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The prevalence of the amphibian pathogen *Batrachochytrium dendrobatidis* in permanent and temporary ponds

Master Thesis of Martina Schenkel

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Table of Contents

Abstract

All over the world amphibians are experiencing declines. The invasive fungus *Batrachochytrium dendrobatidis (Bd)* is considered to be one of the major causes for these declines. Previous studies demonstrated that the prevalence of the pathogen varies among habitat types. In frogs breeding in permanent streams and ponds higher pathogen prevalence was found than in frogs breeding in temporary ponds and streams. Because *Bd* cannot survive desiccation, the summer drying of temporary ponds could be lethal to the fungus and so lead to reduced pathogen prevalence in such habitats. The aim of this master thesis was to investigate whether the hydroperiod of the pond has an influence on the prevalence and infection loads of *Bd* in pond-breeding amphibians, hypothesizing that *Bd* prevalence was lower in temporary than in permanent ponds. *Bd* is known to occur in Swiss amphibian populations, among others, in water frogs (*Pelophylax* spp.) and the alpine newt (*Ichthyosaura alpestris*). Of these two species individuals of 16 ponds (half temporary, half permanent) were tested for infection with *Bd*. Overall, 26.5 % of the 770 amphibians sampled were infected with *Bd*., but no significant difference in infection was found between the two pond hydroperiods. Interestingly, dissimilar patterns were detected in the variables that showed an effect on *Bd* prevalence in *Pelophylax* spp. (body mass) and *I. alpestris* (temperature and sampling day). These results show that the host-pathogen interaction in the case of *Bd* is highly species specific and prevalence and loads are dependent on the physiological characteristics of the host within the context of the external environment.

Introduction

Infectious disease is a relatively new issue in nature conservation (Kilpatrick et al. 2010). Cases of emerging infectious disease have increased over the last 70 years "in an alarming rate" and the majority of these events are caused by pathogens with a wildlife origin (Jones et al. 2008). Their effects on wildlife populations, ecosystem structure and the dynamics of biodiversity are complex (Anderson 1979, Crowl et al. 2008, Blaustein 2012). Population declines, and potentially extinctions, can be a consequence, if variable factors play together and the conditions for severe outbreaks are given (Smith et al. 2009, Voyles et al. 2009). One of the most compelling examples of diseases impacts in vertebrate species is amphibian chytridiomycosis (Daszak et al. 2003, Stuart et al. 2004, Briggs et al. 2010, Collins 2010). Chytridiomycosis is caused by the pathogen *Batrachochytrium dendrobatidis* (*Bd*) (Berger 1998, Longcore 1999). It infects the keratinized parts of the amphibian

skin and lead to epidermal changes, decreased osmotic regulation and a drop of electrolyte blood levels (Longcore 1999, Berger 2004, Voyles 2011), and eventually to death.

Chytridiomycosis is the most important disease related to the ongoing decline in amphibian species (Daszak et al. 2003, Stuart et al. 2004, Kriger & Hero 2007A, Skerratt et al. 2007, Fisher et al. 2009). Amphibians are currently the most declining vertebrate group on earth. According to the IUCN redlist (http://www.iucnredlist.org/initiatives/amphibians/analysis, accessed October 28, 2012) approximately one third (32 %) of the world's amphibian species is threatened or already extinct. Over 42 % of all species experience a decline in population and an improvement of the situation in the near future is not expected. Amphibians are thought to be indicators of general environmental health, hence, causes of declines might also threaten other species (Collins & Storfer 2003). The decline in amphibian species is one of the most compelling conservation issues at the moment (Kilpatrick et al. 2010) and appropriate approaches for disease control are needed (Woodhams et al. 2011).

Still, the environmental drivers of chytridiomycosis are not yet fully understood. In individuals of some species chytridiomycosis leads to rapid death, whereas in individuals of other species only little or no negative effects are visible (Briggs et al. 2010). Impacts range from local mass extinctions and fatal declines, as in Costa Rica 1987 (Lips et al. 2003), and in Spain 1997 (Bosch et al. 2001), to obviously no negative effect in infected midwife toads *Alytes obstetricans* in Switzerland (Tobler et al. 2012). Many environmental, physiological and species specific traits seem to influence *Bd* prevalence and the effects of an infection, for example temperature, elevation, host density, composition of skin peptides or the pond hydroperiod (Fisher et al. 2009, Kilpatrick et al. 2010, Woodhams et al. 2003). Regarding the broad range of species specific responses to *Bd* infection and their rich diversity of life histories and habitats no single solution is appropriate for disease control (Dodd 2010). Knowing that *Bd*-associated mortality is strongly dependent on *Bd* infection loads (Vredenburg et al. 2010, Tobler & Schmidt 2010, Kinney et al. 2011), the main goal for a successful pathogen handling does not have to be to fully extirpate the pathogen. We should rather try to find a strategy to mitigate *Bd* in natural habitats (Woodhams et al. 2011), to provide a refuge from the high infection intensities that cause fatal chytridiomycosis (Vredenburg et al. 2010).

One way to identify potential refuges from infection is to focus on habitats suitable for amphibians, but unsuitable for the pathogen. *Bd* is a waterborne pathogen and as such dependent on water for its survival in the environment. Several studies point out the high dependency on aquatic regimes. In laboratory experiments zoospores managed to survive in sterile water outside a host for up to 7 weeks, but total desiccation leads to death of the spores within 1 and 3 hours (Johnson et al. 2003, Garmyn et al. 2012). Kriger & Hero (2007B) found no evidence of an infection in frogs breeding in temporary water bodies, whereas amphibians breeding in permanent ponds and streams showed a significantly

higher risk of being infected. This study is not fully conclusive as for the different habitat types different species were compared, which could confound habitat and species. But it points out the potential importance of pond hydroperiod in regard to pathogen mitigation. The idea of temporary ponds being derogatory for the pathogen, moreover the summer drying of ephemeral ponds could lead to *Bd* extirpation (Padgett-Flohr & Hopkins 2010) is plausible.

The objective of this study was to find out whether pond hydroperiod influences *Bd* occurrence, prevalence and infection intensity. My expectation was to find *Bd* occurrence, prevalence and load of infection to be lower in temporary ponds than in permanent ponds. For this purpose I investigated populations of water frogs (*Pelophylax* spp.) and alpine newts (*Ichthyosaura alpestris*) in temporary and permanent ponds in Switzerland. *Pelophylax* spp. and *I. alpestris* occur in both pond types and can occupy the same habitats. Including two syntopic species allows a correction for species differences, and the results can be assigned more clearly to pond hydroperiod. The discovery of how *Bd* infection is related to habitat could give important implications for further disease management steps, furthermore, it could lead to a win-win situation in amphibian conservation because temporary ponds are important amphibian habitats (Griffiths 1997, Van Buskirk 2003).

Material and methods

Study design and sampling

Survey species

For this study, I investigated adult individuals of the water frog hybrid complex (*Pelophylax esculentus* spp.) and alpine newts (*Ichthyosaura (Mesotriton) alpestris*). Both species are widely distributed in Switzerland (Grossenbacher 1988) and maintain relatively high *Bd* infection prevalence in northern Switzerland (Tobler et al. 2012). *Pelophylax* spp. is common in a wide range of permanent and temporary pond types (Van Buskirk 2003). It is a highly aquatic species. The mating season reaches its peak in May or June, but first individuals returning from their hibernation space on land can be expected in the ponds from early March onwards (Meyer et al. 2009). *I. alpestris* lives and breeds in almost any kind of small water body and pond type, and even on shallow lakeshores (Grossenbacher 1988). The return to their mating habitats after hibernation on land starts in late February or early March (Meyer et al 2009). *Pelophylax* spp. contains the three species *Pelophylax lessonae* (pool frog), *Pelophylax ridibundus* (lake frog) and *Pelophylax esculentus* (water frog), which is a hybrid between *P. lessonae* and *P. ridibundus* (Schmidt 1993). As a differentiation of the species in the field is not possible with certainty (Meyer et al. 2009), all three species were investigated in my study, and thus the term *Pelophylax* spp. will refer to all three species further in this thesis.

This study focuses on adult individuals only, although this may carry the risk of sampling immigrant animals carrying an already existing infection with *Bd*. Studying tadpoles would ensure that the individuals were infected locally. However, a former study detected no *Bd* infection in over 500 *Pelophylax* spp. tadpoles sampled in 16 ponds in Switzerland, even though infected adults were present in some of the ponds (Lüthi 2011).

Study sites

I investigated 24 ponds in northern Switzerland (cantons Zurich and Thurgau, Fig. 1). In 8 of the water bodies both target species were present and sampled, whereas in the rest of the ponds only one species was present in numbers sufficiently large to sample. For each species, individuals of a total of 16 ponds (of which half were temporary, and half permanent) were sampled (Table 1). I chose the study sites in regard to their hydroperiod (temporary / permanent) and presence of the study species (information provided by Mario Lippuner pers. comm., Lippuner and Rohrbach 2009). The temporary ponds showed hydroperiods of either yearly desiccation, or at least in the rhythm of a few years

(Table 1). I expected *Bd* to occur in the majority of the chosen sites as former studies showed *Bd* prevalence in over 50 ponds in northern Switzerland (U. Tobler and B. R. Schmidt, unpublished data) and in the survey area in particular (Lüthi 2011). None of the ponds was directly adjacent to another water body, but migration of individuals from nearby ponds is possible. All ponds lied within a range theoretically reachable (< 2000 m) for amphibians of proximate habitats (Padgett & Flohr 2010, Jehle & Sinsch 2007, Smith & Green 2005). Water body elevation ranged from 348 to 443 meters above sea level for the water frogs and from 346 to 511 meters a.s.l. for the alpine newts.

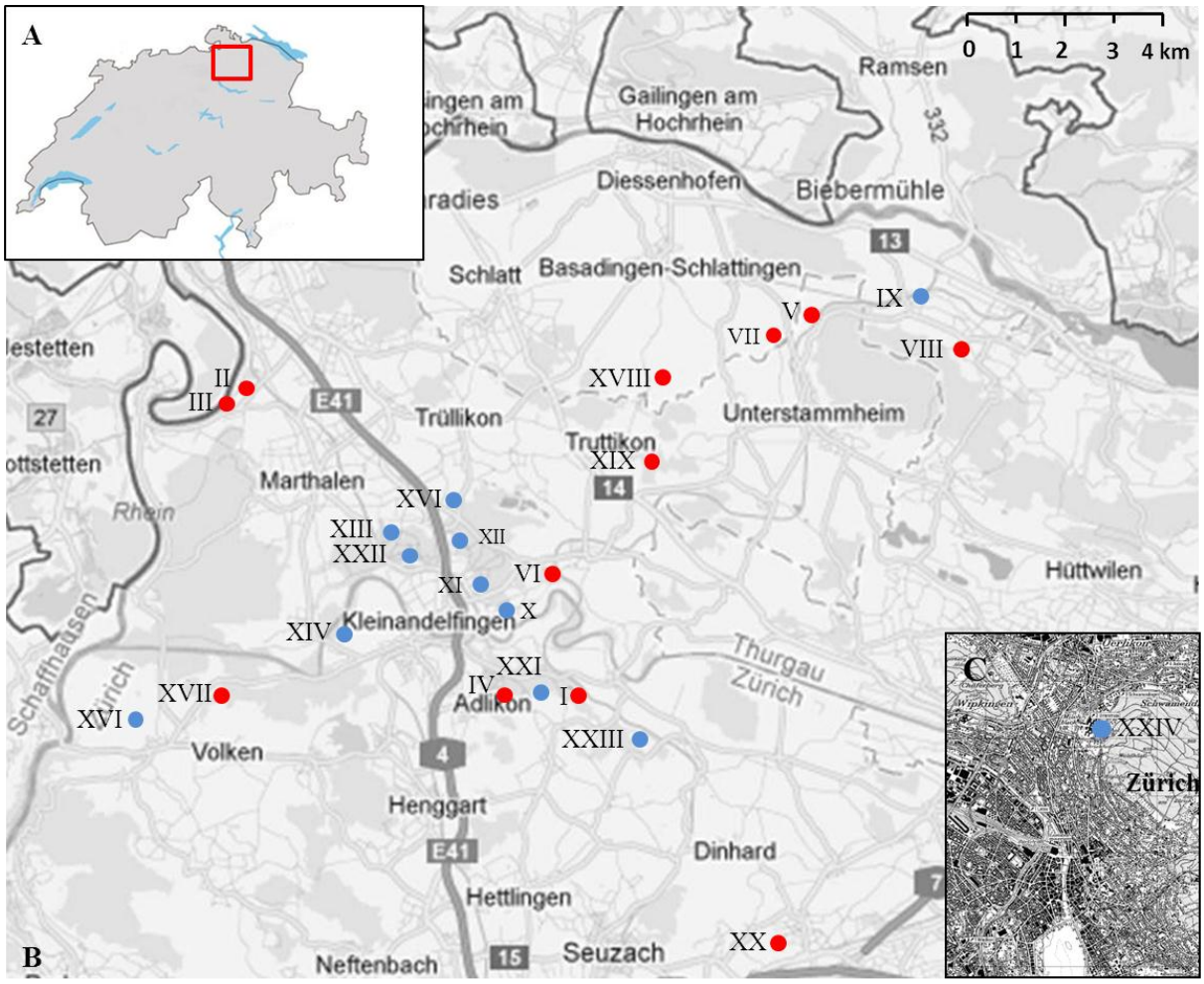

Figure 1. Overview of the sampling sites of *Pelophylax* spp. and *I. alpestris*. **A**: Location of the study area in northern Switzerland. **B**: Study sites are mapped by blue (temporary) and red (permanent) points. The roman numbers refer to the pond numbers of Table 1. **C** shows a map section of Zurich with the location of pond no. XXIV.

Table 1. Study sites for *Pelophylax* spp. and *Ichthyosaura alpestris*.

Pond no.	Pond name§	Hydroperiod	Coordinates (CH1903*)		Elevation	Species sampled (*n*)	
			x	y	(m)	*Pelophylax* spp.	*I. alpestris*
I	Weiher Gütighausen	permanent	696550	271400	412	25	7
II	Grube Oberboden	permanent	689600	277650	394	25	
III	Kiesgrube Rhinauer Feld	permanent	689300	277375	386	25	
IV	Ried bei Adlikon	permanent	694850	270950	429	25	25
V	Seewädeli	permanent	701600	279500	428	25	
VI	Chli Au Ossingen	permanent	696325	273630	369	25	21
VII	Sürch Schlattigen	permanent	700450	279000	414	25	25
VIII	Schutzgebiet Wagenhausen	permanent	704823	278794	443	25	
IX	Tümpel bei Buck	temporary 1	704100	279860	439	25	25
X	Pfaffensee	temporary 2	695000	273100	383	25	
XI	Heinrichsee	temporary 1	694610	273420	396	21	25
XII	Cholgruebsee	temporary 2	693990	274230	411	25	25
XIII	Enteler-Weiher	temporary 2	692400	274700	396	25	25
XIV	Altlauf Inselen	temporary 2	691625	272425	354	25	
XV	Kiesgrube südöstl. Feldhof	temporary 1	687500	270820	348	25	
XVI	Oerlingerried	temporary 4	693700	275500	395	25	
XVII	Tümpel östlich Präuselen	permanent	688950	271300	346		25
XVIII	Kleinweiher bei Brüggli	permanent	698875	278175	444		26
XIX	Tümpel im Junkholz	permanent	698200	276200	467		20
XX	Kiesgrube Ebnet, Sulz	permanent	700910	266838	459		25
XXI	Hättitümpel Oberholz	temporary 4	695530	271290	435		25
XXII	nördl. Räuberichsee	temporary 1	693065	274350	404		25
XXIII	Tümpel im Oberloowald	temporary 4	697950	270400	443		25
XXIV	Bienenhaus Irchel	temporary 3	683812	249934	511		25

§ Pond numbers correspond with the numbers shown in Fig. 1; * Swiss Terrestrial Reference System 1995, n = sample size
Pond desiccation: 1 = yearly; 2 = every few years, last desiccation in autumn 2010; 3 = every few years, last desiccation in spring 2009; 4 = every few years, last desiccation unknown

Sample collection

395 water frogs and 375 alpine newts were captured during April and May 2012. Because the phenology of the two species is different, for *I. alpestris* sampling started one month earlier (April) than for *Pelophylax* spp. Permanent and temporary water bodies were sampled in an alternate order whenever possible. The target sample size was 25 individuals per pond and species, which allows a detection of *Bd* in a population with 10 % pathogen prevalence with 90 % certainty (DiGiacomo & Koepsell 1986). With a few exceptions the target sample size was reached (Table 1). Individuals were caught haphazardly.

Pelophylax spp. individuals were captured by hand during night time using a flashlight to dazzle the frogs. I took the DNA samples for the *Bd* analysis by using a sterile cotton swab (Copan Italia S.p.a., Italy); the underside of the legs, feet and the pelvic patch was swabbed 5 times each in a standardized procedure (Hyatt et al. 2007). Alpine newts were captured by net dipping during daytime and by using newt traps (fine-meshed bow nets equipped with a buoy in order to ensure air supply). Traps were deployed in the early evening hours and surveyed and removed the next morning. Of the alpine newts

the underside of the legs, feet and the belly were swabbed in the same way as for the water frogs. To avoid a bias of different observers ("swabber effect") all samples were taken by the same person.

During the handling process the captured animals were kept individually in plastic bins and were released immediately after sampling the cohort of a pond. Each individual was handled with unused, powder free vinyl gloves. Used swab tubes were stored at -20° C until processing in the lab. All swabs reached the freezer not later than 48 h after a sample was taken. In order to avoid anthropogenic dissemination of *Bd* among the study sites the disinfection protocol by Schmidt et al. (2009) was followed.

Variables measured

Previous studies assumed a range of biotic and abiotic factors to have an influence on *Bd* prevalence and on load of infection. I recorded data for the most discussed, and, according to the literature, most important ones; body mass, water temperature, sampling day and elevation (Woodhams 2003, Berger 2004, Longcore 2007, Pearl 2009. As male and female frogs show different behaviour patterns at breeding sites, the exposure to *Bd* and the risk of infection may be different (Muths et al. 2003). Therefore I recorded sex as an additional factor. These variables were included in the statistical model to account for variation in infection state of populations that is not explained by pond hydroperiod.

Individual-level variables

Of each individual, I measured body mass using a spring scale (to nearest 0.5 g for *Pelophylax spp.* and 0.1 g for *I. alpestris*). Sex was recorded of every adult individual. Of *Pelophylax* spp. 11 juveniles were captured where sex determination was not yet possible.

Population-level variables

For each pond, I recorded temperature, day of sampling, and elevation. A Thermochron® iButton DS1921G data logger was placed in each pond at least 11 days prior to sampling, recording water temperatures every 120 min. The loggers were put in glass tubes and located 15 cm under the water surface, supported by a buoy system (Fig. 7, Appendix). In laboratory culture, 4 - 5 days is the time *Bd* needs for passing a complete life cycle, and for releasing new zoospores. This time span could be longer under suboptimal temperatures, especially in cooler conditions (Voyles et al. 2012). As mean, maximum, and minimum temperatures showed high correlations, only the mean temperature was used for the statistical analysis.

The sampling day for each population was recorded starting at 1 for the first day of sampling and ongoing by counting each calendar day. Site elevations were provided by map.geo.admin.ch (accessed

October 28, 2012). Due to the small range from 346 to 511 meters a.s.l. I did not expect a relevance regarding infection and I did not include the parameter in the analysis. Nevertheless, the information could be interesting for similar studies.

Analysis of *Bd* infection state

The pathogen DNA was extracted from the swab tips using PrepMan Ultra (Applied Biosystems). The occurrence of *Bd* and level of infection were determined using one-step real-time Taqman PCR assays (qPCR), following the protocols of Boyle et al. (2004) and Tobler & Schmidt (2010). The qPCR assays were processed by an Applied Biosystems 7500 Fast Real-Time PCR System. Samples were run in duplicates and the analysis was repeated for probes showing inconclusive results. As a control reaction standard probes containing *Bd* DNA of 100, 10, 1, and 0.1 genomic equivalents (Ecogenics GmbH), and a negative probe containing no DNA template were included in each assay plate. For the qPCR sample extractions were diluted 1:10. The values of genomic equivalents detected were afterwards corrected for the dilution and the term loads of infection refers to the 1:1 values of genomic equivalents further in this thesis. Samples showing an amount of 0.1 genomic equivalents and above were considered positive for *Bd* and accordingly counted as infected.

Statistical analysis

In a first step I tested whether *Pelophylax* spp. and *I. alpestris* showed a difference in prevalence and infection intensity. For the further analysis, I separated the species in order to avoid an influence of species differences.

To test for the influence of explanatory variables on prevalence a general linear mixed effects model (function “glmer” in package “lme4”) for binomial data was used, with the response variable infection state (infected / not infected) of individual amphibians. Pond was included as a random effect, which accounts for non-independence of the individuals within ponds. Only data of adult individuals were used (*Pelophylax* spp.: n=385 / *I. alpestris*: n=374). As fixed effects I included the variables pond type, sex, body mass, temperature and sampling day in the model. Body mass, temperature and sampling day were continuous variables and I standardized the values to the mean of zero and a standard deviation of 1 (by subtracting the respective mean values and dividing by the standard deviation). Pond type and sex were included as factors. Temperature and sampling day showed a high correlation ($r = 0.85$) in *I. alpestris* data. Therefore, I ran the model for the alpine newts again by excluding once the parameter temperature and once the parameter sampling day. Interactions between the fixed effects were tested, but showed no significant effect on prevalence and infection intensity,

except in one case (pond type*sampling day on prevalence in *I. alpestris*). For a better comparability of the models, the interaction was not included in model for the final analysis.

To test for the influence of explanatory variables on infection loads (infection intensity), I used a linear mixed effects model, assuming random intercepts (function “lmer” in package “lme4”). Again, for the analysis the variables pond type, sex (factors), body mass, temperature and sampling day (continuous) were included in the model as fixed effects and pond as a random effect. Influences on infection intensity were calculated among infected individuals, only. The response variable, which was the load of genomic equivalents per individual, was log transformed. Data of body mass, temperature and sampling day were standardized to the mean of zero and a standard deviation of 1. Only data of adult individuals were used (*Pelophylax* spp.: n=133 / *I. alpestris*: n=69). As the correlation between the two parameter temperature and sampling day in *I. alpestris* was notable (r = 0.68), two models were fitted, one with temperature and the other one with sampling day. Interactions between the fixed effects were tested, but did not show a significant effect. All statistical analysis was done using R statistical software (R Core Team 2012).

Results

The pathogen was widespread in the investigated water bodies. In 22 of the 24 (91.7 %) ponds *Bd* was detected, and 204 of the 770 sampled individuals (26.5 %) of *Pelophylax* spp. and *I. alpestris* were found infected. Comparing *Pelophylax* spp. and *I. alpestris Bd* prevalence showed a significant difference (p = 0.0028) in the general linear mixed effects model (Glmer). In contrast, the infection intensity did not differ significantly ($p > 0.05$) in the linear mixed effects model (Lmer).

Pelophylax spp.

Occurrence. *Bd* was detected in water frogs from all 16 sites. Thus, the occurrence did not differ between temporary and permanent ponds.

Prevalence. The number of *Pelophylax* spp. found infected per pond ranged from 2 (8 %) to 22 (88 %) individuals (Fig. 2A). The mean prevalence was almost equal in permanent (34.7 %) and temporary (33.6 %) ponds, and pond type did not show a significant effect on prevalence in the Glmer. The only variable that showed a significant effect on prevalence was body mass (Table 2). Prevalence and body mass were negatively correlated (Fig. 3A).

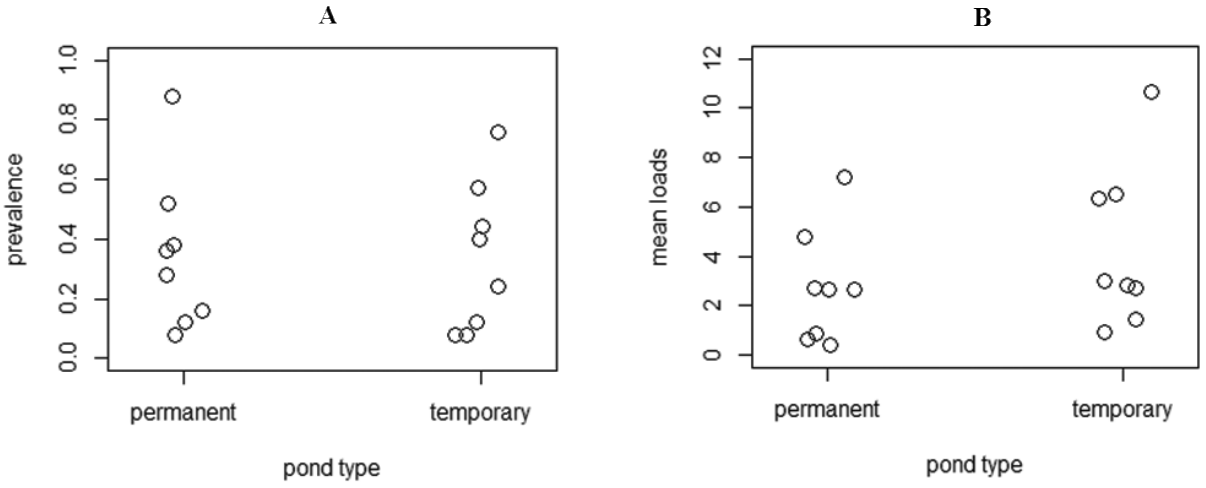

Figure 2. Prevalence and infection intensity in *Pelophylax* spp. did not show a significant difference in permanent and temporary ponds in the (general) linear mixed effects models (p>0.05). **A** shows *Bd* prevalence. Each circle represents one pond. **B** shows the infection intensity per pond. Each circle represents the mean infection intensity detected in one pond. Mean loads were calculated among infected individuals only. The respective standard deviations are shown in Table 5 of the appendix.

Infection intensity. The mean infection intensity per pond did not differ significantly between the two pond types (Fig. 2B). Mean *Bd* loads ranged from 0.40±0.59 to 11±13 genomic equivalents (GE). The individual infection intensity varied from the lower detection limit of 0.1 GE to 53.0±6.0 GE detected in pond no. X. Among infected individuals the variable body mass showed a significant negative effect on infection intensity (Table 2). Thus, individuals with higher body mass showed lower *Bd* loads (Fig. 3B), which is consistent with the effect of body mass on prevalence. Detailed data of prevalence and *Bd* loads in *Pelophylax* spp. are summarized in Table 5 (Appendix).

Table 2. Testing the effect of variables on prevalence and infection intensity in *Pelophylax* spp. in a general linear mixed effects model (Glmer) and a linear mixed effects models (Lmer). The models included the variables pond type, sex, body mass, temperature and sampling day as fixed effects. Data was nested by Pond no, which was included as a random factor. Infection intensity was calculated among infected individuals only. E shows the estimated coefficents, SE the standard error of E. Significant values ($p < 0.05$) are in bold style.

	Prevalence (glmer) (n = 385)			Infection intensity (lmer) § (n = 133)		
Variables	E	SE	*p*	E	SE	*p*
Pond type	-0.28	0.59	0.64	0.34	0.30	0.27
Sex	-0.04	0.34	0.90	0.50	0.32	0.12
Body mass	-0.36	0.16	**0.029**	-0.414	0.135	**0.0027**
Temperature	-0.34	0.31	0.28	0.05	0.15	0.73
Sampling day	0.48	0.31	0.12	0.04	0.15	0.79

§ among infected individuals, excluding negative samples

n = Number of *Pelophylax* spp. included to the analysis

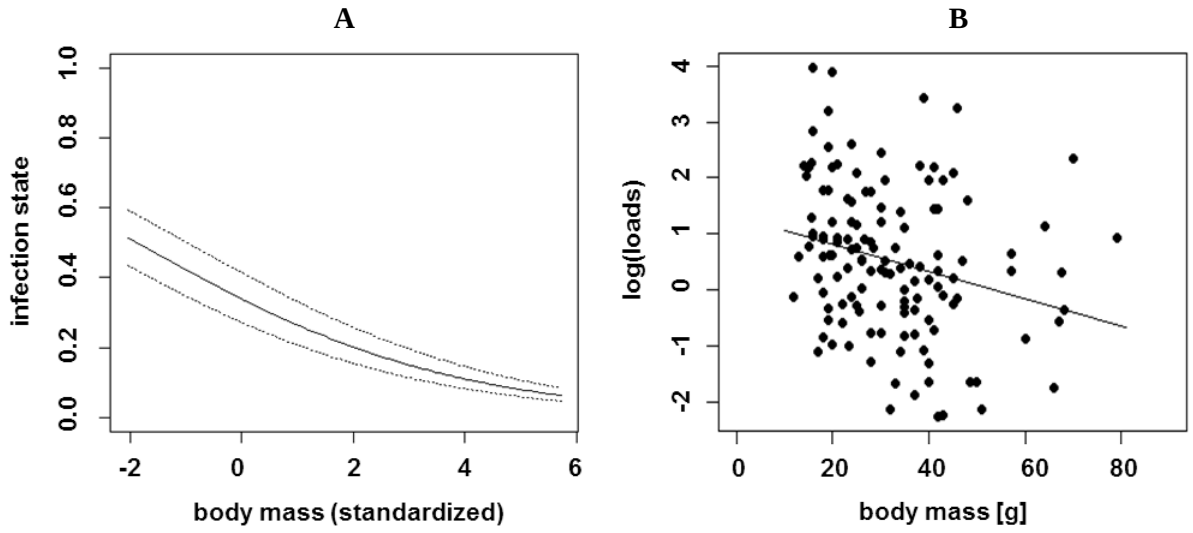

Figure 3. A: Relationship between body mass and infection state in *Pelophylax* spp.; 1=infected and 0=uninfected. The regression line was calculated from the generalized linear mixed effects model including the fixed effects pond type, sex, body mass, temperature and sampling day. The mean of body mass used for the standardization was 34.2 and the standard deviation 16.953. Dotted lines represent the 95 % confidence intervals. **B:** Infection intensity (log(loads)) and body mass showed a negative relationship in *Pelophylax* spp. The equation of the regression line is y = 1.315 - 0.024x. Black dots represent individuals of *Pelophylax* spp.

Ichthyosaura alpestris

Occurrence. In 13 of the 16 ponds sampled *Bd* was detected (81.3 %). Of these ponds 7 were permanent and 6 temporary ones. However, the sample size of pond no. I was very low (*n*=7). It is therefore not possible to infer the absence of *Bd* with certainty (DiGiacomo & Koepsell 1986).

Prevalence. There was no significant difference in prevalence between temporary and permanent ponds (Fig. 4A). The number of infected individuals per pond ranged from 0 to 22 (88 %). In the Glmer analysis including all variables, temperature and sampling day showed a significant effect on prevalence (Table 3). As temperature and sampling day were highly correlated (Fig. 5A) the effects of these parameters were tested using models in which once the variable temperature was excluded, and once the variable sampling day. Sampling day still showed a significant effect on prevalence in the reduced model (Table 3). Lower prevalence was found in *I. alpestris* sampled later in the season (Fig. 5B). Temperature did not show a significant effect on prevalence after the exclusion of the variable sampling day (Table 3).

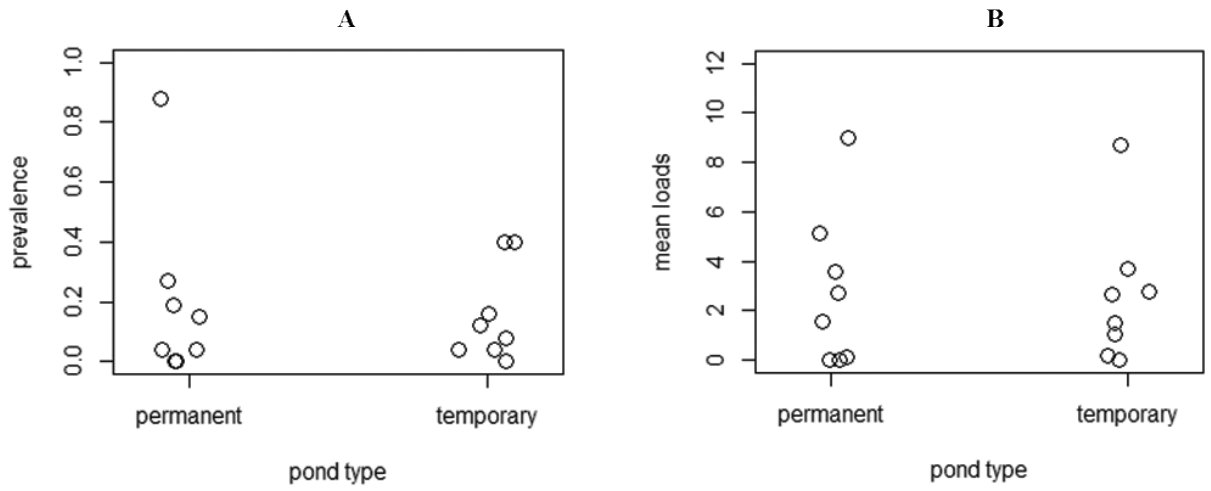

Figure 4. Prevalence and infection intensity in *I. alpestris* did not show a significant difference in permanent and temporary ponds in the (general) linear mixed effects models (p>0.05). **A** shows the *Bd* prevalence. Each circle represents one pond. **B** shows the infection intensity per pond. Each circle represents the mean infection intensity detected in one pond. Mean loads were calculated among infected individuals, only. The respective standard deviations are shown in Table 6 of the appendix.

Table 3. Testing the effect of variables on prevalence in *I. alpestris* in a general linear mixed effects model (Glmer). The model full included the variables pond type, sex, body mass, temperature and sampling day as fixed effects. Data was nested by Pond no, which was included as a random factor. Infection intensity was calculated among infected individuals only. E shows the estimated coefficents, SE the standard error of E. Significant values ($p < 0.05$) are in bold style.

	Prevalence (n = 374) All variables included			Excluding Temperature			Excluding sampling day		
Variables	E	SE	*p*	E	SE	*p*	E	SE	*p*
Pond type	-0.58	0.45	0.21	-0.16	0.62	0.79	0.01	0.86	0.99
Sex	0.38	0.47	0.41	0.39	0.49	0.43	0.38	0.49	0.44
Body mass	0.12	0.22	0.61	0.14	0.24	0.57	0.16	0.24	0.52
Temperature	1.19	0.42	**0.0049**	na	na	na	-0.61	0.43	0.16
Sampling day	-2.09	0.41	**4.49E-07**	-1.12	0.34	**0.00094**	na	na	na

n = Number of *I. alpestris* included to the analysis

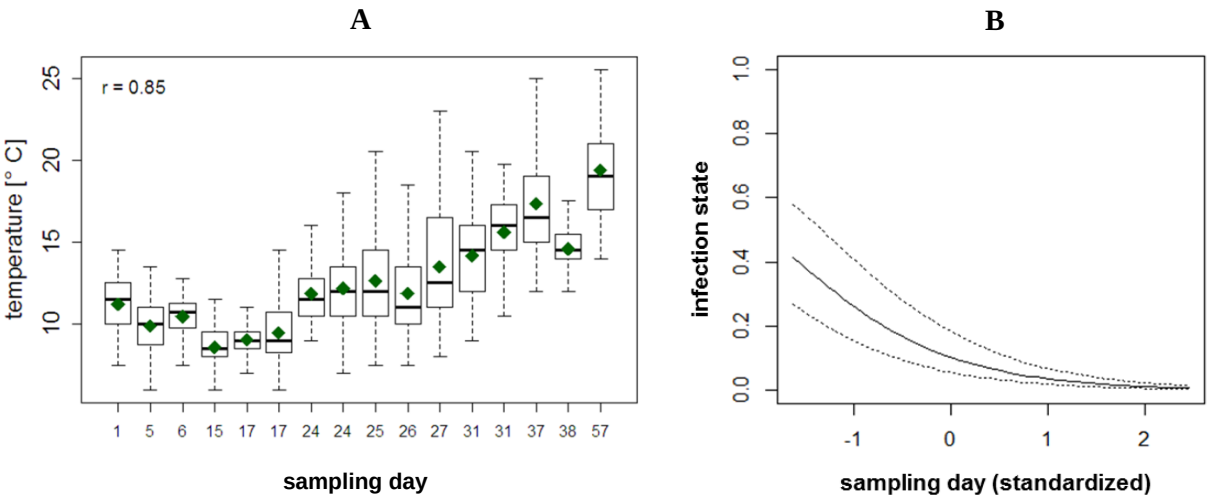

Figure 5. A: Sampling day and temperature in *I. alpestris* data were highly correlated. Each box represents the temperature of one pond. Green crystals represent the mean temperatures, upper whiskers the maximum, and lower whiskers the minimum temperature measured over 11 days prior to sampling. X-axis values correspond to the following dates: 1 = 4 April, 57 = 30 May 2012. **B:** Relationship between sampling day and infection state in *I. alpestris*; 1=infected and 0=uninfected. The regression line was calculated from the general linear mixed effects model including pond type, sex, body mass and sampling day as fixed effects. The mean of body mass used for the standardization was 3.192 and the standard deviation 0.964 Dotted lines represent the 95 % confidence intervals.

Infection intensity. Infection intensity in *I. alpestris* did not differ significantly between temporary and permanent ponds (Table 4). The mean *Bd* load per pond ranged from 0.11±0.07 to 9.0±6.4 GE (Fig. 4B). The individual infection intensity varied from the lower detection limit of 0.1 GE to 20.0±8.4 GE. The latter was found in pond no. XVIII. Among the infected individuals no variables showed a significant effect on infection intensity in the Lmer analysis including all variables. In the model excluding the variable sampling day the effect of temperature was significant and negatively

correlated to infection intensity (Table 4, Fig. 6A). On average, higher infection intensity was found in ponds with cooler mean temperatures. When excluding temperature, the variable sampling day showed a significant negative effect on infection intensity (Table 4, Fig. 6B). This means that higher mean infection intensity was found in populations sampled earlier in the season. The result is consistent with the effect of sampling day on prevalence. Detailed data of prevalence and *Bd* loads in *I. alpestris* are summarized in Table 6 (Appendix).

Table 4. Testing the effect of variables on infection intensity in *I. alpestris* in a linear mixed effects model (Lmer). The full model included the variables pond type, sex, body mass, temperature and sampling day as fixed effects. Data was nested by Pond no, which was included as a random factor. Infection intensity was calculated among infected individuals only. E shows the estimated coefficents, SE the standard error of E. Significant values ($p < 0.05$) are in bold style.

	Infection intensity (n = 69) §								
	All variables included			Excluding Temperature			Excluding sampling day		
Variables	E	SE	*p*	E	SE	*p*	E	SE	*p*
Pond type	0.17	0.52	0.75	-0.04	0.54	0.94	0.20	0.51	0.70
Sex	-0.01	0.33	0.98	0.01	0.33	0.98	-0.01	0.33	0.97
Body mass	-0.08	0.17	0.65	-0.09	0.17	0.58	-0.07	0.16	0.67
Temperature	-0.44	0.35	0.21	na	na	na	-0.53	0.19	**0.0078**
Sampling day	-0.12	0.40	0.76	-0.56	0.24	**0.023**	na	na	na

§ among infected individuals, excluding negative samples

n = Number of *I. alpestris* included to the analysis

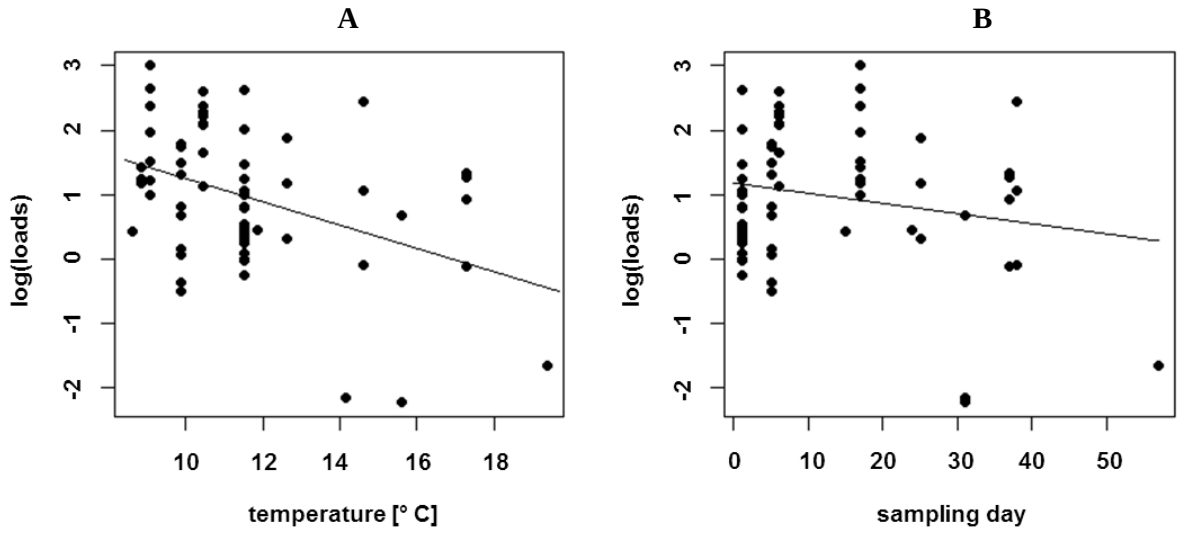

Figure 6. Relationship between infection intensity (log(loads)) and the variables temperature (**A**) and sampling day (**B**) in *I. alpestris*. Temperature and sampling day were highly correlated and therefore tested in separate models. They showed a significant negative effect on infection intensity ($p < 0.05$) in the Lmer analysis. The regression equations are $y = 3.083 - 0.183x$ for temperature and $y = 1.182 - 0.016x$ for the sampling day. Black dots represent individuals of *I. alpestris*.

Discussion

The aim of this study was to find habitat characteristics that are favourable for amphibians but unfavourable for *Bd* such that they may be used to manage amphibian habitats. The habitat characteristic I focused on was pond hydroperiod. Based on published evidence (Kriger & Hero 2007B), I expected that amphibians occupying temporary ponds would show lower occurrence, prevalence, and infection intensity than those in permanent ponds. Although prevalence and infection intensity varied remarkably among the study sites, I did not find a significant difference in occurrence, prevalence, and infection intensity between temporary and permanent ponds. This result contrasts with the result of Kriger & Hero (2007B), who suggested temporary ponds to be habitats with low *Bd* infection risk for amphibians. Besides the absence of a difference in infection between ponds with different hydroperiods, the two study species *Pelophylax* spp. (water frog) and *I. alpestris* (alpine newt) clearly showed differences in pathogen prevalence among the study sites and the effects driving prevalence and infection intensity differed between the two species. For *Pelophylax* spp. I identified body mass to be correlated to prevalence and infection intensity. In *I. alpestris*, on the other hand, *Bd* prevalence and loads of infection were influenced by temperature and sampling day. The results suggest that species responses of syntopic amphibians to a widely distributed pathogen can be variable and solutions for disease management should be developed species and context specific.

Kriger & Hero (2007B) found evidence for a relationship between hydroperiod and *Bd* prevalence. They detected *Bd* infections in frogs breeding in permanent ponds and streams, whereas they found no *Bd* infection in frogs breeding in temporary waters. The results of this study are difficult to interpret because some species only occurred in one of the two types of water bodies. Thus, habitat type and species are confounded. For some species that breed in both kinds of habitat Kriger & Hero (2007B) had low sample size such that prevalence could not be reliably estimated (e.g., n=2 for *Litoria fallax* in ephemeral ponds and n=5 for *Litoria latopalmata* in permanent streams). Therefore, even though the findings of Kriger & Hero (2007B) are highly plausible, the results are not conclusive.

For this thesis I sampled an equal number of ponds for each species and hydroperiod, which allowed me to compare pathogen infection states between species and among populations of the same species. As no difference in occurrence, prevalence or infection intensity was found in regard to pond hydroperiod, the result of Kriger & Hero (2007B) could not be confirmed. This could have several reasons: First, “pond drying” may mean different things in different geographic localities. In Switzerland, pond drying may not reduce humidity in the soil of the dry ponds as much as in Australia.

Therefore, *Bd* may survive in the mud (Johnson & Speare 2005). Second, the interval and time span of desiccation vary among the temporary ponds I investigated. Some of them did not dry in the last season (2011), which may lead to a reduced influence of hydroperiod. Nevertheless, I did not find a pattern in prevalence and infection intensity among temporary ponds with different frequencies of drying. And third, while pond drying may eliminate the pathogen from a pond, *Bd* may recolonize the pond rapidly. Padgett-Flohr & Hopkins (2010) suggested that the presence of *Bd* in temporary ponds may be the result of recurrent immigration, either vectored by amphibians or water fowl (Garmyn et al. 2012). *Pelophylax* spp. and *I. alpestris* show a distinct migration behavior (Holenweg Peter 2001, Perret et al. 2003, Kopecky et al. 2012). As the water bodies of this study were imbedded in a network of habitats in close geographic proximity, immigration of *Bd* through migrating amphibians is thus conceivable. Still, the ponds and streams studied by Kriger & Hero's study (2007B) were all in close proximity such that migration between those study sites may be possible, as well. This leads to the assumption that the observed patterns of *Bd* infection in the different habitat types may result from differences in host specific traits and behavioral dissimilarities of the species rather than from the influence of hydroperiod of the study sites. The findings of lower, respectively no *Bd* infection in temporary ponds by Kriger & Hero (2007B) can thus not be universally expected, but appears to be a context specific result.

The negative effect of body mass on *Bd* prevalence and infection intensity found for *Pelophylax* spp. individuals (Table 2) is consistent with findings in former field and laboratory studies (e.g. Pearl et al. 2009, Woodhams et al. 2012). The relationship between body mass and infection state is not yet fully understood. An open question is whether body size is the cause of reduced infection or a consequence of the infection. Larger animals may have better immunocompetence (Knapp et al. 2011). Alternatively, a high parasite load can reduce amphibian growth rate (Woodhams et al. 2012). Interestingly, an effect of body size was not found in syntopic *I. alpestris*. This implies that the relationship between size and infection and the mechanisms generating the relationship are not general but probably species-specific.

In *I. alpestris* infection was correlated to temperature and sampling day (Table 3 and 4). Because temperature was highly correlated with the day of sampling I tested the two variables in separate models. The correlation of the temperature and sampling day is expected as sampling started in the beginning of April and ended in late May, which is mid spring season in Switzerland. I decided to take both variables into account in my analysis because both factors were identified to have an independent effect on *Bd* (Piotrowski et al. 2004, Ribas et al. 2009, Berger et al. 2004). Both, the amphibian host and the pathogen are dependent on temperature. Temperature affects the metabolic rate, immune responses and behavior of amphibians (Raffel et al. 2006, Knapp et al. 2011), as well as growth and

reproduction rate of *Bd* (Piotrowski et al. 2004, Raffel et al. 2006, Woodhams et al. 2008). *Bd* reaches highest population growth rates in a temperature range of 17 – 25° C, lower in sub-optimal conditions below or above the optimal temperature range (Piotrowski et al. 2004). High temperatures (32 °C upwards) are potentially lethal to *Bd* (Woodhams et al. 2003). If temperature affects prevalence and infection intensity positively or negatively is thus dependent of the temperature range experienced in a study (Eigenbrod et al. 2011). In this study the mean temperatures measured for *I. alpestris* samples ranged from 9 – 19 ° C, reaching optimal *Bd* growth temperatures only in two cases towards the end of the sampling period. In contrast to the upper end of *Bd*'s thermal optimum the effects of the lower sub-optimal temperature ranges are still subject of investigations (Knapp et al. 2011). Therefore, an appropriate comparison with former studies was not possible. A field study of Knapp et al. (2011) did not find an effect of water temperature on *Bd* and suggests that more factors than temperature alone should be taken into account when investigating populations in natural environments. Seasonal fluctuations of infection and mortality of *Bd*, independent of temperature, have already been shown (Kriger & Hero 2007A, Berger et al. 2004). An effect of the variable sampling day, understood as a seasonal indicator, could be caused by many factors that change as the season progresses (e.g., example temperature, community composition and amphibian density, vegetation and ground cover in the pond, water levels, in- and outflow, above-ground plant cover, UV radiation, host behavior and immune response parameters). Thus, seasonal changes could influence host traits and the establishment and development of *Bd* in a population on different levels. In syntopic *Pelophylax* spp., neither an effect of temperature nor an effect of sampling day was found. Therefore, temperature effects seem species-specific. Taking into account the different behavior patterns and the use of the habitat of the two species, it seems plausible that temperature affects species differentially because the species experience the thermal habitat in different ways (Scherrer & Körner 2011). Both species are highly aquatic, but while *I. alpestris* spends most of the time during the mating season in the water, *Pelophylax* spp. uses the pond shore for basking.

The question must be raised, what habitat means for a host and for a pathogen. Is there a shared environment such as a pond and its temperature? Or should we rather consider the host itself as the pathogen's habitat, and environmental conditions have only an indirect influence on *Bd*, perhaps through a modulation of host-pathogen interactions (Blaustein et al. 2012)? The findings of this study point exactly in this direction. The result of a host-pathogen interaction seems to be dependent on the host characteristics within the context of its external environment (Blaustein et al. 2012). The fact that I could not confirm the result of Kriger & Hero (2007B), suggesting an effect of pond hydroperiod on *Bd* prevalence and loads, shows that results cannot be easily transferred to different environments and species. Furthermore, different variables were identified to have an effect on *Bd* prevalence and infection intensity in *Pelophylax* spp. (body mass) and in *I. alpestris* (temperature and sampling day),

which supports the idea that the risk of a *Bd* infection and its impacts are highly species specific (Voyles et al. 2011).

In conclusion, no general statement can be given in regard to the factors influencing *Bd* in different species and environments. The effect of the habitat on pathogen prevalence and infection intensity appears to be species- and context-specific. The environment a host is living in plays an important role, as it can affect host traits and so the host-pathogen relationship. But, one habitat may not lead to the same experienced conditions for one species as it does for another one. For disease management and mitigation in conservational regards it is thus important to identify factors characterising and influencing a host-pathogen relationship to build up appropriate species and context specific strategies.

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Appendix

Appendix A – Additional information on methods

Figure 7. Pictures of the data logger buoy system used for the temperature measurements. **A** shows the glass tube carrying a Thermochron® iButton logger. The logger was put into a water proof, resealable plastic bag and the glass tube was sealed with Parafilm®. **B**: The glass tube is fixed 15 cm under the styrofoam buoy and charged with a stone to hold it at the correct position under water. A tent peg or stone anchored the construct to the ground to hold it in position in the pond.

Appendix B – Data summary of *Pelophylax* spp. and *I. alpestris*

Table 5. Estimates of *Bd* infection prevalence per pond and infection intensity among infected individuals (average loads excluding 0 s) of *Pelophylax spp.* in the 16 ponds sampled. Prevalence = proportion of infected individuals per pond, *Bd* load = infection intensity (mean genomic equivalents) among infected individuals. Temperature was measured 11 over days prior to sampling. Sampling day refers to the first day of sampling, counting every calendar day. Sex shows the number of males, females and juveniles sampled per pond. *n* reflects the number of individuals sampled per pond.

Pond No.	Pond name	*n*	Prevalence	*Bd* load (mean±SD)	Temperature [° C] mean	max	min	Sampling day	Sex m / f (/ j)
I	Weiher Gütighausen *P*	25	0.16	0.67 ± 0.63	12.56	20.50	7.00	24	12 /12 (/ 1)
II	Grube Oberboden *P*	25	0.88	7.19 ± 11.60	17.87	27.25	12.50	48	22 / 3
III	Kiesgrube Rhinauer Feld *P*	25	0.38	2.67 ± 5.26	13.90	23.50	9.00	25	22 / 2 (/ 1)
IV	Ried bei Adlikon *P*	25	0.52	2.73 ± 2.54	15.87	22.50	10.50	48	20 / 4 (/ 1)
V	Seewädeli *P*	25	0.12	2.66 ± 1.60	18.14	23.75	14.25	38	21 / 3 (/ 1)
VI	Chli Au Ossingen *P*	25	0.08	0.40 ± 0.59	17.01	23.13	12.38	55	7 / 13 (/ 5)
VII	Sürch Schlattigen *P*	25	0.36	0.85 ± 0.96	16.19	20.00	11.50	50	23 / 2
VIII	Schutzgebiet Wagenhausen *P*	25	0.28	4.81 ± 5.90	15.27	23.00	10.50	47	10 / 15
IX	Tümpel bei Buck *T*	25	0.12	10.63 ± 13.10	17.34	26.50	12.00	37	10 / 15
X	Pfaffensee *T*	25	0.44	6.34 ± 15.60	15.53	22.50	11.00	47	16 / 9
XI	Heinrichsee *T*	21	0.57	6.49 ± 7.11	13.90	20.00	9.50	49	18 / 3
XII	Cholgruebsee *T*	25	0.76	2.71 ± 2.90	15.64	20.50	10.00	51	17 / 6 (/ 2)
XIII	Enteler-Weiher *T*	25	0.24	2.82 ± 2.76	13.18	17.50	8.00	52	13 /12
XIV	Altlauf Inselen *T*	25	0.08	1.44 ± 0.06	14.59	24.50	9.00	27	25 / 0
XV	Kiesgrube südöstl. Feldhof *T*	25	0.40	3.03 ± 3.53	14.01	23.00	9.00	26	16 / 9
XVI	Oerlingerried *T*	25	0.08	0.91 ± 0.05	18.66	28.50	11.50	38	24 / 1

P = permanent, *T* = temporary

Table 6. Estimates of *Bd* infection prevalence per pond and infection intensity among infected individuals (average loads excluding 0 s) of *I. alpestris* in the 16 ponds sampled. Prevalence = proportion of infected individuals per pond, *Bd* load = infection intensity (mean genomic equivalents) among infected individuals. Temperature was measured over 11 days prior to sampling. Sampling day refers to the first day of sampling, counting every calendar day. Sex shows the number of males and females sampled per pond. *n* reflects the number of individuals sampled per pond.

Pond No.	Pond name	*n*	Prevalence	*Bd* load (mean ± SD)	Temperature [° C] mean	max	min	Sampling day	Sex m / f
I	Weiher Gütighausen *P*	7	0	-	12.11	19.5	7	24	4 / 3
IV	Ried bei Adlikon *P*	25	0.88	2.70 ± 2.88	11.50	14.50	8.25	1	11 / 14
VI	Chli Au Ossingen *P*	21	0.19	3.56 ± 0.40	9.48	15.25	6	17	9 / 12
VII	Sürch Schlattigen *P*	25	0.04	0.11 ± 0.07	14.17	20.5	9	31	16 / 9
XVII	Tümpel östlich Präuselen	25	0	-	13.51	23	8	27	17 / 8
XVIII	Kleinweiher bei Brüggli *P*	26	0.27	8.96 ± 6.41	9.05	11	7	17	14 / 12
XIX	Tümpel im Junkholz *P*	20	0.15	5.13 ± 5.68	14.60	18	12	38	16 / 4
XX	Kiesgrube Ebnet, Sulz *P*	25	0.08	1.56 ± 1.17	11.84	17	9	24	7 / 18
IX	Tümpel bei Buck *T*	25	0.16	2.68 ± 1.31	17.27	26.5	12	37	17 / 8
XI	Heinrichsee *T*	25	0.40	8.67 ± 2.86	10.46	12.75	6.5	6	10 / 15
XII	Cholgruebsee *T*	25	0.08	1.02 ± 1.30	15.62	20	9.5	31	7 / 18
XIII	Enteler-Weiher *T*	25	0.4	2.76 ± 2.05	9.87	14.5	6	5	17 / 8
XXI	Hättitümpel Oberholz *T*	25	0	-	11.88	19	7.5	26	14 / 11
XXII	nördl. Räuberichsee *T*	25	0.04	1.53 ± 0.25	8.59	11.5	6	15	14 / 11
XXIII	Tümpel im Oberloowald	25	0.12	3.69 ± 2.60	12.62	23	7.5	25	17 / 8
XXIV	Bienenhaus Irchel *T*	25	0.04	0.19 ± 0.13	19.39	25.5	14	57	10 / 15

P = permanent, *T* = temporary