Trojan Horses: Colonization of ponds built for amphibian conservation by a pathogen, *Batrachochytrium dendrobatidis.*

Alpine Newt (left) and Common Midwife toad male carrying eggs (right)
Source-Wikipedia

By Aaron Louis Pereira

Supervisors: Dr. Benedikt Schmidt, Dr. Jasmin Winkler & Dr. Arpat Ozgul

Table of Contents

Abstract: Biodiversity is under threat and conservation action is necessary. However conservation efforts are being undermined by a number of issues. One of the relatively understudied issues is infectious diseases. Chytridiomycosis is an infectious disease that is threatening conservation projects for amphibians around the world. It is caused by the fungal pathogen *Batrachochytrium dendrobatidis (Bd)* and studies have shown that it is one of the main causes for the decline of amphibian populations. The study conducted here aims to determine whether current efforts of an amphibian conservation project in Emmental, Switzerland, are being compromised by a deadly pathogen. New breeding ponds are constructed as part of conservation efforts, and these ponds are colonized by amphibians. But this has to be scrutinized as increased movement to ponds by amphibian individuals could lead to an increased risk for the ponds being colonized by *Bd*. We chose two suitable host species, a tolerant host species, *I. alpestris* (the Alpine newt), and a highly susceptible host species, *A. obstetricans* (the Common midwife toad), and determined their roles in the spread and prevalence of the pathogen in ponds. Using a hierarchical Bayesian occupancy model we were able to estimate the effect of the two host species on prevalence of the pathogen within the species. Similarly we aimed to see if the host species densities, species richness, pond characteristics and species specific connectivity among ponds had an effect on the prevalence of the pathogen in ponds. Our results suggest that conservation efforts are being compromised as *Bd* did colonize most of the breeding ponds. *A. obstetricans*, which is also the target species of conservation efforts, had a higher estimated prevalence of the pathogen and infection intensity than *I. alpestris.* The densities of the tolerant species *I. alpestris* had a negative effect on prevalence in ponds, whereas densities of *A. obstetricans* had a large positive effect on prevalence. Species richness seemed to have no observable effect. From the pond characteristics altitude, water temperature and conductivity all had negative effects on prevalence in ponds, while solar exposure was seen to have a positive effect. Species specific connectivity to ponds also appeared to play an important role in the spread of the disease, and for both species it was seen that increase in connectivity among sites had a negative effect on prevalence of the pathogen in ponds. We hope that the findings of our study can help improve current conservation efforts and future conservation strategies can be made by considering all factors that influence and facilitate the spread of *Bd.*

1.0 Introduction

Biodiversity around the world is threatened by a number of issues - habitat loss, climate change and overexploitation have all been directly linked to the global loss of species. Conservation action is very important to help protect biodiversity, but certain issues have the potential to undermine conservation action. A relatively understudied and growing issue in nature conservation is infectious disease (Kilpatrick et al. 2010). The cases of emerging infectious diseases have increased over the last seventy years and were said to do so at an "alarming rate", with the majority being caused by pathogens of a wildlife origin (Jones et al. 2008). Understanding host-pathogen dynamics in the wild is therefore crucial for the success of future conservation efforts.

Chytridiomycosis is an emerging infectious disease that has been directly linked to the on-going decline of amphibians, currently the most vulnerable group of vertebrates (Stuart et al. 2004). The pathogen causing the disease is an aquatic chytridiomycete fungus known as *Batrachochytrium dendrobatidis* (*Bd*) (Berger 1998). It colonizes the keratinized skin layers of amphibians (Longcore et al. 1999), where it causes an overproduction of keratin (hyperkeratosis) and prevents the uptake of essential electrolytes of the amphibian host. Insufficient electrolyte levels can result in cardiac arrest, eventually causing the death of the host (Longcore et al 1999, Voyles et al 2009).

Susceptibility to chytridiomycosis varies among amphibians on a species level as well as on a population level (Daszak et al 2004). Populations of some species have suffered mass mortalities while other populations and species seem to be tolerant of *Bd* infections and rarely develop the disease or are not negatively affected by the disease (Kilpatrick et al 2010). Susceptible host species are those species that are more likely to get the disease than the general population and the pathogen tends to have a high impact on the health of these hosts. Tolerant host species are those that are able to limit the impact of the pathogen on their health or fitness. As both tolerant and susceptible host species can carry the pathogen and spread the infection, both types of species can play an important role in disease dynamics. Tolerant species act as reservoirs for the pathogen and have the potential to maintain a positive force of infection on susceptible host species (McCullum and Dobson

2002, Daszak et al 2004, Vredenburg et al 2010). The Common midwife toad (*Alytes obstetricans,* Laurenti, 1768) is a good example of a susceptible host species as it has a very high risk of being infected by *Bd* and developing chytridiomycosis (Bosch et al 2007, Balaz et al 2013). It has been documented that populations of the Common midwife toad have seen a decline due to the chytrid fungus (Bosch et al 2000). The Alpine newt (*Ichthyosaura alpestris,* Laurenti, 1768) is a good example of a tolerant species. Alpine newts show high levels of *Bd* prevalence (i.e. the proportion of individuals having the disease that are present in a particular population at a given time), but populations of Alpine newts seem to be unaffected by the disease (Ohst et al 2011, Rasmussen et al 2012). Understanding how potential host species, both tolerant and susceptible, play a role in the spread of the pathogen is important when assessing risk in conservation. A number of studies have also suggested that there is a dilution effect in amphibians, i.e. when biodiversity and disease risk are inversely related, where increasing species richness leads to reduced disease risk (Searle et al 2011). Infection dynamics are also under the influence of environmental factors, such as the altitude, water temperature and the connectivity of amphibian breeding ponds (Sapsford et al 2013, Gmur 2011).

To antagonise amphibian declines, many conservationists have implemented different techniques in order to maintain populations of the species under threat. A common method used in amphibian conservation is the building and management of new breeding ponds. This is often done at a terrestrial habitat suitable for one or more target species (Goldberg and Waits 2009). The aim is for the long term maintenance of the amphibian metapopulation, increasing connectivity among these populations and that these constructed breeding ponds would be colonized from the surrounding source populations. However there is a long standing issue that needs to be scrutinized regarding these pond building projects. Integration of isolated populations and the promotion of migration among populations might undermine conservation efforts by boosting the spread of diseases (Hess 1994), and as these ponds are built with suitable habitats and connectivity in mind, pathogens can be a non-target beneficiary of the conservation efforts. There is a documented case where the fungal disease chytridiomycosis has threatened species recovery programs as was seen with the Majorcan midwife toad, *Alytes muletensis* (Walker et al 2008).

To gain a better understanding of disease dynamics in the wild and how it affects conservation programmes, this study investigates the spread and occurrence of *Bd* in a pond creation project for amphibian conservation purposes in the Swiss Emmental (Emmental pond creation project, EPCP). In this context, we want to find out whether the current conservation efforts in the EPCP area are being compromised by the deadly pathogen, *Bd*. To accomplish this objective, I chose to investigate the importance of a tolerant host species, the Alpine newt (*Ichthyosaura alpestris*) and a highly susceptible host species, the Common midwife toad (*Alytes obstetricans*), for the spread of the pathogen in the EPCP area and to see if the species act as vectors for the pathogen. *Alytes obstetricans* is also the target species of conservation efforts, so understanding how the pathogen affects them is important. I determined their role in disease prevalence among different ponds, and how these particular host species' abundance and densities influence prevalence of the disease and infection intensities (the mean number of parasite zoospores found in the infected host) of infected individuals. From previous studies it is shown that both species have high prevalence of the disease (Rosa et al 2012, Rasmussen et al 2012), and therefore I initially expected that the prevalence would increase with increasing densities of both host species. Also I expected infection intensities to differ among the species, with intensities being higher in the Common midwife toad (Bosch et al 2007, Ohst et al 2011). In addition, I also investigated the effect of species richness on disease prevalence in the area. A study by Searle and colleagues (2011) showed that a dilution effect on disease prevalence was a result of high species richness. Host species connectivity to pond sites was also looked at as an important factor that could influence the spread of the pathogen. There is much concern whether increased connectivity among habitats might allow for diseases to spread more easily, and thus it would be expected that increased connectivity for these host species among sites might increase the prevalence of the disease (Hess 1994, Hess 1996).

Not only biotic factors affect pathogen prevalence, abiotic conditions may play a role as well. Pond characteristics such as pond elevation, water temperature, water conductivity and pond solar exposure were also investigated to determine whether these variables affected the prevalence of the disease. It was expected that ponds at lower altitude and ponds with higher water temperatures would have lower prevalence of the disease (Beaskoetxea et al 2015). Conductivity of water, which is directly related to the

concentration of salts dissolved in water, is expected to have a negative effect on prevalence of the disease (Heard et al 2014). Solar exposure, the amount of sunlight at a site, would be expected to have a positive effect on prevalence of *Bd* as it hampers the immune response of amphibian individuals leading to an increase in susceptibility (Garcia et al 2006).

The hope for this study is that the findings will help improve current conservation strategies. By looking at which factors influence the spread of the disease, strategies for the building of new breeding ponds could be based upon the results of this study.

2.0 Methods and Material

2.1 Study Area

The study area is located within the Bernese Emmental region of Switzerland (centered on 62.6° N; 19.6° E, **Figure 1**). The study area comprises a multitude of suitable breeding ponds for *A. obstetricans* and *I. alpestris*. Thirty eight of these ponds were created as part of an amphibian conservation project, the Emmental pond creation project (EPCP), which aims to maintain populations of the endangered Common midwife toad (*Alytes obstetricans*). The ponds are of varying age (see **Figure 2** for a distribution of the age of the ponds). It is known from previous studies that *Bd* is present in some of the source ponds in the area (Gmür 2011) and therefore we knew that the chytrid fungus could potentially be present in the breeding ponds if they were colonized by infected individuals. The names of the ponds and their coordinates are summarized in a table in the appendix.

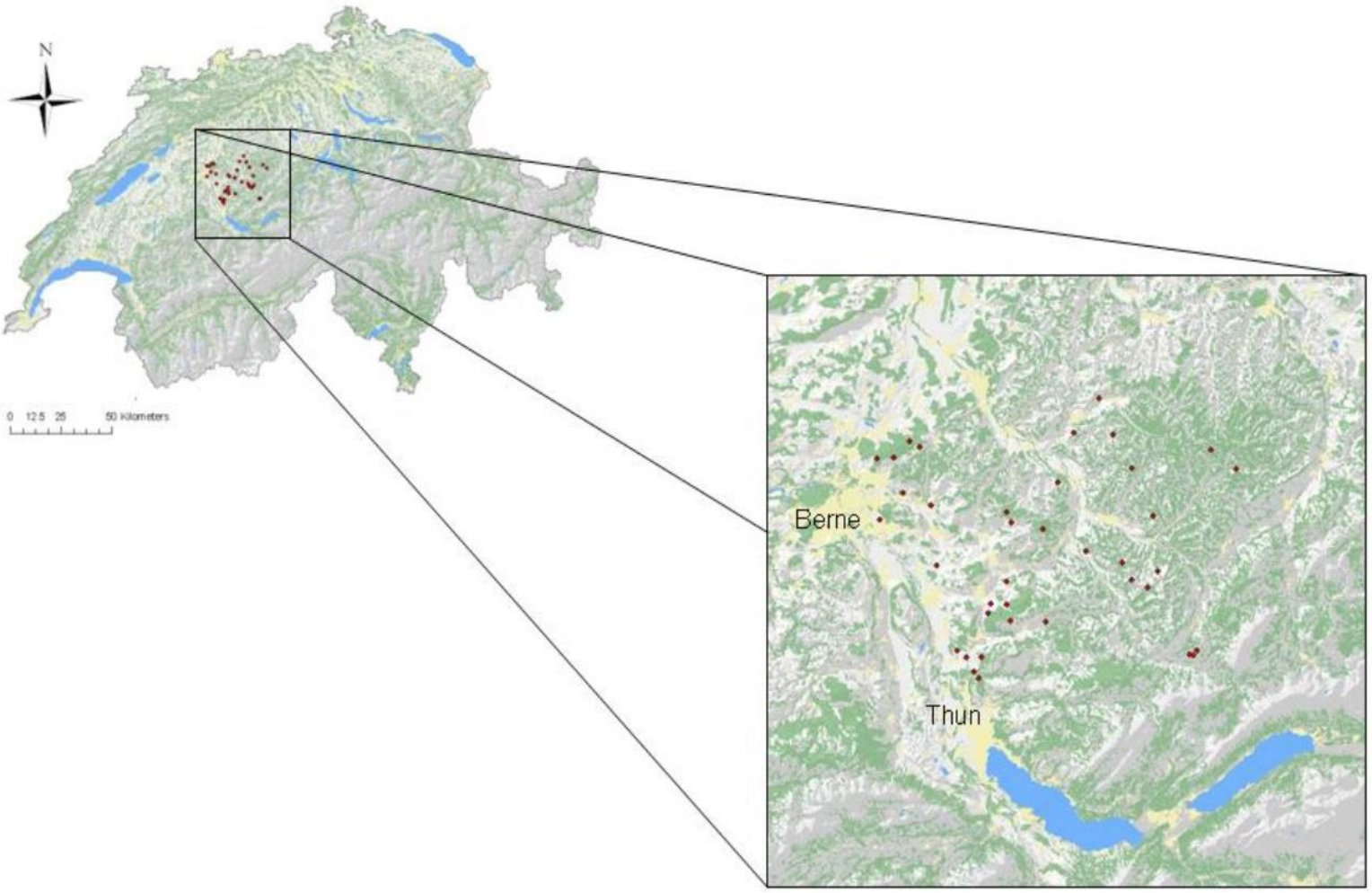

Figure 1: Location of the 38 EPCP ponds in Switzerland

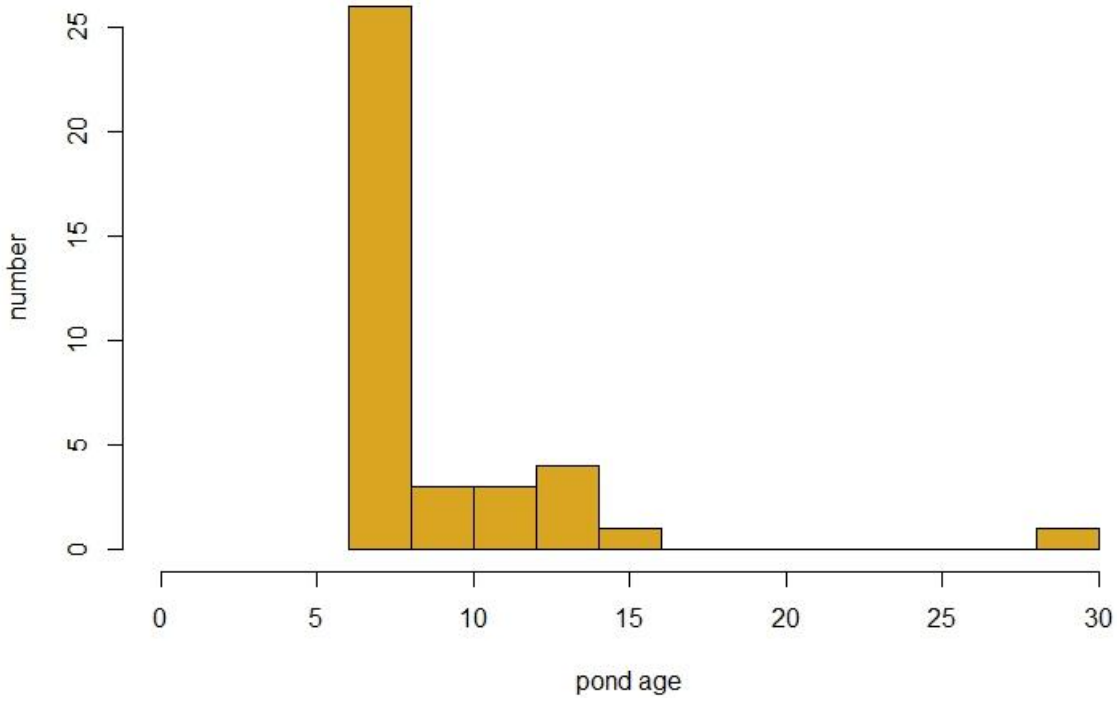

Figure 2: Distribution of the age (years) of the EPCP ponds

2.2 Study Species

2.2.1 Biology of *Bd*

Bd has two life history stages. The reproductive zoosporangium stage is sessile. The reproductive zoosporangium releases zoospores, which once released from the zoosporangium, is motile in water via a single posterior flagellum. The zoospore will directly attach themselves to keratinized layers of its hosts and once attached it matures into a zoosporangium with rhizoids (filamentous extensions used for attachment and assimilation). Within approximately four days the zoosporangium can produce up to 300 zoospores which are released into the environment. This cycle is repeated once the zoospore finds a suitable substrate to settle on. Zoospores can settle on the same host or on a new host if available (Lawrence D. 2008, Berger et al 2005). Isolates of *Bd* grow and reproduce at temperatures between 4– 25 °C and growth is at its maximum at 17–25° C (Piotrowski et al. 2003).

2.2.2 Host Species

The Common midwife toad (*Alytes obstetricans*) is native to Western Europe. It is currently listed as 'endangered' on the Swiss red list. Common midwife toads mate on land and are unique in that the male carries the eggs until embryonic development is completed. Then the adult males look for a pond with suitable conditions where they release the tadpoles. The larvae prefer water temperatures between 22°C - 25°C and larvae usually metamorphose in autumn or after hibernating in the following summer. They reach sexual maturity two to three years after metamorphosis (Meyer et al 2009).

The Alpine newt (*Ichthyosaura alpestris*) is endemic to central Europe and mountainous southern Europe. During the mating season the adults usually stay in ponds, but return to live terrestrially the rest of the year (Andreone et al 1991).

The Common midwife toad has the highest risk of *Bd* infection and of developing chytridiomycosis, compared to other species in the area (Bosch et al 2007, Balaz et al 2013). Therefore, it serves as the susceptible host species in this study. The Alpine newt, according to some studies, shows high levels of *Bd* prevalence but populations of the newt have not

seen a decline (Ohst et al 2011, Rasmussen et al 2012). Therefore the Alpine newt serves as the tolerant host species in this study which carries the pathogen to the ponds they colonize and have the potential to maintain a positive force of infection on the susceptible host species (McCullum and Dobson 2002, Daszak et al 2004, Vredenburg et al 2010).

Apart from these two study species, non-target species of the conservation project such as the Palmate newt (*Lissotriton helveticus,* Razoumowsky 1789), Common toad (*Bufo bufo,* Linnaeus, 1758), Common frog (*Rana temporaria,* Linnaeus, 1758), and Water frogs (*Pelophylax sp,* Linnaeus, 1758) are all found in the EPCP area. These non-target species are mostly not suitable host species for *Bd* apart from the palmate newt, which shows medium levels of infection. The common frog seems relatively resistant to *Bd* infection, while the common toad and water frogs show low to medium levels of *Bd* prevalence (Balaz et al 2013). All potential host species observed at a site were recorded to account for species richness.

2.3 *Bd* sampling

A total of 32 breeding ponds of the EPCP were visited to capture amphibian host species and sample *Bd* located on the hosts' skin tissue during spring and summer 2015. Initially, 38 ponds of the EPCP were chosen to be sampled but a number of ponds had to be excluded from the *Bd* sampling scheme. The reasons for the exclusion was that most of the ponds were dry with failure to capture any amphibian individuals at these sites, and for one of the sites which was under private property, the land owner prohibited us from sampling the pond for the study.

In order to detect at least one infected individual with 95% confidence (i.e. if *Bd* is present), a target of 40 host individuals per pond (pond-level infection) and 20 host individuals per species (species-level infection for both *Alytes obstetricans* and *Ichthyosaura alpestris*) were sampled (DiGiacomo and Koepsell 1986). All individuals were captured by dip-netting, and the two species were held in separate containers in order to prevent cross-species infection. For each pond, at least 50 sweeps were made around the pond or until we had the required amount of host species for the study. There were some ponds where we caught fewer than

20 individuals per species but still we proceeded to sample the few individuals for *Bd* we had caught at the site.

Amphibian skin was swabbed for *Bd* samples using sterile swabs (COPAN Diagnostics, Inc. CA, USA, code 155C). Tadpoles of the Common midwife toad were swabbed on the mouthparts, as this is where zoospores of *Bd* are best detected (Piotrowski et al 2004). Alpine newts were swabbed on the abdomen, underside of the feet and the genitals. Each of the part was swabbed five times in a standardized procedure (Hyatt et al 2007). Samples were stored in a freezer at -22°C.

After each visit, a strict disinfection protocol by Schmidt and colleagues (2009) was followed to limit anthropogenic spread of *Bd* between ponds.

2.4 *Bd* analysis

2.4.1 Chytrid DNA extraction

For detecting the presence of the pathogen and estimating infection intensities of the individuals from skin swabs, we used a quantitative Polymerase Chain Reaction (qPCR) protocol developed by Boyle and colleagues (2004). Chytrid DNA extraction from the swab tips was carried out using 60µl of Prepman Ultra solution (Applied Biosystems, P/N-43189300) and 0.03g of Silicate beads (Biospec) in safe lock tubes (1.5ml, Eppendorf). These tubes were then placed in a bead beater and beat for 40 seconds at the highest frequency (30/s), and right after that tubes were centrifuged for 30 seconds at 14,000 rpm (Eppendorf centrifuge machine). The tubes were then placed in a heating block for 10 minutes at 99°C and were allowed to cool for some time after heating was done followed by centrifuging for 3 minutes at 14,000 rpm. The supernatant in each tube was pipetted out and place into new tubes. From the supernatant 4 µl was pipetted and added to 36 µl of ddH_2O for attenuation.

2.4.2 Real time PCR

To detect the presence/absence of the chytrid and also measure infection intensities we used real time qPCR. After chytrid DNA extraction of all samples, plates for qPCR were prepared. In each well of the plate (one plate had 96 wells) we added a freshly prepared master mix solution. The master mix solution consisted of 474 µl of ddH_2O, 1200 µl of

Master Universal probe solution (Roche), 120 µl of forward primer solution, 6 µl of Probe (Applied Biosystems) and 120 µl reverse primer solution. Primer solutions were made by adding 54 µl of the primer to 246 µl of dd H_2O (Microsynth primers **ITS1:** 5'- CCT TGA TAT AAT ACA GTG TGC CAT ATC TC-3' and **5.8s:** 5'-AGC CAA GAG ATC CGT TGT CAA A-3').
Each plate contained 43 DNA samples that were run in replicates of two and two negative controls. In addition to this there were four positive *Bd* standards used (100, 10, 1 and 0.1 zoospore genomic equivalents) in replicates of two. The plates were sealed using a clear qPCR adhesive seal and placed into the qPCR cycling machine (stage 1: 50°C for 2 min, stage 2: 95°C for 15s and 60°C for 1 min with 60 cycles). All qPCR runs were done using the 7500 Fast Real-time PCR system by Applied Biosystems.

Bd infection status was confirmed if the *Ct* value (the cycle threshold which is defined as the number of cycles required for the fluorescent signal to cross the threshold i.e. exceeds background level) of the sample went above the fluorescence threshold that was generated using these positive *Bd* standards. All qPCR assays were performed using the 7500 Fast Real-Time PCR system by Applied Biosystems at the University of Zurich, Irchel campus. A more comprehensive list of all reagents and materials used during lab analysis is mentioned in the appendix.

2.5 Pond characteristics

Pond temperature was measured using digital data loggers (Onset HOBO® data loggers), which were placed at a constant depth of 25 cm below the surface for one week at each site. The average temperature of the week was taken for use in our analysis. All temperature values were measured in degrees Celsius (°C). The level of sun exposure at each site was measured using a spherical densitometer. It was measured as the percentage of open sun exposure available to a pond. The conductivity of the pond water, which is directly related to the amount of dissolve salts in the water, was measured using a conductivity meter (Consort C530). Conductivity was measured in micro Siemens per centimetre (µS/cm). Measurements for altitude of the pond were taken from the Swiss map website of the Schweizerische Eidgenossenschaft (Swiss Confederation, www.map.geo.admin.ch). Altitude was measured as meters above sea level. Connectivity is defined as "the degree to which the landscape facilitates movements of individuals" (Taylor

et al 1993) and it was calculated based from the occurrence data of the species which was taken from a database provided by Karch (the Swiss Amphibian and Reptile conservation program).

2.6 Statistics

We estimated *Bd* prevalence and infection intensities while accounting for observational uncertainty. The two sources of uncertainty are i) non-detection of infection in sampled individuals and ii) sampling error when quantifying infection intensity for infected individuals. The methods used here were developed by Miller and team (2012), are an extension of occupancy estimation (Mackenzie et al 2002), and address both sources of observation error. At the same time the models account for heterogeneity in detection probability resulting from individual variation in infection intensity. We are able to estimate different parameters using a hierarchical occupancy model and MCMC (Markov chain Monte Carlo) to fit the model.

Within the framework of the Miller and team model, prevalence and infection intensity were modelled as in GLM (Bolker et al 2009). We specified prevalence (Ψ) and infection intensity (δ) to be a function of fixed effects. For example infection intensity could be a function of species, where we used '1' to identify *I. alpestris* and '2' to identify *A. obstetricans*, and specified the relationship as

$$\delta_i = \alpha + \beta_{\delta\ species} * species_i ,$$

with priors specified for each of the coefficients (α, $\beta_{\delta\ species}$). And similarly in another model we made prevalence (Ψ) to be a function of species and specified the relationship as

$$logit(\Psi_i) = \alpha + \beta_{\Psi\ species} * species_i .$$

Estimated mean of prevalence was measured on the logit scale.
Initially we had chosen to fit all effects into one model, but there was a failure to converge for the MCMC sampling. Hence we decided to use simple models and run each covariate effect individually in order for the MCMC to successfully converge. In addition to species

effect on prevalence and infection intensity, we ran separate models to see relationships between species richness and prevalence as well as infection intensity, host species densities with prevalence, pond characteristics (altitude, water temperature, solar exposure and conductivity) with prevalence and host species connectivity with prevalence. All continuous variables had to be standardized and then used in the analysis.

We modified the code created by Miller and team (2012) and used the software JAGS (Just another Gibbs sampler, Plummer 2003) to run the MCMC sampling. This was carried out in R (version 3.2.2) using the 'jagsUI' package (Kellner 2014). From the analysis we get means and credible intervals for posterior distributions of parameters such as the prevalence of *Bd* and infection intensity of individuals for the different covariates.
For all parameters, priors used were uniformly distributed, dunif (-10, 10). The MCMC sampling was run in three chains for 50000 iterations, with a burnin of 20000, and a thinning rate of 200 or 250. Gelman-Rubin statistic Rhat values had to be <1.1 to confirm if convergence was successful.

3.0 Results

We tested in total 695 samples of both *I. alpestris* (n= 544) *and A. obstetricans* (n=151) in a total of 32 sites of the EPCP area. From this a total of 159 (22.87%) sampled individuals from both species tested positive for *Bd* at least once from two replicates in qPCR. For *I. alpestris*, 89 (16.36%) out of 544 individuals tested positive for *Bd* infection and for *A. obstetricans,* 70 (46.35%) out of 151 individuals sampled tested positive*. Bd* was detected in 24 (75%) of the 32 sites sampled. Out of the 29 sites where Alpine newts were found, 22 were positive for *Bd*. And for Midwife toads, 8 of the 10 sites where they were found tested positive for *Bd.* From the data obtained we aimed to observe the difference between the host species *Bd* prevalence and infection intensity for the disease. Also we wanted to see if other factors such as the different pond characteristics (altitude, water temperature, solar exposure and water conductivity), and connectivity among ponds played a role in *Bd* prevalence. All figures stated below in the tables for the posterior distributions of the model are in the logit scale. In order to plot the results on the prevalence scale, values had to be back transformed.

3.1) Differences in prevalence and infection intensity between the two study species

From the hierarchical Bayesian occupancy model it was observed that there were differences in *Bd* prevalence and infection intensity between the two study species. From the posterior distributions of the model for prevalence, it is seen from the intercept and slope values that prevalence of *Bd* was higher in the susceptible host species *A. obstetricans* than in the tolerant host species *I. alpestris* (Table 1) among the sites.
Similarly from the intercept and slope values of the posterior distributions for infection intensity model, it was observed that infection intensities were much higher in *A. obstetricans* as compared with *I. alpestris* (Table 1). The 95% credible intervals for both the intercept and slope do not include zero, hence both effects are significant.

Table 1. Posterior distribution of (A) prevalence and (B) infection intensity parameters from the Bayesian model for both *I. alpestris* and *A. obstetricans*. All values for slope and intercept are on the logit scale.

I. alpestris* vs *A. obstetricans	**Mean**	**2.5%**	**97.5%**
A) Prevalence			
Intercept	-3.345	-4.178	-2.449
Slope	0.694	0.136	1.211
B) Infection intensity			
Intercept	-6.380	-8.103	-5.605
Slope	2.121	1.413	2.903

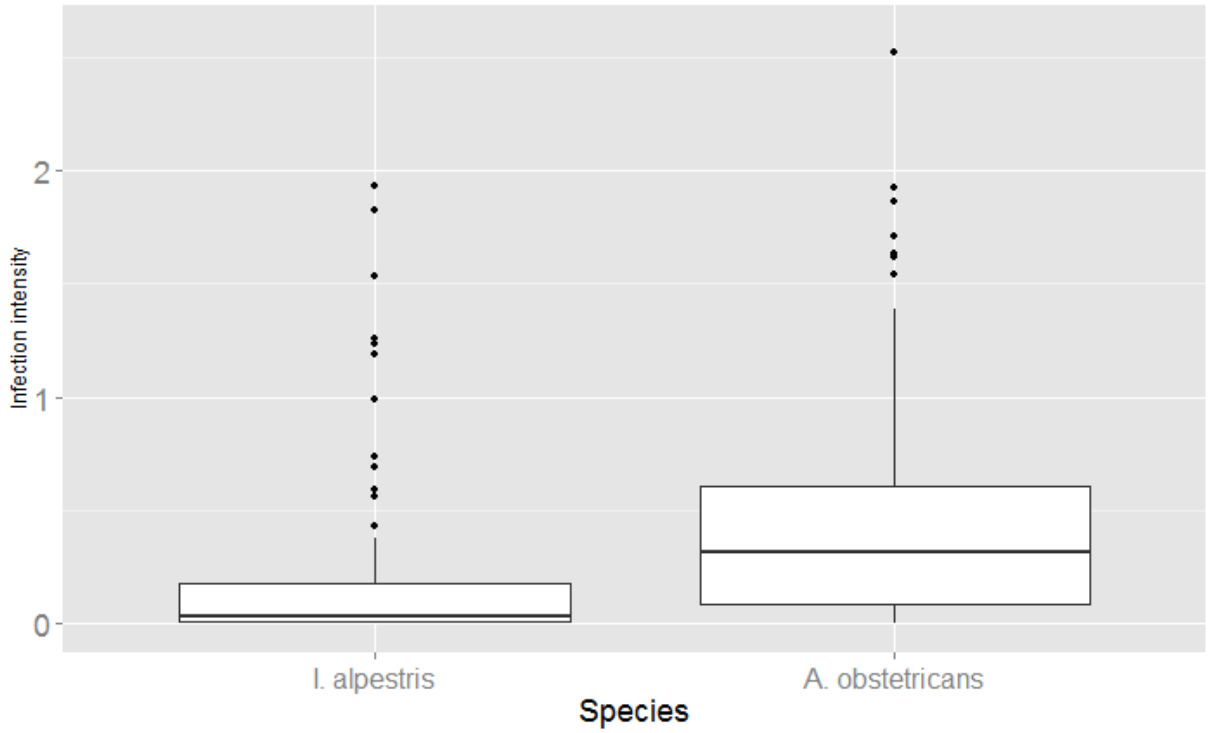

Figure 3. Observed infection intensities from infected individuals of *I. alpestris and A. obstetricans* (four outliers were not included for purposes of better visualization).

3.2) Influence of species densities on *Bd* prevalence

The density of *I. alpestris* had an unexpected negative effect on the prevalence of *Bd* within the species among the sites (Figure 4). The posterior distributions of the Alpine newt density model show that the slope of the relationship between prevalence and newt density is negative (Table 2), hence a decrease in *Bd* prevalence as *I. alpestris* density increased. Whereas for *A. obstetricans* the result was as hypothesized as there was a large positive effect observed on prevalence of *Bd* within the species (Table 2 and Figure 5).

As the 95% credible intervals for both parameters did not include zero, both of these effects were significant.

Table 2. Posterior distribution of prevalence parameters from the Bayesian model for (A) *I. alpestris* and (B) *A. obstetricans* densities. Mean and credible intervals for prevalence as a function of the study host species' density. All values are in the logit scale.

Host Species density	**Mean**	**2.5%**	**97.5%**
A) I. alpestris density			
Intercept	-1.050	-1.261	-0.766
Slope	-0.217	-0.434	-0.022
B) A. obstetricans density			
Intercept	5.497	3.834	6.506
Slope	8.760	6.425	9.955

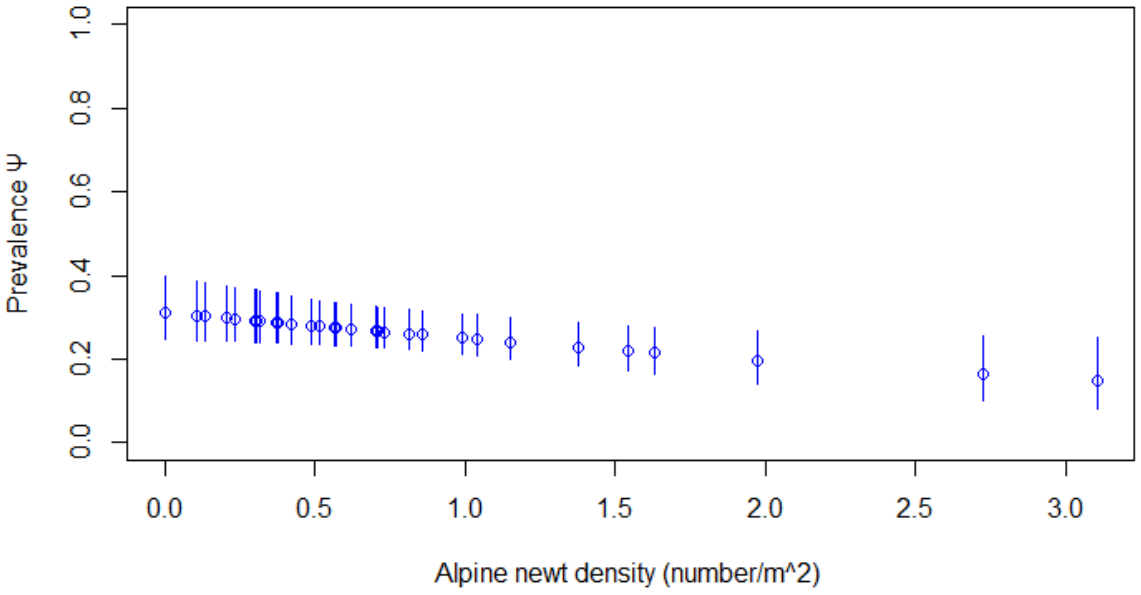

Figure 4. Influence of Alpine newt (*I. alpestris)* density on estimated *Bd* prevalence.

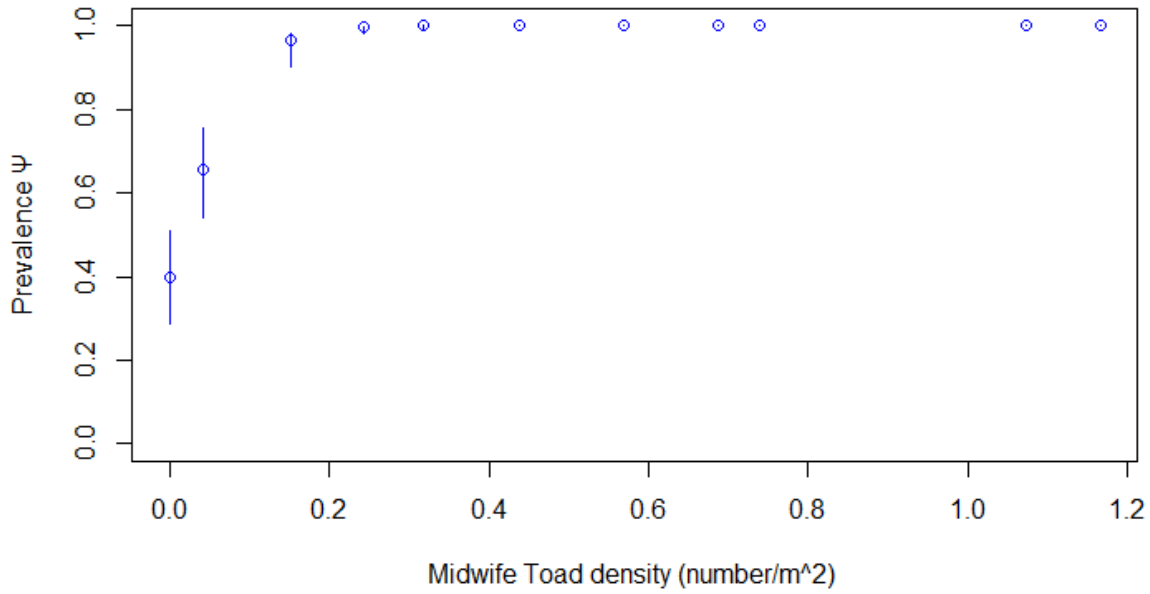

Figure 5. Influence of common midwife toad (*A. obstetricans*) density on estimated prevalence of the pathogen, *Bd*.

3.3) Effect of species richness on prevalence and infection intensity

We tested whether species richness would have an effect on estimated *Bd* prevalence and infection intensities of infected individuals for both species at sites. The model results indicated that there was no real effect of species richness on both estimated *Bd* prevalence and infection intensity as confidence intervals of the slope overlapped zero (Table 3).

Table 3. Posterior distribution of (A) Prevalence and (B) Infection intensity parameters from the Bayesian model for species richness. Mean and credible intervals for prevalence and infection intensity as a function of species richness. All values are in the logit scale.

Species Richness	Mean	2.5%	97.5%
A) Prevalence			
Intercept	-1.050	-1.261	-0.766
Slope	0.075	-0.110	0.265
B) Infection Intensity			
Intercept	5.497	3.834	6.506
Slope	0.057	-0.295	0.409

3.4) Pond Characteristics

Pond characteristic such as altitude, water temperature and solar exposure were all fit into one multilevel Bayesian model as running them individually in separate models resulted in a failure of Markov chains converging. Conductivity of water was run in a separate model. We looked to see whether the different pond characteristics had an effect on the prevalence of both species in general.

Altitude, water temperature and conductivity all had negative effects on prevalence of *Bd* as observed by the value of the slopes from the posterior distributions of the model (Table 4, Figure 6, Figure 7, and Figure 9). While solar exposure had a surprising positive effect on prevalence of *Bd* (Table 4 and Figure 8).

Table 4. The posterior distribution of prevalence parameters of the study host species from the Bayesian model for pond characteristics. Mean and credible intervals for prevalence as a function of pond characteristics (altitude, water temperature, solar exposure, conductivity of water, and pond area). All values are in the logit scale.

Pond Characteristics	**Mean**	**2.5%**	**97.5%**
A) *Altitude, Water temperature & Solar Exposure*			
Intercept	-1.059	-1.321	-0.675
Slope (Altitude)	-0.381	-0.534	-0.115
Slope (Water temp)	-0.321	-0.542	-0.106
Slope (Solar Exposure)	0.381	0.180	0.599
D) Conductivity of water			
Intercept	-1.083	-1.324	-0.789
Slope (Water Conductivity)	-0.451	-0.662	-0.250

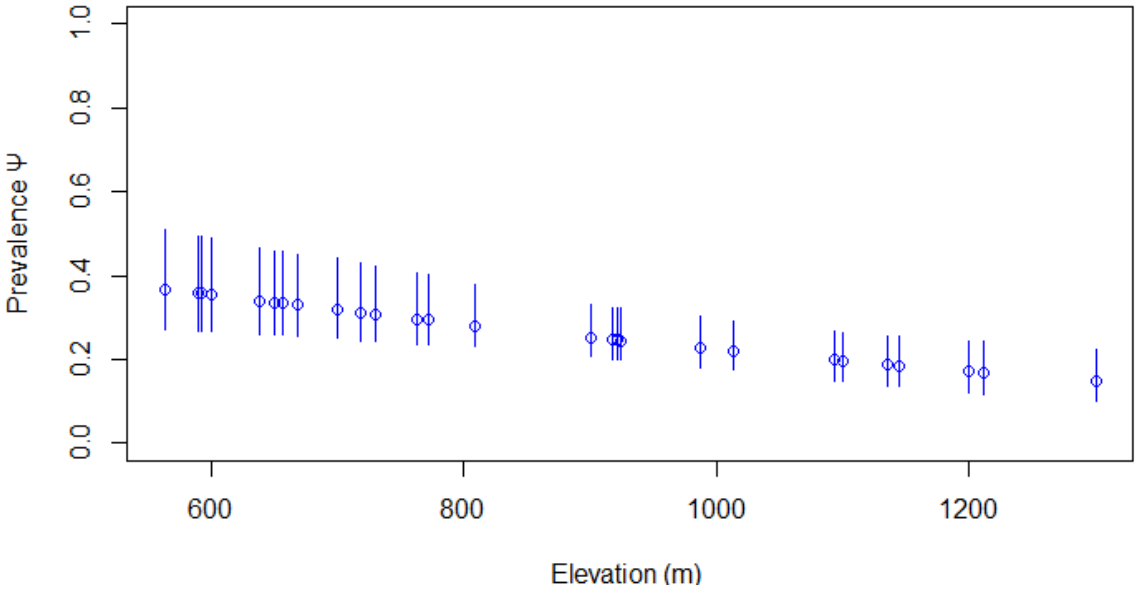

Figure 6. Influence of altitude on estimated prevalence of the pathogen, *Bd*.

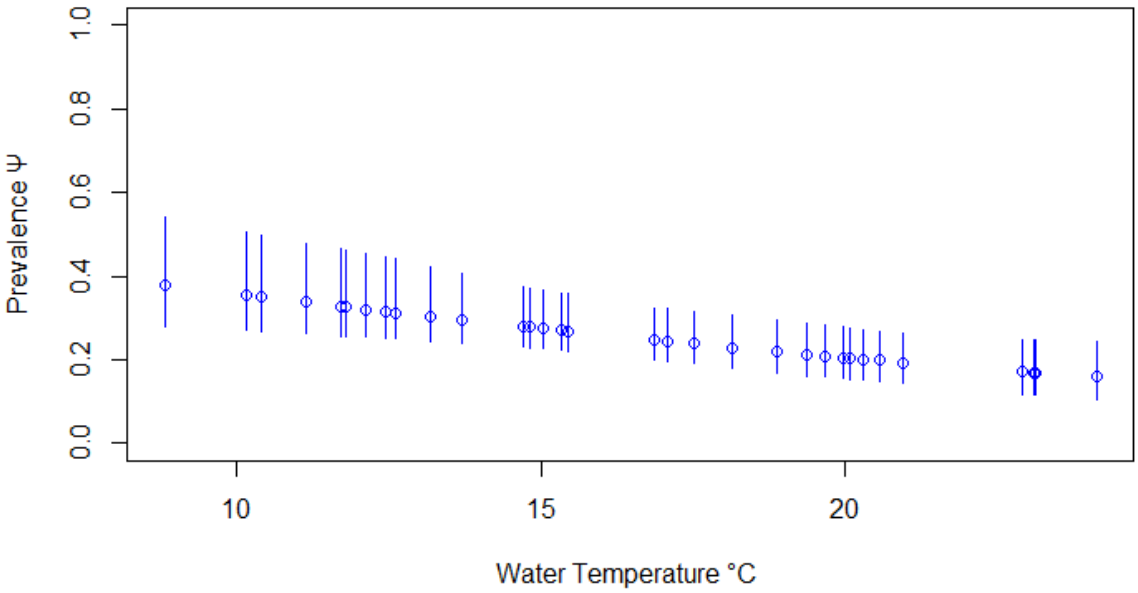

Figure 7. Influence of pond water temperature on estimated prevalence of the pathogen, *Bd*.

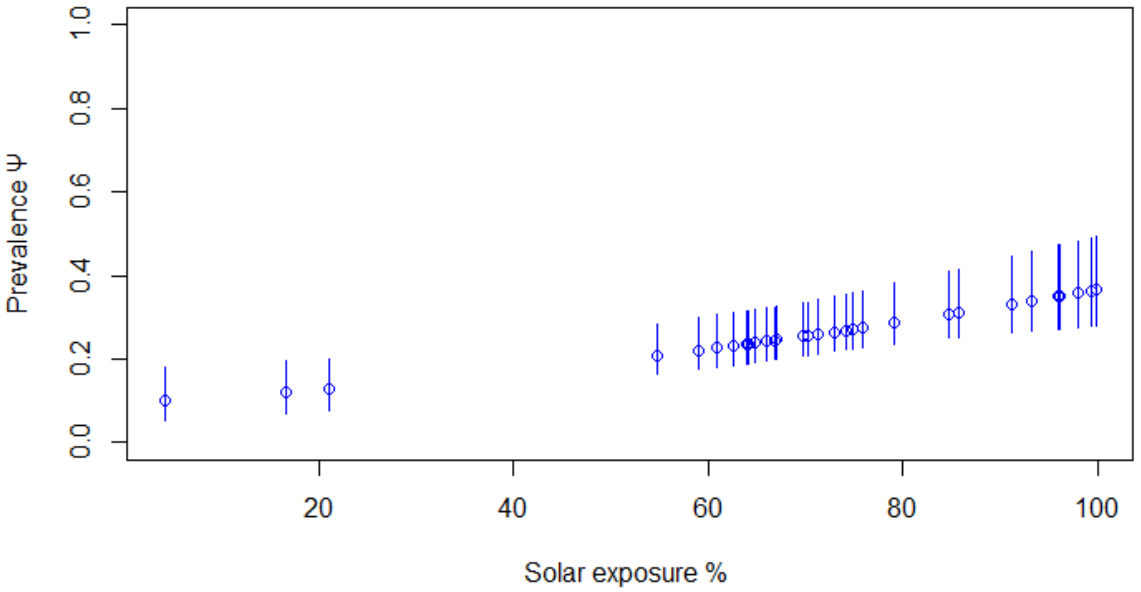

Figure 8. Influence of pond solar exposure on estimated prevalence of the pathogen, *Bd*.

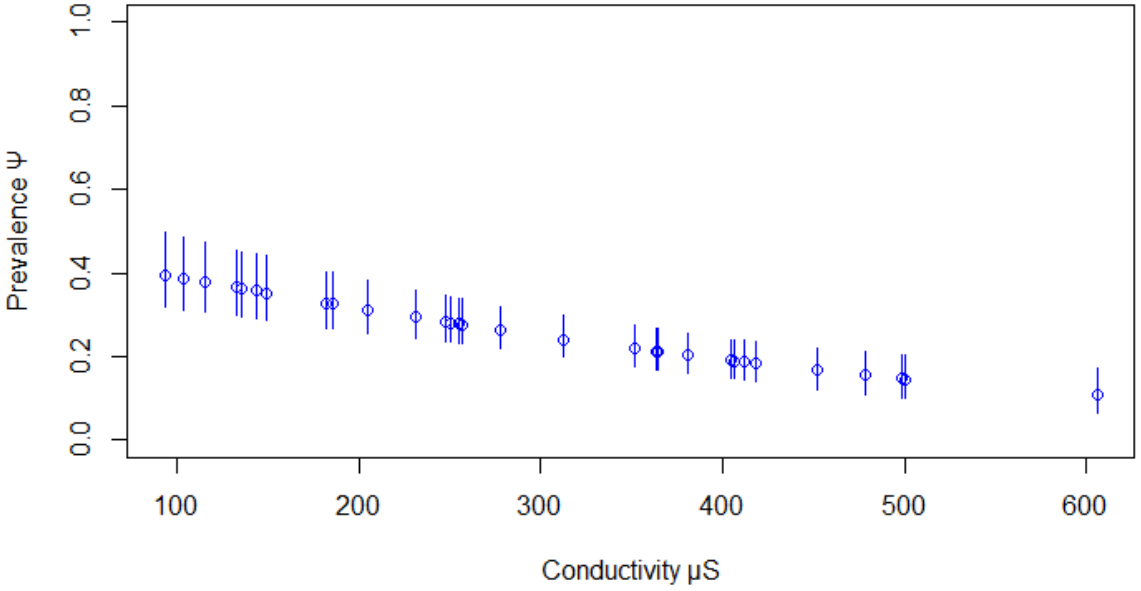

Figure 9. Influence of pond water conductivity on estimated prevalence of the pathogen, *Bd*.

3.5) Influence of species-specific connectivity on *Bd* prevalence

The slope values from the connectivity models for both *I. alpestris* and *A. obstetricans* were negative (refer table 5). This meant that there was negative effect on prevalence of *Bd* for both species as connectivity increased (Figure 10 and Figure 11). As the 95% confidence intervals of both slope values did not overlap zero, the results are statistically significant.

Table 5. Posterior distribution of prevalence parameters from the Bayesian model for both (A) *I. alpestris* and (B) *A. obstetricans* connectivity. Mean and credible intervals for prevalence as a function of connectivity. All values are in the logit scale.

Species specific connectivity	**Mean**	**2.5%**	**97.5%**
A) I. alpestris connectivity			
Intercept	4.954	1.556	9.458
Slope	-3.284	-5.349	-1.572
B) A. obstetricans connectivity			
Intercept	8.325	4.742	9.945
Slope	-4.600	-5.716	-2.712

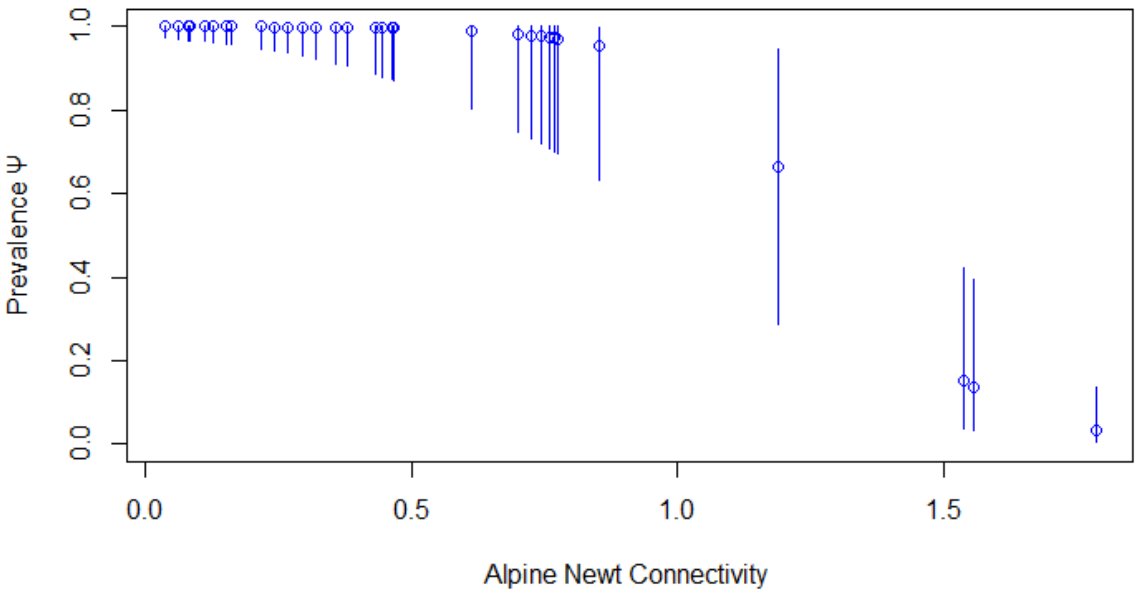

Figure 10. Influence of *I. alpestris* connectivity on estimated prevalence of the pathogen, *Bd*.

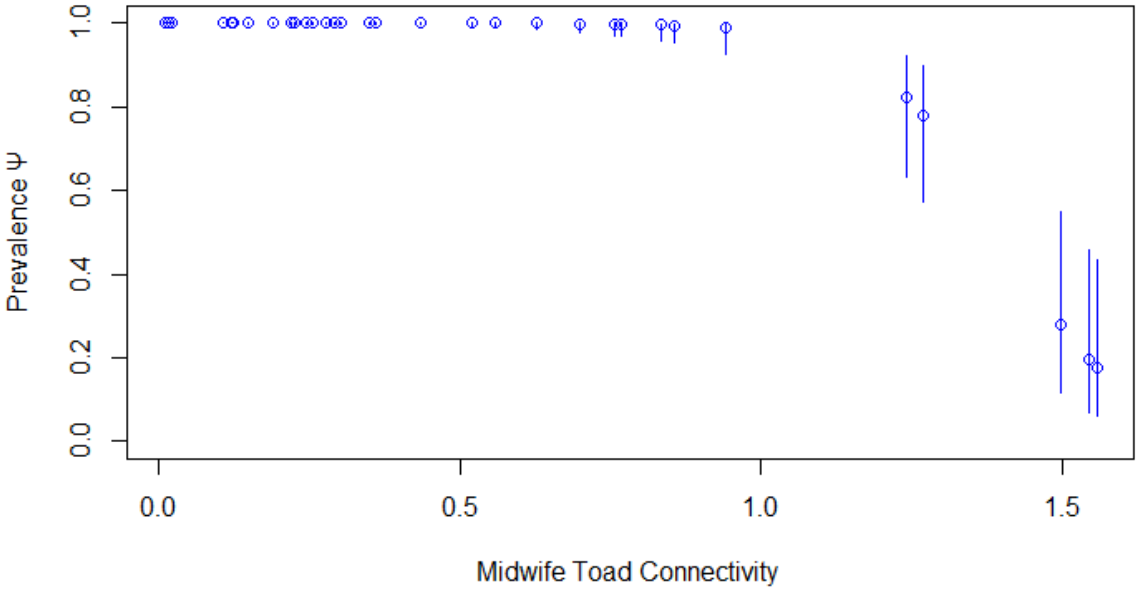

Figure 11. Influence of *A. obstetricans* connectivity on estimated prevalence of the pathogen, *Bd*.

4.0 Discussion

By monitoring the spread of pathogen in a conservation project, we assessed whether the current conservation efforts in the EPCP (Emmental pond creation project) area were compromised by a pathogen, *Batrachochytrium dendrobatidis,* and what factors played a role in the spread of this disease. We found that prevalence and infection intensities differed between the tolerant host species *I. alpestris* and the susceptible host species *A. obstetricans*. The densities of these host species influenced the prevalence of *Bd.* In addition we found that some pond characteristics (abiotic factors) and connectivity influenced the prevalence of the disease.

4.1) Differences between *I.alpestris* and *A. obstetricans*

A. obstetricans had higher prevalence of *Bd* and higher infection intensity levels than *I. alpestris.* This was to be expected as previous studies have shown the high sensitivity of *A. obstetricans* to infection from *Bd* (Bosch et al 2001, 2007). This would also suggest that intra-specific transmission of the infection is high and could also potentially be rapid.

4.2) Host species density

Bd prevalence was expected to increase with host species density (of both tolerant and susceptible host species). This was expected as a tolerant host species (*I. alpestris*) could potentially maintain a positive force of infection on the susceptible species and also carry the pathogen to other ponds in the area, hence increasing prevalence at sites.

A. obstetricans density had a large positive effect on the prevalence of *Bd* as expected. This finding could be possible because intra-species spread of infection and cross-species spread of infection for *A. obstetricans* is quite high, owing to the high sensitivity of the species to infection from *Bd*. This could also mean that tadpoles of *A. obstetricans* are intraspecific reservoirs for the pathogen. For *I. alpestris* however it was surprising to see that increasing density had a negative effect on prevalence of *Bd*. These findings seem to suggest that individuals of *I. alpestris* from this population are not very competent hosts for the

pathogens. Intra-species and even cross-species spread of infection is quite low for *I. alpestris*. Thus even with a higher density of the species, transmission of the pathogen is low. One possible reason for this is that *I. alpestris* could be a potential 'dead end host' for the pathogen, that is when zoospores do not grow to large numbers on the host and cannot be passed on to other individuals. So if the density of *I. alpestris* increases, then zoospores of *Bd* are trapped and eventually die off.

4.3) Species Richness

We expected species richness to have a dilution effect on the prevalence of the pathogen (Schmidt et al 2001, Searle et al 2011). The model results though showed no real effect on prevalence of *Bd,* but this could not be said with certainty as 95% credible intervals from the model results overlapped zero. This could be due to the fact that there is not much variation in species richness in the area. The range of species observed at the sites went from a minimum of 2 species to a maximum of 5 species. The effect of species richness on the prevalence of the pathogen might be seen in a more species rich area.

4.4) Pond characteristics

4.4.1 Altitude

Altitude was expected to have a positive effect on prevalence of the pathogen. At higher altitudes, *Bd* prevalence and its pathogenicity might increase, as higher altitudes might be extreme environments for some amphibians. This could lead to an accumulation of fitness costs which increases the susceptibility of those high altitude individuals. And also at higher altitudes, environmental conditions such as cooler water temperatures seem to favour *Bd* growth (Walker et al 2010). However the results we obtained was contrary to the hypothesis. Altitude had a negative effect on prevalence of the pathogen. The reason I would like to suggest for this happening is that at higher elevations population size of amphibian species might be low as these habitats might be extreme environments for them. And if susceptible host species are low in number at these sites, then prevalence of *Bd* at

these sites would also be low due to inefficient transmission of the pathogen to suitable hosts. Further studies would be needed to determine how exactly altitude affects prevalence and survival of *Bd*.

4.4.2 Water temperature

Temperature was expected to have a significant negative effect on prevalence of *Bd* (Beaskoetxea 2015). From the results we see that this is the case. Both the amphibian host and the pathogen are affected by temperature. Temperature can affect the metabolic rate and immune responses and behaviour of amphibians (Raffel et al. 2006, Knapp et al. 2011). Water temperature can affect the growth rate of *Bd.* The chytrid pathogen reaches its optimal growth rate in a temperature range of 17-25°C, which is lowered in sub optimal conditions below or above the optimal range (Piotrowski et al 2004). The temperatures during the sampling period for this study ranged from 7-25°C which mostly fell in the optimal conditions of *Bd* growth. But to truly look at the effects of temperature on the prevalence and growth of *Bd*, I would suggest that multi-season sampling needs to be carried out. This is because temperature could vary highly from year to year and keeping a record of temperature throughout the year could give a better understanding of how temperature in the area affects *Bd*

.

4.4.3 Solar exposure

Solar exposure had a positive effect on the prevalence of *Bd*. Ultra-violet radiation from the sun has negative impacts on amphibian populations and could potentially hamper the immune systems of certain amphibian species leading to an increase in susceptibility (Garcia et al 2006). Further studies need to be conducted, to observe more closely the effects of sun exposure and ultra-violet radiation levels on the virulence of the pathogen and also on the effect on the immune system of these host species.

4.4.4 Conductivity of water

Conductivity of water is directly related to the concentration of salts dissolved in water. The results correspond with the hypothesis that increased conductivity decreases prevalence. Prevalence decreased sharply at higher conductivity levels. The high salt concentrations seem to disfavour *Bd* growth (Heard et al 2014). I would suggest that more studies need to be conducted that scrutinize the mechanisms involved in the effects of conductivity on the pathogen and also as to what creates the high conductivity in ponds. If high conductivity is caused by agricultural runoff from farm fields then this might have a negative effect on amphibian health.

4.5) Connectivity

When it comes to conservation, there is always the concern that if increasing the connectivity among sites to increase the movement of individuals among meta-populations will increase the chance of diseases being spread (Hess 1994). This was expected to be the case for both the tolerant host species and the susceptible host species. Increasing connectivity of both species would see an increase in prevalence at these sites.

The results for both species were contrary to the hypothesis. There was a negative effect of connectivity of both *I. alpestris and A. obstetricans* on prevalence of the pathogen. A possible reason for this happening is because at ponds with lower connectivity, populations of both species could possibly be isolated with less movement to other sites. This results in *Bd* spreading among these “isolated individuals” and prevalence of the pathogen increasing locally at these sites.

4.6) Conclusion

The effectiveness of conservation programmes need to be monitored and assessed in order to determine if the efforts put into conservation are not being compromised by a pathogen. Our results suggest that current conservation efforts in the EPCP are being compromised by a pathogen as there is high intra-species prevalence of the pathogen, *Bd,* within the target species the Common midwife toad, giving *Bd* the chance to persist in habitats colonized by

the midwife toad and potentially spread to other habitats. From all the Common midwife toad individuals sampled, 46.35% tested positive for infection at least once in the qPCR analysis. The results show that it is important to consider which host species play a role in the spread of *Bd*, as some host species like the Common midwife toad have a higher influence on prevalence of the pathogen than other host species like the Alpine newt. Further studies need to be conducted to see how the spread of the pathogen among these host species could be reduced. Abiotic factors (pond characteristics) also influence prevalence of *Bd* and need to be taken into account before building more breeding ponds for conservation (taking into consideration the altitude, water temperature, conductivity and solar exposure on the pond). Altering of solar exposure could be done on certain ponds by including or removing shade structures. Conductivity also has an influence on the pathogen. If groundwater of the appropriate conductivity is accessible, and if the impacts of this suitable conductivity on biodiversity are low, considering the use of such water to reduce chytrid impacts would be a good conservation experiment (Heard et al. 2014). Possibly further studies need to be conducted to see how exactly conductivity affects the pathogen. Effects of altitude and water temperature also need to be observed closely as these factors also influence prevalence of the pathogen. The findings of this study show that increasing species connectivity among sites might be beneficial and lowers the prevalence of *Bd.* But this result has to be scrutinized and the mechanisms involving connectivity should be better understood, as there is strong evidence from other primary literature that it should increase prevalence. Based on our results we could attempt to predict which ponds are not so suitable for *Bd* and this gives us hope in possibly constructing *'Bd*-unfriendly ponds'. Further monitoring of the project would be needed on a year by year basis, and studies about other factors that could affect the prevalence of the pathogen could be carried out. A recent study suggests that other certain microfauna present in a pond could significantly reduce probability of infection of amphibian individual by the chytrid fungus (Schmeller et al 2014) and this is another factor that could be considered for further research in conservation.

5.0 Acknowledgements

Firstly, I would like to express my sincere gratitude to my supervisors Dr. Benedikt Schmidt and Dr. Jasmin Winkler for their continuous support of my Masters study, for their patience, motivation, and immense knowledge. Their guidance was very valuable in my research and writing of this thesis.

I am also grateful to Dr. Arpat Ozgul and all the members of the Popecol group for all the support they have given me.

My sincere thanks also go to Martina Schenkel, who taught me the protocols in the laboratory and provided support whenever needed, and also to Glauco Camenisch who helped me in the ordering of material for labwork.

A special thanks to Cindy Bregnard and Prof. Maarten Voordouw from the University of Neuchâtel, Switzerland, for providing me with the *Bd* standards that were needed to conduct the qPCR analysis. I appreciate Cindy's efforts to give me the support and information that I needed for lab work.

I also extend my thanks to Sam Cruickshank whose advice was very valuable for my thesis, and to my fellow master's students, also to Bernhard Schmid and Sara Petchey for all their help related to the thesis.

And finally a big thank you to all the land owners in the study area for allowing us to access the ponds, and to the University of Zurich along with the Stiftung Artenschutz for providing us with the funding without which this study could not have been done.

Special note: We had acquired the "**Ausnahmebewilligung Naturschutz**" and "**Fangbewilligung**" permits from the Kanton Bern, which allowed us to capture individuals and collect swab samples.

6.0 References

[1] Kilpatrick AM, Briggs CJ, Daszak P (2010): The ecology and impact of chytridiomycosis: an emerging disease of amphibians. In: *Trends in Ecology & Evolution* 25 (2), S. 109–118.

[2] Jones KE, Patel NG, Levy Marc A, Storeygard A, Balk D, Gittleman JL, Daszak P (2008): Global trends in emerging infectious diseases. In: *Nature* 451 (7181), S. 990–993.

[3] Daszak P, Cunningham AS, Hyatt AD (2003): Infectious disease and amphibian population declines Blackwell Science, Ltd. In: *Diversity and Distributions* 9, S. 141–150.

[4] Hess et al (1994)Conservation Corridors and Contagious Disease GR Conservation Biology Volume: 8 Issue: 1 Pages: 256-262

[5] Longcore JE, Pessier AP, Nichols DK (1999): Batrachochytrium dendrobatidis gen. et sp. nov., a chytrid pathogenic to amphibians. In: *Mycologia* 91 (2), S. 219–227.

[6] Martina Schenkel Master Thesis 2012. The prevalence of the amphibian pathogen Batrachochytrium dendrobatidis in permanent and temporary ponds

[7] Bosch, J., I. Martinez-Solano, and M. Garcia-Paris. 2001. Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas ofcentral Spain. Biological Conservation 97(3): 331–337.

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

[9] Schmidt, B.R., et al., Desinfektion als Maßnahme gegen die Verbreitung der Chytridiomykose bei Amphibien. In: Methoden für Feldherpetologie, p. 229–241 Hachtel, M., et al.. 2009a, Bielefeld: Laurenti Verlag.

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

[11] Jasmin Winkler proposal (unpublished). Trojan Horses in conservation biology: Spatiotemporal epidemiology of an emergent amphibian disease in aquatic habitats created for amphibian conservation. Zoological society of London.

[12] Voyles, J., S. Young, L. Berger, C. Campbell, W. F. Voyles, A. Dinudom, D. Cook, R. Webb,

R. A. Alford, L. F. Skerratt, and R. Speare. 2009. Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. *Science* 326(5952): 582-585.

[13] Goldberg, C. S., and L. P. Waits. 2009. Using habitat models to determine conservation priorities for pond-breeding amphibians in a privately-owned landscape of northern Idaho, USA. Biological Conservation 142:1096–1104.

[14] Walker, S. F., J. Bosch, T. Y. James, A. P. Litvintseva, J. Antonio, O. Valls, S. Piña, G. García, G. A. Rosa, A. A. Cunningham, S. Hole, R. Griffiths, and M. C. Fisher. 2008. Invasive pathogens threaten species recovery programs. Current Biology 18.

[15] Briggs, C. J., R. A. Knapp, and V. T. Vredenburg. 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 107:9695–700.

[16] McCallum, H., and A. Dobson. 2002. Disease, habitat fragmentation and conservation. Proceedings of the Royal Society London, B 269:2041–9.

[17] Bosch, J., L. M. Carrascal, L. Duran, S. Walker, and M. C. Fisher. 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.

[18] Kriger, K.M., F. Pereoglou, and J.M. Hero,Latitudinal variation in the prevalence and intensity of chytrid (*Batrachochytrium dendrobatidis*) infection in Eastern Australia. Conservation Biology, 2007. 21(5): p. 1280-1290.

[19] Berger, L., et al., Effect of season and temperature on mortality in amphibians due to chytridiomycosis. Australian Veterinary Journal, 2004. 82(7): p. 434-439.

[20] Walker, S.F., et al., Factors driving pathogenicity vs. prevalence of amphibian panzootic chytridiomycosis in Iberia. Ecology Letters, 2010 13(3): p. 372-382.

[21] Ribas, L., et al., Expression profiling the temperature-dependent amphibian response to infection by *Batrachochytrium dendrobatidis*. Plos One, 2009. 4(12): e8408.

[22] Matthias Gmur Master's Thesis, Factors driving an emerging pathogen-*Batrachochytrium dendrobatidis* in Switzerland, 2011, University of Zurich.

[23] Madeline Kroepfli Master's Thesis, Factors influencing colonization of created habitats by an endangered amphibian species 3022, University of Zurich.

[24] Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigues A, Fischman D, Waller RW (2004): Status and Trends of Amphibian Declines and Extinctions Worldwide. In: *Science* 306 (5702), S. 1783–1786.

[25] Skerratt LF, Berger L, Speare R, Cashins S, McDonald KR, Phillottt AD, Hines HB (2007): Spread of Chytridiomycosis Has Caused the Rapid Global Decline and Extinction of Frogs.

EcoHealth June 2007, Volume 4, Issue 2, pp 125-134.

[26] Alford RA and Richards SJ (1999): Global Amphibian Declines: A Problem in Applied Ecology. *Annual Review of Ecology and Systematics,* Vol 30 (1999), pp. 133-165.

[27] Berger L, Speare R, Hyatt AD. Chytrid fungi and amphibian declines: overview, implications and future directions. In: Campbell A. (ed) Declines and disappearances of Australian frogs. Environment Australia: Canberra. 1999: 23-3

[28] BALÁŽ, V., VÖRÖS, J., CIVIŠ, P., VOJAR, J., HETTYEY, A., SÓS, E., DANKOVICS, R., JEHLE, R., CHRISTIANSEN, D. G., CLARE, F., FISHER, M. C., GARNER, T. W. J. and BIELBY, J. (2014), Assessing Risk and Guidance on Monitoring of *Batrachochytrium dendrobatidis* in Europe through Identification of Taxonomic Selectivity of Infection. Conservation Biology, 28: 213–223. doi: 10.1111/cobi.12128

[29] Rasmussen C, Eisenberg T, Alfermann D, Kohler J (2012): Presence of *Bd* in amphibians from central and southern Hesse, central Germany: Results from a preliminary regional screening. In: *Salamandra* 48 (3), 166-172.

[30] Searle CL, Biga LM, Spatafora JW, Blaustein AR (2011): A dilution effect in the emerging amphibian pathogen Batrachochytrium dendrobatidis. In: *PNAS vol. 108 no. 39, 16322-16326.*

[31] Geoffrey W. Heard, Michael P. Scroggie, Nick Clemann, and David S. L. Ramsey 2014. Wetland characteristics influence disease risk for a threatened amphibian. Ecological Applications 24:650–662.

[32] Fernández-Beaskoetxea S, Carrascal LM, Fernández-Loras A, Fisher MC, Bosch J (2015) Short Term Minimum Water Temperatures Determine Levels of Infection by the Amphibian Chytrid Fungus in *Alytes obstetricans* Tadpoles. PLoS ONE 10(3): e0120237. doi:10.1371/journal.pone.0120237

7.0 Appendix

1) Coordinates for the ponds sites

CX	CY	SITE
63485	18586	Oberi Lochsiten
63445	18598	Holzweid
61500	19080	Hammenried
61339	18437	Sonnrain
60707	20628	Wannetal
60810	20580	Chräbsloch
63900	20362	Schwesterboden
62557	21037	Oberei
61413	18569	Gollermatte
61695	18915	Schönthalwald
63038	19236	Hohwürz
60417	19887	Lötschenbach
62316	20714	Kösterli Trachselwald
61175	18630	Oppligenmoos
60974	19444	Trimsteinmoos
60919	20023	Stämpach
62430	19583	Aeschau
63656	20545	Enzigrund
63135	19394	Steibächli
61658	19958	Geissrügge
62876	20379	Ober Rämis Langnau
62785	19470	Farneren Eggiwil
61268	18567	Weiher Oppligen
60392	20465	Schwarzkopf Bolligen
60550	20472	Dachshole Bolligen
61470	18991	Undermat Freimettigen
62011	19791	Ofeneggalp Signau
63521	18633	Buebenloui Obersti Lochsiten
62875	19307	Hinter Girsgrat
61383	18371	Wydibühl
61705	19857	Arnigrube
62038	18907	ehem Grube Schlatt
61653	19069	Holz Niederhünigen
60640	20142	Teiche Ferenberg
61653	19921	Hämlismatt
63088	19287	Crümpelhütt Möösli
62692	20691	Chnubel Heimisbach
62156	20241	Sunnberg