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Factors driving an emerging pathogen - *Batrachochytrium dendrobatidis* in Switzerland



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Zusammenfassung

Alarmierend ist der Rückgang der Amphibien welcher derzeit auf globaler Ebene stattfindet. Die Populationsrückgänge sind so massiv dass die Amphibien zur meistbedrohten Klasse der Wirbeltiere wurden. Eine der Ursachen dafür ist der kürzlich beschriebenen Chytridpilz *Batrachochytrium dendrobatidis* (*Bd*). Dieses Pathogen ist in der Schweiz bereits weit verbreitet, wie eine Erhebung zeigte. Ein besseres Verständnis der Faktoren welche *Bd* begünstigen ist daher dringend erforderlich. In dieser Studie wurden 18 bekannte Populationen der Gemeinen Geburtshelferkröte (*Alytes obstetricans*) in der Nordschweiz auf *Bd* hin getestet und mehr als 20 Kovariablen gemessen. In 6 Populationen konnten keine Kaulquappen gefunden werden. Anhand der Infektionsdaten schätzte ich die Prävalenzen der Populationen. Nur eine der zwölf Populationen war noch frei von *Bd*. Die räumlichen Prävalenzunterschiede habe ich mittels eines informationstheoretischen Ansatzes erklärt. Ich konnte als Erster zeigen dass große Teiche in hoher Höhenlage zu hohen Prävalenzwerten neigen. Zur Erklärung dieses Musters schlage ich folgende Mechanismen vor: (1) Grosse Teiche beherbergen andere oder mehr Arten welche als Reservoir für *Bd* dienen; ausserdem erlauben grosse Teiche den Kaulquappen eher zu überwintern und diese Tiere fungieren folglich selbst als Reservoir. (2) In kleinen Teichen könnte *Bd* durch Austrocknung entfernt werden. (3) Kürzere saisonale Warmperioden in großen Höhen führen zu vermehrtem Überwintern der Kaulquappen und so zu einer erhöhten Frequenz von infizierten Kaulquappen beziehungsweise zu erhöhten Übertragungsraten. Des Weiteren habe ich festgestellt, dass die von mir angewandte Methode zur quantitativen Erkennung einer *Bd*-Infektion hochsensibel ist und zwar auch ohne Erfahrung. Dies ermöglicht Erhebungen mit unerfahrenen Helfern. Die Erkenntnis dass die (veränderbare) Teichmorphologie einen starken Einfluss auf die Infektionsrate hat könnte als Strategie zur Bekämpfung von *Bd* eingesetzt werden.

Abstract

An alarming global amphibian decline is occurring. As a consequence, the amphibians are the most endangered class of vertebrates. One reason for these massive population declines is the recently described chytridiomycete fungus *Batrachochytrium dendrobatidis* (*Bd*). A survey in Switzerland showed that *Bd* is already widespread, and a better understanding of the driving factors of this pathogen is urgently needed. In this study I surveyed 18 ponds in northern Switzerland with known populations of the Common Midwife Toad *(Alytes obstetricans)* for *Bd* and recorded more than 20 covariables that are potentially important for the prevalence of the disease. In 6 ponds no tadpoles of the Common Midwife Toad were found. On the basis of the infection data I estimated the prevalences for the populations. Only 1 out of the 12 found populations was still *Bd* free. I also explained the among-pond variation in prevalence with an information theoretic approach. I am the first to show that big ponds at high altitude lead to enhanced prevalence predictions. Also, low temperature enhances *Bd* prevalence predictions. The underlying mechanism for this pattern could be: (1) Different or more reservoir species for *Bd* in big ponds; in addition, big ponds allow tadpoles to hibernate and, thus, these animals could act as a reservoir itself. (2) *Bd* clearance in small ponds by drying. (3) Shorter warm seasons at high altitude trigger hibernation and lead to enhanced transmission rates. In addition, I found that the used swabbing method is highly sensitive for quantitative approaches, even without experience. This allows designing and performing fieldwork with inexperienced observers. The finding that the (modifiable) pond morphology has a strong influence on the prevalence could be used as a tool for conservation strategies.

Introduction

An alarming global amphibian decline is occurring since the 1970`s. Up to 43 % [1] of all 6000+ (http://www.iucnredlist.org/initiatives/amphibians) described amphibian species undergo some form of population decline. Habitat loss, fragmentation, overexploitation and other processes turned the amphibians into a highly endangered class of vertebrates [1]. One of these "other processes" is now identified [2]. It is the recently described chytridiomycete fungus *Batrachochytrium dendrobatidis* (*Bd*) [3, 4]. *Bd* causes a skin disease called chytridiomycosis. Chytridiomycosis goes along with an epidermal change with parakeratotic hyperkeratosis (cell loss, erosion and thickened segments of the stratum corneum) and acanthosis (hyperplasia of the stratum intermedium) [3]. *Bd* is transmitted by zoospores which invades the mouthpart of tadpoles or, in juveniles and adults, the keratinized parts of the amphibian skin [5]. In some amphibian species Chytridiomycosis leads to death.

This is the case for our study species the Common Midwife Toad *(Alytes obstetricans)*. The Common Midwife Toad is highly sensitive to *Bd* [6]. After the first appearance of *Bd* in Spain in 2001 (first described

case of *Bd* in Europe) the Common Midwife Toad has shown a sharp population decline [7-9].

In Switzerland, *Bd* was first described in 2005 [10]. A survey of more than 130 ponds north of the Alps between 2005 and 2009 showed that *Bd* is already widespread (U. Tobler & B. R. Schmidt, unpublished data). This finding shows that a better understanding of the mechanisms of this disease is urgently needed. Many researchers are working on this topic and compiled a growing body of knowledge on the ecology of this disease. This body of research suggests (1) environmental and (2) host specific variables as important drivers for the infection dynamics. (1) With respect to the environment, cold air and water temperatures as a result of altitude, latitude, season and other factors, generally favor *Bd* incidence [11, 12] and enhances the *Bd* prevalence [9] and pathogenicity [12]. Cold environments further reduce the host immune response [13-15] and increase the host mortality [16]. Also, the host breeding habitat is an important factor. Amphibians which prefer permanent and flowing water bodies have a much higher *Bd* risk than those living in temporary and still water bodies [17]. (2) With respect to host traits, small juveniles and early developmental stages show a higher mortality than later stages [18], and bigger juveniles and late developmental stages show a higher infection risk than small and young animals [9, 19]. This knowledge is the base of the presented study.

In this study I determined the incidence and prevalence of *Bd* in northern Switzerland, and I tested the quality of our swabbing method. I monitored 18 different ponds with known populations of the Common Midwife Toad. The ponds had already been visited in 2007 and 2009 (U. Tobler, unpublished data) when *Bd* was found to be widespread. I recorded more than 20 covariables which were chosen based on their effects mentioned above, and I swabbed all tadpoles twice for a quality check of the method. To analyze these data, I used an information theoretic approach and model selection. With this approach I estimated the *Bd* prevalence, explained the among-pond variation in prevalence and I determined the detection probability of our swabbing method.

Material and methods

Field survey

Study sites

In April-June 2010 I surveyed 18 ponds with known populations of the Common Midwife Toad in three regions (Table 1) of northern Switzerland. These wetlands include a range of very small temporary ponds to big permanent ponds with water inflow and ponds that are connected to streams.

Study species

The Common Midwife Toad *(Alytes obstetricans)* is a small anuran (up to 50 mm snout-vent length). It prefers habitats with little vegetation cover and breeds in potholes, runlets or even in flowing water. The Common Midwife Toad mates on land and the male pursues brood care. The offspring is released as small tadpoles. The larvae prefer cool water temperatures

between 22 °C and 25 °C. Depending on the date of eclosion, food supply and temperature they metamorphose in autumn or, after hibernating, in the following summer. They reach sexual maturity two or three years after metamorphosis [20].

Capture method

216 Tadpoles were caught by dip netting [21]. In every pond I did at least 40 sweeps (40 x 70 cm aperture; 1 cm mesh; 2 m long). The 40 sweeps were partitioned proportionally (rough estimation) on the three different microhabitats open water, cane brake and subaqueous vegetation. Recorded covariables were the microhabitat of capture (HAB), the depth of capture (DEPTHc) and the relative density of the tadpoles, measured by their number per sweep (RELps) and the time to capture 20 tadpoles in person minutes (RELppm) (Table 2). The first twenty captured tadpoles were separated in freshwater filled cans and swabbed afterwards. Two persons swabbed the tadpoles and determined the developmental stage (DEVEL) by Gosner`s tables [22]; I recorded the date of observation (DATE) and the observer identity (OBS). To avoid anthropogenic spread of *Bd* I accurately followed the disinfection protocol by Schmidt et al. [23] which does not have negative effects on tadpoles and zooplankton [24]. Tadpoles were only handled with freshwater rinsed powder free vinyl gloves to avoid glove toxicity for the tadpoles [25, 26]. Glove toxicity for *Bd* [27] is not important because also dead zoospores are detectable.

Table 1 List of all visited ponds in Bern (BE), Basel-Landschaft (BL) and St. Gallen (SG) with their coordinates.

Region	Location	Coordinates CH-1903 Cx	Cy
BE	Brandsitengraben	624325	205750
BE	Chnubel	626920	206910
BE	Mattstallwald	622900	209600
BE	Oberrotenbühl	626150	205300
BE	Vorder Birnbaum	624770	203580
BE	Waltisberg	626720	211020
BL	Bickenberg	620800	253850
BL	Chalchofen	624710	258587
BL	Itingen	625990	256600
BL	Reigoldswil	619825	249925
BL	Schleifenberg	624200	259980
BL	Strickrain	627310	259310
BL	Zunzgen Heftelen	627350	254050
SG	Altstätten	758170	249990
SG	Buechholzweiher	763600	253900
SG	Ochsenweid	744100	254740
SG	Sittertobel	743640	251500
SG	Wolfgangweiher	742830	251870

***Bd* Sampling**

Sampling was performed with sterile plain swabs in labeled tubes (COPAN, code 155C). The swabs were smeared a few times in and over the tadpole's mouth. Every tadpole was swabbed twice. The used swabs were kept at room temperature what is adequate enough for quantitative *Bd* detection [28]. After field trips the swabs were frozen at -24 °C.

Pond characteristics

I estimated pond volume (V), surface (A) and depth average (DEPTHav) by measuring the length and width using a rope and the depth at four points using a stick (Figure 1). For the calculation I used the shown formulas (Figure 1). The pond temperatures were measured with loggers (Maxim iButton DS1921G-F5#; Accuracy ±1°C from -30 °C to +70 °C) from June to August. The loggers recorded the temperature every 150 minutes. They were placed at a constant depth (under a buoy) of about 15 cm. The loggers were always placed at the sunny side of the pond and farthest away from in-/outflow if present. From the logger data I obtained different temperature characteristics shown in table 2. The abiotic pond variables were then subjected to a principal component analysis. Temperature data (TEMPav, TEMPmax, TEMPmed, TEMPmin) formed the first principal component (hereafter "temperature spectrum"). The different pond characteristics (V, A, DEPTHav) formed the second principal component (hereafter "pond morphology" (see results)). I further determined the isolation of the ponds (ISOL). Isolation means the

Table 2 Measured covariables from three categories (pond, handling, tadpole) with their abbreviation (bold).

Pond	Handling
(ALT) Altitude	**(DATE)** Date of capture
(ISOL) Isolation	**(OBS)** Observer
(RELppm[a]) Relative tadpole density	**(POND)** Pond identity
(RELps[b]) Relative tadpole density	
(TEMP<9) number of hours < 9 °C	**Tadpole**
(TEMP<10) number of hours < 10 °C	**(DEPTHc)** Depth of capture
(TEMP<11) number of hours < 11 °C	**(DEVEL)** Developmental stage
(TEMP<12) number of hours < 12 °C	**(HAB)** Microhabitat of capture
(TEMP>26) number of hours > 26 °C	
(TEMP>28) number of hours > 28 °C	
(TEMPspect) Temperature spectrum	
(TEMPav) Temperature average	
(TEMPmax) Temperature maxima	
(TEMPmed) Temperature median	
(TEMPmin) Temperature minima	
(PMORPTH) Pond morphology	
(V) Volume	
(A) Surface	
(DEPTHav) Depth average	

[a] per person minute
[b] per sweep

$$A = \pi * \frac{l}{2} * \frac{b}{2}$$

$$DEPTHav = \frac{h_1 + h_2 + h_3 + h_4}{4}$$

$$V_{Pond} = V_1 + V_2 + V_3$$

$$V_1 = \left(\frac{1}{108} * l * b * \pi * h_3\right) + \left(\frac{1}{18} * l * b * (h_1 + h_3)\right) + \left(\frac{1}{108} * l * b * \pi * h_1\right)$$

$$V_2 = \left(\frac{1}{36} * l * b * (h_3 + h_4)\right) + \left(\frac{1}{36} * l * b * (h_1 + h_2 + h_3 + h_4)\right) + \left(\frac{1}{36} * l * b * (h_1 + h_2)\right)$$

$$V_3 = \left(\frac{1}{108} * l * b * \pi * h_2\right) + \left(\frac{1}{18} * l * b * (h_2 + h_4)\right) + \left(\frac{1}{108} * l * b * \pi * h_4\right)$$

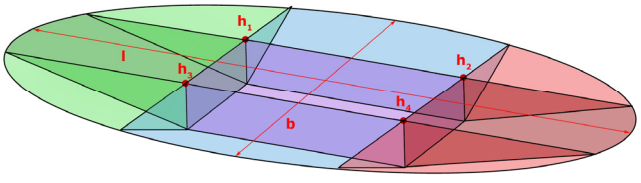

Figure 1 Top: Formulas used to calculate surface (A), volume (V) and depth average (DEPTHav).
Bottom: Sketch illustrating the calculation of the pond volume from width (b), length (l) and depth at four points ($\boldsymbol{h_1 - h_4}$). Colors of the three volumes accord to the colors of the formula frames.

distance to the nearest population of the Common Midwife Toad. If any man-made obstacle that is insurmountable for toads was located between two ponds, the next further population was counted (Data was provided by KARCH). The altitude of the ponds (ALT) was estimated by Google maps elevation web service (Google Inc., accessed October 23, 2010). Scale unit is Swiss height reference system [m.ü.M.].

Bd analysis

Chytrid DNA extraction

To extract the chytrid DNA from the swabs the following protocol was used: Put 0.03 g silicate (0.5 mm Zirconia/Silica Beads (454 g); Biospec; # 11079105z) and 60 µl Prep Man Ultra (Applied Biosystems; P/N 4318930) in a safe-lock Eppendorf tube. Cut off the swab head with a sterile scalpel in a sterile Petri dish. Put the swab head into the Eppendorf tube. Bead beat it 45 s. Centrifuge 30 s (14000 rpm). Bead beat 45 s again. Centrifuge 30 s again (14000 rpm). Heat 10 min at 100 °C. Allow to cool some minutes. Centrifuge for 3 min (14000 rpm). Pipette the supernatant in a new Eppendorf tube. Attenuate 4 µl of the supernatant with 36 µl ddH_2O for the rt-PCR.

rt-PCR process

To detect *Bd* DNA in the extract the following protocol was used. Every extract was analyzed twice to assure the result: Compound 246 µl ddH_2O with 54 µl Primer (Microsynth 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′). Master mix for one plate (96 assays) consists of 474 µl ddH_2O + 1200 µl Taqman Master mix (Applied Biosystems; 4304437) + 120 µl forward primer + 6 µl Probe (Applied Biosystems) + 120 µl reverse primer. Put 20 µl of the master mix in every hole of the plate with 5 µl of the attenuated extraction solution. Cover the plate (ABgene House; Absolute QPCR Seal; Cat. No. AB-1170) and put it into the cycler (stage 1: 50 °C for 2 min; stage 2: 95 °C for 10 min; stage 3: 95 °C for 15 s and 60 °C for 1 min with 50 repetitions). All PCR were done with the 7500 Fast Real-Time PCR System by Applied Biosystems.

Bd infection status verification

To confirm *Bd* infection status both PCR results for one swab had to be identical. Control wells had to be *Bd* negative to exclude contamination. Fluorescence thresholds were individually generated using standards for every run by the SDS program (Version 1.4.0.25).

Statistics

Data analysis

To analyze the *Bd* detection/non-detection data I carried out three analyses. In the first analysis, 11 ponds were included (n=216 tadpoles). Here I (1) estimated the *Bd* prevalence in the different ponds, (2) assessed which covariables (pond/handling-level) explain among-pond variation in prevalence and (3) checked the reliability of our swabbing method (are detection probabilities for first (p_1) and second (p_2) swab events high and identical?).

In the second analysis, only the two ponds (n=40 tadpoles) were included where neither zero nor all

Table 3 Probability for the four possible detection histories of *Bd* at one tadpole after two swab events.

Detection history	Probability
1 1	$\Psi p_1 p_2$
1 0	$\Psi p1(1-p_2)$
0 1	$\Psi(1-p_1)p_2$
0 0	$(1-\Psi) + \Psi(1-p_1)(1-p_2)$

1=*Bd* detected; 0=*Bd* not detected; Ψ=Prevalence of *Bd*; p_1=Probability to detect *Bd* in the first swab event; p_2=Probability to detect *Bd* in the second swab event

tadpoles were infected. In these I (4) assessed which covariables on tadpole-level explain among-pond variation in prevalence.

In the third analysis, observer specific prevalence estimates were calculated for different sample sizes (different numbers of tadpoles by different pond combinations). With these prevalence estimations I (5) demonstrate observer differences by chance due to small sample sizes.

These analyses were implemented using the site occupancy model developed by MacKenzie et al. [29] and were run in PRESENCE 3.0 (available from www.mbr-pwrc.usgs.gov/software/presence.html). In this model site occupancy (Ψ) and detection probability (p) is estimated based on repeated detection/non-detection data from multiple sites. Estimation of site occupancy and detection probability is based on the detection histories. For example, after two visits at a site four detection histories are possible (Table 3). To apply this model on *Bd* infected tadpoles I perceive tadpole as site that is occupied with the species *Bd*. Multiple visits of a "site" means that individual tadpoles are swabbed multiple times. The resulting site occupancy (Ψ) is equivalent to prevalence and the detection probability (p) is the probability to detect *Bd* given that a tadpole is infected. After incorporating the different covariables it is possible to estimate the relationship between the covariables and the prevalence.

Model development and model selection

To explain spatial variation in prevalence, I built a set of candidate models and used the small sample Akaike information criterion (AICc) [30, 31] to determine which model best explains the data. Some of the covariables were included in the candidate models because previous research had shown that they can affect *Bd* prevalence. These are temperature spectrum [9, 12-15], altitude [7, 9], developmental stage [9, 19] and the number of hours in which the temperature was higher than a certain value [32]. Other covariables were likely to affect prevalence but their importance has not yet been tested or verified. These are pond morphology, number of hours in which the temperature was lower than a certain value, pond isolation, tadpole density and depth/microhabitat of capture. Finally, I included some covariables that serve as controls for handling like date of capture, observer and the pond itself.

Modeling

First analysis – All ponds

(1) I evaluated over all prevalence for all ponds as well as individual prevalence for every single pond. (2) Then I integrated the single covariables and the combined covariables (from the PCA, see results) to model Ψ and evaluate the most important covariables and their combinations for found prevalences in two steps. In a first step I modeled Ψ individually for every

available covariable (Table 2) and for all possible pairs of covariables in single runs. In a second step I modeled Ψ for all possible pairs with TEMPspect in addition. As a control I modeled Ψ with pond units (POND), the observers (OBS) and the date of capture (DATE) as covariables. (3) I further assessed the quality of our swabbing method. For this purpose I modeled p_1 (Quality of first swab event on a tadpole) and p_2 (Quality of second swab event on a tadpole) on the basis of the found detection history data of all ponds without any covariables.

Second analysis – Partially infected ponds

(4) In the second analysis I used the detection histories of the tadpoles from the two ponds where only a fraction of tadpoles was infected (n=40). I modeled Ψ with all covariables on tadpole-level in single runs. So I evaluated those covariables that best explained the found prevalences. As a control I modeled Ψ with pond units (POND), the observers (OBS) and the date of capture (DATE) as covariables.

Third analysis – Effects of sample size on estimates of prevalence

(5) In this analysis I calculated observer specific estimates of Ψ for observer 1, observer 2 and both observers together. In every visited pond both observers swabbed 10 out of the 20 caught tadpoles. So I calculated three prevalence estimates for all ponds (n=108, n=108, n=216), for partially infected ponds (n=20, n=20, n=40) and for Itingen (n=10, n=10, n=20) and Chalchofen (n=10, n=10, n=20). With these estimates I demonstrate differences in prevalence estimation by observers due to small sample sizes.

Results

Principal component analysis

Temperature minimum, median, average and maximum were combined into the first principal component "temperature spectrum" (Figure 3) and depth average, surface and volume were combined into the second principal component "pond morphology" (Figure 4). These two new variables temperature spectrum and pond morphology (Figure 5) explain together 82.6 % (Figure 6) of the formerly seven covariables.

First analysis – All ponds

***Bd* prevalence**

(1) In 2010 I detected tadpoles of the Common Midwife Toad at 12 out of 18 previously occupied sites. At 5 sites neither tadpoles nor adults of the Common Midwife Toad were seen, and at 1 site I found adults only. At 3 of the 12 *Bd*-positive sites *Bd* was detected for the first time and only 1 site was still *Bd* free. The overall *Bd* prevalence was 0.79 ($se = 0.0276$). The prevalence estimates for single ponds in 2010 (Table 4) as well as data from 2007 and 2009 (U. Tobler, unpublished data) are shown in figure 2. Note that data from 2007 is from summer and prevalences in summer are usually lower.

Prevalence affecting covariables

(2) I found that a model including pond morphology and altitude best described the prevalence (Table 5). A model that included additionally the temperature

spectrum also described the data well (Table 5). Thus, the lower the altitude, the smaller the pond, and the higher the temperature is, the lower is the predicted prevalence (Figure 7 & 8, table 6).

Quality of the swabbing method

(3) The described and commonly used swabbing method turned out to be highly sensitive and accurate. In a total of 216 swabbed tadpoles, 171 tested positive. The detection probability for the first swab event was $p_1 = 1.00$ $(se = 0.0)$ and for the second swab event $p_2 = 0.98$ $(se = 0.01)$ (Table 7). This means that the second swab never was *Bd* positive if the first swab was *Bd* negative. Conversely, only three cases were found where the first swab was *Bd* positive and the second swab was *Bd* negative.

Second Analysis – Partially infected ponds

(4) I found that a model including developmental stage best described the prevalences in partially infected ponds (Table 8 & figure 9). But note that over all ponds also early developmental stages were infected (Figure 10).

Third Analysis – Effects of sample size on estimates of prevalence

(5) Figure 11 shows that the estimates strongly depends on sample size. The estimates for the individual observers differ markedly from each other and they differ from the estimates based on both observers.

Table 4 First analysis (1): Prevalence of *Bd* at the different sites (where tadpoles of the Common Midwife Toad were found) and the most important covariables.

Pond	n	Prevalence	ALT m.ü.M	TEMPmin °C	TEMPmax °C	TEMPmed °C	TEMPav °C	V m^3	A m^2	DEPTHav m
Brandsitengraben	20	**1.00 (se=0.0000)**	745	10.0	17.0	14.5	14.2	44.62	328	0.76
Chnubel	20	**1.00 (se=0.0000)**	864	8.5	15.0	12.5	12.3	10.53	67	0.88
Vorder Birnbaum	20	**1.00 (se=0.0000)**	825	12.5	26.5	18.5	18.9	84.00	42	2.00
Waltisberg	20	**1.00 (se=0.0000)**	854	9.5	20.0	13.5	13.8	7.04	111	0.36
Chalchofen	20	**0.55 (se=0.1112)**	395	13.0	32.0	21.5	21.5	1.59	40	0.22
Itingen	20	**0.20 (se=0.0894)**	413	12.5	24.5	19.0	18.6	26.85	191	0.79
Zunzgen	20	**1.00 (se=0.0000)**	483	10.0	23.5	16.0	16.5	1103.03	3987	1.55
Altstätten	20	**1.00 (se=0.0000)**	543	8.0	27.5	17.0	16.7	0.42	11	0.22
Buechholzweiher	3	**1.00 (se=0.0000)**	537	a	a	a	a	3.92	244	0.09
Ochsenweid	16	**1.00 (se=0.0000)**	575	9.5	28.5	17.0	17.0	71.07	468	0.85
Sittertobel	20	**0.00 (se=0.0000)**	608	11.5	23.5	17.0	16.8	1.45	12	0.69
Wolfgangweiher	20	**1.00 (se=0.0000)**	677	11.0	24.0	18.5	18.1	380.54	1292	1.65

a logger defect

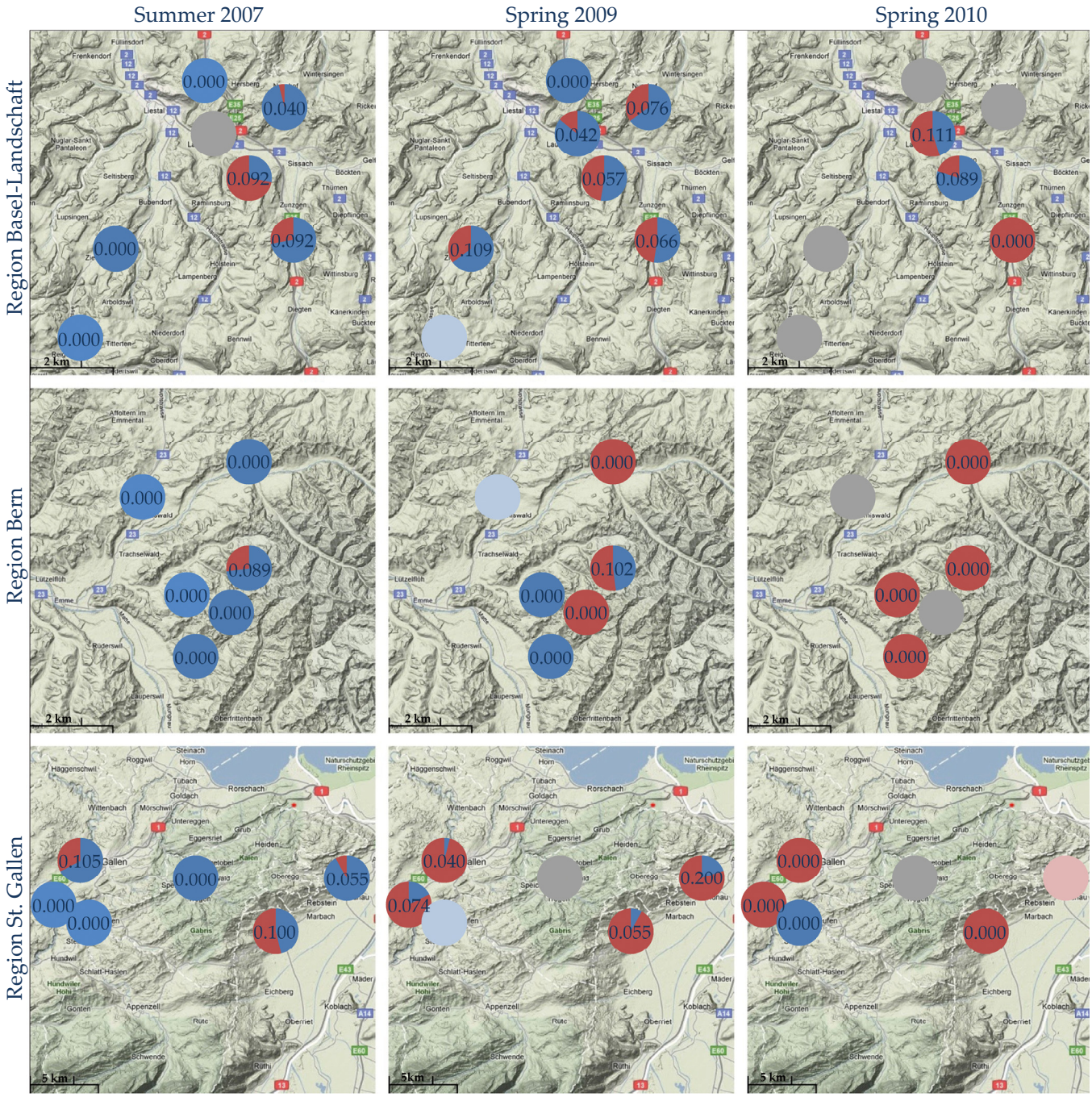

Figure 2 First analysis (1): *Bd* prevalence estimates of the ponds in the three regions indicated by pie charts. Data from 2007 (left), 2009 (middle) and 2010 (right) of the three regions Basel-Landschaft (top), Bern (middle) and St. Gallen (bottom). Data from 2007 and 2009 were provided by U. Tobler (unpublished data).

% *Bd* positive (Prevalence) | % *Bd* negative | *Bd* present | *Bd* probably absent | No tadpoles found
Numbers in the pie charts indicate the standard errors of prevalence.
Picture credits: ©2010 Google – Kartendaten ©2010 Tele Atlas.

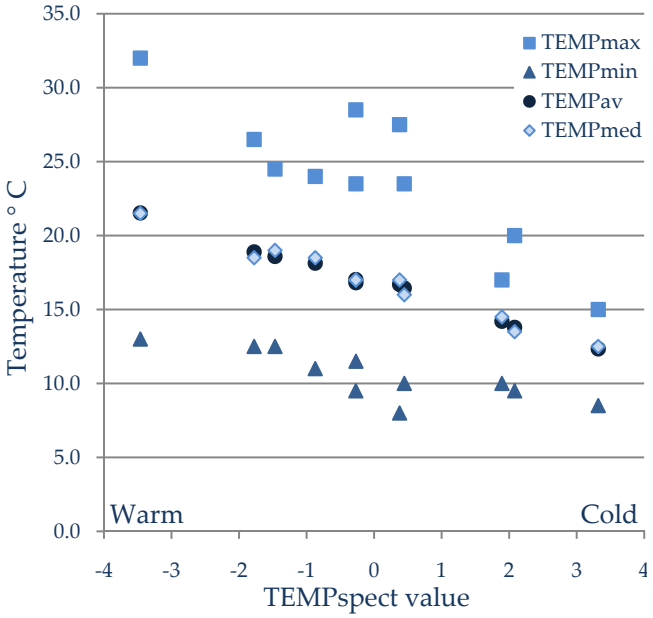

Figure 3 Original values for temperature maximum, minimum, average and median (y-axis) in relation to factor scores for the first principal component TEMPspect (x-axis) that resulted from the PCA.

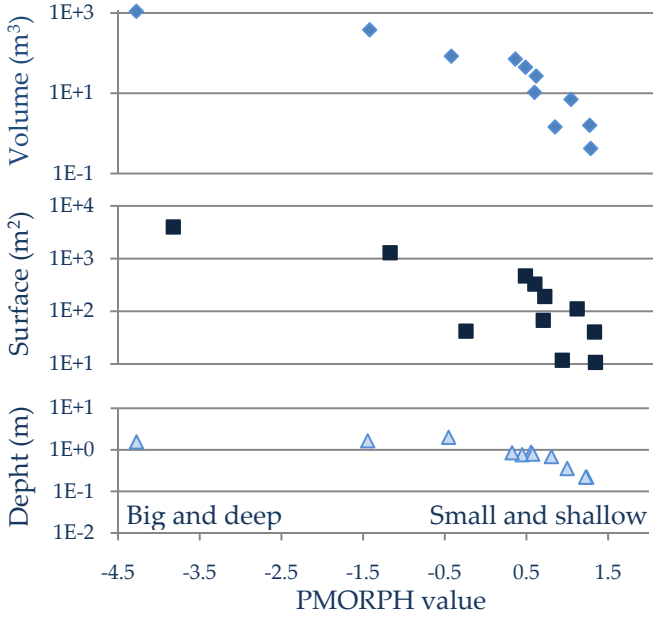

Figure 4 Original values for pond depth average, surface and volume (y-axis) in relation to factor scores for the second principal component PMORPH (x-axis) that resulted from the PCA.

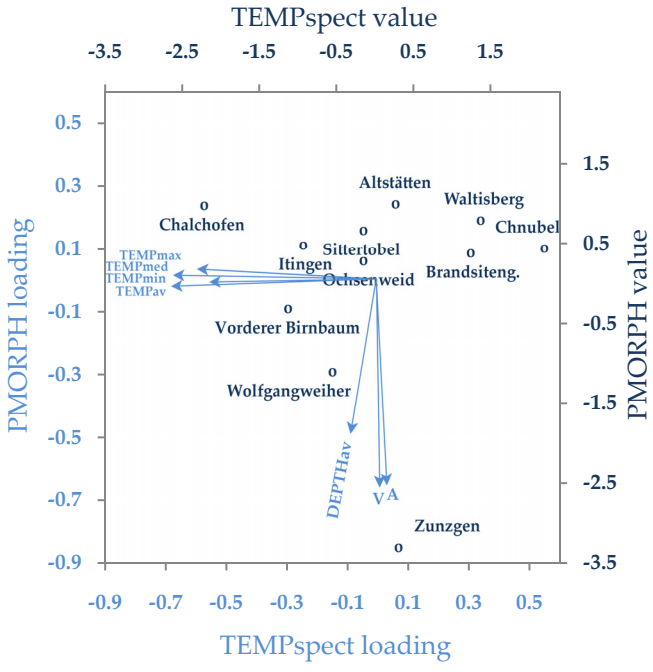

Figure 5 Result of the principal component analysis. TEMPspect is the first principal component, PMORPH is the second principal component. Light blue: Component loadings of TEMPspect (prim. x-axis) and PMORPH (prim. y-axis) for the different covariables V, A, DEPTHav, TEMPmin, TEMPmax, TEMPav and TEMPmed. Dark blue: Values of the different ponds for the principal components TEMPspect (sec. x-axis) and PMORPH (sec. y-axis).

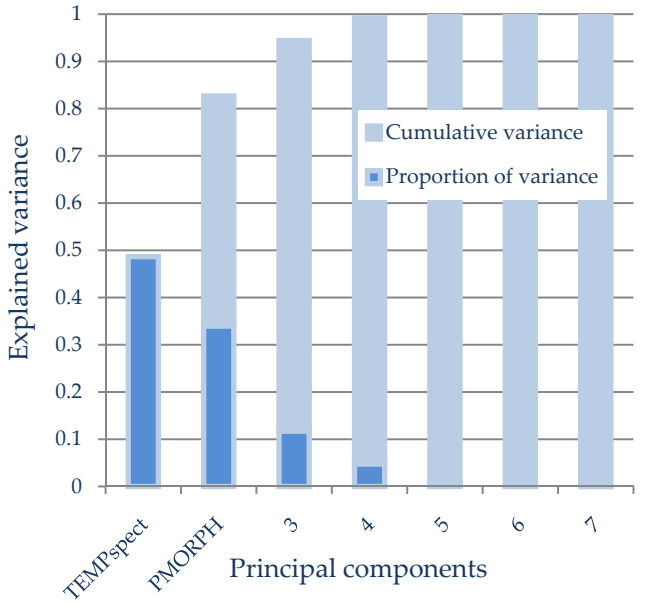

Figure 6 Explained variance in percent (y-axis) by the principal components (x-axis) as proportion (dark blue) and cumulative proportion (light blue). Labeled are the chosen two principal components TEMPspect and PMORPH which together explain 82.6 % of variance.

Table 5 First analysis (2): Best supported models for the observed *Bd* prevalences. The three models at the bottom serve as controls.

Model	-2log-Likelihood	K	AICc	ΔAICc	Akaike weight
Ψ(PMORPH & ALT)p(.)	188.93	4	197.12	0.00	0.6950
Ψ(TEMPspect & PMORPH & ALT)p(.)	188.63	5	198.92	1.8	0.2971
Control: Ψ(POND)p(.)	a				
Control: Ψ(OBS)p(.)	254.15	3	260.26	76.88	0.0000
Control: Ψ(DATE)p(.)	310.59	3	316.70	133.32	0.0000

PMORPH=Pond morphology; TEMPspect=Temperature spectrum; ALT=Altitude; OBS=Observer; DATE=Date of observation; POND=Pond identity
[a] Model did not reach convergence

Table 6 Estimates of the different betas for the two models Ψ(PMORPH & ALT)p(.) (Figure 7) and Ψ(TEMPspect & PMORPH & ALT)p(.) (Figure 8) with their respective standard errors.

Model	Formula	β-estimate	se(β)	According covariable
Ψ(PMORPH & ALT)p(.)	$\Psi = \frac{\exp(\beta_0 + \beta_1 * x_1 + \beta_2 * x_2)}{1 - \exp(\beta_0 + \beta_1 * x_1 + \beta_2 * x_2)}$	$\beta_0 = -3.135755$	0.896257	
		$\beta_1 = -0.689919$	0.237725	$x_1 = PMORPH$
		$\beta_2 = 8.090770$	1.541146	$x_2 = ALT$
Ψ(TEMPspect & PMORPH & ALT)p(.)	$\Psi = \frac{\exp(\beta_0 + \beta_1 * x_1 + \beta_2 * x_2 + \beta_3 * x_3)}{1 - \exp(\beta_0 + \beta_1 * x_1 + \beta_2 * x_2 + \beta_3 * x_3)}$	$\beta_0 = -2.490498$	1.459407	
		$\beta_1 = -0.688687$	0.247534	$x_1 = PMORPH$
		$\beta_2 = 7.037821$	2.400960	$x_2 = ALT$
		$\beta_3 = 0.108275$	0.194899	$x_3 = TEMPspect$

PMORPH=Pond morphology; TEMPspect=Temperature spectrum; ALT=Altitude

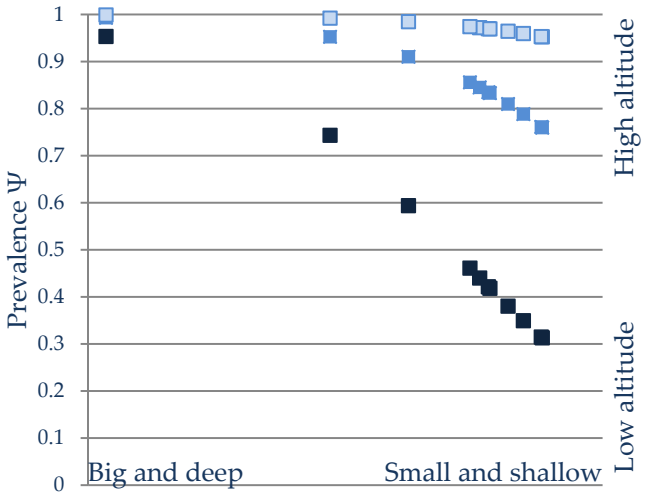

Figure 7 Predicted *Bd* prevalence values (y-axis) for different pond morphologies (x-axis) in relation to pond altitude. Different altitudes are indicated by color (Maximum □; average ■ and minimum ■ of found altitude). Underlying function with predicted beta values and their respective standard errors are shown in table 6.

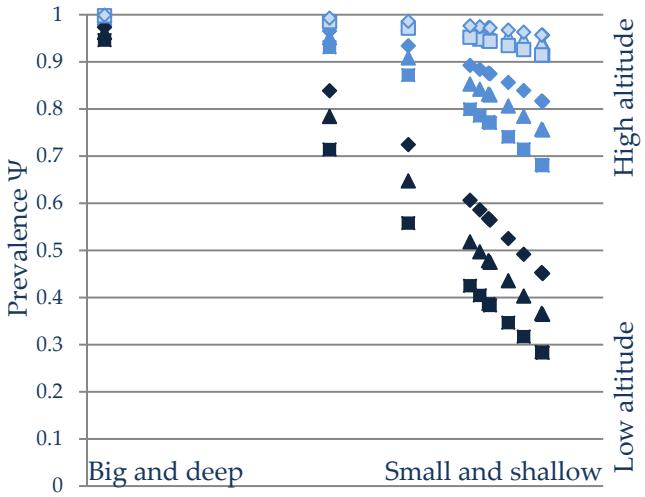

Figure 8 Predicted *Bd* prevalence values (y-axis) for different pond morphologies (x-axis) in relation to pond temperature spectrum and altitude. Different pond temperature spectrums are indicated by shape (Maximum ◇, average △ and minimum □ of found temperature spectrum. Note that high values mean cold ponds (Figure 3)). Different altitudes are indicated by color (Maximum □, average ■ and minimum ■ of found altitude). Underlying function with predicted beta values and their respective standard errors are shown in table 6.

Table 7 First analysis (3): Quality of the swabbing method.

Model	-2log-Likelihood	K	AICc	ΔAICc	Akaike weight
Ψ(.)p(survey-specific)	251.28	3	257.39	0.00	0.7465
Ψ(.)p(constant)	255.44	2	259.50	2.10	0.2535

Table 8 Second analysis: Most supported model for the found *Bd* prevalences. Models at the bottom serve as control.

Model	-2log-Likelihood	K	AICc	ΔAICc	Akaike weight
Ψ(DEVEL)p(.)	45.47	3	52.14	0.00	0.9987
Control: Ψ(POND)p(.)	62.09	3	68.76	16.62	0.0002
Control: Ψ(DATE)p(.)	62.09	3	68.76	16.62	0.0002
Control: Ψ(OBS)p(.)	66.51	3	73.18	21.04	0

DEVEL=Developmental stage of the tadpoles; DATE=Date of observation; OBS=Observer; POND=Pond identity

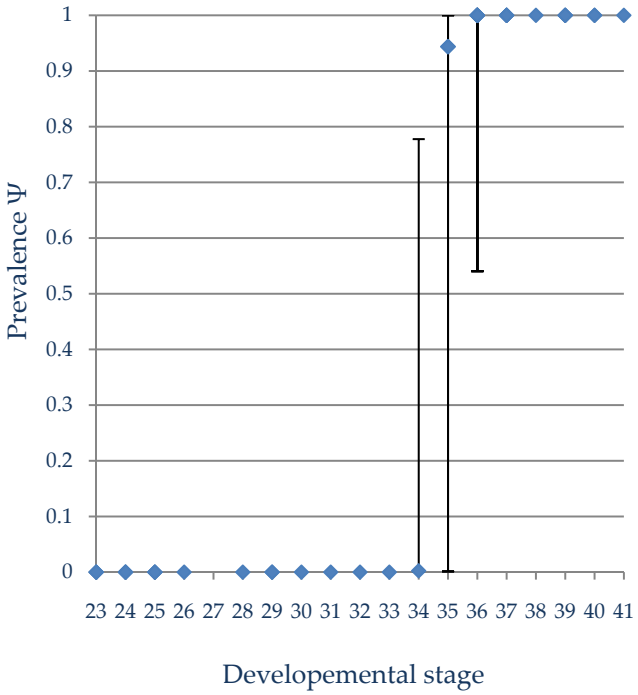

Figure 9 Predicted *Bd* prevalence values (y-axis) for different developmental stages (x-axis, Gosner stages) in partially infected ponds.
Error bars indicate the standard errors.

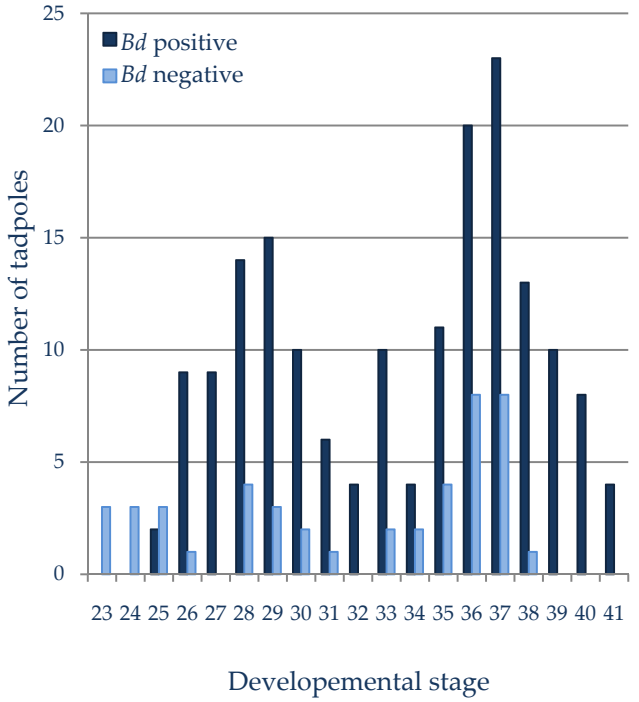

Figure 10 Number of *Bd* positive (y-axis, dark blue) and *Bd* negative (y-axis, light blue) tested tadpoles in 2010 for different developmental stages (x-axis, Gosner stages). Data from all ponds was used.

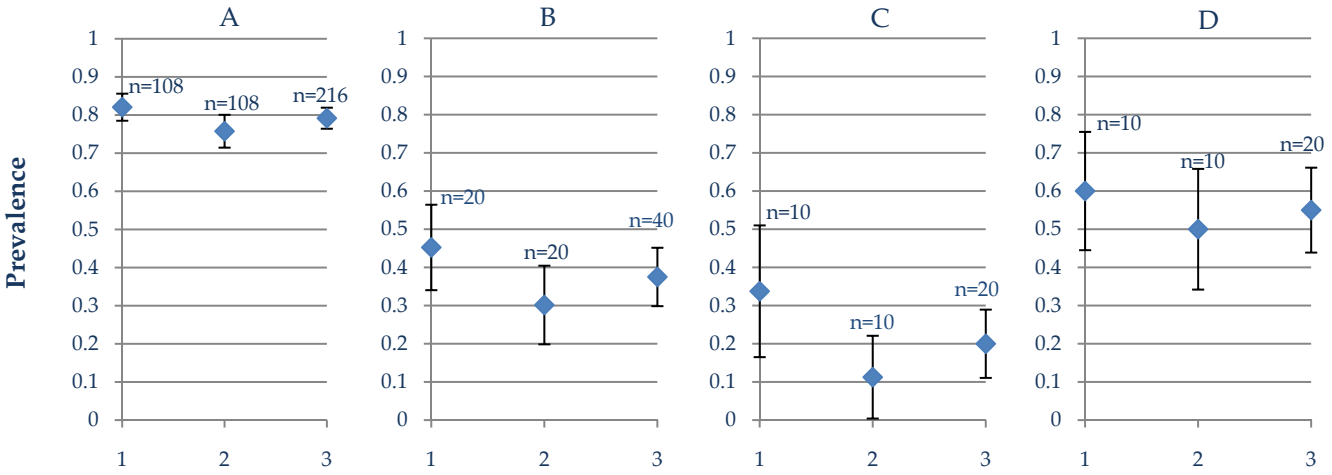

Figure 11 *Bd* prevalence estimates (y-axis) for the different observers (x-axis: 1=Observer 1, 2=Observer 2, 3=Both observers) and their combination. Letters at the top shows pond identities: A=all ponds; B=partially infected ponds; C= Itingen; D= Chalchofen.
Error bars indicates the standard error and numbers the sample size (n).

Discussion

I used the site occupancy model to estimate (1) the *Bd* prevalence and (2) explained among-pond variation in prevalence caused by environmental and tadpole characteristics. I also checked (3) the reliability of the widely used swabbing method, and I showed (4) random observer specific differences in prevalence.

Bd in northern Switzerland

(1) In northern Switzerland *Bd* is widespread. Although prevalences are fluctuating they are mainly increasing or reached already high levels. In 2010, *Bd* propagated into 3 ponds where it had not been observed before and only 1 pond escaped invasion till now (Figure 2). This shows that the distribution of *Bd* is not static.

Bd driving variables

(2) The variables that determined prevalence were pond morphology, altitude and temperature spectrum (Table 5). Big deep ponds at high altitude showed the highest prevalence (Figure 7). What are the mechanisms that created this pattern? I suggest here two possibilities for pond morphology effects: (i) *Bd* reservoirs and (ii) probability of drying; and I reject a third one: (iii) density dependent transmission.

(i) *Bd* reservoirs: Pond morphology goes along with physical factors (drying, winter anoxia) and biotic effects (predation) [33] and this leads to different communities. Big ponds seem to hold communities with species that act as reservoirs for *Bd*. Another plausible reservoir for *Bd* are the tadpoles of the Common Midwife Toad itself because they often hibernate. Hibernation is less possible in small ponds due to total freezing. Thus the next generation faces *Bd* in big ponds with a higher probability.

(ii) Probability of drying: In dry summers ponds with very small volumes tend to dry out. As far as we know *Bd* cannot survive in dry ponds [34] and is rare in ephemeral ponds [17]. If pond drying eliminates *Bd* from the pond, then *Bd* infection risk may be reduced

once the pond fills again. (iii) Density dependence: It is unlikely that small ponds have enhanced transmission rates. An enhanced transmission rate is imaginable in small ponds (tadpole crowding) if the transmission would be density dependent. In my analysis no density effects were found. Similar findings were made by Rachowicz et al. [35] and Raffel et al. [36].

The influence of altitude on prevalence is also important (Figure 7 & 8). A lot of papers describe effects of altitude on *Bd*, but they always suggest that the altitude effect is in fact an effect of temperature [9, 12]. In our case, however, temperature and altitude were independent factors. Thus, there is likely a direct effect of altitude that is independent of temperature (e.g. exposition to solar radiation). A possible explanation for an effect of altitude could be that, because of shorter warm seasons at higher altitudes, more tadpoles have to hibernate. If a lot of tadpoles hibernate, they run a high risk to get infected. This is due to the long exposure time and the low temperatures in winter [13, 14]. A high frequency of infected tadpoles in turn results in a high transmission rate to the next generation [35]. This may lead to an upward spiral of *Bd* prevalence and could be an important cause for the already observed fatal chytridiomycosis outbreaks at high altitudes [7, 9].

The finding that the temperature spectrum is a predictor for *Bd* incidence was already known. As a new result I found that spatial variation in prevalence is also predictable by temperature (Figure 8). Possible explanations for this finding are (i) the temperature dependent appearance or disappearance of *Bd* [5, 37] maybe due to a combination of enhanced host resistance by high temperatures [16] and inhibition of the innate defense mechanism (peptides) by cold temperatures [13, 15, 38], (ii) increased susceptibility for pathogens due to changing temperatures [39] and (iii) enhanced pathogenicity of *Bd* at low temperatures [12]. Temperature effects also contributes to the seasonality of this (and other) infection diseases [40].

Interestingly, I did not find evidence that a certain duration of high temperatures leads to a reduction of *Bd* prevalence. A loss of *Bd* infection after prolonged exposure to high temperature was found in a laboratory experiment [32]. It is possible that these effects would be visible with extended monitoring periods.

In partially infected ponds with low prevalences developmental stage (or time of exposure) may play a role (Figure 9). It seems that the longer the tadpoles are exposed to *Bd* the more likely they are to be infected. This shows that an inspection of the tadpoles of a pond to determine the prevalence always should include older individuals. Investigating only early developmental stages could lead to an underestimation of *Bd* prevalence.

Swabbing method

(3) I found that the used swabbing method is highly sensitive and independent of operator experience or developmental stage of the tadpoles. Out of 216 tadpoles 171 were tested positive for *Bd*. Estimated detection probability was 1.

This finding contrasts the results obtained by the innovators of this method (Retallick et al. 2006).

Retallick et al. supposed that the method has a detection probability of around 0.5 [41]. The reason for this low detection probability is most likely founded in the different swabbing methods. Retallick et al. used toothpicks whereas I used cotton swabs. With usage of sterile cotton swabs the sensitivity of this method is extremely improved. Also in contrast to Retallick et al. I did not find evidence that the person handling the tadpoles influences the result (if the sample sizes are big enough) because the detection probability over all first swab events was 1, including the initial observations whereat the observers had no experience at all.

Thus, I assert that this swabbing method is a very good choice to determine the quantitative *Bd* state of tadpoles because it is a supremely sensitive, non-lethal sampling method which is independent of observers and experience.

Prevalence estimation

(4) I showed that accurate prevalence estimation depends on the sample size (Figure 11). Low sample sizes lead to fluctuations in the estimates. At first sight this is a trivial conclusion and there are different mathematical approaches to calculate the required sample sizes for a requested confidence interval in prevalence estimates without [42] and with known population sizes [43]. But in practice it is easily forgotten because of a lack of time, money or patience and this should be kept in mind.

Conclusion

This study suggests that pond temperature, altitude and pond morphology are important predictors of *Bd* prevalence. It was already shown that *Bd* incidence is predictable with temperature and altitude data but I am the first to show that altitude also predicts prevalence. In addition I report a hitherto unknown driving factor for *Bd*: the pond morphology. Pond morphology could be used as a tool for conservation strategies, because it is modifiable. I further found strong evidence that the used swabbing method is highly sensitive for quantitative approaches and its result does not depend on the experience of the person applying it. This allows designing and conducting extensive fieldwork with inexperienced volunteers or employees. Based on our results I predict that small, shallow and sun exposed ponds which are situated on low elevations should generally exhibit low *Bd* risks.

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