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Life history tradeoffs influence mortality associated with the amphibian pathogen *Batrachochytrium dendrobatidis*

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Fatal amphibian chytridiomycosis has typically been associated with the direct costs of infection. However the relationship between exposure to the pathogen, infection and mortality may not be so straightforward. Using results from both field work and experiments we report how exposure of common toads to *Batrachochytrium dendrobatidis* influences development and survival and how developmental stage influences host responses. Our results show that costs are accrued in a dose dependent manner during the larval stage and are expressed at or soon after metamorphosis. Exposure to *B. dendrobatidis* always incurs a growth cost for tadpoles and can lead to larval mortality before or soon after metamorphosis even when individuals do not exhibit infection at time of death. In contrast, exposure after metamorphosis almost always results in infection, but body size dictates survival to a greater extent than does dose. These data show that amphibian survival in the face of challenge by an infectious agent is dependent on host condition as well as life history stage. Under current models of climate change, many species of amphibia are predicted to increasingly occur outside their environmental optima. In this case, condition-dependent traits such as we have demonstrated may weigh heavily on species survival.

Microbial pathogenesis is the outcome of a multifactorial context-dependent interaction between the host and its pathogen that results in costs to the host (Casadevall and Pirofski 2003). Costs may include physical damage to the host (Casadevall and Pirofski 1999, Graham et al. 2005) but investment in host responses to a pathogen also negatively correlates with investment in other components of fitness (Festa-Bianchet 1989, Sheldon and Verhulst 1996). These tradeoffs can impact a host's ability to meet the demands of somatic maintenance, development, growth and reproduction, but may or may not correlate with visible damage to a host. If reallocation to pathogen response substantially reduces growth rate, host survival is questionable even if an infection has been resolved or prevented. Similarly, host resource availability also dictates the ability to meet the demands imposed by pathogen insult. If host condition is poor or heavy demands on resources are unavoidable before the onset of the host–pathogen interaction, the ability to mount a successful immune response will be impaired (Roy and Kirchner 2000).

Chytridiomycosis, caused by the chytridiomycete fungus *Batrachochytrium dendrobatidis* (*Bd*), is a highly virulent disease of amphibians and is known to be a major driver of amphibian declines. Within the *Bd* literature there is the tendency to use the terms 'disease' and 'mortality' synonymously. In reality, various grades of costs should exist. The aetiology of disease will be dictated to some extent by general demands on resources not directly related to host immunity as well as the direct accumulation of costs from *Bd* exposure and infection. Evidence for context-dependent virulence and patterns of infection support this (Parris 2004, Green and Sherman 2001, Blaustein et al. 2005, Kriger and Hero 2006a). For example, *Bd* exhibits extraordinary host generality (Green and Sherman 2001, Bonaccorso et al. 2003, La Marca et al. 2005, Scherer et al. 2005, Bosch and Martínez-Solano 2006, Kriger and Hero 2006b, Bosch et al. 2007) contrasted against host-specific patterns of mortality (Parris 2004, Blaustein et al. 2005, Woodhams et al. 2007). This is illustrated by the patterns of mortality recorded at the index site of *Bd* emergence in Europe, Peñalara Natural Park (Bosch et al. 2001, 2007, Bosch and Martínez-Solano 2006). At Peñalara, three species of amphibians, all with complex life histories, have experienced mass die-offs due to chytridiomycosis. *Bd*-driven mortality events were first detected in the anuran *Alytes obstetricans* and subsequent mortality in a caudate species, *Salamandra salamandra*, lagged several years behind (Bosch et al. 2001, Bosch and Martínez-Solano 2006). Most recently, mass mortalities of common toads, *Bufo bufo*, have begun to occur (this study). These

data indicate that *Bd* has been accumulating in Peñalara and sequentially overwhelmed species with differing context-dependent thresholds for disease.

In all three species, mortality was associated with the onset or completion of metamorphosis. Amphibian metamorphosis requires substantial investment of resources as larval morphologies are adjusted for terrestrial, and in the case of most anurans, from predominantly herbivorous to carnivorous, lifestyles (Duellman and Trueb 1986). The costs of metamorphosis are high (Steiner and van Buskirk 2008) and can overwhelm individuals that lack sufficient resources. Poor growth during larval development and small body size at metamorphosis often leads to mortality during or soon after metamorphosis (Altwegg 2002a,b, Merilä et al. 2000, Lane and Mahony 2002, Altwegg and Reyer 2003) and experimental manipulation of food availability or environmental stress exacerbates growth and mortality effects (Merilä et al. 2000, Altwegg 2002a,b, Lane and Mahony 2002, van Buskirk 2002, Altwegg and Reyer 2003, Ficetola and De Bernardi 2006, Steiner and van Buskirk 2008). This suggests that imposing the physiological cost of exposure to a pathogen during early amphibian development would incur mortality even in the absence of pathogenesis. By extension, if condition is poor after metamorphosis, the additional costs of responding to a pathogen, even if successful, may also decrease the probability of host survival. In support of this, investments in amphibian metamorphosis and reproduction are known to correlate with depressed immune function (Robert et al. 1995, McCallum and Trauth 2007, Gervasi and Foufopoulos 2008).

Here, we present field data and laboratory experiments describing how one of Peñalara's threatened amphibians, the common toad *Bufo bufo*, responded to *Bd* exposure during development. We used challenge experiments to examine how different doses of *Bd* affected larval developmental rate and survival both during and after the larval period. We examined how *Bd* dose was related to patterns of infection at several developmental stages and also challenged recently metamorphosed toadlets to determine how condition after metamorphosis affects the probability of survival.

Methods

Field study

The field study was conducted in Peñalara Natural Park, (768 ha, 40°50′N, 3°57′W), an alpine region located between 1800–2200 m a.s.l. in the Sierra de Guadarrama mountain range, central Spain (Fig. 1). Peñalara has been the site of *Bd*-driven amphibian mass mortalities of pre- and post-metamorphic *Alytes obstetricans* since the late 1990s (Bosch et al. 2001). High larval densities of *Bufo bufo* are known at three of the ponds located within the *Bd* zone of infection: Laguna Grande de Peñalara (LGP), Laguna de Mariposa (LM) and Laguna de Pajaraos (LP) (Fig. 1). In the breeding seasons of 2004 and 2005 we recorded mass die-offs of *B. bufo* metamorphs at two localities (LGP and LP). We collected dead toadlets and 2–3 mm toe-clips from live individuals in 2004 and 2005 at both locations where mass mortality was detected. Larvae between Gosner stages 26 and 40 (Gosner 1960) were collected and euthanized with an overdose of MS222 (tricaine methanesulfonate). Samples of toe clips and larvae were also collected at the third location (LM) where no mortalities were detected. Tissue samples were stored in 70% ethanol until DNA extraction and eventually screened using a quantitative real-time polymerase chain reaction protocol (qPCR, Boyle et al. 2004). Larval mouthparts, toe clips and the combined skin of the pelvic region, including the pelvic patch, and complete hind limbs of dead metamorphs were all extracted according to Boyle et al. (2004). Extractions were diluted 1/10 before real time PCR amplification, performed in duplicate, and with *Bd* genomic equivalent (GE) standards of 100, 10, 1 and 0.1 GE. In the event that only one replicate from any sample did not amplify, this sample was run a third time. If this third amplification attempt did not result in an amplification profile, the sample was scored as negative for infection.

We used an internal positive control (IPC) to measure PCR inhibition in most of the samples derived from both the field and the larval dose response experiment that tested negative for *Bd* infection. Following the methodology of Hyatt et al. (2007), a VICTM labeled synthetic amplicon was used as the IPC (VICTM dye, Applied Biosystems No.

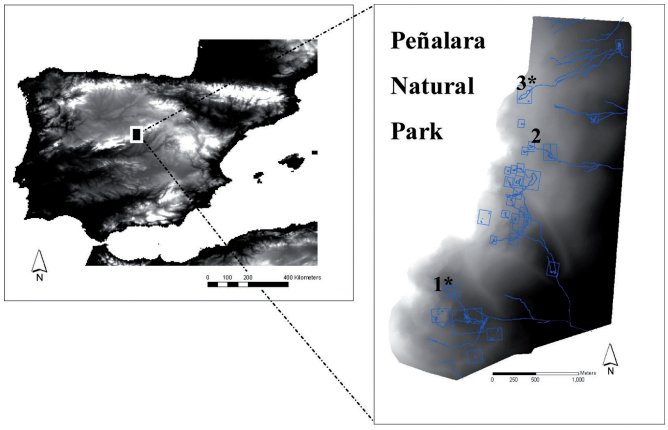

Figure 1. The locality of Peñalara Natural Park in central Spain and ponds used for surveillance within the park. 1 = Laguna Grande de Peñalara, 2 = Laguna de Mariposa, 3 = Laguna de Pajaros. * = site of mass mortality

4308323). The IPC was included in one of each duplicate well as 1 μl 10 × Exo IPC mix and 0.5 μl 50 × Exo IPC DNA. Samples with Ct values at least two cycles greater than that of the comparable control were identified as being influenced by PCR inhibition. Here, differences of three cycles represented approximately a ten- fold difference in the calculated number of zoospores.

Response and patterns of infection during larval development

Fifteen common toad egg strings were subsampled in England and allowed to hatch in captivity. Once tadpoles had reached Gosner stage 23, tadpoles were pooled into a single tank to average any possible genetic variation among clutches and avoid any untoward influence of a single clutch on results. Once tadpoles had fully developed opercula and no visible gills (Gosner stage 25), we randomly assigned thirty tadpoles to reference, euthanized them with an overdose of MS222 and transferred to 70% ethanol. These references served as confirmation of developmental stage at the start of the experiment and were also examined for signs of *Bd* infection of mouthparts using qPCR analysis.

Experimental tadpoles ($n = 105$) were transferred to individual Nunc 75 cm^2 EasY Flasks without lids. Flasks were assigned randomly to treatment and dosed according to one of three treatments: repeated high doses (3000–15 000 zoospores total per exposure) of active *Bd* culture, repeated low doses (1/100 of high dose) of active *Bd* culture and repeated sham exposure using culture filtrate. We used an isolate of the fungus (isolate IA2004 043) generated from a dead *Alytes obstetricans* metamorph collected from a mass mortality event at Ibon Acherito, Spain (40°53′N, 0°42′W, 1869 m). Zoospore concentration of *Bd* cultures was assayed prior to infection using a haemocytometer and by counting only visibly active zoospores. We diluted an aliquot of stock culture by 1/100 using filter-purified media to obtain the 1/100 low dose infection. Negative controls (sham infections) were obtained by filtering active *Bd* culture using a 25 ml sterile syringe and a 0.2 μm sterile filter disk.

Tadpoles were exposed to *Bd* every four days when water in the flask was changed by adding *Bd* to the freshly changed water. Tadpoles were fed 400 mg of ground Tetra Tabi Min every second day. Mortality was recorded every day. Once the forelimbs of a tadpole had emerged the water in the flask was reduced by 70% and the flask tilted to provide both aquatic and terrestrial habitat. Once a flask was tilted we ceased exposing the individual to *Bd*. When an individual's tail had receded to a stub (Gosner stage 45), we classified the individual as a metamorph, removed it from the flask and transferred it to a 1 l box lined with moist paper towel. The entire experiment was completed in a climate-controlled room kept at 18°C and with a 12:12 h day/night light schedule. The experiment lasted 81 days after administering the initial infectious dose.

To ascertain individual *Bd* infection status and burden, we either extracted the entire mouthparts from tadpoles pre-Gosner stage 42 or one of the hindlimb feet of more advanced tadpoles lacking keratinized mouthparts as well as metamorphosed individuals, following the protocol used for analyses of field surveillance samples. All animals lacking emergent limbs that tested negative for infection in the first screen were examined further by re-dilution of extraction and qPCR. In the case of tadpoles with emergent limbs and keratinized mouthparts which were negative on the first examination, we re-extracted one complete hind limb foot and, in a separate extraction and where available, both forelimb feet. In the case of animals Gosner stage 42 or greater, both the entire drink patch and an entire hind limb were used for one re-extraction and both forelimbs for a second. Each extraction was used for separate qPCR amplification attempts.

We assessed infection status as both a binary response (infected vs uninfected) and a continuous variable. The latter was estimated as genomic equivalents (GE) from the standard curves generated as described above. Prevalence levels were calculated together with their 95% Clopper–Pearson confidence intervals and comparisons between groups were made using χ^2 and Fisher's exact tests. We plotted Kaplan–Meier survivorship functions to illustrate the effect of treatment on survival to metamorphosis and survival post-metamorphosis in the dose response experiment. The significance of the difference in the shape of the survivorship functions was assessed in R using log rank tests. The effect of treatment on weight at metamorphosis was assessed using the Wilcoxon rank sum test. The results of these tests informed further survivorship analyses for individuals post-metamorphosis. We used a Cox proportional hazards model to obtain parameter estimates for the effect of treatment and to examine the additional affect of the covariate, weight at metamorphosis, on survival post-metamorphosis; in so doing we were able to adjust treatment estimates for weight at metamorphosis and examine any interaction between treatment and weight.

We infected a further subset of larvae at Gosner stages 25–27 in 2006 to assess the progression of infection visually using histology. We followed the protocol for infection described in the larval challenge experiment (high dose 3000–7000 zsp $dose^{-1}$; low dose 30–70 zsp $dose^{-1}$); tadpoles and metamorphs were removed at several developmental stages suitable for infection detection due to the presence of keratin (Fig. 2). We sampled tadpoles at four Gosner stages nested within five categories where diagnosis of infection should be straightforward and diagnostic (Pessier et al. 1999, Berger et al. 2005, Fig. 2, categories i–v). Individuals removed from treatments were euthanized and preserved in 10% formalin. Tissues removed for histological preparations were dorsal and ventral skin (i–v), mouthparts (i–iii), entire hindlimbs (i–v) and entire forelimbs (ii–v, Fig. 2). Tissues were embedded in wax blocks, decalcified where necessary, sectioned at 6 μm thickness at three depths and stained with haemotoxylin and eosin (Berger et al. 1998). Infection was diagnosed for each Gosner stage in each treatment category by the presence of sporangia and immature bodies within keratinized tissues or tissue layers.

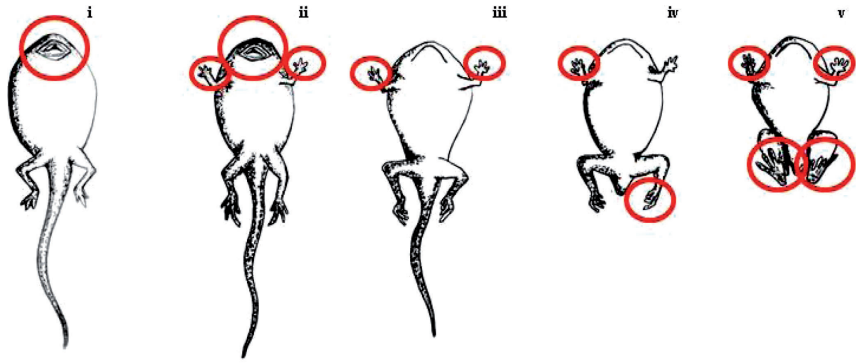

Figure 2. Gosner stages sampled for histological detection of infection during larval and early post-larval development. i) Gosner stages 38–40, hind leg toe development completed or near-completion with or without subarticular tubercules present, cloacal tail piece still present and tadpole mouthparts present; ii) Gosner stage 42, forelimbs emerged no obvious reconstructuring of mouthparts; iii) Gosner stage 44, forelimbs emerged, mouthparts restructured for terrestrial foraging (teeth present, mouth fully formed) but tail stub still present.; iv) Gosner stage 46, metamorphosis just complete; v) Gosner stage 46, at least four days after metamorphosis completed. Numbers indicate sample sizes for each category as per dose (L = low, H = high). Those in brackets indicate number of samples where infection was detected. Areas of infection are circled.

Response to exposure after the completion of metamorphosis

We weighed 30 recently metamorphosed toadlets and distributed them evenly according to weight into threegroups (n = 10). Each group was assigned to one of three treatments: a single high (5000 zsp), low (50 zsp) or sham dose, on day 0. Each metamorph was placed for 5 h in a lidded petri dish containing 30 ml of aged tap water and 200 µl of the appropriate dose of *Bd*. The experiment was performed in a climate-controlled room kept at 18°C and with a 12:12 h day/night light schedule. After the bath exposure, metamorphs were housed as before. The experiment lasted for a total of 40 days post-exposure, following which we euthanized and preserved all surviving animals. We again used Kaplan–Meier survivorship functions to illustrate the effect of treatment on survival to metamorphosis, but further subdivided the data within each dose treatment into large- (>mean mass) and small- (<mean mass) sized metamorphs. We used a Cox proportional hazards model to obtain parameter estimates for the effect of dose and to examine the additional effect of the covariate mass at time of exposure on survival post-exposure.

Results

Field study

Some metamorphs collected at mass mortalities at Laguna Grande de Peñalara (LGP) and Laguna de Pajaraos (LP) and toe clips from living metamorphs sampled at all three sites tested positive for *Bd* infection both in 2004 and 2005. However, a large proportion of metamorphs found dead at LGP and LP did not (Table 1). More than half of the dead metamorps collected at LGP tested negative for *Bd* in both years, while over 30% tested negative at LP in both years. Surprisingly few of the destructively sampled tadpoles from LGP and LP exhibited a molecular signal of infection (Table 1). Infection was detected in metamorphosed toads at our no-mortality site, Laguna de Mariposa (LM), but

Table 1. Site-based summary statistics for *B. dendrobatidis* field screens. LHS = life history stage (LV = larva, Met = metamorph); L = live, D = dead; E = euthanased. 95% CPCI = 95% Clopper Pearson confidence interval

Locality	Year	LHS	L/D	No. screened	No. positive	% prevalence (95% CPCI)
Laguna Grande	2004	Met	D	28	13	46.4 (27.5–66.1)
Laguna Grande	2004	Met	L	12	8	68.8 (34.9–90.1)
Laguna de Pajaros	2004	Met	D	16	11	68.8 (41.3–89.0)
Laguna de Pajaros	2004	Met	L	5	2	40.0 (5.3–85.3)
Mariposa	2004	Met	L	2	1	50.0 (1.3–98.8)
Laguna Grande	2005	Met	D	15	6	40.0 (16.3–67.7)
Laguna Grande	2005	Met	L	NA	NA	NA
Laguna Grande	2005	LV	E	25	1	4.0 (0.1–20.4)
Laguna de Pajaros	2005	Met	D	25	16	64.0 (42.5–82.0)
Laguna de Pajaros	2005	Met	L	NA	NA	NA
Laguna de Pajaros	2005	LV	E	16	1	6.3 (1.5–30.2)
Mariposa	2005	Met	L	24	2	8.3 (1.0–27.0)
Mariposa	2005	Met	D	NA	NA	NA
Mariposa	2005	LV	L	10	0	0 (0.0–30.9)
Mariposa	2004	LV	L	13	0	0 (0–24.7)

only in the 2005 sample, and far fewer were infected than at either of the locations of mortalities. None of the larval mouthparts from LM tested positive for *Bd*.

Response and patterns of infection during larval development

Tadpoles from the high dose treatment died at extremely early developmental stages (Gosner stages 26–40, 23% mortality) and all were infected with *Bd* (100% prevalence, 95% Clopper–Pearson confidence interval (CPCI) 63.05–100, Table 2 for data on infection). However, in the low dose treatment 13% of the animals died at very early developmental stages (Gosner stages 26 and 40) and none of these were infected (prevalence = 0%, 95% CPCI 0.0000–0.7076). Mortality of control tadpoles that did not reach Gosner stage 42 was 8% and all tested negative for infection. All the remaining premetamorphic deaths were individuals which had reached Gosner stages 42 to 44. In the high dose experiment, 100% of those animals that failed to reach Gosner stage 45 tested positive for *Bd* and carried buccal or skin infections. In sharp contrast, only 27% of the larvae exposed to low doses that failed to reach Gosner stage 45 were carrying infections at time of death. For those animals that survived to Gosner stage 45, the prevalence of infection was significantly different between high and low exposure treatments (Fisher's exact test $p = 0.001$). All the toadlets (Gosner stage 45) screened from the high exposure treatment that were dead by the end of the experiment tested positive for *Bd* (95% CPCI: 88.78–100). In the low exposure treatment only 64% (95% CPCI: 35.43–84.81) of metamorphosed and dead animals were infected: there was no significant difference in the prevalence of infection among live (71%, 95% CPCI: 29.04–96.34) and dead (56%, 95% CPCI: 21.20–86.31) animals exposed to low doses (Fisher's exact test, $p > 0.05$). None of the control animals that died during the experiment or that survived to the end tested positive for infection.

Histological examinations revealed that category i and category v animals exposed to high doses of *Bd* consistently exhibited infection (Fig. 2). Infection of category i tadpoles occurred only in association with keratinized mouthparts: all other tissues lacked visible *Bd* and keratin. Category v animals were infected only on limbs, but not on dorsal or ventral skin sections. Intermediate stages exposed to high doses (ii–iv) were not consistently infected: category ii animals had infected mouthparts and forelimbs, while only the forelimbs of category iii animals were infected (Fig. 2). *Bd* was observed on the forelimb of one category iv animal and on a hind limb of the other. The sites of infection detected in low dose animals were consistent with those of high dose animals with respect to category, as was the shifting pattern of prevalence amongst categories (e.g. category iii animals exhibiting the lowest prevalence of all categories). However, the two categories consistently infected in high dose animals (category i and v) were not consistently infected when low doses were applied. Odds ratio tests pooling categories within dose treatments reflected this: odds of infection for low dose animals were 0.64 greater than controls (95% CI 0.25–1.64) and were 2 times greater for animals exposed to high doses (95% CI 0.75–5.33). Risk of infection was not homogeneous (test of homogeneity $\chi^2 = 14.57$, $p = 0.0007$) and was higher with higher doses (trend of odds $\chi^2 = 14.42$, $p = 0.0001$).

In the first dose experiment, the overall proportion of animals reaching metamorphosis varied from 92% in control animals to 69% and 60% in high and low exposure animals, respectively (Table 2). Survival to metamorphosis of controls was significantly different from both high and low treatments (Fig. 3, log-rank $p < 0.01$). Furthermore, animals exposed to *Bd* that survived through metamorphosis had significantly reduced chances of survival during the terrestrial period (Fig. 3) compared to sham-infected animals. Survival to the end of the experiment varied from 80% among control animals to 39% and 2% among low and high exposure animals, respectively (Table 2). Using the predictor 'days post-metamorphosis', Kaplan Meier survivorship functions were significantly different for each of the following pairs: high–low ($p < 0.01$); high–control ($p <$ low–control $p < 1 \times 10^{-12}$); low–control ($p < 0.001$). Sixty-eight percent of mortalities among high exposure animals occurred between 8 and 11 days post-metamorphosis. Cox proportional hazards regression with the predictor days post-metamorphosis was consistent with the statistically significant effect of treatment previously described; the relative instantaneous mortality hazard of death was increased by 18.67 times ($p < 0.0001$) and 5.85 times ($p < 0.0001$) in the high and low treatments, respectively.

Exposure to *Bd* significantly reduced mass at metamorphosis (Fig. 4, Table 2, Wilcoxon signed rank $p < 0.01$) but not amongst high and low dose treatments (Wilcoxon signed rank $p = 0.057$). Interestingly, regression analyses revealed no significant relationship between mass at metamorphosis of animals from the high dose treatment and the infection load at the time of death (adjusted $r^2 = -0.00887$, $p = 0.395$) and exposure to *B. dendrobatidis* had no significant effect on the duration of the larval period (unpubl.). Adjusting the treatment-based survival estimates for weight at metamorphosis and allowing for an

Table 2. Mass at metamorphosis, time to metamorphosis, mortality and infection amongst categories in larval challenge experiment. TTM = time to metamorphosis; TM/P = percent of total number of animals in a treatment dying/percent of total number of animals in a treatment testing positive for infection; PREM/P = same as TM/P but for animals dying before metamorphosis; PSTM/P = same as TM/P and PREM/P but for animals dying after metamorphosis

Treatment	Mass (mg)	TTM (days)	TM/P	PREM/P	PST/MP
Control	119.71 ± 2.90	64.09 ± 0.26	20/0	8/0	11/0
Low	104.67 ± 2.36	61.07 ± 0.56	71/40	31/27	40/64
High	110.67 ± 2.49	62.73 ± 0.18	98/100	37/100	60/100
All	113.5 ± 1.47	63.01 ± 6.58			

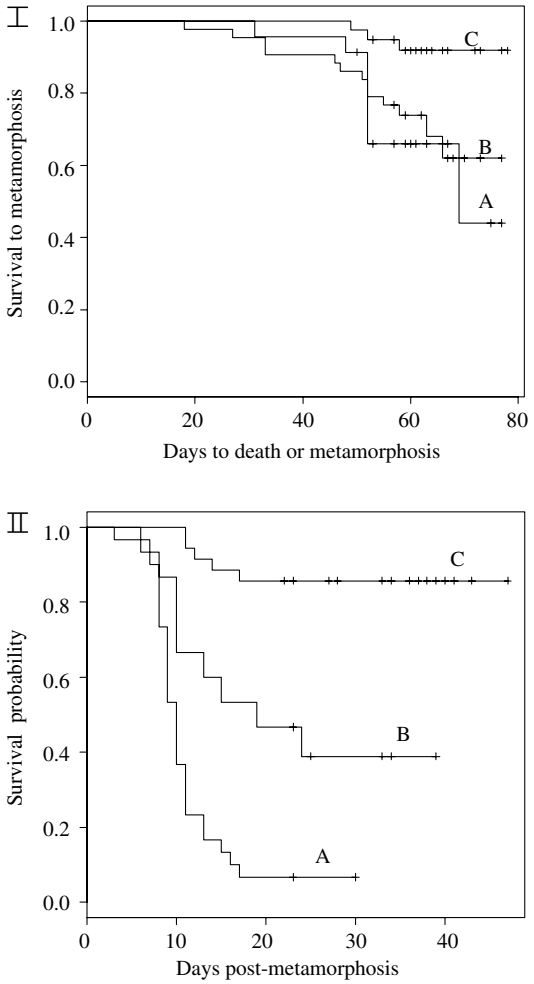

Figure 3. Kaplan–Meier cohort-based survivorship functions illustrating the effect of treatment on: (I) 'survival to metamorphosis'; (II) 'survival post-metamorphosis'. A = high dose, B = low dose, C = sham infection.

interaction between weight and treatment demonstrated a marginally significant interaction between high-exposure and weight ($p = 0.058$). This interaction predicts that high exposure animals that are lighter at metamorphosis have reduced prospects of survival, or die more quickly than their heavier counterparts.

Response to exposure after the completion of metamorphosis

All metamorphs exposed to high doses of *Bd* were infected at the end of this experiment, and only a single animal exposed to a low dose of *Bd* successfully evaded infection. The relative instantaneous mortality hazard was 3.64 ($p = 0.076$) times higher in low dose animals relative to controls and 7.29 ($p = 0.0057$) times higher in high dose animals relative to controls. Body mass before exposure, however, had the greatest effect on survival (Fig. 5). Small toadlets died, even when exposed to sham doses of *Bd*, and exposure to *Bd* appeared to merely accelerate, rather than dictate, mortality in small animals (Fig. 5). By contrast, larger than average toadlets exposed to *Bd* exhibited a more dose-dependent pattern and survived well when exposed to low doses even though infected (Fig. 5) The mass-dependent relative instantaneous mortality hazard was 0.98, i.e. 2% reduction per mg increase in mass ($p = 0.00071$).

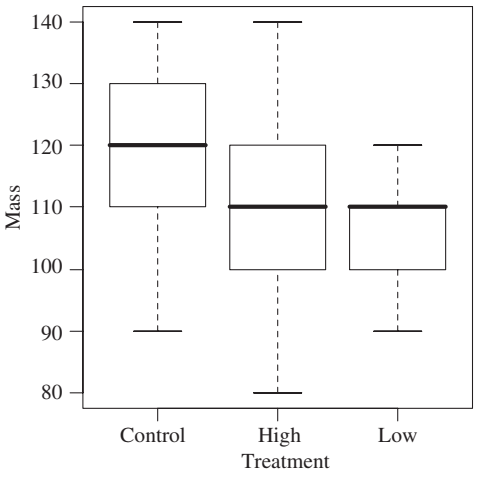

Figure 4. Variation in mass at metamorphosis (g) between treatments.

Discussion

The aetiology of an infectious agent in the manifestation of disease and its diagnosis is complex (Casadevall and Pirofski 2003, Steurer et al. 2006) and does not necessarily fit the standard definition of an infectious disease. The most striking result from our study was the consistent evidence that mortality caused by exposure to *Bd* is commonly dissociated from detectable infection at time of death in both experimental and natural settings. Whether or not the infection status of an exposed individual can be quantified

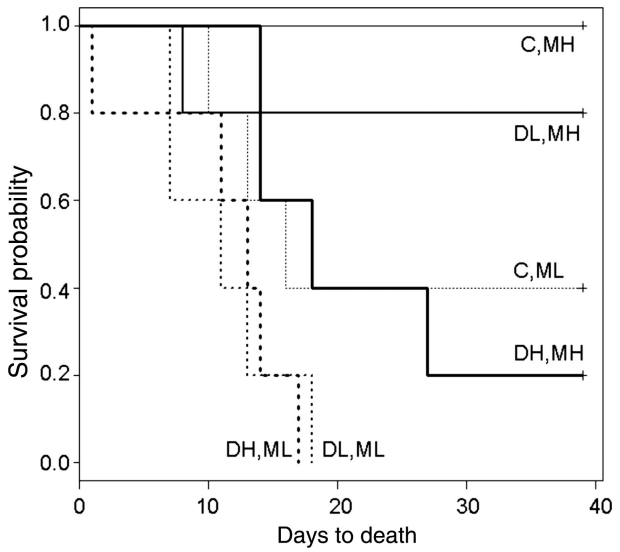

Figure 5. Kaplan–Meier survivorship functions illustrating the effects of mass and *Bd* dose on toad survival. Dotted lines refer to low mass animals, solid lines refer to large mass animals. DH = high dose, DL = low dose, C = sham infection (negative control), MH = high mass, ML = low mass.

accurately is dependent upon the sensitivity and type of screening or diagnostic protocols used. To address the issue of sensitivity we tested animals in replicate, we employed a molecular test that can detect less than a single pathogen genome and we used internal positive controls (IPCs). IPCs showed that there was no evidence for PCR inhibition in any of our experimental animals. PCR inhibition was present in only 9 of the 29 DNA extractions from field samples testing negative for infection and therefore could not explain the lack of detectable infection in the majority of larval and metamorphic field mortalities. We maximised our chance of detecting *Bd* infection by targeting tissues known to be preferentially infected by *Bd*, namely the front and rear digits and pelvic patch (Pessier et al. 1999, Berger et al. 2005). Here, histological examinations confirmed that extracting tadpole mouthparts and limbs of later Gosner stages were appropriate targets for molecular detection, as well as providing further evidence of lack of infection predominantly associated with low dose. Of course, as is the case with any method for detecting infection, error is assumed. Nevertheless, the large number of animals testing negative in field surveys and experiments, the sensitivity of the molecular test we employed and our stringency in examining tissues that are targets for infection leads us to conclude that any undetected infection in mortalities was nominal and could not alone be responsible for the observed levels of mortality.

This is not to say infection and mortality are decoupled. Rather, it appears that multiple factors contribute to toad mortality following exposure to *Bd*. We can conceive of two hypotheses to account for the lack of detectable infection we observed in both larval challenges and field data. First, low intensity infections of *Bd* may occur at low doses and be resolved by the host. In this situation the mechanism(s) by which infection is cleared, while effective at limiting reproduction of *Bd*, involve cost(s) that reduce the probability of survival. The most likely form of cost would be immunopathological, for which numerous examples are known (Graham et al. 2005), but immunopathologies have not been reported for *Bd* and chytridiomycosis is noted for its lack of obvious physical traumas (Pessier et al. 1999). *Bd* infection does elicit epidermal hyperplasia and hyperkeratosis in metamorphosed amphibians, but neither of these changes was detected in our histological examinations. Our second hypothesis is that infection is prevented at low doses, but investment in the prevention of infection overwhelms early developmental processes and leads either directly or indirectly to mortality. Precedence for this may be found in studies of larval life history tradeoffs where tadpoles are exposed to predator cues produced by caged predators. In these studies tradeoffs directly or indirectly affect larval survival and survival in the early post-metamorphic period due to exposure to the perceived risk of a predator, even though the predator does not have the capability to attack experimental animals (Altwegg 2002a,b, Lane and Mahony 2002, Altwegg and Reyer 2003, Steiner and van Buskirk 2008). A host response to a pathogen that has not yet entered the body of the host may seem improbable as immune responses are generally associated with pathogen intrusion. However chytridiomycete fungi form attachments to hosts before cell penetration (Deacon and Saxena 1997, Ibelings et al. 2004) and enter host cells through the enzymatic degradation of host cell walls (Lopez-Llorca and Hernandez 1996) Since cell infection results in host cell death (Kagami et al. 2007), selection should favour strong host mechanisms to inhibit zoospore attachment. Such innate immune responses present at the skin surface have been described (Woodhams et al. 2006a,b, 2007).

Whether infection is controlled or successfully prevented, our experimental data show that the host response during the larval period imposed a significant direct cost due to exposure to *Bd*, as exposure to *Bd* significantly reduced the probability of surviving through metamorphosis and both high and low doses of the fungus led to significantly reduced mass at metamorphosis. Again, this indicates tradeoffs are occurring. Increased skin or mouthpart shedding (Marinelli and Tejedo 1988, Pessier et al. 1999, Vredenburg and Summers 2001), the production of physiologically costly non-specific, innate immune responses (Woodhams et al. 2006a,b, 2007) and any other response to the presence of infectious *Bd* zoospores could be traded off against larval growth and condition at metamorphosis. If so, a successful response to pathogen insult may still result in mortality even if infection itself is prevented, cleared or limited as long as sufficient costs have been incurred. Such tradeoffs would be evolutionarily maintained if there is some chance of survival, with or without infection, a chance which we showed to be present in low dose animals that successfully completed metamorphosis and survived to the end of the larval challenge experiment.

The picture is radically altered if exposure to *Bd* occurs soon after metamorphosis. Infection is, to all intents and purposes, unavoidable: all but one low dose toadlet tested positive for *Bd*. Body size is a more important predictor of survival than *Bd* dose: a significant effect of body mass on survival among boreal toadlets (*Bufo boreas*) exposed to *Bd* after metamorphosis has previously been reported (Carey et al. 2006). If survival and infection of larger metamorphs reported in this study are both maintained over winter during hibernation, these 'young of the year' have the ability to act as among-year reservoirs of *Bd*, a situation which, in theory, has the potential to lead rapidly to host population extinction (Mitchell et al. 2008). Even though the infective stage of *Bd* is water-borne, this could mean that infection dynamics beyond the pond may have a greater ability to dictate host population responses to disease in temperate zone amphibians.

Increasing global temperature has been invoked as an important covariate influencing the virulence of *Bd* (Bosch et al. 2007). As toad breeding sites are brought into the climate envelope that facilitates *Bd* growth and thus zoospore density, as has happened in Peñalara (Bosch et al. 2007), we would expect mass mortalities dominated by recently metamorphosed toadlets, as we have reported here for Peñalara populations. Depending on the environmental burden of active zoospores, a proportion of these animals may test negative for infection and some surviving animals will carry infections after the majority of mortality has occurred, which we also observed in the field. The availability of infectious zoospores in the aquatic environment preceding tadpole hatch date may be mediated by alternative hosts: in Peñalara the host initially identified as suffering from lethal chytridiomycosis, *Alytes obstetricans*, commonly overwinters as tadpoles that are infected with *Bd*

(Bosch et al. 2001, Bosch and Martínez-Solano 2006). Increasing global temperature may also serve to move some toad breeding sites beyond this optimal *Bd* climate envelope and thus decrease the likelihood of such a mass mortality event occurring. However, increasing average winter temperature also affects the overwintering regime of temperate zone common toad females and causes poor condition at spring emergence as well as poor quality clutches (Reading 2007). Poorer female condition causes reduced egg size in temperate anurans and environmental stressors are known to negatively influence maternal effects that influence egg size (Kaplan 1989, Laugen et al. 2002, Pakkasmaa et al. 2003, Räsänen et al. 2005). Smaller eggs lead to smaller size at metamorphosis (Kaplan 1989, Laugen et al. 2002, Pakkasmaa et al. 2003, Räsänen et al. 2005), thus if common toad females are in poorer condition, we would expect toadlets to metamorphose at a decreased body size. If these animals subsequently encounter environmental *Bd*, even at low doses, exposure could result in substantial, even catastrophic, losses.

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