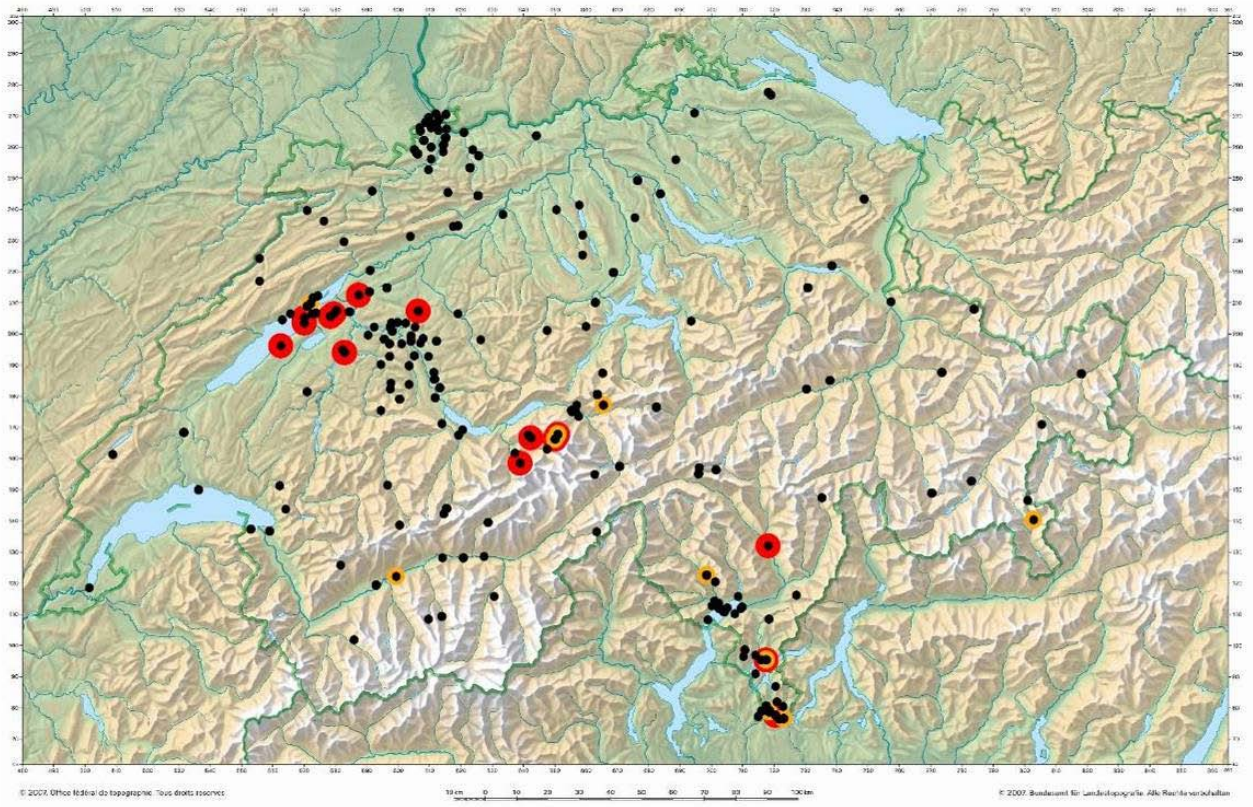
Master Thesis

Historical evidence for the presence of the emerging amphibian pathogen *Batrachochytrium dendrobatidis* (Longcore et al. 1999) in Switzerland



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Abstract

Emerging infectious diseases are threatening the global ecosystem and can cause huge economic damage. Worldwide, amphibians are at a high risk of decline or even extinction due to the fungal pathogen *Batrachochytrium dendrobatidis*. Its origin remains obscure and its global distribution is underestimated because of its cryptic spread.

To investigate the historical status and distribution of this pathogen in Switzerland, I made a retrospective survey on preserved amphibians from natural history museum collections.

I took skin scrapings from 788 specimens of all 20 Swiss species from 252 different locations, collected over a time span from 1853 until 2009.

The fungal DNA was detected by real time quantitative polymerase chain reaction in 21 specimens from 15 different locations ranging from 260 to 1960 meters above sea level. The earliest finding of the pathogen is from 1901 followed by evidences from the 1980ties.

Because of the fact that locations in all biogeographic regions and at the deepest and highest elevations are infected I assume that the pathogen can potentially occur everywhere in Switzerland where amphibians occur. The pathogen seems to be at least in some populations human introduced, which reveals the importance to prevent further spread by humans. The actual situation and future directions of the infection status need to be surveyed further to prevent possible negative impacts on the Swiss amphibian community.

Introduction

Cryptic or novel and rapidly spreading diseases are massive threat to biodiversity and are considered as the most important causes for extinctions after habitat loss (Vitousek et al. 1997). Recently discovered emerging pathogens are responsible for many population declines in many different species. The White Nose Syndrome in bats caused by the snowy-white fungus (*Geomyces destructans*) first appeared in 2006 and caused a population decline of up to 97% in hibernation sites of some bat species in the USA (Blehert et al. 2009). Another disease affecting ash trees (*Fraxinus* spp.) in large parts of Europe is known since 1851 but its recent emergence remains enigmatic (Queloz et al. 2010). Another dramatic case of an emerging disease takes place in the amphibians.

92.5% of the amphibians that are critically endangered are not threatened by classical threats such as habitat loss and overexploitation (Stuart et al. 2004; Mohneke et al. 2009). Many of these species occur in protected and therefore mainly undisturbed areas and the reasons for population declines were partly unknown in the beginning (Daszak et al. 1999; Skerratt et al. 2007; Rosenblum et al. 2010). Mass mortality events in Australia and Central America during 1993-1997 causing significant and long term population declines lead 1998 to the first report of a parasitic chytridiomycota on vertebrates (Berger et al. 1998). It is now generally accepted that many of the rapid enigmatic declines are driven by this fungus (Skerratt et al. 2007) and that it has been responsible for mass mortality events in Costa Rica in 1987 (Lips 1999) and in Spain in 1997 (Bosch et al. 2001; Walker et al. 2010).

This new member of the chytridiomycota was classified as *Batrachochytrium dendrobatidis* (Longcore et al. 1999) [Bd]. Transmission experiments proved the lethal effect of the pathogen (Berger et al. 1998/2002; Longcore et al. 1999; Tobler & Schmidt 2010). A global survey found 387 species in 37 families of frogs, toads and salamanders infected with Bd (Fisher et al. 2009). A recently developed species distribution model showed that the total distribution of one sixth of all and half of the range of over 50% of all known amphibian species fall into regions potentially suitable for Bd. Almost all of the 397 species identified under high risk of decline due to Bd are listed as threatened with extinction under the IUCN Red List of Threatened Species (Rödder et al. 2009).

Bd has been considered “as the worst infectious disease ever recorded among vertebrates in terms of the number of species impacted, and its propensity to drive them to extinction” and “there is growing consensus among scientists that the spread of chytridiomycosis has driven and will continue to drive amphibian species to extinction at a rate unprecedented in any taxonomic group in human history” (Gascon et al. 2007).

Where Bd comes from is still not clear and there is an ongoing debate on the ancestral origin of Bd (Daszak et al. 1999; Weldon et al. 2004; Garner et al. 2006; Rachowicz et al. 2005; Skerratt et al. 2007). The earliest documented evidence of Bd infection in wild amphibians is from Africa from 1933 (Soto-Azat et al. 2010), followed by North America from 1961 (Ouellet et al. 2005), Australia from 1978 (Skerratt et al. 2007), South America from 1980 (Lips et al. 2008), Europe from 1997 (Bosch et al. 2001) and Mainland Asia (Yang et al. 2009). In Switzerland the earliest confirmation of Bd in the wild to date is from 2005 (Garner et al. 2005). But these dates often do not necessarily show the first occurrence in these regions. Instead, they often just represent the date of the first research done. As the pathogen is found in almost every region once researchers start to look for it, the global distribution of Bd seems to be underestimated and the timing of spread wrongly dated (Skerratt et al. 2007; Lips et al. 2008; Rödder et al. 2009).

A recently initiated Global Mapping Project found evidence of Bd in 45 of 78 (58%) countries sampled. 1168 (48%) of 2449 sites examined were infected with Bd (Fisher et al. 2009). In Europe, Bd has already been found in France, Spain, Portugal, Italy, Great Britain and Switzerland, infecting many different amphibian species (Garner et al. 2005/2006). In Switzerland the infection prevalence has been studied intensively and was considered exceptionally high (Garner et al. 2005) and Bd is widespread (Tobler & Schmidt 2010).

There are two main hypotheses on the origin of chytridiomycosis:

1) The *Novel or Spreading Pathogen Hypothesis* proposes that a pathogen has spread into new areas, where the hosts are not adapted to it. This thesis is supported by the fact that in most of the cases there was no evidence of Bd before die-offs (Berger et al. 1999; Green & Sherman 2001; Ouellet et al. 2005; Simoncelli et al. 2005). The first evidence of Bd (Soto-Azat et al. 2010) and a high prevalence without observations of mass die-offs have been reported from Africa; this may suggest a long co-evolutionary history between African hosts and Bd (Kielgast et al. 2009). Therefore the pathogen is considered to have its origin in Africa (Weldon et al. 2004; Soto-Azat et al. 2009/2010; Kielgast et al. 2009). The spreading of Bd presumably takes place by the global dislocation of animals, plants and raw material by humans (Daszak et al. 2003; Rachowicz et al. 2005; Garner et al. 2006) leading to its widespread but patchy distribution (Rödder et al. 2009; Walker et al. 2010). Genetic analyses found low global genetic variation which could be due to recent Bd population mixing by translocation between regions (Daszak et al. 2003), by single origin (Fisher et al. 2009) or recent, rapid and repeated dispersal of the pathogen (James et al. 2009).

2) The *Emerging Endemic Pathogen Hypothesis* in contrast states that the endemic pathogen has become either more pathogenic, virulent and transmissible or rather the hosts became more susceptible to the pathogen (Daszak et al. 1999; Rachowicz et al. 2005; Skerratt et al. 2007). Evidence of environmental factors influencing the outbreak of the disease (Fisher et al. 2009), the fact that some species are less susceptible than others and that Bd can be present in a population before declines happen (Garner et al. 2005/2006; Ouellet et al. 2005) point toward this second hypothesis.

However, both hypotheses may not be mutually exclusive and might not as well apply to all species or all areas (Rachowicz et al. 2005; Kilpatrick et al. 2009). It is still unknown how and when Bd was introduced into protected and undisturbed habitats (Vredenburg et al. 2010; Rosenblum et al. 2010).

In order to decide whether Bd is a novel or endemic pathogen, I need to know for how long it has been present outside its putative area of origin (i.e. Africa). Here I present the first historical retrospective survey in Switzerland and Europe to investigate the historical status and distribution of Bd. To do so, I tested museum specimens for the presence of Bd. A historic survey of Bd is also important because conservation action will differ substantially depending how long it is present in an area and if the pathogen is novel or endemic.

Material & Methods

Short review: Diagnostic Methods

Zoospores and sporangia of Bd are most likely found in the skin of the ventral abdomen, the pelvis, on hind limbs and feet (Longcore et al. 1999; Pessier et al. 1999; Green & Sherman 2001; Berger et al. 1999/2005b). In tadpoles, the fungus can only be found in the keratinised mouthparts (Vredenburg & Summers 2001; Garner et al. 2005; Hyatt et al. 2007).

There are several methods how Bd and/or chytridiomycosis can be detected. Hyatt et al. (2007) and Smith (2007) point out that the evidence of zoospores from Bd does not necessarily imply evidence of the disease chytridiomycosis as some species seem to be healthy carriers of the fungus. For this study this differentiation is not essential, as I primarily want to find the first evidence of infection with Bd and not the first outbreak of the disease.

Scanning (Berger et al. 1998; Berger et al. 2005a) and transmission electron microscopy (Pessier et al. 1999; Ouellet et al. 2005; Berger et al. 2005a) is used to identify Bd by means of the ultra structure of the zoospores and chytridiomycosis by means of skin anomalies. This method is not useful for screening a lot of samples for infections. It is very time consuming and because of the small area examined, light infections can be missed. Different techniques have been used for detecting Bd and chytridiomycosis, respectively.

Routine histological methods have been used frequently for detecting intracellular sporangia in amphibian skin samples and a wide variety of different staining procedures have been tested. One of the simplest methods is to just scrape the skin of the specimen under investigation, to stain the probe with Congo Red and to look for stained zoospores and sporangia (Briggs 2003; Briggs & Burgin 2004). Staining of formalin fixed, paraffin-embedded and thin sectioned tissues with haematoxylin and eosin [H&E] was the first and most frequent method used (Berger et al. 1998; Longcore et al. 1999; Daszak et al. 1999). With these approaches, the chytrid sporangia could be confounded with developmental stages of Protozoans (Pessier et al. 1999), and thus skilled experts are needed for effective detection and accurate identification of the fungus (Van Ells et al. 2003; Briggs 2003).

A more sensitive method for detecting the chytrid fungus, which also decreases the need for skilled diagnosticians, has been established by the use of polyclonal antibodies together with an immunoperoxidase [IPX] stain (Berger et al. 2002). This method has been used frequently (Van Ells et al. 2003; Olsen et al. 2004).

One problem with all these methods is, that usually only a small part of the animal's epidermis such as a single toe clip is examined and therefore only late stages and heavy infections can be detected routinely (Kriger et al. 2006). Serial examination of many slides

improves the sensitivity but is even more time consuming (Hyatt et al. 2007). Also, the invasive sampling can be problematic, especially in valuable museum specimens and live animals of rare species (Boyle et al. 2004; Soto-Azat et al. 2009). Furthermore, in old preserved samples a lack of the upper keratinised epidermis can occur because of partial erosion (Ouellet et al. 2005; Walker et al. 2008); consequently only few or no sporangia may be left in the sample. This problem can be solved partially by counterstaining the epidermal keratin, leading to increased confidence in a negative diagnosis where keratin is present but not the fungus (Olsen et al. 2004).

DNA based diagnostic assays have been established and used to screen for the fungus (Boyle et al. 2004; Annis et al. 2004; Simoncelli et al. 2005). A bigger skin surface can be examined by using skin scrapings (Pessier et al. 1999), swabs (Berger et al. 1998) or by bathing the specimen (Hyatt et al. 2007) and therefore the risk of missing the infection is reduced compared to the use of toe clips.

Methods differ in their sensitivity. An overview of the sensitivity of the different methods is given by Hyatt et al. (2007). Histological methods are not able to detect the fungus until 14 days after infection, while a real time quantitative polymerase chain reaction [qPCR] assay is able to find evidence already after 7 days. Hence, qPCR can also detect light infections. The diagnostic sensitivity of qPCR using bath sampling is 77% and of qPCR with toe clipping 58%. With histological methods and the use of fungal stains (H&E) the sensitivity was only 29.5% and 33.3% with the use of polyclonal antibodies (IPX) respectively (Hyatt et al. 2007). Also Kriger et al. (2006) showed, that qPCR with swab sampling is at least twice as sensitive to detect Bd as histology of toe clips. Little is known about the variability between levels of infection on toe clips (Boyle et al. 2004). For archived museum specimens preserved with formalin, it could be a challenge to extract the fungal DNA and use it for a qPCR assay (Miething et al. 2006; Wandeler et al. 2007). There are methods to extract DNA from archival tissue (Jackson et al. 1990; Kallio et al. 1991; Sepp et al. 1994; Shedlock et al. 1997) and whole genome amplification was feasible with tissue stored in formalin for 17 years (Walker et al. 2008).

Pilot study

To investigate the appropriate method for our purposes, I tested different protocols: staining and two kinds of PCR.

Staining skin with Congo Red (Briggs 2003; Briggs & Burgin 2004), the simplest, most rapid and most cost effective method was not convincing in a test with infected specimens. The presence of Bd could be foreshadowed, but without experience and expert knowledge, the proof of a Bd infection remains vague. Different species of fungi cannot be distinguished using this method and I assume that light infections are easily missed.

Initial tests with PCR were more promising. The most sensitive and specific diagnostic approaches detect fungal DNA by real time quantitative polymerase chain reaction [qPCR] (Boyle et al. 2004; Simoncelli et al. 2005; Walker et al. 2008). I did preliminary tests to reveal if these methods can also be used with non invasive sampling methods (skin scrapings) and with formalin preserved specimens. I sampled three ethanol (BLZ 17, BLZ 28, SGA 36) and five formalin (BLZ 02, BLZ 04, SGA 47, SGA 50, BLI 31) fixed *Alytes obstetricans* from the Zoological Museum of the University of Zurich, which were tested positive for Bd by Ursina Tobler (personal communication) in other studies. I used skin scrapings taken with a scalpel razor blade from the ventral abdomen/pelvis, the ventral hind limbs and the plantar surface of the hind feet. This procedure is minimally invasive and does not cause visible damage to the specimens, which is important for valuable museum collections. The DNA was extracted following the methods used in Boyle et al. (2004), Simoncelli et al. (2005) and Walker et al. (2008). Here, a similar kit for the Whole Genome Amplification was used: WGA5 GenomePlex Tissue Whole Genome Amplification Kit, Sigma Aldrich. The qPCR according to Boyle et al. (2004) was duplicated for every extraction method and an internal negative control and standards (100; 10; 1; 0.1 zoospore standards) were used. All the samples from ethanol stored specimens were tested positive with both methods, while from the formalin fixed samples, only 20 percent of the tested specimens could be tested positive with the method of Simoncelli et al. (2005) and the other method yielded no positive results (Appendix: Table 4). Many specimens from the Natural Museum Bern had been stored in formalin first and then transferred into ethanol (Lüps et al. 2005). Therefore and because the costs are lower, I used the extraction method from Boyle et al. (2004) for museum specimens stored in ethanol and the extraction method from Simoncelli et al. (2005) only for formalin fixed specimens and for specimens where it is not clear how they were stored. Manufacturer protocols were followed.

Data Collection

I contacted 14 natural history museums in Switzerland and from four of them I received the catalogues of their amphibian collections (Appendix: Table 5). The other museums do not have Swiss amphibians in their collection or the specimens are stored in a matter that does not make them suitable for my study. In total 1414 specimens have complete records in terms of species, sampling year and location (Appendix: Table 5: specimens available). All 20 Swiss species are available (Schmidt & Zumbach 2005). Specimens from 252 different locations over a time span from 1853 until 2009 are available. Needless to say the available museum specimen are somewhat clumped in terms of species, time and location because of differing collecting effort (Pyke & Ehrlich 2010). If a lot of individuals from the same species were sampled at the same location and year, I excluded some specimens and used only about ten adult animals per location and year. This results in a total of 788 specimens examined (Appendix: Table 5: specimens used).

Interesting is one specimen of the African Clawed Frog (*Xenopus laevis*) from a breeding programme of the pharmaceutical company Hoffman La Roche in Basel collected in 1948. This species is known to be healthy carriers of Bd (Berger et al. 1999; Daszak et al. 1999; Wake & Vredenburg 2008) and is suspected to have spread the pathogen via international trade (Daszak et al. 2003; Weldon et al. 2004; Schloegel et al. 2010).

Data collection included taking the skin scrapings, noting the collection location of the specimens, the date of collecting and the altitude and coordinates of the sampling location. To prevent cross-contamination between the specimens, I used a fresh pair of disposable gloves and a new scalpel blade for every single individual. Before taking the skin scrape, the animal was rinsed with demineralised water to remove most of the storage solution and placed in a fresh Petri dish for examination. This was important in particular for jars containing multiple specimens of the same species. In the Natural History Museum of Basel, a lot of animals were stored in this manner, while in the other museums every specimen was stored in an individual container. The skin scrapings were transferred into Eppendorf tubes and stored at -20° degrees until DNA extraction.

DNA Extraction

The DNA from the skin scrapings were extracted by using the method described in Boyle et al. (2004) for ethanol stored specimens (Museums Basel, Neuenburg, Zürich) and the method described in Simoncelli et al. (2005) for specimens with unknown fixation and / or storage history (Museum Bern). The protocols from the manufacturers were followed.

DNA Analysis

The protocol by Boyle et al. (2004) was used for the qPCR, but only duplicates instead of triplicates were run for each sample. A sample was considered positive if both replicates yielded a clear sigmoid amplification curve (Soto-Azat et al. 2010; Kielgast et al. 2009). If only one PCR well from one sample returned a positive result, the qPCR was repeated once, and if the repetition returned no positive result, the sample was considered as negative. As the extractions for the ethanol stored specimens were diluted 1:10, the qPCR results in genomic equivalents were multiplied by the factor 10 (Boyle et al. 2004). Because Simoncelli et al. (2005) use a different extraction method with several purification and cleaning steps, it was not clear in the beginning how the results of these two methods could be compared. Therefore I tested ten infected and ethanol stored specimens of *Alytes obstetricans* from the Zoological Museum of the University of Zurich with both methods to investigate the dilution factor for the method used by Simoncelli (2005). This test resulted in a multiplication factor of 80 (comparable to Vredenburg et al. 2010 and Briggs et al. 2010).

Results

Of the 788 specimens examined (Appendix: Table 5: specimens used), 21 were tested positive, ten specimens yielded equivocal results and were considered as equivocal cases of Bd infections (Table 1, 2, 3) and 757 yielded a negative qPCR result.

Table 1: Tested species (No. = Number of specimens examined; Pos. = Number of positive results; Eq. = Number of equivocal results)

Family	**Species**	**No.**	**Pos.**	**Eq.**
Salamandridae	Alpine Salamander (*Salamandra atra*, Laurenti 1768)	52	0	1
	Fire Salamander (*Salamandra salamandra*, Linnaeus, 1758)	40	0	1
	Great Crested Newt (*Triturus cristatus*, Laurenti 1768)	12	0	0
	Italian Crested Newt (*Triturus carnifex*, Laurenti 1768)	2	0	0
	Alpine Newt (*Mesotriton alpestris*, Laurenti 1768)	179	0	1
	Palmate Newt (*Lissotriton helveticus*, Razoumowsky, 1789)	31	0	0
	Common Newt (*Lissotriton vulgaris*, Linnaeus, 1758)	21	0	0
Alytidae	Midwife Toad (*Alytes obstetricans*, Laurenti, 1768)	32	0	0
Bombinatoridae	Yellow-bellied Toad (*Bombina variegata*, Linnaeus, 1758)	26	2	0
Bufonidae	Common Toad (*Bufo bufo*, Linnaeus, 1758)	117	5	3
	Natterjack (*Bufo calamita*, Laurenti, 1768)	20	0	0
	Green Toad (*Bufo viridis*, Laurenti, 1768)	3	1	0
Hylidae	Common Tree Frog (*Hyla arborea*, Laurenti 1758)	14	2	0
	Italian Tree Frog (*Hyla intermedia*, Boulenger, 1882)	4	1	0
Ranidae	Pool Frog (*Pelophylax lessonae*, Camerano, 1882)	11	1	2
	Common Waterfrog (*Pelophylax esculentus*, Linnaeus, 1758)	56	2	0
	Laughing Frog (*Pelophylax ridibundus*, Pallas, 1771)	11	3	0
	Common Frog (*Rana temporaria*, Linnaeus, 1758)	113	2	2
	Agile Frog (*Rana dalmatina*, Bonaparte, 1840)	27	2	1
	Italian Agile Frog (*Rana latastei*, Boulenger, 1879)	15	0	0

Table 2: Positive tested specimens

GENUS	SPECIES	CANTON	COUNTY	LOCALNAME	X COORDINATES	Y COORDINATES	ELEVATION	YEAR	MUSEUM	MUSEUM NR	AGE	SEX	qPCR	GE
PELOPHYLAX	LESSONAE	Ticino	Lugano	Lugano	717570	95510	260	1901	NMBE	1019420	-	-	pp	7.36
PELOPHYLAX	LESSONAE	Ticino	Lugano	Lugano	717570	95510	260	1901	NMBE	1019423	-	-	pn	0.26
RANA	TEMPORARIA	Ticino	Maggia	-	698435	122554	600	1968	NMBS	7182	AD	-	pn	0.10
SALAMANDRA	ATRA	Berne	Innertkirchen	Gental	665515	177248	1300	1972	NMBE	1016699	AD	-	pn	0.72
SALAMANDRA	SALAMANDRA	Valais	Saint-Léonard	-	599409	122278	500	1980	NMBE	1016766	AD	F	pn	29.62
RANA	DALMATINA	Ticino	Iragna	Iragna	718096	132067	270	1982	NMBE	1019226	AD	M	pp	0.98
BOMBINA	VARIEGATA	Berne	Müntschemier	Grube	578417	205648	430	1983	NMBE	1017446	-	-	pp	1.23
PELOPHYLAX	ESCULENTUS	Berne	Witzwil	Reservat Fanel	570089	203576	430	1984	NMBE	1019401	JUV	-	pp	19.96
BOMBINA	VARIEGATA	Vaud	Portalban	-	562684	196240	430	1987	NMNE	90.268	-	-	pp	0.80
PELOPHYLAX	RIDIBUNDUS	Fribourg	Kleinbösingen	Auried	582930	194131	490	1988	NMBE	1019417	JUV	-	pp	0.02
RANA	TEMPORARIA	Berne	Bäriswil	Bermoos	606567	207256	550	1992	NMBE	1019674	AD	M	pp	0.07
RANA	DALMATINA	Ticino	Chiasso	Sotto Penz	723040	76611	300	1994	NMBE	1021805	AD	M	pn	1.02
BUFO	VIRIDIS	Ticino	Novazzano	Torazza (Industrie)	720540	77711	280	1995	NMBE	1049111	JUV	-	pp	28330.16
RANA	TEMPORARIA	Berne	Treiten	Treitenweiher	579542	206492	440	1995	NMBE	1029072	SUBAD	-	pp	2.08
MESOTRITON	ALPESTRIS	Berne	Gals	Ruefgumme, Müligumme, Lätti	572038	209610	470	1995	NMBE	1029081	AD	M	pn	0.18
RANA	TEMPORARIA	Grisons	Poschiavo	Pozzulasc	802966	140363	1560	1995	NMBE	1037749	AD	M	pn	3.92
BUFO	BUFO	Ticino	Genestrerio	Colombera	718404	78755	340	1996	NMBE	1053643	AD	M	pn	0.04
PELOPHYLAX	RIDIBUNDUS	Berne	Gampelen	Seewald	570139	205242	430	1999	NMBE	1048874	AD	M	pp	0.19
PELOPHYLAX	RIDIBUNDUS	Fribourg	Kleinbösingen	Auried	582930	194131	490	1999	NMBE	1048875	AD	-	pp	0.28
HYLA	INTERMEDIA	Ticino	Novazzano	Novazzano	720540	77711	280	2002	NMBE	1053498	AD	-	pp	0.29
BUFO	BUFO	Berne	Grindelwald	Loichbiel	650124	166278	1700	2004	NMBE	1054136	AD	M	pp	0.03
BUFO	BUFO	Berne	Grindelwald	Grosse Scheidegg, Chalberboden	651018	167664	1920	2004	NMBE	1054140	AD	M	pp	6.41
BUFO	BUFO	Berne	Grindelwald	Grosse Scheidegg, Chalberboden	651018	167664	1920	2004	NMBE	1054141	AD	M	pp	16.65
BUFO	BUFO	Berne	Grindelwald	Buessalp	642457	166645	1740	2004	NMBE	1054144	AD	M	pp	0.06
RANA	DALMATINA	Berne	Kappelen	Katzenstyl	587471	212487	440	2004	NMBE	1054023	AD	M	pp	0.48
BUFO	BUFO	Berne	Lauterbrunnen	Wengernalp	639084	158534	1960	2004	NMBE	1054150	AD	M	pp	0.50
BUFO	BUFO	Berne	Grindelwald	Loichbiel	650124	166278	1700	2004	NMBE	1054034	AD	F	pn	0.05
BUFO	BUFO	Berne	Grindelwald	Grosse Scheidegg, Chalberboden	651018	167664	1920	2004	NMBE	1054139	AD	M	pn	0.01
PELOPHYLAX	ESCULENTUS	Berne	Kappelen	Katzenstyl	587471	212487	440	2005	NMBE	1054760	JUV	-	pp	4585.75
HYLA	ARBOREA	Fribourg	Kleinbösingen	Auried	582930	194131	490	2005	NMBE	1054759	AD	M	pp	0.04
HYLA	ARBOREA	Fribourg	Kleinbösingen	Auried	582930	194131	490	2007	NMBE	1056711	AD	-	pp	2392.44

X / Y COORDINATES = swiss coordinate system (Swiss grid)

ELEVATION: Level above sea (m)

AGE: AD = adult; SUBAD = subadult; JUV = juvenile

MUSEUM: NMBE = Natural History Museum of Bern; NMBS = Natural History Museum of Basel; NMNE = Natural History Museum of Neuchâtel

SEX: M = male; F = female

qPCR: pp = both replicates positive; pn = only one replicate positive

GE = Bd genomic equivalents

Table 3: Distribution of positive specimens over decades examined

Decade	Positive	Equivocal	Tested	% Positive
1850-1859	0	0	10	0
1860-1869	0	0	0	0
1870-1870	0	0	70	0
1880-1889	0	0	51	0
1890-1899	0	0	7	0
1900-1909	1	1	54	1.9
1910-1919	0	0	15	0
1920-1929	0	0	13	0
1930-1939	0	0	13	0
1940-1949	0	0	37	0
1950-1959	0	0	30	0
1960-1969	0	1	78	0
1970-1979	0	1	61	0
1980-1989	5	1	183	2.7
1990-1999	5	4	74	6.8
2000-2009	10	2	92	10.9
Total	21	10	788	2.7

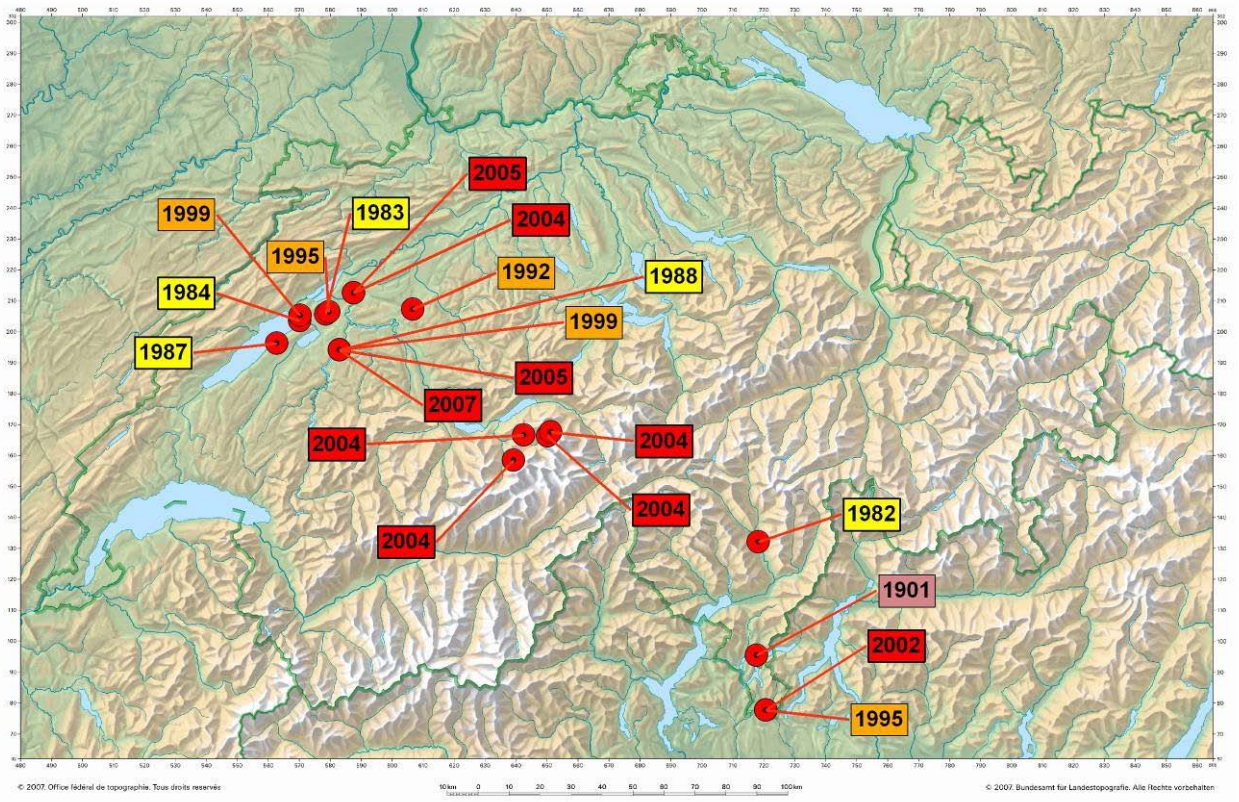

Figure 1: Map showing the locations of the positive tested specimens (red circles) with year of collection (boxes). The colours of the boxes indicate different decades.

I found evidence for Bd at 22 out of 252 sites where museum specimens were collected (Figure 2) ranging from 260 to 1960 meter above sea level (Table 2). From 3 of these locations both positive and equivocal, from 12 only positive and from 7 only equivocal results from specimens examined were found.

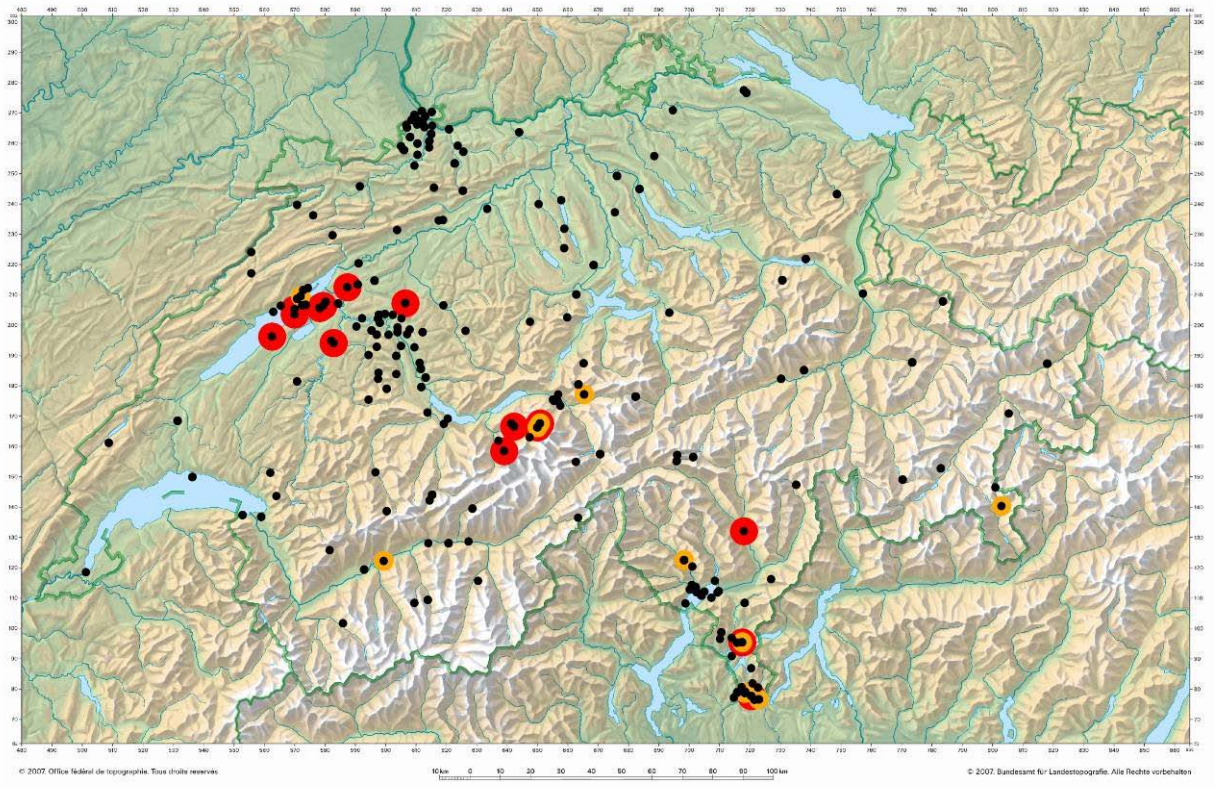

Figure 2: Map showing collection sites of examined museum specimens (black dots) and positive (red dots) or equivocal (orange dots) tested locations respectively.

Discussion

Bd could serve as a model species for understanding emergent and rapidly spreading pathogens (Rosenblum et al. 2009). It is important to investigate the historical distribution of the fungus to evaluate possible threats for species in so far unaffected regions and to find out if chytridiomycosis is a novel or endemic disease. The conservation and research efforts should be different for both cases.

Here I present the earliest evidence of Bd in the wild for Switzerland, Europe and maybe even worldwide. The fungus has been present in Switzerland for at least 28 years and presumably even for more than a century. The pathogen has been in the main biogeographic regions of Switzerland and at all altitudes where amphibians occur for decades (Figure 2). This suggests that Bd was as widespread in the 1980ties as it is nowadays (Garner et al. 2005, U. Tobler & B.R. Schmidt, unpublished data), both in terms of geography and number of host amphibian species. The percentages of positive tested specimens increased in the decades since the 1980ties from 2.7 over 6.8 to 10.9 (Table 3), indicating a growing infection prevalence. But this could be also just due to difficulties in detecting degraded DNA from older samples.

Despite the fact that many of the positive qPCR results yielded only very low levels of Bd genomic equivalents (GE), these results were constant and repeatable as in other studies (Hyatt et al. 2007; Vredenburg et al. 2010; Briggs et al. 2010; Murray et al. 2009). As I minimized the risks of contamination, the low levels could be due to two reasons. First, DNA can get degraded over time and depending on the storage conditions (Miething et al. 2006; Wandeler et al. 2007; Walker et al. 2008; Soto-Azat et al. 2009/2010). Second, if the animals had only very light and aclinical infections, the number of zoospores is low which makes detection difficult (Hyatt et al. 2007; Briggs et al. 2010; Murray et al. 2009).

In my survey I tested 2.5% of 788 specimens positive for Bd. This is comparable with similar studies (Soto-Azat et al. 2010), but the real historical prevalence and distribution of the pathogen is certainly larger due to the difficulties to detect the fungus in museum specimens. It has to be noted that a negative result is not necessarily a proof of an uninfected specimen (McClintock et al. 2010). Bd infection is not always detected and this is certainly true for museum specimens.

Because Bd was detected in populations on both sides of the Alps, of all biogeographic regions and at the deepest and highest elevations and in half of the occuring species, there may be no environmental or taxonomic limits to the distribution of Bd in Switzerland. This supports the prediction of the distribution model of Rödder et al. (2009) which showed that with the exception of the highest mountains most of Switzerland is suitable for Bd.

Several observations of Bd merit a special commentary. A green toad (*Bufo viridis*) collected in 1995 in Novazzano, canton Ticino, in southern Switzerland was tested positive for Bd. This observation is interesting for three reasons. First, the population of green toads went extinct (Grossenbacher 2003). It is not possible to tell, however, whether Bd was causing the local extinction. Second, at the time when the specimen was collected, local herpetologists were worried about a viral disease in syntopic agile frogs (*Rana dalmatina*) that caused clearly visible symptoms (Grossenbacher 1997). The herpetologists did not worry, however, about Bd, another, but invisible pathogen that was present at the same time (and was not even known to science). Third, perhaps the green toad did not get infected in Novazzano. Before it was deposited in the museum, it was kept in captivity for several years (K. Grossenbacher, personal communication). It may have become infected in captivity. Such individual histories of museum specimens may affect inference from museum-based research. However, since an Italian treefrog (*Hyla intermedia*) collected at the same site in 2002 was also tested positive for Bd, it seems likely that the green toad was infected with Bd in its natural habitat.

My study also provides some indications on possible dispersal pathways for the pathogen. One is found in the situation in Auried, Fribourg in western Switzerland. Here, Bd occurred since 1988 in the Laughing Frog (*Pelophylax ridibundus*), which is a non-native, human introduced and invasive species. The first infected native animal, a Common Tree Frog (*Hyla arborea*) was found at the same location not until 17 years later. At some locations, the pathogen may also have been directly human introduced through scientists which reveals the importance to follow recommended hygiene standards, like disinfection of the used equipment (Schmidt et al. 2009) while doing research in the field. The highest reproducing population of the common toad (*Bufo bufo*) in Grindelwald has been studied since 20 years and has been declining (Hemelaar et al. 1988; Grossenbacher 2002). Another reason for the decline than Bd has not yet been found (K. Grossenbacher, personal communication). Our findings suggest that the arrival of Bd may be responsible for the decreased growth rate as it has been documented in other montane toad populations (Pilliod et al. in press). That there have never been found exceptionally high numbers of dead or moribund animals and that no remarkable declines were observed could be an indication that the pathogen may already have become enzootic in this population. Therefore all individuals of this population will be tested on Bd in 2010 to elucidate the role of the pathogen in this decline. The continuation of this valuable long term population study could reveal possible impacts of Bd on survival and mortality, on the establishment of resistance against the disease and long term persistence of the pathogen in a population.

We cannot give a final answer if Bd is an endemic or novel pathogen in Switzerland. There were no positive specimens for 81 years since my earliest finding from 1901 which would not be expected if the pathogen is endemic in Switzerland since then. Although this specimen was retested several times and the qPCR results were constantly positive, I cannot definitively exclude a possible contamination e.g. during the long storage period in the museum (Wandeler et al. 2007). Therefore, the specimen from 1901 will also be examined with histological methods at the Natural History Museum in Berne to confirm the infection status. If sporangia of Bd can be found in the skin of this specimen, there will be no doubt anymore. Anyhow, the earliest evidence of the pathogen in a region does not necessarily reflect the earliest occurrence. It rather reflects the first investigation done and the actual distribution is often underestimated (Garner et al. 2005; Lips et al. 2008).

Conservation action will differ substantially depending on how long it is present in an area and if the pathogen is novel or endemic. If Bd is endemic, it could just be a "normal" pathogen among others, affecting only on the individual level and not on population level (Tobler & Schmidt 2010), and further knowledge is needed about the onset of the disease, the variability of infection susceptibility of different species (Kielgast et al. 2009; Briggs et al. 2010; Kilpatrick et al. 2009; Queloz et al. 2010). If Bd is newly introduced, reasons for its low actual virulence have to be found. The situation in Switzerland seems to be different than in Spain and South America because in Switzerland no die-off's have been observed so far. The climate may not support an endemic progression of the pathogen or the local strain may be less virulent than others but there is the danger that with the climate change an outbreak of the disease can still happen (Fisher et al. 2009) also in Switzerland. For example, the Midwife Toad (*Alytes obstetricians*) has declined dramatically and disappeared from many locations in Switzerland (Schmidt & Zumbach 2005), and it is still not completely clear why in Switzerland this species seems less vulnerable to Bd (Tobler & Schmidt 2010) than the same species in Spain (Bosch et al. 2001).

This study shows how important biological collections of museums are and how broad the research potential in this stored information is (Green & Sherman 2001; Ouellet et al. 2005; Wandeler et al. 2007; Soto-Azat et al. 2009/2010; Pyke & Ehrlich 2010). Museum collections provide long term records of information and this preserved evolutionary history can help to solve environmental questions. Temporal patterns of disease prevalence or historical species distribution are only some examples how such research can induce and guide conservation actions. Potential distribution for alien species before their introduction and possible resulting impacts on indigenous biodiversity could be modeled, enhancing the value of biological

collections. However, museums mainly collect specimens for taxonomic and phylogenetic research. The utility of museum collections for ecological and environmental research could be enhanced if museums adopted a more systematic collection strategy (Pyke & Ehrlich 2010).

The need for further investigation on the role of Bd in amphibian declines is vitally important. Of 6683 amphibian species known to date (June 25, 2010; www.amphibiaweb.org), almost one third are threatened or extinct and 43% show declining populations. Up to 168 species may already have gone extinct (IUCN 2008). Many more species presumably have gone extinct without ever having been discovered and it is speculated that we are entering the sixth mass extinction (Vitousek et al. 1997; Daszak et al. 2000; Wake & Vredenburg 2008). Also most of the 20 Swiss amphibian species are declining, 70% are listed on the red list and some experienced strong declines since the 1980ties (Schmidt & Zumbach 2005). Amphibians reflect the current trends of biodiversity on this planet and are „symptomatic of an accelerating collapse of the global ecosystem" (Halliday 2008). Simon Stuart, senior director of the IUCN/Conservation International Biodiversity Assessment Unit, depicted the current situation of extinction threat to amphibians: “More amphibian species are declining more rapidly, over a wider geographic scale than is the case for any other group of species. With amphibians, the extinction crisis is no longer theoretical. It’s happening right before our eyes.”
(http://www.iucn.org/about/work/programmes/species/news_events/?3268/Amphibian-Global-Action-Team-needed-to-avert-an-extinction-catastrophe).

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References

Annis SL, Dastoor FP, Ziel H, Daszak P, Longcore JE. 2004. A DNA-based assay identifies *Batrachochytrium dendrobatidis* in amphibians. Journal of Wildlife Diseases. 40(3): 420–428.

Berger L, Hyatt AD, Olsen V, Hengstberger SG, Boyle D, Marantelli G, Humphreys K, Longcore JE. 2002. Production of polyclonal antibodies to *Batrachochytrium dendrobatidis* and their use in an immunoperoxidase test for chytridiomycosis in amphibians. Diseases of Aquatic Organisms. 48(3): 213-220.

Berger L, Hyatt AD, Speare R , Longcore JE. 2005a. Life cycle stages of the amphibian chytrid *Batrachochytrium dendrobatidis*. Diseases of Aquatic Organisms. 68(1): 51-63.

Berger L, Speare R, Daszak P, Green D, Cunningham A, Goggin L, Slocombe R, Ragan M, Hyatt A, McDonald K, Hines H, Lips K, Marantelli G, Parkes H. 1998. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. Proceedings of the National Acadamy of Science. 95: 9031-9036.

Berger L, Speare R, Hyatt AD. 1999. Chytrid fungi and amphibian declines: overview, implications and future directions. Ed. A. Campbell. Environment Australia: Canberra. 23-33.

Berger L, Speare R, Skerratt LF. 2005b. Distribution of *Batrachochytrium dendrobatidis* and pathology in the skin of green tree frogs *Litoria caerulea* with severe chytridiomycosis. Diseases of Aquatic Organisms. 68: 65-70.

Blehert DS, Hicks AC, Behr M, Meteyer CU, Berlowski-Zier BM, Buckles EL, Coleman JTH, Darling SR, Gargas A, Niver R, Okoniewski JC, Rudd RJ, Stone WB. 2009. Bat White-Nose Syndrome: An Emerging Fungal Pathogen? Science. 323 (5911): 227.

Bosch J, Martínez-Solano I, García-París M. 2001. Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain. Biological Conservation 97: 331-7.

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

Briggs C, Burgin S. 2004. Congo Red, an effective stain for revealing the chytrid fungus, *Batrachochytrium dendrobatidis*, in epidermal skin scrapings from frogs. Mycologist. 18(3): 98-103.

Briggs C. 2003. A rapid technique to detect chytrid infection in adult frogs. Herpetological Review. 34(2): 124-126.

Briggs CJ, Knapp RA, Vredenburg VT. 2010. Enzootic and epizootic dynamics of the chytrid fungal pathogen of amphibians. Proceedings of the National Academy of Sciences. 107 (21): 9695-9700.

Daszak P, Berger L, Cunningham AA, Hyatt AD, Green DE, Speare R. 1999. Emerging infectious diseases and amphibian population declines. Emerging Infectious Diseases. 5(5): 735-748.

Daszak P, Cunningham AA, Hyatt AD. 2000. Emerging infectious diseases of wildlife - threats to biodiversity and human health. Science. 287: 1756-1756.

Daszak P, Cunningham AA, Hyatt AD. 2003. Infectious disease and amphibian population declines. Diversity and Distributions. 9: 141-150.

Di Rosa I, Simoncelli F, Fagotti A, Pascolini R. 2007. The proximate cause of frog declines? Nature. 447(31): E4-E5.

Fischer MC, Garner TWJ. 2007. The relationship between the emergence of *Batrachochytrium dendrobatidis*, the international trade in amphibians and introduced amphibian species. Fungal Biology Reviews. 2 (1): 2-9.

Fisher MC, Garner TWJ, Walker SF. 2009. Global emergence of *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis in space, time, and host. Annual Review of Microbiology. 63: 291-310.

Garner TWJ, Walker S, Bosch J, Hyatt AD, Cunningham AA, Fisher MC. 2005. Chytrid fungus in europe. Emerging Infectious Diseases. 11(10): 1639-1641.

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

Gascon C, Collins JP, Moore RD, Church DR, McKay JE, Mendelson JR III (eds). 2007. Amphibian conservation action plan. IUCN/SSC Amphibian Specialist Group. Gland, Switzerland and Cambridge, UK. 64pp.

Green DE, Sherman CK. 2001. Diagnostic histological findings in Yosemite toads (*Bufo canorus*) from a die-off in the 1970s. Journal of Herpetology. 35(1):92-103.

Grossenbacher K. 1997. Eine Springfroschkrankheit auf der Alpensüdseite. In: Krone A, Kühnel KD, Berger H. (Hrsg.): Der Springfrosch (*Rana dalmatina*) - Ökologie und Bestandssituation. Rana Sonderheft. 2: 203-205.

Grossenbacher K. 2002. First results of a 20-year-study on Common toad *Bufo bufo* in the Swiss Alps. Biota. 3: 43-48.

Grossenbacher K. 2003. Die Wechselkröte (*Bufo viridis*) in der Schweiz: Historische und aktuelle Situation. Podloucky R. & Manzke U. (Hrsg.): Verbreitung, Ökologie und Schutz der Wechselkröte (*Bufo viridis*). Mertensiella. 14: 147-152.

Halliday TR. 2008. Why amphibians are important. International Zoo Yearbook. 42: 7-14.

Hemelaar A. 1988. Age, growth and other population characteristics of *Bufo bufo* from different latitudes and altitudes. Journal of Herpetology. 22 (4): 369-388.

Hyatt AD, Boyle DG, Olsen V, Berger L, Obendorf D, Dalton A, Kriger K, Hero M, Hines H, Phillott R, Campbell R, Marantelli G, Gleason F, Colling A. 2007. Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. Diseases of Aquatic Organisms. 73(3): 175-192.

IUCN, Conservation International, and NatureServe. 2008. An analysis of amphibians on the 2008 IUCN Red List. www.iucnredlist.org/amphibians. Downloaded on July, 22 2009.

Jackson DP, Lewis FA, Taylor GR, Boylston AW, Quirke P. 199. Tissue extraction of DNA and RNA and analysis by the polymerase chain reaction. Journal of Clinical Pathology. 43: 499-503.

James TY, Litvintseva AP, Vilgalys R, Morgan JAT, Taylor JW, Fisher MC, Berger L, Weldon C, du Preez L, Longcore JE. 2009. Rapid global expansion of the fungal disease chytridiomycosis into declining and healthy amphibian populations. PLoS Pathogens. 5(5): e1000458. doi:10.1371/journal.ppat.1000458.

Kallio P, Tervahauta A, Syrjänen K. 1991. A simple method for isolation of DNA from formalin-fixed paraffin-embedded samples for PCR. Journal of Virological Methods. 35: 39-47.

Kielgast J, Rödder D, Veith M, Lötters S. 2010. Widespread occurrence of the chytrid fungus in Kenya. Animal Conservation. 13 (Suppl. 1): 1-8.

Kilpatrick AM, Briggs CJ, Daszak P. 2009. The ecology and impact of chytridiomycosis: an emerging disease of amphibians. Trends in Ecology and Evolution. 25: 109-118.

Kriger KM, Hines HB, Hyatt AD, Boyle DG, Hero JM. 2006. Techniques for detecting chytridiomycosis in wild frogs: comparing histology with real-time Taqman PCR. Diseases of Aquatic Organisms. 71: 141-148.

Lips KR, Diffendorfer J, Mendelson JR, Sears MW. 2008. Riding the wave: Reconciling the roles of disease and climate change in amphibian declines. PLoS Biology. 6(3): e72. doi:10.1371/journal.pbio.0060072.

Lips KR. 1999.Mass mortality of the anuran fauna at an upland site in Panama. Conservation Biology. 13: 117-125.

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

Lüps P, Güntert M, Althaus R, Blöchlinger B, Grossenbacher K, Nussbaumer NA, Schmid P. 2005. Vertebrate collections at the Natural History Museum Bern (NMBE) – a synopsis. Jahrbuch Naturhistorisches Museum Bern. 14: 3–97.

McClintock BT, Nichols JD, Bailey LL, MacKenzie DI, Kendall WL, Franklin AB. 2010. Seeking a second opinion: uncertainty in disease ecology. Ecology Letters. 13 (6): 659-674.

Miething F, Hering S, Hanschke B, Dressler J. 2006. Effect of fixation to the degradation of nuclear and mitochondrial DNA in different tissues. Journal of Histochemistry & Cytochemistry. 54(3): 371-374.

Mohneke M, Onadeko AB, Rödel MO. 2009. Exploitation of frogs – a review with a focus on West Africa. Salamandra. 45: 193-202.

Murray KA, Skerratt LF, Speare R, McCallum H. 2009. Impact and dynamics of disease in species threatened by the amphibian chytrid fungus, *Batrachochytrium dendrobatidis*. Conservation Biology. 23:1242-52.

Mutschmann F, Berger L, Zwart P, Gaedicke C. 2000. Chytridiomycosis in amphibians- -first report in Europe. Berliner und Münchener Tierärztliche Wochenschrift. 113(10): 380-383.

Olsen V, Hyatt AD, Boyle DG, Mendez D. 2004. Co-localisation of *Batrachochytrium dendrobatidis* and keratin for enhanced diagnosis of chytridiomycosis in frogs. Diseases of Aquatic Organisms. 61: 85-88.

Ouellet M, Mikaelian I, Pauli BD, Rodrigue J, Green DM. 2005. Historical evidence of widespread chytrid infection in North American amphibian populations. Conservation Biology. 19(5): 1431-1440.

Pessier AP, Nichols DK, Longcore JE, Fuller MS. 1999. Cutaneous chytridiomycosis in poison dart frogs (*Dendrobates spp*.) and White's tree frogs (*Litoria caerulea*). Journal of Veterinary Diagnostic Investigation. 11: 194-199.

Pilliod DS, Muths E, Scherer RD, Bartelt PE, Corn PS, Hossack BR, Lambert BA, McCaffery R, Gaughan C. 2010. Effects of amphibian chytrid fungus on individual survival probability in wild boreal toads. Conservation Biology. In press.

Puechmaille SJ, Verdeyroux P, Fuller H, Ar Gouilh M, Bekaert M, Teeling EC. 2010. White-nose syndrome fungus (*Geomyces destructans*) in bat, France. Emerging Infectious Diseases. doi: 10.3201/eid1602.091391

Pyke GH; Ehrlich PR. 2010. Biological collections and ecological/environmental research: a review, some observations and a look to the future. Biological Reviews. 85: 247–266.

Queloz V, Grünig CR, Berndt R, Kowalski T, Sieber TN, Holdenrieder O. 2010. Cryptic speciation in *Hymenoscyphus albidus*. Forest Pathology. doi: 10.1111/j.1439-0329.2010.00645.x

Rachowicz LJ, Hero JM, Alford RA, Taylor JW, Morgan JAT, Vredenburg VT, Collins JP, Briggs CJ. 2005. The novel and endemic pathogen hypotheses: Competing explanations for the origin of emerging infectious diseases of wildlife. Conservation Biology. 19(5): 1441-1448.

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

Rosenblum EB, Fisher MC, James TY, Stajich JE, Longcore JE, Gentry LR, Poorten TJ. Preprint 2009. A molecular perspective: biology of the emerging pathogen *Batrachochytrium dendrobatidis*. Diseases of Aquatic Organisms. doi: 10.3354/dao02179

Rosenblum EB, Voyles J, Poorten TJ, Stajich JE. 2010. The deadly chytrid fungus: A story of an emerging pathogen. PLoS Pathogens 6(1): e1000550. doi:10.1371/journal.ppat.1000550

Schloegel LM, Daszak P, Cunningham AA, Speare R, Hill B. 2010. Two amphibian diseases, chytridiomycosis and ranaviral disease, are now globally notifiable to the World Organization for Animal Health (OIE): an assessment. Diseases of Aquatic Organisms. doi: 10.3354/dao02140

Schmidt BR, Zumbach S. 2005. Rote Liste der gefährdeten Amphibien der Schweiz. Hrsg. Bundesamt für Umwelt, Wald und Landschaft, Bern, und Koordinationsstelle für Amphibien- und Reptilienschutz in der Schweiz, Bern. BUWAL-Reihe: Vollzug Umwelt. 48 S.

Schmidt BR, Furrer S. Kwet A, Lötters S, Rödder D, Sztatecsny M, Tobler U, Zumbach S. 2009. Desinfektion als Maßnahme gegen die Verbreitung der Chytridiomykose bei Amphibien. Zeitschrift für Feldherpetologie, Supplement. 15: 229–241.

Sepp R, Szabó I, Uda H, Sakamoto H. 1994. Rapid techniques for DNA extraction from routinely processed archival tissue for use in PCR. Journal of Clinical Pathology. 47: 318-323.

Shedlock AM, Haygood MG, Pietsch TW, Bentzen P. 1997. Enhanced DNA extraction and PCR amplification of mitochondrial genes from formalin-fixed museum specimens. BioTechniques. 22: 394-400.

Simon N, Chanson JS, Cox NA, Young BE, Rodrigues ASL, Fischman DL, Waller RW. 2004. Status and trends of amphibian declines and extinctions worldwide. Science. 306: 1783-1785.

Simoncelli F, Fagotti A, Dall'Olio R, Vagnetti D, Pascolini R, Di Rosa I. 2005. Evidence of *Batrachochytrium dendrobatidis* infection in Water Frogs of the *Rana esculenta* complex in Central Italy. EcoHealth. 2: 307-312.

Skerratt LF, Berger L, Speare R, Cashins S, McDonald KR, Phillott AD, Hines HB, Kenyon N. 2007. Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. Ecohealth. 4(2): 125-134.

Smith, KG. 2007. Use of quantitative PCR assay for amphibian chytrid detection: comment on Kriger et al. Diseases of Aquatic Organisms. 73: 253-255.

Smith KG, Lips KR, Chase JM. 2009. Selecting for extinction: nonrandom disease-associated extinction homogenizes amphibian biotas. Ecology Letters. 12: 1069-1078.

Soto-Azat C, Clarke BT, Fisher MC, Walker SF, Cunningham AA. 2009. Non-invasive sampling methods for the detection of *Batrachochytrium dendrobatidis* in archived amphibians. Diseases of Aquatic Organisms. 84: 163-166.

Soto-Azat C, Clarke BT, Poynton JC, Cunningham AA. 2010. Widespread historical presence of *Batrachochytrium dendrobatidis* in African pipid frogs. Diversity and Distributions 16: 126-131.

Stuart S, Chanson JS, Cox NA, Young BE, Rodrigues ASL, Fishman DL, Waller RW. 2004. Status and trends of amphibian declines and extinctions worldwide. Science. 306: 1783-1786.

Tobler U, Schmidt BR. 2010. Within- and Among-Population Variation in chytridiomycosis-Induced Mortality in the Toad *Alytes obstetricians*. PLoS ONE 5(6): e10927. doi:10.1371/journal.pone.0010927

Van Ells T, Stanton J, Strieby A, Daszak P, Hyatt AD, Brown C. 2003. Use of immunohistochemistry to diagnose chytridiomycosis in dyeing poison dart frogs (*Dendrobates tinctorius*). Journal of Wildlife Diseases. 39(3): 742-745.

Vitousek PM, Mooney HA, Lubchenco J, Melillo JM. 1997. Human domination of earth's ecosystems. Science. 277: 494-499.

Vredenburg VT, Summers AP. 2001. Field identification of chytridiomycosis in *Rana muscosa* (Camp 1915). Herpetological Review. 32: 151-152.

Vredenburg VT, Knapp RA, Tunstall TS, Briggs CJ. 2010. Dynamics of an emerging disease drive large-scale amphibian population extinctions. Proceedings of the National Academy of Sciences. 107 (21): 9689-9694.

Wake DB, Vredenburg VT. 2008. Are we in the midst of the sixth mass extinction? A view from the world of amphibians. Proceedings of the National Academy of Sciences. 105 (Suppl):11466-11473.

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

Walker SF, Bosch J, Gomez V, Garner TW, Cunningham AA, Schmeller DS, Ninyerola M, Henk DA, Ginestet C, Arthur CP, Fisher MC. 2010. Factors driving pathogenicity vs. prevalence of amphibian panzootic chytridiomycosis in Iberia. Ecology Letters. 13 (3): 372-82.

Wandeler P, Hoeck PAE, Keller LF. 2007. Back to the future: museum specimens in population genetics. Trends in Ecology and Evolution. 122: 634-642.

Weldon C, du Preez LH, Hyatt AD, Muller R, Speare R. 2004. Origin of the amphibian chytrid fungus. Emerging Infectious Diseases. 10(12): 2100-2105.

Yang HJ, Baek HJ, Speare R, Webb R, Park SK, Kim TH, Lasater KC, Shin SP, Son SH, Park JH, Min MS, Kim YJ, Na K, Lee H, Park SC. 2009. First detection of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in free-ranging populations of amphibians on mainland Asia: survey in South Korea. Diseases of Aquatic Organisms. 86 (1): 9-13.

Appendix

Table 4: Percentage positive results from the pilot study (n=qPCR wells)

	Ethanol stored		**Formalin stored**	
Sampling Method	Skin scrape	Toe clip	Skin scrape	Toe clip
Simoncelli	100% (n=10)	100% (n=8)	20% (n=10)	20% (n=10)
Boyle	100% (n=4)	100% (n=4)	0% (n=4)	0% (n=4)
Walker	(not tested)	(not tested)	0% (n=12)	0% (n=10)

Table 5: Museums and numbers of specimens in the collections

Museum	**Preservative**	**No. of specimens used (available)**
Basel (NMBS)	Alcohol	390 (514)
Bern (NMBE)	Alcohol (formerly Formalin)	384 (861)
Neuenburg (NMNE)	Alcohol	8 (26)
Zürich (NMZH)	Alcohol/Formalin	6 (13)