

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

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9 **Claude Miaud • Tony Dejean • Karine Savard • Annie Millery • Alice Valentini • Trenton**
10 **W.J. Garner**

11
12
13
14
15
16 9 Claude Miaud

17 10 *Université de Savoie-Mont-Blanc, UMR CNRS 5553, Laboratoire d'Écologie Alpine, 73376*
18 11 *Le-Bourget-du-Lac, France*

19 12 *Centre d'Ecologie Fonctionnelle et Évolutive (UMR 5175), Ecole Pratique des Hautes*
20 13 *Etudes, Biogéographie et Ecologie des Vertébrés, Campus CNRS, 34293 Montpellier, France*

21 14 e-mail: claude.miaud@cefe.cnrs.fr

22 15 Tél: 33 4 67 61 33 43

23 16
24 17 Tony Dejean

25 18 *Université de Savoie-Mont-Blanc, UMR CNRS 5553, Laboratoire d'Écologie Alpine, 73376*
26 19 *Le-Bourget-du-Lac, France*

27 20 *Parc naturel régional Périgord-Limousin, 24450 La Coquille, France*

28 21 tony.dejean@spygen.com

29 22
30 23 Karine Savard

31 24 *Université de Savoie-Mont-Blanc, UMR CNRS 5553, Laboratoire d'Écologie Alpine, 73376*
32 25 *Le-Bourget-du-Lac, France*

33 26 *Agriculture and Agri-Food Canada, Ottawa, Ontario K1A 0C6, Canada*

34 27 karine.savard@agr.gc.ca

35 28
36 29 Annie Millery

37 30 *Université de Savoie-Mont-Blanc, UMR CNRS 5553, Laboratoire d'Écologie Alpine, 73376*
38 31 *Le-Bourget-du-Lac, France*

39 32 Annie.Millery@univ-savoie.fr

40 33
41 34
42 35 Alice Valentini

43 36 *Université de Savoie-Mont-Blanc, UMR CNRS 5553, Laboratoire d'Écologie Alpine, 73376*
44 37 *Le-Bourget-du-Lac, France*

45 38 alice.valentini@spygen.com

46 39
47 40 Nadine Curt Grand Gaudin

48 41 *Université de Savoie, UMR CNRS 5553, Laboratoire d'Écologie Alpine, 73376 Le-Bourget-*
49 42 *du-Lac, France*

50 43 Nadine.Cristina@univ-savoie.fr

1
2 Trenton W.J. Garner

3 *Institute of Zoology, Zoological Society of London, Regents Park, NW1 4RY, London, UK*

4 *Environmental Sciences and Development, Northwest University, Private Bag X6001,*

5 *Potchefstroom, 2531, South Africa*

6 Trent.Garner@ioz.ac.uk

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8 **Abstract** Invasive species can be a threat to native species in several ways, including

9 transmitting lethal infections caused by the parasites they carry. However, invasive species

10 may also be plagued by novel and lethal infections they acquire when invading, making

11 inferences regarding the ability of an invasive host to vector disease difficult from field

12 observations of infection and disease. This is the case for the pathogenic fungus

13 *Batrachochytrium dendrobatidis* (Bd) in Europe and one invasive host species, the North

14 American bullfrog *Lithobates catesbeianus*, hypothesized to be responsible for vectoring

15 lethal infection to European native amphibians. We tested this hypothesis experimentally

16 using the alpine newt *Ichthyosaura alpestris* as our model native host. Our results show that

17 infected bullfrog tadpoles are effective vectors of Bd. Native adult newts co-housed with

18 experimentally infected bullfrog tadpoles became Bd infected (molecular and histological

19 tests). Moreover, the exposed adult newts suffered mortality while the majority of infected

20 Bullfrog tadpoles survived until metamorphosis. These results cannot resolve the historical

21 role of alien species in establishing the distribution of Bd across Europe or other regions in

22 the world where this species was introduced, but they show its potential role as a Bd reservoir

23 capable of transmitting lethal infections to native amphibians. Finally, our results also suggest

24 that the removal of infected bullfrogs from aquatic environments may serve to reduce the

25 availability of Bd in European amphibian communities, offering another justification for

26 bullfrog eradication programmes that are currently underway or may be considered.

1 **Keywords** Introduced amphibian • disease • cross-contamination • fungus • American
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1 Introduction

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5 3 Invasive, non-native species are considered to be one of the greatest threats to biodiversity
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7 4 and threaten native species through a variety of mechanisms. The co-introduction of parasites
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10 5 capable of eliciting significant pathogenesis in naïve native hosts is thought to be one of the
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12 6 major mechanisms behind biodiversity loss attributable to invasive species (Daszak et al.
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15 7 2000; Prenter et al. 2004; Crowl et al. 2008). Indeed, parasites that are transported with
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17 8 invasive species tend to reach equivalent prevalence in native species (Torchin et al. 2003),
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19 9 sometimes with devastating consequences (Martel et al. 2014; Doddington et al. 2013; Bosch
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22 10 et al. 2013). However, invasive species may carry significantly reduced parasite diversity
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24 11 when invading (Torchin et al. 2003) and commonly become infected with parasites that occur
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27 12 in endemic residents (Colautti et al., 2004; Bürgi and Mills 2014). Invasive species that are
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29 13 infected with resident parasites can suffer costs exceeding those experienced by the native
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32 14 host species (Wolfe et al. 2004) or equivalent to those experienced by native species infected
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34 15 with newly introduced parasites (Heger and Jeschke 2014). The unpredictability of these
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37 16 relationships means that patterns of parasite infection and disease in native and invasive hosts
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39 17 do not always indicate which host may be serving as a vector for the parasite.

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42 18 *Batrachochytrium dendrobatidis* (Bd), a global fungal pathogen of amphibians, is
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44 19 presumed to be an invasive parasite in many parts of its range (Farrer et al. 2011). Bd
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46 20 invasion is commonly attributed to the release of infected, asymptomatic species that have
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49 21 been displaced as a result of trade (Hanselmann et al. 2004). A prime example is that of the
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51 22 North American bullfrog *Lithobates catesbeianus* (Hanselmann et al. 2004). Due to their
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53 23 ubiquity as a traded species infected with Bd (Bai et al. 2010; Schloegel et al. 2009), their
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56 24 distribution and a consistent pattern of infection with Bd (Garner et al. 2006), they have been
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59 25 proposed to be important vectors of Bd into native amphibians. Bullfrogs may contribute to

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

9 Bullfrogs have been widely introduced in Europe in an uncoordinated, multinational
10 effort to establish viable populations for the trade in frog legs (Ficetola et al. 2007). Invasive
11 bullfrog populations were consistently founded by a small number of adults directly
12 transported from their native range and much of the current distribution in Europe probably
13 arose through translocation from these founder populations (Ficetola et al. 2008). This small
14 number of potential transport vectors is incompatible with the widespread, pervasive
15 distribution of Bd across Europe (Olson et al. 2013) and the patterns of Bd invasion in areas
16 of Europe where bullfrogs are absent (Bielby et al. 2013; Bosch et al. 2013; Walker et al.
17 2008; 2010). Introduced bullfrogs can potentially transmit Bd to native amphibians, but spill-
18 over can be from native hosts to invasive bullfrogs. If this were the case, invasive bullfrogs
19 would be accruing infection from native hosts, and we would predict that native species
20 commonly infected with Bd would be relatively tolerant of infection while bullfrogs would
21 exhibit costs such as the post-metamorphic mortality of bullfrogs experiencing strong
22 infections in their native range, as described by Gervasi et al. (2013). The possibility does
23 remain that bullfrogs act as vectors of infection and disease in Europe, and can act as
24 significant reservoir hosts. Indeed, Bd has been documented infecting bullfrog populations
25 across Europe (Garner et al. 2006). In this instance, we predict that native European

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

4 In this paper, we experimentally determine if invasive bullfrogs are significant vectors
5 of Bd to European amphibians. We cohoused experimentally infected bullfrog tadpoles with
6 adult native amphibians, in this case, the alpine newt. We selected the alpine newt because it
7 is known to be infected with Bd across Europe (Zampiglia et al. 2013; Sztatecsny and Glaser
8 2011) but little is known about its susceptibility to lethal chytridiomycosis. We considered
9 bullfrog tadpoles as the appropriate life history stage for assessing reservoir status of the
10 species because tadpoles with prolonged larval periods are commonly cited as significant
11 reservoirs of infection (Briggs et al. 2010; Walker et al. 2010). We recorded infection status,
12 determined through molecular diagnostics and histology, burden of infection and survival in
13 both bullfrog tadpoles and adult alpine newts. For bullfrogs, we measured mortality rates
14 until the onset of metamorphosis, as significant costs associated with larval infection initially
15 manifest when metamorphosis is near to completion (Gervasi et al. 2013; Walker et al. 2010;
16 Garner et al. 2009).

18 **Materials and methods**

19
20 One clutch of American bullfrog (*Lithobates catesbeianus*) spawn was collected in June 2009
21 at an artificial pond in Ambarès in southwestern France (44°56'22" N, 0°31'04" E; 20 m
22 a.s.l.). Eggs were hatched and larvae reared in the laboratory in three plastic containers (400 x
23 600 x 200 mm) each containing approximately 35 L of aged tap water. Larvae were fed
24 flaked goldfish food provided *ad libitum* during this and the subsequent exposure periods (see
25 below). In May 2010, 30 of the 250 available tadpoles (Gosner stages 26–30; Gosner 1960)

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

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

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

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

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

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

3 Statistical analyses were performed with the Program R (R Development Core Team,
4 2010). We used log rank tests to test for differences amongst treatment groups for both
5 bullfrog tadpoles and alpine newts. We also assessed the differences in alpine newt mortality
6 between the 3 treatments using survival analysis (Kaplan-Meier estimate) with 'time until
7 death' as the response variable. Individuals without a corresponding time until death (i.e.,
8 survived to the end of the experiment, n = 23) were removed from the analysis.

10 **Results**

12 **Bullfrog tadpole Bd status and survival**

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

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

1 Survival of bullfrog tadpoles did not differ across tadpole treatment groups (20 tadpoles
2 housed alone and the two newt experiment treatments: Fig. 1a; Log rank test, Chi square test
3 = 1.3, $df = 2$, $p = 0.526$).

5 Alpine newt Bd status and survival

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

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

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

1 26, 9 days after bullfrog tadpoles were removed (Fig. 1b). Cohousing newts with infected
2 tadpoles significantly affected the mortality rate (Log rank test, Chi square = 21.7, df = 2, $p =$
3 1.91×10^{-05}). At day 30, survival of newts cohoused with infected tadpoles was reduced by
4 25 % when compared to newts cohoused with uninfected tadpoles or newts reared alone
5 (Table 2). At day 38, survival of newts cohoused with infected tadpoles was reduced by 80 %
6 compared to newts cohoused with uninfected tadpoles and 70 % compared to those reared
7 alone (Table 2).

9 **Discussion**

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

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

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

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

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

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

9 These experimental findings are the first evidence of death attributable to exposure to
10 and infection with Bd in alpine newts, adding to an ever-growing list of European amphibian
11 species that may be deleteriously affected through interactions with this fungus (Baláz et al.
12 2014; Bosch et al. 2013; Garner et al. 2013; Luquet et al. 2012; Bielby et al. 2009; Garner et
13 al. 2009; Bosch et al. 2001). Additional surveillance for Bd-related newt mortality in the wild
14 is called for to investigate potential disease-associated decline under natural conditions.

16 **Conclusion**

18 We conclude from our experiment that invasive bullfrogs are effective reservoirs of Bd,
19 capable of transmitting infections to native hosts. Infections with the Bd-GPL lineage
20 transmitted by invasive bullfrogs can be sustained for weeks after initial exposure and have
21 the capacity to cause significant mortality in native species. Although we cannot resolve the
22 debate regarding the role invasive hosts have played in introducing Bd to Europe, we do
23 conclude that infected, invasive bullfrog tadpoles will increase the likelihood that infection
24 with Bd will be maintained in a European amphibian community (Spitzen-van der Sluijs et al.
25 2014). Experimental results (this study) do not always reflect field conditions, but spill-over

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

12
13 **Summary statement** All experimental work done here was ethically reviewed at Université
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1 **References**

- 2
3
4 3 Bai C, Garner TWJ, Yiming L (2010) First evidence of *Batrachochytrium dendrobatidis* in
5
6 4 China: discovery of chytridiomycosis in introduced American bullfrogs and native
7
8 5 amphibians in the Yunnan Province, China. *Ecohealth* 7: 127-134
9
- 10 6 Baláz V, Vörös J, Civiš P, Voja J, Hettyey A, Sós E, Dankovics R, Jehle R, Christiansen DG,
11
12 7 Clare F, Fisher MC, Garner TWJ, Bielby J (2014) Assessing risk and guidance on
13
14 8 monitoring of *Batrachochytrium dendrobatidis* in Europe through identification of
15
16 9 taxonomic selectivity of infection. *Conserv Biol* 28: 213-223
17
- 18 10 Bataille A, Fong JJ, Cha M, Wogan GOU, Baek HJ, Lee H, Min M-S, Waldman B (2013)
19
20 11 Genetic evidence for a high diversity and wide distribution of endemic strains of the
21
22 12 pathogenic chytrid fungus *Batrachochytrium dendrobatidis* in wild Asian amphibians.
23
24 13 *Mol Ecol* 22: 4196-4209
25
- 26 14 Bielby J, Bovero S, Sotgiu G, Tessa G, Favelli M, Angelini C, Doglio S, Clare F, Gazzaniga
27
28 15 E, Lapietra F, Garner TWJ (2009) Fatal chytridiomycosis in the Tyrrhenian painted
29
30 16 frog. *Ecohealth* 6: 27-32
31
- 32 17 Bielby J, Bovero S, Sotgiu G, Tessa G, Favelli M, Angelini C, Perkins M, Garner TWJ
33
34 18 (2013) Geographic, taxonomic and ecological variation in *Batrachochytrium*
35
36 19 *dendrobatidis* infection within a highly endemic amphibian community. *Divers &*
37
38 20 *Distrib* 19: 1153-1163
39
- 40 21 Bielby J, Fisher MC, Clare FC, Rosa GM, Garner TWJ (2015) Host species vary in infection
41
42 22 probability, sub-lethal effects, and costs of immune response when exposed to an
43
44 23 amphibian parasite. *Sci. Reports* 5: 20828
45
- 46 24 Bosch J, Garcia D, Fernandez-Beaskoetxea S, Fisher MC, Garner TWJ (2013) Evidence for
47
48 25 the introduction of lethal chytridiomycosis affecting wild Betic midwife toads (*Alytes*
49
50 26 *dickhilleni*). *Ecohealth* 10: 82-89
51
- 52 27 Bosch J, Martínez-Solano I, Garcia-Paris M (2001) Evidence of a chytrid fungus infection
53
54 28 involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected
55
56 29 areas of central Spain. *Biol Conserv* 97: 331-337
57
58
59
60
61
62
63
64
65

1 Boyle DG, Boyle DB, Olson V, Morgan JAT, Hyatt AD (2004) Rapid quantitative detection
2 of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using
3 real-time Taqman PCR assay. *Dis Aquat Organ* 60: 141-148
4
5
6 4 Briggs CJ, Knapp RA, Vredenburg VT (2010) Enzootic and epizootic dynamics of the
7 chytrid fungal pathogen of amphibians. *Proc Nat Acad Sc U.S.A.* 107: 9695-9700
8
9
10 6 Bürgi LP, Mills NJ (2014) Lack of enemy release for an invasive leafroller in California:
11 temporal patterns and influence of host plant origin. *Biol Inv* 16: 1021-1034
12
13
14
15 8 Cashins SD, Grogan LF, McFadden M, Hunter D, Harlow PS, Berger L, et al. (2013) Prior
16 Infection Does Not Improve Survival against the Amphibian Disease
17 Chytridiomycosis. *PLoS ONE* 8(2): e56747.
18
19
20 10
21
22 11 Colautti R, Ricciardi A, Grigorovich IA, MacIsaac HJ (2004) Is invasion success explained
23 by the enemy release hypothesis? *Ecol Lett* 7(8): 721–733
24
25
26
27 13 Crowl TT, Crist TO, Parmenter RR, Belovsky G, Lugo AE (2008) The spread of invasive
28 species and infectious disease as drivers of ecosystem change. *Frontiers in Ecology*
29 and the Environment 6(5): 238–246
30
31
32
33
34 16 Daszak P, Cunningham AA, Hyatt AD (2000) Emerging infectious diseases of wildlife –
35 threats to biodiversity and human health. *Science* 287: 443-449
36
37
38
39 18 Doddington BJ, Bosch J, Oliver JA, Grassly NC, Garcia G, Garner TWJ, Fisher MC (2013)
40 Context-dependent amphibian host population response to an invading pathogen.
41 *Ecology* 94: 1795-1804
42
43
44
45 21 Farrer RA, Henk DA, Garner TWJ, Balloux F, Woodhams DC, Fisher MC (2013)
46 Chromosomal copy number variation, selection and uneven rates of recombination
47 reveal cryptic genome diversity linked to pathogenicity. *PLoS Genetics* 9(8):
48 e1003703
49
50
51
52
53 25 Farrer RA, Weinert LA, Bielby J, Garner TWJ, Balloux F, Clare F, Bosch J, Cunningham
54 AA, Weldon C, du Preez LH, Anderson L, Kosakovsky Pond SL, Shahar-Golan R,
55 Henk DA, Fisher MC (2011) Multiple emergences of amphibian chytridiomycosis
56
57
58
59
60
61
62
63
64
65

- 1 include a globalised hypervirulent recombinant lineage. Proc Nat Acad Sc U.S.A.
2 108: 18732-18736
- 3 Ficetola GF, Bonin A, Miaud C (2008) Population genetics reveals origin and number of
4 founders in a biological invasion. Mol Ecol 17: 773-782
- 5 Ficetola GF, Coïc C, Detaint M, Berroneau M, Lorvelec O, Miaud C (2007) Pattern of
6 distribution of the American bullfrog *Rana catesbeiana* in Europe. Biol Inv 9: 767-772
- 7 MC, TWJ. Garner (2007) The relationship between the emergence of *Batrachochytrium*
8 *dendrobatidis*, the international trade in amphibians and introduced amphibian
9 species. Fungal biology reviews 21: 2 – 9
- 10 Garner TWJ, Walker S, Bosch J, Hyatt AD, Cunningham AA, Fisher MJ (2005) Chytrid
11 fungus in Europe. Emerg. Infect. Dis. 11: 1639-1641
- 12 Garner TWJ, Perkins M, Govindarajulu P, Seglie D, Walker SJ, Cunningham AA , Fisher
13 MC (2006) The emerging amphibian pathogen *Batrachochytrium dendrobatidis*
14 globally infects introduced populations of the North American bullfrog, *Rana*
15 *catesbeiana*. Biol Letters 2: 455-459
- 16 Garner TWJ, Walker S, Bosch J, Leech S, Rowcliffe JM, Cunningham AA, Fisher MC
17 (2009) Life history trade-offs influence mortality associated with the amphibian
18 pathogen *Batrachochytrium dendrobatidis*. Oikos 118: 783-791
- 19 Garner TWJ, Martel A, Bielby J, Bosch J, Anderson L, Meredith A, Cunningham AA, Fisher
20 MC, Henk DA, Pasmans F (2013) Infectious diseases that may threaten Europe's
21 amphibians. Pp. 1-41 *In: Amphibian Biology, Volume 11, Issue 3: Diseases, Declines*
22 *and Conservation of Amphibians in Seven Western European Countries. Pelagic*
23 *Publishing Ltd.*
- 24 Gervasi SS, Urbina J, Hua J, Chestnut T, Relyea RA, Blaustein AR (2013) Experimental
25 evidence for American bullfrog (*Lithobates catesbeianus*) susceptibility to chytrid
26 fungus (*Batrachochytrium dendrobatidis*). Ecohealth 10: 166-171
- 27 Gosner KL (1960) A simplified table for staging anuran embryos and larvae with notes on
28 identification. Herpetol 16: 183-190
- 29 Greenspan SE, Calhoun AJ, Longcore JE, Levy MG (2012) Transmission of
30 *Batrachochytrium dendrobatidis* to wood frogs (*Lithobates sylvaticus*) via a bullfrog
31 (*L. catesbeianus*) vector. J Wild Diseases 48: 575-582

- 1 Hanselmann R, Rodríguez A, Lampo M, Fajardo-Ramos L, Aguirre AA, Kilpatrick AM,
2 Rodríguez JP, Daszak P (2004) Presence of an emerging pathogen of amphibians in
3 introduced bullfrogs *Rana catesbeiana* in Venezuela. *Biol Conserv* 120: 115-119
4
5
6 Heger T, Jeschke JM (2014) The enemy release hypothesis as a hierarchy of hypotheses.
7
8 *Oikos* 123: 741-750
9
10 Kupferberg SJ (1997) Bullfrog (*Rana catesbeiana*) invasion of a California river: the role of
11 larval competition. *Ecology* 78: 1736-1751
12
13
14 Lawler SP, Dritz D, Strange T, Holyoak M (1999) Effects of introduced mosquitofish and
15 bullfrogs on the threatened California red-legged frog. *Conserv Biol* 13: 613-622
16
17
18 Luquet E, Garner TWJ, Léna J-P, Bruel C, Joly P, Lengagne T, Grolet O, Plénet S (2012)
19 Genetic erosion in wild populations makes resistance to a pathogen more costly.
20
21 *Evolution* 66: 1942-1952
22
23
24 Martel A, Blooi M, Adriaensen C, Van Rooij P, Beukema W, Fisher MC, Farrer RA, Schmidt
25 BR, Tobler U, Goka K, Lips KR, Muletz C, Zamudio K, Bosch J, Lötters S,
26 Wombwell E, Garner TWJ, Spitzen-van der Sluijs A, Salvidio S, Ducatelle R,
27 Nishikawa K, Nguyen TT, Van Bocxlaer I, Bossuyt F, Pasmans F (2014) Recent
28 introduction of a chytrid fungus endangers Western Palearctic salamanders. *Science*
29
30 346: 630-631
31
32
33
34
35
36 McMahon TA, Sears BF, Venesky MD, Bessler SM, Brown JM, Deutsch K, Halstead NT,
37 Lentz G, Tenouri N, Young S, Civitello DJ, Ortega N, Fites JS, Reinert LK, Rollins-
38 Smith LA, Raffel TR, Rohr JR (2014) Amphibians acquire resistance to live and dead
39 fungus overcoming fungal immunosuppression. *Nature* 511:224–227
40
41
42
43
44 Michelin G, Epain-Henry C, Beguin D (2004) Grenouille taureau *Lithobates catesbeianus*
45 (Shaw, 1802) Bilan des prospections et phase opérationnelle 2014. Comité
46
47 Départemental de la Protection de la Nature et de l'Environnement, Syndicat
48
49 d'Entretien du Bassin du Beuvron (Eds), 54 p.
50
51
52
53 Murray KA, Skerratt LF, Speare R, Callum HMC (2009) Impact and Dynamics of Disease in
54
55 Species Threatened by the Amphibian Chytrid Fungus, *Batrachochytrium*
56
57 *dendrobatidis*. *Conserv Biol* 23: 1242–1252
58
59
60
61
62
63
64
65

- 1 Olson DH, Aanensen DM, Ronnenberg KL, Powell CI, Walker SF, Bielby J, Garner TWJ,
2 Weaver G, the *Bd* Mapping Group, Fisher MC (2013) Mapping the global emergence of
3 *Batrachochytrium dendrobatidis*, the amphibian chytrid fungus. PLoS ONE 8(2): e56802
4
5 Ouellet, M., Mikaelian I, Pauli BD, Rodrigue J, Green DM (2005) Historical evidence of
6 widespread chytrid infection in North American amphibian populations. Conserv Biol
7 19:1431–1440
8
9 Pasmans F, Van Rooij P, Blooi M, Tessa G, Bogaerts S, Sotgiu G, Garner TWJ, Fisher MC,
10 Woeltjes T, Beukema W, Bovero S, Adriaensen C, Oneto F, Ottonello D, Martel A,
11 Salvidio S (2013) Resistance to Chytridiomycosis in the European Plethodontid of the
12 Genus *Speleomantes*. PLoS ONE 8(5): e63639
13
14 Peterson AC, McKenzie VJ (2014) Investigating Differences across Host Species and Scales
15 to Explain the Distribution of the Amphibian Pathogen *Batrachochytrium*
16 *dendrobatidis*. PLoS ONE 9(9): e107441.
17
18 Prenter J, MacNeil C, Dick JTA, Dunn AM (2004) Roles of parasites in animal invasions.
19 Trends in Ecology and Evolution 19(7): 385-390.
20
21 Rachowicz LJ, Vredenburg VT (2004) Transmission of *Batrachochytrium dendrobatidis*
22 within and between amphibian life stages. Dis Aquat Organ 61: 75-83
23
24 Ramsey JP, Reinert LK, Harper LK, Woodhams DC, Rollins-Smith LA (2010) Immune
25 defenses against *Batrachochytrium dendrobatidis*, a fungus linked to global
26 amphibian declines, in the South African Clawed Frog, *Xenopus laevis*. Infect Immun
27 78:3981–3992
28
29 R Development Core team (2010) R: A language and environment for statistical computing.
30 Vienna, Austria
31
32 Rollins-Smith LA (2009) The role of amphibian antimicrobial peptides in protection of
33 amphibians from pathogens linked to global amphibian declines. Biochim Biophys
34 Acta 1788:1593–1599
35
36 Richardson JML, Govindarajulu P, Anholt BR (2014) Distribution of the disease pathogen
37 *Batrachochytrium dendrobatidis* in non-epidemic amphibian communities in western
38 Canada. Ecography 37: 883-893
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

- 1 Schloegel LM, Ferreira CM, James TY, Hipolito M, Longcore JE, Hyatt AD, Yabsley M,
2 Martins AMCRPF, Mazzoni R, Davies AJ, P. Daszak (2010) The North American
3 bullfrog as a reservoir for the spread of *Batrachochytrium dendrobatidis* in Brazil.
4 Anim Conserv 13: 53-61
- 5 Schloegel LM, Picco AM, Kilpatrick AM, Davies AJ, Hyatt AD, Daszak P (2009) Magnitude
6 of the US trade in amphibians and the presence of *Batrachochytrium dendrobatidis*
7 and ranavirus infection in imported North American bullfrogs (*Rana catesbeiana*).
8 Biol Conserv 142: 1420-1426
- 9 Searle CL, Biga L, Spatafora JW, Blaustein AR (2011) A dilution effect in the emerging
10 amphibian pathogen *Batrachochytrium dendrobatidis*. Proc. Natl. Acad. Sci. U.S.A.
11 108: 16322-16326
- 12 Spitzen - van der Sluijs A, Martel A, Hallmann CA, Bosman W, Garner TWJ, van Rooij P,
13 Jooris R, Haesebrouck F, Pasmans F (2014) Environmental determinants promote
14 recent endemism of *Batrachochytrium dendrobatidis* infections in amphibian
15 assemblages in Northwestern Europe in the absence of disease outbreak. Conserv Biol
16 28: 1302-1311
- 17 Sztatecsny M, Glaser F (2011) From the eastern lowlands to the western mountains: first
18 records of the chytrid fungus *Batrachochytrium dendrobatidis* in wild amphibian
19 populations from Austria. Herpetol J 21: 87-90
- 20 Torchin ME, Lafferty KD, Dobson AP, McKenzie VJ, Kuris AM (2003) Introduced species
21 and their missing parasites. Nature 421: 628-630
- 22 Walker SF, Bosch J, Gomez V, Garner TWJ, Cunningham AA, Schmeller DS, Ninyerola M,
23 Henk D, Ginestet C, Christian-Philippe A, Fisher MC (2010) Factors driving
24 pathogenicity versus prevalence of the amphibian pathogen *Batrachochytrium*
25 *dendrobatidis* and chytridiomycosis in Iberia. Ecol Letters 13: 372-382
- 26 Walker SF, Bosch J, James TY, Litvintseva AP, Valls JAO, Piña S, Garcia G, Rosa GA,
27 Cunningham AA, Hole S, Griffiths R, Fisher MC (2008) Invasive pathogens threaten
28 species recovery programs. Current Biol 18: R853-R854
- 29 Wolfe LM, Elzinga JA, Biere A (2004) Increased susceptibility to enemies following
30 introduction in the invasive plant *Silene latifolia*. Ecol Letters 7: 813-820

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
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45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 Woodhams DC, Ardipradja K, Alford RA, Marantelli G, Reinert LK, Rollins-Smith LA
2 (2007) Resistance to chytridiomycosis varies among amphibian species and is
3 correlated with skin peptide defenses. *Anim Conserv* 10:409–417

4 Zampiglia M, Canestrelli D, Chiocchio A, Nascetti G (2013) Geographic distribution of the
5 chytrid pathogen *Batrachochytrium dendrobatidis* among mountain amphibians along
6 the Italian peninsula. *Dis Aquat Organ* 107: 61-68

7
8
9

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

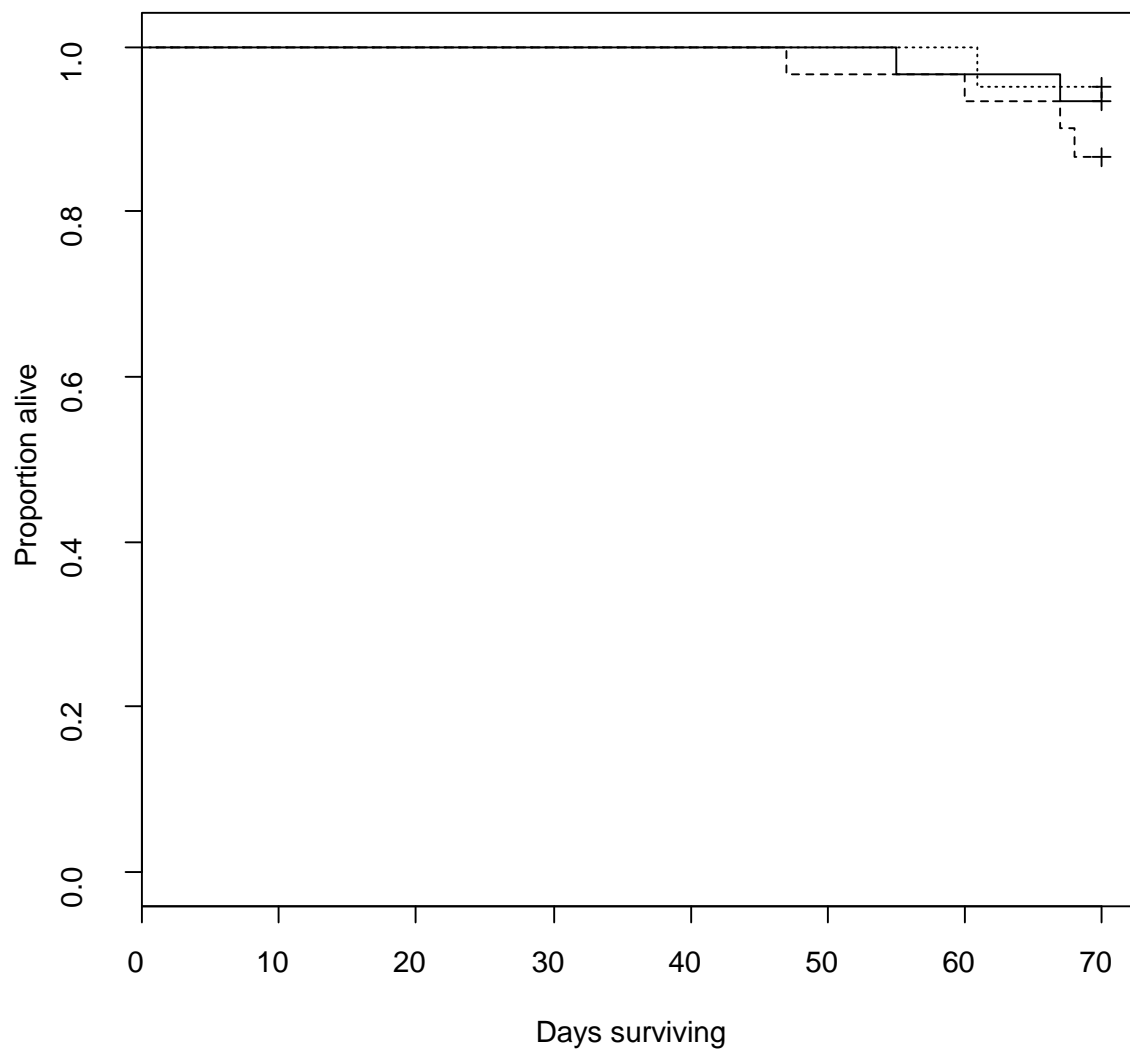


Fig. 1b

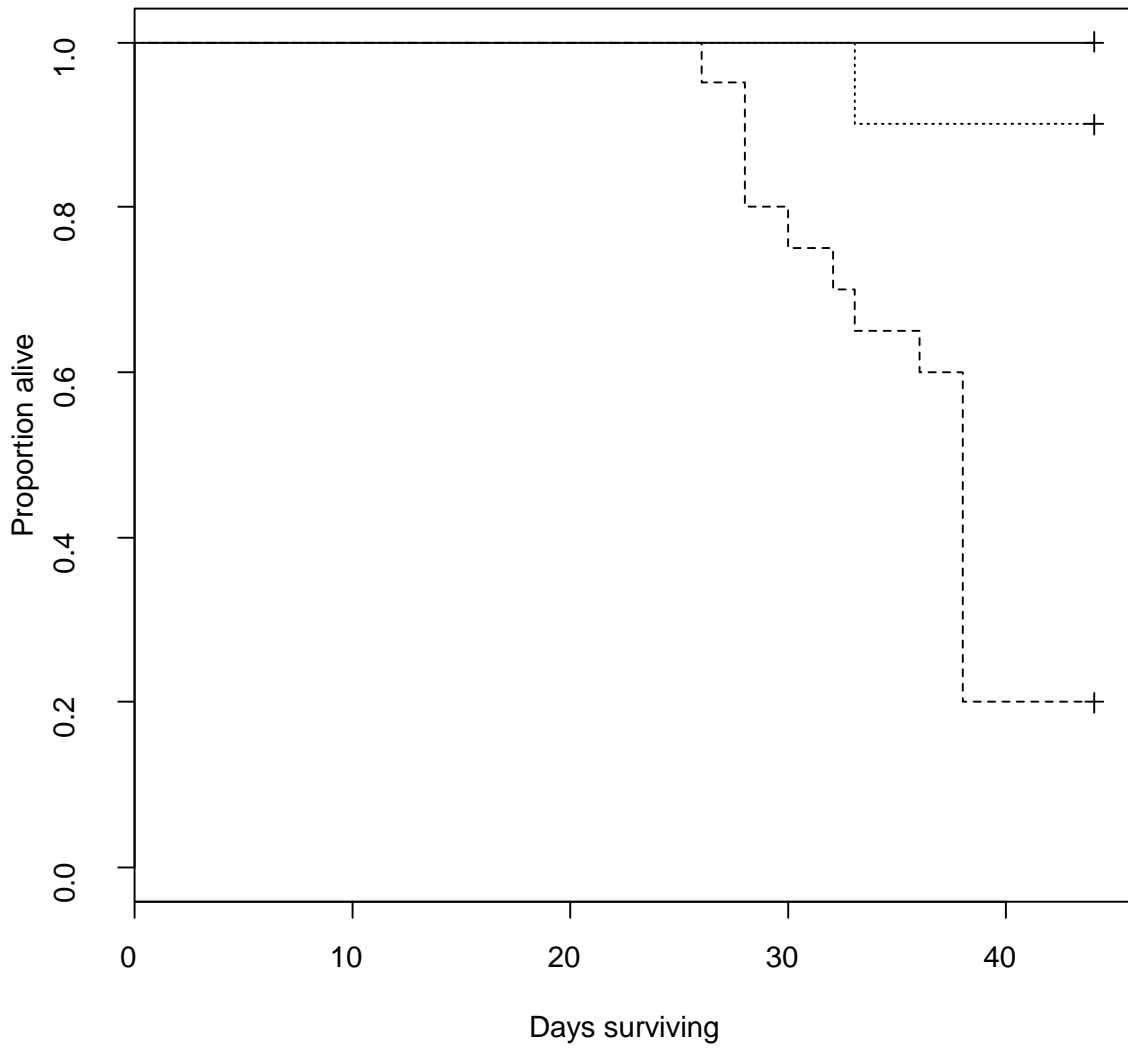


Table 1 Design and results of the cross contamination experiment, the co-housing of alpine newt *Ichthyosaura alpestris* with American bullfrog tadpoles *Lithobates catesbeianus* infected by the fungus *Batrachochytrium dendrobatidis*.

	qPCR test			
	Bd+	Bd-		
<i>Before the experiment</i>				
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	
<i>During the experiment</i>	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.