Invasive North American bullfrogs transmit lethal fungus *Batrachochytrium dendrobatidis* infections to native amphibian host species

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Abstract Invasive species can be a threat to native species in several ways, including transmitting lethal infections caused by the parasites they carry. However, invasive species may also be plagued by novel and lethal infections they acquire when invading, making inferences regarding the ability of an invasive host to vector disease difficult from field observations of infection and disease. This is the case for the pathogenic fungus *Batrachochytrium dendrobatidis* (Bd) in Europe and one invasive host species, the North American bullfrog *Lithobates catesbeianus*, hypothesized to be responsible for vectoring lethal infection to European native amphibians. We tested this hypothesis experimentally using the alpine newt *Ichthyosaura alpestris* as our model native host. Our results show that infected bullfrog tadpoles are effective vectors of Bd. Native adult newts co-housed with experimentally infected bullfrog tadpoles became Bd infected (molecular and histological tests). Moreover, the exposed adult newts suffered mortality while the majority of infected Bullfrog tadpoles survived until metamorphosis. These results cannot resolve the historical role of alien species in establishing the distribution of Bd across Europe or other regions in the world where this species was introduced, but they show its potential role as a Bd reservoir capable of transmitting lethal infections to native amphibians. Finally, our results also suggest that the removal of infected bullfrogs from aquatic environments may serve to reduce the availability of Bd in European amphibian communities, offering another justification for bullfrog eradication programmes that are currently underway or may be considered.

Keywords Introduced amphibian • disease • cross-contamination • fungus • American bullfrog • Alpine newt

Introduction

Invasive, non-native species are considered to be one of the greatest threats to biodiversity and threaten native species through a variety of mechanisms. The co-introduction of parasites capable of eliciting significant pathogenesis in naïve native hosts is thought to be one of the major mechanisms behind biodiversity loss attributable to invasive species (Daszak et al. 2000; Prenter et al. 2004; Crowl et al. 2008). Indeed, parasites that are transported with invasive species tend to reach equivalent prevalence in native species (Torchin et al. 2003), sometimes with devastating consequences (Martel et al. 2014; Doddington et al. 2013; Bosch et al. 2013). However, invasive species may carry significantly reduced parasite diversity when invading (Torchin et al. 2003) and commonly become infected with parasites that occur in endemic residents (Colautti et al., 2004; Bürgi and Mills 2014). Invasive species that are infected with resident parasites can suffer costs exceeding those experienced by the native host species (Wolfe et al. 2004) or equivalent to those experienced by native species infected with newly introduced parasites (Heger and Jeschke 2014). The unpredictability of these relationships means that patterns of parasite infection and disease in native and invasive hosts do not always indicate which host may be serving as a vector for the parasite.

Batrachochytrium dendrobatidis (Bd), a global fungal pathogen of amphibians, is presumed to be an invasive parasite in many parts of its range (Farrer et al. 2011). Bd invasion is commonly attributed to the release of infected, asymptomatic species that have been displaced as a result of trade (Hanselmann et al. 2004). A prime example is that of the North American bullfrog *Lithobates catesbeianus* (Hanselmann et al. 2004). Due to their ubiquity as a traded species infected with Bd (Bai et al. 2010; Schloegel et al. 2009), their distribution and a consistent pattern of infection with Bd (Garner et al. 2006), they have been proposed to be important vectors of Bd into native amphibians. Bullfrogs may contribute to

maintain Bd in native amphibian community (Peterson and McKenzie 2014), whereas the distribution of invasive bullfrogs appears as a poor predictor of Bd distributions (Richardson et al. 2014; Bataille et al. 2013). Native bullfrogs do have the ability to transmit infection to species that occur within their natural range (Greenspan et al. 2012), invasive bullfrogs tended to produce a higher number of Bd zoospores relative to native species (Peterson and McKenzie 2014) but do not appear to sustain infections for prolonged periods of time and can die from heavy infections (Gervasi et al. 2013). The evidence that invasive bullfrogs can act as significant vectors of chytridiomycosis to native hosts is relatively weak.

Bullfrogs have been widely introduced in Europe in an uncoordinated, multinational effort to establish viable populations for the trade in frog legs (Ficetola et al. 2007). Invasive bullfrog populations were consistently founded by a small number of adults directly transported from their native range and much of the current distribution in Europe probably arose through translocation from these founder populations (Ficetola et al. 2008). This small number of potential transport vectors is incompatible with the widespread, pervasive distribution of Bd across Europe (Olson et al. 2013) and the patterns of Bd invasion in areas of Europe where bullfrogs are absent (Bielby et al. 2013; Bosch et al. 2013; Walker et al. 2008; 2010). Introduced bullfrogs can potentially transmit Bd to native amphibians, but spill-over can be from native hosts to invasive bullfrogs. If this were the case, invasive bullfrogs would be accruing infection from native hosts, and we would predict that native species commonly infected with Bd would be relatively tolerant of infection while bullfrogs would exhibit costs such as the post-metamorphic mortality of bullfrogs experiencing strong infections in their native range, as described by Gervasi et al. (2013). The possibility does remain that bullfrogs act as vectors of infection and disease in Europe, and can act as significant reservoir hosts. Indeed, Bd has been documented infecting bullfrog populations across Europe (Garner et al. 2006). In this instance, we predict that native European

amphibians would be susceptible to lethal chytridiomycosis caused by transmission from infected bullfrogs, while bullfrogs would not exhibit significant costs associated with exposure to and infection with Bd.

In this paper, we experimentally determine if invasive bullfrogs are significant vectors of Bd to European amphibians. We cohoused experimentally infected bullfrog tadpoles with adult native amphibians, in this case, the alpine newt. We selected the alpine newt because it is known to be infected with Bd across Europe (Zampiglia et al. 2013; Sztatecsny and Glaser 2011) but little is known about its susceptibility to lethal chytridiomycosis. We considered bullfrog tadpoles as the appropriate life history stage for assessing reservoir status of the species because tadpoles with prolonged larval periods are commonly cited as significant reservoirs of infection (Briggs et al. 2010; Walker et al. 2010). We recorded infection status, determined through molecular diagnostics and histology, burden of infection and survival in both bullfrog tadpoles and adult alpine newts. For bullfrogs, we measured mortality rates until the onset of metamorphosis, as significant costs associated with larval infection initially manifest when metamorphosis is near to completion (Gervasi et al. 2013; Walker et al. 2010; Garner et al. 2009).

Materials and methods

One clutch of American bullfrog (*Lithobates catesbeianus*) spawn was collected in June 2009 at an artificial pond in Ambarès in southwestern France (44°56’22” N, 0°31’04” E; 20 m a.s.l.). Eggs were hatched and larvae reared in the laboratory in three plastic containers (400 x 600 x 200 mm) each containing approximately 35 L of aged tap water. Larvae were fed flaked goldfish food provided *ad libitum* during this and the subsequent exposure periods (see below). In May 2010, 30 of the 250 available tadpoles (Gosner stages 26–30; Gosner 1960)

were selected randomly and examined for evidence of infection by swab-sampling their mouthparts (swab ref. M01-MW100, Kitvia Co.) and testing DNA extracted from these swabs using the TaqMan Assay described by Boyle et al. (2004). Because the extraction reagent is a PCR inhibitor, samples were diluted by a ratio of 1:10 prior to attempted PCR amplification. For all molecular assessments of infection, amplifications yielding quantitative scores of 0.1 genomic equivalents (GE; untransformed value) or greater were considered Bd-positive, allowing us to assign individuals as either 'infected' or 'not infected'.

Twenty tadpoles were transferred to plastic containers (240 x 160 x 144 mm) filled with approximately 2 L of aged tap water and maintained as such until the end of the experiment as negative controls for infection with Bd. Over the course of the next 20 days, we individually exposed another 120 of the remaining tadpoles five times to 30,000 zoospores using a Bd culture isolated from a dead, recently metamorphosed *Alytes obstetricans*. The dead *Alytes* was collected at a recurrent *A. obstetricans* mass mortality site located in the French Pyrenees where only the global pandemic lineage (Bd-GPL) is known to occur and was genotyped as such (Farrer et al. 2011; 2013). Before each exposure, 40 of the 160 mL of water in each tadpole container were replaced and all visible tadpole faeces removed using a disposable sterile pipette. Seven days after the fifth exposure, all tadpoles were again swab-sampled and tested for evidence of infection using the qPCR molecular diagnostic.

At the same time, we collected 40 male alpine newts (*Ichthysaura alpestris*) from artificial ponds located on the Bourget-du-Lac campus of the University of Savoie-Mont-Blanc (45°38'30" N, 5°52'02" E; 240 m a.s.l.). Newts (mean mass ± SD = 1.8 ± 0.23 g) were also housed individually, swab sampled over the fore- and hindlimbs, abdomen and cloaca and swabs tested for evidence of infection using the qPCR molecular diagnostic. Individual newts were then housed in 40 plastic containers (240 x 160 x 144 mm) containing 1.5 liters of

aged tap water. Ten of these experimental units were left as is, containing only a single newt. We added 3 bullfrog tadpoles to all of the other 30 replicates; 10 with unexposed and presumably uninfected tadpoles, and 20 with exposed and presumably infected tadpoles. Tadpoles and newts were cohoused for 15 days at 20.1 ± 1.0°C and on a 16hr/8hr artificial day/night schedule. Water levels were assessed daily and topped up when needed with aged tap water. On day 15, tadpoles were removed, water levels reduced by 500 mL and containers tilted to allow newts to have access to a terrestrial area (a plastic box, 140 x 140 mm, placed within the experimental unit). While cohoused with tadpoles and during the post-exposure period, newts were fed chironomid larvae every 48 hours. Newt containers were cleaned every day with a disposable, sterile plastic pipette to remove feces and food remains. For 29 days after tadpoles were removed we recorded newt mortality and all newts were again swab sampled after death or as survivors at the end of the experiment. Tadpoles that were cohoused with newts (1 per replicate involving exposed tadpoles, n = 30, and 2 per unexposed replicates, n = 20) were rehoused individually in plastic containers as per the negative control tadpoles. Tadpoles were maintained as such until the onset of metamorphosis (Gosner stage 42; Gosner 1960) and then swab-sampled across the epidermis for evidence of infection. We switched swab sampling to skin at this stage because tadpoles have shed keratinized mouthparts which are the target of infection earlier in development, and because keratinization of the *stratum corneum* that occurs at this time becomes the new target for Bd infection.

Dead newts were stored in 70° alcohol. The four newts exposed to Bd infected bullfrog tadpoles alive at the end of the experiment were sacrificed with an overdose of 10 mL/L of phenoxyethanol. Ten cross sections (4 µm) were taken from skin sampled from the interior proximal part of the hind foot of each newt. The skin was embedded in tissue-teck (Sakura Fineteck, USA) and frozen at -18°C. Cross sections were cut using a LEICA CM3050 S

freezing microtome, stained with Ehrlich's haematoxylin and examined for evidence of infection with Bd using light microscopy.

Statistical analyses were performed with the Program R (R Development Core Team, 2010). We used log rank tests to test for differences amongst treatment groups for both bullfrog tadpoles and alpine newts. We also assessed the differences in alpine newt mortality between the 3 treatments using survival analysis (Kaplan-Meier estimate) with 'time until death' as the response variable. Individuals without a corresponding time until death (i.e., survived to the end of the experiment, n = 23) were removed from the analysis.

Results

Bullfrog tadpole Bd status and survival

The 30 tadpoles from which unexposed tadpoles were selected for cohousing with newts (n=10) tested negative for Bd DNA (Table 1). The 60 tadpoles experimentally exposed to Bd zoospores were comprehensively infected with Bd (mean GE $\pm$ 1 SD: 58.6 $\pm$ 32.8) at the start of the cohousing period with newts (n=20, table 1). Exposed tadpoles that were removed from experimental replicates all tested positive for infection on day 15 (n = 60, mean GE $\pm$ 1 SD: 39.6 $\pm$ 23.3), while those from unexposed replicates did not test positive. Twenty of the 26 tadpoles from the exposed replicates surviving to the end of the experiment also tested positive (n = 20, mean GE $\pm$ 1 SD: 49.7 $\pm$ 29.3), whereas no unexposed tadpoles tested positive on day 44.

Tadpoles started to metamorphose (Gosner stage 42) on day 70. Only seven tadpoles did not survive to this date, among them 4 exposed to Bd and cohoused with alpine newt, 2 unexposed to Bd and cohoused with alpine newt, and 1 control (unexposed to Bd and alone).

Survival of bullfrog tadpoles did not differ across tadpole treatment groups (20 tadpoles housed alone and the two newt experiment treatments: Fig. 1a; Log rank test, Chi square test = 1.3, df = 2, $p = 0.526$).

Alpine newt Bd status and survival

All 40 male alpine newts tested negative for Bd before cohousing (Table 1). Newts housed alone (n=10) or with unexposed bullfrog tadpoles (n=10) for 15 days tested negative for Bd at the end of the experiment. Alternatively, 14 newts cohoused with infected bullfrog tadpoles tested positive for infection either at time of death or at the end of the experiment (mean GE $\pm$ 1 SD: 7.6 $\pm$ 6.2). The remaining 6 newts cohoused with infected bullfrog tadpoles tested negative for Bd DNA during this experiment. Among the 16 newts cohoused with infected bullfrog tadpoles which died during the experiment, 11 tested PCR positive for infection. Of the 4 newts cohoused with infected bullfrog tadpoles that survived, 3 were positive for Bd (respectively 3.15, 4.4 and 46.4 GE).

Histological examinations were performed on the skin of all the newts which were exposed to infected bullfrog tadpoles (16 dead newts and the 4 newts alive at the end of the experiments, table 1). Intracellular thalli and zoosporangia at various stages of maturation were observed in the 11 newts that died and tested PCR positive for Bd, in 3 of the 5 dead newts that tested PCR negative for Bd, and in the 3 newts PCR positive for Bd which survived to the end of the experiment. One newt that survived and PCR tested negative for Bd also had no observable thalli and zoosporangia.

Significant variation of mortality occurred among newt treatments: 16 of 20 newts exposed to Bd-infected bullfrog tadpoles were dead by the end of the experiment, while only one of the 20 newts that were not exposed to Bd or tadpoles died. Newts began dying on day

26, 9 days after bullfrog tadpoles were removed (Fig. 1b). Cohousing newts with infected tadpoles significantly affected the mortality rate (Log rank test, Chi square = 21.7, df = 2, p = 1.91 x 10^{-05}). At day 30, survival of newts cohoused with infected tadpoles was reduced by 25 % when compared to newts cohoused with uninfected tadpoles or newts reared alone (Table 2). At day 38, survival of newts cohoused with infected tadpoles was reduced by 80 % compared to newts cohoused with uninfected tadpoles and 70 % compared to those reared alone (Table 2).

Discussion

Genetic and genomic data have been used to describe geographically widespread Bd and endemic Bd lineages (Farrer et al. 2011; 2013). The contact between allopatric populations of Bd could allow recombination, generation of virulent lineages and lead to contemporary amphibian disease emergence (Farrer et al. 2011). Increased sampling and analysis confirmed that Bd is composed of multiple divergent lineages, but which appear endemic in some parts of its range and novel (i.e. emerging) in others (Rosenblum et al. 2013). Perhaps more relevant to this study, patterns of mutation, recombination and aneuploidy make resolving historical relationships of isolates, even within lineages, problematic (Farrer et al. 2013). Because of this, it is questionable if the relationship between invasive amphibian hosts and history of Bd invasion can ever be clearly elucidated.

Nevertheless, introducing infected hosts of any kind to naïve amphibian communities increases host density, elevating transmission rates (Rachowicz and Vredenburg 2004), and prevalence of infection which may be vectored into susceptible species. The American bullfrog is a good candidate to fulfil this role: it has been globally introduced (review in Ficetola et al. 2007) and carries Bd in native (Ouellet et al. 2005) and introduced populations

in Asia (Bai et al. 2010), Europe (Garner et al. 2006), North (Peterson and McKenzie 2014) and South America (Hanselman et al. 2004; Schloegel et al. 2010). Direct evidence of the role of bullfrog as a reservoir of local Bd lineages and/or introduction of allopatric lineages to native amphibian communities are lacking, but in Colorado, amphibian communities invaded by non-native bullfrogs were more likely to support Bd infected individuals (Peterson and McKenzie 2014). The transmission of Bd from native American bullfrog juveniles to syntopic wood frog tadpoles (*Lithobates sylvaticus*) was shown experimentally by Greenspan et al. (2012). Extending on their work, our experiment shows that infected and non-native bullfrog tadpoles can transmit Bd to adult alpine newts under experimental conditions. Newts exposed to infected bullfrog tadpoles in our study readily developed infections in a matter of days and sustained these infections for weeks after exposure without any need for re-exposure beyond the 15 days of cohousing. In the wild, bullfrog populations are well-established in France and geographically overlap with native alpine newt populations in one region (Ficetola et al. 2007). Temporally the potential for spill over exists, as breeding by adult newts coincides with the presence of bullfrog tadpoles for a period of months (Michelin et al. 2014) and tadpoles we experimentally exposed were still infected 70 days after initial exposure, with no significant decrease in infection burden. This was strong enough to transmit infection to at least of 70% of the cohoused newts. Newly metamorphosed bullfrogs are not always an efficient reservoir species for Bd and may experience heavy mortality (Gervasi et al. 2013), but bullfrog metamorphs in our study did not suffer mortality from this virulent Bd-GPL lineage. Range overlap, persistent and strong burdens of infection and high prevalence, in this case across life history stages: these are all key traits of a competent vector, as transmission is more likely when infectious particles are available for transmission over a longer time span (Murray et al. 2009).

In nature newts commonly leave water and stay on land for significant periods of time. Behavioural avoidance of aquatic zoospores has been described in another species (e.g. McMahon et al. 2014) and our experiment offered no opportunity for newts to escape from the water. The aquatic environment is important for transmission of zoospores, and the heavy infections consistently generated in our study by cohousing with bullfrog tadpoles were of similar strength to burdens estimated from newts captured from aquatic environments in the wild at sites where newts occur at high densities (Garner et al. 2005). Bullfrogs have not been detected at the newt study sites sampled by Garner et al. (2005) and mortality of alpine newts attributable to chytridiomycosis has never been reported. The impact of host community structure on probability of infection and strength of infection with Bd is a common theme in amphibian host/chytrid systems, where increased density of hosts harbouring the heaviest infections is expected to elicit greater prevalence and heavier infections (Searle et al. 2011; but see Bielby et al. 2015). Infections of tadpoles were far stronger than newts in our study, which may go some way towards explaining why experimental newts experienced significant mortality while newts occupying ponds lacking a heterospecific reservoir exhibiting stronger infections appear not to. Further study of the relationships between habitat choice, host community composition and susceptibility of alpine newts to infection and chytridiomycosis is certainly warranted.

Some newts that died did not exhibit detectable infection using either diagnostic. Several studies have reported increased risk of mortality during prolonged exposure to Bd even with no evidence of infection at time of death (Luquet et al. 2012; Garner et al. 2009). Resisting infection with Bd is probably costly, potentially increasing the mortality risk of these individuals. But not in all cases: of the four survivors, at least 3 exhibited significant levels of infection. One hypothesis for this could be inter-individual variation in immune defence. Innate immunity in the form of skin antimicrobial peptides secretions can act as first

line of defence against Bd, and has been shown to allow tolerance of infection (Woodhams et al. 2007; Rollins-Smith 2009; Ramsey et al. 2010). Whatever component of immunity may be responsible for tolerance or resistance, repeated exposure to Bd has been shown immunize against subsequent costs (McMahon et al. 2014, but see Cashings et al. 2013. This seems unlikely in our case as we have never detected infection in the source population for the newts we used (102 adults tested with qPCR, C. Miaud, unpublished data) and none of our experimental animals tested positive before exposure. These are strong indications that the alpine newts used in the experiment were Bd naïve.

These experimental findings are the first evidence of death attributable to exposure to and infection with Bd in alpine newts, adding to an ever-growing list of European amphibian species that may be deleteriously affected through interactions with this fungus (Baláž et al. 2014; Bosch et al. 2013; Garner et al. 2013; Luquet et al. 2012; Bielby et al. 2009; Garner et al. 2009; Bosch et al. 2001). Additional surveillance for Bd-related newt mortality in the wild is called for to investigate potential disease-associated decline under natural conditions.

Conclusion

We conclude from our experiment that invasive bullfrogs are effective reservoirs of Bd, capable of transmitting infections to native hosts. Infections with the Bd-GPL lineage transmitted by invasive bullfrogs can be sustained for weeks after initial exposure and have the capacity to cause significant mortality in native species. Although we cannot resolve the debate regarding the role invasive hosts have played in introducing Bd to Europe, we do conclude that infected, invasive bullfrog tadpoles will increase the likelihood that infection with Bd will be maintained in a European amphibian community (Spitzen-van der Sluijs et al. 2014). Experimental results (this study) do not always reflect field conditions, but spill-over

from bullfrog tadpoles to native European amphibians has the potential to drive mortality in native species. Removal of invasive bullfrogs as a conservation strategy has been adopted in several European countries based on the conclusion that bullfrogs can cause native species declines due to competition and predation (Kupferberg 1997; Lawler et al. 1999). Our study further justifies these efforts. Even if removal may not eliminate infections in native hosts, any reduction in density of infected hosts capable of transmitting to susceptible hosts should reduce the likelihood of infections reaching potentially lethal thresholds (Peterson and McKenzie 2014). Exposure duration, zoospore load and virulence can dictate the severity of the costs associated with exposure and infection (e.g. Briggs et al. 2010) and the removal of infected bullfrogs has a strong likelihood of reducing the impact of Bd on other, native susceptible host species.

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Fig. 1. Survival curves for American bullfrog tadpoles *Lithobates catesbeianus* tadpoles (a) and alpine newt *Ichthyosaura alpestris* (b). For both figures, animals housed singly and not exposed to the fungus *Batrachochytrium dendrobatidis* (Bd) are represented by the dotted line, animals cohoused with uninfected animals are represented by the solid line and cohoused animals where tadpoles were infected with Bd are represented by broken line.

Fig. 1a

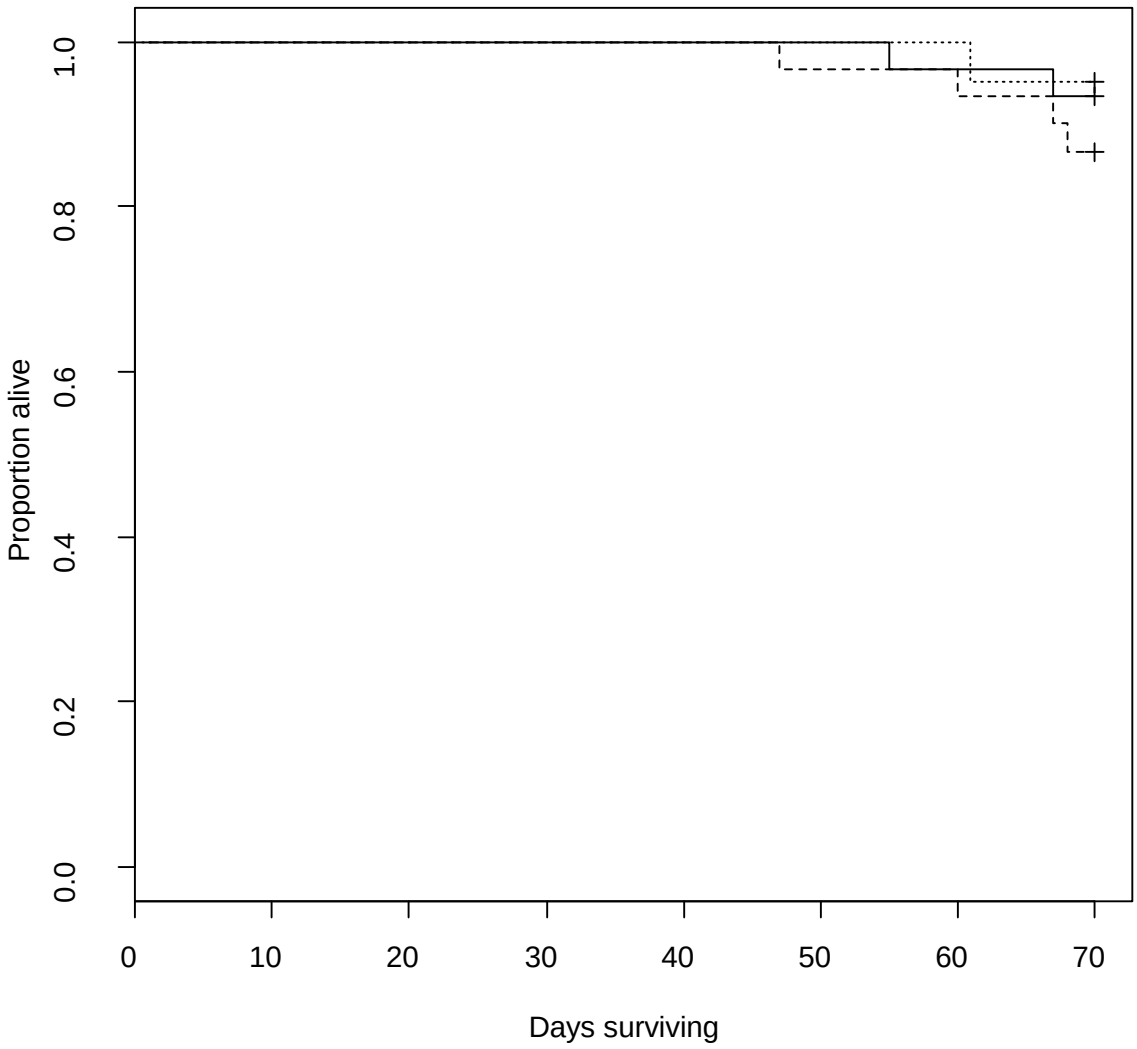

Fig. 1b

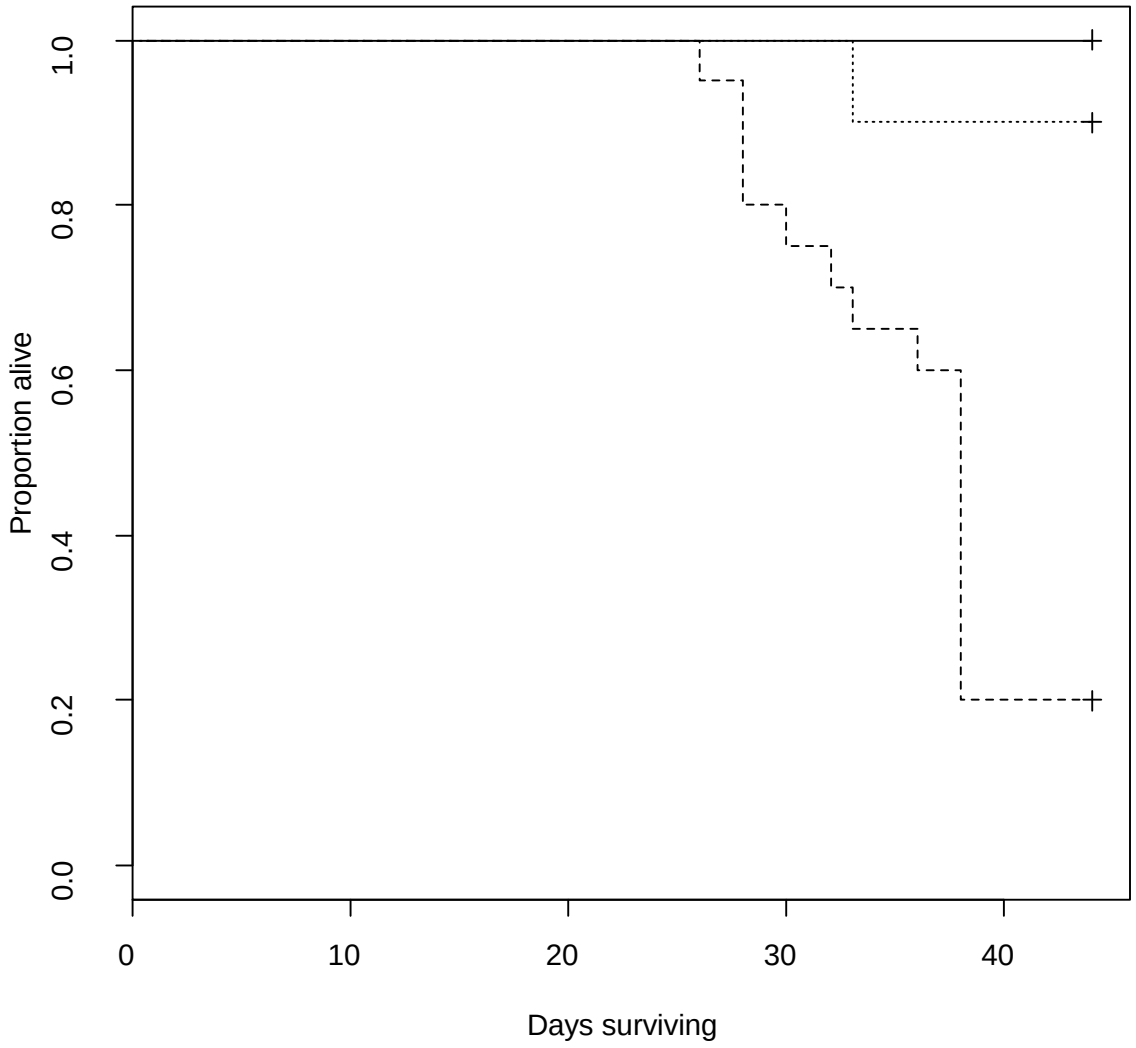

Table 1 Design and results of the cross contamination experiment, the co-housing of alpine newt *Ichthyosaura alpestris* with American bullfrog tadpoles *Lithobates catesbeianus* infected by the fungus *Batrachochytrium dendrobatidis.*

	qPCR test			
Before the experiment	Bd+	Bd-		
Alpine newts alone (n=40)	0	40		
Bullfrog tadpoles alone (n=20)	0	20		
Bullfrog tadpoles Bd+ (n=60)	60	0		
Bullfrog tadpoles Bd- (n=30)	0	30		
	qPCR test		**Histological test**	
During the experiment	Bd+	Bd-	Bd+	Bd-
Alpine newt alone (n=10)	0	10	-	-
Alpine newt with Bd- tadpoles (n=10)	0	10	-	-
Alpine newts with Bd+ tadpoles (n=20) overall	14	6	17	3
Alpine newts with Bd+ tadpoles which died (n=16)	11	5	14	2
Alpine newts with Bd+ tadpoles which survived (n=4)	3	1	1	2

Bullfrog tadpoles Bd+ = tadpole experimentally infected with Bd and co-housed with alpine newts. Bullfrog tadpoles Bd- = tadpole Bd- and co-housed with alpine newts. Alpine newt with Bd- or Bd+ tadpoles = 1 alpine newt adult is co-housed with 3 American Bullfrog tadpoles.

Table 2 Survival of alpine newts *Ichthyosaura alpestris* co-housed with American bullfrog tadpoles *Lithobates catesbeianus* infected by the fungus *Batrachochytrium dendrobatidis.*

Treatment = newts with Bd positive tadpoles

Time	n	n death	survival	std.err	lower 95% CI	upper 95% CI
26	20	1	0.95	0.0487	0.8591	1.000
28	19	3	0.80	0.0894	0.6426	0.996
30	16	1	0.75	0.0968	0.5823	0.966
32	15	1	0.70	0.1025	0.5254	0.933
33	14	1	0.65	0.1067	0.4712	0.897
36	13	1	0.60	0.1095	0.4195	0.858
38	12	8	0.20	0.0894	0.0832	0.481

12 dead in this treatment

Treatment = newt as control (newt alone)

Time	n	n death	survival	std.err	lower 95% CI	upper 95% CI
30	10	0	1.0	-	-	-
33	10	1.0	0.90	0.0949	0.7320	1.0000
38	10	1.0	0.9	0.0949	0.7320	1.0000

1 dead in the control at day 33

Time = days since the beginning of the experiment, n = number of alive alpine newts, n death = number of dead alpine newt along the last 24h.