EcoHealth 10, 82–89, 2013
DOI: 10.1007/s10393-013-0828-4

EcoHealth

Original Contribution

Evidence for the Introduction of Lethal Chytridiomycosis Affecting Wild Betic Midwife Toads (*Alytes dickhilleni*)

Jaime Bosch,[1] David García-Alonso,[2] Saioa Fernández-Beaskoetxea,[1] Matthew C. Fisher,[3] and Trenton W. J. Garner[4]

[1]*Museo Nacional de Ciencias Naturales, CSIC, José Gutiérrez Abascal 2, 28006 Madrid, Spain*
[2]*Bioparc Fuengirola, Camilo José Cela 6, Fuengirola, 29640 Malaga, Spain*
[3]*Department of Infectious Disease Epidemiology, Imperial College London, St. Mary's Hospital, Norfolk Place, London W2 1PG, UK*
[4]*Institute of Zoology, Zoological Society of London, Regents Park, London NW1 4RY, UK*

Abstract: *Batrachochytrium dendrobatidis* is an unpredictable pathogen for European amphibian species, and existing field surveillance studies likely underestimate the scope of its distribution and effects. Mass mortality episodes recorded in Europe indicate that investigations of unstudied species should focus on members of the frog family Alytidae. Here, we report the combined results of a field survey and laboratory observations of field collected *Alytes dickhilleni*. Our data support the hypothesis that *B. dendrobatidis* has recently emerged in at least two disjunct locations in the species range and populations across much of the species range lack evidence of infection pathogen. Tadpoles taken into the laboratory from sites with infection experienced 70% mortality, unlike those taken into the laboratory from uninfected sites, and both infection and strength of infection was associated with mortality in animals collected from infected locations. Several conservation interventions are underway in response to our study, including the establishment of a captive assurance colony, a public awareness campaign, and experimental tests of disease mitigation schemes.

Keywords: *Batrachochytrium dendrobatidis*, chytridiomycosis, Betic midwife toad, *Alytes dickhilleni*

Introduction and Purpose

The chytridiomycete fungus *Batrachochytrium dendrobatidis* is an extremely important pathogen of wildlife with respect to biodiversity conservation. The fungus is responsible for amphibian mass mortality, population declines, and possible species extinction at an intercontinental scale (Fisher et al. 2009). Disease emergence across the Neotropics and Australia are the worst on record and have caused catastrophic declines of numerous hosts in both regions (Berger et al. 1998; Lips et al. 2006, 2008; Skerratt et al., 2007; Fisher et al., 2009). In contrast, since its detection in Europe in the late 1990s, infection with *B. dendrobatidis* has been associated with mortality of a handful of European species in the wild; far fewer than are known to carry infections (Bosch et al. 2001; Bosch and Martínez-Solano 2006; Garner et al. 2006; Bovero et al. 2008; Walker et al. 2008; Garner et al. 2009a; Bielby et al. 2009; Ohst et al. 2011; Sztatecsny and Glaser 2011; Rosa et al. 2012). Published sampling for Europe is strongly spatially biased, includes only a subset of European amphibian species diversity (www.bd-maps.eu/) and many studies are extremely limited in scope and effort (Adams

Published online: March 15, 2013

Correspondence to: Jaime Bosch, e-mail: bosch@mncn.csic.es

et al. 2008; Federici et al. 2008; Ficetola et al. 2011). This fact suggests that the documented list of European amphibians known to be infected with *B. dendrobatidis* and experiencing mortality is underestimated. Alternatively, some un- or under-sampled species may truly be resistant to infection (e.g., Bielby et al. 2008; Luquet et al. 2012), but this cannot be distinguished from lack of exposure to *B. dendrobatidis* without combined spatial surveillance and experimentation.

At the species level, the link between infection and disease is not straightforward (Garner et al. 2011; Luquet et al. 2012). Even when detectable mortality of a highly susceptible host species has been reported for multiple locations (Bosch et al. 2001; Walker et al. 2010; Pasmans et al. 2010; Rosa et al. 2012), the same species may not suffer from the lethal form of the disease at other sites where it is infected at high prevalence (Walker et al. 2010). Conversely, at locations where infection is detected but lethal disease has not been observed, cryptic mortality may occur (Tobler and Schmidt 2010). Studies restricted to a spatial investigation of infection therefore present uncertainties when attempting to assess the risk that infection presents to a host species. Combining spatial studies of prevalence of infection with in situ or ex situ experimental tests of virulence can potentially distinguish between tolerance of infection versus unobserved lethal chytridiomycosis. For obvious reasons, clarifying the relationship between infection with *B. dendrobatidis* and amphibian mortality has greater conservation merit than simply describing parasite distribution (Garner et al. 2012).

Developing the database for resistance, tolerance, or susceptibility for all of Europe's amphibians is an enormous research task. The potential conservation threat chytridiomycosis may pose to the European amphibian fauna calls for a more rapid, but still reasoned approach to risk assessment. Previous mass mortalities episodes and population declines recorded in Europe showed that the family Alytidae is a strong candidate for detecting both infection and lethal disease, if they occur. Mortality in one member of the genus *Alytes* (*A. obstetricans*) is the flagship case of lethal chytridiomycosis in Europe (Bosch et al. 2001, 2007; Walker et al. 2010; Rosa et al. 2012). Infection and death has also been described for two other members of the family, *Alytes muletensis* (Walker et al. 2008) and *Discoglossus sardus* (Bielby et al. 2009). Other members of the genus *Alytes* exhibit traits (prolonged larval period and restricted species range) that should predispose them to declines due to chytridiomycosis (Martínez-Solano et al. 2004; Gonçalves et al. 2007; Bielby et al. 2008), which strongly argues for targeted studies of previously unsampled *Alytes* spp.

The Betic midwife toad (*Alytes dickhilleni*) is a recently described species (Arntzen and García-Paris 1995) that is the sister taxon to *A. muletensis* and possibly *Alytes maurus* (Martínez-Solano et al. 2004; Gonçalves et al. 2007). *A. dickhilleni* is distributed across an extremely restricted species range, occurring in mountains and the nearby plains of six provinces located in the southeast of Spain. It is listed as vulnerable by the IUCN, but no attempt has been made to ascertain if *B. dendrobatidis* is infecting this species or capable of killing post-metamorphic animals, as is true for both *A. obstetricans* and *A. muletensis* (Bosch et al. 2001; Walker et al. 2008, 2010). In this manuscript, we describe a two-phase study of the distribution of infection and lethal disease in *A. dickhilleni*. In the first phase, we surveyed for the presence of infection in wild populations of *A. dickhilleni*, a study that covered the entire species range. In the second, after determining where infection was located, we used an ex situ experimental approach (Tobler and Schmidt 2010) to determine if the presence of infection was associated with mortality due to chytridiomycosis in animals near to, or completing, metamorphosis.

Methods

To ascertain the distribution and prevalence of infection in *A. dickhilleni* we first selected 30 sample sites covering the entire species range (Fig. 1). We focussed on sampling overwintered larvae because this life history stage has proven to be the most appropriate life history stage for detecting infection in two congeners (Walker et al. 2008, 2010). This was not possible at several sites, so young of the year tadpoles were sampled as an alternative (Table 1). To collect samples for molecular diagnosis of infection we swabbed the oral disc of each tadpole using a sterile swab (MW100, Medical Wire UK), a method proven to be reliable in previous studies of *Alytes* spp. tadpoles (Walker et al. 2008, 2010; Tobler and Schmidt 2010). At one location oral disks were excised from euthanized tadpoles and used for analysis. Our intention was to sample a minimum of 20 tadpoles however this could not be achieved comprehensively due to poor tadpole abundance at some locations and an absence of tadpoles at others. At these latter sites we sampled smaller numbers of recently metamorphosed animals or adults by taking toe clips (Table 1).

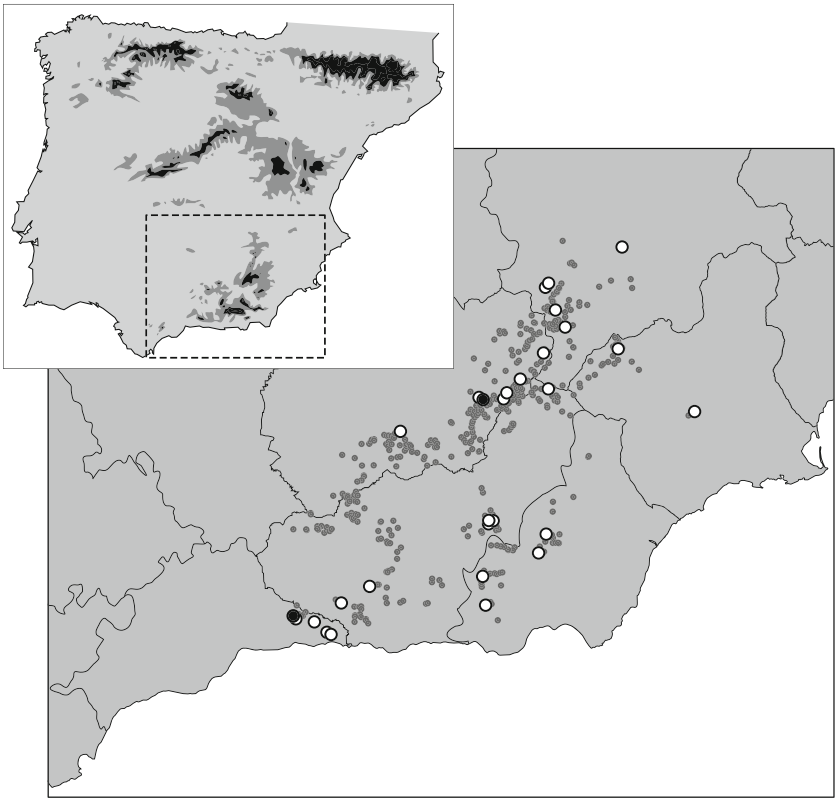

Figure 1. Sampling locations, including sites where *Batrachochytrium dendrobatidis* infection was detected (*filled*, *black circles*) and those where it was not (*open circles*). Small *grey points* show all known breeding sites for the species (from Bosch and González-Miras 2012).

In all cases recently metamorphosed animals were dead at time of sampling, and discovered that way. Swabs were refrigerated and stored dry while toe clips were stored in ethanol until DNA extraction and qPCR amplification as per Boyle et al. (2004). Extractions were diluted 1:10 before real-time PCR amplification, performed in duplicate, with *B. dendrobatidis* genomic equivalent (GE) standards of 100, 10, 1, and 0.1 GE. When only one replicate from any sample amplified, we ran this sample a third time. If the third amplification did not result in an amplification profile, we considered the sample negative for infection. We used a Potthoff–Whittinghill's test of overdispersion to determine if risk of disease was homogeneous among sites (Potthoff and Whittinghill 1966). To do this, we condensed the UTM values for each sampled location into 10×10 km^2 cells. For the null hypothesis, we assumed that the number of infections per cell followed an expected Poisson distribution.

We assessed the consequences of infection ex situ by broadly following the experimental protocol of Tobler and Schmidt (2010). Overwintered tadpoles were collected on the third week of September of 2009 from three locations, one where we had detected infected tadpoles (La Rahige in Sierra Tejeda), and two where we had not detected any evidence of infection (two sites located in Cazorla, Segura y Las Villas Natural Park). To minimize any possible effects of time in captivity, we restricted the collection of experimental tadpoles to 1 day per location. Consequences of this collection approach included uneven sample sizes among groups in the laboratory observations (Sierra Tejeda, $n = 16$; 2 sites in Cazorla, Segura y Las Villas Natural Park, $n = 19$ or 10).

We transported tadpoles to Bioparc Fuengirola where they were housed individually in plastic cups containing 1 l of aged tap water. Tadpoles were fed every two days and water was changed at each feed. Temperature in the experimental unit was kept at a constant 18°C and on a 12:12-h light schedule. Tadpoles were maintained in this manner until 14 days post-metamorphosis or death. Metamorphosis was defined as the day the tail was first observed to be resorbed (dark tail stub present, but stub not protruding beyond the "heels" of the hind limbs). To determine infection status, one hind toe was clipped from each animal either 7 days after metamorphosis or, in the event the animal died before 7 days post-metamorphosis, on the day of death. Toe clips were subjected to the same laboratory procedure, as were field surveillance samples. Animals that survived for 14 days post-metamorphosis were treated with Itraconazole (Itrafungol, Esteve) following Garner et al. (2009b) and returned to their site of

Table 1. Summary of Sampling Effort and qPCR Analysis of Field Samples (Swabs of Oral Disks were Collected from Alive Tadpoles at 25 Sites, Excised Oral Mouth disks from Euthanized Tadpoles were used at One Site and Toe Clips were Collected from Dead Metamorphosed Animals at Four Sites)

Site-province	UTM*x*	UTM*y*	Altitude (m)	Sample size/type	LHS	Prevalence (99% CPCI)	GE mean	VMR
Albacete 1	547775	4264778	1321	20 Swabs	YOY	0	–	–
Albacete 2	548263	4262150	1124	20 Swabs	YOY	0	–	–
Albacete 3	548976	4262444	1118	3 Swabs	YOY	0	–	–
Albacete 4	551157	4251097	1358	20 Swabs	YOY	0	–	–
Albacete 5	554814	4242480	1154	20 Swabs	YOY	0	–	–
Almería 1	506909	4103323	2130	20 Swabs	OW	0	–	–
Almería 2	510777	4087945	1549	20 Swabs	OW	0	–	–
Almería 3	542640	4118482	1830	3 Toe clips	metamorph	0	–	–
Almería 4	546738	4127735	1482	20 Swabs	OW	0	–	–
Granada 1	436927	4087844	1316	17 Swabs	larvae	0	–	–
Granada 2	450349	4100057	1377	20 Swabs	OW	0	–	–
Granada 3	512655	4137144	2023	20 Swabs	OW	0	–	–
Granada 4	513184	4135332	1995	10 Swabs	OW	0	–	–
Granada 5	513522	4136045	2030	8 Swabs	OW	0	–	–
Granada 6	547507	4210131	1482	3 Toe clips	metamorph	0	–	–
Jaén 1	463077	4178001	1500	6 Oral disks	YOY	0	–	–
Jaén 2	510055	4201811	1205	20 Swabs	OW	0.95 (0.68–1.00)	653.68	740.69
Jaén 3	510617	4200161	1257	20 Swabs	larvae	0	–	–
Jaén 4	521022	4203158	1668	20 Swabs	OW	0	–	–
Jaén 5	522555	4206471	1719	20 Swabs	OW	0	–	–
Jaén 6	528958	4210948	1677	20 Swabs	YOY	0	–	–
Jaén 7	540949	4224510	1600	9 Toe clips	metamorph	0	–	–
Málaga 1	404929	4082251	1080	20 Swabs	OW	1 (0.77–1.00)	100.00	27.41
Málaga 2	405361	4081292	701	11 Swabs	larvae	0.36 (0.07–0.77)	469.5	856.76
Málaga 3	414736	4079961	947	20 Swabs	YOY	0	–	–
Málaga 4	420409	4072607	289	20 Swabs	larvae	0	–	–
Málaga 5	421703	4071624	374	2 Toe clips	adult	0	–	–
Murcia 1	583085	4278154	905	20 Swabs	YOY	0	–	–
Murcia 2	586330	4228098	1216	20 Swabs	YOY	0	–	–
Murcia 3	628110	4196409	900	19 Swabs	YOY	0	–	–

LHS refers to life history stage (*YOY* young of year larvae, *OW* overwintered larvae, *larvae* undetermined age). For populations where *Bd* was detected prevalence is presented with 99% Clopper Pearson confidence intervals. VMR is variance to mean ratio of genomic equivalent scores.

collection after two months' quarantine and no evidence of infection was detected using molecular diagnostic techniques applied after quarantine. We used a Cox proportional hazards (CPH) model to determine whether time to metamorphosis, infection with *Bd* as a categorical value (infected yes/no) or mean GE were significant predictors of survival of tadpoles collected from the field. Because infection data were unavailable for one of the Sierra Tejeda animals that died on day 42, it was excluded from the CPH model.

Results

We sampled a mean of 14.4 individuals (min. 3, max. 20) at 30 breeding sites distributed across the six provinces where *A. dickhilleni* is endemic (Fig. 1; Table 1). The risk of infection was not homogeneously distributed across sites (Potthoff–Whittinghill's test of overdispersion; $T = 11004.33$, $P = 0.001$) and infection was detected at three sites, clustered in two 10 × 10 grid squares (Fig. 1). The

grid square containing two of the sites where infection was detected includes the recreational area of La Rahige located in the Sierra Tejeda, Málaga. One of these two sites is a completely artificial breeding site (cattle tank, La Rábita Fountain, site Málaga 1, Table 1), while the other is a man-made pool (La Rahige, site Málaga 2, Table 1) located in small, slow-moving, naturally occurring and permanent stream within the recreational area. Stream flow downstream from La Rahige has been partially dammed to increase water depth in the pool for recreational purposes. The third positive location is an undisturbed stream (Guadahornillos, site Jaén 2, Table 1) located near to the Roblehondo Biological Station CSIC in Cazorla, Segura y Las Villas Natural Park, located in another grid cell several cells away from the cell containing La Rábita Fountain and La Rahige.

Prevalence at the two Sierra Tejeda sites varied: tadpoles at La Rábita Fountain were 100% infected, while at La Rahige only 36% of tadpoles were infected. In comparison, tadpoles at Guadahornillos were more similar to La Rábita Fountain in terms of prevalence (95%). Burden of infection was, on average, high at all three locations and mean GE was similar amongst sites (Table 1). All recently metamorphosed juveniles and the two adults that were tissue sampled also tested negative for infection.

Survival of post-metamorphic *A. dickhilleni* in captivity was not consistent across treatments. Experimental tadpoles from the Sierra Tejeda were the only animals that tested positive for infection at time of death or seven days post-metamorphosis and experienced substantial mortality. Tadpoles collected from the sites where infections were not detected during the field survey experienced significantly less mortality when compared to the Sierra Tejeda group (Fig. 2). After excluding the one animal for which we lacked infection data, animals that died in the Sierra Tejeda group (12/15) were almost all infected (10/12) and the majority died within 60 days after the start of the laboratory observations (n = 10, minimum number of days to death = 6, mean number of days to death ± SD = 24.6 ± 15.4, mean GE ± SD = 12,741.4 ± 10,734.8, range GE = 0–29,367.6). The two other tadpoles from Sierra Tejeda that died did so 143 and 261 days after the start of the laboratory observations and exhibited infectious burdens of, respectively, 0.1 and 3.9 mean GE. Cox proportional hazards analysis revealed that infection status (yes/no, $P = 0.002$) but not mean GE ($P = 0.470$) nor time to metamorphosis ($P = 0.329$) significantly increased probability of death. Being infected increased the relative instantaneous mortality hazard by a factor of 15.7 times.

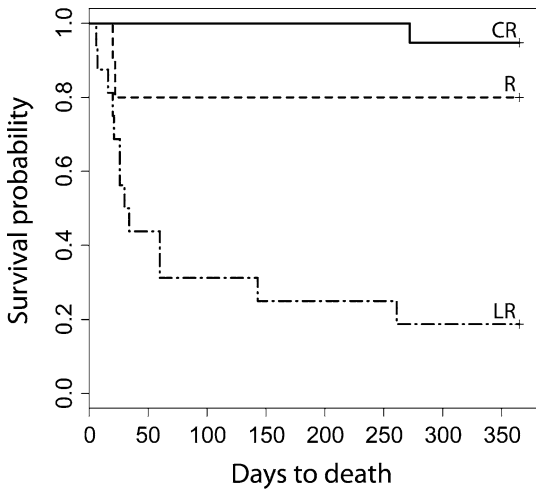

Figure 2. Survival curves for tadpoles sampled at three locations, two within Cazorla Segura y Las Villas Natural Park (CR, R) and at Sierra Tejeda (LR).

Discussion

The spatial pattern and number of infected populations we detected strongly suggests that *B. dendrobatidis* is a recently emerged parasite of *A. dickhilleni*. There are few robust spatial studies of *B. dendrobatidis* distribution available for European hosts, but of those that are published the majority involve congeners of our study species, and report a significantly greater proportion of sites with infections than we found (Walker et al. 2008, 20% sites with infected animals; Walker et al. 2010, 25% sites with infected animals; Tobler et al., 2012, 61.5% sites with infected animals; this study, 10% of sites with infected animals). Walker et al. (2008, 2010) identified other Iberian locations where recent introductions of *B. dendrobatidis* into *Alytes* spp. populations had occurred, including human-mediated introduction into populations of *A. muletensis* in the 1990s with little or no evidence of post-introduction dispersal (Walker et al. 2008). The pattern described by our study fits this latter case: two highly distinct foci of infection where prevalence is at or near to saturation in overwintered tadpoles (La Rábita Fountain and Guadahornillos), indicative of two recent introductions, and limited evidence of fungal dispersal to nearby sites (La Rahige: located near to a saturated population, but with significantly lower prevalence). Both of our proposed sites of introduction are locations where human activity that could promote pathogen introduction is common. Guadahornillos is located just 1.5 km away from an important location for biological research and Sierra Tejeda Natural Park is a multi-use

recreational park frequently visited by amateur herpetologists looking for the species at its *terra typica*.

Our spatial study revealed no observable evidence of disease-linked mortality in recently metamorphosed juveniles. Mass mortality at metamorphosis is the signature of lethal chytridiomycosis in *A. obstetricans* (Bosch et al. 2001, 2007; Walker et al. 2010) and some field mortality has been observed in *A. muletensis* (Walker et al. 2008; JB personal observations), but we never encountered large numbers of dead animals of any life history stage at any location. The few recently metamorphosed and dead animals that we did encounter in nature did not test positive for infection (Table 1). The results of laboratory observations of field-collected animals, however, showed that it is highly probable that *A. dickhilleni* metamorphs at Sierra Tejeda are experiencing substantial, cryptic mortality as nearly 70% of overwintered tadpoles collected from this area died in captivity. Mortality was significantly associated with infection status, typically occurred soon after the onset of the laboratory observations and was commonly associated with heavy burdens of infection.

These results also provide the first experimental evidence that infected tadpoles are capable of maintaining infections over long periods of time without external forcing of infection (Briggs et al. 2010) and dying as a result. This finding is important because increased exposure to *B. dendrobatidis* through forcing of infection can be strongly influenced by transmission from other infected hosts, and has been linked to increasing risk of mortality through the accumulation of fungal load (Briggs et al. 2010; Vredenburg et al. 2010) but the relationship between host density and transmission is unclear (Rachowicz and Briggs 2007). Because our experimental animals were housed individually we can exclude transmission among hosts as an influence, removing the effect of persistent external forcing from the individual disease dynamic. Most of the Sierra Tejeda tadpoles spent more than 20 days without being exposed to any transmission vector, two greater than 140 days, and still died after metamorphosis. Infections acquired earlier during development were therefore sufficient to elicit death long after the initial transmission event, as was the case for *A. obstetricans* tadpoles (Tobler and Schmidt 2010). This may be an indication that costs accrued by tadpoles during this time relate to post-metamorphic death, which has been seen in species with relatively short-larval periods (Garner et al. 2009a; Luquet et al. 2012). It seems sensible, therefore, that a more prolonged larval period with an associated prolonged period of sustained infection should be costly to the infected host.

Studies of chytridiomycosis in *Alytes* spp. do not always provide a definitive link between mortality caused by chytridiomycosis and species decline. In Peñalara Natural Park, *A. obstetricans* was driven locally extinct due to disease emergence (Bosch et al. 2001; Martínez-Solano et al. 2003; Bosch and Rincón 2008). A recent publication by Tobler et al. (2012) outlines the alternative and describes a lack of a clear relationship between the presence of infection and host population dynamics although the authors do postulate that their system may have suffered historical declines due to disease. We lack quantitative data on population responses at locations where *A. dickhilleni* is infected. Irrespective of the inconsistencies, evidence for introduction of *B. dendrobatidis* into populations of a high risk species coupled with evidence for substantial mortality due to disease is cause for conservation intervention. Bioparc Fuengirola and its Foundation, in association with the Amphibian Ark, have established a biosecure facility for the maintenance of a disease-free captive assurance colony of *A. dickhilleni*. This is coupled with more general conservation activities including habitat restoration and improvement, local education programs, and other efforts to raise awareness regarding amphibian decline, conservation, and chytridiomycosis. Disease monitoring and the development of methods to mitigate infection in the wild are underway, as part of the in situ, ''Betic Midwife Toad Conservation Project'' lead by Bioparc Fuengirola and in collaboration with the National Museum of Natural Sciences of Madrid (CSIC).

Acknowledgments

Funding for this study was provided from the BiodiverERsa project RACE and a Zero Project supported by the Fundación General CSIC and the Banco de Santander. The authors also acknowledge EAZA and Amphibian Ark for funding part of the field component of the study. E. Gonzalez-Miras, E. Albert, M. Benítez and M. Tejedo helped during fieldwork or provided tissues samples. The Consejería de Medio Ambiente of Junta de Andalucía, Castilla La Mancha and Región de Murcia provided permits for field study.

References

Adams MJ, Galvan S, Scalera R, Grieco C, Sindaco R (2008) *Batrachochytrium dendrobatidis* in amphibian populations in Italy. *Herpetological Review* 39:324–326

Arntzen JW, García-Paris M (1995) Morphological and allozyme studies of midwife toads (Genus *Alytes*), including the description of two new taxa from Spain. *Contributions to Zoology* 65:5–34

Berger L, Speare R, Daszak P, Green DE, Cunningham AA, Goggin CL, Slocombe R, Ragan MA, Hyatt AD, McDonald KR, Hines HB, Lips KR, Marantelli G, Parkes H (1998) Chytridiomycosis causes amphibian mortality associated with population declines in the rainforests of Australia and Central America. *Proceedings of the National Academy of Sciences of the United States of America* 95:9031–9036

Bielby J, Cooper N, Cunningham AA, Garner TWJ, Purvis A (2008) Predictors of rapid decline in frog species. *Conservation Letters* 1:82–90

Bielby J, Bovero S, Sotgiu G, Tessa G, Favelli M, Angelini C, Doglio S, Clare F, Gazzaniga E, Lapietra F, Garner TWJ (2009) Fatal chytridiomycosis in the Tyrrhenian painted frog. *Ecohealth* 6:27–32

Bosch J, González-Miras E (2012) Seguimiento de Alytes dickhilleni: informe final. Monografía SARE. Asociación Herpetológica Española—Ministerio de Agricultura, Alimentación y Medio Ambiente, Madrid

Bosch J, Martínez-Solano I (2006) Chytrid fungus infection related to unusual mortalities of *Salamandra salamandra* and *Bufo bufo* in the Peñalara Natural Park, Spain. *Oryx* 40:84–89

Bosch J, Rincón PA (2008) Chytridiomycosis-mediated expansion of *Bufo bufo* in a montane area of Central Spain: an indirect effect of the disease. *Diversity and Distributions* 14:637–643

Bosch J, Martínez-Solano I, García-Paris M (2001) Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas in central Spain. *Biological Conservation* 97:331–337

Bosch J, Carrascal LM, Durán L, Walker S, Fisher MC (2007) Climate change and outbreaks of amphibian chytridiomycosis in a montane area of Central Spain; is there a link? *Proceedings of the Royal Society, Series B* 274:253–260

Bovero S, Sotgiu G, Angelini C, Doglio S, Gazzaniga E, Cunningham AA, Garner TWJ (2008) Detection of chytridiomycosis caused by *Batrachochytrium dendrobatidis* in the endangered Sardinian newt *Euproctus platycephalus* in Southern Sardinia, Italy. *Journal of Wildlife Diseases* 44:712–715

Boyle DG, Boyle DB, Olsen V, Morgan JAT, Hyatt AD (2004) Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Diseases of Aquatic Organisms* 60:141–148

Briggs CJ, Knapp RA, Vredenburg VT (2010) Enzootic and epizootic dynamics of the chytrid fungal pathogen of amphibians. *Proceedings of the National Academy of Sciences of the United States of America* U107:9695–9700

Federici S, Clemenzi S, Favelli M, Tessa G, Andreone F, Casiraghi M, Crottini A (2008) Identification of the pathogen *Batrachochytrium dendrobatidis* in amphibian populations of a plain area in the Northwest of Italy. *Herpetology Notes* 1:33–37

Ficetola FG, Valentini A, Miaud C, Noferini A, Mazzotti S, Dejean T (2011) *Batrachochytrium dendrobatidis* in amphibians from the Po River Delta, Northern Italy. *Acta Herpetologica* 6:297–302

Fisher MC, Garner TWJ, Walker SF (2009) The global emergence of *Batrachochytrium dendrobatidis* in space, time and host. *Annual Review of Microbiology* 63:291–310

Garner TWJ, Perkins MW, Govindarajulu P, Seglie D, Walker SF, Cunningham AA, Fisher MC (2006) The emerging amphibian pathogen *Batrachochytrium dendrobatidis* globally infects introduced populations of the North American bullfrog, *Rana catesbeiana*. *Biology Letters* 2:455–459

Garner TWJ, Walker SF, Bosch J, Leech S, Rowcliffe JM, Cunningham AA, Fisher MC (2009a) Life history trade-offs influence mortality associated with the amphibian pathogen *Batrachochytrium dendrobatidis*. *Oikos* 118:783–791

Garner TWJ, Garcia G, Carroll B, Fisher MC (2009b) Using itraconazole to clear *Batrachochytrium dendrobatidis* infection and subsequent depigmentation of *Alytes muletensis* tadpoles. *Diseases of Aquatic Organisms* 83:257–260

Garner TWJ, Rowcliffe JM, Fisher MC (2011) Climate, chytridiomycosis or condition: an experimental test of amphibian survival. *Global Change Biology* 17:667–675

Garner TWJ, Briggs CJ, Bielby J, Fisher MC (2012) Determining when parasites of amphibians are conservation threats to their hosts: methods and perspectives. In: *New Directions in Conservation Medicine: Applied Cases of Ecological Health*, Aguirre A, Ostfeld R, Daszak P (editors), New York: Oxford University Press, pp 521–538

Gonçalves HA, Martínez-Solano I, Ferrand N, García-Paris M (2007) Conflicting phylogenetic signal of nuclear vs. mitochondrial DNA markers in midwife toads (Anura, Discoglossidae, *Alytes*): deep coalescence or ancestral hybridization? *Molecular Phylogenetics and Evolution* 44:494–500

Lips KR, Brem F, Brenes R, Reeve JD, Alford RA, Voyles J, Carey C, Livo L, Pessier AP, Collins JP (2006) Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proceedings of the National Academy of Sciences of the United States of America* 103:3165–3170

Lips KR, Diffendorfer J, Mendelson JR III, Sears MW (2008) Riding the wave: reconciling the roles of disease and climate change in amphibian declines. *PLoS Biology* 6:e72

Luquet E, Garner TWJ, Léna JP, Bruel C, Joly P, Lengagne T, Grolet O, Plénet S (2012) Genetic erosion in wild populations makes resistance to a pathogen more costly. *Evolution* 66:1942–1952

Martínez-Solano I, Bosch J, García-París M (2003) Demographic trends and community stability in a montane amphibian assemblage. *Conservation Biology* 17:238–244

Martínez-Solano I, Gonçalves HA, Arntzen JW, García-París M (2004) Phylogenetic relationships and biogeography of midwife toads (Discoglossidae: *Alytes*). *Journal of Biogeography* 31:603–618

Ohst T, Gräser Y, Mutschmann F, Plötner J (2011) Neue erkenntnisse zur gefährdung europäischer amphibian durch den hautpilz *Batrachochytrium dendrobatidis*. *Zeitschrift für Feldherpetologie* 18:1–17

Pasmans F, Muijsers M, Maes S, Van Rooij P, Brutyn M, Ducatelle R, Haesebrouck F, Martel A (2010) Chytridiomycosis related mortality in a midwife toad (*Alytes obstetricans*) in Belgium. *Vlaams Diergeneeskundig Tijdschrift* 79:461–463

Potthoff RF, Whittinghill M (1966) Testing for homogeneity: II. The Poisson distribution. *Biometrika* 53:183–190

Rachowicz LJ, Briggs CJ (2007) Quantifying the disease transmission function: effects of density on *Batrachochytrium dendrobatidis* transmission in the mountain yellow-legged frog *Rana muscosa*. *Journal of Animal Ecology* 76:711–721

Rosa GM, Anza I, Moreira PL, Conde J, Martins F, Fisher MC, Bosch J (2012) Evidence of chytrid-mediated population declines in common midwife toads (*Alytes obstetricans*) in Serra da Estrela Portugal. *Animal Conservation* . doi:10.1111/j.1469-1795.2012.00602.x

Skerratt LF, Berger L, Speare R, Cashins S, McDonald KR, Phillott AD, Hines HB, Kenyon N (2007) Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *Ecohealth* 4:125–134

Sztatecsny M, Glaser F (2011) From the eastern lowlands to the western mountains: first records of the chytrid fungus *Batrachochytrium dendrobatidis* in wild amphibian populations from Austria. *Herpetological Journal* 21:87–90

Tobler U, Schmidt BR (2010) Within- and among-population variation in chytridiomycosis-induced mortality in the toad *Alytes obstetricans*. *PLoS ONE* 5:e10927

Tobler U, Borgula A, Schmidt BR (2012) Populations of a susceptible amphibian species can grow despite the presence of a pathogenic chytrid fungus. *PLoS ONE* 7:e34667

Vredenburg VT, Knapp RA, Tunstall TS, Briggs CJ (2010) Dynamics of an emerging disease drive large-scale amphibian population extinctions. *Proceedings of the National Academy of Sciences of the United States of America* 107:9689–9694

Walker SF, Bosch J, James TY, Litvintseva AP, Valls JAO, Piña S, Garcia G, Rosa GA, Cunningham AA, Hole S, Griffiths RA, Fisher MC (2008) Invasive pathogens threaten species recovery programs. *Current Biology* 18:R853–R854

Walker SF, Bosch J, Gomez V, Garner TWJ, Cunningham AA, Schmeller DS, Ninyerola M, Henk D, Ginestet C, Christian-Philippe A, Fisher MC (2010) Factors driving pathogenicity versus prevalence of the amphibian pathogen *Batrachochytrium dendrobatidis* and chytridiomycosis in Iberia. *Ecology Letters* 13:372–382