DIFFERENTIAL RESPONSES ON INDIVIDUAL- AND POPULATION-LEVEL TO A FUNGAL PATHOGEN: *BD* INFECTION IN THE MIDWIFE TOAD *ALYTES OBSTETRICANS*

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DIFFERENTIAL RESPONSES ON INDIVIDUAL- AND POPULATION-LEVEL TO A FUNGAL PATHOGEN: *BD* INFECTION IN THE MIDWIFE TOAD *ALYTES OBSTETRICANS*

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ZUSAMMENFASSUNG

Infektionskrankheiten werden zu einer immer stärkeren Bedrohung der Biodiversität, und die Anzahl bekannter Wildtierkrankheiten, die sich ausbreiten, nimmt dramatisch zu (Daszak *et al.* 2000, Smith *et al.* 2009). Die Problematik kommt daher, dass Krankheitserreger, die ein breites Wirtsspektrum haben, in naive Populationen eingeschleppt werden, zusammen mit ihren relativ resistenten ursprünglichen Wirten, die als Überträger dienen (Daszak *et al*. 2004).

Gemäss der IUCN sind Amphibien die am stärksten bedrohte Wirbeltiergruppe; rund ein Drittel der Arten ist bedroht (Gascon *et al*. 2007). Eine Pilzerkrankung, die Chytridiomykose, wurde als eine wichtige Ursache der Bestandesrückgänge identifiziert. Deren Erreger, der Pilz *Batrachochytrium dendrobatidis* (*Bd*) ist ein Chytridiomycet, der die keratinisierte Haut von Amphibien befällt und mit dem lokalen und globalen Aussterben von Amphibien auf mehreren Kontinenten in Verbindung gebracht wird (Fisher *et al.* 2009b, Kilpatrick *et al.* 2010). *Bd* wird hauptsächlich im Wasser übertragen und ein langes Kaulquappenstadium, wie es bei der Geburtshelferkröte vorkommt, erhöht somit das Ansteckungsrisiko. Infektionen bei Kaulquappen sind auf die Mundfelder beschränkt, was keine Krankheitssymptome bewirkt. Im Gegensatz dazu sind die Tiere kurz nach der Metamorphose am anfälligsten, wenn die gesamte Amphibienhaut von Keratin überzogen wird und das Immunsystem erst schwach ausgebildet ist (Rollins-Smith 1998). Nebst Unterschieden in der Krankheitsanfälligkeit verschiedener Lebensstadien beobachtet man auch Unterschiede aufgrund von Hautpeptiden, der Lebensweise und dem Verhalten; auch bakterielle Symbionten auf der Amphibienhaut und klimatische Bedingungen dürften eine Rolle spielen (Fisher *et al.* 2009b, Kilpatrick *et al.* 2010). So sind manche Arten relativ resistent und erkranken nicht, während bei anderen Arten Massensterben auftreten. Geburtshelferkröten-Populationen in einem Nationalpark in Spanien nahe Madrid nahmen beispielsweise drastisch ab, nachdem *Bd* dort erstmals nachgewiesen wurde (Bosch *et al.* 2007, Bosch *et al.* 2001); daher ist die Annahme begründet, dass *Bd* auch in anderen Verbreitungsgebieten der Art zu Bestandesabnahmen führt.

In der Schweiz hat die Geburtshelferkröte in den letzten drei Jahrzehnten massive Bestandesrückgänge erlitten (Schmidt & Zumbach 2005). Vielen lokalen Aussterbeereignissen kann keine offensichtliche Ursache zugewiesen werden und daher ist

eine eher kryptische Ursache wie eine Infektionskrankheit eine plausible alternative Erklärung.

Im **ersten Kapitel** meiner Dissertation bestimme ich den geographischen Massstab, in dem Geburtshelferkröten-Populationen organisiert sind. Die meisten Arten sind in Populationen organisiert, die ihrerseits Netzwerke aus Subpopulationen sind. Diese Netzwerke zeichnen sich durch einen unterschiedlichen Grad an Genfluss zwischen einzelnen Subpopulationen aus (Wright 1965). In meiner Arbeit untersuchte ich die genetische Struktur von Geburtshelferkröten in vier Regionen der Schweiz. Alle vier Regionen beherbergen Netzwerke von Subpopulationen, die sich in der Stärke der Bestandesabnahmen, im Grad ihrer Vernetzung und einer Reihe weiterer Ursachen, die die genetische Struktur beeinflussen können, unterscheiden. Anhand neutraler Mikrosatellitenmarker bestimmte ich die genetische Diversität und Differenzierung innerhalb der Regionen. Mittels Modellselektion suchte ich die Faktoren, die die Unterschiede in der genetischen Struktur zwischen den Regionen am besten erklären. Wir fanden keine Hinweise darauf, dass die Stärke der Bestandesabnahmen mit der genetischen Diversität korreliert. Ebenso wenig war die genetische Differenzierung mit der Isolation der Subpopulationen korreliert; alle Populationen waren genetisch isoliert, selbst über geringe Distanzen. Nur die Höhenlage hatte einen Einfluss auf die genetische Diversität: Sie nahm mit zunehmender Höhenlage zu. Fluktuierende Umweltbedingungen in grösseren Höhenlagen sind eine mögliche Erklärung für diese Beobachtung (Fisher 1930, Munwes *et al.* 2010). Das Fehlen eines Zusammenhangs zwischen Bestandesrückgängen und genetischer Zusammensetzung deutet darauf hin, dass Geburtshelferkröten-Subpopulationen als relative unabhängige Einheiten funktionieren und Genfluss zwischen Subpopulationen relativ unwichtig ist (Beebee 2005, Frankham *et al.* 2002). Daher sollten sich Auswirkungen der Infektionskrankheit anhand ihres Effekts auf einzelne Populationen zeigen lassen, unabhängig vom Infektionsstatus benachbarter Populationen.

In **Kapitel 2** quantifiziere ich die Mortalität von mit *Bd* infizierten Geburtshelferkröten nach der Metamorphose. Dazu fing ich natürlicherweise infizierte Kaulquappen von drei verschiedenen Populationen und zog sie im Labor auf. Die Kaulquappen wurden in drei Behandlungsgruppen eingeteilt: 1) Die *Bd*-negative Kontrolle befreite ich durch die Behandlung mit dem fungiziden Medikament Itraconazol von der Infektion (Garner *et al.* 2009a). 2) Die Stresskontroll-Gruppe behandelte ich nach dem gleichen Protokoll wie die *Bd*-

negative Kontrolle, verzichtete aber auf das Itraconazol während der Behandlung. Diese Gruppe erfuhr also denselben Behandlungsstress wie die Itraconazol-behandelte Gruppe, blieb aber infiziert. 3) Die letzte Gruppe wurde nicht behandelt und nur die regulären Wasserwechsel und Fütterungen, die bei allen Versuchsgruppen stattfanden, wurden vorgenommen. Auch diese Gruppe blieb demnach infiziert. Die Resultate zeigen, dass die Mortalität durch *Bd*-Infektion hoch war (44.4% aller infizierten Tiere starben), aber dass es grosse Unterschiede zwischen den verschiedenen Populationen gab (die Sterblichkeit schwankte zwischen 27% und 90%). Die Unterschiede, die wir beobachteten, könnten auf unterschiedliche symbiotische Hautbakterien, unterschiedliche Erregerstämme oder Umwelteffekte vor dem Fang im natürlichen Lebensraum zurückzuführen sein. Umwelteffekte lassen jedoch über kurze Zeit nach (Van Buskirk 2002) und Hautbakterien und Erregerstämme wurden aufgrund unseres Laborprotokolls zwischen den Populationen vermutlich vermischt. Daher gehen wir davon aus, dass genetische Unterschiede zwischen den Populationen die Unterschiede in der Mortalität bewirkt haben.

Wie die hohe Sterblichkeit, die wir im 2. Kapitel beobachteten, zeigt, sind Auswirkungen von *Bd* auf die Überlebenswahrscheinlichkeit der Populationen anzunehmen. Daher versuche ich im **3. Kapitel** einen Zusammenhang zwischen dem Vorkommen von *Bd* in einer Population und dem lokalen Aussterben der Geburtshelferkröte herzustellen. Dazu benützten wir Daten aus der Erhebung, die im Rahmen der Aktualisierung der Roten Liste in 2003 und 2004 durchgeführt wurde (Schmidt & Zumbach 2005). Anhand dieser Daten wählten wir 79 Teiche, verteilt über das gesamte Verbreitungsgebiet der Geburtshelferkröte in der Schweiz, von denen wir wussten, dass sie um ca. 1985 Geburtshelferkröten-Populationen beherbergt hatten. Wir besuchten diese Teiche in 2008 und testeten sie auf das Vorkommen von *Bd*, indem wir Hautabstriche von Amphibien sammelten, die wir an den Teichen fingen. Diese Abstriche wurden im Labor mittels real-time PCR auf *Bd* getestet (Boyle *et al*. 2004). Gleichzeitig führten wir eine erneute Erhebung über die Anwesenheit von Geburtshelferkröten an den Teichen durch. Die Anwesenheit von *Bd* und Geburtshelferkröten wurden dann in einem hierarchischen Modell analysiert, das die Vorkommenswahrscheinlichkeit mehrerer Arten während mehrerer Erhebungsperioden modelliert. Bei dieser Art von Modellen wird die Antreffwahrscheinlichkeit einer Art mitberücksichtigt. Ich modellierte also gleichzeitig die Vorkommenswahrscheinlichkeit von *Bd* in 2008 und die Vorkommenswahrscheinlichkeit der Geburtshelferkröte während den

zwei Erhebungen in 2003/2004 und 2008 in einem einzigen Modell. Dieses Modell berechnete ich in mit dem Programm WinBUGS (Kéry 2010, Royle & Dorazio 2008), das auf dem Bayes'schen Theorem bedingter Wahrscheinlichkeiten beruht. Dadurch war es mir möglich, die Wahrscheinlichkeit, dass die Geburtshelferkröte vorkommt, ausgestorben ist oder überlebt hat an einem Teich, und die Wahrscheinlichkeit, dass *Bd* vorkommt, in Abhängigkeit von einander modellieren und dabei jeweils den Fehler der geschätzten Wahrscheinlichkeiten berücksichtigen (Waddle *et al*. 2010). Dabei zeigte sich, dass das Vorkommen bzw. die lokale Aussterbewahrscheinlichkeit der Geburtshelferkröte unabhängig vom Vorkommen von *Bd* ist. Dieses überraschende Ergebnis wird durch die Resultate aus meinem nächsten Kapitel gestützt.

Im **4. Kapitel** untersuche ich die Wachstumsraten von Geburtshelferpopulationen in An- oder Abwesenheit von *Bd*. Adrian Borgula stellte grosszügigerweise die jährlichen Ruferzählungen von 26 Teichen im Kanton Luzern von 2002 bis 2009 zur Verfügung. Für jede Population modellierte ich den Populationstrend in Abhängigkeit des Vorkommens von *Bd* und der Häufigkeit nachgewiesener Fortpflanzung. Dieses Modell wurde wiederum im Programm WinBUGS beruhend auf dem Bayes'schen Wahrscheinlichkeitstheorem berechnet. Der Vorteil dieses Ansatzes gegenüber konventionellen Maximum-Likelihood Schätzung ist, dass bei der Analyse der Fehler im Populationstrend, der durch den Beobachtungsprozess und den Prozess der Analyse entsteht, berücksichtigt wird. Wiederum fanden wir keinen negativen Zusammenhang zwischen dem Vorkommen von *Bd* und den Wachstumsraten der Geburtshelferkröten-Populationen. Das Fehlen eines Zusammenhangs zwischen dem Vorkommen von *Bd* und lokalen Bestandesabnahmen (Kapitel 4) oder lokalem Aussterben (Kapitel 3) der Geburtshelferkröte kann durch mehrere Mechanismen zustande kommen, die einander nicht zwingend ausschliessen: 1) Möglicherweise begünstigen die herrschenden Umweltbedingungen keine Krankheitsausbrüche (Bosch *et al.* 2007, Rohr *et al.* 2008, Walker *et al.* 2010). 2) Andererseits kann *Bd* sowohl als enzootische als auch als epizootische Infektion vorliegen (Briggs *et al.* 2010). Während epizootische Krankheitsdynamik meist additive, d.h. zusätzliche, Mortalität bewirkt, besteht bei enzootischer Dynamik die Möglichkeit, dass die Mortalität kompensiert wird. *Bd* in der Schweiz könnte mehrheitlich enzootische Krankheitsdynamik bewirken. 3) *Bd* verringert möglicherweise nicht die Überlebenswahrscheinlichkeit, sondern wirkt sich auf andere Fitnessparameter von Individuen aus. Ist das Leistungsvermögen in einem Fitnessbereich

reduziert, kann möglicherweise eine gesteigerte Effizienz in anderen Fitnessbereichen für die Verluste kompensieren (Jolles *et al.* 2005). 4) Möglich ist auch, dass *Bd* zu Beginn nach dem ersten Auftreten eine Bestandesabnahme bewirkt hat und dass sich die Populationen jetzt auf einem geringeren Niveau stabilisiert haben (Briggs *et al*. 2005).

Die Prävalenz und Abundanz von *Bd* variiert stark zwischen verschiedenen Populationen. Massensterben treten nur auf, wenn die Abundanz von *Bd* sehr hoch ist (Briggs *et al.* 2010, Vredenburg *et al.* 2010). Daher versuche ich in **Kapitel 5** diese Unterschiede durch die Umwelteigenschaften der Gewässer zu erklären. Dazu wählte ich 19 Teiche in den drei Gebieten, die ich in Kapitel 1 bereits beprobt hatte und in denen *Bd* vorkam, nämlich BE, BL und SG. An diesen 19 Teichen beprobte ich im Frühling und Sommer Kaulquappen der Geburtshelferkröte auf *Bd* und mass von April bis Oktober Umweltdaten. Anhand eines hierarchischen Bayes-Modells schätzte ich die Auswirkungen der Amphibiendichte, der Wassertemperatur, der genetischen Diversität der Wirtspopulation und der Zooplanktondichte auf die Entdeckungs- und Vorkommenswahrscheinlichkeit und Abundanz des Erregers ab. Da Abundanzmodelle eine grosse Anzahl an Iterationen benötigen, bis sie konvergieren, sind die Ergebnisse, die in diesem Kapitel präsentiert werden, erst vorläufig und ändern möglicherweise noch, bis ein Modell gefunden ist, das optimal konvergiert. Die Effekte der Kovariaten Amphibiendichte, Wassertemperatur und Zooplanktondichte sollten qualitativ robust sein, auch wenn die Schätzwerte noch ändern können. *Bd* war häufiger in wärmeren Teichen, vermutlich weil sogar warme Gewässer in unseren Breitengraden sich nie für längere Zeit über das Wachstumsoptimum von *Bd* hinaus erhitzen. Eine höhere Kaulquappendichte korrelierte mit einer höheren Abundanz von *Bd*. Die Ergebnisse belegen, dass Umwelteigenschaften einen Einfluss auf die Abundanz von *Bd* haben. Dieses Wissen könnte wichtig werden, wenn es darum geht, Habitatmanagement zu betreiben, um die Auswirkungen von *Bd* zu reduzieren.

Während meiner Dissertation gelang es mir zu zeigen, das *Bd* auf Individuenebene ein ernst zu nehmender Krankheitserreger sein kann und hohe Mortalität bewirkt. Auf Populationsebene jedoch beobachten wir keine negativen Auswirkungen von *Bd*. Entweder können die Verluste kompensiert werden oder die Mortalität ist gering, da die Umweltbedingungen keine Krankheitsausbrüche begünstigen. Da die Abundanz des Erregers von den Umweltbedingungen abhängt, kann die Manipulation des Lebensraums der

Geburtshelferkröte eine sinnvolle Massnahme zur Verringerung der Auswirkungen von *Bd* in Zukunft darstellen.

Literaturnachweis

Beebee, T. J. C. (2005). Conservation genetics of amphibians. *Heredity* 95(6): 423-427.

Bosch, J., Carrascal, L. M., Duran, L., Walker, S. and Fisher, M. C. (2007). Climate change and outbreaks of amphibian chytridiomycosis in a montane area of Central Spain; is there a link? *Proceedings of the Royal Society Biological Sciences Series B* 274(1607): 253-260.

Bosch, J., Martínez-Solano, I. and García-París, M. (2001). Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain. *Biological Conservation* 97(3): 331-337.

Boyle, D. G., Boyle, D. B., Olsen, V., Morgan, J. A. T. and Hyatt, A. D. (2004). Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Diseases of Aquatic Organisms* 60(2): 141-148.

Briggs, C. J., Knapp, R. A. and Vredenburg, V. T. (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* 107(21): 9695-9700.

Briggs, C. J., Vredenburg, V. T., Knapp, R. A. and Rachowicz, L. J. (2005). Investigating the population-level effects of chytridiomycosis: An emerging infectious disease of amphibians. *Ecology* 86(12): 3149-3159.

Daszak, P., Cunningham, A. A. and Hyatt, A. D. (2000). Wildlife ecology - Emerging infectious diseases of wildlife - Threats to biodiversity and human health. *Science* 287(5452): 443-449.

Daszak, P., Strieby, A., Cunningham, A. A., Longcore, J. E., Brown, C. C. and Porter, D. (2004). Experimental evidence that the bullfrog (*Rana catesbeiana*) is a potential carrier of chytridiomycosis, an emerging fungal disease of amphibians. *Herpetological Journal* 14(4): 201-207.

Fisher, M. C., Garner, T. W. J. and Walker, S. F. (2009). Global emergence of *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis in space, time, and host. *Annual Review of Microbiology* 63: 291-310.

Fisher, R. A. (1930). The genetical theory of natural selection. Oxford, Clarendon Press.

Frankham, R., Ballou, J. D. and Briscoe, D. A. (2002). Introduction to Conservation Genetics. Cambridge, Cambridge University Press.

Garner, T. W. J., García, G., Carroll, B. and Fisher, M. C. (2009). Using itraconazole to clear *Batrachochytrium dendrobatidis* infection, and subsequent depigmentation of *Alytes muletensis* tadpoles. *Diseases of Aquatic Organisms* 83(3): 257-260.

Gascon, C., Collins, J. P., Moore, R. D., Church, D. R., McKay, J. E. and Mendelsson, J. R. e. (2007). Amphibian conservation action plan. Gland, The World Conservation Union (IUCN).

Jolles, A. E., Cooper, D. V. and Levin, S. A. (2005). Hidden effects of chronic tuberculosis in African buffalo. *Ecology* 86(9): 2358-2364.

Kéry, M. (2010). Introduction to WinBUGS for Ecologists. London, Elsevier.

Kilpatrick, A. M., Briggs, C. J. and Daszak, P. (2010). The ecology and impact of chytridiomycosis: an emerging disease of amphibians. *Trends in Ecology & Evolution* 25(2): 109-118.

Munwes, I., Geffen, E., Roll, U., Friedmann, A., Daya, A., Tikochinski, Y. and Gafny, S. (2010). The change in genetic diversity down the core-edge gradient in the eastern spadefoot toad (*Pelobates syriacus*). *Molecular Ecology* 19(13): 2675-2689.

Rohr, J. R., Raffel, T. R., Romansic, J. M., McCallum, H. and Hudson, P. J. (2008). Evaluating the links between climate, disease spread, and amphibian declines. *Proceedings of the National Academy of Sciences of the United States of America* 105(45): 17436-17441.

Rollins-Smith, L. A. (1998). Metamorphosis and the amphibian immune system. *Immunological Reviews* 166: 221-230.

Royle, J. A. and Dorazio, R. M. (2008). Hierarchical Modeling and Inference in Ecology: The Analysis of Data from Populations, Metapopulations and Communities. London, Elsevier.

Schmidt, B. R. and Zumbach, S. (2005). Rote Liste der gefährdeten Amphibien der Schweiz. Bundesamt für Umwelt, Wald und Landschaft, Bern, und Koordinationsstelle Amphibien- und Reptilienschutz in der Schweiz, Bern.

Smith, K. F., Acevedo-Whitehouse, K. and Pedersen, A. B. (2009). The role of infectious diseases in biological conservation. *Animal Conservation* 12(1): 1-12.

Van Buskirk, J. (2002). Phenotypic lability and the evolution of predator-induced plasticity in tadpoles. *Evolution* 56(2): 361-370.

Vredenburg, V. T., Knapp, R. A., Tunstall, T. S. and Briggs, C. J. (2010). Dynamics of an emerging disease drive large-scale amphibian population declines. *Proceedings of the National Academy of Sciences of the United States of America* 107(21): 9689-9694.

Waddle, J. H., Dorazio, R. M., Walls, S. C., Rice, K. G., Beauchamp, J., Schuman, M. J. and Mazzotti, F. J. (2010). A new parameterization for estimating co-occurrence of interacting species. *Ecological Applications* 20(5): 1467-1475.

Walker, S. F., Bosch, J., Gomez, V., Garner, T. W. J., Cunningham, A. A., Schmeller, D. S., Ninyerola, M., Henk, D., Ginestet, C., Arthur, C.-P. and Fisher, M. C. (2010). Factors driving pathogenicity vs. prevalence of amphibian panzootic chytridiomycosis in Iberia. *Ecology Letters* 13(3): 372-382.

Wright, S. (1965). The interpretation of population-structure by F-statistics with special regard to systems of mating. *Evolution* 19(3): 395-420.

SUMMARY

Infectious diseases are becoming an important hazard to biodiversity, and the number of reports on newly emerging diseases of wildlife is increasing dramatically (Daszak *et al.* 2000, 2001, Smith *et al.* 2009). The threat arises mostly from the spread of generalist pathogens into naïve populations along with their relatively resistant native hosts that act as carriers (Daszak *et al.* 2004, de Castro & Bolker 2005).

According to the IUCN, amphibians are the most threatened vertebrate taxon with about one third of all known species threatened by extinction (Gascon *et al*. 2007). A fungal disease called chytridiomycosis has been identified as a main contributor to the declines. The pathogen causing it, *Batrachochytrium dendrobatidis* (*Bd*), is a chytridiomycete fungus that infects the keratinised amphibian skin and has been implicated in local and global extinctions of amphibians on several continents (Fisher *et al.* 2009b, Kilpatrick *et al.* 2010). *Bd* is primarily transmitted in water and a prolonged larval stage as it is found in *Alytes* thus increases infection risk. While infection in tadpoles is restricted to the mouthparts and this does not cause disease, susceptibility is highest shortly after metamorphosis when the skin becomes keratinised and the amphibian immune system is depressed (Rollins-Smith 1998). Apart from variation in susceptibility among life stages, susceptibility varies with skin peptide defences, species ecology and behaviour; bacterial skin symbionts and climatic conditions have also been invoked in explaining the different outcomes of *Bd* presence in amphibian populations (Fisher *et al.* 2009b, Kilpatrick *et al.* 2010). Hence, while some species are resistant to chytridiomycosis, others suffer mass mortalities. For example, *Alytes obstetricans* populations have strongly declined after the invasion of the pathogen in Central Spain (Bosch *et al.* 2007, Bosch *et al.* 2001), and it is therefore likely that *Bd* also contributes to population declines and extinctions in other areas of the species distribution.

In Switzerland, *Alytes obstetricans* has suffered severe declines during the last three decades (Schmidt & Zumbach 2005). Many local extirpations had no obvious cause, and a more cryptic cause like an infectious disease is therefore a plausible explanation.

In the **first chapter** of my thesis, I determine the spatial scale at which *Alytes obstetricans* populations are organised. Most species are organised in populations that are networks of subpopulations with varying degrees of connectivity among them. The genetic

structure of such a population is determined by levels of gene flow among the subpopulations (Wright 1965). I studied genetic structure in four regions of Switzerland that harbour a network of subpopulations. The regions vary in the degree of population decline of midwife toads, population isolation and a number of other traits that may affect genetic structure. Using neutral microsatellite loci, I quantified genetic diversity and differentiation within the regions and used a model selection approach to determine which factors best explain the differences in genetic structure observed. We found no evidence that the strength of decline was correlated with genetic diversity. Also, all populations were strongly isolated even across small geographic scales and population isolation was not correlated with genetic differentiation among populations. Only elevation had an effect on genetic composition: genetic diversity increased with increasing elevation. This can be explained by balancing selection caused by environmental fluctuation at higher elevation (Fisher 1930, Munwes *et al.* 2010). The lack of an effect of decline and isolation on genetic composition suggests that *Alytes obstetricans* populations function as relatively independent units within a region with gene flow among them being of minor importance (Beebee 2005, Frankham *et al.* 2002). Thus, effects of infectious disease should be visible at the single-population level, independent of whether neighbouring populations are infected or not.

In **chapter 2,** I quantify *Bd*-induced mortality in *Alytes obstetricans* after completion of metamorphosis. Tadpoles from three different populations were caught naturally infected after hibernation and brought to the lab where I raised them through metamorphosis. I assigned them to three different treatments: 1) In the *Bd*-negative control I cleared them from infection using the antifungal drug itraconazole (Garner *et al.* 2009a). 2) In the treatment control I used the same protocol as for the itraconazole treatment but did not use the antifungal drug. These tadpoles thus experienced the same stress as those in the itraconazole treatment, but remained infected. 3) The last group was not handled, apart from regular water changes and feeding that were applied to all treatments, and remained infected with *Bd*. The results showed that *Bd*-induced mortality was high (44.4% of all infected individuals died), but that there was considerable variation among populations (mortality ranged from 27% to 90%). The differences we observed might be attributed to differences in skin microbiota, different pathogen strains or environmental effects the tadpoles experienced before being brought to the laboratory. However, because environmental effects fade out quickly (Van Buskirk 2002) and effects of microbiota or

pathogen strains should have been homogenised during the laboratory rearing due to our handling protocol, we argue that the variation in survival most likely resulted from genetic differences among the study populations.

Given the high mortality determined in chapter 2, effects of *Bd* presence on local population survival are likely. Hence, I try to establish a link between the occurrence of *Bd* at a site and the local extinction of *Alytes* in **chapter 3**. We used the Red List survey conducted in 2003/2004 (Schmidt & Zumbach 2005) to select 79 ponds across the species distribution in Switzerland where we know of *Alytes* presence/absence since around 1985. We visited all ponds during spring 2008 and tested them for *Bd* by collecting skin swabs from amphibians caught from the ponds. The swabs were tested for *Bd* using rt-PCR (Boyle *et al*. 2004). At the same time, we conducted another survey for the presence of *Alytes*. The presence of both *Bd* and *Alytes* were then modelled in a multi-season multi-species hierarchical site occupancy model taking into account imperfect detection. Using a Bayesian model implemented in the program WinBUGS (Kéry 2010, Royle & Dorazio 2008), I jointly simulated the distribution of *Bd* in a single sampling event (2008) and the distribution of *Alytes* in two sampling events (2003/2004, and 2008). This allowed the estimation of *Alytes* occupancy, extinction and survival probability in the presence and absence of *Bd*, taking into account the error in the estimate for *Bd* presence (Waddle *et al*. 2010). The results suggest that the occurrence or local extinction of *Alytes* is independent from *Bd* presence at the site. This surprising finding is supported by the results from the next chapter of my thesis.

In **chapter 4,** I study population trends of *Alytes* populations with or without *Bd*. Adrian Borgula kindly provided annual counts of calling males from 26 sites in the canton Lucerne from 2002 to 2009. For every population, I modelled the population trend as a function of *Bd* presence and the frequency of reproduction. This model was again implemented in the program WinBUGS in a Bayesian framework. The advantage of this approach over conventional maximum likelihood methods is that it allows the simultaneous estimation of the population trend and the associated errors in the observation and trend estimation process. Again, there was no negative effect of *Bd* presence on population trends. The lack of correlation between *Bd* presence and population declines (chapter 4) or local extinctions (chapter 3) can be explained by several explanations that do not necessarily exclude each other: 1) Environmental conditions may currently not favour disease outbreaks (Bosch *et al.* 2007, Rohr *et al.* 2008, Walker *et al.* 2010). 2) *Bd* may be enzootic rather than epizootic and

mortality under enzootic disease dynamics may be compensatory rather than additive (Briggs *et al.* 2010). 3) *Bd* may not affect survival, but vital rates of individuals, such that a decreased performance in one life history trait may be compensated by increased performance in another (Jolles *et al.* 2005). 4) *Bd* may have caused an initial decline following invasion of the pathogen, but populations may since have established at a lower equilibrium (Briggs *et al*. 2005).

The prevalence and abundance of *Bd* varies strongly among populations, and high infection loads are a requirement for disease outbreaks (Briggs *et al.* 2010, Vredenburg *et al.* 2010). Therefore, in **chapter 5,** I try to explain these differences based on small-scale environmental characteristics of the study sites. I selected 19 ponds in the three study regions from chapter 1 that harboured *Bd*, namely BE, BL and SG. From these ponds, I sampled swabs from *Alytes* tadpoles during spring and summer 2009 and sampled environmental data throughout spring, summer and fall 2009. I used a Bayesian hierarchical model to estimate the effects of amphibian density, water temperature, genetic diversity, and zooplankton density on *Bd* detection and occurrence probability and abundance. Because abundance models require a large number of iterations to converge, the results presented in this chapter are only preliminary and may still change quantitatively until the final model with good convergence is achieved. However, for the effects of the covariates amphibian density, water temperature and zooplankton the results should be qualitatively robust. I found that *Bd* abundance is higher in warmer ponds, probably because even warm ponds in this area never exceed *Bd* growth optima for prolonged time periods. Higher *Alytes* tadpole density was correlated with higher *Bd* abundance. Overall, the results suggest that characteristics of the local environment have an effect on *Bd* abundance and that this knowledge may be useful to implement habitat management measurements to mitigate effects of *Bd* presence.

In conclusion, I was able to show that at the individual level *Bd* can be a devastating pathogen causing high mortality. However, at the population level these losses can either be compensated for or mortality is limited under current environmental conditions since we do not observe negative effects of pathogen presence on populations. Since the abundance of the pathogen depends on environmental conditions, habitat manipulation can provide a useful tool to mitigate the effects of *Bd* in the case of future disease outbreaks.

References

Beebee, T. J. C. (2005). Conservation genetics of amphibians. *Heredity* 95(6): 423-427.
Bosch, J., Carrascal, L. M., Duran, L., Walker, S. and Fisher, M. C. (2007). Climate change and outbreaks of amphibian chytridiomycosis in a montane area of Central Spain; is there a link? *Proceedings of the Royal Society Biological Sciences Series B* 274(1607): 253-260.
Bosch, J., Martínez-Solano, I. and García-París, M. (2001). Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain. *Biological Conservation* 97(3): 331-337.
Boyle, D. G., Boyle, D. B., Olsen, V., Morgan, J. A. T. and Hyatt, A. D. (2004). Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Diseases of Aquatic Organisms* 60(2): 141-148.
Briggs, C. J., Knapp, R. A. and Vredenburg, V. T. (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* 107(21): 9695-9700.
Briggs, C. J., Vredenburg, V. T., Knapp, R. A. and Rachowicz, L. J. (2005). Investigating the population-level effects of chytridiomycosis: An emerging infectious disease of amphibians. *Ecology* 86(12): 3149-3159.
Daszak, P., Cunningham, A. A. and Hyatt, A. D. (2000). Wildlife ecology - Emerging infectious diseases of wildlife - Threats to biodiversity and human health. *Science* 287(5452): 443-449.
Daszak, P., Strieby, A., Cunningham, A. A., Longcore, J. E., Brown, C. C. and Porter, D. (2004). Experimental evidence that the bullfrog (*Rana catesbeiana*) is a potential carrier of chytridiomycosis, an emerging fungal disease of amphibians. *Herpetological Journal* 14(4): 201-207.
Fisher, M. C., Garner, T. W. J. and Walker, S. F. (2009). Global emergence of *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis in space, time, and host. *Annual Review of Microbiology* 63: 291-310.
Fisher, R. A. (1930). The genetical theory of natural selection. Oxford, Clarendon Press.
Frankham, R., Ballou, J. D. and Briscoe, D. A. (2002). Introduction to Conservation Genetics. Cambridge, Cambridge University Press.
Garner, T. W. J., García, G., Carroll, B. and Fisher, M. C. (2009). Using itraconazole to clear *Batrachochytrium dendrobatidis* infection, and subsequent depigmentation of *Alytes muletensis* tadpoles. *Diseases of Aquatic Organisms* 83(3): 257-260.
Gascon, C., Collins, J. P., Moore, R. D., Church, D. R., McKay, J. E. and Mendelsson, J. R. e. (2007). Amphibian conservation action plan. Gland, The World Conservation Union (IUCN).
Jolles, A. E., Cooper, D. V. and Levin, S. A. (2005). Hidden effects of chronic tuberculosis in African buffalo. *Ecology* 86(9): 2358-2364.
Kéry, M. (2010). Introduction to WinBUGS for Ecologists. London, Elsevier.
Kilpatrick, A. M., Briggs, C. J. and Daszak, P. (2010). The ecology and impact of chytridiomycosis: an emerging disease of amphibians. *Trends in Ecology & Evolution* 25(2): 109-118.
Munwes, I., Geffen, E., Roll, U., Friedmann, A., Daya, A., Tikochinski, Y. and Gafny, S. (2010). The change in genetic diversity down the core-edge gradient in the eastern spadefoot toad (*Pelobates syriacus*). *Molecular Ecology* 19(13): 2675-2689.

Rohr, J. R., Raffel, T. R., Romansic, J. M., McCallum, H. and Hudson, P. J. (2008). Evaluating the links between climate, disease spread, and amphibian declines. *Proceedings of the National Academy of Sciences of the United States of America* 105(45): 17436-17441.

Rollins-Smith, L. A. (1998). Metamorphosis and the amphibian immune system. *Immunological Reviews* 166: 221-230.

Royle, J. A. and Dorazio, R. M. (2008). Hierarchical Modeling and Inference in Ecology: The Analysis of Data from Populations, Metapopulations and Communities. London, Elsevier.

Schmidt, B. R. and Zumbach, S. (2005). Rote Liste der gefährdeten Amphibien der Schweiz. Bundesamt für Umwelt, Wald und Landschaft, Bern, und Koordinationsstelle Amphibien- und Reptilienschutz in der Schweiz, Bern.

Smith, K. F., Acevedo-Whitehouse, K. and Pedersen, A. B. (2009). The role of infectious diseases in biological conservation. *Animal Conservation* 12(1): 1-12.

Van Buskirk, J. (2002). Phenotypic lability and the evolution of predator-induced plasticity in tadpoles. *Evolution* 56(2): 361-370.

Vredenburg, V. T., Knapp, R. A., Tunstall, T. S. and Briggs, C. J. (2010). Dynamics of an emerging disease drive large-scale amphibian population declines. *Proceedings of the National Academy of Sciences of the United States of America* 107(21): 9689-9694.

Waddle, J. H., Dorazio, R. M., Walls, S. C., Rice, K. G., Beauchamp, J., Schuman, M. J. and Mazzotti, F. J. (2010). A new parameterization for estimating co-occurrence of interacting species. *Ecological Applications* 20(5): 1467-1475.

Walker, S. F., Bosch, J., Gomez, V., Garner, T. W. J., Cunningham, A. A., Schmeller, D. S., Ninyerola, M., Henk, D., Ginestet, C., Arthur, C.-P. and Fisher, M. C. (2010). Factors driving pathogenicity vs. prevalence of amphibian panzootic chytridiomycosis in Iberia. *Ecology Letters* 13(3): 372-382.

Wright, S. (1965). The interpretation of population-structure by F-statistics with special regard to systems of mating. *Evolution* 19(3): 395-420.

GENERAL INTRODUCTION

Infectious diseases are becoming a major concern in conservation biology (Altizer *et al.* 2003, Daszak *et al.* 2000, 2001, Smith *et al.* 2009, Smith *et al.* 2006). Although host extinction by a pathogen is usually a rare event, a number of newly emerging diseases have brought many species to the brink of extinction. This is possible because 1) emerging diseases are spread into novel host populations that are naïve to infection (Biek & Real 2010, Daszak *et al.* 2001, Smith *et al.* 2009) and 2) these newly introduced pathogens often have a competent reservoir that is resistant to disease (Daszak *et al.* 2004, Rushton *et al.* 2006).

However, establishing the link between the invasion of a pathogen and host declines is not always straight forward, not even in the case of emerging diseases: Both the host and the pathogen are subject to complex interactions with the environment (Daszak *et al.* 2001), and the absence of disease-induced mortality does not prove the absence of population-level effects or *vice versa*. Individual-level effects can strongly differ from population-level responses (Buenestado *et al.* 2009, Jolles *et al.* 2006, Meagher 1999), depending on which individuals within a population are mostly affected and succumb to disease. 1) If mortality is additive, the prey or host density will be altered, which can result in population cycling, or a decrease to a lower population density (Anderson & May 1979, Hudson *et al.* 1998, Ibelings *et al.* 2004). 2) If predator- or pathogen-induced mortality is compensatory (i.e. only individuals die that would have died for another reason in the absence of the predator or pathogen), no change in the density of the prey or host species is expected (Burnham & Anderson 1984, Errington 1946, Jolles *et al.* 2006).

One of the most devastating wildlife diseases is the fungal disease chytridiomycosis. It is caused by the chytridiomycete fungus *Batrachochytrium dendrobatidis* (*Bd*), and threatens amphibian populations around the globe. *Bd* infects keratinised amphibian skin and can cause disease and death in susceptible amphibian species (Longcore *et al.* 1999, Voyles *et al.* 2009). While the infection is restricted to the mouthparts in tadpoles, where it does not cause disease, infection can spread during metamorphosis when the skin becomes keratinised. At the same time, the amphibian immune system is depressed during and shortly after metamorphosis (Rollins-Smith 1998). Therefore, freshly metamorphosed individuals are at greatest risk of succumbing to disease. Nevertheless, in many parts of the

world amphibian populations have collapsed within very short time after the arrival of the pathogen, indicating that also a large proportion of adult individuals must have died from disease (Lips *et al.* 2006, Skerratt *et al.* 2007). Because sympatric resistant species still carry the infection, but do not develop disease, they act as reservoirs that maintain the pathogen in the system (Daszak *et al.* 2004). However, even in susceptible species some populations can persist despite pathogen presence (Briggs *et al.* 2005, Longcore *et al.* 2007, Retallick *et al.* 2004), and a variety of mechanisms have been invoked to explain these differences (Bosch *et al.* 2007, Briggs *et al.* 2010, Harris *et al.* 2009, Pounds *et al.* 2006). Some may give rise to treatments against *Bd* in the future (Harris *et al.* 2009, Lubick 2010), but although methods are currently being tested (Lubick 2010, Woodhams *et al.* 2011) there is no mitigation strategy for wild amphibians available so far. Hence, it is of crucial importance to determine whether species of conservation concern are at risk.

A species of great conservation concern in central Europe is the common midwife toad *Alytes obstetricans*. The genus *Alytes* has a reproductive strategy that is quite unique for temperate anurans. The species is strongly terrestrial in its adult life stage and the males attract females by a high-pitched call from land. After the mating, which takes place on land, the male wraps the egg strings around its hind legs and carries them until the tadpoles are ready to hatch (Meyer *et al*. 2009). The tadpoles are then released into the breeding water body where they may hibernate as tadpoles, depending on water temperature (Thiesmeier 1992). Consequently, overwintered tadpoles grow to a large size and several cohorts of tadpoles can be found in the breeding water body at the same time. The evolutionary distinctiveness of the genus *Alytes* makes these species an important target for conservation.

Populations of *Alytes obstetricans* have declined in Switzerland by more than 50% within the past 25 years (Schmidt & Zumbach 2005). Many of the declines cannot be directly linked to the loss of suitable habitat (Borgula & Zumbach 2003). A more cryptic explanation like infectious disease may be a plausible cause of declines. Since the presence of *Bd* has caused massive declines of the species in Spain (Bosch *et al.* 2001), the same pathogen may contribute to declines in Switzerland. During my PhD I investigated whether and how the presence of *Bd* affects the survival of *Alytes obstetricans* populations in Switzerland. I studied the effects of *Bd* both at the individual- and population level and determined under which environmental conditions *Bd* may impose a threat to populations.

In chapter 1, I determined the spatial scale at which *Alytes* populations function. For this purpose, I studied the population genetic structure of *Alytes obstetricans* in four regions of Switzerland. These regions harbour clusters of either declining or stable populations. Using a model selection approach, I tried to identify whether declines, population isolation or other features of the study regions and populations best explain the differences in genetic structure observed. The results suggest that every breeding site acts as a relatively independent unit and that populations are strongly isolated from each other. Hence, the relevant spatial scale to study disease effects is the level of breeding sites.

When I started my PhD, only four dead metamorphs that tested positive for *Bd* had been found in the field in Switzerland; it was thus unknown whether Swiss midwife toads are susceptible to chytridiomycosis and to what extent. In chapter 2, I quantified *Bd*-induced mortality of freshly metamorphosed *Alytes obstetricans* in a laboratory setting. I found substantial mortality at the individual level with up to 90% mortality in infected individuals immediately after metamorphosis. Such substantial mortality may well translate into effects at the population level; hence, in chapter 3 I set out to test whether there is a link between the occurrence of *Bd* and local extinctions of *Alytes obstetricans* in the field. I did not find a correlation between the presence of *Bd* and local *Alytes* extinction. However, *Bd* may suppress populations to smaller sizes that are then more prone to demographic stochasticity (Briggs *et al*. 2005). Thus, chapter 4 tries to establish a link between declining population trends based on long-term counts of calling males and the presence of *Bd* in the respective populations. Surprisingly, populations with *Bd* have equal or even higher growth rates than *Bd*-free populations. Therefore, *Alytes* populations may either be able to compensate for *Bd*-induced mortality or environmental conditions do not benefit disease outbreaks. Field surveys and model results suggest that outbreaks of chytridiomycosis are strongly linked to infection loads (Briggs *et al.* 2010, Vredenburg *et al.* 2010). To study the link between environmental conditions and *Bd* abundance, I attempt to determine pond characteristics that may explain the huge variation in *Bd* prevalence in *Alytes obstetricans* tadpoles in chapter 5. Although the results are only preliminary and need to be interpreted with care, *Bd* seems to be more abundant in ponds with higher tadpole density and warmer temperatures. Knowledge of environmental conditions that favour/disfavour the pathogen can give rise to mitigation strategies by habitat manipulation in the future.

In conclusion, I was able to show that there is a strong contrast in individual- and population-level responses of *Alytes obstetricans* to *Bd*. While individual-level mortality is high, this does currently not translate into reduced population growth rates or survival probabilities of local populations. This could be due to compensatory mechanisms counterbalancing *Bd*-induced mortality of early life stages, or environmental conditions that do not favour disease outbreaks in the wild. In case that this will change in the future, habitat manipulation may provide a tool to mitigate *Bd* effects in the wild. Whatever management actions are taken, they may be directed towards single infected populations since our genetic data suggest that breeding sites act as independent units and are the relevant population level for the implementation of conservation measures.

References

Altizer, S., Harvell, D. and Friedle, E. (2003). Rapid evolutionary dynamics and disease threats to biodiversity. *Trends in Ecology & Evolution* 18(11): 589-596.

Anderson, R. M. and May, R. M. (1979). Population biology of infectious diseases: Part I. *Nature* 280: 361-367.

Biek, R. and Real, L. A. (2010). The landscape genetics of infectious disease emergence. *Molecular Ecology* 19(17): 3515–3531.

Borgula, A. and Zumbach, S. (2003). Verbreitung und Gefährdung der Geburtshelferkröte (*Alytes obstetricans*) in der Schweiz. *Zeitschrift für Feldherpetologie* 10: 11-26.

Bosch, J., Carrascal, L. M., Duran, L., Walker, S. and Fisher, M. C. (2007). Climate change and outbreaks of amphibian chytridiomycosis in a montane area of Central Spain; is there a link? *Proceedings of the Royal Society Biological Sciences Series B* 274(1607): 253-260.

Bosch, J., Martínez-Solano, I. and García-París, M. (2001). Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain. *Biological Conservation* 97(3): 331-337.

Briggs, C. J., Knapp, R. A. and Vredenburg, V. T. (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* 107(21): 9695-9700.

Briggs, C. J., Vredenburg, V. T., Knapp, R. A. and Rachowicz, L. J. (2005). Investigating the population-level effects of chytridiomycosis: An emerging infectious disease of amphibians. *Ecology* 86(12): 3149-3159.

Buenestado, F. J., Ferreras, P., Blanco-Aguiar, J. A., Tortosa, F. S. and Villafuerte, R. (2009). Survival and causes of mortality among wild Red-legged Partridges *Alectoris rufa* in southern Spain: implications for conservation. *Ibis* 151(4): 720-730.

Burnham, K. P. and Anderson, D. R. (1984). Tests of compensatory vs. additive hypotheses of mortality in mallards. *Ecology* 65(1): 105-112.

Daszak, P., Cunningham, A. A. and Hyatt, A. D. (2000). Wildlife ecology - Emerging infectious diseases of wildlife - Threats to biodiversity and human health. *Science* 287(5452): 443-449.

Daszak, P., Cunningham, A. A. and Hyatt, A. D. (2001). Anthropogenic environmental change and the emergence of infectious diseases in wildlife. *Acta Tropica* 78(2): 103-116.

Daszak, P., Strieby, A., Cunningham, A. A., Longcore, J. E., Brown, C. C. and Porter, D. (2004). Experimental evidence that the bullfrog (*Rana catesbeiana*) is a potential carrier of chytridiomycosis, an emerging fungal disease of amphibians. *Herpetological Journal* 14(4): 201-207.

Errington, P. L. (1946). Predation and vertebrate populations. *Quarterly Review of Biology* 21(2): 144-177.

Harris, R. N., Brucker, R. M., Walke, J. B., Becker, M. H., Schwantes, C. R., Flaherty, D. C., Lam, B. A., Woodhams, D. C., Briggs, C. J., Vredenburg, V. T. and Minbiole, K. P. C. (2009). Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *ISME Journal* 3(7): 818-824.

Hudson, P. J., Dobson, A. P. and Newborn, D. (1998). Prevention of population cycles by parasite removal. *Science* 282(5397): 2256-2258.

Ibelings, B. W., De Bruin, A., Kagami, M., Rijkeboer, M., Brehm, M. and van Donk, E. (2004). Host parasite interactions between freshwater phytoplankton and chytrid fungi (*Chytridiomycota*). *Journal of Phycology* 40(3): 437-453.

Jolles, A. E., Etienne, R. S. and Olff, H. (2006). Independent and competing disease risks: Implications for host populations in variable environments. *American Naturalist* 167(5): 745-757.

Lips, K. R., Brem, F., Brenes, R., Reeve, J. D., Alford, R. A., Voyles, J., Carey, C., Livo, L., Pessier, A. P. and Collins, J. P. (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(9): 3165-3170.

Longcore, J. E., Pessier, A. P. and Nichols, D. K. (1999). *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. *Mycologia* 91(2): 219-227.

Longcore, J. R., Longcore, J. E., Pessier, A. P. and Halteman, W. A. (2007). Chytridiomycosis widespread in anurans of Northeastern United States. *Journal of Wildlife Management* 71(2): 435-444.

Lubick, N. (2010). Emergency medicine for frogs. *Nature* 465: 680-681.

Meagher, S. (1999). Genetic diversity and *Capillaria hepatica* (Nematoda) prevalence in Michigan deer mouse populations. *Evolution* 53(4): 1318-1324.

Meyer, A., Zumbach, S., Schmidt, B. R. and Monney, J.-C. (2009). Auf Schlangenspuren und Krötenpfaden. Bern, Haupt.

Pounds, J. A., Bustamante, M. R., Coloma, L. A., Consuegra, J. A., Fogden, M. P. L., Foster, P. N., La Marca, E., Masters, K. L., Merino-Viteri, A., Puschendorf, R., Ron, S. R., Sanchez-Azofeifa, G. A., Still, C. J. and Young, B. E. (2006). Widespread amphibian extinctions from epidemic disease driven by global warming. *Nature* 439(7073): 161-167.

Retallick, R. W. R., McCallum, H. and Speare, R. (2004). Endemic infection of the amphibian chytrid fungus in a frog community post-decline. *PLoS Biology* 2(11): 1965-1971.

Rollins-Smith, L. A. (1998). Metamorphosis and the amphibian immune system. *Immunological Reviews* 166: 221-230.

Rushton, S. P., Lurz, P. W. W., Gurnell, J., Nettleton, P., Bruemmer, C., Shirley, M. D. F. and Sainsbury, A. W. (2006). Disease threats posed by alien species: the role of a poxvirus in the decline of the native red squirrel in Britain. *Epidemiology and Infection* 134(3): 521-533.

Schmidt, B. R. and Zumbach, S. (2005). Rote Liste der gefährdeten Amphibien der Schweiz. Bundesamt für Umwelt, Wald und Landschaft, Bern, und Koordinationsstelle Amphibien- und Reptilienschutz in der Schweiz, Bern.

Skerratt, L. F., Berger, L., Speare, R., Cashins, S., McDonald, K. R., Phillot, A. D., Hines, H. B. and Kenyon, N. (2007). Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *EcoHealth* 4: 125-134.

Smith, K. F., Acevedo-Whitehouse, K. and Pedersen, A. B. (2009). The role of infectious diseases in biological conservation. *Animal Conservation* 12(1): 1-12.

Smith, K. F., Sax, D. F. and Lafferty, K. D. (2006). Evidence for the role of infectious disease in species extinction and endangerment. *Conservation Biology* 20(5): 1349-1357.

Thiesmeier, B. (1992). Daten zur Larvalentwicklung der Geburtshelferkröte *Alytes o. obstetricans* (Laurenti, 1768) im Freiland. *Salamandra* 28(1): 34-48.

Voyles, J., Young, S., Berger, L., Campbell, C., Voyles, W. F., Dinudom, A., Cook, D., Webb, R., Alford, R. A., Skerratt, L. F. and Speare, R. (2009). Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. *Science* 326(5952): 582-585.

Vredenburg, V. T., Knapp, R. A., Tunstall, T. S. and Briggs, C. J. (2010). Dynamics of an emerging disease drive large-scale amphibian population declines. *Proceedings of the National Academy of Sciences of the United States of America* 107(21): 9689-9694.

Woodhams, D. C., Bosch, J., Briggs, C. J., Cashins, S., Davis, L. R., Lauer, A., Muths, E., Puschendorf, R., Schmidt, B. R., Sheafor, B. and Voyles, J. (2011). Mitigating amphibian disease: strategies to maintain wild populations and control chytridiomycosis. *Frontiers in Zoology* 8(8).

Comparative population genetic analysis of toad populations in regions with and without declines

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Abstract

Genetic diversity is crucial for population persistence. Population decline ranks among the most potent processes causing a reduction in genetic diversity and an increase in genetic differentiation among subpopulations. However, while the theory behind this is well-developed, empirical evidence from wild populations is inconsistent. Using neutral microsatellite markers, we compare the genetic structure of populations of an amphibian species, the midwife toad *Alytes obstetricans*, in regions where the species has either suffered no declines or severe declines. To account for region-specific effects on neutral genetic diversity, we also estimated the impact of connectivity, peripheral location, elevation, or location close to a stream on genetic structure and used a model selection approach to find the best model. We did not find differences in genetic structure between regions with and without declines. However, allelic richness was increased at higher elevation where environmental conditions may fluctuate strongly. Our study may provide support for Fisher's hypothesis of fluctuating selection in range edge populations. The absence of genetic imprints of decline on genetic differentiation may suggest that midwife toad populations function as independent units and conservation efforts should thus focus on decreasing local extinction probability rather than increasing connectivity.

Key words:

Alytes obstetricans, genetic diversity, geographic variation, microsatellites, population isolation, population structure

Introduction

Genetic diversity is considered to be of crucial importance for population persistence because a lack or reduction of genetic diversity is a threat to long-term population survival (Evans & Sheldon 2008, Frankham 2005). Many factors can influence the genetic structure of populations, but a sharp population decline over a short time span and the disruption of gene flow are considered the most potent processes for reducing measurable genetic diversity within a population and for increasing differentiation among subpopulations (Frankham 1995, 2005). For example, loss of populations diminishes rates of gene flow across the range, resulting in decreased genetic diversity through genetic drift. Given sufficient time, among-population differentiation would be higher in areas where population loss is significant than in areas where local extirpation has had a lower impact (Frankham 2005, Lande 1998). While the theory is well-developed, empirical evidence is not as consistent. Although species categorised as threatened usually have smaller population sizes (Evans & Sheldon 2008) and support lower genetic diversity than unthreatened sister taxa (Spielman *et al.* 2004b), a number of studies have found no such relationship (Booy *et al.* 2000).

Most studies describing a link between loss of genetic diversity and declines are unreplicated (Blomqvist *et al.* 2010, Johnson *et al.* 2010, Westemeier *et al.* 1998). That is, they were conducted at a single site or within a single geographic area. Hence, such studies may be unrepresentative of the general relationship between rapid decline and loss of genetic diversity. The inconsistencies associated with single population or location studies suggest that a better approach may be to measure genetic effects across a replicate of populations or metapopulations. Here, we compare the population genetic structure of an endangered amphibian in four regions where the species experienced severe declines and regions where there were no declines.

The global amphibian decline (Houlahan *et al.* 2000, Stuart *et al.* 2004) could be viewed as an interesting opportunity for investigating the links between decline and population structure. Amphibian populations usually function over limited spatial scales and population dynamics are thus influenced by local environmental processes rather than large-scale processes (Beebee 2005). Adopting the classic definitions of Wright (1965), a region across which amphibian breeding ponds are distributed acts as a population, with each pond being a subpopulation. A suitable amphibian species to study the link between decline and genetic

structure would have the following traits: 1) discrete subpopulations; 2) data available on subpopulation persistence across comparable ranges; 3) variable rates of subpopulation extinction across comparable ranges. Such a model exists in the common midwife toad, *Alytes obstetricans*, in Switzerland, where the distribution of the species is well known (Borgula & Zumbach 2003, Grossenbacher 1988). *Alytes obstetricans* exhibits a typical complex life history with an aquatic larval stage and a terrestrial adult stage, so breeding ponds act as defined subpopulations. The species has suffered strong declines in Switzerland: since the mid-1980ties. 50% of subpopulations have been extirpated, and *A. obstetricans* is categorized as "endangered" on the most recent Swiss Red List (Schmidt & Zumbach 2005). The strength of declines varies among regions and in many cases extirpation cannot be attributed to habitat loss (Borgula & Zumbach 2003, Schmidt & Zumbach 2005).

In this study, we take advantage of existing knowledge regarding the spatial distribution of recent losses of *A. obstetricans* subpopulations. We ask whether population genetic structure and diversity measured in subpopulations and regions varies and can be attributed to among-region variation in rates of recent species decline. To do this, we sampled four regions where the common midwife toad is found and where quantitative evidence of variation in subpopulation loss is available. To distinguish between the effects of declines and region-specific characteristics on genetic diversity, we modelled whether other factors such as connectivity, peripheral location, elevation, or location close to a stream contributed to observed genetic patterns. We used microsatellite polymorphisms to measure within- and among-subpopulation genetic variability. We expected that declines, i.e. the loss of local populations, should increase genetic differentiation among populations, which on a longer term will also cause reduced genetic diversity due to increased drift (Lande 1988). We predicted similar effects for lower geographic connectivity. However, because geographic connectivity is not necessarily paralleled by declines in local population size, we predicted no or only weak effects on allelic richness, heterozygosity and F_{IS} (Cushman 2006). Peripheral habitats are often marginally suitable, leading to decreased population size and connectivity (Eckert *et al.* 2008). We thus predicted negative effects of peripheral location on genetic diversity, heterozygosity and $F_{IS,}$ and increased population differentiation. We considered elevation as a special case of marginal habitat and hence predicted similar effects as those resulting from range periphery on high-elevation populations (Giordano *et al.* 2007). Finally, because streams may act as corridors for gene flow either downstream (tadpoles) or

upstream (adults; Grant *et al.* 2010, Mullen *et al.* 2010), we predicted lower genetic differentiation among populations along identical catchments (Table 1.1).

Materials and Methods

Study populations and field sampling

We collected genetic samples from four regions in Switzerland during spring and summer 2007: Baselland (BL), Bern (BE), Lucerne (LU), and St. Gallen (SG; Figure 1.1). Without *a priori* knowledge on population structure, we will refer to clusters of breeding sites as regions and to breeding sites as subpopulations (Beebee 2005) throughout the study. BL is located in the Jura Mountains and supports a comparatively dense network of *A. obstetricans* populations. Most of these are located in disturbed habitats and reproduction commonly takes place in man-made water bodies, including disused quarries and artificial ponds. Subpopulations of *A. obstetricans* in BL are relatively persistent, i.e. of 74 known sites, 51 were revisited in 2009 and species presence was confirmed at 45 sites. In addition, many hitherto unreported subpopulations were detected (Schmidt *et al.* 2010). The mean Euclidian distance between all known sites is 0.89 km (Schmidt *et al.* 2010, Table 1.2). In BE, subpopulations of *A. obstetricans* are also relatively densely distributed (mean distance among subpopulations: 1.3 km; B. Lüscher, pers. comm.) and the species mostly utilises water reservoirs or artificial ponds for breeding and larval development. *A. obstetricans* has suffered moderate declines in BE, and while colonisation of new ponds is reported, it is at a low rate: of 149 total populations known since 1970, 43 went extinct until 2003 and 12 were newly colonised (Ryser *et al.* 2003, Table 1.2). LU is a pre-alpine region and the distances between subpopulations are relatively large with a mean Euclidian distance of 17.7 km among subpopulations (A. Borgula, pers. comm.). Relatively more subpopulations of *A. obstetricans* in LU have been lost (51 subpopulations reported in 1980 down to 23 reported in 2002) than in the previous two subpopulations (BL and BE) and there are only 3 reports of colonisation of new ponds (Borgula & Zumbach 2003). Breeding habitat for *A. obstetricans* in LU includes natural water bodies such as small streams or ponds, located in pre-alpine and alpine meadows. SG, the second region with severe declines, is also a pre-alpine region. Subpopulation extirpation is common: 68 out of 118 recorded subpopulations have been

Table 1.1: Predictions of how factors are expected to affect allelic richness AR, expected heterozygosity, H_e, inbreeding coefficient F_{IS} and population differentiation F_{ST}, , and the observed effect on the genetic measures.

factor	levels	Prediction	observed effect
decline	2 (0, 1)	low gene flow among populations 1. decrease in AR due to random loss of alleles through genetic drift 2. only slight reduction in H_e because rare alleles lost by drift contribute little to H_e 3. increase in F_{IS} due to inbreeding 4. stronger genetic differentiation among subpopulations due to increased drift	1. no difference in AR 2. no difference in H_e 3. no difference in F_{IS} 4. no difference in F_{ST}
geographic connectivity	45 (mean pairwise geographic distances)	low gene flow among subpopulations, but less strong effects than under decline 1. no or only slight decrease in AR due to random loss of alleles through genetic drift 2. no reduction in H_e 3. no effect on F_{IS} 4. increased genetic differentiation	1. no difference in AR 2. no difference in H_e 3. no difference in F_{IS} 4. no difference in F_{ST}
peripheral location	2 (0, 1)	larger distance between less suitable habitat patches 1. decrease in AR due to random loss of alleles by genetic drift in smaller populations at range margins 2. slight decrease in H_e because H_e degrades more slowly than allelic richness 3. increased F_{IS} 4. stronger genetic differentiation among subpopulations due to lower connectivity	1. no difference in AR 2. no difference in H_e 3. no difference in F_{IS} 4. no difference in F_{ST}
elevation	45 (elevation of study sites)	larger distance between less suitable habitat patches 1. decrease in AR due to random loss of alleles by genetic drift in smaller populations at high elevation 2. slight decrease in H_e because H_e degrades more slowly than allelic richness 3. increased F_{IS} 4. stronger genetic differentiation among subpopulations due to lower connectivity	1. increase in AR with increasing elevation 2. no difference in H_e 3. no difference in F_{IS} 4. no difference in F_{ST}
location along stream	2 (0, 1)	increased connectivity 1. increased or equal AR due to enhanced gene flow along streams 2. no difference in H_e 3. decreased F_{IS} if neighbouring populations contributing immigrants are significantly differentiated, otherwise no effect on F_{IS} 4. lower F_{ST}	1. increase in AR with increasing elevation 2. no difference in H_e 3. no difference in F_{IS} 4. no difference in F_{ST}

Table 1.2: Characteristics of the four study regions

	region			
	BE	BL	LU	SG
number of populations	11	15	11	8
declines	none	none	strong	strong
peripheral location	no	no	yes (south)	yes (east)
elevation of study sites [mean (range)]	790 masl (590 – 940)	485 masl (400 – 590)	878 masl (590 – 1540)	543 masl (450 – 680)
number of populations in/along streams	0	0	6	2
distance among study populations [mean (range)]	4.3 km (0.6 – 9.0)	5.7 km (0.9 – 13.2)	13.1 km (1.2 – 25.9)	11.1 km (0.9 – 20.8)

lost and another 23 potentially extirpated (Barandun 2004). Breeding habitat in SG is composed of a combination of pre-alpine streams and artificial ponds; mean distance among subpopulations in this region is 19.8 km (J. Barandun, pers. comm.).

In each region we selected 8 to 15 ponds previously reported to harbour *A. obstetricans* subpopulations. We selected the study populations by haphazardly choosing an area within a study region that covered 10 to 15 *Alytes* subpopulations. Yet, many subpopulations that we visited were so small that we could not find any tadpoles; hence we extended our search for study sites until we had a sufficient number of subpopulations per region. From each site, we caught tadpoles by dip-netting and collected genetic material by cutting off less than 3mm from the tail tip. We tried to sample at least 25 tadpoles per site for tail tissue. The tadpoles were released into the ponds immediately thereafter.

Microsatellite development

Microsatellite primers were developed commercially by Ecogenics GmbH (Zurich, Switzerland) using *Alytes obstetricans* tissue samples from central Spain, France and Switzerland. An enriched library was made from the DNA of one Swiss *A. obstetricans*: size selected genomic DNA was ligated to SAULA/SAULB-linker (Armour *et al.* 1994) and enriched by magnetic bead selection with biotin-labelled $(GT)_{13}$, $(CT)_{13}$, $(GATA)_7$, $(GTAT)_7$, $(ACAG)_7$ and $(GCGT)_7$ oligonucleotide repeats (Gautschi *et al.* 2000). Of 1893 recombinant colonies screened, 241 gave a positive signal after hybridization. Plasmids from 187 positive clones were sequenced and primers were designed for 29 microsatellite inserts, of which 21 were tested for polymorphism. We selected a set of 12 primers that exhibited clear and reliable

Table 1.3: Microsatellite primer sequences, repeat types, size range, allele numbers and concentrations used in multiplex PCR reactions.

multiplex	locus	primer sequence 5'-3'	modification 5'	repeat type[1]	size bp EU[2]	# of alleles EU[2]	size bp CH[3]	# of alleles CH[3]	concentration per reaction
1	Alyobs3	F CCAACATGTTCACTTTATAGAGCAG	Yakima Yellow	$(TATC)_{28}$	203-227	11	182-279	18	1.25 µM
		R GGAACCTTGAATCTCGAAAGC							1.25 µM
1	Alyobs4	F TTTTCCCTTGCTAAATCCTCAG	ATTO565	$(CTGT)_{11}$	123-167	7	134-143	3	0.75 µM
		R AAAGTGTTGATGCACATTTTCC							0.75 µM
1	Alyobs7	F AAGGACGTGCTTCTATCTGC	FAM	$(TATC)_{16}(TG)_3(TA)_3(TC)(TA)_4$	116-268	13	124-210	14	1.25 µM
		R AGTTCGCACACATTACATTGC							1.25 µM
1	Alyobs28	F CCAGTGCTGTGGTTTTCTCA	Yakima Yellow	$(GT)_{13}(GA)_3(GTGA)_3$	100-136	10	103-109	3	1.88 µM
		R AAATATCAAGAGCCTTAGCTAACATTT							1.88 µM
2	Alyobs8	F TGAGGGGTCAGTGGAAGATATAC	FAM	$(ACAG)_{11}(AG)_2(ACAG)(ATAG)_{23}$	223-332	13	158-340	24	2.00 µM
		R GGACAAATTCCAGCATAAAGAAC							2.00 µM
2	Alyobs16	F CAATGGCTGTACACAAGGAAAC	FAM	$(GT)_{17}$	134-150	9	132-146	7	7.80 µM
		R CCTATAGAAATGTAAACATGCACAC							7.80 µM
3	Alyobs17	F TTCTCTTCAGCTGGGCAATC	Yakima Yellow	$(GT)_{13}$	137-161	6	148-165	6	2.50 µM
		R TGGAACTGAAGAGCGAGGAC							2.50 µM
3	Alyobs19	F TGAATGTGCCGGTGAAGAC	FAM	$(GT)_{12}$	69-103	8	76-82	3	2.50 µM
		R AAACACATATGAACAGGTGAAAAGAG							2.50 µM
3	Alyobs20	F GATGCAGCACATTTCTGAGC	ATTO565	$(GT)_{12}$	96-111	4	113-113	1	0.42 µM
		R GGTGCATCTGCCATAGTGTG							0.42 µM
3	Alyobs23	F TGCAGAGCTCAGCCACTTAG	ATTO550	$(GT)_{13}$	206-244	5	207-215	4	0.50 µM
		R TGACCAATCCAATCATCCAG							0.50 µM
3	Alyobs24	F TCCTCAAAATCTTGTGATGTGC	ATTO550	$(CA)_{28}$	79-134	13	102-147	17	0.50 µM
		R ATGGCCAGATGTCCCAATAC							0.50 µM
3	Alyobs25	F CCTTCTGTCTACCTTGTCATATTTCC	ATTO565	$(GT)_{16}$	138-160	6	154-167	5	0.50 µM
		R AAAGCGACTAATACAGAACACTGC							0.50 µM

1: based on sequenced clone
2: based on 15 individuals from Europe
3: based on 1389 individuals from Switzerland

amplification, polymorphism and no evidence of null alleles in preliminary tests for generating population genetics data (Table 1.3).

Microsatellite amplification

We extracted DNA from tail clips using the BioSprint 96 DNA Blood Kit (Qiagen, Hombrechtikon, Switzerland), following the protocol for tissue extraction. Polymerase chain reactions (PCR) were performed with fluorescent-labelled primers in three separate multiplexes (Table 1.3). Each well contained 1.25 µl Multiplex PCR Kit (Qiagen), the respective primer volumes (Table 1.3) and 1µl of template DNA. 80% of the forward primer concentration consisted of colour-labelled primers. PCRs were carried out on a TC-412 Thermal Cycler (Barloworld Scientific, USA) with polymerase activation at 95°C for 15 min, followed by 33 cycles (multiplex 1) or 30 cycles (multiplexes 2 & 3) of denaturing for 0.30 min at 94°C, annealing for 1.30 min at 52°C (multiplex 1) or 56°C (multiplexes 2 & 3) and extension for 1.00 min at 72°C, followed by a final extension for 30 min at 60°C. PCR products were run on an ABI 3730 Avant Capillary Sequencer (Applied Biosystems, Rotkreuz, Switzerland) with internal size standard GeneScan-500 LIZ; peaks were visually scored using GENEMAPPER 3.7 (Applied Biosystems 2004).

Statistical analysis of population genetic structure

Microsatellite loci were tested for the presence of null alleles, stuttering and large allelic dropout using MICROCHECKER (Van Oosterhout *et al.* 2004). Because we sampled tadpoles, which increases the risk of sampling siblings and therefore may induce bias in estimates of genetic diversity and gene frequencies (Goldberg & Waits 2010), we tried to identify siblings using COLONY 3.1 (Wang 2004). However, because consecutive COLONY runs based on the same data could not consistently recognise full siblings (less than 50% agreement) or half siblings (less than 20% agreement) we could not exclude siblings from the analyses (J. Wang, pers. comm.).

We used ARLEQUIN 3.1 (Excoffier *et al.* 2005) to test for linkage disequilibrium and deviations from Hardy-Weinberg (HW) equilibrium. We tested all microsatellites for selection using the program FDIST (Beaumont & Nichols 1996), which plots F_{ST} against

expected heterozygosity and designates all markers that fall outside the 95% confidence interval as affected by selection. All population genetic diversity indices (see below) were calculated with and without the markers identified by FDIST as candidates for selection.

Because *Alytes* tadpoles can hibernate facultatively as tadpoles and only metamorphose in the year after hatching, we calculated F_{ST} values treating spring (overwintered tadpoles from 2006) and summer (young-of-the-year tadpoles from 2007) cohorts as populations in all ponds where date on two cohorts was available. This analysis tests whether reproduction among cohorts was strongly skewed towards a few parental individuals (Savage *et al.* 2010; P. Wandeler, personal communication). Because none of the F_{ST}-values between cohorts were significant (results not shown), we assume that our measures of genetic diversity within subpopulations are representative.

Measures of genetic diversity per subpopulation and region (observed heterozygosity (H_o), expected heterozygosity (H_e), inbreeding coefficient F_{IS} (Weir & Cockerham 1984) and the frequency of private alleles) were calculated using GENETIX 4.05.2 (Belkhir *et al.* 1996-2004) and a sample-size corrected estimate of allelic richness (AR) was obtained using

Figure 1.1: Map of Switzerland showing the distribution of *Alytes* (green dots) and the location of the study populations (white dots). Data sources: Swisstopo and KARCH (Koordinationsstelle für Amphibien- und Reptilenschutz in der Schweiz, www.karch.ch)

FSTAT 2.9.3.2 (Goudet 2001). The low variability and high fixation rate of the microsatellite markers in our study populations did not allow the estimation of effective subpopulation size.

We calculated pairwise F_{ST}-values as a measure of among-subpopulation differentiation and tested the significance with an exact test with 1000 permutations (Excoffier *et al.* 2005). We tested for isolation by distance (IBD) across and within regions using $F_{ST}/(1- F_{ST})$-transformed F_{ST} values and log-transformed Euclidian distances (Excoffier *et al.* 2005). To determine the amount of genetic variation distributed within and among subpopulations/regions, we ran an Analysis of Molecular Variance (AMOVA) in Arlequin 3.1 (Excoffier *et al.* 2005).

Model selection

We used a model selection approach (Burnham & Anderson 2002) to identify whether severity of declines, geographic connectivity, peripheral location, altitude, or location near a stream best explained differences in genetic structure (Cushman 2006, Eckert *et al.* 2008, Giordano *et al.* 2007, Grant *et al.* 2010, Lande 1988, Mullen *et al.* 2010). We extracted data on elevation and pairwise Euclidian distances among subpopulations within a region from the national map 1:25000 (Swisstopo). Populations were considered peripheral if they were located in the regions LU or SG, and as stream populations if they were within 200 m or located in a stream.

We used AR, H_e, F_{IS}, and the mean subpopulation F_{ST} as response variables in separate linear models; we did not use all variables of genetic diversity that are commonly used in population genetic studies because all measures of allelic diversity (number of private alleles, fixed loci, and AR) were correlated (all $r > 0.45$), and the same was true for H_e and H_o ($r = 0.89$). We calculated a mean subpopulation F_{ST} as the mean of all pairwise F_{ST} values of a subpopulation to all other subpopulations within the same region. In contrast to using the global F_{ST} for every region, this provided us with subpopulation-level F_{ST}-values and thus higher statistical resolution during the model selection analysis.

The linear models included one of the following fixed effects as explanatory variables: 1) region, 2) severity of decline, 3) peripheral location), 4) altitude, 5) location close to a stream, or 6) population isolation (mean pairwise Euclidian distance to all other

subpopulations in the same region). Additional candidate models included the factors region plus altitude, location at a stream, or subpopulation isolation, respectively, and the interaction between region and the other factors (Table 1.6). We used an intercept-only model as a null model. We standardised elevation and isolation for the use in linear models using z-transformation.

We ranked models based on Akaike's information criterion for small sample sizes (AICc; Burnham & Anderson 2002) and report parameter estimates for models within 2 ΔAICc units of the best model. Model assessment was also based on inspection of the parameter estimates and their standard errors; i.e., only parameters of which the confidence interval did not include 0 were considered important (Burnham *et al.* 2010). All linear models were fitted to the data in R 2.8.1. (R Development Core Team 2008).

Results

Genetic diversity

We did not detect null alleles, large allele dropout or stuttering at any microsatellite locus (all $p>0.05$). Although we could not exclude siblings *a priori* from the data set, we found no deviations from HW equilibrium after Bonferroni correction and very little and inconsistent linkage at few markers in few subpopulations. This indicates that the (possible) presence of siblings in the genotype data did not negatively affect our results (Rasmussen 1979). Four markers were identified by FDIST as being potentially non-neutral. Alyobs23 was designated as a candidate for directional selection, while Alyobs3, Alyobs4 and Alyobs8 were identified as candidates for balancing selection (Beaumont & Nichols 1996). Excluding these markers from the calculation of our measures of genetic diversity estimates did not alter the model selection results (results not shown). We thus report the estimates of genetic diversity based on all 11 polymorphic markers.

Locus Alyobs20 was monomorphic in all study populations and excluded from the analyses. For the remaining 11 loci, average allelic richness (AR) was 2.38 (range: 1.58 – 3.58). Overall, 6.3 ± 1.2 loci were fixed in every subpopulation while on average we detected one private allele in every second subpopulation. Average H_e was 0.27 and ranged from 0.15 – 0.40; the difference between H_e and H_o never exceeded 0.12. F_{IS} was on average slightly negative (-0.05, range: -0.41 – 0.16, Figure 4; summary statistics for subpopulations and regions are given in Table 1.4).

Table 1.4: Measures of genetic diversity in all study populations. AR: allelic richness (sample size corrected), H_e: expected heterozygosity, H_o: observed heterozygosity, F_{IS}: inbreeding coefficient

	location	SS	total # of alleles	# of fixed loci	# private alleles	AR	H_e	H_o	F_{IS}	mean F_{ST} [1]
BE	Brandsiten	62	25	8	0	2.17	0.21	0.22	-0.05	0.248
	Chnubel	47	25	7	0	2.17	0.27	0.31	-0.15	0.253
	Hinter Schwarzenegg	15	23	8	0	2.00	0.25	0.36	-0.40	0.199
	Laternengraben	23	18	4	0	1.58	0.20	0.17	0.16	0.253
	Mattstallwald	52	26	5	1	2.25	0.21	0.22	-0.06	0.342
	Ober Fürten	30	21	5	0	1.83	0.25	0.28	-0.11	0.173
	Ober Rotenbühl	35	28	7	0	2.42	0.29	0.30	-0.02	0.147
	Rüegsau	6	21	5	1	1.83	0.24	0.36	-0.41	0.189
	Süerisguethalden	20	21	5	0	1.83	0.26	0.33	-0.24	0.176
	Voder Birnbaum	50	31	9	0	2.67	0.29	0.30	-0.01	0.133
	Waltisberg	61	28	5	1	2.42	0.22	0.24	-0.05	0.247
	mean BE		25.6	6.4	0.4	2.22	0.25	0.28	-0.11	0.215
BL	Bickenberg	51	38	7	2	3.25	0.33	0.32	0.05	0.205
	Brunnmatt	19	27	6	0	2.33	0.33	0.35	-0.05	0.228
	Chalchofen	31	37	6	0	3.17	0.27	0.27	0.01	0.242
	Chienberg	24	32	6	2	2.75	0.28	0.28	0.02	0.237
	Hard	21	30	6	0	2.58	0.28	0.31	-0.11	0.234
	Heftelen	57	37	6	1	3.17	0.31	0.33	-0.06	0.190
	Huppergrube	20	42	7	0	3.58	0.40	0.41	-0.01	0.177
	Itingen	52	28	7	2	2.42	0.20	0.20	-0.02	0.324
	Niederdorf	27	39	8	2	3.33	0.34	0.33	0.05	0.181
	Reigoldswil	48	24	6	0	2.08	0.26	0.26	0.02	0.398
	Schleifenberg	45	36	7	0	3.08	0.36	0.36	0.02	0.204
	Seewen	25	24	4	2	2.08	0.24	0.26	-0.06	0.276
	Strickrain alt	11	29	7	0	2.50	0.32	0.35	-0.05	0.268
	Strickrain neu	47	39	9	2	3.33	0.32	0.32	0.01	0.246
	Wildenstein	14	18	3	0	1.58	0.15	0.16	-0.07	0.431
	mean BL		33.3	6.8	1.0	2.86	0.29	0.30	-0.02	0.256
LU	Aemmenmatt	16	23	7	0	2.00	0.33	0.33	0.01	0.222
	Bahngleis	23	25	7	0	2.17	0.34	0.32	0.11	0.184
	Chalchloch	24	21	6	0	1.83	0.18	0.19	-0.05	0.215
	Egghütten	21	30	7	1	2.58	0.29	0.29	0.04	0.157
	Fontanne	20	27	6	0	2.33	0.28	0.31	-0.10	0.194
	Hilferenmättili	24	28	7	0	2.42	0.31	0.35	-0.08	0.155
	Hinter Rohren	25	27	6	0	2.33	0.22	0.22	0.05	0.271
	Hinter Rüchi	24	29	6	0	2.50	0.25	0.26	-0.02	0.201
	Räschenhus	22	22	5	0	1.92	0.25	0.30	-0.14	0.325
	Ribihütte	32	33	6	1	2.83	0.31	0.32	0.00	0.155
	Rossei	24	25	6	0	2.17	0.23	0.24	-0.04	0.186
	mean LU		26.8	6.4	0.2	2.31	0.28	0.29	-0.02	0.206
SG	Altstätten	26	27	7	1	2.33	0.33	0.36	-0.08	0.218
	Buechholz	25	22	5	0	1.92	0.19	0.19	0.00	0.316
	Goldach	21	26	7	0	2.25	0.36	0.38	-0.05	0.202
	Lochmüli	17	27	7	0	2.33	0.30	0.32	-0.04	0.203
	Ochsenweid	21	33	7	2	2.83	0.34	0.35	0.01	0.163
	Sittertobel	28	20	5	0	1.75	0.18	0.21	-0.13	0.299
	Thal	7	19	6	0	1.67	0.25	0.27	-0.02	0.264
	Wolfgangweiher	20	32	6	1	2.75	0.28	0.30	-0.05	0.187
	mean SG		25.6	6.3	0.5	2.23	0.28	0.30	-0.05	0.231

[1] mean pairwise F_{ST} to all other populations within the same region

Table 1.6: Model selection results. ΔAICc values of models for allelic richness (AR), expected heterozygosity (H_e), inbreeding coefficient (F_{IS}), and mean population F_{ST}.

Model	df	ΔAICc AR	He	Fis	Fst
intercept	2	8.75	0.00	1.46	2.03
region	5	0.99	2.02	0.00	4.34
decline	3	8.87	2.28	2.77	3.03
peripher	3	8.87	2.28	2.77	3.03
region + elevation	6	0.00	4.37	2.21	6.20
region + stream	6	3.25	4.10	2.65	6.46
region + isolation	6	3.07	7.92	5.00	10.43
elevation	3	10.66	1.77	3.68	0.00
stream	3	9.65	1.95	3.14	2.36
isolation	3	10.13	5.43	5.50	7.15

Table 1.5: Analysis of molecular variance (AMOVA) of 11 microsatellite loci among the study regions (BE, BL, LU, and SG), among subpopulations within regions, and within subpopulations.

source of variation	degrees of freedom	sums of squares	% of variation
among regions	4	1121.847	18.5199
among subpopulations within	43	1320.666	19.47277
within subpopulations	1326	4407.606	62.00733

Table 1.7: Parameter estimates [mean (95% CI)] for models within 2 ΔAICc units of the best model for the effects of region, decline, peripheral location, elevation, location near a stream, and geographic isolation.

	model	ΔAICc	intercept	region BE	BL	LU	SG	elevation	stream
AR	region+elevation	0.00	-	2.024 (1.983 – 2.065)	2.868 (2.829 – 2.906)	2.138 (2.093 – 2.183)	2.314 (2.267 – 2.362)	0.164 (0.138 – 0.191)	-
	region	0.99	-	2.106 (2.067 – 2.146)	2.749 (2.715 – 2.783)	2.280 (2.240 – 2.320)	2.229 (2.182 – 2.275)	-	-
H_e	intercept	0.00	0.277 (0.275 – 0.280)	-	-	-	-	-	-
	elevation	1.77	0.277 (0.275 – 0.280)	-	-	-	-	-0.006 (-0.009 – -0.004)	-
	stream	1.95	0.275 (0.272 – 0.278)	-	-	-	-	-	0.013 (0.006 – 0.020)
F_{IS}	region	0.00	-	-0.122 (-0.131 – -0.113)	-0.017 (-0.024 – -0.009)	-0.020 (-0.029 – -0.011)	-0.045 (-0.055 – -0.035)	-	-
	intercept	1.46	-0.048 (-0.053 – -0.044)	-	-	-	-	-	-
F_{ST}	elevation	0.00	-0.048 (-0.053 – -0.044)	-	-	-	-	-0.004 (-0.009 – 0.000)	-

Population structure

The global F_{ST} of the four regions ranged from 0.209 to 0.261 (BE: 0.239, BL: 0.261, LU: 0.209, SG 0.232). The global F_{ST} of all regions was 0.352. Pairwise F_{ST} values among subpopulations within regions ranged from 0.035 to 0.534; all of them were significant. Isolation by distance was highly significant across all regions (p<0.001), although variance was high (Figure 1.2). Isolation by distance within regions was significant for BE (p=0.017), BL

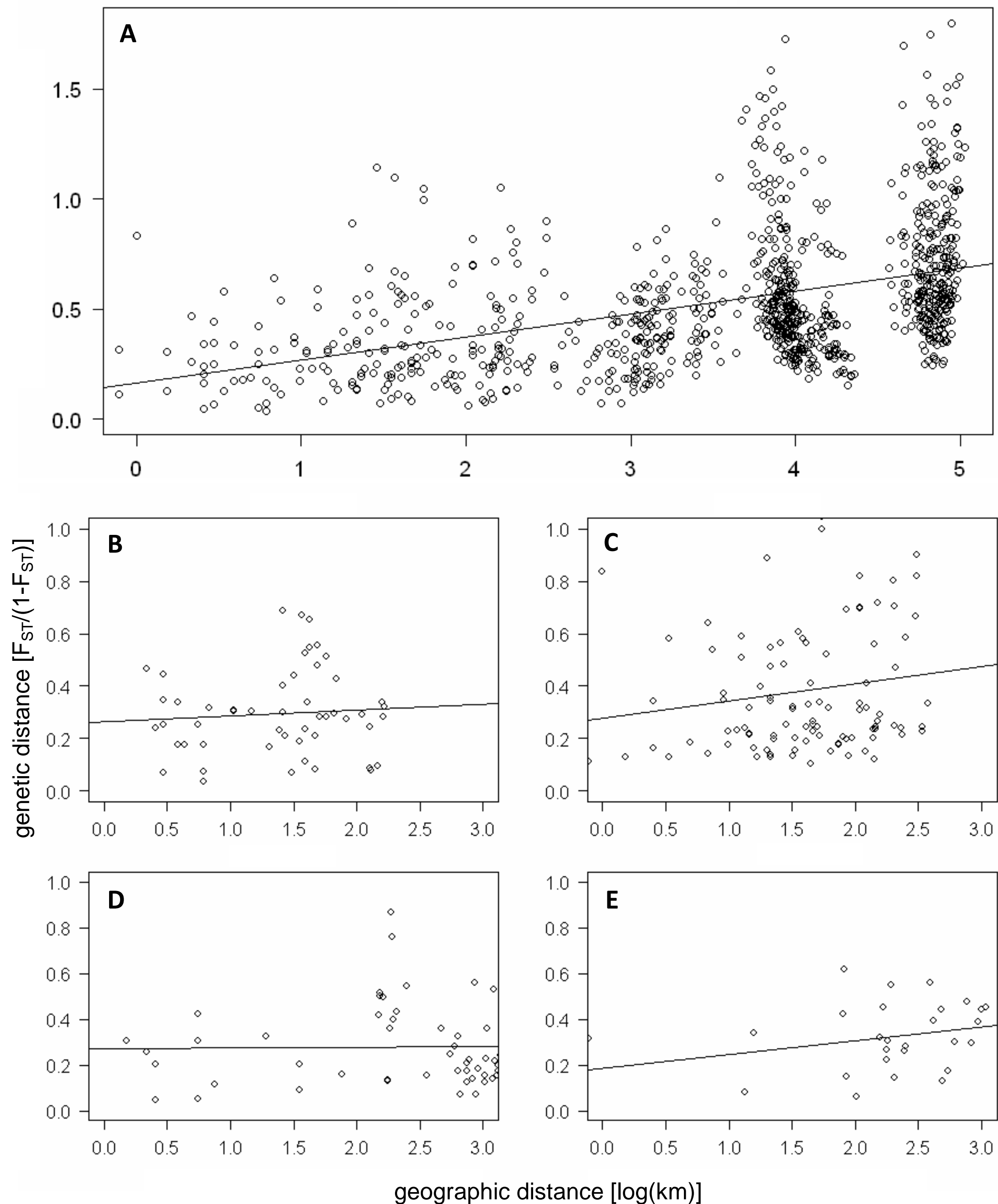

Figure **1.2**: pairwise F_{ST} values plotted against pairwise distances between populations (isolation by distance [IBD]). A: IBD across all regions. B: IBD within region BE. C: IBD within region BL. D: IBD within region LU. E: IBD within region SG

(p=0.048), LU (p<0.001) but not SG (p=0.109). The AMOVA revealed that most genetic diversity was found within populations with a considerable amount of variation found among regions (Table 1.5)

Model selection results

Different models were the most parsimonious explanations for the different measures of genetic structure. Allelic richness was best explained by differences among regions and in elevation (Table 1.6) with subpopulations at higher elevation having higher allelic richness (Table 1.7, Figure 1.3). H_e was best explained by the null model, but several other models were within ~2 ΔAICc values (Table 1.6). However, the confidence interval of the estimates of the explanatory variables in these models included zero. Thus, these factors have little explanatory power at best (Table 1.7). For F_{IS}, differences among regions explained most of the variation observed, although the intercept model also ranked among the models with less than 2 ΔAICc units from the best model (Table 1.6, Figure 1.4). F_{ST} was best explained by elevation (Table 1.6), but the confidence interval for the elevation effect included zero (Table 1.7). Elevation had thus no explanatory power for F_{ST}. The intercept only model was the second-best model and seems more appropriate for inference.

Discussion

We expected to find differences in the population genetic structure and diversity among subpopulations and regions. Yet, even though the four regions differed in a number of factors, there was very little variation in genetic structure and diversity that could be attributed to the factors that we studied. In particular, we found no difference in genetic structure between regions with and without population declines. Variation in expected heterozygosity, inbreeding coefficient F_{IS} and subpopulation differentiation F_{ST} were best explained by differences among regions or could not be accounted for by any of the factors used in this study. However, we found an increase in allelic richness at higher elevation.

Strong genetic differentiation in all regions

Subpopulations in regions undergoing strong declines had similar levels of genetic diversity as subpopulations in regions without declines. In addition, there was strong genetic

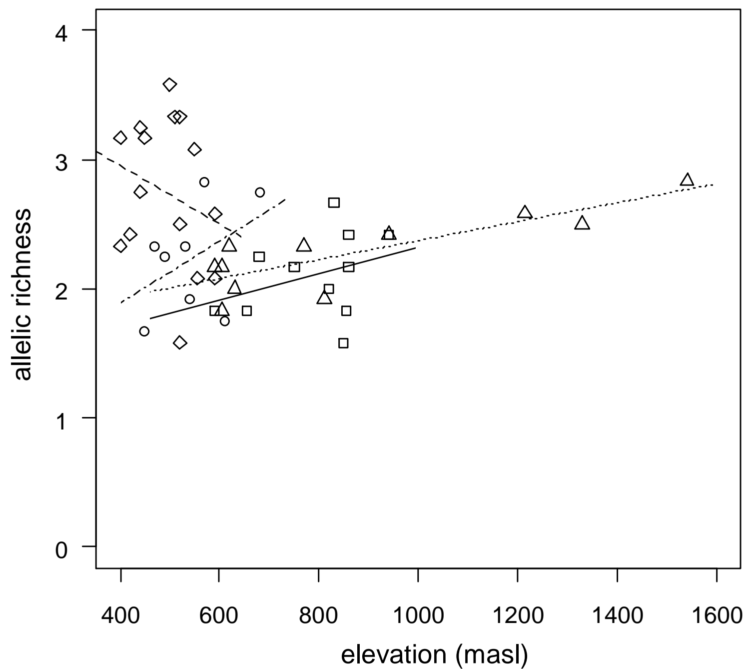

Figure 1.3: Allelic richness varies among regions and increases with altitude. Squares and solid line: region BE, diamonds and dashed line: region BL, triangles and dotted line: region LU, circles and dash-dotted line: region SG

differentiation among subpopulations even across small geographical scales. Although genetic differentiation increased with Euclidian distance (Figure 1.2), distances of less than 500m resulted in significant F_{ST}-values. Similarly high levels of population differentiation and allelic fixation have been observed in other species of midwife toads (Gonçalves *et al.* 2009, Kraaijeveld-Smit *et al.* 2005).

The very high F_{ST}-values and allelic fixation rates observed suggest very low rates of gene flow and are suggestive of very poor dispersal abilities. Moreover, high F_{ST}-values indicate that every breeding assemblage acts indeed as a relatively independent unit (Beebee 2005). Gene flow may be reduced in human-dominated landscapes or in marginal habitats and it may be enhanced in the presence of natural dispersal corridors. However, in our study neither geographic distance among subpopulations nor location at the periphery of the range or elevation had an influence on population differentiation. Also, proximity to streams that facilitates gene flow in some stream-dwelling species (Grant *et al.* 2010, Morrissey & de Kerckhove 2009, Mullen *et al.* 2010) did not affect population differentiation. We could not detect effects of streams on gene flow within catchments in *Alytes*. Thus, gene flow was not higher along natural migration routes than in human-dominated landscapes.

Amphibian species differ strongly in their dispersal abilities (Beebee 2005, Semlitsch 2008). All the above evidence indicates that *Alytes* has very low dispersal abilities and therefore subpopulations are isolated (Kraaijeveld-Smit *et al.* 2005). We argue that this is a result of the species' biology rather than landscape processes. Natural population structures are a continuum reaching from continuous populations to metapopulations to isolated populations (Frankham *et al.* 2002). In isolated populations, breeding sites act as independent units and demographic and genetic processes in every population are uncoupled from the neighbouring populations (Frankham *et al.* 2002). Consequently, extirpation of a neighbouring population would not affect immigration rates, as it does not contribute to decoupling populations further. In such a system, the loss of local populations associated with species declines may not lead to increased genetic differentiation of populations. Accordingly, we argue that geographic connectivity is relatively unimportant in shaping genetic structure in this species even though it is often found to be an important determinant of pond occupancy in other amphibian species (Zanini *et al.* 2009). Further evidence for the strong isolation of subpopulations comes from the fact that an equal amount of genetic variation is found among subpopulations within regions as among regions (Table 1.5); the small geographic distances separating subpopulations are thus as efficient in preventing migration as the large geographic distances among regions. Hence, *Alytes*

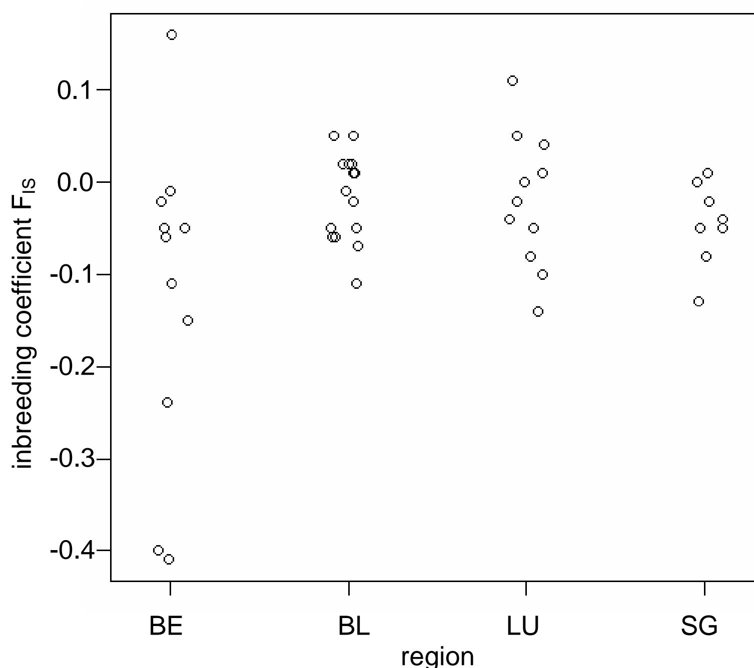

Figure 1.4: Scatterplot of the F_{IS}-values in the different regions. Each dot represents the F_{IS}-value of a subpopulation clustered by region.

subpopulations function as relatively independent units and demographic stochasticity may be more important in shaping the population genetic structure than geographic isolation.

Increased genetic diversity at high elevation

Low-elevation and high-elevation subpopulations differed in allelic richness. Because suitable habitat patches are often scarcer both at range edges and at high elevations, high elevation subpopulations may experience conditions similar to range edges and similar population genetic processes may occur (Giordano *et al.* 2007, Segelbacher & Storch 2002). Thus, the usual expectation is that genetic variation is reduced in high altitude or edge populations. We found the opposite pattern: allelic richness was higher in subpopulations at higher elevation. In all regions but BL, genetic diversity increased with increasing altitude (Figure 1.3). BL is the region with highest genetic diversity in our study, but has no considerable elevational gradient, which may explain why we did not find a positive correlation of genetic diversity with elevation in this region. For the other three regions that show an elevational gradient, genetic diversity increased linearly with elevation. Recently, Munwes *et al.* (2010) showed that another amphibian species had higher levels of genetic diversity at the range edge. When explaining the pattern, Munwes *et al*. (2010) referred to Fisher (1930) who postulated that genetic diversity may be higher in edge populations because the populations experience varying environmental conditions. At high elevation, environmental conditions fluctuate strongly because there is strong spatial and temporal variation in temperature, humidity or UV-radiation (Körner 2007, Scherrer & Körner 2011). Such strong fluctuation in environmental conditions may select for increased genetic variability to allow individuals to cope with extremes encountered over short temporal time spans. Although we can only speculate about the mechanism behind the observation, it is unlikely that the pattern has been caused by heterozygote advantage. First, despite an excess of heterozygotes in many subpopulations (negative F_{IS}), this was not linked to elevation. Second, all subpopulations were in Hardy-Weinberg equilibrium and heterozygote proportions did not significantly deviate from proportions expected under neutrality. It hence seems more likely that fluctuating environmental conditions lead to balancing selection. In contrast, constant conditions are predictable and may lead to selection that is more directional towards specific traits. We can exclude that the increased genetic diversity at higher elevation arises from higher migration rates because neither elevation nor

population isolation did affect population differentiation. Our result may constitute further support for Fisher's (1930) hypothesis of fluctuating selection in edge populations.

Conservation implications

Matocq & Villablanca (2001) pointed out the importance of suitable reference groups when interpreting population genetic data. Had we analysed only the population genetic structure from regions where *Alytes obstetricans* is declining, we would have concluded that the declines are associated with low levels of genetic diversity and strong population differentiation. With reference populations in regions where the species is not declining, we find no evidence that decline and genetic structure are associated. Based on the analysis of a single region, we might have recommended translocations to enhance genetic diversity. With our comparative approach, it is clear that translocation as a conservation strategy would be unlikely to halt population declines.

Yet, the strong isolation of subpopulations is of concern because demographic stochasticity can be an important threat to population survival (Lande 1993). If there is a choice between conservation actions that decrease local subpopulation extinction probability and conservation actions that aim at increasing connectivity among subpopulations, then we would recommend the former. Given our analyses of the genetic structure of *Alytes* populations, this would probably be the best conservation strategy.

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Lucerne, and the Amt für Raumentwicklung of the canton St. Gallen. We thank S. Angelone for comments on an earlier version of this manuscript and P. Hedrick, P. Wandeler and J. Wang for helpful discussions.

References

Armour, J. A. L., Neumann, R., Gobert, S. and Jeffreys, A. J. (1994). Isolation of Human Simple Repeat Loci by Hybridization Selection. *Human Molecular Genetics* 3(4): 599-605.

Barandun, J. (2004). Neue Chance für die Geburtshelferkröte in der Region St. Gallen - Appenzell. unpublished action plan - final report. St. Gallen, Ökonzept GmbH. 15 pp.

Beaumont, M. A. and Nichols, R. A. (1996). Evaluating loci for use in the genetic analysis of population structure. *Proceedings of the Royal Society Biological Sciences Series B* 263(1377): 1619-1626.

Beebee, T. J. C. (2005). Conservation genetics of amphibians. *Heredity* 95(6): 423-427.

Belkhir, K., Borsa, P., Chikhi, L., Raufaste, N. and Bonhomme, F.-G., logiciel sous Windows TM pour la génétique des populations. Laboratoire Génome, Populations, Interactions, CNRS UMR 5000, Université de Montpellier II, Montpellier (France). (1996-2004). GENETIX 4.05, logiciel sous Windows TM pour la génétique des populations. *Laboratoire Génome, Populations, Interactions, CNRS UMR 5000, Université de Montpellier II, Montpellier (France).* .

Blomqvist, D., Pauliny, A., Larsson, M. and Flodin, L.-Å. (2010). Trapped in the extinction vortex? Strong genetic effects in a declining vertebrate population. *BMC Evolutionary Biology* 10: 33.

Booy, G., Hendriks, R. J. J., Smulders, M. J. M., Van Groenendael, J. M. and Vosman, B. (2000). Genetic diversity and the survival of populations. *Plant Biology* 2(4): 379-395.

Borgula, A. and Zumbach, S. (2003). Verbreitung und Gefährdung der Geburtshelferkröte (*Alytes obstetricans*) in der Schweiz. *Zeitschrift für Feldherpetologie* 10: 11-26.

Burnham, K. P., Anderson, C. W. and Huyvaert, K. P. (2010). AIC model selection and multimodel inference in behavioral ecology: some background, observations, and comparisons. *Behavioural Ecology and Sociobiology* 65(1): 23-35.

Burnham, K. P. and Anderson, D. R. (2002). Model selection and multimodel inference: a practical information-theoretic approach. New York, Springer-Verlag.

Cushman, S. A. (2006). Effects of habitat loss and fragmentation on amphibians: A review and prospectus. *Biological Conservation* 128(2): 231-240.

Eckert, C. G., Samis, K. E. and Lougheed, S. C. (2008). Genetic variation across species' geographical ranges: the central-marginal hypothesis and beyond. *Molecular Ecology* 17(5): 1170-1188.

Evans, S. R. and Sheldon, B. C. (2008). Interspecific patterns of genetic diversity in birds: Correlations with extinction risk. *Conservation Biology* 22(4): 1016-1025.

Excoffier, L., Laval, G. and Schneider, S. (2005). Arlequin (version 3.0): An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics* 1: 47-50.

Fisher, R. A. (1930). The genetical theory of natural selection. Oxford, Clarendon Press.

Frankham, R. (1995). Conservation genetics. *Annual Review of Genetics* 29: 305-327.

Frankham, R. (2005). Genetics and extinction. *Biological Conservation* 126(2): 131-140.

Frankham, R., Ballou, J. D. and Briscoe, D. A. (2002). Introduction to Conservation Genetics. Cambridge, Cambridge University Press.

Gautschi, B., Tenzer, I., Muller, J. P. and Schmid, B. (2000). Isolation and characterization of microsatellite loci in the bearded vulture (*Gypaetus barbatus*) and cross-amplification in three Old World vulture species. *Molecular Ecology* 9(12): 2193-2195.
Giordano, A. R., Ridenhour, B. J. and Storfer, A. (2007). The influence of altitude and topography on genetic structure in the long-toed salamander (*Ambystoma macrodactylum*). *Molecular Ecology* 16: 1625-1637.
Goldberg, C. S. and Waits, L. P. (2010). Quantification and reduction of bias from sampling larvae to infer population and landscape genetic structure. *Molecular Ecology Resources* 10: 304-313.
Gonçalves, H., Martínez-Solano, I., Pereira, R. J., Carvalho, B., García-París, M. and Ferrand, N. (2009). High levels of population subdivision in a morphologically conserved Mediterranean toad (*Alytes cisternasii*) result from recent, multiple refugia: evidence from mtDNA, microsatellites and nuclear genealogies. *Molecular Ecology* 18(24): 5143-5160.
Goudet, J. (2001). FSTAT, a program to estimate and test gene diversities and fixation indices version 2.9.3. . *Available from* http://www.unil.ch/izea/softwares/fstat.html.
Grant, E. H. C., Nichols, J. D., Lowe, W. H. and Fagan, W. F. (2010). Use of multiple dispersal pathways facilitates amphibian persistence in stream networks. *Proceedings of the National Academy of Sciences of the United States of America* 107(15): 6936-6940.
Grossenbacher, K. (1988). Verbreitungsatlas der Amphibien der Schweiz. *Documenta faunistica helvetiae* 7: 1-207.
Houlahan, J. E., Findlay, C. S., Schmidt, B. R., Meyer, A. H. and Kuzmin, S. L. (2000). Quantitative evidence for global amphibian population declines. *Nature* 404(6779): 752-755.
Johnson, W. E., Onorato, D. P., Roelke, M. E., Land, E. D., Cunningham, M., Belden, R. C., McBride, R., Jansen, D., Lotz, M., Shindle, D., Howard, J., Wildt, D. E., Penfold, L. M., Hostetler, J. A., Oli, M. K. and O'Brien, S. J. (2010). Genetic restoration of the Florida Panther. *Science* 329(5999): 1641-1645.
Körner, C. (2007). The use of 'altitude' in ecological research. *Trends in Ecology & Evolution* 22(11): 569-574.
Kraaijeveld-Smit, F. J. L., Beebee, T. J. C., Griffiths, R. A., Moore, R. D. and Schley, L. (2005). Low gene flow but high genetic diversity in the threatened Mallorcan midwife toad *Alytes muletensis*. *Molecular Ecology* 14(11): 3307-3315.
Lande, R. (1988). Genetics and demography in biological conservation. *Science* 241(4872): 1455-1460.
Lande, R. (1993). Risks of population extinction from demographic and environmental stochasticity and random catastrophes. *American Naturalist* 142(6): 911-927.
Lande, R. (1998). Anthropogenic, ecological and genetic factors in extinction and conservation. *Researches on population ecology* 40(3): 259-269.
Matocq, M. D. and Villablanca, F. X. (2001). Low genetic diversity in an endangered species: recent or historic pattern? *Biological Conservation* 98(1): 61-68.
Morrissey, M. B. and de Kerckhove, D. T. (2009). The maintenance of genetic variation due to asymmetric gene flow in dendritic metapopulations. *American Naturalist* 174(6): 875-889.
Mullen, L. B., Woods, H. A., Schwartz, M. K., Sepulveda, A. J. and Lowe, W. H. (2010). Scale-dependent genetic structure of the Idaho giant salamander (*Dicamptodon aterrimus*) in stream networks. *Molecular Ecology* 19(5): 898-909.

Munwes, I., Geffen, E., Roll, U., Friedmann, A., Daya, A., Tikochinski, Y. and Gafny, S. (2010). The change in genetic diversity down the core-edge gradient in the eastern spadefoot toad (*Pelobates syriacus*). *Molecular Ecology* 19(13): 2675-2689.

R Development Core Team (2008). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.

Rasmussen, D. I. (1979). Clusters and Genotype Frequencies. *The American Naturalist* 113(6): 948-951.

Ryser, J., Lüscher, B., Neuenschwander, U. and Zumbach, S. (2003). Geburtshelferkröten im Emmental, Schweiz. *Zeitschrift für Feldherpetologie* 10(1): 27-35.

Savage, W. K., Fremier, A. K. and Shaffer, H. B. (2010). Landscape genetics of alpine Sierra Nevada salamanders reveal extreme population subdivision in space and time. *Molecular Ecology* 19(16): 3301–3314.

Scherrer, D. and Körner, C. (2011). Topographically controlled thermal-habitat differentiation buffers alpine plant diversity against climate warming. *Journal of Biogeography* 38(2): 406-416.

Schmidt, B. R., Tobler, U. and Ramseier, P. (2010). Die Verbreitung der Geburtshelferkröte (*Alytes obstetricans*) im Kanton Basel-Landschaft (NW-Schweiz). *Mitteilungen der Naturforschenden Gesellschaften beider Basel* 12: 75-84.

Schmidt, B. R. and Zumbach, S. (2005). Rote Liste der gefährdeten Amphibien der Schweiz. Bundesamt für Umwelt, Wald und Landschaft, Bern, und Koordinationsstelle Amphibien- und Reptilienschutz in der Schweiz, Bern.

Segelbacher, G. and Storch, I. (2002). Capercaillie in the Alps: genetic evidence of metapopulation structure and population decline. *Molecular Ecology* 11(9): 1669-1677.

Semlitsch, R. D. (2008). Differentiating migration and dispersal processes for pond-breeding amphibians. *Journal of Wildlife Management* 72(1): 260-267.

Spielman, D., Brook, B. W. and Frankham, R. (2004). Most species are not driven to extinction before genetic factors impact them. *Proceedings of the National Academy of Sciences of the United States of America* 101(42): 15261-15264.

Stuart, S. N., Chanson, J. S., Cox, N. A., Young, B. E., Rodrigues, A. S. L., Fischman, D. L. and Waller, R. W. (2004). Status and trends of amphibian declines and extinctions worldwide. *Science* 306(5702): 1783-1786.

Swisstopo Landeskarte der Schweiz 1:25000 (2003 - 2009). Bundesamt für Landestopographie. 3084 Wabern.

Van Oosterhout, C., Hutchinson, W. F., Wills, D. P. M. and Shipley, P. (2004). micro-checker: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes* 4(3): 535 - 538.

Wang, J. L. (2004). Sibship reconstruction from genetic data with typing errors. *Genetics* 166(4): 1963-1979.

Weir, B. S. and Cockerham, C. C. (1984). Estimating F-Statistics for the Analysis of Population-Structure. *Evolution* 38(6): 1358-1370.

Westemeier, R. L., Brawn, J. D., Simpson, S. A., Esker, T. L., Jansen, R. W., Walk, J. W., Kershner, E. L., Bouzat, J. L. and Paige, K. N. (1998). Tracking the long-term decline and recovery of an isolated population. *Science* 282(5394): 1695-1698.

Wright, S. (1965). The interpretation of population-structure by F-statistics with special regard to systems of mating. *Evolution* 19(3): 395-420.

Zanini, F., Pellet, J. and Schmidt, B. R. (2009). The transferability of distribution models across regions: an amphibian case study. *Diversity and Distributions* 15(3): 469-480.

WITHIN- AND AMONG- POPULATION VARIATION IN CHYTRIDIOMYCOSIS-INDUCED MORTALITY IN THE TOAD *ALYTES OBSTETRICANS*

(PLoS ONE 5(6): e10927)

Ursina Tobler, Benedikt R. Schmidt

Abstract

Chytridiomycosis is a fungal disease linked to local and global extinctions of amphibians. Susceptibility to chytridiomycosis varies greatly between amphibian species, but little is known about between- and within-population variability. However, this kind of variability is the basis of resistance evolution to disease. In a common garden experiment, we measured chytridiomycosis-induced mortality after metamorphosis of *Alytes obstetricans* naturally infected with *Batrachochytrium dendrobatidis*. Mortality rates differed significantly among populations and ranged from 27 to 90%. Within populations, mortality strongly depended on mass at and time trough metamorphosis. Although we cannot rule out that the differences observed resulted from differences in skin microbiota, different pathogen strains or environmental effects experienced by the host or the pathogen prior to the start of the experiment, we argue that genetic differences between populations are a likely source of this variation. To our knowledge, this is the first study showing differences in survival between and within populations under constant laboratory conditions. Our results suggest that the potential for the evolution of resistance, which might allow population persistence, exists.

Key words: *Alytes obstetricans*, *Batrachochytrium dendrobatidis*, between and within population variation, body size, chytridiomycosis, disease resistance, metamorphosis, mortality

Introduction

Emerging infectious diseases can represent an important threat to biodiversity and wildlife (Daszak *et al.* 2000). Although extinction by disease is a rare event and unlikely in most systems, certain conditions can increase the risk of local or even global extinction by disease: a sympatric host that acts as a reservoir for the pathogen, disease transmission that is frequency-dependent, or disease that drives local populations to such low densities that stochastic factors become important (de Castro & Bolker 2005).

Chytridiomycosis, a disease caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd* hereafter), is a disease of amphibians that may cause extinctions because it fulfils all three conditions: It infects a variety of hosts with different susceptibilities, some of them acting as reservoirs (Fisher *et al.* 2009b) , transmission is frequency-dependent at low densities (Rachowicz & Briggs 2007), and amphibians often occur in small populations that are prone to stochastic effects (Green 2003). Accordingly, chytridiomycosis has been linked to local and global extinctions of amphibians in Central America, Australia and Europe since its discovery in 1998 (Skerratt *et al*. 2007).

Susceptibility to *Bd* and chytridiomycosis varies greatly among amphibian species (Kilpatrick *et al.* 2010) and much research has been devoted to the identification of the causes of this variability (e.g. Brucker *et al.* 2008, Rowley & Alford 2007, e.g. Woodhams *et al.* 2006). This line of research helps to identify which species are at risk from *Bd* (Bielby *et al*. 2008). While some species seem to have disappeared completely following *Bd* invasion (Lips *et al*. 2006), populations of other species have persisted (Briggs *et al.* 2005, Murray *et al.* 2009, Retallick *et al.* 2004), suggesting that there is variation in susceptibility to disease within species. Here we focus on within-species variation in susceptibility to chytridiomycosis because this kind of variability is the raw material for the possible evolution of resistance to *Bd*, and hence population persistence (Bell & Collins 2008, Miller & Vincent 2008, Spielman *et al.* 2004a). As no methods are available yet to treat amphibian populations in the field against *Bd*, susceptible species may persist only where conditions are not favourable for *Bd* or for disease outbreaks (Bosch *et al.* 2007, Walker *et al.* 2010), or when they can evolve an evolutionary response to the threat imposed by the emergence of chytridiomycosis. Nevertheless, only few studies have addressed within-species variation in susceptibility to this disease and none has looked at the genetic basis of this variation (Kilpatrick *et al.* 2010).

One of the species that are known to be highly susceptible to chytridiomycosis is the common midwife toad, *Alytes obstetricans*. The species is widespread across Europe and has suffered substantial declines throughout its range (Bosch *et al*. 2008). It was the first species to decline in Europe as a result of mass die-offs after the emergence of *Bd* (Bosch *et al.* 2001). Mortality in Spain almost exclusively affected recently metamorphosed individuals of this species, as the adults are strongly terrestrial and have a low risk of infection. Chytridiomycosis-induced mortality is strongly associated with cold and moist conditions at the time of metamorphosis (Bosch *et al.* 2007, Walker *et al.* 2010). Midwife toads have also suffered strong declines in Switzerland, where more than 50% of the populations went extinct over the last quarter century (Schmidt & Zumbach 2005). *Bd* is present and widespread in Switzerland (Garner *et al*. 2005). Yet, in the field, no mass mortality has been observed. Only four metamorphs whose death was caused by *Bd* have been found so far (B. R. Schmidt & U. Tobler, unpublished data).

The aim of our study was to quantify the impact of *Bd* and variation thereof on midwife toad populations by measuring post-metamorphic survival of infected individuals in a common garden laboratory experiment. We demonstrate that *Bd* causes mortality under laboratory conditions and that mortality varies greatly among populations. We also identify correlates of mortality among individuals within populations.

Materials and Methods

Study sites

We collected one-year old tadpoles of *Alytes obstetricans* shortly after hibernation in 2008 from three sites in Switzerland, two of which were located in canton Baselland and one in canton St. Gallen. Standard hygiene protocols were followed during field work to avoid the spread of *Bd* and other pathogens (Schmidt *et al.* 2009a). The sites were chosen because 1) we knew they sustained large tadpole numbers and 2) they were positive for *Bd* (Schmidt *et al.* 2009a). The first site in Baselland (7.783306°E, 47.459667°N, 410 masl, hereafter referred to as “BLI”) is located in a former quarry and consists of three main water bodies, in only two of which tadpoles were caught. The larger pond is about 16 m^2 in size, 0.8 m deep and is densely vegetated; the smaller pond is 8 m^2 in size, 0.5 m in depth, and has very little vegetation but has reed along the edges. The second site in Baselland (7.801185°E, 47.436677°N, 480 masl, hereafter referred to as “BLZ”) is a forest pond approximately 300

m^2 in size and 2.5 m in depth. The site in St. Gallen (9.533416°E, 47.381742°N, 540 masl, hereafter referred to as "SGA") is a garden at the south-eastern distribution border of the species. The population is larger than 20 calling males, and three small garden ponds (2 – 5 m^2 in size, 0.2 -0.5 m in depth) serve as breeding sites.

Laboratory experiment: treatments

Bd only infects keratinised skin of amphibians (Longcore *et al.* 1999) and infection in tadpoles is restricted to the mouthparts, which does not cause disease. However, during metamorphosis, the skin becomes keratinised, and the pathogen can then spread over the whole body and cause hyperkeratosis and osmotic imbalance, leading to death (Berger *et al.* 1998, Voyles *et al.* 2009). Recently metamorphosed individuals are most susceptible to disease, supposedly due to a downregulation of immune defences during metamorphosis (Rollins-Smith 1998). Thus, we measured differences in post-metamorphosis survival of *Bd*-positive and -negative *Alytes* tadpoles from the three populations. Because all tadpoles were tested for *Bd* infection with rt-PCR (Boyle *et al*. 2004) and confirmed positive initially, we assigned them to three different treatments in order to obtain one *Bd*-negative control: 1) The "Itraconazole treatment" group served as a *Bd*-free control. Individuals were treated with Itraconazole (1mg/L; Sporanox, Janssen-Cilag) for 5 minutes per day over the course of seven days to clear the infection. We followed the protocol developed by Garner *et al*. (2009a) which had been successfully used for the treatment of *Alytes muletensis* tadpoles against *Bd*; unlike Garner *et al*. (2009a), we observed no depigmentation. The tadpoles were returned to the same container after treatment, which had been rinsed with boiling water during the Itraconazole bath. 2) Because the tadpoles were exposed to low water levels and frequent capture during the Itraconazole treatment, we designed the "sham treatment" as a control for stress during handling. The tadpoles were treated in exactly the same way as the Itraconazole treatment, but instead of an Itraconazole solution, tap water was used. 3) The "no handling" group was not handled apart from the regular water change and feeding and was left infected throughout the experiment.

To test for differences in survival among populations, we assigned equal numbers of tadpoles from the three populations to the three treatments. We balanced body mass among treatments within populations.

Laboratory experiment: procedures

We caught 143 tadpoles (50 from BLI, 43 from BLZ and 50 from SGA) in April 2008 and brought them to the laboratory, where they were placed in individual one-litre plastic containers filled with tap water and a single dried beech tree (*Fagus spp*.) leaf. The laboratory was equipped with full spectrum sunlight lamps, and we used a daylength of 16 hours and kept the room at 19 - 21°C. Throughout the course of the experiment, we changed the water in the containers twice a week and added food containing *Spirulina* algae (Tetra Pleco Wafer, Tetra Germany, Spectrum Brands Inc.) ad libitum three times a week. In the week following capture, the tadpoles were weighed to the nearest 0.01 g (Scaletec Instruments, Heiligenstadt, Germany), measured from the snout to the beginning of the tail muscle to the nearest 0.1 mm, and swabbed over the mouth parts with a sterile cotton swab (Copan Italia S.p.A., Brescia, Italy). Further swabs were taken when metamorphosis was finished (Gosner stage 46, Gosner 1960) and (3) upon death or 30 days after stage 42. Size measurements was repeated three times during the experiment: (1) when the larvae entered metamorphosis (Gosner stage 42), (2) when metamorphosis was finished (Gosner stage 46) and (3) upon death or 30 days after stage 42. We used separate pairs of gloves for handling and separate plastic beakers for weighing each tadpole. When the tadpoles entered metamorphosis (Gosner stage 42), the containers were drained of most of the water and tilted, so that both land and water were available to the toadlets. We put a moist paper towel at the bottom of each container. When the tail was fully resorbed (Gosner stage 46), we fed the toadlets crickets of adequate size *ad libitum* three times a week. For each individual, the experiment was ended 30 days after stage 42 or upon death.

rt-PCR

We analysed the swabs for the presence of *Bd* with *Bd*-specific primers in rt-PCR following the protocol by Boyle *et al*. (2004) with slight modifications: samples were run in duplicates and when the two PCR-wells retuned inconsistent results, the analysis was repeated. Reactions yielding 0.1 genomic equivalents or above were considered *Bd*-positive.

Statistical analyses

We tested for differences in body length among the different populations at the beginning of the experiment, at stage 42 and at stage 46 using ANOVA and we tested for

correlations between body mass and length at stage 42, 46 and the end of the experiment. Because mass and length were strongly correlated, we only used body mass for subsequent analyses.

To analyse survival data we used the Cox proportional hazard model. This model assumes an underlying hazard function describing how hazard changes over time, and fits effect parameters using Cox's likelihood. Individual survival times are censored, which means that individuals may die after the end of the study period. We tested for overall differences in mortality between treatments and populations using daily counts on the number of survivors until 30 days after beginning of metamorphosis (Gosner stage 42). To test for the effects of individual condition and environmental effects on hazard risk, we did a Cox proportional hazard model with the two infected treatments only. The variables on individual condition included were body mass at the beginning of the experiment, at stage 42, 46 and at the end or death, and time through metamorphosis; the variables for environmental effects were zoospore loads at the beginning, at stage 46, at the end and time to metamorphosis. We also included interactions between zoospore loads and population.

Because individuals that survived until the end of the experiment had more time to gain mass, we tested the correlation between body mass and time since metamorphosis with a Pearson correlation test. All tests were performed in R, version 2.8.1 (R Development Core Team 2008).

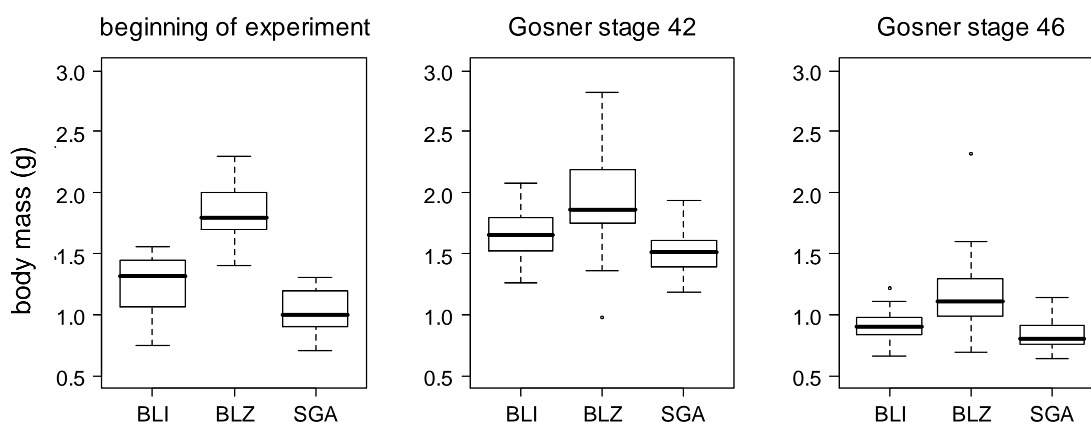

Figure 2.1: Boxplots showing body mass of the different populations at the beginning of the experiment, at Gosner stage 42 and at stage 46.

Results

Populations differed significantly in body mass at the beginning the experiment (ANOVA for mass beginning stage 42: $p<0.001$) but not at metamorphosis (stage 46: $p=0.144$; Figure 2.1). Body length and mass were strongly correlated at all stages (Pearson correlation for beginning, stage 42, stage 46 and end of experiment; $r_{beginning}=0.83$, $r_{42}=0.76$, r46=0.30, $r_{end}=0.90$; all $p<0.001$).

All 143 overwintered tadpoles were infected with *Bd* at the time of capture. When they reached stage 46, all Itraconazole-treated tadpoles were *Bd*-negative while all untreated or sham treated tadpoles were still infected. Eight tadpoles died during the course of the experiment before undergoing metamorphosis: Two tadpoles from BLI died before treatment assignment within a week after capture, and another tadpole from BLI died in the no handling group. Two tadpoles from BLZ died in the Itraconazole group, and from SGA one tadpole died in the Itraconazole treatment and two from the sham treatment, the last five all within 1.5 weeks after treatment. Because chytridiomycosis usually does not cause mortality during the larval stage and most deaths were probably caused by transportation or handling stress, these 8 tadpoles were excluded from the analyses. The remaining 135 tadpoles reached Gosner stage 42 (beginning of metamorphosis) 25 to 129 days after 25 April, when the first swab was collected (and 32-136 days after capture). From stage 42 on, it took them seven to 18 days (mean ± SD: 12 ± 2) to fully resorb the tail.

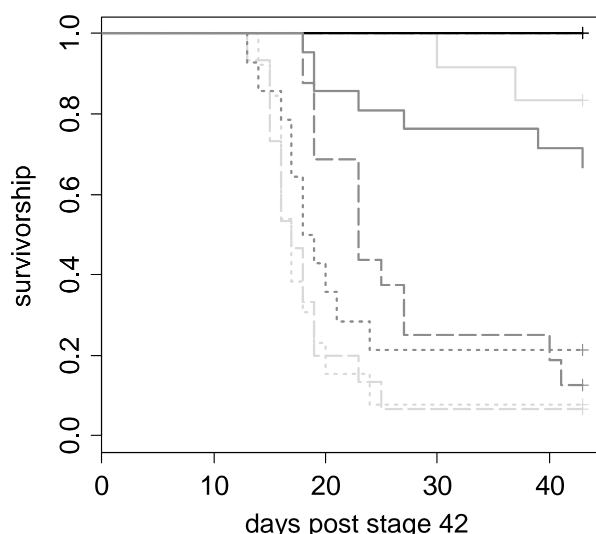

Figure 2.2: Cox regression on survival depending on treatment and population. The black line represents the Itraconazole treatment for all populations. Light grey = sham treatment, dark grey = no handling. Continuous line = BLI, dashed line = BLZ, dotted line = SGA.

Table 2.1: Mean values and Cox proportional hazard test results analysing the impact of body mass at the beginning, at Gosner stages 42, 46 and at the end of experiment, time to and trough metamorphosis and zoospore load (genomic equivalents GE) at the beginning, stage 46 and the end of the experiment.

	mean ± SD		test statistic				
	survivors	non-survivors	coef	exp(coef)	se(coef)	z	p
population	-	-	4.37	244.10	1.05	4.03	0.000
mass beginning	1.31 ± 0.33	1.37 ± 0.41	-5.18	0.01	1.83	-2.83	0.005
mass stage 42 (g)	1.72 ± 0.29	1.69 ± 0.31	2.71	15.00	1.33	2.04	0.041
mass stage 46 (g)	0.98 ± 0.30	0.96 ± 0.22	-1.62	0.20	1.50	-1.08	0.280
mass end (g)	1.36 ± 0.28	0.93 ± 0.29	-1.06	0.35	0.79	-1.35	0.180
time to stage 42 (days)	64.84 ± 31.59	52.85 ± 13.13	0.00	1.00	0.00	-0.04	0.970
time from stage 42 to 46 (days)	12.87 ± 2.81	12.28 ± 2.58	0.00	1.00	0.00	3.41	0.001
zoospore load beginning (GE)	661.20 ± 2081.79	677.79 ± 1008.66	0.00	1.00	0.00	2.49	0.013
zoospore load stage 46 (GE)	155.96 ± 376.63	8496.52 ± 23779.21	-0.04	0.96	0.02	-2.22	0.027
zoospore load end (GE)	28.19 ± 113.21	9700.93 ± 21726.15	0.00	1.00	0.07	-0.01	0.990
population*zoospore load stage 46	-	-	0.00	1.00	0.00	-3.36	0.001
population*zoospore load end	-	-	0.00	1.00	0.00	-2.44	0.016

Animals that died showed disease symptoms typical of chytridiomycosis shortly before death (NSW National Parks and Wildlife Service 2001). Diseased individuals stopped feeding approximately one day prior to death but did not show any other symptoms until less than a day before they died. Only within a few hours to death they would become lethargic and loose their righting reflex, at the same time they started to shed skin heavily when touched.

The Cox proportional hazard test revealed a significant effect of treatment ($p<0.001$) and population ($p<0.001$) on survival (Figure 2.2). Survival differed between infected and uninfected individuals, but not between the untreated and the sham treated group (no handling: 33.5 ± 29.1%, sham treatment: 32.6 ± 43.9%). Population BLI differed from the two other populations in survival among infected individuals (i.e. confidence intervals did not overlap; BLI: 75 ± 2.0% (SE), BLZ: 14.6 ± 1.9%, SGA: 9.6 ± 0.7%), but there was no difference in survival between SGA and BLZ. Because the sham and no handling group did not differ in survival, they were pooled for subsequent analyses.

In the infected treatment groups, heavier individuals survived better (Cox proportional hazard test, Figure 2.3, Table 2.1); body mass at the end of the experiment strongly correlated with life span since stage 46 (Pearson correlation, $p<0.001$). Survivors completed metamorphosis slightly slower than individuals that died later on (survivors: 12.9 ± 2.8 (SD) days, non-survivors: 12.3 ± 2.6 days, Table 2.1). Although infection load at any stage did not affect individual hazard risk (Cox proportional hazard test, Table 2.1), non-

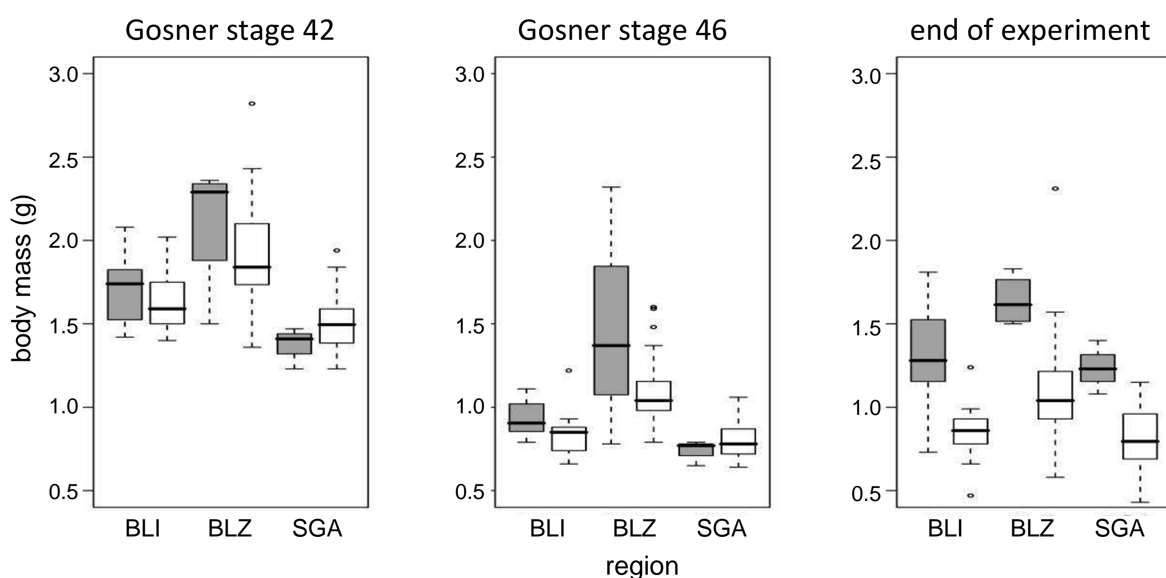

Figure 2.3: Boxplots showing body mass of infected individuals at the beginning of metamorphosis (stage 42), at the end of metamorphosis (stage 46) and at the end of the experiment or death. Grey boxes = survivors, white boxes = non-survivors

survivors had on average higher zoospore loads (9701 GE ± 21726.2) than survivors (28.2 GE ± 113.2; Figure 2.4) at death or at the end of the experiment, respectively. There was an interaction between zoospore load and population (Table 2.1, Figure 2.4). Three individuals out of 31 survivors had cleared infection below the detection threshold.

Discussion

Here, we show that under laboratory conditions, mortality in *Bd*-infected metamorphs was population-specific and varied from 27% to 90%. Our experiment thus confirms that *Bd* can cause disease and substantial mortality in *Alytes* toadlets from populations where no mass mortality events have been reported. If the observed mortality is representative of *Bd*-induced mortality in the field, such high levels of mortality may lead to populations declines (Hels & Nachman 2002, Lampo & De Leo 1998).

How could such populations persist? Sensitivity-analyses of amphibian life stages suggest that post-metamorphic juvenile survival appears to determine the fate of the populations. Such high levels of mortality may lead to population declines (Hels & Nachman 2002, Lampo & De Leo 1998). In contrast, Briggs *et al.* (2005) modelled the effects of *Bd* on population dynamics and predicted population persistence if some infected individuals survive. Because no data on population trends are available for the populations that we studied, we cannot tell whether they currently undergo declines. To predict the fate of our study populations, more data would be necessary. For example, it is unknown (1) for how long *Bd* has been present in these populations, (2) how long after the onset of chytridiomycosis the adult populations are expected to decline, (3) whether and how strongly mortality rates differ between years and (4) whether density dependence in the adult stage could dampen the effects of *Bd*-associated mortality immediately after metamorphosis.

To our knowledge, this is the first study to show differences in the survival of *Bd*-infected individuals both within and between populations in the laboratory. We do not know why individuals and populations differed in susceptibility. Some variation may be attributable to differences in body mass (Garner *et al.* 2009b, Figure 2.3), although the large difference in body mass at the end of the experiment mostly resulted from the longer lifespan of survivors (Figure 2.4). Variation in mass and survival may have been caused by

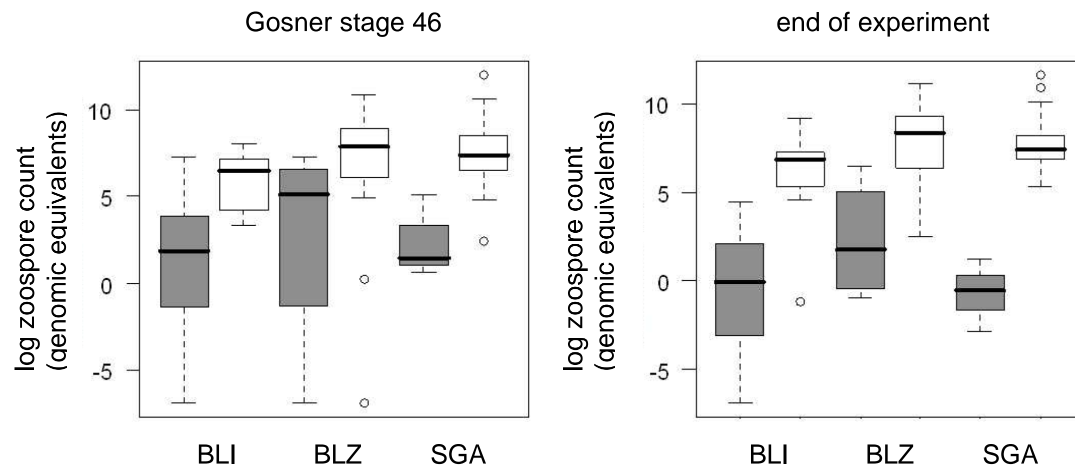

Figure 2.4: Infection load, measured in genomic equivalents from rt-PCR reactions and logarithmically transformed, in individuals from the infected treatments at the end of experiment. The black line represents the median, the box represents the interquartile range containing 50% of the values, and whiskers mark the 1.5 fold interquartile range. Outliers are marked with circles. Grey boxes = survivors, white boxes = non-survivors

differential environmental conditions early in the larval stage, by variation in the tadpole immune system (i.e. genetic variation at disease resistance loci, antimicrobial peptides andsymbiotic bacteria), because of differences between *Bd* strains from the tadpoles' sites of origin or because of infection of single vs. multiple *Bd* strains.

We do not expect that environmental conditions experienced early in life affected the outcome of the experiment. The tadpoles were captured after hibernation. We kept them in the laboratory under common garden conditions for 4 to 18 weeks before they started metamorphosis. Because effects of previously experienced environmental conditions often fade out quickly (Van Buskirk 2002) and because the common conditions in the laboratory experiment would minimise environmental variation, the effects of previously experienced environmental conditions in the ponds of origin should be minimal.

The immunocompetence is likely to have varied among individuals and populations. While we know little about the genetic basis of the immune system in amphibians (Richmond *et al*. 2009), we know that antimicrobial peptides and symbiotic bacteria that are active against *Bd* may vary both among individuals and populations (Harris *et al.* 2009, Tennessen & Blouin 2008). Strains of *Bd* are known to differ in how much mortality they inflict on amphibians (Fisher *et al.* 2009a) and multiple strains of *Bd* may occur within the same locality (Goka *et al.* 2009, Walker *et al.* 2010). Infection with multiple strains might affect virulence (Alizon 2008, Ebert *et al.* 2000). We did not test whether tadpoles in our experiment had the same antimicrobial peptides, bacteria or *Bd* strains. We know, however,

that the host populations are genetically differentiated (pairwise F_{ST} based on microsatellites ≥ 0.19, U. Tobler & B. R. Schmidt, unpublished data) and that the bacterial communities differ between the populations BLI and BLZ (L. Davis, personal communication). For logistic reasons during experimental work, the same equipment was used for water changes for all populations within one treatment, and thus bacterial communities may have been homogenised. Considering all this, we suggest that genetic differences among populations may at least partially explain the observed differences in mortality, as it is often the case in host-parasite associations (Cory & Myers 2009, Ebert *et al.* 1998).

A number of experimental studies reported that many *Bd*-infected amphibians can survive or even clear infection (see electronic appendix to Kilpatrick *et al.* 2010). For example, Fisher *et al.* (2009a) reported that host survival varied in a dose-dependent manner among *Bd* strains. In most cases, a substantial proportion of hosts survived (as in our experiment; Figure 2.1). Nevertheless, *Bd* imposes strong selection on amphibian hosts. If variation for susceptibility to *Bd* has a genetic basis, we would expect to see genetic changes in the host population and ultimately the evolution of resistance to *Bd* (Haag & Ebert 2004, Zbinden *et al.* 2008). Such pathogen-mediated selection is known to occur in amphibians: Tennessen and Blouin (2008) and Teacher *et al.* (2009) showed that there is natural selection on the genetic diversity of antimicrobial peptides and MHC alleles, respectively. One interpretation of among-population variation in *Bd*-associated mortality reported in Figure 2.1 is that the populations we studied may already differ in their degree of resistance to *Bd*.

While strategies to manage *Bd* in the wild are still being developed (Fisher *et al.* 2009b, Kilpatrick *et al.* 2010), we suggest that enhancing an evolutionary response of amphibians to *Bd* may be the worthwhile conservation strategy to mitigate the effects of the disease (Kilpatrick 2006). The model by Briggs *et al.* (2005) suggests that amphibian populations can persist or even recover if some individuals loose the infection. Such a "waiting for evolution to occur" strategy may be risky, however, as some hosts may fail to evolve adaptations to novel pathogens (Bell & Collins 2008). First, rapid disease emergence may cause amphibian populations to go extinct in many places before resistance allowing for population persistence can evolve (Bell & Collins 2008, Gomulkiewicz & Houle 2009, Orr & Unckless 2008). Second, *Bd* is likely to evolve counteradaptations to host resistance (Fisher *et al.* 2009a). Yet, if *Bd* and amphibian hosts would enter a coevolutionary process, then extinction – as commonly observed in areas where *Bd* emerged – may become a less likely outcome.

In summary, our experiment demonstrates that within a species, mortality can greatly differ both at the population and individual level, and that these different mortalities are not necessarily due to climatic variation at the time of metamorphosis because in this case they were observed under stable laboratory conditions. The results also show that *Bd*-associated mortality can be substantial in an area where *Bd* is widespread (Garner *et al.* 2005, U. Tobler & B. R. Schmidt, unpublished data, Schmidt *et al.* 2009a) but where no mass mortalities or *Bd*-associated population declines have been reported (Schmidt & Zumbach 2005). Nevertheless, the high mortality rates we observed are likely to affect populations and *Bd* may be a cryptic driver of amphibian population dynamics. The mechanisms how amphibian populations can cope with additional mortality due to chytridiomycosis are unknown. We argue that in many situations, global or local extinction will only occur if aided by other threats such as habitat degradation, demographic stochasticity or unusual weather conditions. We suggest that conservation measures should prioritise populations that have high resistance against chytridiomycosis to prevent the loss of these populations by other threats. In the long term it is desirable to determine what factors are involved in population level disease resistance in order to allow the transfer of resistance mechanisms, such as resistant genotypes or symbiotic skin microbiota, into non-resistant populations. This may enable the management of amphibian populations, or their habitat, to increase survival rates and thus allow long-term population survival in the presence of novel disease threats.

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References

Alizon, S. (2008). Decreased overall virulence in coinfected hosts leads to the persistence of virulent parasites. *American Naturalist* 172(2): E67-E79.

Bell, G. and Collins, S. (2008). Adaptation, extinction and global change. *Evolutionary Applications* 1(1): 3-16.

Berger, L., Speare, R., Daszak, P., Green, D. E., Cunningham, A. A., Goggin, C. L., Slocombe, R., Ragan, M. A., Hyatt, A. D., McDonald, K. R., Hines, H. B., Lips, K. R., Marantelli, G. and Parkes, H. (1998). Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proceedings of the National Academy of Sciences of the United States of America* 95(15): 9031-9036.

Bielby, J., Cooper, N., Cunningham, A. A., Garner, T. W. J. and Purvis, A. (2008). Predicting susceptibility to future declines in the world's frogs. *Conservation Letters* 1: 82-90.

Bosch, J., Beebee, T., Schmidt, B. R., Tejedo, M., Martínez-Solano, I., Salvador, A., García-París, M., Gil, E. R., Arntzen, J. W., Diaz Paniagua, C. and Marquez, R. (2008). *Alytes obstetricans*. *in: IUCN 2009. IUCN Red List of Threatened Species Version 2009.2* (www.iucnredlist.org): downloaded on 19 January 2010.

Bosch, J., Carrascal, L. M., Duran, L., Walker, S. and Fisher, M. C. (2007). Climate change and outbreaks of amphibian chytridiomycosis in a montane area of Central Spain; is there a link? *Proceedings of the Royal Society Biological Sciences Series B* 274(1607): 253-260.

Bosch, J., Martínez-Solano, I. and García-París, M. (2001). Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain. *Biological Conservation* 97(3): 331-337.

Boyle, D. G., Boyle, D. B., Olsen, V., Morgan, J. A. T. and Hyatt, A. D. (2004). Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Diseases of Aquatic Organisms* 60(2): 141-148.

Briggs, C. J., Vredenburg, V. T., Knapp, R. A. and Rachowicz, L. J. (2005). Investigating the population-level effects of chytridiomycosis: An emerging infectious disease of amphibians. *Ecology* 86(12): 3149-3159.

Brucker, R. M., Harris, R. N., Schwantes, C. R., Gallaher, T. N., Flaherty, D. C., Lam, B. A. and Minbiole, K. P. C. (2008). Amphibian chemical defense: antifungal metabolites of the microsymbiont *Janthinobacterium lividum* on the salamander *Plethodon cinereus*. *Journal of Chemical Ecology* 34(11): 1422-1429.

Cory, J. S. and Myers, J. H. (2009). Within and between population variation in disease resistance in cyclic populations of western tent caterpillars: a test of the disease defence hypothesis. *Journal of Animal Ecology* 78(3): 646-655.

Daszak, P., Cunningham, A. A. and Hyatt, A. D. (2000). Wildlife ecology - Emerging infectious diseases of wildlife - Threats to biodiversity and human health. *Science* 287(5452): 443-449.

de Castro, F. and Bolker, B. (2005). Mechanisms of disease-induced extinction. *Ecology Letters* 8(1): 117-126.

Ebert, D., Zschokke-Rohringer, C. D. and Carius, H. J. (1998). Within- and between-population variation for resistance of *Daphnia magna* to the bacterial endoparasite *Pasteuria ramosa*. *Proceedings of the Royal Society Biological Sciences Series B* 265(1410): 2127-2134.

Ebert, D., Zschokke-Rohringer, C. D. and Carius, H. J. (2000). Dose effects and density-dependent regulation of two microparasites of *Daphnia magna*. *Oecologia* 122(2): 200-209.

Fisher, M. C., Bosch, J., Yin, Z., Stead, D. A., Walker, J., Selway, L., Brown, A. J. P., Walker, L. A., Gow, N. A. R., Stajich, J. E. and Garner, T. W. J. (2009a). Proteomic and phenotypic profiling of the amphibian pathogen *Batrachochytrium dendrobatidis* shows that genotype is linked to virulence. *Molecular Ecology* 18(3): 415-429.

Fisher, M. C., Garner, T. W. J. and Walker, S. F. (2009b). Global emergence of *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis in space, time, and host. *Annual Review of Microbiology* 63: 291-310.

Garner, T. W. J., García, G., Carroll, B. and Fisher, M. C. (2009a). Using itraconazole to clear *Batrachochytrium dendrobatidis* infection, and subsequent depigmentation of *Alytes muletensis* tadpoles. *Diseases of Aquatic Organisms* 83(3): 257-260.

Garner, T. W. J., Walker, S., Bosch, J., Hyatt, A. D., Cunningham, A. A. and Fisher, M. C. (2005). Chytrid fungus in Europe. *Emerging Infectious Diseases* 11(10): 1639-1641.

Garner, T. W. J., Walker, S., Bosch, J., Leech, S., Rowcliffe, J. M., Cunningham, A. A. and Fisher, M. C. (2009b). Life history tradeoffs influence mortality associated with the amphibian pathogen *Batrachochytrium dendrobatidis*. *Oikos* 118(5): 783-791.

Goka, K., Yokoyama, J., Une, Y., Kuroki, T., Suzuki, K., Nakahara, M., Kobayashi, A., Inaba, S., Mizutani, T. and Hyatt, A. D. (2009). Amphibian chytridiomycosis in Japan: distribution, haplotypes and possible route of entry into Japan. *Molecular Ecology* 18(23): 4757-4774.

Gomulkiewicz, R. and Houle, D. (2009). Demographic and genetic constraints on evolution. *American Naturalist* 174(6): E218-E229.

Gosner, K. L. (1960). A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetological Journal* 16: 183-190.

Green, D. M. (2003). The ecology of extinction: population fluctuation and decline in amphibians. *Biological Conservation* 111(3): 331-343.

Haag, C. R. and Ebert, D. (2004). Parasite-mediated selection in experimental metapopulations of *Daphnia magna*. *Proceedings of the Royal Society Biological Sciences Series B* 271(1553): 2149-2155.

Harris, R. N., Brucker, R. M., Walke, J. B., Becker, M. H., Schwantes, C. R., Flaherty, D. C., Lam, B. A., Woodhams, D. C., Briggs, C. J., Vredenburg, V. T. and Minbiole, K. P. C. (2009). Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *ISME Journal* 3(7): 818-824.

Hels, T. and Nachman, G. (2002). Simulating viability of a spadefoot toad *Pelobates fuscus* metapopulation in a landscape fragmented by a road. *Ecography* 25(6): 730-744.

Kilpatrick, A. M. (2006). Facilitating the evolution of resistance to avian malaria in Hawaiian birds. *Biological Conservation* 128: 475-485.

Kilpatrick, A. M., Briggs, C. J. and Daszak, P. (2010). The ecology and impact of chytridiomycosis: an emerging disease of amphibians. *Trends in Ecology & Evolution* 25(2): 109-118.

Lam, B. A., Walke, J. B., Vredenburg, V. T. and Harris, R. N. (2010). Proportion of individuals with anti-*Batrachochytrium dendrobatidis* skin bacteria is associated with population persistence in the frog *Rana muscosa*. *Biological Conservation* 143(2): 529-531

Lampo, M. and De Leo, G. A. (1998). The invasion ecology of the toad *Bufo marinus*: from South America to Australia. *Ecological Applications* 8(2): 388-396.

Laugen, A. T., Kruuk, L. E. B., Laurila, A., Räsänen, K., Stone, J. and Merilä, J. (2005). Quantitative genetics of larval life-history traits in *Rana temporaria* in different environmental conditions. *Genetical Research* 86(3): 161-170.

Lips, K. R., Brem, F., Brenes, R., Reeve, J. D., Alford, R. A., Voyles, J., Carey, C., Livo, L., Pessier, A. P. and Collins, J. P. (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(9): 3165-3170.

Longcore, J. E., Pessier, A. P. and Nichols, D. K. (1999). *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. *Mycologia* 91(2): 219-227.

Miller, M. P. and Vincent, E. R. (2008). Rapid natural selection for resistance to an introduced parasite of rainbow trout. *Evolutionary Applications* 1(2): 336-341.

Murray, K. A., Skerratt, L. F., Speare, R. and McCallum, H. (2009). Impact and dynamics of disease in species threatened by the amphibian chytrid fungus, *Batrachochytrium dendrobatidis*. *Conservation Biology* 23(5): 1242-1252.

NSW National Parks and Wildlife Service (2001). Hygiene protocol for the control of disease in frogs. NSW National Parks and Wildlife Service, H. N., Australia., NSW National Parks and Wildlife Service.

Orr, A. H. and Unckless, R. L. (2008). Population extinction and the genetics of adaptation. *American Naturalist* 172(2): 160-169.

R Development Core Team (2008). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.

Rachowicz, L. J. and Briggs, C. J. (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(4): 711-721.

Retallick, R. W. R., McCallum, H. and Speare, R. (2004). Endemic infection of the amphibian chytrid fungus in a frog community post-decline. *PLoS Biology* 2(11): 1965-1971.

Richmond, J. Q., Savage, A. E., Zamudio, K. R. and Rosenblum, E. B. (2009). Toward immunogenetic studies of amphibian chytridiomycosis: linking innate and acquired immunity. *Bioscience* 59(4): 311-320.

Rollins-Smith, L. A. (1998). Metamorphosis and the amphibian immune system. *Immunological Reviews* 166: 221-230.

Rowley, J. J. L. and Alford, R. A. (2007). Behaviour of Australian rainforest stream frogs may affect the transmission of chytridiomycosis. *Diseases of Aquatic Organisms* 77(1): 1-9.

Roy, B. A. and Kirchner, J. W. (2000). Evolutionary dynamics of pathogen resistance and tolerance. *Evolution* 54(1): 51-63.

Schmidt, B. R., Furrer, S., Kwet, A., Lötters, S., Rödder, D., Sztatecsny, M., Tobler, U. and Zumbach, S. (2009). Desinfektion als Maßnahme gegen die Verbreitung der Chytridiomykose bei Amphibien. Methoden der Feldherpetologie. Hachtel, M., Schlüpmann, M., Thiesmeier, B. and Weddeling, K. Bielefeld, Laurenti Verlag. 229–241

Schmidt, B. R. and Zumbach, S. (2005). Rote Liste der gefährdeten Amphibien der Schweiz. Bundesamt für Umwelt, Wald und Landschaft, Bern, und Koordinationsstelle Amphibien- und Reptilienschutz in der Schweiz, Bern.

Skerratt, L. F., Berger, L., Speare, R., Cashins, S., McDonald, K. R., Phillot, A. D., Hines, H. B. and Kenyon, N. (2007). Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *EcoHealth* 4: 125-134.

Spielman, D., Brook, B. W., Briscoe, D. A. and Frankham, R. (2004). Does inbreeding and loss of genetic diversity decrease disease resistance? *Conservation Genetics* 5(4): 439-448.

Teacher, A. G. F., Garner, T. W. J. and Nichols, R. A. (2009). Evidence for directional selection at a novel Major Histocompatibility Class I marker in wild Common frogs (*Rana temporaria*) exposed to a viral pathogen (*Ranavirus*). *PLoS ONE* 4(2): e4616.

Tennessen, J. A. and Blouin, M. S. (2008). Balancing selection at a frog antimicrobial peptide locus: fluctuating immune effector alleles? *Molecular Biology and Evolution* 25(12): 2669-2680.

Tennessen, J. A., Woodhams, D. C., Chaurand, P., Reinert, L. K., Billheimer, D., Shyr, Y., Caprioli, R. M., Blouin, M. S. and Rollins-Smith, L. A. (2009). Variations in the expressed antimicrobial peptide repertoire of northern leopard frog (*Rana pipiens*) populations suggest intraspecies differences in resistance to pathogens. *Developmental and Comparative Immunology* 33(12): 1247-1257.

Van Buskirk, J. (2002). Phenotypic lability and the evolution of predator-induced plasticity in tadpoles. *Evolution* 56(2): 361-370.

Voyles, J., Young, S., Berger, L., Campbell, C., Voyles, W. F., Dinudom, A., Cook, D., Webb, R., Alford, R. A., Skerratt, L. F. and Speare, R. (2009). Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. *Science* 326(5952): 582-585.

Walker, S. F., Bosch, J., Gomez, V., Garner, T. W. J., Cunningham, A. A., Schmeller, D. S., Ninyerola, M., Henk, D., Ginestet, C., Arthur, C.-P. and Fisher, M. C. (2010). Factors driving pathogenicity vs. prevalence of amphibian panzootic chytridiomycosis in Iberia. *Ecology Letters* 13(3): 372-382.

Woodhams, D. C., Rollins-Smith, L. A., Carey, C., Reinert, L., Tyler, M. J. and Alford, R. A. (2006). Population trends associated with skin peptide defenses against chytridiomycosis in Australian frogs. *Oecologia* 146(4): 531-540.

Zbinden, M., Haag, C. R. and Ebert, D. (2008). Experimental evolution of field populations of *Daphnia magna* in response to parasite treatment. *Journal of Evolutionary Biology* 21(4): 1068-1078.

NO ASSOCIATION BETWEEN THE PRESENCE OF AN EMERGING PATHOGEN AND LOCAL EXTINCTIONS OF A SUSCEPTIBLE HOST SPECIES: INSIGHTS FROM A HOST-PATHOGEN DISTRIBUTION MODEL

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Abstract

Emerging infectious diseases are a growing risk to the survival of threatened species. One of the pathogens with most devastating effects is the amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*). Epizootic outbreaks of chytridiomycosis, the associated disease, have caused dramatic declines and extinctions of amphibian species world-wide, but do not occur everywhere where the pathogen is present. The question hence becomes whether *Bd* can also cause amphibian declines and population extirpation in an apparently enzootic stage. We use long-term data on the presence or absence of *Alytes obstetricans*, a species that has suffered *Bd*-induced mass-mortalities and population declines, at 78 ponds across Switzerland. All ponds had been occupied by the species in the past, and were tested for the presence of *Bd* in 2008. We built a hierarchical Bayesian multi-season, multi-species model to jointly estimate the occupancy for *Bd* and the probability of *Alytes* occurrence and survival separately for sites with and without *Bd*. *Bd* was found in more than 50% of all ponds, but our model results suggest that *Alytes* declines in Switzerland are unrelated to the presence of the pathogen. Several mechanisms may explain the lack of negative effects of the pathogen on the host in this system where *Bd* is apparently enzootic: The current environmental conditions in Switzerland may not favour disease outbreaks, populations may be able to compensate for disease-induced mortality or they have stabilised at lower density following an initial decline after invasion of the pathogen. In conclusion, our data demonstrate that the same host-pathogen system can result in different disease dynamics ranging from mass-mortalities to apparently benign.

Key words: *Alytes obstetricans*, *Batrachochytrium dendrobatidis*, decline, detection probability, disease, extinction probability, hierarchical model, multi-species site occupancy model

Introduction

Newly emerging diseases of wildlife are reported regularly (Brasier & Kirk, Buchen 2010, Porter *et al.* 2008) and infectious diseases are increasingly recognised as threats to biodiversity (Daszak *et al.* 2000, Smith *et al.* 2006). Because newly emerging pathogens or pathogens spreading into new areas encounter naïve hosts, their effects can be devastating. Even if emerging diseases do not lead to massive and obvious die-offs, pathogens may significantly reduce survival, fecundity or population growth rate of a species (Anderson & May 1979, Jolles *et al.* 2005, May & Anderson 1979, Pilliod *et al.* 2010) and thus increase extinction probability of populations where the pathogen is present.

Chytridiomycosis is a fungal disease of amphibians that is causing population declines and extinctions globally (Berger *et al.* 1998, Bosch *et al.* 2001, Lips *et al.* 2006, Rachowicz *et al.* 2006). In many regions, species have disappeared completely following the introduction of *Batrachochytrium dendrobatidis* (*Bd* hereafter), the pathogen causing the disease. In other regions, the distribution of the pathogen is more patchy and widespread and seems to be enzootic rather than cause epizootic disease outbreaks (Briggs *et al.* 2010, Longcore *et al.* 2007). Although a number of explanations are put forward, to date it is largely unresolved why the pathogen exerts devastating effects on amphibian populations in some regions and why it is enzootic with mild effects in other regions (Fisher *et al.* 2009b). The model developed by Briggs *et al*. (2010) suggests that the different outcomes may be explained solely by variation in host-pathogen dynamics. Empirical data suggest that even when prevalence is relatively high and *Bd* infection causes a reduction in survival, the reduction in population growth rate may be moderate (Pilliod *et al*. 2010).

In the case of the midwife toad *Alytes obstetricans*, severe die-offs were observed after the initial arrival of *Bd* in Spain (Bosch *et al.* 2001). The same species has suffered severe declines in Switzerland and roughly 50% of populations have been extirpated since the mid 1980s (Schmidt & Zumbach 2005); the loss of local populations is apparently unrelated to habitat loss (Borgula & Zumbach 2003). However, *Bd* is widespread and present in Switzerland since at least the 1980s (Peyer 2010), suggesting that it is enzootic in Switzerland. In a laboratory setup, mortality among freshly metamorphosed *A. obstetricans* from Switzerland that were infected with *Bd* was high (Tobler & Schmidt 2010). The question hence becomes whether *Bd* can cause amphibian declines in a supposedly enzootic stage.

In generalist pathogens infecting multiple hosts such as *Bd*, detectability of the pathogen may differ among different host species. Generally, reservoir hosts are competent but able to control infection and therefore often have lower infection loads than susceptible hosts (Begon 2008). Detectability of the pathogen in a reservoir host is therefore often reduced compared to the probability of detection in a host that develops disease. On the other hand, susceptible hosts may die when infected and thus prevalence in susceptible hosts may be lower than in reservoir hosts. When trying to assess the risk of local extinction by disease of a susceptible species, it is therefore often difficult to compare disease status of sites where the focal species is present or absent: The pathogen is more likely to be overlooked at sites where the susceptible focal species is extinct. Thus, when modelling the distribution and effects of a multi-host pathogen, one must explicitly account for the host species on which the pathogen was detected. Moreover, the detection of the host may differ depending on infection status (Jennelle *et al.* 2007, McClintock *et al.* 2010).

Occupancy models are a tool to overcome this problem because they allow the estimation of host and pathogen occurrence in a two-step process where detection probability and occupancy probability are estimated separately (Kéry & Schmidt 2008). Recent extensions allow the separate estimation of detectability in the presence or absence of another species. In the model of Waddle *et al*. (2010), the detection, occurrence and survival of a subordinate species (the susceptible host) depends on the presence of a dominant species (in our case the multi-host pathogen). This modelling approach eliminates detection bias that may lead to false conclusions about the occurrence of the subordinate species.

In the present study, we aimed to determine whether there is an association between local extinctions of the susceptible host species *Alytes obstetricans* and the presence of the pathogen *Bd*. The distribution of *Alytes obstetricans* is well known in Switzerland (Grossenbacher 1988). We surveyed 76 ponds which were occupied by *Alytes* during the 1980ies and from which data on *Alytes* presence in 2003/2004 were available (Schmidt & Zumbach 2005), covering the whole distribution of the species in Switzerland. The 76 ponds were randomly selected from all known populations recorded in the Swiss amphibian distribution data base (*n*=1147) maintained by the Koordinationsstelle für Amphibien- und Reptilienschutz in der Schweiz (karch). These 76 sites were revisited in a new survey in 2008. The goal of this later survey was to determine which amphibian species were present and to

test them for the presence of *Bd*. We expected that *Bd* would be commonly found in ponds where *Alytes* had gone locally extinct, but only rarely in those ponds where the species was still present. Because we expect the occurrence of *Alytes* to depend on the presence of *Bd*, we used a multispecies-occupancy model similar to the one of Waddle *et al*. (2010) to estimate occurrence and survival probability of *Alytes* separately for sites with and without the pathogen. We extended the model to account for the fact that *Bd* may be more likely to be detected in heavily infected susceptible species such as *Alytes* by including a covariate on detection that indicates whether the sample was taken from *Alytes* or not and a covariate on occupancy that indicates whether *Alytes* was currently present at the pond or not.

Materials and Methods

We surveyed 76 amphibian breeding sites in 2008 for *Bd* and amphibians that were already surveyed for amphibians in 2003/2004 as part of the update of the Swiss amphibian Red List (Schmidt & Zumbach 2005). For the Red List update, sites were selected randomly from the Swiss amphibian distribution data base maintained by the Koordinationsstelle für Amphibien- und Reptilienschutz in der Schweiz. The data base holds records of more than 10’000 amphibian breeding sites across Switzerland. Since the distribution of *Alytes obstetricans* is well known (Borgula & Zumbach 2003, Grossenbacher 1988), the sample is representative. For all 76 sites, we knew that *Alytes obstetricans* occurred in the past. Hence, all absences in 2003/2004 and/or 2008 are local extinctions. Field work in 2003/2004 was conducted by professional and amateur herpetologists. All 76 sites were visited 4 times in either 2003 or 2004. Site visits that occurred before the annual reproductive cycle of *Alytes obstetricans* had begun or after it had ended were discarded and not used for statistical analysis (MacKenzie *et al*. 2002). During April and May 2008, the 76 ponds were revisited three times and surveyed for the presence of *Alytes obstetricans*, other amphibians and *Bd* by a crew of five field workers that had not taken part in the 2003/2004 survey.

Bd *sampling*

Bd samples were taken during the first visit to a site which took place during daytime. Because *Alytes* had gone extinct from more than half of the ponds (Schmidt & Zumbach 2005), we sampled for *Bd* from adults of all amphibian species that were present. The only

larval amphibians we sampled for *Bd* were *Alytes* tadpoles. We tried to catch a number of 25 individuals per site, which allows the detection of the pathogen with good confidence even if prevalence is low (DiGiacomo & Koepsell 1986). However, in some of the sites no or fewer amphibians were caught and *Bd* sample size was accordingly smaller. We swabbed all animals that were caught with a sterile cotton swab (Copan Italia S.p.A., Brescia, Italy). In tadpoles, we swabbed the keratinised mouthparts, and in adult amphibians we moved the swab with slight pressure five times each over all feet, the drink patch and the tights in a standardised way. We recorded amphibian species, the developmental stage (Gosner 1960) in tadpoles, and sex (if possible) in adults. Standard hygiene protocols were followed during field work (Schmidt *et al.* 2009a).

Upon return from the field, we stored the swabs in the fridge until analysis. We extracted and analysed the swabs according to the protocol by Boyle *et al*. (2004) with slight modifications as described in Tobler and Schmidt (2010). A detection threshold of 0.1 genomic equivalents was applied to avoid false positives.

Habitat characteristics and amphibian species assemblage

During the first site visit, we collected additional information on the site characteristics: elevation, pond surface area and depth, the number of ponds, presence of a stream going into or out of the pond, canopy cover (in percentage of pond area covered by canopy), pond type (forest, quarry or other), and presence of fish (Table 3.1).

We extracted information on climatic conditions for each site from the WorldClim database (Hijmans *et al*. 2005) using the program DIVA-GIS 7.1.7.2. The available climate data represent means from years 1950 to 2000 in a roughly 1 km resolution extrapolated from global weather stations. We based the choice of variables describing climate on the results of Rödder *et al.* (2008) and Walker *et al.* (2010): maximum temperature of warmest months, minimum temperature of coldest month, mean annual temperature, mean annual precipitation, seasonality, and isothermality. Some climate variables were eliminated because they were highly correlated with others (maximum temperature of warmest months, minimum temperature of coldest month and isothermality).

Alytes and amphibian survey

The second and third visits to a site took place during the first half of the night and each visit lasted between 20 and 60 min. We determined the presence of amphibian species by visual search, dip-netting and call surveys (Dodd 2009).

Statistical analysis

We modelled the distributions of *Bd* and *Alytes* and the effect of *Bd* on *Alytes* using a multi-season multi-species site occupancy model (MacKenzie *et al.* 2004, MacKenzie *et al.* 2003, Waddle *et al.* 2010). Site occupancy models allow taking imperfect detection into account (MacKenzie *et al*. 2002). We jointly modelled the distribution of *Bd* in 2008, the distribution of *Alytes* in 2003/2004 and extinction and colonization of sites in between 2003/2004 and 2008 using a hierarchical Bayesian approach implemented in the program WinBUGS (Kéry 2010, Royle & Dorazio 2008). We specified a model with site-specific detection and occurrence probabilities for the pathogen of the form:

$$\text{logit}(p^{Bd}_{ij}) = \alpha + \beta_{\text{Alytes}} * \text{cov.Alytes}_{ij} + \beta_1 * \text{cov}_{1,ij} + ...$$

$$\text{logit}(\psi^{Bd}_{i}) = \alpha 1 * z^{Ao}_{i,2} + \alpha 0 * (1 - z^{Ao}_{i,2}) + \beta 1 * \text{cov}_{i1} + ...$$

p^{Bd}_{ij} corresponds to the detection probability of *Bd* at site *i* during visit *j*. The detection covariate cov.Alytes_{ij} accounts for differences in detection probabilities between swabs taken from an *Alytes* tadpole and from an adult of another species (Table 3.1). The occurrence probability of *Bd* at site *i* (ψ^{Bd}_{i}) is conditional on the true occurrence of *Alytes* at site *i* during the 2nd *Alytes* survey in 2008 $z^{Ao}_{i,2}$. It incorporates the effects that the presence of *Alytes* tadpoles at a pond may have on *Bd* occurrence probability by providing a competent aquatic host throughout the year. β_1 is the slope of the first covariate in the model (cov_1). Further covariates were entered into the model in the same way (Table 3.1).

The model for *Alytes* included the effects of *Bd* occurrence on *Alytes* similar to the multi-species model developed by Waddle *et al.* (2010). We defined the occurrence probability of *Alytes* at site *i* ψ^{Alob}_{i} in year k=1 as a function of the true occurrence state (Royle & Kery 2007) of *Bd* at site *i* z^{Bd}_{i} and other covariates. We estimated separate intercepts for the occurrence of *Alytes* in the presence (α1) and absence (α0) of *Bd*.

$$\text{logit}(\psi^{Alytes}_{i1}) = \alpha 1 * z^{Bd}_{i} + \alpha 0 * (1 - z^{Bd}_{i}) + \beta * \text{cov}_{i1}$$

The occurrence probability of *Alytes* at site *i* in year 1 (ψ^{Alytes}_{i1}) is conditional on the probability of *Bd* occurrence at site *i* z^{Bd}_{i}. Because the presence of a pathogen is likely to affect the extinction probability of a susceptible host, we included site-specific extinction and colonisation probabilities for *Alytes*. The occurrence probability of *Alytes* at site *i* in consecutive years *k* depends on its true occupancy state in year 1 times the probability that the species persisted given it was present, plus the probability it went extinct given it was absent:

$$\text{logit}(\psi^{Alytes}_{ik}) = z^{Alytes}_{i1} * \phi^{Alytes}_{i} + (1- z^{Alytes}_{i1}) * \varepsilon^{Alytes}_{i}$$

where

$$\text{logit}(\phi^{Alytes}_{i}) = \alpha1 * z^{Bd}_{i} + \alpha0 * (1-z^{Bd}_{i}) + \beta * \text{cov}_{i}$$

and

$$\text{logit}(\varepsilon^{Alytes}_{i}) = \alpha1 * z^{Bd}_{i} + \alpha0 * (1-z^{Bd}_{i}) + \beta * \text{cov}_{i}$$

Two separate regressions were fitted for both the survival probability ϕ^{Alytes}_{i} and the extinction probability ε^{Alytes}_{i} in the presence [$\alpha1 * z^{Bd}_{i}$] and absence [$\alpha0 * (1-z^{Bd}_{i})$] of *Bd*.

Because *Bd* infection does not cause disease symptoms in the tadpoles of this species and is therefore not expected to lead to different encounter rates between infected and uninfected individuals, we considered detection probability of *Alytes* independent of *Bd* occurrence at a site.

$$\text{logit}(p^{Alytes}_{ikj}) = \alpha_k + \beta * \text{cov}_{ikj}$$

p^{Alytes}_{ikj} denotes the detection probability of *Alytes* at site *i* in year *k* during visit *j*. Two separate regression lines were fitted for the survey in 2003/2004 and for the survey in 2008 as indicated by the subscript *k* for the intercept α_k.

We fitted a model with the covariates shown in table 3.1. After initial runs, three covariates had to be excluded because we never achieved satisfactory convergence: diurnal range for *Bd* occupancy probability and elevation and elevation^2 for *Alytes* occupancy. We ran three parallel chains starting from different initial values. The chains were run for 25'000 iterations; we discarded the first 5000 as burn-in and applied a thinning factor of 5. Covariates were considered important if their 95% credible interval (CRI) did not include zero.

Table 3.1: List of covariates used in the model selection approach. p_{Bd} and ψ_{Bd} denotes parameters that were used to optimise detection probability and occupancy probability of *Bd*, respectively. p_{Alytes}, ψ_{Alytes} and ϕ_{Alytes} denote parameters that were used for detection, occupancy and survival probability of *Alytes obstetricans*.

Covariate	use	range	description
detection			
Alytes	p_{Bd}	0, 1	1=swab from an *Alytes* tadpole
swabber	p_{Bd}	categorical	swabber identity
date	p_{Bd}, p_{Alytes}	13 March – 5 July	Julian date[1]
$(date)^2$	p_{Bd}, p_{Alytes}		Julian date squared
04/08	p_{Alytes}	0, 1	estimation of separate intercepts for surveys in 2003/2004 and in 2008
method	p_{Alytes}	categorical	dip-netting or calling survey
Climate			
masl	ψ_{Bd}, ψ_{Alytes}	305 – 1099 masl	altitude
$(masl)^2$	ψ_{Bd}, ψ_{Alytes}	305 – 1099 masl	altitude squared
meantemp	ψ_{Bd}	6.1 – 10.4 °C	mean temperature[1] (mean annual air temperature)
diurrange	ψ_{Bd}	7.3 – 9.2 °C	mean diurnal temperature range[1] (monthly max – monthly min)
meanprecip	ψ_{Bd}	804 – 1296 mm	mean annual precipitation[1]
amphibian species			
Alytes	ψ_{Bd}	0, 1	1= detection of *Alytes obstetricans*
Meal	ψ_{Bd}	0, 1	1= detection of *Mesotriton alpestris*
Pelo	ψ_{Bd}, ψ_{Alytes}., ϕ_{Alytes}	0, 1	1= detection of *Pelophylax spp.*
habitat characteristics			
area	ψ_{Alytes}	1.5 – 50600 m^2	pond surface area
depth	ψ_{Alytes}	0.1 – 10 m	pond depth
shadiness	ψ_{Alytes}	1 – 3	degree of pond shadiness (1=complete shade, 3=complete sun)
fish	ψ_{Alytes}, ε_{Alytes}	0, 1	1=presence of fish (irrespective of species)
runwater	ψ_{Bd}, ψ_{Alytes}	0, 1	1= running water (in- and/or outflow)
# ponds	ψ_{Bd}, ψ_{Alytes}	1 – 12	number of ponds at a site
type	ψ_{Bd}, ψ_{Alytes}	categorical	pond location ($type_{forest}$, $type_{quarry}$)
multi-species interaction			
Bd	ψ_{Alytes}, ϕ_{Alytes}, γ_{Alytes}	0-1	*Bd* occupancy probability as estimated simultaneously in the model
Alytes	ψBd	0-1	*Alytes* occupancy probability as estimated simultaneously in the model

[1] z-standardised

Results

Results of the amphibian and Bd *survey*

The ponds covered an altitudinal range from 305 to 1099 m above sea level (median: 492 m) and varied in size and depth from 1.5 to 50’600 m^2 (median: 216 m^2) and from 0.1 to 10 m (median: 1 m), respectively. In 2008, *Alytes* was found in 27 ponds (35.5 %) while we detected *Bd* in 30 ponds (39.5 %) and in 236 (15.2 %) out of 1555 amphibians sampled (Table

Table 3.2: Prevalence and infection loads (genomic equivalents) of the all sampled species in *Bd*-positive ponds. $N_{individuals}$ is the number of individuals tested, N_{ponds} gives the number of ponds the species was sampled from.

species	$N_{individuals}$	N_{ponds}	prevalence (± SD)	infection load (± SD)
Alytes obstetricans	104	9	0.577 (± 0.496)	308.976 ± 629.457
Bombina variegata	56	6	0.250 (± 0.437)	5.514 ± 8.664
Bufo bufo	9	6	0	0
Bufo calamita	18	4	0.389 (± 0.502)	0.981 ± 1.203
Hyla arborea	17	4	0.059 (± 0.243)	1.550 ± 4.027
Pelophylax spp.	126	16	0.183 (± 0.388)	8.590 ± 16.246
Rana temporaria	27	8	0	0
Salamandra salamandra	16	2	0	0
Mesotriton alpestris	244	25	0.209 (± 0.407)	2.650 ± 2.988
Triturus cristatus	2	1	0	0
Lissotriton helveticus	74	15	0.054 (± 0.228)	3.773 ± 4.696
Lissotriton vulgaris	24	8	0.292 (± 0.464)	7.887 ± 1.439

3.2). *Alytes* occurred 13 times in the absence of *Bd* and 13 times in the presence of *Bd*. The species was extinct 28 times from *Bd*-negative ponds and 18 times from *Bd*-positive ponds. For four ponds no data on *Bd* presence or absence were available. Compared to the survey in 2003/2004, where *Alytes* had been present in 39 (51 %) of the ponds, it had disappeared from another 12 ponds within four years. These raw statistics do not take imperfect detection into account. *Bd* occurrence data will be made available on www.bd-maps.eu.

Based on model results that take imperfect detection into account, *Bd* was present in (mean [0.025 – 0.975 quartiles of CRI]) 36.1 [13.0 – 67.8] of ponds, while *Alytes* occupied 40.9 [39.0 – 43.0] ponds during 2003/2004 and 35.4 [27.0 – 44.0] ponds during 2008. These estimates are 8% higher for *Bd* and 2.5 (2003/2004) and 11% (2008) for *Alytes* than the naïve estimates based on raw observation (Table 3.3).

Swabs taken from an *Alytes* tadpole were more likely to be positive for *Bd* than swabs from another amphibian species or stage (detection probability for swabs from *Alytes* tadpoles: $p = 0.893$ [0.833 – 0.933]; for all other amphibians: $p = 0.136$ [0.098 – 0.184]). Of the other detection covariates for *Bd*, only swabber identity accounted for some variation in detection probability; however, not all swabbers differed (Table 3.3, Figure 3.1).

None of the climatic variables had any explanatory power in explaining pathogen occurrence, and also the presence of *Mesotriton alpestris* and *Pelophylax spp.*, two widespread species that maintained relatively high *Bd* infection prevalence in this study and may therefore act as reservoir species, did not affect *Bd* occurrence probability. There was also no effect of *Alytes* presence on *Bd* occupancy.

Table 3.3: Parameter estimates on the logit scale for covariates for *Bd* detection p_{Bd}, *Bd* occurrence ψ_{Bd}, *Alytes* detection p_{Alytes}, *Alytes* occurrence ψ_{Alytes} , *Alytes* survival ϕ_{Alytes} and *Alytes* colonisation γ_{Alytes}. q 0.025 and q 0.975 are the lower and upper percentiles of the 95% CRI, Rhat is a measure of parameter convergence (at convergence, Rhat = 1).

	mean	**sd**	**q 0.025**	**q 0.975**	**Rhat**
Bd *detection*					
intercept	-1.587	0.283	-2.202	-1.078	1.001
Alytes	**2.115**	**0.267**	**1.599**	**2.646**	**1.001**
Date	0.247	0.186	-0.117	0.617	1.001
$(\text{date})^2$	0.029	0.082	-0.132	0.19	1.001
swabber1	-0.035	0.316	-0.642	0.602	1.001
swabber2	0.589	0.392	-0.177	1.379	1.001
swabber3	-1.569	0.458	-2.479	-0.675	1.001
swabber4	-0.255	0.316	-0.854	0.390	1.001
Bd *occupancy*					
intercept without *Alytes*	-1.521	1.114	-3.791	0.580	1.002
intercept with *Alytes*	0.054	1.268	-2.490	2.562	1.001
masl	0.640	0.881	-1.321	1.916	1.001
$(\text{masl})^2$	-0.570	0.346	-1.328	0.030	1.001
meantemp	0.096	0.867	-1.753	1.593	1.001
meanprecip	-0.537	0.321	-0.978	0.198	1.001
Alytes	-0.061	1.182	-2.357	2.340	1.001
Pelo	1.309	0.724	-0.084	2.743	1.002
Meal	0.395	0.862	-1.264	2.112	1.002
Alytes *detection*					
intercept 2003/04	0.950	0.305	0.368	1.571	1.001
intercept 2008	-0.028	0.357	-0.587	0.921	1.003
Date	**-0.987**	**0.167**	**-1.333**	**-0.677**	**1.002**
$(\text{date})^2$	**-0.323**	**0.154**	**-0.624**	**-0.024**	**1.001**
method	**-1.627**	**0.615**	**-2.904**	**-0.485**	**1.001**
Alytes *occupancy*					
intercept without *Bd*	0.017	1.642	-3.056	3.440	1.002
intercept with *Bd*	-2.112	1.642	-5.293	1.073	1.002
area	1.092	0.648	-0.032	2.352	1.001
depth	**-0.768**	**0.399**	**-1.589**	**-0.019**	**1.001**
shade	0.816	0.551	-0.271	1.832	1.001
fish	0.448	0.800	-1.078	2.036	1.002
runwater	0.864	0.723	-0.554	2.234	1.001
nrponds	-0.206	0.160	-0.539	0.095	1.001
type forest	1.062	0.833	-0.586	2.644	1.001
type quarry	1.184	0.805	-0.432	2.689	1.001
Pelo	**-2.075**	**0.853**	**-3.858**	**-0.486**	**1.001**
Alytes *survival*					
intercept without *Bd*	12.956	9.645	-0.581	29.15	1.001
intercept with *Bd*	6.604	4.575	-0.089	14.56	1.002
fish	1.947	4.735	-8.139	9.585	1.002
Pelo	1.346	5.045	-8.810	9.558	1.001
Alytes *colonisation*					
intercept without *Bd*	-8.346	3.889	-14.70	-1.718	1.001
intercept with *Bd*	-6.024	4.044	-14.41	-1.247	1.001

For *Alytes*, detection probability depended on date and on the method used (Table 3.3, Figure 3.2). Dip-netting for tadpoles was less suitable to detect *Alytes* than calling surveys.

Because *Alytes* occurred in all ponds in the past, occupancy indicates in which ponds it did not go extinct until 2003/04. *Alytes* persisted more often in large and shallow ponds. The

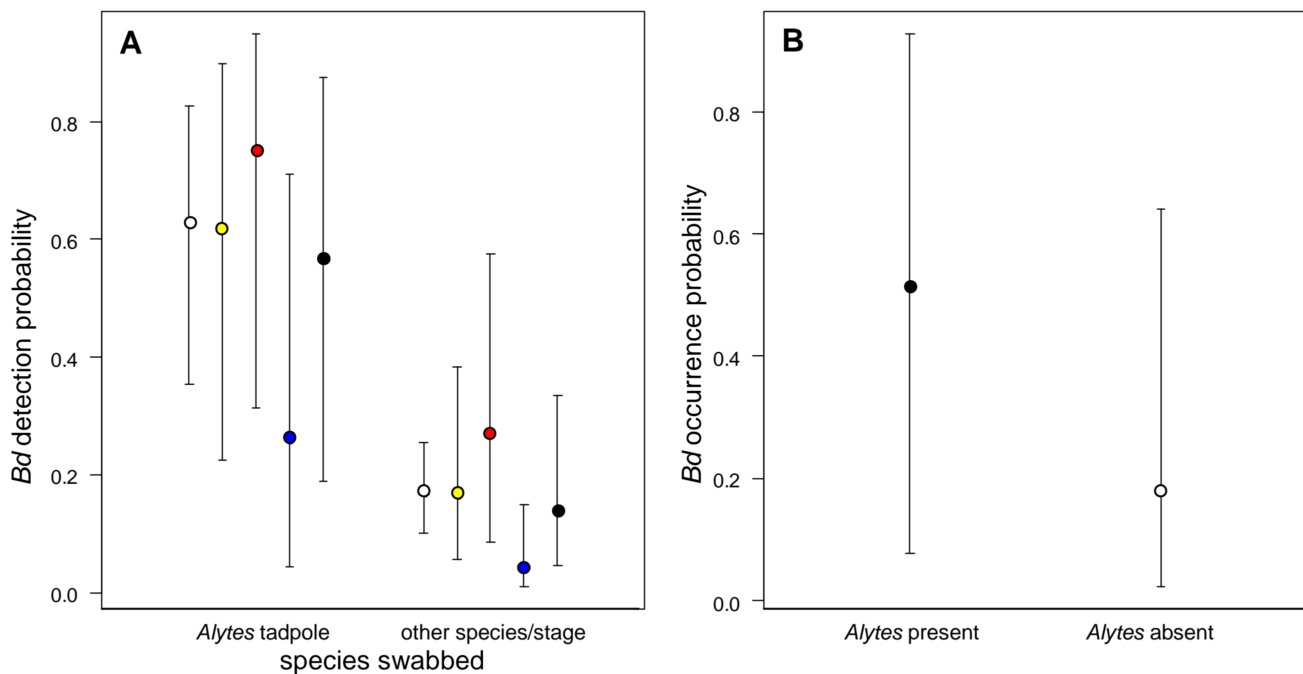

Figure 3.1: Model predictions for *Bd* detection and occurrence probability. A: Detection probability depending on whether the swab was taken from an *Alytes* tadpole or another species/stage. Different colours denote different swabbers. B: *Bd* occurrence probability. Error bars indicate the credible interval.

presence of *Pelophylax spp.* decreased persistence, but the presence of *Bd* did affect neither the occurrence, survival nor the colonisation probability of single populations from 2003/2004 to 2008 (Table 3.3, Figure 3.2).

Discussion

The generally widespread occurrence of *Bd* in Switzerland suggests that the pathogen is enzootic in the area (Briggs *et al.* 2010, Longcore *et al.* 2007, Peyer 2010). Our results suggest that the declines of *Alytes* reported by Schmidt and Zumbach (2005) are ongoing in Switzerland, but that they are seemingly unrelated to *Bd*. The presence of *Alytes* was independent of pathogen presence (and *vice versa*), and the same was true for survival probability.

Bd was more likely to be detected in swabs from *Alytes* tadpoles than in swabs from any other species or life stage. This finding may indicate that *Alytes* tadpoles are particularly susceptible to *Bd* infection, but it certainly indicates that any analysis of *Bd* incidence or

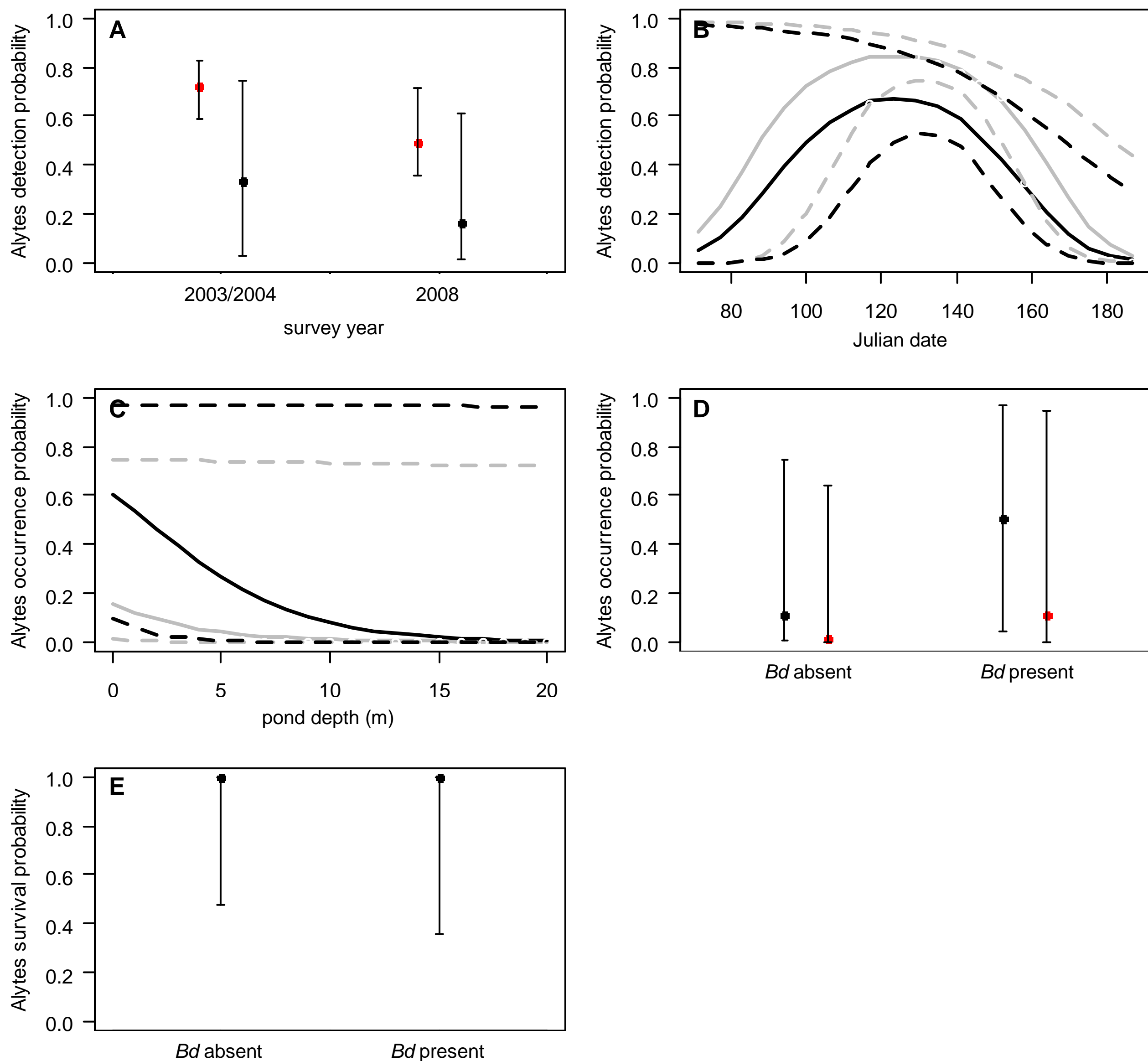

Figure 3.2: Model predictions for *Alytes* detection, occurrence and survival probability. A: Predictions for detection probability depending on survey year and method used; red: dip-netting, black: caller counts. B: Predictions for detection probability depending on date; grey: survey 2003/2004, black: survey 2008. C: Predictions for occurrence probability depending on *Bd* presence and pond depth; grey: *Bd* absent, black: *Bd* present. D: Predictions for occurrence probability depending on the presence of *Bd* and *Pelophylax spp*.; black: *Pelophylax* absent, red: *Pelophylax* present. E: Predictions for survival probability based on *Bd* presence or absence. Error bars and broken lines indicate the 95% CRI.

prevalence should take host-specific detection probabilities into account or else inference may be erroneous.

Because *Alytes* is probably the most susceptible host in the system and because hibernating tadpoles may act as reservoirs for *Bd*, it may be possible that the pathogen fades out after this host went locally extinct. However, this is not what we observed. *Bd* was not

more likely to occur in ponds where *Alytes* has persisted than in ponds from where *Alytes* has been extirpated. We can thus exclude a positive effect of *Alytes* presence on pathogen persistence which might have biased our assessment of the effects of *Bd* on *Alytes*.

Bd was found in all climate regimes and at all elevations in our study area, suggesting a wide tolerance of pathogen occurrence to climate regimes. *Bd* has been present in Switzerland since at least the 1980s (Peyer 2010) and has colonised all areas and altitudes in Switzerland that are inhabited by amphibians. Peyer (2010) found the pathogen at ~2000 m elevation. The wide distribution of the pathogen and its broad tolerance of conditions are in agreement with the model by Rödder *et al*. (2009) which predicts that all areas of Switzerland except for the high-alpine range are suitable for the pathogen.

Despite its high susceptibility, we did not find a negative association between the occurrence of *Bd* and *Alytes*. This is surprising because *Bd* causes mass mortality and massive population declines of *Alytes* in Spain (Bosch *et al.* 2001, Walker *et al.* 2010), and a laboratory experiment shows that Swiss *Alytes* are susceptible to *Bd* and chytridiomycosis (Tobler & Schmidt 2010). We can rule out that our result is a methodological artefact for two reasons. First, our model accounts for imperfect detection of both host and pathogen. Second, even if *Bd* prevalence strongly declined after *Alytes* went locally extinct from a pond, this would not introduce bias. We modelled *Bd* and *Alytes* simultaneously, thus taking into account the error of the probability of *Bd* occurrence when using *Bd* as a covariate for *Alytes* occupancy or extinction probability (McClintock *et al.* 2010, Waddle *et al.* 2010).

The most devastating effects of *Bd* presence on amphibian populations are expected if the pathogen causes massive die-offs as it has been reported for many parts of the world (Berger *et al.* 1998, Lips *et al.* 2006, Rachowicz *et al.* 2006). Although such mass mortality events have been observed in Spanish *Alytes obstetricans* populations, no more than a few dead individuals infected with *Bd* have been found in Switzerland (C. Geiger, B. Schmidt, and U. Tobler, unpublished data). In Australia, Central America, and Spain, a link between environmental conditions and disease outbreaks has been observed (Kriger & Hero 2008, La Marca *et al.* 2005, Pounds *et al.* 2006, Walker *et al.* 2010). While its broad thermal tolerance may allow *Bd* to invade any amphibian community, outbreaks of chytridiomycosis may be limited to certain favourable conditions that do not occur in our study region. On the other hand, evidence is accumulating that strains differ in virulence (Fisher *et al.* 2009a) and phylogenetic analysis of a Swiss *Bd* isolate places it closely to the relatively avirulent

Mallorcan strain (R. Farrer and M. Fisher, pers. comm.). Further, vastly different host-pathogen dynamics may result even in the absence of variation in host susceptibility or pathogen virulence (Briggs *et al.* 2010). In their model, Briggs *et al*. were able to show that variation in density-dependence in the host-pathogen system alone can result in both endemic and epidemic pathogen dynamics.

A lack of association between the presence of *Bd* and the extirpation of *Alytes* is not conclusive evidence for the lack of influence of the pathogen on the host. Enzootic pathogens do not necessarily lead to dramatic declines of host species, but may suppress host populations at a lower abundance (Briggs *et al*. 2005). After the initial invasion of a susceptible host population by the pathogen, it may cause an initial decline in host density. Thereafter, the pathogen may either fade out and the host population can recover, or the host population may remain at a lower level regulated by the pathogen (Briggs *et al*. 2005). In the case of *Bufo boreas* populations positive for *Bd*, the pathogen did not cause a dramatic initial population reduction, but causes a continuous and steady decline of the populations compared to a *Bd*-negative population (Pilliod *et al*. 2010). Alternatively, populations may be able to compensate for pathogen-induced mortality (Anderson & May 1979, Burnham & Anderson 1984, Jolles *et al.* 2006), e.g. via increased survival of uninfected juveniles.

In conclusion, our study provides further evidence that *Bd* does not necessarily cause massive die-offs of susceptible hosts and that the same host-pathogen system can result in vastly different outcomes of disease dynamics. Extirpation of *Alytes* was not associated with *Bd* presence, despite strong negative effects at the individual level and disease-induced mass-mortality of the same species in Spain (Bosch *et al.* 2001, Tobler & Schmidt 2010). In the future, it will be most important to know what mechanisms lead to these different outcomes of disease in order to mitigate *Bd*. In the case of differences in virulence among pathogen strains, preventing the spread of highly virulent strains is of highest priority. A different approach has to be taken if epidemic and endemic disease dynamics are the result of density-dependent mechanisms of host and pathogen (Briggs *et al.* 2010); in this case, manipulating infected host density may provide relief by preventing epidemic disease (Lubick 2010, Woodhams *et al.* 2011). If environmental or climatic conditions determine host-pathogen dynamics, habitat manipulation may provide a valuable tool to mitigate effects of *Bd* presence on amphibian populations.

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References

Anderson, R. M. and May, R. M. (1979). Population biology of infectious diseases: Part I. *Nature* 280: 361-367.

Begon, M. (2008). Effects of host diversity on disease dynamics. Infectious disease ecology: effects of ecosystems on disease and of disease on ecosystems. Ostfeld, R. S., Keesing, F. and Eviner, V. T. Princeton & Oxford, Princeton University Press. 12-29.

Berger, L., Speare, R., Daszak, P., Green, D. E., Cunningham, A. A., Goggin, C. L., Slocombe, R., Ragan, M. A., Hyatt, A. D., McDonald, K. R., Hines, H. B., Lips, K. R., Marantelli, G. and Parkes, H. (1998). Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proceedings of the National Academy of Sciences of the United States of America* 95(15): 9031-9036.

Borgula, A. and Zumbach, S. (2003). Verbreitung und Gefährdung der Geburtshelferkröte (*Alytes obstetricans*) in der Schweiz. *Zeitschrift für Feldherpetologie* 10: 11-26.

Bosch, J., Martínez-Solano, I. and García-París, M. (2001). Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain. *Biological Conservation* 97(3): 331-337.

Boyle, D. G., Boyle, D. B., Olsen, V., Morgan, J. A. T. and Hyatt, A. D. (2004). Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Diseases of Aquatic Organisms* 60(2): 141-148.

Brasier, C. M. and Kirk, S. A. Rapid emergence of hybrids between the two subspecies of Ophiostoma novo-ulmi with a high level of pathogenic fitness. *Plant Pathology* 59(1): 186-199.

Briggs, C. J., Knapp, R. A. and Vredenburg, V. T. (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* 107(21): 9695-9700.

Briggs, C. J., Vredenburg, V. T., Knapp, R. A. and Rachowicz, L. J. (2005). Investigating the population-level effects of chytridiomycosis: An emerging infectious disease of amphibians. *Ecology* 86(12): 3149-3159.

Buchen, L. (2010). Disease epidemic killing only US bats. *Nature* 463: 144-145.

Burnham, K. P. and Anderson, D. R. (1984). Tests of compensatory vs. additive hypotheses of mortality in mallards. *Ecology* 65(1): 105-112.

Daszak, P., Cunningham, A. A. and Hyatt, A. D. (2000). Wildlife ecology - Emerging infectious diseases of wildlife - Threats to biodiversity and human health. *Science* 287(5452): 443-449.

DiGiacomo, R. F. and Koepsell, T. D. (1986). Sampling for detection of infection or disease in animal populations. *Journal of the American Veterinary Medical Association* 189(1): 22-23.

Dodd, K., C. JR. (2009). Amphibian Ecology and Conservation. Oxford, Oxford University Press.

Fisher, M. C., Bosch, J., Yin, Z., Stead, D. A., Walker, J., Selway, L., Brown, A. J. P., Walker, L. A., Gow, N. A. R., Stajich, J. E. and Garner, T. W. J. (2009a). Proteomic and phenotypic profiling of the amphibian pathogen *Batrachochytrium dendrobatidis* shows that genotype is linked to virulence. *Molecular Ecology* 18(3): 415-429.

Fisher, M. C., Garner, T. W. J. and Walker, S. F. (2009b). Global emergence of *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis in space, time, and host. *Annual Review of Microbiology* 63: 291-310.

Gosner, K. L. (1960). A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetological Journal* 16: 183-190.

Grossenbacher, K. (1988). Verbreitungsatlas der Amphibien der Schweiz. *Documenta faunistica helvetiae* 7: 1-207.

Hijmans, R. J., Cameron, S. E., Parra, J. L., Jones, P. G. and Jarvis, A. (2005). Very high resolution interpolated climate surfaces for global land areas. *International Journal of Climatology* 25(15): 1965-1978.

Jennelle, C. S., Cooch, E. G., Conroy, M. J. and Senar, J. C. (2007). State-specific detection probabilities and disease prevalence. *Ecological Applications* 17(1): 154-167.

Jolles, A. E., Cooper, D. V. and Levin, S. A. (2005). Hidden effects of chronic tuberculosis in African buffalo. *Ecology* 86(9): 2358-2364.

Jolles, A. E., Etienne, R. S. and Olff, H. (2006). Independent and competing disease risks: Implications for host populations in variable environments. *American Naturalist* 167(5): 745-757.

Kéry, M. (2010). Introduction to WinBUGS for Ecologists. London, Elsevier.

Kéry, M. and Schmidt, B. R. (2008). Imperfect detection and its consequences for monitoring for conservation. *Community Ecology* 9(2): 207-216.

Kriger, K. M. and Hero, J. M. (2008). Altitudinal distribution of chytrid (*Batrachochytrium dendrobatidis*) infection in subtropical Australian frogs. *Austral Ecology* 33(8): 1022-1032.

La Marca, E., Lips, K. R., Lotters, S., Puschendorf, R., Ibanez, R., Rueda-Almonacid, J. V., Schulte, R., Marty, C., Castro, F., Manzanilla-Puppo, J., García-Perez, J. E., Bolanos, F., Chaves, G., Pounds, J. A., Toral, E. and Young, B. E. (2005). Catastrophic population declines and extinctions in neotropical harlequin frogs (Bufonidae: *Atelopus*). *Biotropica* 37(2): 190-201.

Lips, K. R., Brem, F., Brenes, R., Reeve, J. D., Alford, R. A., Voyles, J., Carey, C., Livo, L., Pessier, A. P. and Collins, J. P. (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(9): 3165-3170.

Longcore, J. R., Longcore, J. E., Pessier, A. P. and Halteman, W. A. (2007). Chytridiomycosis widespread in anurans of Northeastern United States. *Journal of Wildlife Management* 71(2): 435-444.

Lubick, N. (2010). Emergency medicine for frogs. *Nature* 465: 680-681.

MacKenzie, D. I., Bailey, L. L. and Nichols, J. D. (2004). Investigating species co-occurrence patterns when species are detected imperfectly. *Journal of Animal Ecology* 73(3): 546-555.

MacKenzie, D. I., Nichols, J. D., Hines, J. E., Knutson, M. G. and Franklin, A. B. (2003). Estimating site occupancy, colonization, and local extinction when a species is detected imperfectly. *Ecology* 84(8): 2200-2207.

MacKenzie, D. I., Nichols, J. D., Lachman, G. B., Droege, S., Royle, J. A. and Langtimm, C. A. (2002). Estimating site occupancy rates when detection probabilities are less than one. *Ecology* 83(8): 2248-2255.

May, R. M. and Anderson, R. M. (1979). Population biology of infectious diseases: Part II. *Nature* 280: 455-461.

McClintock, B. T., Nichols, J. D., Bailey, L. L., MacKenzie, D. I., Kendall, W. L. and Franklin, A. B. (2010). Seeking a second opinion: uncertainty in disease ecology. *Ecology Letters* 13(6): 659-674.

Peyer, N. F. (2010). Historical evidence for the presence of the emerging amphibian pathogen Batrachochytrium dendrobatidis (Longcore *et al*. 1999) in Switzerland. M.Sc. thesis. Zurich, Switzerland, University of Zurich.

Pilliod, D. S., Muths, E., Scherer, R. D., Partelt, P. E., Corn, P. S., Hossack, B. R., Lambert, B. A., McCaffery, R. and Gaughan, C. (2010). Effects of amphibian chytrid fungus on individual survival probability in wild boreal toads. *Conservation Biology* 24(5): 1259-1267.

Porter, J. W., Lipp, E. K., Sutherland, K. P. and Mueller, E. (2008). The ecology of an infectious coral disease in the Florida Keys: from pathogens to politics. Infectious disease ecology: effects of ecosystems on disease and of disease on ecosystems. Ostfeld, R. S., Keesing, F. and Eviner, V. T. Princeton & Oxford, Princeton University Press. 387-403.

Pounds, J. A., Bustamante, M. R., Coloma, L. A., Consuegra, J. A., Fogden, M. P. L., Foster, P. N., La Marca, E., Masters, K. L., Merino-Viteri, A., Puschendorf, R., Ron, S. R., Sanchez-Azofeifa, G. A., Still, C. J. and Young, B. E. (2006). Widespread amphibian extinctions from epidemic disease driven by global warming. *Nature* 439(7073): 161-167.

Rachowicz, L. J., Knapp, R. A., Morgan, J. A. T., Stice, M. J., Vredenburg, V. T., Parker, J. M. and Briggs, C. J. (2006). Emerging infectious disease as a proximate cause of amphibian mass mortality. *Ecology* 87(7): 1671-1683.

Rödder, D., Kielgast, J., Bielby, J., Schmidtlein, S., Bosch, J., Garner, T. W. J., Veith, M., Walker, S. F., Fisher, M. C. and Lötters, S. (2009). Global amphibian extinction risk assessment for the panzootic chytrid fungus *Diversity* 1: 52-66.

Rödder, D., Veith, M. and Lötters, S. (2008). Environmental gradients explaining the prevalence and intensity of infection with the amphibian chytrid fungus: the host's perspective. *Animal Conservation* 11(6): 513-517.

Royle, J. A. and Dorazio, R. M. (2008). Hierarchical Modeling and Inference in Ecology: The Analysis of Data from Populations, Metapopulations and Communities. London, Elsevier.

Royle, J. A. and Kery, M. (2007). A Bayesian state-space formulation of dynamic occupancy models. *Ecology* 88(7): 1813-1823.

Schmidt, B. R., Furrer, S., Kwet, A., Lötters, S., Rödder, D., Sztatecsny, M., Tobler, U. and Zumbach, S. (2009). Desinfektion als Maßnahme gegen die Verbreitung der Chytridiomykose bei Amphibien. Methoden der Feldherpetologie. Hachtel, M., Schlüpmann, M., Thiesmeier, B. and Weddeling, K. Bielefeld, Laurenti Verlag. 229–241

Schmidt, B. R. and Zumbach, S. (2005). Rote Liste der gefährdeten Amphibien der Schweiz. Bundesamt für Umwelt, Wald und Landschaft, Bern, und Koordinationsstelle Amphibien- und Reptilienschutz in der Schweiz, Bern.

Smith, K. F., Sax, D. F. and Lafferty, K. D. (2006). Evidence for the role of infectious disease in species extinction and endangerment. *Conservation Biology* 20(5): 1349-1357.

Tobler, U. and Schmidt, B. R. (2010). Within- and among-population variation in chytridiomycosis-induced mortality in the toad *Alytes obstetricans*. *PLoS ONE* 5(6): e10927.

Waddle, J. H., Dorazio, R. M., Walls, S. C., Rice, K. G., Beauchamp, J., Schuman, M. J. and Mazzotti, F. J. (2010). A new parameterization for estimating co-occurrence of interacting species. *Ecological Applications* 20(5): 1467-1475.

Walker, S. F., Bosch, J., Gomez, V., Garner, T. W. J., Cunningham, A. A., Schmeller, D. S., Ninyerola, M., Henk, D., Ginestet, C., Arthur, C.-P. and Fisher, M. C. (2010). Factors driving pathogenicity vs. prevalence of amphibian panzootic chytridiomycosis in Iberia. *Ecology Letters* 13(3): 372-382.

Woodhams, D. C., Bosch, J., Briggs, C. J., Cashins, S., Davis, L. R., Lauer, A., Muths, E., Puschendorf, R., Schmidt, B. R., Sheafor, B. and Voyles, J. (2011). Mitigating amphibian disease: strategies to maintain wild populations and control chytridiomycosis. *Frontiers in Zoology* 8(8).

POPULATIONS OF A SUSCEPTIBLE AMPHIBIAN SPECIES CAN GROW DESPITE THE PRESENCE OF A PATHOGENIC CHYTRID FUNGUS

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Abstract

Diseases can be important drivers of host population dynamics and disease epidemics can cause severe host population declines. *Batrachochytrium dendrobatidis* (*Bd*), the pathogen causing amphibian chytridiomycosis, can occur both as an epidemic and enzootic stage and causes diverse negative effects on amphibian populations. While effects of *Bd* epidemics are well documented, the effects of enzootic *Bd* have rarely been described. We test whether population trends of a species highly susceptible to *Bd*, the midwife toad *Alytes obstetricans*, are negatively affected by the enzootic presence of the pathogen, and whether habitat management can ameliorate effects of *Bd* on the populations. Unexpectedly, *Bd* had no negative effect on population trends although the species is known to be susceptible to chytridiomycosis. This suggests that negative effects of disease on individuals do not necessarily translate into negative effects at the population level. In conclusion, populations of amphibian species that are susceptible to the emerging disease chytridiomycosis can persist despite the enzootic presence of the pathogen.

Key words: additive and compensatory mortality, *Alytes obstetricans*, *Batrachochytrium dendrobatidis,* chytridiomycosis, count data, disease, population trend

Introduction

Parasites and pathogens can be important drivers of host population dynamics by altering host behaviour, demography or genetics (Anderson & May 1979, Ibelings *et al.* 2004, Jolles *et al.* 2006, Tompkins & Begon 1999). The most extreme way how a pathogen can affect its host is host extinction (de Castro & Bolker 2005). However, host extinction is rare for two main reasons. First, parasites and their hosts generally share a common history and extinction of either antagonist is unlikely (Ebert & Hamilton 1996). Second, when host density declines, the pathogen's transmission rate is expected to drop, too, unless there is frequency-dependent transmission or a reservoir host (de Castro & Bolker 2005). However, emerging diseases are different from established pathogens because parasite interactions with novel hosts are unpredictable and can sometimes lead to host population declines and extinctions (Ebert 1994, Read 1994). Unfortunately, while effects of disease on vital rates of captive populations are well documented, the effects on dynamics of wild populations are still poorly understood (Deem *et al.* 2008, Jolles *et al.* 2006, Tompkins & Begon 1999, Tompkins *et al.* 2001). For example, a reduction in population size is only expected if pathogen-induced mortality is additive rather than compensatory (Burnham & Anderson 1984, Jolles *et al.* 2006). If pathogen-induced mortality is additive, then overall mortality is the sum of pathogen-induced mortality plus mortality inflicted by all other causes. In contrast, if mortality is compensatory, then increased mortality due to the presence of *Bd* is countered by a reduction in mortality due to other causes, often in a density-dependent manner (Lebreton 2005). Here, our goal is to contribute to a better understanding of an emerging infectious disease on host population dynamics.

An emerging pathogen of amphibians, the fungus *Batrachochytrium dendrobatidis* (hereafter *Bd*), contributes to the global amphibian declines and extinctions on many continents (Fisher *et al.* 2009b, Skerratt *et al.* 2007, Stuart *et al.* 2004). The pathogen is spreading in a wave-like fashion and amphibian populations collapsed after emergence of the pathogen (Lips *et al.* 2006, Wake & Vredenburg 2008). However, host extinction is not the only outcome of *Bd* emergence in new localities. In many places, the pathogen is widespread at sometimes high prevalence. Thus, it is apparently enzootic without causing much harm to amphibian populations (Longcore *et al.* 2007, Retallick *et al.* 2004, Walker *et al.* 2010). The amphibian host – chytrid pathogen models by Briggs *et al*. (2010, 2005) suggest that enzootic *Bd*-infection may cause initially a reduction in abundance but

thereafter populations remain stationary. Yet, due to a lack of time series data on abundance of amphibian populations with enzootic *Bd*, we cannot state with certainty that enzootic *Bd*-infection causes no harm to amphibian populations. In contrast to the model predictions, the mark-recapture analysis of Pilliod *et al*. (2010) showed that *Bd* depressed both individual survival of *Bd*-infected toads and population growth rates, leading to a steady population decline.

Our goal was to quantify the effects of enzootic *Bd* on populations of an amphibian species that is known to be susceptible to *Bd* (Bosch *et al.* 2001, Tobler & Schmidt 2010, Walker *et al.* 2010). Ideally, one would compare population sizes before and after the emergence of *Bd* in a population. Unfortunately, such data are rarely available (Laurance *et al.* 1996, Lips *et al.* 2006, Teacher *et al.* 2010). We compare population monitoring data from sites where *Bd* is present with population monitoring data from sites where *Bd* is absent. We analyse short series of caller count data from 26 populations of the common midwife toad, *Alytes obstetricans*, in the Swiss canton Lucerne. The genus *Alytes* has an unusual reproductive strategy that may make it especially susceptible to *Bd*-induced declines: 1) Reproductive output is low because females only lay between 5 to 77 eggs per clutch and up to threee clutches per year. However, hatching success is relatively high due to paternal care. 2) Tadpoles grow to a large size and can spend more than a year in the aquatic phase; they commonly hibernate especially in cold water bodies (Nöllert & Nöllert 1992). This increases infection risk due to a prolonged exposure to *Bd* in the water. Assuming that *Bd* leads to chytridiomycosis-induced mortality of individuals (Bosch *et al.* 2001, Tobler & Schmidt 2010, Walker *et al.* 2010) and that mortality is additive rather than compensatory (Burnham & Anderson 1984, Jolles *et al.* 2006), we expect to find declining populations in presence of the pathogen while populations free of the pathogen should be either stationary or growing. The pathogen has been present in Switzerland since at least the early 1980s (Peyer 2010), is widespread and prevalence often high (Garner *et al.* 2005, Schmidt *et al.* 2009a; U. Tobler & B. R. Schmidt, unpublished data). Yet, no chytridiomycosis-induced mass mortality as it was reported from Spain has been observed in Switzerland, including our study area. However, a few dead metamorphs were detected at two sites in the study area, and they tested positive for *Bd* (U. Tobler, C. Geiger & B. R. Schmidt, unpublished data).

Alytes obstetricans is a red-listed species in Switzerland and therefore the target of conservation actions (Borgula & Zumbach 2003, Schmidt & Zumbach 2005). Knowing

whether and to what degree *Bd* poses a threat to *Alytes* survival in this area is vital to apply suitable conservation strategies. Currently, no habitat mitigation methods are available barring ex-situ captive breeding although mitigation methods using antifungal chemicals or bacterial treatments are currently being tested (Lubick 2010, Woodhams *et al.* 2011).

Materials and Methods

Study sites

Based on the availability of population count data, 26 sites in canton Lucerne, Switzerland, were included in the analysis. All sites are situated between 46.871° and 47.258° N and 7.882° and 8.382° E (Figure 4.1) and along an altitudinal gradient raging from 402 to 1330 masl. Mean summer temperatures range from 12.3 to 17.5°C (Hijmans *et al.* 2005). Habitat types include quarries, ponds in open meadows, garden ponds, fire water reservoirs, or alpine and pre-alpine streams.

Population monitoring

Populations of *Alytes obstetricans* were regularly monitored as part of the midwife toad action plan of the Swiss canton Lucerne (Amt für Natur- und Landschaftsschutz 2000, Borgula & Zuberbühler 2010). Annual counts of the number of calling *Alytes obstetricans* males were obtained from 2002 to 2008 except for four sites where *Alytes obstetricans* was reintroduced and callers only occurred after 2002. Every site was visited at least twice a year by experienced volunteers. Volunteers were free to choose the nights for the monitoring, but advised to do so during optimal warm and humid nights when detectability, i.e. calling activity, of *Alytes obstetricans* is high (Schmidt 2005). The number of calling males, an index to population size (Schmidt 2004), was recorded during every visit. We used the highest number of calling males within a year in the analysis. In our analysis of population trends, detectability may vary among years but we assume that detectability shows no temporal trend such that population trends can be reliably estimated from caller counts (Bart *et al.* 2004, Kéry & Schmidt 2008). Trend estimates are unlikely to depend on *Bd* infection status because observers were unaware of *Bd* infection status of a site. Hence, we can exclude an interaction between *Bd* presence and detectability that would have biased our conclusion on

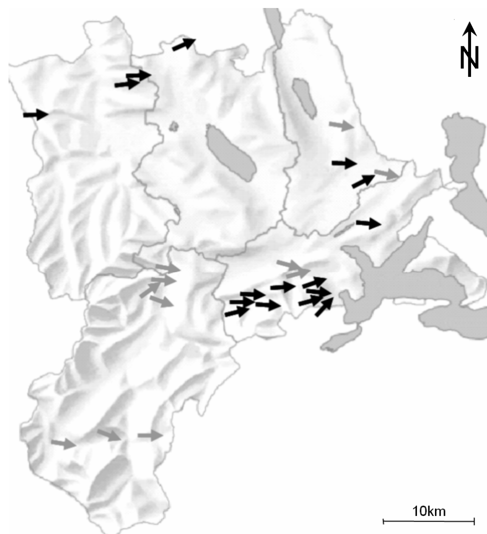

Figure 4.1: Topographic map of the canton Lucerne and study sites. Lakes are shaded in grey. Grey arrows mark *Bd*-negative sites; black arrows mark *Bd*-positive sites. The steepness of the arrow represents population trend. Population trends were scaled such that the population with the largest population trend has an arrow pointing upwards in a 45° angle.

the impact of *Bd* on population trends. In addition to the caller count data, presence or absence of tadpoles (based on visual encounter surveys, Skelly & Richardson 2009) was noted; this provided us with the variable reproduction, which indicates the number of years tadpoles were observed in the breeding water body.

Sampling for Bd

During summer 2007 and spring 2008 or 2009, all sites were sampled for the presence of *Bd*. 16 to 47 (mean ± SD: 26.0 ± 6.6) amphibians were caught and swabbed with a sterile cotton swab (Copan Italia S.p.A., Brescia, Italy). Exceptions are sites "Sagerhüsli", where only two dead *Alytes* metamorphs were sampled, and "Hombrig", where sample size was six. Because both these sites tested positive for *Bd* we do not expect the small sample sizes to affect our results because the goal was to determine presence or absence of *Bd.* Because sample sizes were large at all other sites (mean ± SD for sites that tested negative: 26.5 ± 7.6), we are confident that we detected *Bd* where it occurred at an ecologically relevant prevalence (DiGiacomo & Koepsell 1986 and results section). Sampling was done

opportunistically, i.e. all available amphibian species were sampled. Apart from midwife toads, the other amphibians sampled were the fire-bellied toad *Bombina variegata*, the

Table 4.1: Estimates of infection prevalence and intensity in *Alytes* tadpoles and in all other species (pooled) for all study ponds. Prevalence is denoted in proportion of infected individuals, infection intensity is denoted as mean zoospore equivalents (genomic equivalents) per individual ± 1 SE.

	Alytes		other species	
population	prevalence	infection intensity	prevalence	infection intensity
Ämmenmatt	0		0	
Ballwil	-	-	0	0.025 (± 0.026)
Chalchloch	0		-	-
Chräuel	0.786	0.686 (± 0.725)	0.118	0.171 (± 0.283)
Egghütten	0	-	-	-
Ehrendingen	0	-	-	-
Einsamkeit	-	-	0.407	0.868 (± 1.022)
Fontanne	0	-	-	-
Grisigen	0.950	244.676 (± 276.418)	0.167	12.260 (± 0.000)
Hergiswald	0.647	2.158 (± 2.601)	0.154	0.142 (± 0.141)
Hilferenmättili	0	-	0	-
Hiltbrunnen	0.750	17.553 (± 2.789)	0.478	1.699 (± 2.607)
Hinter Rohren	0.333	21.596 (± 5.852)	0	-
Hochrüti	-	-	0	-
Hohenrain	-	-	0	-
Hombrig	0.200	2.92 (± 0.000)	0	-
Lätten	0	-	0.061	0.186 (± 0.195)
Linden	-	-	0	-
Ottigenbüel	-	-	0.080	0.416 (± 0.032)
Pfaffwil	-	-	0.654	1.823 (± 2.739)
Räschenhus	0.667	6.659 (± 8.018)	0.105	0.111 (± 0.060)
Rossei	0	-	-	-
Sagerhüsli	1.000	20.735 (± 1.888)	-	-
Schauensee	0.800	603.22 (± 360.954)	0.450	21.397 (± 61.641)
Schlagweiher	-	-	0.167	0.301 ± (0.408)
Unter Utigen	1.000	-	0.222	4.297 (± 8.557)

waterfrogs *Pelophylax lessonae* and *Pelophylax esculentus* (the two taxa were pooled because they form a hybridogenetic complex, Schmidt 1993), the alpine newt *Mesotriton alpestris* and the palmate newt *Lissotriton helveticus*. In 6 out of 11 sites that were *Bd*-negative, samples were exclusively obtained from *Alytes* tadpoles. Due to the prolonged larval period *Alytes* tadpoles are more likely to be infected than any other species or life stage in this system (see results). Standard hygiene recommendations were followed during field work (Schmidt *et al.* 2009a, b).

Extraction and analysis for *Bd*-DNA were done following the rt-PCR protocol by Boyle *et al.* (2004) using *Bd*-specific primers and standards to quantify the amount of DNA (the latter corresponds to infection load). We ran each sample twice and the PCR was repeated if the

two wells returned unequal results. We report untransformed values and reactions below 0.1 genomic equivalents were scored *Bd*-negative (Tobler & Schmidt 2010).

Statistical analysis

We tested for differences in prevalence of *Bd* among the sampled species using a generalised linear mixed model (glmm) with a binomial error distribution. We tested for differences in infection intensity among species using a linear mixed model (lmm) with a normal error distribution. In both analyses, we used site as grouping (random) variable. Both analyses were done in R 2.8.1. (R Development Core Team 2008).

We used a Bayesian state-space model implemented in WinBUGS 1.4 (Lunn *et al*. 2000) to explore the effects of *Bd* presence and the frequency of reproduction observed on population trends. State-space models disentangle the effects of the biological process and the observation component on an observed time series of counts. A process and observation error can be included that account for biological variability not explained by the model and imperfect detection in the observed counts (Kéry & Schaub *in preparation*). For every population, we built a model that estimates the observation and biological process on two hierarchical levels:

```
count[i,t] ~ dnorm(N.est[i,t], tau.obs[i])        observation process

N.est[i,t+1] <- N.est[i,t] * lambda[i,t]          biological process
```

Hence, the counts at site *i* during observation *t* are modelled as normally distributed draws from a distribution with mean *N.est* and precision *tau.obs*. *tau.obs* accounts for variation in the counts due to an imperfect observation process. The estimated true number of individuals *N.est* during visit *t+1* at site *i* is a function of *N.est* at time *t* times the population growth rate *lambda*. *lambda* was defined to be year- and site-specific and had mean of *mean.lambda* and precision *tau.lambda*:

```
lambda[i,t] ~ dnorm(mean.lambda[i], tau.lambda[i])
```

mean.lambda was the average population trend of a population during the survey period. Values below 1 indicate declining populations while values above 1 indicate growing population sizes. *tau.lambda* accounts for biological variation in population size that is not explained by the model. The effect of *Bd* presence and reproduction on population trends were calculated as follows:

```
mean.lambda[i] <- alpha + beta.Bd * Bd[i] + beta.repro * repro[i]
```

Table 4.2: Population trends as calculated by the Bayesian model. *Bd* is the *Bd* status of a site (1=positive) mean lambda is the average population growth rate across all 8 survey years. sigma[growth rate] gives the standard deviation associated with the error in the estimation of mean lambda, sigma[observation] gives the error in the observation process. All estimates are given as means ± standard deviation with the 95% CRI in brackets.

	Bd	mean lambda	sigma[growth rate]	sigma[observation]
Aemmenmatt	0	0.871 ± 0.063 (0.740 - 0.989)	5.271 ± 12.056 (0.122 - 43.832)	3.140 ±21.863 (0.001 - 18.802)
Ballwil	1	1.013 ± 0.052 (0.921 - 1.123)	0.536 ± 1.391 (0.002 - 2.828)	43.395 ± 57.075 (0.153 - 190.000)
Chalchloch	0	0.968 ± 0.094 (0.757 - 1.128)	4.654 ± 11.069 (0.008 - 38.782)	68.574 ± 91.777 (1.212 - 327.005)
Chräuel	1	1.037 ± 0.045 (0.953 - 1.132)	0.592 ± 1.802 (0.002 - 3.098)	13.555 ± 33.003 (0.007 - 83.283)
Egghütten	0	0.895 ± 0.067 (0.752 - 1.017)	0.462 ± 1.054 (0.002 - 2.515)	8.220 ± 10.648 (0.014 - 35.720)
Ehrendingen	0	0.968 ± 0.094 (0.757 - 1.128)	0.092 ± 0.246 (0.001 - 0.446)	10.466 ± 33.411 (0.003 - 58.931)
Einsamkeit	1	0.964 ± 0.074 (0.843 - 1.121)	7.355 ± 13.905 (0.005 - 52.242)	11.565 ± 18.070 (0.491 - 52.482)
Fontanne	0	0.920 ± 0.074 (0.756 - 1.049)	7.130 ± 12.229 (0.008 - 45.040)	190.983 ± 214.982 (0.056 - 761.725)
Grisigen	1	1.110 ± 0.052 (1.015 - 1.212)	1.657 ± 2.398 (0.107 - 6.936)	146.905 ± 225.389 (0.032 - 791.812)
Hergiswald	1	1.086 ± 0.045 (1.001 - 1.174)	0.518 ± 1.668 (0.000 - 3.637)	140.024 ± 120.709 (13.609 - 493.800)
Hilferenmättili	0	0.920 ± 0.074 (0.756 - 1.049)	0.321 ± 1.214 (0.000 - 1.946)	3.417 ± 3.903 (0.437 - 12.890)
Hiltbrunnen	1	1.061 ± 0.043 (0.980 - 1.144)	0.072 ± 0.107 (0.000 - 0.308)	3.015 ± 6.509 (0.002 - 13.750)
Hinter Rohren	1	1.135 ± 0.062 (1.021 - 1.256)	0.098 ± 0.121 (0.005 - 0.371)	9.704 ± 23.339 (0.003 - 58.094)
Hochrüti	0	0.773 ± 0.079 (0.610 - 0.936)	1.137 ± 1.770 (0.209 - 4.371)	0.030 ± 0.417 (0.000 - 0.248)
Hohenrain	0	0.773 ± 0.079 (0.610 - 0.936)	0.061 ± 0.114 (0.000 - 0.288)	0.501 ± 1.150 (0.000 - 2.653)
Hombrig	1	1.086 ± 0.045 (1.001 - 1.174)	0.166 ± 0.323 (0.000 - 0.901)	16.112 ± 22.979 (0.084 - 70.225)
Lätten	1	1.061 ± 0.043 (0.980 - 1.144)	1.006 ± 2.917 (0.001 - 5.459)	18.746 ± 31.239 (0.035 - 90.531)
Linden	1	1.013 ± 0.052 (0.921 - 1.123)	0.745 ± 1.570 (0.004 - 3.461)	4.973 ± 8.869 (0.006 - 25.180)
Ottigenbüel	1	0.964 ± 0.074 (0.843 - 1.121)	0.098 ± 0.356 (0.000 - 0.464)	0.297 ± 0.716 (0.001 - 1.614)
Pfaffwil	0	0.846 ± 0.062 (0.724 - 0.966)	1.044 ± 2.237 (0.005 - 5.817)	91.589 ± 107.273 (1.827 - 405.707)
Räschenhus	1	1.086 ± 0.045 (1.001 - 1.174)	0.766 ± 2.305 (0.019 - 4.071)	6.120 ± 9.027 (0.456 - 26.250)
Rossei	0	0.846 ± 0.062 (0.724 - 0.966)	3.107 ± 8.811 (0.001 - 26.770)	8.242 ± 31.220 (0.006 - 54.762)
Sagerhüsli	1	1.110 ± 0.052 (1.015 - 1.212)	0.114 ± 0.197 (0.001 - 0.488)	8.125 ± 14.127 (0.023 - 38.550)
Schauensee	1	1.037 ± 0.045 (0.953 - 1.132)	0.114 ± 0.198 (0.004 - 0.464)	6.438 ± 14.275 (0.002 - 37.001)
Schlagweiher	1	1.086 ± 0.045 (1.001 - 1.174)	4.180 ± 6.715 (0.030 - 21.820)	76.575 ± 130.830 (0.024 - 457.207)
Unter Utigen	1	1.037 ± 0.045 (0.953 - 1.132)	1.731 ± 3.615 (0.010 - 10.011)	13.619 ± 24.959 (0.003 - 71.705)

The average population trend of a population hence was modelled as a function of *Bd*-presence and the frequency of reproduction observed at a site with a constant intercept alpha per site.

Results

We detected *Bd* in 16 out of the 26 (61.5 %) sites and in 16.5 % of sampled amphibians. *Bd* was not found in any site in the Entlebuch valley, which encompassed the south-western cluster of populations (Figure 4.1). The two dead metamorphs collected at Sagerhüsli in 2007 tested positive for *Bd* with an average infection intensity of 19.4 ± 4.5 genomic equivalents. 4 out of 5 dead metamorphs collected at Schauensee in 2010 tested positive with an average infection intensity of 2.9 ± 1.8 genomic equivalents (U. Tobler, C. Geiger & B. R. Schmidt, unpublished data).

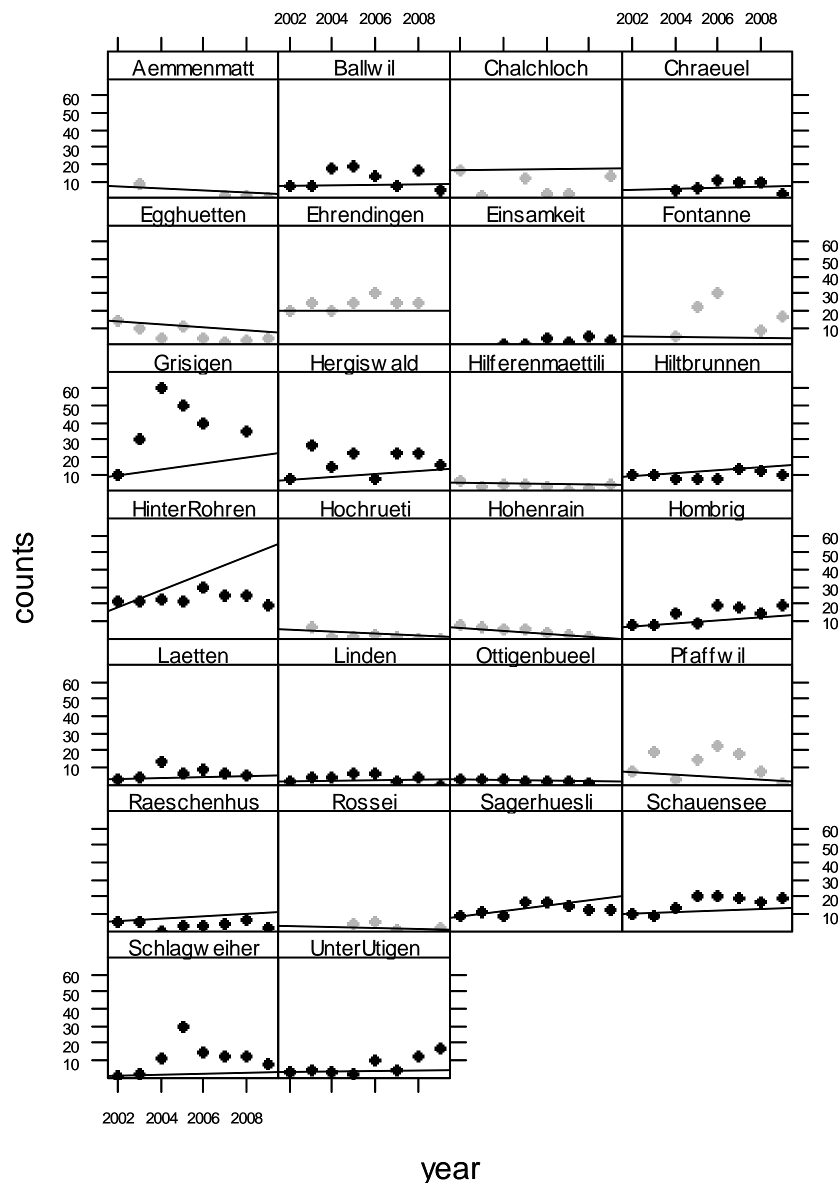

Figure 4.2: Observed counts per population and year. The dots mark the real counts, the solid line shows the predicted growth rate based on the model results. *Bd*-positive sites are in black, *Bd*-negative sites are in grey.

Alytes tadpoles had higher infection prevalence (mean proportion of individuals carrying *Bd* per pond (mean, [range]): *A. obstetricans:* 0.57 [0 – 0.95]; *Pelophylax* spp.: 0.27 [0.18 – 0.68]; *M. alpestris*: 0.24 [0 – 0.67]; *L. helveticus*: 0.14 [0 – 0.60]) and infection intensity than the other species (*A. obstetricans:* 112.4 ± 215.0 genomic equivalents, *Pelophylax* spp.: 4.2 ± 5.7, *M. alpestris*: 9.4±29.0, *L. helveticus*: 0.9 ± 1.3). Within positive sites, the species differed significantly in infection prevalence (glmm, z=-4.250, p=0.0175) and intensity (=*Bd* genomic equivalents; lmm, t=-3.551, p<0.001) respectively (Table 4.1).

Alytes populations had an average of 10.8 ± 9.1 (SD) calling males (range within single years: 0-75 callers; range of mean number of callers per population: 2-43). Between 2002 and 2009, tadpoles were observed in 22 out of the 26 ponds during at least one visit; in

Table 4.3: Parameter estimates for the effects of the presence of *Bd* and reproduction on population growth rates.

parameter	mean	SD	95% CRI
intercept	0.773	0.079	0.610 - 0.936
Bd	0.191	0.076	0.046 - 0.353
repro	0.024	0.015	-0.007 - 0.051
deviance	518.831	149.031	219.997 - 768.407

those ponds where tadpoles were observed, they were seen in 4.8 ± 1.7 years on average out of the 7 study years.

The mean population growth rates of all populations varied from 0.773 to 1.135; the average population growth rate of all populations during the 8-year study period was (mean ± SD) 0.987 ± 0.105 (Table 4.2). This means that on average the number of calling males in the region remained stable. Populations that tested *Bd*-positive had an increased population growth rate that was by 0.191 (95% CRI 0.046 – 0.353) higher than that of *Bd*-negative populations. Populations where reproduction had been observed in a higher number of study years also had an increased growth rate compared to populations where reproduction was not or only rarely observed; every year of observed reproduction increased the growth rate by 0.024 (95% CRI -0.007 – 0.051; Table 4.3).

Discussion

Bd-positive populations did not show more negative population trends than those that tested negative for the pathogen. This result is unexpected. The absence of negative effects of *Bd* on population trends contrasts strongly with many studies that report dramatic negative effects of *Bd* on amphibian populations, including global extinction of species (Lips *et al.* 2006, Schloegel *et al.* 2006, Skerratt *et al.* 2007). It is even more surprising since *Alytes obstetricans* is known to be highly susceptible to *Bd* (Bosch *et al.* 2001, Tobler & Schmidt 2010, Walker *et al.* 2010). The result is robust even if we should have missed *Bd* in some populations. If all populations that we scored as *Bd*-negative would be *Bd*-positive, then the average population trend would still be positive.

Population models suggest that high juvenile mortality lowers population growth rates in species with complex life cycles (Conroy & Brook 2003, Hels & Nachman 2002, Lampo & De Leo 1998). Hence, we expected that high chytridiomycosis-induced juvenile mortality in *Alytes obstetricans* (Tobler & Schmidt 2010) would lead to population declines. Apparently, and even though we observed some *Bd*-infected dead metamorphs in the field, juvenile

mortality did not translate into effects at the population level. There are several possible explanations why there were no population-level effects of *Bd* despite the strong individual-level effects reported by Tobler and Schmidt (2010). The explanations are not mutually exclusive.

The first explanation is based on the fact that environmental conditions, especially those related to altitude, may mediate the effects of *Bd* on amphibian populations (Bosch *et al.* 2007, Fisher *et al.* 2009b, Walker *et al.* 2010). It may be that environmental conditions, especially climate, in our study area were such that *Bd* did not cause mortality in the field. Also, *Bd* infection intensity observed was relatively low in all species. However, we do not think that the populations that we studied experience environmental conditions where *Bd* has no effect on amphibian populations. First, some dead and *Bd*-positive metamorphs were observed at two of our study sites. Second, one fifth of our populations were within the summer temperature range within which fatal chytridiomycosis is observed in Spain (Walker *et al.* 2010). Further, because *Bd* occurred at all elevations and thus all climate regimes within our study region, we can exclude that altitude was confounded with *Bd* presence in a way that e.g. high altitude sites were both *Bd* negative and had declining population trends.

A second explanation may be that *Bd*-induced mortality could be compensatory rather than additive when *Bd* is enzootic. A decline in population size is only expected if mortality due to disease is additive, i.e. individuals die that would not die for other reasons in the absence of the disease (Burnham & Anderson 1984). While disease-induced mortality during *Bd* epidemics is obviously additive (e.g. when *Bd* epidemics caused *Alytes obstetricans* population declines in Spain; Bosch *et al.* 2001), this may not be the case for enzootic *Bd*. Compensatory mortality can result in a lack of disease effects on host abundance (Burnham & Anderson 1984, Jolles *et al.* 2006). If mortality is compensatory, increased mortality due to the presence of *Bd* would have to be countered by a reduction in mortality due to other causes, often in a density-dependent manner (Lebreton 2005). Density-dependence in the terrestrial stages of amphibian populations may indeed occur (Altwegg 2003, Berven 2009, Patrick *et al.* 2008).

The third explanation may be that *Bd* had no effects on abundance but on vital rates of individuals. Jolles *et al*. (2005) reported that chronic tuberculosis caused lower survival of African buffalo but no detectable effects at the population level because fecundity was increased. Yet, a population model showed that population growth rate was lowered

because of the pathogen. Jolles *et al*. (2005) therefore suggested that buffalo populations were less resilient to disturbance.

The fourth explanation may be that effects of *Bd* on abundance occurred in the past and are no longer measurable. The Briggs *et al*. (2010, 2005) host-pathogen models suggests that amphibian populations may decline to lower abundance after the emergence of *Bd* but remain stationary at a smaller size after *Bd* became enzootic. Indeed, strong *Alytes obstetricans* population declines were observed in our study area in the 1980ties and 1990ties (Borgula & Zumbach 2003). It may be that *Bd* contributed to these declines. Today, the *Alytes obstetricans* populations may have reached the stationary state predicted by the models such that an effect of *Bd* on abundance is no longer detectable. This is also supported by the low infection intensities observed in the field today. If populations stabilized at lower abundance, they may now be more prone to environmental and/or demographic stochasticity because of reduced abundance.

Although there are possible explanations why we did not observe the expected negative effect of *Bd* on abundance, the question remains open why *Bd*-positive populations had higher growth rates than *Bd*- populations. The more often reproduction was observed at a given site, the more likely the population was to grow. This may suggest that recruitment determines population growth (Grafe *et al.* 2004, Lampo & De Leo 1998). Hence, the compensatory mechanism that we alluded to above may simply be increased juvenile production as it was described by Jolles *et al*. (2005) for African buffalo. *Bd*-positive populations of *Bufo boreas* may be able to survive if they have high recruitment (E. Muths, personal communication).

In conclusion, we demonstrated that populations of a species that is susceptible to an emerging pathogen can grow despite a high prevalence of the pathogen. Evidently, individual-level effects of disease (mortality of individuals) did not translate into population-level effects (negative population trends). Our results are phenomenological and we do not know the mechanisms that allow the populations to persist. Understanding why an amphibian species that is known to be susceptible to *Bd* can have growing populations despite high prevalence of the pathogen would be a key to successful mitigation of the effects of chytridiomycosis.

Acknowledgements

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References

Altwegg, R. (2003). Multistage density dependence in an amphibian. *Oecologia* 136(1): 46-50.

Amt für Natur- und Landschaftsschutz (2000). Artenhilfsprogramm Geburtshelferkröte 2000-2009. unpublished action plan. Luzern, Kanton Luzern. unpublished report: 32.

Anderson, R. M. and May, R. M. (1979). Population biology of infectious diseases: Part I. *Nature* 280: 361-367.

Bart, J., Droege, S., Geissler, P., Peterjohn, B. and Ralph, C. J. (2004). Density estimation in wildlife surveys. *Wildlife Society Bulletin* 32(4): 1242-1247.

Berven, K. A. (2009). Density dependence in the terrestrial stage of wood frogs: evidence from a 21-year population study. *Copeia* (2): 328-338.

Borgula, A. and Zuberbühler, N. (2010). Artenhilfsprogramm Geburtshelferkröte 2010-2019. unpublished action plan. Luzern, Kanton Luzern. unpublished report: 15.

Borgula, A. and Zumbach, S. (2003). Verbreitung und Gefährdung der Geburtshelferkröte (*Alytes obstetricans*) in der Schweiz. *Zeitschrift für Feldherpetologie* 10: 11-26.

Bosch, J., Carrascal, L. M., Duran, L., Walker, S. and Fisher, M. C. (2007). Climate change and outbreaks of amphibian chytridiomycosis in a montane area of Central Spain; is there a link? *Proceedings of the Royal Society Biological Sciences Series B* 274(1607): 253-260.

Bosch, J., Martínez-Solano, I. and García-París, M. (2001). Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain. *Biological Conservation* 97(3): 331-337.

Boyle, D. G., Boyle, D. B., Olsen, V., Morgan, J. A. T. and Hyatt, A. D. (2004). Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Diseases of Aquatic Organisms* 60(2): 141-148.

Briggs, C. J., Knapp, R. A. and Vredenburg, V. T. (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* 107(21): 9695-9700.

Briggs, C. J., Vredenburg, V. T., Knapp, R. A. and Rachowicz, L. J. (2005). Investigating the population-level effects of chytridiomycosis: An emerging infectious disease of amphibians. *Ecology* 86(12): 3149-3159.

Burnham, K. P. and Anderson, D. R. (1984). Tests of compensatory vs. additive hypotheses of mortality in mallards. *Ecology* 65(1): 105-112.

Conroy, S. D. S. and Brook, B. W. (2003). Demographic sensitivity and persistence of the threatened white- and orange-bellied frogs of Western Australia. *Population Ecology* 45(2): 105-114.

de Castro, F. and Bolker, B. (2005). Mechanisms of disease-induced extinction. *Ecology Letters* 8(1): 117-126.

Deem, S. L., Ezenwa, V. O., Ward, J. R. and Wilcox, B. A. (2008). Research frontiers in ecological systems: evaluating the impacts of disease on ecosystems. Infectious disease ecology: effects of ecosystems on disease and of disease on ecosystems. Ostfeld, R. S., Keesing, F. and Eviner, V. T. Princeton & Oxford, Princeton University Press. 304-318.

DiGiacomo, R. F. and Koepsell, T. D. (1986). Sampling for detection of infection or disease in animal populations. *Journal of the American Veterinary Medical Association* 189(1): 22-23.

Ebert, D. (1994). Virulence and local adaptation of a horizontally transmitted parasite. *Science* 265(5175): 1084-1086.

Ebert, D. and Hamilton, W. D. (1996). Sex against virulence: the coevolution of parasitic diseases. *Trends in Ecology & Evolution* 11(2): A79-A82.

Fisher, M. C., Garner, T. W. J. and Walker, S. F. (2009). Global emergence of *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis in space, time, and host. *Annual Review of Microbiology* 63: 291-310.

Garner, T. W. J., Walker, S., Bosch, J., Hyatt, A. D., Cunningham, A. A. and Fisher, M. C. (2005). Chytrid fungus in Europe. *Emerging Infectious Diseases* 11(10): 1639-1641.

Grafe, T. U., Kaminsky, S. K., Bitz, J. H., Lussow, H. and Linsenmair, K. E. (2004). Demographic dynamics of the afro-tropical pig-nosed frog, *Hemisus marmoratus*: effects of climate and predation on survival and recruitment. *Oecologia* 141(1): 40-46.

Hels, T. and Nachman, G. (2002). Simulating viability of a spadefoot toad *Pelobates fuscus* metapopulation in a landscape fragmented by a road. *Ecography* 25(6): 730-744.

Hijmans, R. J., Cameron, S. E., Parra, J. L., Jones, P. G. and Jarvis, A. (2005). Very high resolution interpolated climate surfaces for global land areas. *International Journal of Climatology* 25(15): 1965-1978.

Ibelings, B. W., De Bruin, A., Kagami, M., Rijkeboer, M., Brehm, M. and van Donk, E. (2004). Host parasite interactions between freshwater phytoplankton and chytrid fungi (*Chytridiomycota*). *Journal of Phycology* 40(3): 437-453.

Jolles, A. E., Cooper, D. V. and Levin, S. A. (2005). Hidden effects of chronic tuberculosis in African buffalo. *Ecology* 86(9): 2358-2364.

Jolles, A. E., Etienne, R. S. and Olff, H. (2006). Independent and competing disease risks: Implications for host populations in variable environments. *American Naturalist* 167(5): 745-757.

Kéry, M. and Schaub, M. Bayesian Population Analysis using WinBUGS/OpenBUGS - a hierarchical perspective *in preparation*.

Kéry, M. and Schmidt, B. R. (2008). Imperfect detection and its consequences for monitoring for conservation. *Community Ecology* 9(2): 207-216.

Lampo, M. and De Leo, G. A. (1998). The invasion ecology of the toad *Bufo marinus*: from South America to Australia. *Ecological Applications* 8(2): 388-396.

Laurance, W. F., McDonald, K. R. and Speare, R. (1996). Epidemic disease and the catastrophic decline of Australian rain forest frogs. *Conservation Biology* 10(2): 406-413.

Lebreton, J. D. (2005). Dynamical and statistical models for exploited populations. *Australian & New Zealand Journal of Statistics* 47(1): 49-63.

Lips, K. R., Brem, F., Brenes, R., Reeve, J. D., Alford, R. A., Voyles, J., Carey, C., Livo, L., Pessier, A. P. and Collins, J. P. (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(9): 3165-3170.

Longcore, J. R., Longcore, J. E., Pessier, A. P. and Halteman, W. A. (2007). Chytridiomycosis widespread in anurans of Northeastern United States. *Journal of Wildlife Management* 71(2): 435-444.

Lubick, N. (2010). Emergency medicine for frogs. *Nature* 465: 680-681.

Lunn, D. J., Thomas, A., Best, N. and Spiegelhalter, D. (2000). WinBUGS -- a Bayesian modelling framework: concepts, structure, and extensibility. *Statistics and Computing* 10: 325-337.

Nöllert, A. and Nöllert, C. (1992). Die Amphibien Europas: Bestimmung, Gefährdung, Schutz. Stuttgart 1992, Franckh-Kosmos Verlags-GmbH & Co.

Patrick, D. A., Harper, E. B., Hunter, M. L. and Calhoun, A. J. K. (2008). Terrestrial habitat selection and strong density-dependent mortality in recently metamorphosed amphibians. *Ecology* 89(9): 2563-2574.

Peyer, N. F. (2010). Historical evidence for the presence of the emerging amphibian pathogen Batrachochytrium dendrobatidis (Longcore *et al*. 1999) in Switzerland. M.Sc. thesis. Zurich, Switzerland, University of Zurich.

Pilliod, D. S., Muths, E., Scherer, R. D., Partelt, P. E., Corn, P. S., Hossack, B. R., Lambert, B. A., McCaffery, R. and Gaughan, C. (2010). Effects of amphibian chytrid fungus on individual survival probability in wild boreal toads. *Conservation Biology* 24(5): 1259-1267.

R Development Core Team (2008). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.

Read, A. F. (1994). The evolution of virulence. *Trends in Microbiology* 72(3): 73-76.

Retallick, R. W. R., McCallum, H. and Speare, R. (2004). Endemic infection of the amphibian chytrid fungus in a frog community post-decline. *PLoS Biology* 2(11): 1965-1971.

Schloegel, L. M., Hero, J. M., Berger, L., Speare, R., McDonald, K. and Daszak, P. (2006). The decline of the sharp-snouted day frog (*Taudactylus acutirostris*): The first documented case of extinction by infection in a free-ranging wildlife species? *EcoHealth* 3(1): 35-40.

Schmidt, B. R. (1993). Are hybridogenetic frogs cyclical parthenogens. *Trends in Ecology & Evolution* 8(8): 271-273.

Schmidt, B. R. (2004). Declining amphibian populations: The pitfalls of count data in the study of diversity, distributions, dynamics, and demography. *Herpetological Journal* 14(4): 167-174.

Schmidt, B. R. (2005). Monitoring the distribution of pond-breeding amphibians when species are detected imperfectly. *Aquatic Conservation-Marine and Freshwater Ecosystems* 15(6): 681-692.

Schmidt, B. R., Furrer, S., Kwet, A., Lötters, S., Rödder, D., Sztatecsny, M., Tobler, U. and Zumbach, S. (2009a). Desinfektion als Maßnahme gegen die Verbreitung der Chytridiomykose bei Amphibien. Methoden der Feldherpetologie. Hachtel, M., Schlüpmann, M., Thiesmeier, B. and Weddeling, K. Bielefeld, Laurenti Verlag. 229–241

Schmidt, B. R., Geiser, C., Peyer, N., Keller, N. and von Rutte, M. (2009b). Assessing whether disinfectants against the fungus *Batrachochytrium dendrobatidis* have negative effects on tadpoles and zooplankton. *Amphibia-Reptilia* 30(3): 313-319.

Schmidt, B. R. and Zumbach, S. (2005). Rote Liste der gefährdeten Amphibien der Schweiz. Bundesamt für Umwelt, Wald und Landschaft, Bern, und Koordinationsstelle Amphibien- und Reptilienschutz in der Schweiz, Bern.

Skelly, D. K. and Richardson, J. L. (2009). Larval sampling. Amphibian ecology and conservation: a handbook of techniques. Dodd, C. K. Oxford, Oxford University Press. 55-70.

Skerratt, L. F., Berger, L., Speare, R., Cashins, S., McDonald, K. R., Phillot, A. D., Hines, H. B. and Kenyon, N. (2007). Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *EcoHealth* 4: 125-134.

Stuart, S. N., Chanson, J. S., Cox, N. A., Young, B. E., Rodrigues, A. S. L., Fischman, D. L. and Waller, R. W. (2004). Status and trends of amphibian declines and extinctions worldwide. *Science* 306(5702): 1783-1786.

Teacher, A. G. F., Cunningham, A. A. and Garner, T. W. J. (2010). Assessing the long-term impact of *Ranavirus* infection in wild common frog populations. *Animal Conservation* 13: 514-522.

Tobler, U. and Schmidt, B. R. (2010). Within- and among-population variation in chytridiomycosis-induced mortality in the toad *Alytes obstetricans*. *PLoS ONE* 5(6): e10927.

Tompkins, D. M. and Begon, M. (1999). Parasites can regulate wildlife populations. *Parasitology Today* 15(8): 311-313.

Tompkins, D. M., Dobson, A. P., Arneberg, P., Begon, M. E., Cattadori, I. M., Greenman, J. V., Heesterbeek, J. A. P., Hudson, P. J., Newborn, D., Pugliese, A., Rizzoli, A. P., Rosà, R., Rosso, F. and Wilson, K. (2001). Parasites and host population dynamics. The ecology of wildlife diseases. Hudson, P. J., Rizzoli, A., Grenfell, B. T., Heesterbeek, H. and Dobson, A. P. Oxford, Oxford University Press. 45-62.

Wake, D. B. and Vredenburg, V. T. (2008). Are we in the midst of the sixth mass extinction? A view from the world of amphibians. *Proceedings of the National Academy of Sciences of the United States of America* 105: 11466-11473.

Walker, S. F., Bosch, J., Gomez, V., Garner, T. W. J., Cunningham, A. A., Schmeller, D. S., Ninyerola, M., Henk, D., Ginestet, C., Arthur, C.-P. and Fisher, M. C. (2010). Factors driving pathogenicity vs. prevalence of amphibian panzootic chytridiomycosis in Iberia. *Ecology Letters* 13(3): 372-382.

Woodhams, D. C., Bosch, J., Briggs, C. J., Cashins, S., Davis, L. R., Lauer, A., Muths, E., Puschendorf, R., Schmidt, B. R., Sheafor, B. and Voyles, J. (2011). Mitigating amphibian disease: strategies to maintain wild populations and control chytridiomycosis. *Frontiers in Zoology* 8(8).

AMONG-POPULATION VARIATION OF INFECTION LOADS OF THE PATHOGEN *BATRACHOCHYTRIUM DENDROBATIDIS* IN POPULATIONS OF A THREATENED EUROPEAN AMPHIBIAN SPECIES

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Abstract

Emerging infectious diseases of wildlife are a major threat to biodiversity. In many cases, the effect of the pathogens on host populations depends on pathogen abundance which can be quantified as either prevalence or infection load. Since pathogen abundance is often subject to environmental conditions, mitigation may be possible by modification of environmental characteristics of the habitat. In this study, we aimed to identify environmental correlates of the small-scale, among-population variation in abundance of the emerging fungal pathogen *Batrachochchytrium dendrobatidis* (*Bd*) in populations of the threatened midwife toad *Alytes obstetricans*. In this species pathogen-induced mortality is strongly linked to high infection loads. We determined pathogen prevalence and infection load in *Alytes obstetricans* tadpoles in 19 populations in three distinct regions of Switzerland. We fitted zero-inflated hierarchical models with biotic and abiotic covariates measured at the pond level as explanatory variables to the data. Our results suggest that both pathogen occurrence and prevalence are independent of the covariates we studied. However, pathogen loads were dependent on small-scale characteristics of the ponds and highest in spring among overwintered tadpoles. High infection loads were associated with high tadpole density and warmer water temperatures. This knowledge can be used to mitigate negative effects of the pathogen on the host.

Key words: *Alytes obstetricans*, amphibian, *Batrachochytrium dendrobatidis*, disease mitigation, ecological conditions, habitat characteristics, hierarchical Bayesian model, pathogen load, prevalence, zero-inflation

Introduction

Pathogens can be important regulators of host populations (Daszak *et al.* 2003, Pedersen *et al.* 2007, Smith *et al.* 2009). Emerging infectious diseases are particularly important because they can cause dramatic population declines or even extinction of host species (Daszak *et al.* 2000). Yet, in many cases, the effects of emerging diseases on host populations vary strongly among regions (Buchen 2010, Fisher *et al.* 2009b, Rushton *et al.* 2006). For some diseases, the impact on host populations varies even among populations within the same species (Rudicell *et al.* 2010, Walker *et al.* 2010).

Chytridiomycosis is such an emerging disease of amphibians for which a variety of outcomes of infection have been described, ranging from mild enzootic infections with little mortality to complete host extinction (Briggs *et al.* 2005, La Marca *et al.* 2005, Longcore *et al.* 2007, Rachowicz *et al.* 2006). Chytridiomycosis is caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) (Fisher *et al.* 2009b, Longcore *et al.* 1999). In populations infected with *Bd*, prevalence and infection load have been identified as key determinants of the outcome of infection. Mass mortality events are only observed if prevalence and infection load exceed a certain threshold (Briggs *et al.* 2010, Vredenburg *et al.* 2010). Prevalence and infection load in turn are strongly dependent on environmental factors (Kriger & Hero 2007, Murphy *et al.* 2009).

A large number of studies show environmental correlates of *Bd* presence, prevalence or infection loads. The majority of studies describing a link between environment and prevalence or infection load use climate data across large geographical scales (Bosch *et al.* 2007, Murray *et al.* 2011, Rödder *et al.* 2009, Rödder *et al.* 2008, Walker *et al.* 2010), explaining *Bd* occurrence at global or continent-wide scales. Studies working at smaller geographical scales and including not only climate but also habitat characteristics analysed the presence of *Bd* only (Adams *et al.* 2010, Padgett-Flohr & Hopkins 2010). Since mortality is strongly dependent on infection loads at individual and pond level (Briggs *et al.* 2010, Tobler & Schmidt 2010), studies of environmental correlates should focus on prevalence and infection loads rather than pathogen presence. Because the effects of *Bd* are population-specific (Tobler & Schmidt 2010), environmental characteristics should be measured at pond-level.

Previous analysis of small-scale environmental correlates of infection prevalence or load showed that both biotic and abiotic factors are important (Hall *et al.* 2010). If the selection of parameters is focused on environmental characteristics that can be manipulated relatively easily, such analyses can give rise to mitigation strategies (Woodhams *et al.* 2011). Given the strong dependence of *Bd* growth and survival on temperature (Piotrowski *et al.* 2004), abiotic climatic conditions such as temperature are certainly important also at small geographic scales (Richards-Zawacki 2010, Woodhams *et al.* 2003). However, biotic factors may be important as well (Belden & Harris 2007, Hall *et al.* 2010, Keesing *et al.* 2006). The community ecology context is essential (Johnson & Thieltges 2010). The presence of reservoir species or life stages (Adams *et al.* 2010, Daszak *et al.* 2004, Rachowicz & Vredenburg 2004) or amphibian density (Briggs *et al.* 2005, Rachowicz & Briggs 2007) affect infection prevalence and load. Also, the presence of non-host species may be important: In a related chytridiomycete fungus infecting the diatom *Asterionella*, high densities of *Daphnia* spp. can prevent epidemics by ingestion of zoospores and hence reduce transmission rates (Kagami *et al.* 2004). An analogous mechanism may operate in the case of *Bd* that has similar-sized zoospores as the algal parasite above (Berger *et al.* 2005). Additionally, host immune defences can play a role, but so far only skin peptides and microbiota have been studied (Woodhams *et al.* 2007a, Woodhams *et al.* 2007b). In many systems, genetic diversity is crucial in disease resistance and may determine the susceptibility to infection (Acevedo-Whitehouse *et al.* 2003, Coltman *et al.* 1999, Meagher 1999, Pearman & Garner 2005). The role of genetic diversity in resistance to *Bd* infection or chytridiomycosis has so far largely been ignored (but see May *et al.* 2011).

A suitable system to study environmental correlates of *Bd* infection prevalence and load are populations of the common midwife toad, *Alytes obstetricans*, in Switzerland. *Bd* is widespread in Swiss populations of *Alytes* and infection prevalence and load vary strongly among populations (chapters 3 & 4). *Alytes* tadpoles commonly have the highest prevalence and infection load among amphibians present at a pond (chapters 3 & 4). Most importantly, during the larval stage they do not develop disease and die. This allows estimating the prevalence and load without the bias arising from mortality of highly infected individuals (McCallum 2005).

In this study, we aim to determine whether ecological factors and host genetic diversity determine *Bd* prevalence and infection load in natural ponds. We collected data on

prevalence and infection load of *Bd* in 19 *Alytes* populations from three regions in Switzerland; we restricted our sampling to tadpoles because they have a high infection risk but do not die from infection even if infection loads are high. We use a zero-inflated model to test the effects of abiotic and biotic environmental conditions on *Bd* prevalence and infection load. Because all these habitat characteristics that we included in the model as explanatory variables can be manipulated artificially, our results may give rise to mitigation strategies for *Bd* in *Alytes* populations (Woodhams *et al.* 2011).

Materials and Methods

Study sites

We selected 19 populations in three distinct *Bd*-positive regions: Baselland in north-western Switzerland (BL), Bern in east-central Switzerland (BE), and St.Gallen in north-eastern Switzerland (SG). All sites harbour relatively large *Alytes* populations where tadpoles can be encountered regularly. The habitat types range from quarries, fire water reservoirs, and garden ponds to forest ponds. The sites were initially visited during summer 2007 to obtain genetic samples (chapter 1) and to determine the *Bd* infection status of every site and then repeatedly visited during 2009 to sample for *Bd* prevalence and environmental data. Standard hygiene protocols were followed during all parts of field work (Schmidt *et al.* 2009a).

Alytes *genetic samples and analyses*

We collected tissue samples from tadpole tail clips in summer 2007. We tried to sample at least 25 tadpoles per pond and to sample different-sized tadpoles to minimise the risk of sampling full-siblings. Tissue samples were stored in 99.8% ethanol and extracted using the BioSprint 96 DNA Blood Kit (Qiagen, Hombrechtikon, Switzerland), following the protocol for tissue extraction. Polymerase chain reactions (PCR) were run in three separate multiplexes with fluorescent-labelled primers in three separate multiplexes according to the protocol described in chapter 1. PCR products were run on an ABI 3730 Avant capillary sequencer (Applied Biosystems, Rotkreuz, Switzerland) with internal size standard GeneScan-500 LIZ; peaks were visually scored using GENEMAPPER 3.7 (Applied Biosystems 2004).

We tested the loci for the presence of null alleles, stuttering and large allelic dropout using MICROCHECKER (Van Oosterhout *et al.* 2004). Due to a high allelic fixation rate we were unable to exclude siblings from the data set using the program COLONY 3.1 (Wang 2004). However, because we found no deviations from Hardy-Weinberg (HW) equilibrium, the potential presence of siblings in our data set did not affect our results. As measures of genetic diversity, we calculated the number of fixed private alleles per population, a sample-size corrected estimate of allelic richness (A) using FSTAT 2.9.3.2. (Goudet 2001) and the percentage of polymorphic loci (P), observed (Ho) and expected (He) heterozygosity, the inbreeding coefficient F_{IS}, and pairwise F_{ST} using GENETIX 4.05.2. (Belkhir *et al.* 1996-2004). From the pairwise F_{ST} values, we calculated the mean F_{ST} of a population to all populations within the same region. These genetic measures were used in a principal component analysis in R 2.8.1 (R Development Core Team 2008). The first two principal components which together explained 93% of the variation were used for modelling.

Bd *prevalence and abundance data*

We visited all ponds twice during 2009 to sample for *Bd* prevalence and abundance. The first visit took place during March and April when only overwintered (OW) tadpoles are present. The second visit took place during July and August and aimed at sampling young of the year (YOY) tadpoles. During both visits, we captured up to 25 tadpoles per pond by dip-netting and stored them in individual containers filled with aged tap water. We measured the body length of every tadpole with a sliding caliper and determined the developmental stage (Gosner 1960). To test for *Bd*, we took one buccal swab per tadpole by rubbing a sterile cotton swab (Copan Italia S.p.A., Brescia, Italy) over the mouthparts. The swabs were stored in the fridge upon return from the field and until processing. We extracted and analysed the swabs for the presence of *Bd* following the protocol by Boyle *et al*. (2004) with the modifications described in Tobler & Schmidt (2010). We applied a threshold of 0.1 genomic equivalents in the rt-PCR output below which we considered a swab negative to avoid false positives.

Environmental data

Starting in March 2009, all ponds were visited 8 times in roughly three week intervals

Table 5.1: List of covariates used in the ZIB and ZIP model for *Bd* detection, occurrence and prevalence/ abundance.

covariate	range[2]	description
detection		
Gosner stage	25 – 45	Gosner stage of tadpole development (Gosner 1960)
occurrence		
mean *Alytes* OW density[1]	0 – 205 individuals/m^2	average density of overwintered tadpoles from April to September
mean *M. alpestris* density[1]	0 – 88 individuals/m^2	average density of *Mesotriton alpestris* from April to September
mean *L. helveticus* density[1]	0 – 5 individuals/m^2	average density of *Lissotriton helveticus* from April to September
mean water temperature[1]	12.4 – 20.4°C	mean water temperature from April to September
infection load		
slope of pond edge[1]	0.05 – 2.00 m	depth of the pond 1m from the pond edge
pond surface area[1]	1.5 – 230 m^2	pond surface area in m^2
genetic diversity PC1		*Alytes* genetic diversity, first principal pomponent
genetic diversity PC2		*Alytes* genetic diversity, second principal component
water temperature[1]	8.1 – 24.1°C	water temperature in the month before the spring/summer sampling event
mean zooplankton density[1]	6.4 – 776.9 individuals/L^3	mean zooplankton density from April to September
density of *Alytes* tadpoles[1]	0 – 309 individuals/m^2	density of *Alytes* tadpoles(both OW and YOY) during the visit before the spring/summer sampling event
density of newts[1]	0 – 88 individuals/m^2	density of newts (both *M. alpestris and L. helveticus*) during the visit before the spring/summer sampling event

[1] z-standardised
[2] in case of z-standardised variables: range before transformation

throughout spring, summer and early fall. During the first visit, we measured pond surface area, maximum depth and slope of the pond edge (depth 1m from the edge), and during every visit, we collected data on the amphibian community present and zooplankton density.

The amphibian community data were obtained by doing 10 dip-net strokes of 2 m in length around the pond in large ponds and 5 strokes in small ponds. All amphibians caught in the net were counted by species and released only after the last stroke. We did not count tadpoles of *Rana temporaria* and *Bufo bufo* because they are relatively unlikely to harbour *Bd* infection due to the short larval period (chapter 3). During the summer when both

overwintered and freshly hatched tadpoles of *Alytes* were present, we distinguished between OW and YOY tadpoles based on body size (Thiesmeier 1992).

To estimate zooplankton density we sunk a zooplankton net of 19.5 cm in diameter to the pond bottom approximately 1 m from the pond edge in a place with little or no vegetation. Each time, the depth to which the net sunk was noted. This procedure was repeated 10 times per visit around the pond for large ponds and 5 times for small ponds. During each visit, we filtered a water volume of 3 to 530 L depending on pond depth. The zooplankton was filtered through a sieve and stored in ethanol until counting. For counting, five 1 mL subsamples of every sample were transferred to a Petri dish with five 1x1cm grids. All copepods in the grid cells were counted using a binocular. From the filtered water volume and the dilutions applied during ethanol storage we calculated the original zooplankton density in the pond.

All ponds were equipped with one HOBO H8 temperature data logger (Onset Computer Corporation, Bourne MA, USA) that recorded water and air temperature four times a day between 1 April and 30 September 2009. The loggers were placed in parts of the ponds where high tadpole densities were observed during field work in 2007.

Statistical modelling

We built two separate zero-inflated models to model prevalence and abundance (Martin *et al.* 2005). In a zero-inflated model, occupancy and prevalence/abundance parameters are only estimated for sites that are suitable for the species of interest. For both models, we added the same covariates to each hierarchical level to calculate covariate effects (Table 5.1).

For the prevalence model, we used a zero-inflated binomial (ZIB) model implemented in WinBUGS 14 (Kéry 2010) to model prevalence, occurrence and detection probability of *Bd*. Every swab was considered an independent sampling event *j*, i.e. a "visit *j*" to a site *i*. The spring and summer sampling events were considered as different seasons *k* with the *Bd* population being open to changes in prevalence and colonisation/extinction between them. The resulting model had three hierarchical levels:

true presence of *Bd* at pond *i* in season *k* $Y_{ik} \sim \text{Bernoulli}(\text{psi}_{ik})$

true prevalence at pond *i* in season *k* $N.pos_{ik} \sim binomial(q_{ik}, nswabs_{i,k})$

detection at pond *i* in season *k* on swab *j* $y_{ijk} \sim Bernoulli(p_{ikj})$

y[i,k,j] is the detection of *Bd* on swab *j* during season *k* at pond *i* and has a Bernoulli distribution with a success probability *p[i,k,j]*. *p[i,k,j]* has a season-specific intercept *alpha.p[k]* modified by the effect of covariates. $beta.cov_1$ is the slope of the first covariate in the model (cov_1). Further covariates were entered into the model in the same way (Table 5.1). In the WinBUGS language, this becomes

```
y[i,k,j] ~ dbern(p[i,k,j])
p[i,k,j] <- Y[i,k] * (exp(lin1[i,k,j]) / (1 + exp(lin1[i,k,j])))
where
lin1[i,k,j] <- alpha.p[k] + beta.cov1 * cov1[i,k,j]
```

Multiplication of the expit function including the linear predictor for the covariate effects *lin1[i,k,j]* with *Y[i,k]* enables the estimation of covariate effects only for ponds where *Bd* occurs since *Y[i,k]* is the true occurrence of *Bd* during season *k* at pond *i*. *Y[i,k]* followed a Bernoulli distribution with success probability *psi[i,k]* and covariates were entered in the same way as for the detection probability of *Bd*:

```
Y[i,k] ~ dbern(psi[i,k])
logit(psi[i,k]) <- Bd.psi[k]+ beta.cov2 * cov2[i] + ...
```

The true site-specific prevalence *N.pos[i,k]* follows a binomial distribution with success probability q_{ik} and $nswabs_{ik}$ trials:

```
N.pos[i,k] ~ dbin(q[i,k], nswabs[i,k])
q[i,k] <- Y[i,k] * (exp(alpha.p[k] + lin2[i,k])/(1 + lin2[i,k] + exp(alpha.p[k])))
where
lin2[i,k] <- alpha.prev + + beta.cov3 * cov3[i] + ...
```

Again, multiplication of the expit function including the intercept for detection probability *alpha.p[k]* and the linear predictor for prevalence parameters *lin1[i,k]* with *Y[i,k]* allowed the estimation of covariate effects for *Bd*-positive sites only. We ran three parallel chains starting from different initial values. The chains were run for 20’000 iterations; we discarded the first 5000 as burn-in and applied a thinning factor of 5. Covariates were considered important if their 95% credible interval (95% CRI) did not include zero.

For the abundance model, we used a zero-inflated hierarchical Poisson (ZIP) model in WinBUGS 14 to model abundance, occurrence and detection probability of *Bd*. The estimation of detection probability was analogous to the estimation in the prevalence model. On each sampling event (i.e. tadpole buccal swab), *Bd* could be detected with a certain probability *p*, and if it was present, have a certain abundance *N*. Abundance *N* was equivalent to the genomic equivalents determined by rt-PCR. However, for the modelling, we limited the maximum count per swab to 1000 zoospore equivalents because the highest standard in the rt-PCR reaction was 100 GE and any estimate beyond the tenfold amount of the highest standard is unreliable.

Analogous to the prevalence model, the spring and summer sampling events were considered as independent seasons *k*. The model consists of three hierarchical levels:

true presence of *Bd* at pond *i* in season *k* $z_{ik} \sim \text{Bernoulli}(\psi)$

true abundance at pond *i* in season *k* $N_{ik} \sim \text{Poisson}(z_{ik} * \lambda_k)$

infection load at pond *i* in season *k* on swab *j* $c_{ijk} \sim \text{binomial}(N_{ik}, p_{ijk})$

C[i,k,j] is the estimated number of zoospores (genomic equivalents from rt-PCR reactions) on swab *j* during season *k* at pond *i* and has a binomial distribution with a success probability *p[i,k,j]* and *N[i,k]* trials. The success probability *p[i,k,j]* is a function of the season-specific intercept *Bd.p[k]* modified by the effect of covariates. For each hierarchical level, we added covariates to calculate covariate effects. *beta.cov1* is the slope of the first covariate in the model (*cov1*). Further covariates were entered into the model in the same way (Table 5.1). In the WinBUGS language, this becomes

```
C[i,k,j] ~ dbin(p[i,k,j], N[i,k])
logit(p[i,k,j]) <- Bd.p[k] + beta.cov1 * cov1[i,k,j] + ...
```

Occurrence probability *occ[i,k]* during season *k* at pond *i* followed a Bernoulli distribution with success probability *psi[i,k]* and covariates were entered in the same way as for the detection probability of *Bd*:

```
occ[i,k] ~ dbern(psi[i,k])

logit(psi[i,k]) <- Bd.psi[k]+ beta.cov2 * cov2[i] + ...
```

The true site-specific abundance *N[i,k]* follows a Poisson distribution with density parameter lambda, which in turn is defined by the season-specific intercept *Bd.lambda[k]* and covariates:

N[i,k] ~ dpois(lambda[i,k])

log(lambda[i,k]) <- Bd.lambda[k] + beta.cov3 * cov3[i] + ...

We ran three parallel chains starting from different initial values. The chains were run for 450'000 iterations; we discarded the first 350'000 as burn-in and applied a thinning factor of 18. Covariates were considered important if their 95% credible interval (95% CRI) did not include zero.

We parameterised both models with covariates on detection and occupancy probability and on prevalence or abundance of *Bd* (Table 5.1). Because tadpole stage and size were correlated (r = 0.698), we used only Gosner stage as detection covariate. As covariates for occupancy probability, we used the density of overwintered tadpoles, because they might act as reservoir for *Bd* during winter when most amphibians have left the pond. We further included the density of the newts *Mesotriton alpestris* and *Lissotriton helveticus* because they are species commonly found at many ponds and have relatively high *Bd* prevalence (chapter 3), and mean water temperature during the survey period. Because slope of the pond edge was strongly correlated with pond depth (r = 0.814) but not with pond surface area (r = -0.182), we used surface area and slope, but not depth, as covariates for prevalence or abundance. None of the other covariates were strongly correlated (all r ≤ ± 0.368). All continuous explanatory variables were standardised to a mean of 0 and standard deviation of 1 prior to modelling.

Results

Bd *occurrence, prevalence and infection load*

In summer 2009, *Bd* was found in 4 out of 6 ponds in BE. In BL, 6 out of 7 ponds were *Bd*-positive and in SG, 4 out of 6 ponds were *Bd*-positive. Mean prevalence among overwintered tadpoles in spring 2009 ranged from 0.14 to 1.00 in *Bd*+ ponds with a mean of 0.65 ± 0.30 (SD). Mean prevalence among young-of-the-year tadpoles in *Bd*+ ponds in summer 2009 ranged from 0.04 to 0.76 with a mean of 0.19 ± 0.20 (Table 5.2). The average infection load of overwintered tadpoles per pond ranged from 7.296 ± 2.101 to 1526.891 ± 5033.919 with a mean of 241.051 ± 471.517 zoospores per tadpole among all ponds. Among the young-of-the-year tadpoles mean infection per pond ranged from 0.277 ± 0.345 to 12.626 ± 12.404 with a mean of 3.415 ± 3.443 zoospore equivalents per tadpole (Table 5.2).

Table 5.2: Prevalence (proportion of individuals tested positive) and infection loads (genomic equivalents resulting from rt-PCR analyses) among *Alytes* tadpoles for all study ponds. The spring sample includes overwintered tadpoles only, the summer sample young-of-the-year tadpoles only. All values are given as mean ± SD.

		spring 2009			summer 2009		
region	Pond	N	prevalence	infection load	N	prevalence	infection load
BE	Brandsiten	26	0	-	25	0	-
	Chnubel	25	0.52 ± 0.51	9.0 ± 11.3	25	0.24 ± 0.44	2.7 ± 2.5
	Mattstallwald	0	-	-	25	0	-
	Oberrotenbühl	26	1	22.8 ± 22.5	14	0.14 ± 0.36	0.5 ± 0.5
	Vorder Birnbaum	29	0	-	25	0.08 ± 0.28	3.1 ± 4.2
	Waltisberg	28	1	105.7± 74.7	25	0.76 ± 0.44	6.6 ± 6.5
BL	Bickenberg	20	0.35 ± 0.49	17.4 ± 20.2	0	-	-
	Chalchofen	71	0.14 ± 0.35	96.8 ± 192.5	25	0.16 ± 0.37	0.8 ± 0.6
	Heftelen	59	0.48 ± 0.50	210.7 ± 292.0	25	0.04 ± 0.20	1.1
	Itingen	78	0.51 ± 0.50	863.3 ± 1478.4	25	0.08 ± 0.28	4.9 ± 3.9
	Reigoldswil	0	-	-	21	0	-
	Schleifenberg	25	0	-	25	0.08 ± 0.28	0.3 ± 0.3
	Strickrain	40	0.35 ± 0.48	1526.9 ± 5033.9	0	-	-
SG	Altstätten	25	0.92 ± 0.28	10.0 ± 11.0	25	0.24 ± 0.44	3.4 ± 3.7
	Buechholz	5	0.80 ± 0.45	7.3 ± 2.1	25	0.28 ± 0.46	12.6 ± 12.4
	Ochsenweid	25	0.96 ± 0.20	7.7 ± 8.8	21	0.19 ± 0.40	2.0 ± 3.4
	Sittertobel	0	-	-	24	0	-
	Thal	0	-	-	3	0	-
	Wolfgangweiher	28	0.82 ± 0.39	15.2 ± 12.1	25	0.04 ± 0.20	2.9

Parameter estimates of the ZIB model

The ZIB model on prevalence revealed that there was no difference in detection and occurrence probability or prevalence among seasons (95% CRI for spring and summer sampling overlap in Table 5.3). Also, the 95% CRI of all covariates included zero, indicating that none of the covariates for detection, occurrence or prevalence were important (Table 5.3).

Parameter estimates of the ZIP model

The 95% CRI of the estimates of the spring and summer detection probability reached from 0 to 1, suggesting that the estimate of detection probability is imprecise and that there may be no difference in detection probability of *Bd* among seasons or depending on tadpole stage (Table 5.4, Figure 5.1). The same was true for the estimate of occurrence probability; also, occurrence probability was independent of any covariate that we measured.

Model convergence for infection load covariates was partly poor (Table 5.4). Convergence was particularly a problem for the density of both young-of-the-year and

Table 5.3: ZIB model estimates for the parameters and their respective covariates on the logit scale for *Bd* detection, occurrence and prevalence. q0.025 and q0.975 are the 2.5% and 97.5% percentiles of the 95% credible interval (CRI), Rhat is the scale reduction factor (at convergence, Rhat = 1).

parameter	mean	sd	q0.025	q0.975	Rhat
detection[spring]	*0.805*	*1.629*	*0.057*	*1.565*	*1.001*
detection[summer]	*-0.501*	*0.869*	*-0.998*	*0.103*	*1.001*
tadpole stage	1.311	0.182	0.955	1.670	1.001
occurrence[spring]	*1.176*	*1.020*	*-0.709*	*3.342*	*1.001*
occurrence[summer]	*1.114*	*1.050*	*-0.746*	*3.479*	*1.001*
mean density OW tadpoles	-5.343	2.539	-9.677	0.268	1.001
mean density of *M. alpestris*	1.925	2.325	-2.388	6.746	1.001
mean density of *L. helveticus*	0.548	0.837	-0.892	2.495	1.001
mean water temperature	0.252	0.583	-0.867	1.444	1.001
prevalence[spring]	*0.023*	*2.887*	*-4.748*	*4.741*	*1.001*
prevalence[summer]	*0.088*	*2.874*	*-4.724*	*4.752*	*1.001*
density of *Alytes* tadpoles	-0.002	4.038	-6.651	6.600	1.002
density of newts	0.001	4.028	-6.610	6.644	1.001
pond surface area	-0.024	4.078	-6.669	6.673	1.001
slope of pond edge	0.018	4.043	-6.640	6.660	1.001
zooplankton density	0.062	4.035	-6.640	6.661	1.002
water temperature	-0.044	4.005	-6.646	6.624	1.001
genetic diversity PC1	0.065	4.059	-6.633	6.678	1.001
genetic diversity PC2	0.007	4.048	-6.633	6.669	1.001

Table 5.4: ZIP model estimates for the parameters and their respective covariates for *Bd* detection, occurrence and abundance. q0.025 and q0.975 are the 2.5% and 97.5% percentiles of the 95% credible interval (CRI), Rhat is the scale reduction factor (at convergence, Rhat = 1). Please not that model estimates for detection and occurrence and their covariates are on the logit scale, estimates for abundance and its covariates are on the log scale.

parameter	mean	sd	q0.025	q0.975	Rhat
detection[spring]	*-0.946*	*15.060*	*-27.370*	*23.590*	*1.001*
detection[summer]	*0.262*	*14.970*	*-27.240*	*23.740*	*1.001*
tadpole stage	2.653	12.640	-21.580	23.630	1.001
occurrence[spring]	*-0.040*	*11.600*	*-19.030*	*19.030*	*1.001*
occurrence[summer]	*0.151*	*11.540*	*-19.030*	*19.000*	*1.001*
mean density OW tadpoles	-0.043	14.470	-23.730	23.710	1.001
mean density of *M. alpestris*	0.018	14.490	-23.780	23.760	1.001
mean density of *L. helveticus*	0.120	14.460	-23.730	23.810	1.001
mean water temperature	0.149	14.380	-23.760	23.610	1.001
abundance[spring]	*10.820*	*0.038*	*10.760*	*10.880*	*1.003*
abundance[summer]	*9.186*	*0.039*	*9.117*	*9.248*	*1.020*
density of *Alytes* tadpoles	0.031	0.017	0.001	0.056	1.658
density of newts	-0.053	0.016	-0.074	-0.027	1.793
pond surface area	0.225	0.012	0.200	0.249	1.456
slope of pond edge	-0.532	0.004	-0.540	-0.524	1.224
zooplankton density	0.093	0.004	0.086	0.101	1.075
water temperature	0.292	0.007	0.281	0.301	1.088
genetic diversity PC1	0.052	0.429	-0.081	0.116	1.833
genetic diversity PC2	0.203	0.884	-0.096	0.310	2.665

overwintered tadpoles, pond surface area, pond slope, and both genetic diversity indices. However, except for the estimates of the genetic diversity indices, all chains agreed on the

covariate effect being either positive or negative, although the exact estimate differed by chain. Hence, the results should be qualitatively robust except for the genetic diversity indices, but nevertheless interpreted with care. To improve model convergence, we will further reduce the model complexity and introduce season-specific estimates of infection load covariate effects before publication. We will further try to combine prevalence and infection load in one single model.

The preliminary results show that infection load was higher for the spring sampling event than for the summer sampling with infection loads being about 5 times higher in spring. Infection loads were positively correlated with pond surface area and decreased with increasing slope of the pond edge. Water temperature was positively correlated with *Bd* loads. Biotic factors were also important in explaining infection loads: *Bd* was more abundant in ponds with higher zooplankton and *Alytes* tadpole density. The opposite was true for the density of newts. Neutral genetic diversity of the host population did not have an effect on *Bd* infection load (Table 5.4).

Discussion

Bd was present in the majority of our study populations with considerable variation among sites in prevalence and abundance. Although none of the covariates were important in explaining the presence or absence of *Bd* or its prevalence at a site, we found that infection loads were explained by variation among ponds in biotic and abiotic factors.

A large number of studies have found climatic correlates of *Bd* occurrence or prevalence across large geographical scales (Kriger *et al.* 2007, Murray *et al.* 2011, Rödder *et al.* 2008, Walker *et al.* 2010). In contrast, our study could not find any such correlations in among-population variation in pathogen occurrence and prevalence. This may be due to lower variation in climatic conditions on small geographical scales. Within a suitable environment, the presence or prevalence is patchy and not all suitable habitats are occupied (Adams *et al.* 2010, Longcore *et al.* 2007, Skerratt *et al.* 2007). Walker *et al*. (2010) found that even among sites where the pathogen is present, disease outbreaks occur only under certain environmental conditions. Given that the outbreaks of chytridiomycosis are linked to high infection loads (Briggs *et al.* 2010, Vredenburg *et al.* 2010), there is a need to model infection load rather than occurrence of the pathogen.

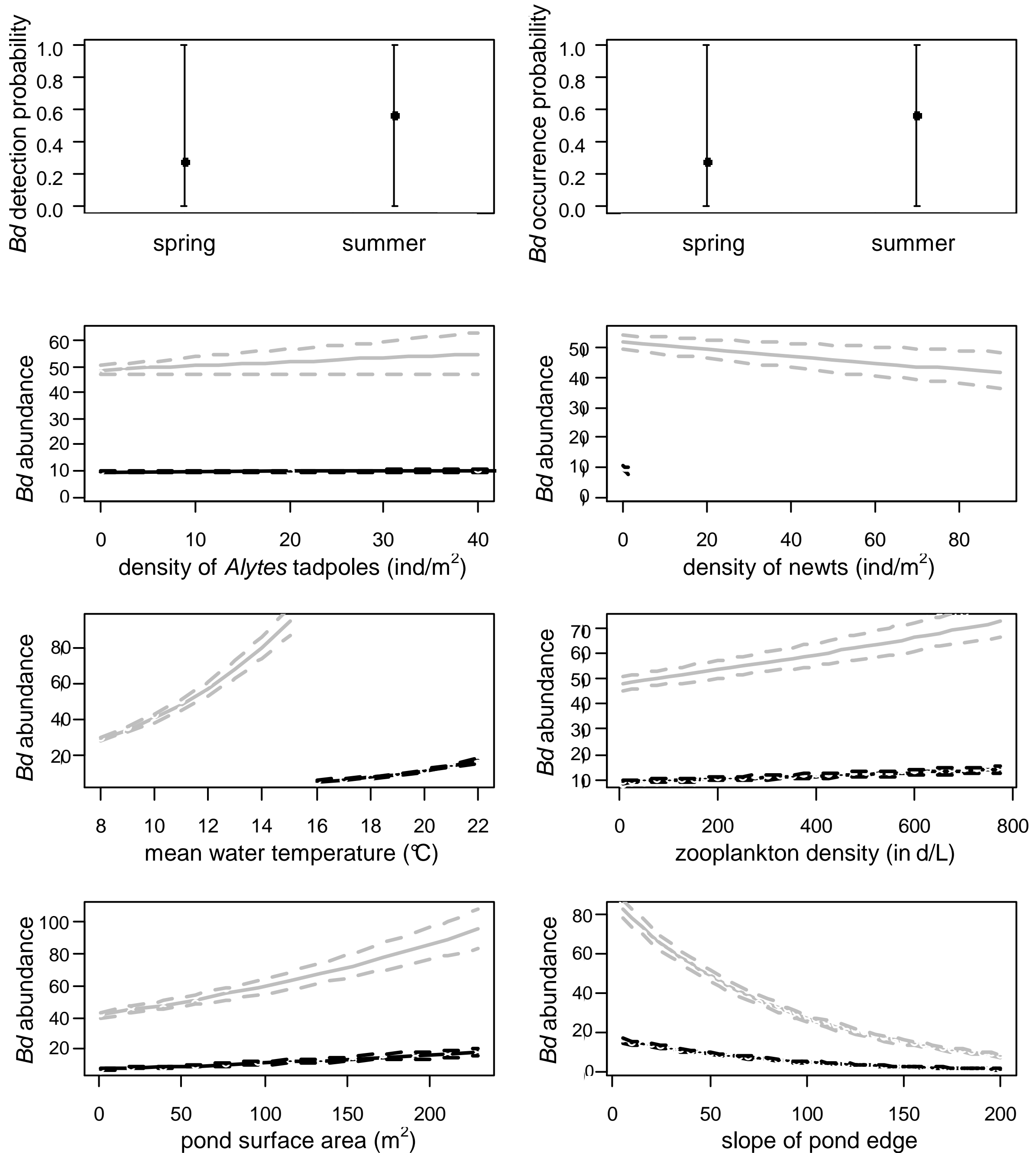

Figure 5.1: model predictions for *Bd* detection and occupancy probabilities and abundance. A: detection probability depending on season. B: occurrence probability depending on season. C: abundance depending on the density of *Alytes* tadpoles. D: abundance depending on the density of newts. E: abundance depending on water temperature. F: abundance depending on zooplankton density. G: abundance depending on pond surface area. H: abundance depending on slope of the pond edge (in cm one meter from the pond edge). *Bd* abundance is the sum of the predicted zoospore loads of all tadpoles in a pond in genomic equivalents x 10^3. Error bars and dashed lines represent the 95% CRI. Grey: spring sampling event, black: summer sampling event

Seasonal variation in infection loads in our study was substantial with infection loads in spring being about five times higher than in summer (Table 5.2, Figure 5.1). *Bd* infection commonly peaks under seasonally recurring climate that provides optimal growth conditions for the pathogen (Kriger & Hero 2007, Longo *et al.* 2009, Woodhams & Alford 2005). Yet, in our system, infection loads were higher in spring after prolonged exposure to temperatures that are seemingly too cold for *Bd* growth (Longcore *et al.* 1999). Nevertheless, the overwintered tadpoles carried much higher loads in spring than the young-of-the-year tadpoles prior to hibernation. We do not know currently when and why infection loads increase during hibernation. A possible explanation may include the prolonged exposure time of tadpoles during hibernation. Also, the tadpoles probably aggregate in deeper areas of the pond (C. Geiger and U. Tobler, own observation) where transmission and reinfection within- and among tadpoles may be high (Han *et al.* 2008, Longcore *et al.* 1999, Weldon & Du Preez 2006).

Abiotic characteristics of the ponds were important in explaining among-pond variation in infection loads. Generally, *Bd* was more abundant in warmer water. Water temperatures in early spring may be at the lower limit for *Bd*, but approach optimal growth conditions around 22°C later in summer. Although shallow pond edges may reach temperatures of up to 40°C, this only happens on few very hot summer days (data not shown) and is not sufficient to heat up the deeper pond areas or larger ponds beyond the upper temperature limit of *Bd* which is around 30°C (Longcore *et al*. 1999). Also, large sized ponds and ponds with shallow edges had higher infection loads than smaller ponds or ponds with steep edges. Basin shape was identified as an important factor in explaining epidemiology of a fungal pathogen in *Daphnia dentifera* (Hall *et al.* 2010). A number of complex interactions of basin shape with abiotic (e.g. mixing of water layers) and biotic (e.g. presence of predators and dilution hosts) were invoked in explaining the differences in infection prevalence among lakes. Similar effects may occur in different-sized water bodies with varying pond edge slopes in *Alytes*-inhabited ponds. Furthermore, on warm and sunny days, tadpoles often aggregate along shallow edges (Dupré & Petranka 1985, Han *et al.* 2008). Tadpoles aggregating along the edge for sunbathing and foraging may facilitate transmission, giving rise to a halo effect (Greer & Collins 2007).

Biotic characteristics of the pond were also important in explaining among-pond variation in infection loads. The abundance of *Bd* increased slightly with increasing density of

Alytes tadpoles, which would be expected since transmission of *Bd* seems to be density dependent (Rachowicz & Briggs 2007). The density of newts, however, was negatively correlated with *Bd* abundance. Such an effect may arise from newts feeding on young *Alytes* tadpoles, hereby reducing the density of competent hosts (Hall *et al.* 2010). Alternatively, newts may be less competent hosts and act as dilution hosts, although this hypothesis requires experimental evaluation (Keesing *et al.* 2006). In contrast to our expectation, high zooplankton density was correlated with higher *Bd* infection loads. Zooplankton may not be able to consume *Bd* zoospores at a scale that is important in reducing pathogen abundance, and not all species may be large enough to do so at all (D. Ebert, pers.comm.). Nevertheless, our results suggest that species composition of ponds is important in explaining variation in infection loads.

We had hypothesised that *Bd* infection loads may be higher when genetic diversity of the host population is lower. We did not find any correlation between genetic diversity and pathogen abundance (and the model converged poorly for those covariates) even though Pearman and Garner (2005) reported such a relationship in an anuran. Possibly, genetic composition only becomes important during and after metamorphosis since tadpoles have a limited immune response compared to post-metamorphic anurans (Rollins-Smith 1998). Hence, they may simply be unable to fight infection before metamorphosis. Yet, genetics may become important in determining which individuals develop disease and which can raise a sufficient immune response after metamorphosis (May *et al.* 2011, Rollins-Smith 1998, Tobler & Schmidt 2010).

Overall, our results suggest that *Bd* infection loads are subject to environmental conditions. As infection loads are strongly associated with disease outbreaks (Briggs *et al.* 2010, Vredenburg *et al.* 2010), this opens the possibility for disease mitigation via habitat manipulation. Although it will not be possible to eradicate *Bd* from a pond simply by manipulating its characteristics, reducing infection loads may be sufficient to mitigate *Bd* and prevent disease outbreaks (Woodhams *et al.* 2011). Based on our modelling results, what recommendations can be given? Reducing the density of infected hosts by culling or removal of a portion of tadpoles from the pond to raise them in captivity could be an option. However, *ex-situ* upbringing of tadpoles is labour-intensive and carries the risk of pathogen spread upon release (Walker *et al.* 2008), and culling is not very popular in threatened

species. Most importantly, given the relatively small effect of tadpole density on *Bd* abundance observed in our study, reducing tadpole density may not be very effective.

A more promising approach could be to exploit the seasonal variation of *Bd* infection loads in *Alytes* tadpoles. Overwintered tadpoles have far higher infection loads than young-of-the-year tadpoles, putting them at higher risk of developing disease (Briggs *et al.* 2010). Since overwintered tadpoles are still present in the ponds when young-of-the-year tadpoles enter the system, their high *Bd* loads may also facilitate the rapid infection of freshly hatched tadpoles (Rachowicz & Vredenburg 2004). Reducing the *Bd* loads in spring may thus be the best way forward. This could be achieved by either reducing infection loads among overwintered tadpoles or reducing the proportion of hibernating tadpoles. A reduction in the proportion of hibernating tadpoles can be achieved by raising the water temperature since more tadpoles hibernate in colder ponds (Thiesmeier 1992). A slight increase in pond water temperature could be relatively easily achieved by the occasional removal of shading vegetation or one-time structural modification of the pond bottom tread. Since we currently do not know which factors cause the accumulation of infection loads during hibernation, the manipulation of infection loads among overwintered tadpoles is not possible at present.

The seasonal variation of *Bd* loads in *Alytes* tadpoles could be further exploited in planning the timing of mitigation measurements such as the treatment of whole ponds or individual amphibians with fungicidal chemicals (Lubick 2010, Woodhams *et al.* 2011). The prospect of eradicating the pathogen from a pond may be highest during summer when infection loads are low already. However, since *Bd* can survive on amphibian hosts outside the pond (Andre *et al.* 2008), long-term eradication may not be possible. Because disease outbreak is mostly dependent on infection load prior to metamorphosis, a higher impact may be achieved by treating overwintered tadpoles when temperatures start to rise in spring because this is when *Bd* loads and the risk for (re)infection are highest. Whether the treatment of individuals or ponds in spring when overwintered tadpoles are present may disrupt the accumulation of *Bd* loads and thereby translate into a long-term reduction in *Bd* abundance also in young-of-the-year tadpoles is currently being tested (C. Geiger, pers. comm.).

In conclusion, our study shows that there are environmental factors explaining among-pond variation in *Bd* infection loads. Since the habitat characteristics we identified can be manipulated experimentally, our model results provide a basis for the planning and testing

of mitigation strategies. Also, the large number of characteristics identified may allow disease mitigation in varying environments. However, due to complex interactions of biotic and abiotic factors in determining pathogen loads (Hall *et al.* 2010), disease mitigation by habitat manipulation may be an intricate task. Nevertheless, the results of this study can help designing experimental tests of mitigation strategies that aim to reduce infection loads. Because the outbreak of chytridiomycosis is strongly linked to high infection loads (Briggs *et al.* 2010, Vredenburg *et al.* 2010), a reduction of fungal loads may be sufficient to efficiently mitigate the impact of *Bd* on amphibian populations (Woodhams *et al.* 2011).

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References

Acevedo-Whitehouse, K., Gulland, F., Greig, D. and Amos, W. (2003). Disease susceptibility in California sea lions. *Nature* 422(6927): 35-35.

Adams, M. J., Chelgren, N. D., Reinitz, D., Cole, R. A., Rachowicz, L. J., Galvan, S., McCreary, B., Pearl, C. A., Bailey, L. L., Bettaso, J., Bull, E. L. and Leu, M. (2010). Using occupancy models to understand the distribution of an amphibian pathogen, *Batrachochytrium dendrobatidis*. *Ecological Applications* 20(1): 289-302.

Andre, S. E., Parker, J. and Briggs, C. J. (2008). Effect of temperature on host response to *Batrachochytrium dendrobatidis* infection in the mountain yellow-legged frog (*Rana muscosa*). *Journal of Wildlife Diseases* 44(3): 716-720.

Belden, L. K. and Harris, R. N. (2007). Infectious diseases in wildlife: the community ecology context. *Frontiers in Ecology and the Environment* 5(10): 533-539.

Belkhir, K., Borsa, P., Chikhi, L., Raufaste, N. and Bonhomme, F.-G., logiciel sous Windows TM pour la génétique des populations. Laboratoire Génome, Populations, Interactions, CNRS UMR 5000, Université de Montpellier II, Montpellier (France). (1996-2004). GENETIX 4.05, logiciel sous Windows TM pour la génétique des populations. *Laboratoire Génome, Populations, Interactions, CNRS UMR 5000, Université de Montpellier II, Montpellier (France).* .

Berger, L., Hyatt, A. D., Speare, R. and Longcore, J. E. (2005). Life cycle stages of the amphibian chytrid *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms* 68(1): 51-63.

Bosch, J., Carrascal, L. M., Duran, L., Walker, S. and Fisher, M. C. (2007). Climate change and outbreaks of amphibian chytridiomycosis in a montane area of Central Spain; is there a link? *Proceedings of the Royal Society Biological Sciences Series B* 274(1607): 253-260.

Boyle, D. G., Boyle, D. B., Olsen, V., Morgan, J. A. T. and Hyatt, A. D. (2004). Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Diseases of Aquatic Organisms* 60(2): 141-148.

Briggs, C. J., Knapp, R. A. and Vredenburg, V. T. (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* 107(21): 9695-9700.

Briggs, C. J., Vredenburg, V. T., Knapp, R. A. and Rachowicz, L. J. (2005). Investigating the population-level effects of chytridiomycosis: An emerging infectious disease of amphibians. *Ecology* 86(12): 3149-3159.

Buchen, L. (2010). Disease epidemic killing only US bats. *Nature* 463: 144-145.

Coltman, D. W., Pilkington, J. G., Smith, J. A. and Pemberton, J. M. (1999). Parasite-mediated selection against inbred Soay sheep in a free-living, island population. *Evolution* 53(4): 1259-1267.

Daszak, P., Cunningham, A. A. and Hyatt, A. D. (2000). Wildlife ecology - Emerging infectious diseases of wildlife - Threats to biodiversity and human health. *Science* 287(5452): 443-449.

Daszak, P., Cunningham, A. A. and Hyatt, A. D. (2003). Infectious disease and amphibian population declines. *Diversity and Distributions* 9(2): 141-150.

Daszak, P., Strieby, A., Cunningham, A. A., Longcore, J. E., Brown, C. C. and Porter, D. (2004). Experimental evidence that the bullfrog (*Rana catesbeiana*) is a potential carrier of chytridiomycosis, an emerging fungal disease of amphibians. *Herpetological Journal* 14(4): 201-207.

Dupré, R. K. and Petranka, J. W. (1985). Ontogeny of Temperature Selection in Larval Amphibians. *Copeia* (2): 462-467.

Fisher, M. C., Garner, T. W. J. and Walker, S. F. (2009). Global emergence of *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis in space, time, and host. *Annual Review of Microbiology* 63: 291-310.

Gosner, K. L. (1960). A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetological Journal* 16: 183-190.
Goudet, J. (2001). FSTAT, a program to estimate and test gene diversities and fixation indices version 2.9.3. . *Available from http://www.unil.ch/izea/softwares/fstat.html*.
Greer, A. L. and Collins, J. P. (2007). Habitat fragmentation as a result of biotic and abiotic factors controls pathogen transmission throughout a host populations. *Journal of Animal Ecology* 77(2): 364-369.
Hall, S. R., Smyth, R., Becker, C. R., Duffy, M. A., Knight, C. J., MacIntyre, S., Tessier, A. J. and Cáceres, C. E. (2010). Why are *Daphnia* in some lakes sicker? Disease ecology, habitat structure, and the plankton. *Bioscience* 60: 363-375.
Han, B. A., Bradley, P. W. and Blaustein, A. R. (2008). Ancient behaviors of larval amphibians in response to an emerging fungal pathogen, *Batrachochytrium dendrobatidis*. *Behavioral Ecology and Sociobiology* 63(2): 241-250.
Johnson, P. T. J. and Thieltges, D. W. (2010). Diversity, decoys and the dilution effect: how ecological communities affect disease risk. *Journal of Experimental Biology* 213(6): 961-970.
Kagami, M., Van Donk, E., de Bruin, A., Rijkeboer, M. and Ibelings, B. W. (2004). *Daphnia* can protect diatoms from fungal parasitism. *Limnology and Oceanography* 49(3): 680-685.
Keesing, F., Holt, R. D. and Ostfeld, R. S. (2006). Effects of species diversity on disease risk. *Ecology Letters* 9(4): 485-498.
Kéry, M. (2010). Introduction to WinBUGS for Ecologists. London, Elsevier.
Kriger, K. M. and Hero, J. M. (2007). Large-scale seasonal variation in the prevalence and severity of chytridiomycosis. *Journal of Zoology* 271(3): 352-359.
Kriger, K. M., Pereoglou, F. and Hero, J. M. (2007). Latitudinal variation in the prevalence and intensity of chytrid (*Batrachochytrium dendrobatidis*) infection in Eastern Australia. *Conservation Biology* 21(5): 1280-1290.
La Marca, E., Lips, K. R., Lotters, S., Puschendorf, R., Ibanez, R., Rueda-Almonacid, J. V., Schulte, R., Marty, C., Castro, F., Manzanilla-Puppo, J., García-Perez, J. E., Bolanos, F., Chaves, G., Pounds, J. A., Toral, E. and Young, B. E. (2005). Catastrophic population declines and extinctions in neotropical harlequin frogs (Bufonidae: *Atelopus*). *Biotropica* 37(2): 190-201.
Longcore, J. E., Pessier, A. P. and Nichols, D. K. (1999). *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. *Mycologia* 91(2): 219-227.
Longcore, J. R., Longcore, J. E., Pessier, A. P. and Halteman, W. A. (2007). Chytridiomycosis widespread in anurans of Northeastern United States. *Journal of Wildlife Management* 71(2): 435-444.
Longo, A. V., Burrowes, P. A. and Joglar, R. L. (2009). Seasonality of *Batrachochytrium dendrobatidis* infection in direct-developing frogs suggests a mechanism for persistence. *Diseases of Aquatic Organisms* DAO Special 4: pp2
Lubick, N. (2010). Emergency medicine for frogs. *Nature* 465: 680-681.
Martin, T. G., Wintle, B. A., Rhodes, J. R., Kuhnert, P. M., Field, S. A., Low-Choy, S. J., Tyre, A. J. and Possingham, H. P. (2005). Zero tolerance ecology: improving ecological inference by modelling the source of zero observations. *Ecology Letters* 8(11): 1235-1246.
May, S., Zeisset, I. and Beebee, T. J. C. (2011). Larval fitness and immunogenetic diversity in chytrid-infected and uninfected natterjack toad (Bufo calamita) populations. *Conservation Genetics* 12(3): 805-811.
McCallum, H. (2005). Inconclusiveness of chytridiomycosis as the agent in widespread frog declines. *Conservation Biology* 19(5): 1421-1430.

Meagher, S. (1999). Genetic diversity and *Capillaria hepatica* (Nematoda) prevalence in Michigan deer mouse populations. *Evolution* 53(4): 1318-1324.
Murphy, P. J., St-Hilaire, S., Bruer, S., Corn, P. S. and Peterson, C. R. (2009). Distribution and pathogenicity of *Batrachochytrium dendrobatidis* in boreal toads from the grand teton area of Western Wyoming. *EcoHealth* 6(1): 109-120.
Murray, K. A., Retallick, R. W. R., Puschendorf, R., Skerratt, L. F., Rosauer, D., McCallum, H. I., Berger, L., Speare, R. and VanDerWal, J. (2011). Assessing spatial patterns of disease risk to biodiversity: implications for the management of the amphibian pathogen, Batrachochytrium dendrobatidis. *Journal of Applied Ecology* 48(1): 163-173.
Padgett-Flohr, G. E. and Hopkins, R. L. (2010). Landscape epidemiology of *Batrachochytrium dendrobatidis* in central California. *Ecography* 33: 688-697.
Pearman, P. B. and Garner, T. W. J. (2005). Susceptibility of Italian agile frog populations to an emerging strain of *Ranavirus* parallels population genetic diversity. *Ecology Letters* 8(4): 401-408.
Pedersen, A. B., Jones, K. E., Nunn, C. L. and Altizer, S. (2007). Infectious diseases and extinction risk in wild mammals. *Conservation Biology* 21(5): 1269-1279.
Piotrowski, J. S., Annis, S. L. and Longcore, J. E. (2004). Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. *Mycologia* 96(1): 9-15.
R Development Core Team (2008). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.
Rachowicz, L. J. and Briggs, C. J. (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(4): 711-721.
Rachowicz, L. J., Knapp, R. A., Morgan, J. A. T., Stice, M. J., Vredenburg, V. T., Parker, J. M. and Briggs, C. J. (2006). Emerging infectious disease as a proximate cause of amphibian mass mortality. *Ecology* 87(7): 1671-1683.
Rachowicz, L. J. and Vredenburg, V. T. (2004). Transmission of *Batrachochytrium dendrobatidis* within and between amphibian life stages. *Diseases of Aquatic Organisms* 61(1-2): 75-83.
Richards-Zawacki, C. L. (2010). Thermoregulatory behaviour affects prevalence of chytrid fungal infection in a wild population of Panamanian golden frogs. *Proceedings of the Royal Society Biological Sciences Series B* 277(1681): 519-528.
Rödder, D., Kielgast, J., Bielby, J., Schmidtlein, S., Bosch, J., Garner, T. W. J., Veith, M., Walker, S. F., Fisher, M. C. and Lötters, S. (2009). Global amphibian extinction risk assessment for the panzootic chytrid fungus *Diversity* 1: 52-66.
Rödder, D., Veith, M. and Lötters, S. (2008). Environmental gradients explaining the prevalence and intensity of infection with the amphibian chytrid fungus: the host's perspective. *Animal Conservation* 11(6): 513-517.
Rollins-Smith, L. A. (1998). Metamorphosis and the amphibian immune system. *Immunological Reviews* 166: 221-230.
Rudicell, R. S., Jones, J. H., Wroblewski, E. E., Learn, G. H., Li, Y. Y., Robertson, J. D., Greengrass, E., Grossmann, F., Kamenya, S., Pintea, L., Mjungu, D. C., Lonsdorf, E. V., Mosser, A., Lehman, C., Collins, D. A., Keele, B. F., Goodall, J., Hahn, B. H., Pusey, A. E. and Wilson, M. L. (2010). Impact of simian immunodeficiency virus infection on chimpanzee population dynamics. *Plos Pathogens* 6(9).

Rushton, S. P., Lurz, P. W. W., Gurnell, J., Nettleton, P., Bruemmer, C., Shirley, M. D. F. and Sainsbury, A. W. (2006). Disease threats posed by alien species: the role of a poxvirus in the decline of the native red squirrel in Britain. *Epidemiology and Infection* 134(3): 521-533.

Schmidt, B. R., Furrer, S., Kwet, A., Lötters, S., Rödder, D., Sztatecsny, M., Tobler, U. and Zumbach, S. (2009). Desinfektion als Maßnahme gegen die Verbreitung der Chytridiomykose bei Amphibien. Methoden der Feldherpetologie. Hachtel, M., Schlüpmann, M., Thiesmeier, B. and Weddeling, K. Bielefeld, Laurenti Verlag. 229–241

Skerratt, L. F., Berger, L., Speare, R., Cashins, S., McDonald, K. R., Phillot, A. D., Hines, H. B. and Kenyon, N. (2007). Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *EcoHealth* 4: 125-134.

Smith, K. F., Acevedo-Whitehouse, K. and Pedersen, A. B. (2009). The role of infectious diseases in biological conservation. *Animal Conservation* 12(1): 1-12.

Thiesmeier, B. (1992). Daten zur Larvalentwicklung der Geburtshelferkröte *Alytes o. obstetricans* (Laurenti, 1768) im Freiland. *Salamandra* 28(1): 34-48.

Tobler, U. and Schmidt, B. R. (2010). Within- and among-population variation in chytridiomycosis-induced mortality in the toad *Alytes obstetricans*. *PLoS ONE* 5(6): e10927.

Van Oosterhout, C., Hutchinson, W. F., Wills, D. P. M. and Shipley, P. (2004). micro-checker: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes* 4(3): 535 - 538.

Vredenburg, V. T., Knapp, R. A., Tunstall, T. S. and Briggs, C. J. (2010). Dynamics of an emerging disease drive large-scale amphibian population declines. *Proceedings of the National Academy of Sciences of the United States of America* 107(21): 9689-9694.

Walker, S. F., Bosch, J., Gomez, V., Garner, T. W. J., Cunningham, A. A., Schmeller, D. S., Ninyerola, M., Henk, D., Ginestet, C., Arthur, C.-P. and Fisher, M. C. (2010). Factors driving pathogenicity vs. prevalence of amphibian panzootic chytridiomycosis in Iberia. *Ecology Letters* 13(3): 372-382.

Walker, S. F., Bosch, J., James, T. Y., Litvintseva, A. P., Valls, J. A. O., Piña, S., García, G., Rosa, G. A., Cunningham, A. A., Hole, S., Griffiths, R. and Fisher, M. C. (2008). Invasive pathogens threaten species recovery programs. *Current Biology* 18(18): R853-R854.

Wang, J. L. (2004). Sibship reconstruction from genetic data with typing errors. *Genetics* 166(4): 1963-1979.

Weldon, C. and Du Preez, L. H. (2006). Quantitative measurement of Batrachochytrium dendrobatidis in amphibian skin. *Diseases of Aquatic Organisms* 72(2): 153-161.

Woodhams, D. C. and Alford, R. A. (2005). Ecology of chytridiomycosis in rainforest stream frog assemblages of tropical Queensland. *Conservation Biology* 19(5): 1449-1459.

Woodhams, D. C., Alford, R. A. and Marantelli, G. (2003). Emerging disease of amphibians cured by elevated body temperature. *Diseases of Aquatic Organisms* 55(1): 65-67.

Woodhams, D. C., Ardipradja, K., Alford, R. A., Marantelli, G., Reinert, L. K. and Rollins-Smith, L. A. (2007a). Resistance to chytridiomycosis varies among amphibian species and is correlated with skin peptide defenses. *Animal Conservation* 10(4): 409-417.

Woodhams, D. C., Bosch, J., Briggs, C. J., Cashins, S., Davis, L. R., Lauer, A., Muths, E., Puschendorf, R., Schmidt, B. R., Sheafor, B. and Voyles, J. (2011). Mitigating amphibian disease: strategies to maintain wild populations and control chytridiomycosis. *Frontiers in Zoology* 8(8).

Woodhams, D. C., Vredenburg, V. T., Simon, M.-A., Billheimer, D., Shakhtour, B., Shyr, Y., Briggs, C. J., Rollins-Smith, L. A. and Harris, R. N. (2007b). Symbiotic bacteria contribute to innate immune defenses of the threatened mountain yellow-legged frog, *Rana muscosa*. *Biological Conservation* 138: 390-398.

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I dedicate this thesis to all amphibians that got sampled during the project.

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Tobler U, Schmidt BR (2010) Within- and among-population variation in chytridiomycosis-induced mortality in the toad *Alytes obstetricans*. PLoS ONE. 5(6): e10927. doi:10.1371/journal.pone.0010927

Tobler U, Schmidt BR, Geiger CC (2010) *Batrachochytrium dendrobatidis*: ein Chytridpilz, der zum weltweiten Amphibiensterben beiträgt. Schweizerische Zeitschrift für Pilzkunde 88(3): 112-116.

Schmidt BR, Tobler U, Ramseier P (2010) Die Verbreitung der Geburtshelferkröte (*Alytes obstetricans*) im Kanton Basel-Landschaft (NW-Schweiz). Mitteilungen der Naturforschenden Gesellschaften beider Basel 12: 75-84.

Schmidt BR, Furrer S, Kwet A, Lötters S, Rödder D, Sztatecsny M, Tobler U, Zumbach S (2009) Desinfektion als Maßnahme gegen die Verbreitung der Chytridiomykose bei Amphibien. In: Hachtel, M., Schlüpmann, M., Thiesmeier, B. and Weddeling, K. (eds.) Methoden der Feldherpetologie. Laurenti Verlag, Bielefeld, pp. 229–241.