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Presence of *Batrachochytrium dendrobatidis* in amphibians from central and southern Hesse, central Germany: results from a preliminary regional screening

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Chytridiomycosis is an emerging infectious amphibian disease that has been associated with population declines in different regions of the globe, but the geographic distribution and actual effect of the fungus *Batrachochytrium dendrobatidis* (*Bd*) is still insufficiently known (Fisher et al. 2009, Ohst et al. 2011). Mass mortalities are apparently restricted to certain regions, whereas in other cases, infected populations barely appear to suffer from their infection (e.g., Woodhams et al. 2007b, Kilpatrick et al. 2010). Recently, Farrer et al. (2011) identified different strains of *Bd* and suggested that one highly virulent strain might be responsible for the most dramatic consequences of the disease. However, specific differences in susceptibility of host species, individual fitness, and/or environmental conditions might also play important roles in the effects of *Bd* infections (e.g., Blaustein et al. 2005, Woodhams et al. 2007a, Hossak et al. 2010, Kilpatrick et al. 2010, Tobler & Schmidt 2010, Ohst et al. 2011). Variable effects have even been observed within individual populations of the same species (Tobler et al. 2010).

Bd currently occurs on all continents (except Antarctica) and has just recently been detected in Asia (Mutschmann et al. 2000, Garner et al. 2005, Bosch et al. 2007, Bielby et al. 2009, Kielgast et al. 2010, Ohst et al. 2011, Swei et al. 2011). Concerning Central Europe, the disease is well studied in Switzerland, where *Bd* could be found at half of all water bodies north of the Alps (Garner et al. 2005, Tobler & Schmidt 2010). Given the data from Switzerland, it can be assumed that *Bd* is also widespread and common in Germany, but the knowledge about *Bd* distribution and its impact at national level is very fragmentary. Between 2003 and 2010, Ohst et al. (2011) tested 2,100 samples from 156 German localities for *Bd*, including ten localities within Hesse. Of these ten localities, four tested positive for the presence of *Bd* (T. Ohst pers. comm.).

Given the fragmentary knowledge in Germany and the high potential threat of *Bd*, our small-scale study aimed at a first preliminary screening for *Bd* within a limited geographical region. To assess the prevalence of *Bd* and estimate infection rates among amphibian species and populations, we selected certain localities and target taxa, hoping to provide useful new data for future in-depth research and conservation planning.

The field survey included 14 collection sites in central and southern Hesse, Germany (Fig. 1). All collection sites range in altitude between 88 and 350 m above sea level. Geographical coordinates were geo-referenced by using GoogleEarth (Tab. 1). We collected samples from 221 individuals of adult amphibians representing eight species, as well as three samples from juvenile brown frogs, between February and June 2011. Additionally, we included 60 preserved tissue samples from toe clippings collected during a field survey at Heusenstamm in 2007. For easier sampling we mainly selected locations with fences established for amphibian protection. During an earlier study conducted by Ohst et al. (2011) between 2003 and 2010, four collection sites in Hesse had showed *Bd* infection (Fischborn, Wiesbaden-Auringen 1, Wiesbaden-Auringen 4, and Eppertshausen; T. Ohst pers. comm.), two of which (Fischborn, Eppertshausen) were again included in this study. To investigate the situation of the regionally endangered *Rana arvalis*, we took samples from a spawning area in Rodgau and a pond in Riedstadt-Erfelden near a spawning site, the latter being located within the conservation area Kühkopf-Knoblochsaue.

Individuals were captured with dip-nets, fish traps or along the fences with pitfall traps. To avoid the further spread of *Bd*, sampling was subjected to the recommendations of Schmidt et al. (2009). We used Virkon S (2 g/l) for disinfecting dip-nets, fish traps and waders, and 70% ethanol for smaller items like torch lights. For collecting zoospores, we used disposable gloves and sterile cotton swabs (Sarstedt, Nümbrecht, Germany), which were firmly swept over the skin of ventral and dorsal surfaces, thighs and webbing of the foot as described by Kriger et al. (2006). Dry swabs were stored in tubes and frozen at -20°C following sampling. Tissue samples obtained by toe clippings in 2007 were preserved in small tubes (1.5 ml) containing 90% ethanol.

Swabs were eluted in 1.0 ml PBS buffer by an ultrasound treatment for 5 min. After removal of the cotton tip, a centrifugation step (7,500 rpm for 10 min.) was performed and the supernatant carefully removed. Toe clippings were diluted 1:5 in PBS buffer and homogenised in vials containing 1.4 mm ceramic spheres (Lysing Matrix D; mp biomedicals, Eschwege, Germany) for 45 s with intensity 6.5 using a cell disrupter (Ribolyser Fastprep 120 Bio101, Thermo Electron, Osterode, Germany). One millilitre of the sample solution was then centrifuged (7,500 rpm for 10 min.) and the cell pellet further processed for nucleic acid extraction. DNA was extracted from all samples using a commercial kit (Qiagen DNeasy Blood & Tissue Kit, Hilden, Germany) according to the manufacturer's recommendations (methods not specifically adapted for *Bd*). Briefly, the cell pellet was suspended in 200 µl reaction buffer with proteinase K, incubated at 56°C for 60 min and centrifuged (8,000 rpm for 1 min.) following the addition of buffer and ethanol. Eluted DNA was extracted from DNeasy columns after several centrifugation steps and 5 µl template DNA were utilized for PCR. A realtime PCR protocol for the specific detection of *Bd* was employed according to Boyle et al. (2004) with the minor modifications mentioned below. The final reaction volume of 25 µl contained templates

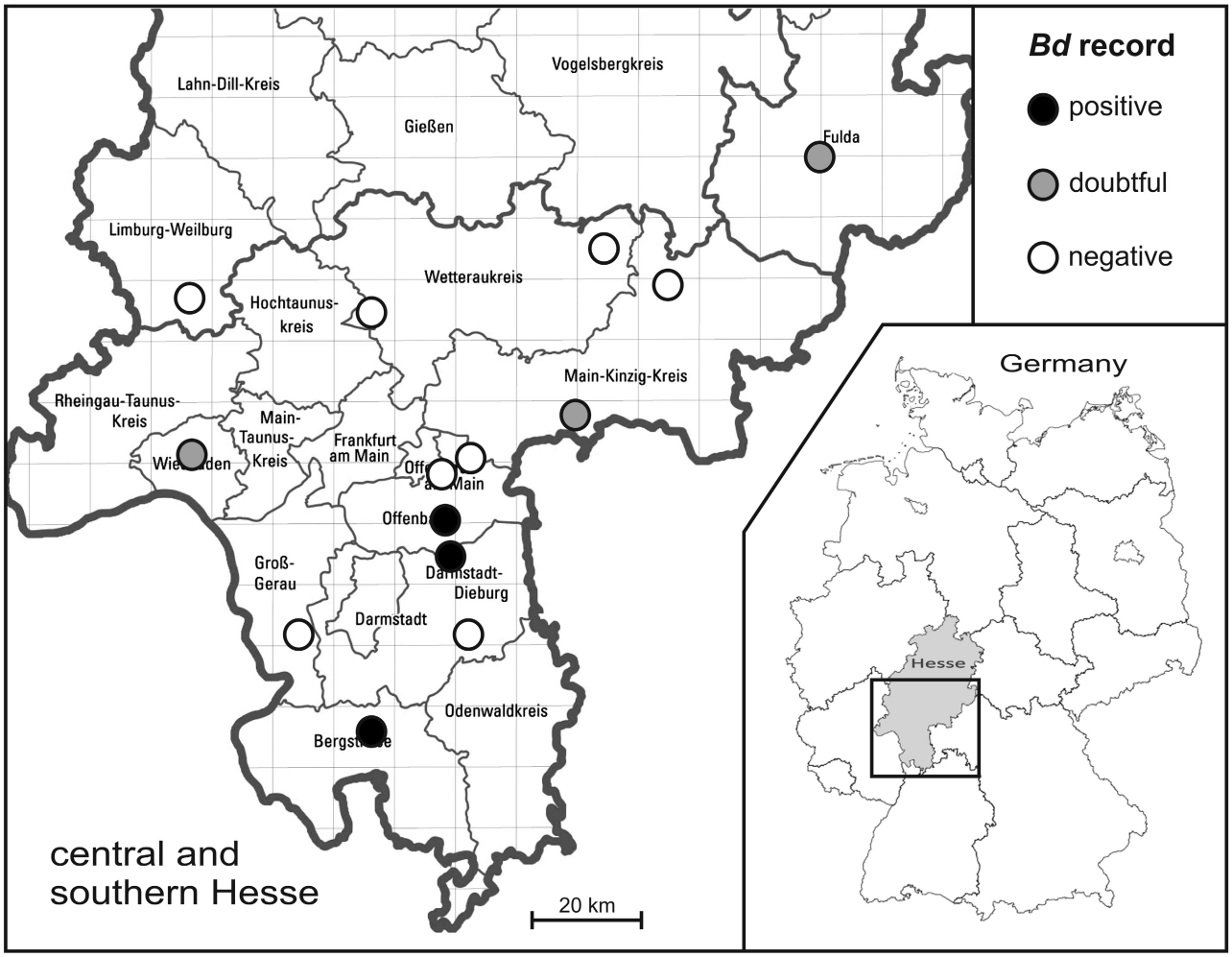

Figure 1. Schematic map of Germany (lower right) and southern Hesse (left) showing the sampled areas. Localities with positive results for *Batrachochytrium dendrobatidis* (*Bd*) are indicated by black dots, those with doubtful results by grey dots, and those without positive results by circles, respectively.

Table 1. Number of samples taken per locality and species. Abbreviations used refer to *B.b.* (*Bufo bufo*), *L.v.* (*Lissotriton vulgaris*), *M.a.* (*Mesotriton alpestris*), *P.*spp. (*Pelophylax* spp.), *R.a.* (*Rana arvalis*), *R.d.* (*Rana dalmatina*), *R.t.* (*Rana temporaria*), *P.f.* (*Pelobates fuscus*) and juv. (juvenile *Rana temporaria*). *) Tissue samples collected by toe clipping in 2007.

Locality/coordinates	*B.b.*	*L.v.*	*M.a.*	*P.* spp.	*P.f.*	*R.a.*	*R.d.*	*R.t.*	juv.	total
Gedern 50°26'40" N, 9°11'10" E	6	6	4	0	0	0	0	0	0	**16**
Fischborn 50°23'15" N, 9°17'43" E	6	0	6	6	0	0	0	6	0	**24**
Fulda 50°35'52" N, 9°42'26" E	6	6	6	0	0	0	0	5	0	**23**
Bad Camberg 50°18'03" N, 8°19'18" E	10	0	0	0	0	0	0	0	0	**10**
Rosbach 50°16'58" N, 8°42'31" E	13	0	1	0	0	0	0	0	0	**14**
Freigericht 50°08'11" N, 9°10'07" E	10	0	0	0	0	0	0	0	0	**10**
Wiesbaden 50°05'49" N, 8°11'09" E	12	5	1	0	0	0	0	6	0	**24**
Mühlheim am Main 50°06'22" N, 8°50'46" E	0	0	0	0	0	0	0	12	3	**15**
Rodgau 49°59'10" N, 8°51'56" E	0	3	0	7	0	1	2	9	0	**22**
Eppertshausen 49°56'58" N, 8°49'23" E	6	0	8	6	0	0	6	4	0	**30**
Riedstadt 49°50'27" N, 8°26'42" E	0	0	0	12	0	0	0	0	0	**12**
Reinheim 49°49'07" N, 8°50'26" E	10	0	0	0	0	0	0	0	0	**10**
Bensheim 49°43'21" N, 8°33'12" E	4	3	2	1	2	0	0	2	0	**14**
Heusenstamm* 50°03'45" N, 8°47'46" E	0	0	40	0	0	0	13	7	0	**60**
	83	**23**	**68**	**32**	**2**	**1**	**21**	**51**	**3**	**284**

or controls (5 µl), primers (each 1.1 µl with 20 pmol/µl), and taqman probe (0.6 µl with 10 pmol/µl) as well as an internal control reaction (Hoffmann et al. 2006), and 2x Quantitect Probe PCRmaster mix (Qiagen). After centrifugation (7,000 rpm for 20 s), the PCR was processed on a realtime thermocycler (iCycler, Bio-Rad Laboratories Inc., Munich, Germany; initial denaturation 95°C for 15 min., 45 cycles with 94°C for 60 s and 60°C for 60 s, and finally 40°C for 30 s) without quantification standard. To calculate 95% confidence intervals (CI) for *Bd* prevalences, we used the software Quantitative Parasitology 3.0 and Sterne's exact method (Rózsa et al. 2000, Reiczigel 2003).

In total, 284 samples were analysed for *Bd*, with samples from *Bufo bufo* and *Mesotriton alpestris* representing the vast majority with 83 and 68 samples, respectively, followed by *Rana temporaria* with 51 samples. Smaller sampling sizes were obtained from the remaining amphibian species sampled, including, e.g., only two samples from *Pelobates fuscus* and a single sample from *Rana arvalis* (Tab. 1).

We detected *Bd* at three out of the fourteen sampling sites (Figs. 1, 2). The samples from Eppertshausen, near the conservation area Rallenteich, were notable for their high detection rates with a prevalence of approximately 50% (CI 32–68). In addition, *Bd* was recorded in one water frog (*Pelophylax* sp.) at the *Rana arvalis* spawning site in Rodgau, but samples from a pond near the large conservation area Kühkopf-Knoblochsaue tested negative. The affected sites Rodgau/Nieder-Roden (5% prevalence, CI 0–22) and Eppertshausen are located very close to each other, i.e., within approximately 5 km. The third locality where *Bd* was detected belongs to Bensheim-Langwaden (20% prevalence, CI 6–47) and is located >50 km apart from Rodgau and Eppertshausen. Furthermore, we had suspicious results in samples from four localities (Eppertshausen, Fulda, Freigericht, Wiesbaden) with amplifications at Ct values above 38. Repeated amplification plots showed the same results and therefore we tentatively classify these samples as positive. We did not find any *Bd*-

positive samples in eight of the surveyed collection sites (Fig. 1).

We detected *Bd* in 19 out of 284 individuals (6.7%). Six out of eight species were infected: *Bufo bufo, Pelophylax* spp., *Rana dalmatina*, *R. temporaria*, *Lissotriton vulgaris* and *Mesotriton alpestris* (Tab. 2). The highest *Bd* prevalence was noted in *Pelophylax* spp. and *Rana dalmatina* with 18.8% and 14.3%, respectively, followed by the alpine newt (*M. alpestris*) with 7.4% (Tab. 2). Three tested juvenile *R. temporaria* (snout–vent lengths of about 1 cm) metamorphosed in 2011 showed negative results for *Bd*. In summary, our results indicate that *Bd* has a patchy distribution in the sampled area as well as among the sampled amphibian species.

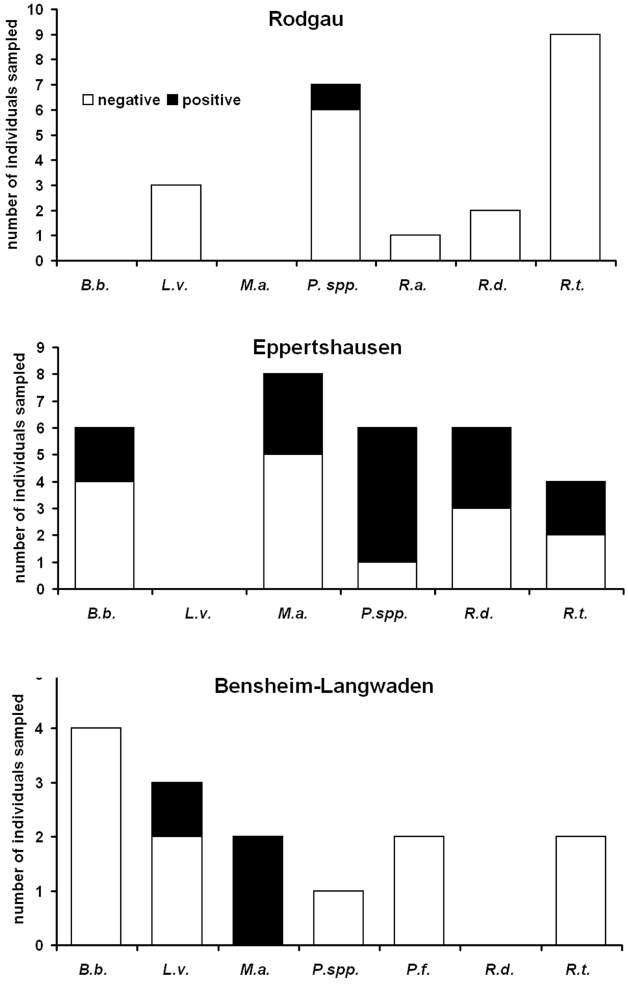

Figure 2. Diagrams showing the number of sampled individuals per species at three study sites and the distribution of samples testing positive for *Batrachochytrium dendrobatidis* (*Bd*). Black bars indicate positive records for *Bd*, white bars indicate negative samples. Abbreviations used refer to *B.b.* (*Bufo bufo*), *L.v.* (*Lissotriton vulgaris*), *M.a.* (*Mesotriton alpestris*), *P.* spp. (*Pelophylax* spp.), *R.a.* (*Rana arvalis*), *R.d.* (*Rana dalmatina*), *R.t.* (*Rana temporaria*), and *P.f.* (*Pelobates fuscus*), respectively.

Batrachochytrium dendrobatidis is widespread in Switzerland (Tobler et al. 2010), and by starting our screening in Hesse, we expected to find most localities to be affected as well. However, we could detect *Bd* with certainty at three sites only. There are various possible explanations for the surprisingly low number of *Bd* records. Small sample size is certain to be an important factor. As a consequence of limited funding available, we tried to sample at least six individuals per species at each site, but even obtaining this limited number of samples was impossible in some cases due to the low abundance of individuals. With the actual *Bd* prevalence within a certain population being unknown, such a small sample size may easily lead to false-negative results. The data at hand leaves us unable to exclude the possibility that *Bd* is also present at sites without positive records. Another problematic factor could be the field conditions and swab sampling by different, partly little-experienced persons. After sampling, many swabs are inevitably covered with various microorganisms and traces of dirt, which can degrade *Bd* DNA; less careful application of the sampling method may result in rather low amounts of *Bd* spores on the swab. It is also possible that we failed to detect *Bd* in some cases because of both very low prevalences and very low amounts of fungal DNA in the samples. In addition, some of the involved tissue samples had been stored in 90% ethanol at about 10–15°C for four years. Such storage conditions could have led to the degradation of high amounts of DNA (Spigelmann et al. 2001, T. Ohst unpubl.).

We cannot exclude the possibility that *Bd* is in fact heterogeneously distributed within the studied region and among taxa, as has also been found by other studies (compare e.g., Swei et al. 2011, Lötters et al. 2012). Nevertheless, the reasons for such a patchy and uneven distribution of the chytrid fungus remain poorly understood, as are those for the different degrees of prevalences observed. Dilution effects, with a greater diversity of species at a certain locality reducing the risk for disease, and climatic conditions have been proposed (or rejected) to be responsible for the latter phenomenon (Rohr et al. 2008, Fisher et al. 2009, Murray et al. 2009, Searle et al. 2011). We did not conduct any histopathological examinations of amphibian skin, but we never noticed any symptoms of chytridiomycosis in the tested animals. We can therefore only speculate on the fungus' role. It is possible that the investigated populations are unaffected by the presence of *Bd* infection (e.g. Lötters et al. 2012) or that the disease is endemic at a low level in the area (Kriger et al. 2007). Even commensal host-pathogen relationships have been suggested (Goka et al. 2009).

However, the amphibian populations from Eppertshausen, a locality that had tested positive for *Bd* in a previous study (Ohst et al. 2011), show a declining trend, particularly in the species *Bufo bufo* (Fig. 3; Reinecke 2010). Ohst et al. (2011) analysed 25 samples of four amphibian species from Eppertshausen and at that time found a prevalence of *Bd* infection of 4%. Among the 30 studied samples from five species from the same locality, we now de-

Table 2. Number of sampled and positively-testing amphibian individuals and populations, and the prevalences of *Batrachochytrium dendrobatidis* in the respective taxa and populations.

	Mesotriton alpestris	*Lissotriton vulgaris*	*Bufo bufo*	*Pelobates fuscus*	*Pelophylax* spp.	*Rana arvalis*	*Rana dalmatina*	*Rana temporaria*
individuals								
N	68	23	83	2	32	1	21	51
N infected	5	1	2	0	6	0	3	3
prevalence [%]	7.4	4.3	2.4	0	18.8	0	14.3	5,9
95% confidence interval	3–16	0–21	0–8	–	9–36	–	4–35	2–16
populations								
N	11	6	8	1	5	1	2	8
N infected	1	1	2	0	2	0	1	2
% infected	9.1	16.7	25.0	0	40.0	0	50.0	25.0

tected a *Bd* prevalence of 50% (Tab. 2). Despite some indications, we are unable to prove a causal link between *Bd* presence and the observed declines in this particular case, because of the small sample size and other potential factors (intrinsic dynamics in *Bd* prevalence and infection intensity), but comparable population declines and dramatic outbreaks of chytridiomycosis in *B. bufo* have already been reported from other European regions (Bosch et al. 2001, Bosch & Martínez-Solano 2006).

In Germany, *Bd* was previously known from almost all amphibian species (Ohst et al. 2011), so we were not surprised to find *Bd*-positive individuals in six of the studied species. The study of Ohst et al. (2011) found high *Bd* prevalences in water frogs (*Pelophylax* spp.) and alpine newts (*M. alpestris*), which is a result confirmed by our study. However, contrary to Ohst et al. (2011) we found remarkably high *Bd* prevalences in *Rana dalmatina*, particularly at Eppertshausen. However, we cannot completely exclude the possibility that these high prevalences are partly due to cross infection during sampling by dip-netting and pitfall traps, as might be indicated by relatively high amplification Ct values (35.04–40.96) in these samples.

In summary, our short-term small-scale screening provides further records of *Bd* in German amphibian populations. Due to the limited number of localities and samples studied, as well as some other factors mentioned, it is hardly possible to draw sound conclusions from the results obtained, though. Particularly the impact of *Bd* on populations testing positive remains unknown. Nevertheless, there seems to be some indication that *Bd* has a patchy distribution among the localities and populations studied. Future long-term monitoring of population dynamics and the presence of *Bd* at Eppertshausen is probably qualified to prove or disprove a connection of *Bd* prevalence, an outbreak of chytridiomycosis, and amphibian population declines. Such a monitoring effort should include *Bd* sam-

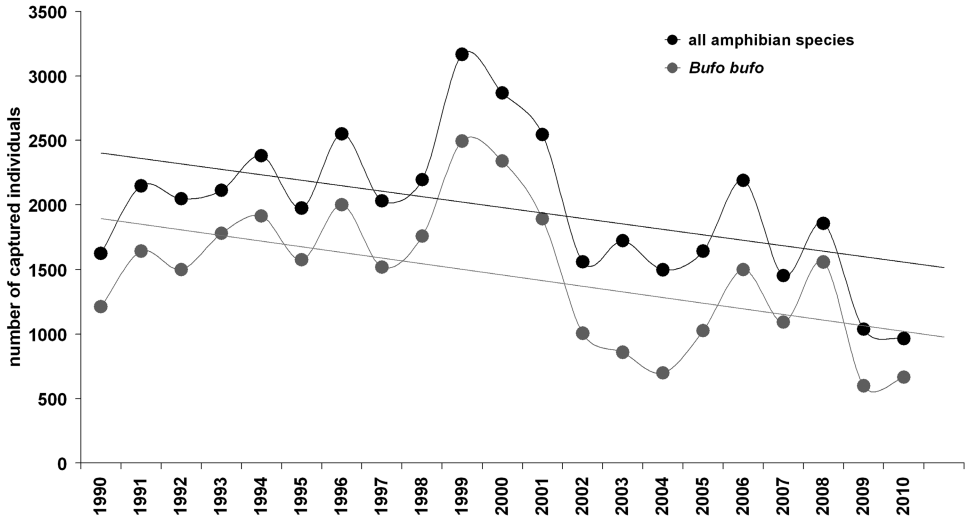

Figure 3. Graph showing total numbers of captured amphibian individuals and those of *Bufo bufo* from 1990 to 2010 at Eppertshausen (data from Reinecke 2010), a locality testing positive for *Batrachochytrium dendrobatidis* and showing high prevalences.

pling from amongst tadpoles, as larval stages have shown to contain higher prevalences (e.g., Rachowicz & Vredenburg 2004, Woodhams & Alford 2005) and mortality has often been noted to occur at metamorphosis (e.g., Walker et al. 2010). Obviously, metamorph mortality may result in a decrease of recruitment and thus poor reproductive success negatively impacting on population size.

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