by treating in 2009 *A. muletensis* tadpoles inhabiting two permanent pond sites in one of the two infected drainages, Cocó de sa Bova (Fig.S1), with the antifungal itraconazole. We used a treatment protocol previously shown to eliminate infection in tadpoles [7]. Treatments were applied *ex situ*, and prior to post-treatment release the two ponds were completely drained of water and naturally dried by the arid environment that typifies Mallorca. We had previously determined that *Bd* is absent from the other two ephemeral water bodies in this drainage, and environmental *Bd* is not thought to persist during periods of drying [16]. The two ponds naturally refilled during the autumn rainy season. At no point during this prolonged period of captivity did we detect any evidence of infection in the treated tadpoles. The following spring, qPCR analysis showed that all treated animals had contracted infections not significantly different from what had been recorded at the location before treatment [17] (Fig.1). Repeating the protocol in the spring of 2012, this time without draining the breeding sites, and with tadpole release only 7 days after treatment, was again not associated with reduction in the prevalence of infection or reduced burdens of infection in the following spring (Fig.1).

In contrast, at three breeding sites utilized by the species in the second drainage, Torrent des Ferrerets (Fig.S2), we could not detect infection in any animals sampled at the location in 2013 after treatment of tadpoles and whatever terrestrial *A. muletensis* life stages we could capture with itraconazole, draining the sites and then treating the environment with Virkon S (Fig.S3-4), (Fig.1). Replication of this protocol at Cocó de sa Bova in 2013 and application of Virkon S solution to the rock crevices located around the ponds where metamorphosed *A. muletensis* reside again cleared infection in the larger population of tadpoles resident in the larger pond at this location. Residual infection was detected in tadpoles occupying the smaller permanent pond site. Data from samples taken at Torrent des Ferrerets two years after chemical disinfection showed that the effect of environmental application of Virkon S twinned with itraconazole treatment of tadpoles carried over across years, as again no evidence of infection was detected in 2014 (Fig.1).

4. Discussion and conclusions

We cannot say with certainty why direct treatment of tadpoles with antifungals without environmental disinfection failed to resolve infection at Cocó de sa Bova, but the most likely explanation is that infection reinvaded tadpoles from post-metamorphic animals that we could not access in their terrestrial refuges. We do occasionally discover corpses of juveniles exhibiting a strong molecular signal of infection. Like other amphibian species, *Alytes* spp. tadpoles scavenge from corpses, and this process is presumed to be a factor in transmission of *Bd* from corpses to tadpoles in another species [18,19]. Irrespective, our application of Virkon S at Torrent des Ferrerets provided proof-of-principle that environmental application of fungicides and other chemical treatments may be a better approach when combined with antimicrobial treatment of infected hosts. This initial conclusion was reinforced when we recapitulated our result by clearing infection in Cocó de sa Bova the following year. In our case, combining chemical disinfection twinned with antifungal treatment of tadpoles proved the better strategy, eliminating infection and preventing spill-back over the short term at four of the five pools where we attempted mitigation.

The development of disinfection strategies alone cannot eliminate the threat of chytridiomycosis, as evidence continues to accumulate that lethal amphibian-associated chytrid fungi are frequently being introduced into Europe and beyond [12,20]. Clearing site-level infection is no guarantee against pathogen reintroduction or the introduction of novel pathogens. However, to cope with the existing, recurring and future threats of chytridiomycosis, rapid response strategies require cheap, simple and transferrable methods for mitigating infection that can be employed as soon as the threat has been identified. We acknowledge that Virkon S is a controversial chemical to use environmentally and our use of it was driven by the urgency of midwife decline on Mallorca. Virkon S is only one of several chemical treatments known to have antifungal properties against chytrid fungi [21,22] and antifungal treatments do not require extensive investment in time and effort. We argue that research informing efforts to combat chytridiomycosis should include in-depth investigations of the impact of antifungals and anti-*Bd* chemicals on amphibian health without discarding attempts to develop immunization and other methods of disease control. Research on the application of these chemicals for control of wildlife diseases must also include investigation of the potential impacts of chemical application to other biodiversity, the environment and associated ecosystem services.

Ethics. The work was carried out under the Govern de les Illes Balears's permit # CEP 43/2015.

Data accessibility. Data available in the supplementary material.

Author contributions. J.B., T.W.J.G. and M.C.F. designed and wrote the paper, with contributions from E.S.T. Data were collected and/or analysed by E.S.T., A.F.L. and J.A.O; all authors provided intellectual input and edited/approved the manuscript.

Competing interests. We declare we have no competing interests.

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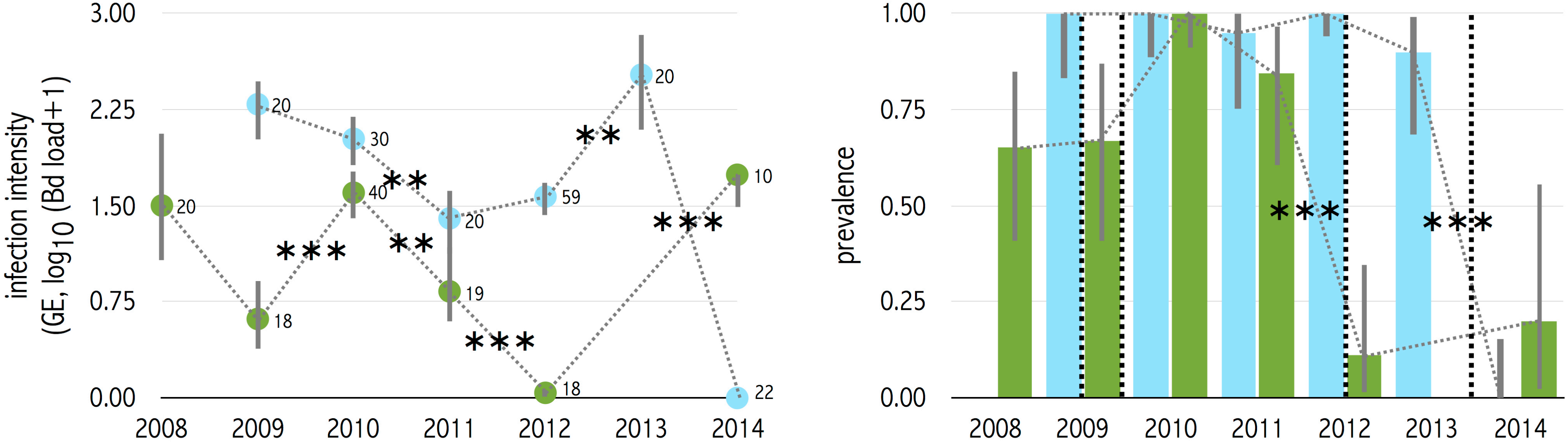
Figure legend

Figure 1. Infection intensity (left panels; mean +/- 95% CI by the BCa method with 2000 bootstrap replications) and prevalence (on the right; mean +/- 95% Clopper-Pearson CI) over 2 pond sites at the Cocó de sa Bova (combined in top panels) and 3 at the Torrent des Ferrerets (combined in bottom panels), over the course of the study. Blue are values derived from spring sampling, green for summer. Pairwise comparisons (Wilcoxon signed rank tests for infection intensities and Fisher exact tests for prevalence) are represented by dashed lines and significant differences represented with *(p < 0.05), **(p < 0.01) and ***(p < 0.001) after a sequential Bonferroni adjustment. Sample sizes are shown in left panels. Dashed vertical lines in right panels indicate when treatments were implemented.

Cocó de sa Bova



Torrent des Ferrerets

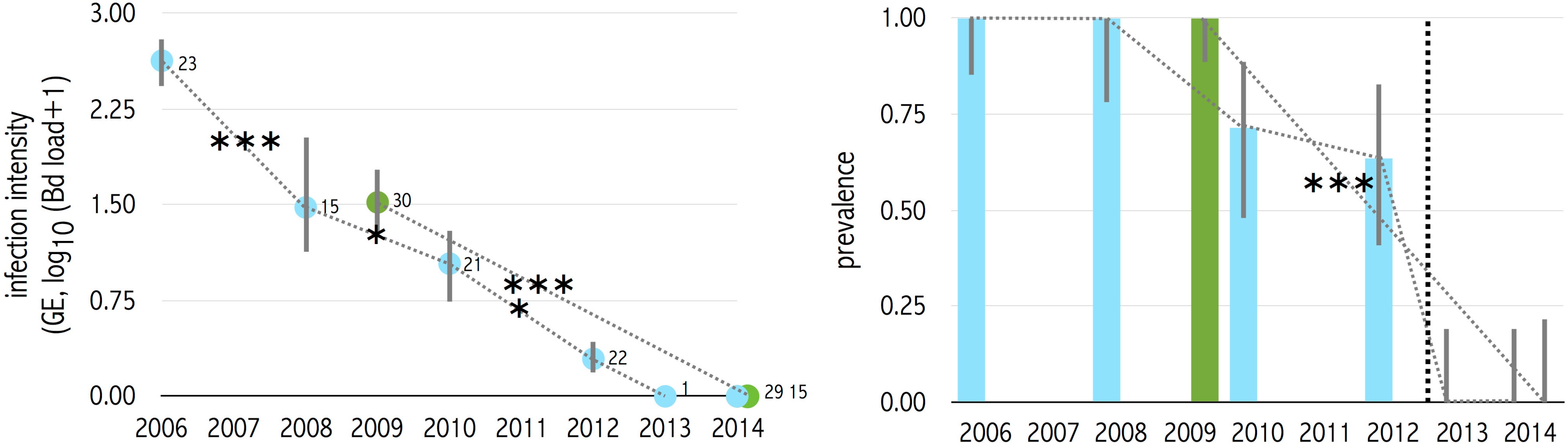


Figure captions

S1. Larger of the two pools that make up Coco di sa Bova.

S2. One of the three permanent water bodies that make up Torrent de Ferrerets.

S3. A pool prepared for treatment with Virkon S after draining most of the water and collecting every tadpole.

S4. A pool after treatment with Virkon S.

S1

S2

S3

S4

Cocó de sa Bova

SUM08	SPR09	SUM09	SPR10	SUM10	SPR11	SUM11	SPR12	SUM12	SPR13	SPR14
0.0	5.5	0.0	5.0	0.3	0.5	0.0	1.9	0.0	0.0	0.0
0.0	34.5	0.0	7.3	0.4	1.3	0.0	2.6	0.0	0.0	0.0
0.0	40.3	0.0	8.5	11.0	10.1	0.0	3.1	0.0	5.5	0.0
0.0	79.2	0.0	17.7	14.2	40.0	0.4	4.4	0.0	8.6	0.0
0.0	114.6	0.0	22.7	16.8	4.0	1.3	4.6	0.0	30.0	0.0
0.0	119.6	0.0	53.8	23.2	13.0	0.6	5.5	0.0	70.0	0.0
0.0	137.8	0.1	60.1	29.1	83.0	2.6	5.5	0.0	200.0	0.0
2.9	199.8	0.2	63.1	41.4	29.0	3.1	6.2	0.0	210.0	0.0
4.2	227.5	0.7	64.8	48.8	32.0	3.2	7.0	0.0	228.7	0.0
4.5	259.5	1.1	68.2	51.7	9.0	3.7	8.0	0.0	270.0	0.0
5.2	280.8	1.1	74.2	53.2	48.0	4.2	8.2	0.2	320.0	0.0
6.3	306.0	1.4	80.3	54.6	171.0	4.2	9.4	0.0	380.0	0.0
6.9	309.4	2.2	84.6	58.6	58.0	5.8	10.3	0.0	490.0	0.0
7.2	422.5	4.9	99.6	62.5	19.0	10.0	10.6	0.0	740.0	0.0
34.0	455.2	5.7	133.8	65.1	12.5	10.0	12.5	0.0	990.0	0.0
49.4	461.2	8.7	140.0	68.0	142.0	20.0	15.3	0.0	1900.0	0.0
85.0	489.7	26.9	150.6	72.7	0.0	22.4	19.9	0.0	2370.0	0.0
221.4	509.2	29.6	157.8	73.3	99.0	30.0	20.4	0.1	2558.9	0.0
1123.1	523.3		167.5	73.5	45.8	260.0	21.4		3150.0	0.0
2261.9	1125.1		198.2	267.0	39.6		22.5		3370.0	0.0
			221.9	0.4			23.4			0.0
			226.8	3.2			31.5			0.0
			252.6	3.2			39.1			0.0
			260.0	22.7			40.2			0.0
			270.3	25.2			41.0			0.0
			283.7	27.3			41.0			0.0
			292.8	31.4			42.6			0.0
			535.6	34.3			43.2			0.0
			560.0	34.8			43.9			0.0
			788.9	39.4			44.6			0.0
				73.8			52.5			30.0
				93.6			55.9			100.0
				98.1			58.3			
				106.4			59.6			
				112.8			60.6			
				146.9			60.6			
				152.8			66.4			
				213.2			77.5			
				284.1			80.1			
				471.3			80.3			
							83.8			
							85.8			
							88.2			
							94.3			
							94.5			
							100.0			
							102.4			
							106.9			
							120.2			
							134.3			
							137.1			
							138.1			
							147.7			
							148.6			
							151.9			
							153.0			
							153.2			
							175.0			
							572.2			

Torrent des Ferrerets

SPR06	SPR08	SUM09	SPR10	SPR12	SPR14	SUM14
228.9	2.9	666.6	0.0	0.0	0.0	0.0
109.5	4.2	645.3	0.0	0.0	0.0	0.0
320.4	4.5	383.8	0.0	0.1	0.0	0.0
563.1	5.2	382.6	0.0	0.1	0.0	0.0
968.8	6.3	301.4	0.0	0.1	0.0	0.0
552.4	6.9	125.8	0.0	0.3	0.0	0.0
68.3	7.2	118.2	0.4	0.6	0.0	0.0
190.9	18.6	75.4	0.5	0.8	0.0	0.0
952.6	24.4	69.1	2.1	1.0	0.0	0.0
396.1	34.0	59.4	2.7	1.1	0.0	0.0
1261.3	49.4	58.8	3.9	1.3	0.0	0.0
2021.9	85.0	49.4	3.9	1.5	0.0	0.0
450.2	221.4	44.8	7.9	3.7	0.0	0.0
101.0	1123.1	42.0	17.6	4.7	0.0	0.0
683.8	2261.9	29.9	18.1	0.0	0.0	0.0
435.8		26.8	19.9	0.0	0.0	
51.9		24.3	20.1	0.0	0.0	
125.8		20.0	39.2	0.0	0.0	
399.7		19.2	42.7	0.0	0.0	
515.2		17.0	48.9	0.0	0.0	
1672.2		14.3	53.1	1.0	0.0	
2425.8		12.6		2.9	0.0	
1133.5		11.4			0.0	
		10.2			0.0	
		6.2			0.0	
		5.0			0.0	
		4.0			0.0	
		1.5			0.0	
		0.7			0.0	
		0.5				