Final Report

Survey and Risk Assessment of the Salamander-killing Chytrid Fungus in Illinois

IDNR project # T-103-R-1, grant #F15AF01064

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Summary

The purpose of grant # F15AF01064 titled "Survey and Risk Assessment of the Salamander-killing Chytrid Fungus in Illinois" was to determine whether the virulent amphibian pathogens *Batrachochytrium salamandrivorans* (Bsal) and *B. dendrobatidis* (Bd) are present in Illinois salamanders, and to quantify its virulence for salamander native species in Greatest Need of Conservation in Illinois. We surveyed 14 communities of Illinois salamanders and assayed skin swabs of 791 salamanders for presence of both pathogens through a duplex quantitative PCR protocol. We did not detect Bsal in any salamander or site in Illinois. We found Bd in nine of 14 screened salamanders. Patterns of Bd prevalence varied among species, seasonally, and geographically. Two species, *Ambystoma tigrinum* and *Notophthalmus viridescens*, had high infection prevalences of 43% and 38% respectively. Prevalence of Bd was highest during spring and lowest during summer. The widespread occurrence of Bd in Illinoisan salamanders is similar to patterns of Bd infection in Illinoisan frogs (Talley 2014), although at most sites the prevalence of Bd appeared to be lower for salamanders than for frogs. Intensities of infections of Bd-positive salamanders were below the critical threshold commonly associated with lethal chytridiomycosis or mass die-offs. We were unable to complete susceptibility experiments planned for four species of *Ambystoma* of Greatest Need of Conservation in Illinois. However, recently published and ongoing susceptibility experiments suggest that most species of *Ambystoma* are not or weakly susceptible to developing chytridiomycosis. Recent federal regulation under the Lacey Act prohibit import of 201 salamander species known to be carriers of Bsal. However, inter-state transport of these salamanders continue to be permitted, and the import of known frog hosts (and potential carriers) of Bsal in Europe is currently not regulated. We recommend periodic screening of captive and wild salamanders for Bsal to prevent the introduction and spread of the highly virulent Bsal pathogen.

Purpose

The purpose of grant # F15AF01064 titled "Survey and Risk Assessment of the Salamander-killing Chytrid Fungus in Illinois" was to determine whether the highly virulent salamander pathogen *Batrachochytrium salamandrivorans* (Bsal) is present in Illinois, and to quantify its virulence for salamander native species in Greatest Need of Conservation in Illinois. While the project was successful at surveying nearly 800 salamanders of 14 species at the originally proposed study sites and at additional sites, the proposed susceptibility experiments were not completed, and funds associated with these experiments were not spent. The inability to complete this part of the project is largely due to the exceptionally difficult budgetary circumstances experienced during the period from 2016 to 2017.

The state budget impasse and subsequent budgetary constraints effectively put the project on hold from May 2016 to August 2017, because SIUC did not allow any expenses for this type of grant requiring reimbursement from a state agency. The project had access to funds from the period of January-April 2016, and again after September 2017. The salary of the field researcher, master student Josh Parrott, was covered by the Department of Zoology under a Teaching Assistantship contract from May 2016 to August 2017, but the scope of the activities were scaled down due to the lack of access to funds to cover travel and other research expenses. Once SIUC again allowed spending to resume in August 2017, Mr. Parrott restarted working full time for this project. However, the PI, Dr. Alessandro Catenazzi left SIUC at the end of December 2017, and the contract was assigned to a new PI. Dr. Catenazzi traveled to Carbondale (on his own funds) in February and April 2018 to meet with Mr. Parrott and discuss progress on this grant. During the second meeting, Mr. Parrott communicated his decision to resign his position at the end of April. Considering the short time remaining to complete the project, the time of season,

and the fact that Mr. Parrott had been rearing larval salamanders in preparation for the susceptibility experiments and had secured all collecting permits under his name, we were unable to replace Mr. Parrott to complete the susceptibility experiments component of the contract.

Therefore, the present report emphasizes results of our disease surveys, which in addition to Bsal screened salamanders for the amphibian chytrid disease *Batrachochytrium dendrobatidis* (Bd). We also put our results in the context of previous surveys of Bd in frog communities in Illinois (data collected in 2008 and 2009; Talley 2014).

Specific objectives

The specific objectives, as stated in the original proposal, were to:

1. Quantify prevalence and intensity of infection by *B. salamandrivorans* (Bsal) in native populations of salamanders in Illinois.
2. Quantify prevalence and intensity of infection by Bsal in captive populations of salamanders in Illinois.
3. Determine the geographic distribution of Bsal in Illinois/Identify potential routes of pathogen introduction and generate recommendations to avoid introduction and spread of disease.
4. Assess the susceptibility to infection (i.e., reduction in survivorship) for salamander species of Greatest Need of Conservation.

Introduction

Amphibians are the most threatened vertebrates, with ~18% of the 7,530 described species listed as endangered or critically endangered (IUCN 2014). A major cause of global amphibian declines is chytridiomycosis, a disease caused by the fungal pathogens *Batrachochytrium dendrobatidis* (Bd) (Daszak et al. 2003; Longcore et al. 1999; Wake and Vredenburg 2008) and *Batrachochytrium salamandrivorans* (Bsal) (Martel et al. 2014). Chytridiomycosis caused by Bd is implicated in the extinction, or serious population declines, of more than 200 amphibian species worldwide (Crawford et al. 2010; Skerratt et al. 2007). Bd has been detected at 48% of localities sampled from around the world, representing every continent where amphibians live (James et al. 2015). Fungal-borne diseases affecting both wild and domestic organisms are emerging at rates not seen with bacterial or viral-borne disease (Fisher et al. 2012). In addition, generalist fungal pathogens, such as Bd and Bsal, can persist in host communities over the long-term due to varying levels of host susceptibility. These also have the ability to reproduce very quickly during epizootics, potentially driving some host species to extinction (Fisher et al. 2012).

The recently discovered "salamander-eating fungus", Bsal is emerging as a highly virulent pathogen of salamanders (Martel et al. 2014) and has recently spread to, and devastated, wild populations of *Salamandra salamandra* in the Netherlands and Belgium (Martel et al. 2013; Spitzen-van der Sluijs et al. 2013). The pathogen has been progressing through the Netherlands and neighboring Belgium and Germany. Researchers report Bsal at more than a dozen separate areas in these countries where it infects at least three species of salamanders (Spitzen-van der Sluijs et al. 2016). In Europe, three-fourths of the species of salamander belong to Salamandridae

(AmphibiaWeb 2016); many of which have been shown to be susceptible to Bsal infection (Martel et al. 2014). Many of these will disperse seasonally, reproduce aquatically, and are associated with seeps, pools, and riparian areas. Aquatic zoospore dispersal, as seen in Bd, could allow Bsal to easily spread in water bodies and stream networks, and infect many species of salamanders. These factors make the Bsal threat a very serious conservation concern in Europe and beyond. Switzerland has banned importation of all salamanders to prevent introduction of Bsal (Schmidt 2016).

Bsal has not been reported in the US, but it represents a substantial threat to native salamander species. Therefore there is great risk of pathogen spillover from the live amphibian trade, as well as from pet owners who release infected newts outside of their native ranges. North America, and particularly Mexico and Apalachia and surrounding regions in the southeastern US, hosts a rich and diverse salamander fauna. Salamanders are unique among vertebrates in displaying richer family and species diversity at temperate rather than tropical latitudes; much of this unique biogeographic pattern is explained by high species richness along the Apalachian mountain range. Should *Bsal* spread in North America, it could cause the collapse of native communities of salamanders (Yap et al. 2015). The Chinese fire belly newt (*Cynops orientalis*) were among the most commonly imported salamander in the US (Kolby et al. 2014). Fire belly newts are an ancient and immunocompetent host for Bsal (Martel et al. 2014) and likely can carry Bsal. Given the serious threat of Bsal introduction into North America, importation of 201 salamander species was restricted by the U.S. federal government in 2016 (http://www.fws.gov/policy/library/2016/2016-00452.pdf). The reasoning was that imported salamanders which are hosts for Bsal represent sources of harm to endemic U.S. salamanders under the Lacey Act (1990; 16U.S.C. §§ 3371–3378 and 18 U.S.C. §§ 42–43). The

rule was originally intended to ban both international importation and interstate transport of listed salamanders. However, in United States Association of Reptile Keepers, Inc. v. Zinke, No. 15-5199 (D.C. Cir. UPDATE: Implementation of the D.C. Circuit Court Decision in April 7, 2017), the D.C. Circuit reached a definitive judgment on the shipment clause's meaning included in the Lacey Act. The shipment clause does not prohibit interstate transport within the continental United States, and thus the current rule concerning salamanders prohibits import of injurious salamanders (the 201 species known to be carriers of Bsal) into the United States, as well as transport of injurious salamanders between the listed jurisdictions in the shipment clause (the continental United States, the District of Columbia, Hawaii, the Commonwealth of Puerto Rico, and any possession of the United States).

In Illinois, all 19 species of salamanders (including the eight salamander species in Greatest Need of Conservation; Appendix 1) in IL could be affected by the introduction of Bsal into the state. Disease outbreaks could be especially devastating for populations of species with restricted geographic distribution, such as the Silvery and Jefferson salamanders, and the Spotted Dusky salamander. More generally, the spread of this virulent disease in North America, the region with the richest diversity of salamanders, would have dramatic consequences on global amphibian biodiversity and is a pressing conservation priority (Martel et al. 2014). There is an urgent need to screen live amphibians for infection with diseases known to cause population declines and extirpations (Yap et al. 2015). We have the capacity to develop cost-effective surveillance programs that can quickly detect infected individuals.

Survey and study of Bsal are conservation priorities (Grant et al. 2015; Sleeman 2013). Informed entities must facilitate a rapid response to Bsal outbreaks should they occur (Gray et al. 2015). We missed this opportunity with the earliest Bd epizootics, and only recently have

phylogenetic studies been able to pinpoint to the likely source of the pathogen in the Korean peninsula (O'Hanlon et al. 2018). Bsal is similarly thought to have originated from Asia, possibly from China, but we currently have little evidence to support this hypothesis other than the general observation that Bsal seems to be widespread in China (Yuan et al. 2018), where it appears to show little pathogenicity towards native salamanders. This observation suggests a shared evolutionary history between Bsal and Chinese salamanders.

Given that Bsal may be easily missed without sufficient breadth of taxonomic and geographic coverage, surveys over broad geographic scales and large sample sizes are preferable. Bd is known to occur widely in frogs in Illinois (Talley 2014), where it has been present since at least 1888. The Bsal strain responsible for the epizootic in Europe shows a thermal preference lower than Bd under laboratory conditions, with optimal growth occurring between 10 and 15°C; the pathogen is sensitive to temperatures above 20°C (Martel et al. 2013) and infection is cleared after prolonged host exposure to 25°C in laboratory trials (Blooi et al. 2015). Because of known affinity for low temperature, we can hypothesize that Bsal thrives in temperate climates, and that Illinois presents suitable conditions for its growth.

Methods

Field surveys

We conducted field surveys at 30 sites and 59 wetlands distributed in northern, central and southern Illinois (**Figure 1**). We generally used surveys techniques aimed at maximizing sample size, for example by sampling at times and seasons of high salamander activity (during wet nights within breeding season, etc.). Most surveys consisted of visual searches by walking around ponds, ditches, flipping cover objects such as logs, rocks, etc., and by searching along

roadways. We also used funnel traps overnight at several sites in southern Illinois (Oakwood Bottoms, Cache, Wildcat), and pitfall traps during migration events at the Pomona site. Finally, mudpuppies sampled at and near Wolf Lake were trapped under water as part of a project supported by the Shedd Aquarium (swabs provided by Alicia Beattie; Beattie 2016). Much of our data was collected over a short timeframe: a single breeding event, season, or quarter. These short-term data complement our efforts by providing an increase in taxonomic breadth and geographic coverage.

The areas surveyed for salamander pathogens include: a wetland complex at Pomona, several wetlands at the Cache River National Park, Cypress Pond, Bowman's Bottoms, Oakwood Bottoms NF, Lincoln Trail SP, Kickapoo Creek Rec Area, Pine Dunes FP, Wolf Lake, and several wetlands in McLean and Woodford Counties (Figure 2). At some locations (Cache/Wildcat Bluffs, Oakwoods, and Cypress Pond) several communities of salamanders were monitored monthly to represent the changing seasons and to help elucidate Bd-host seasonal dynamics (Table 4).

Site locations were chosen based upon wetland accessibility and the identity/availability of salamander inhabitants. Larger sites were delineated into wetlands that are at least 1.5 km from each other; this strategy allows us to assume with good probability that we are working with separate communities of salamanders associated with those wetlands.

Historic surveys

We screened 37 museum specimens of four species previously preserved in the herpetological collection at Southern Illinois University Carbondale, prior to their transfer to the current storage location at the Illinois Natural History Survey. We used the same swabbing

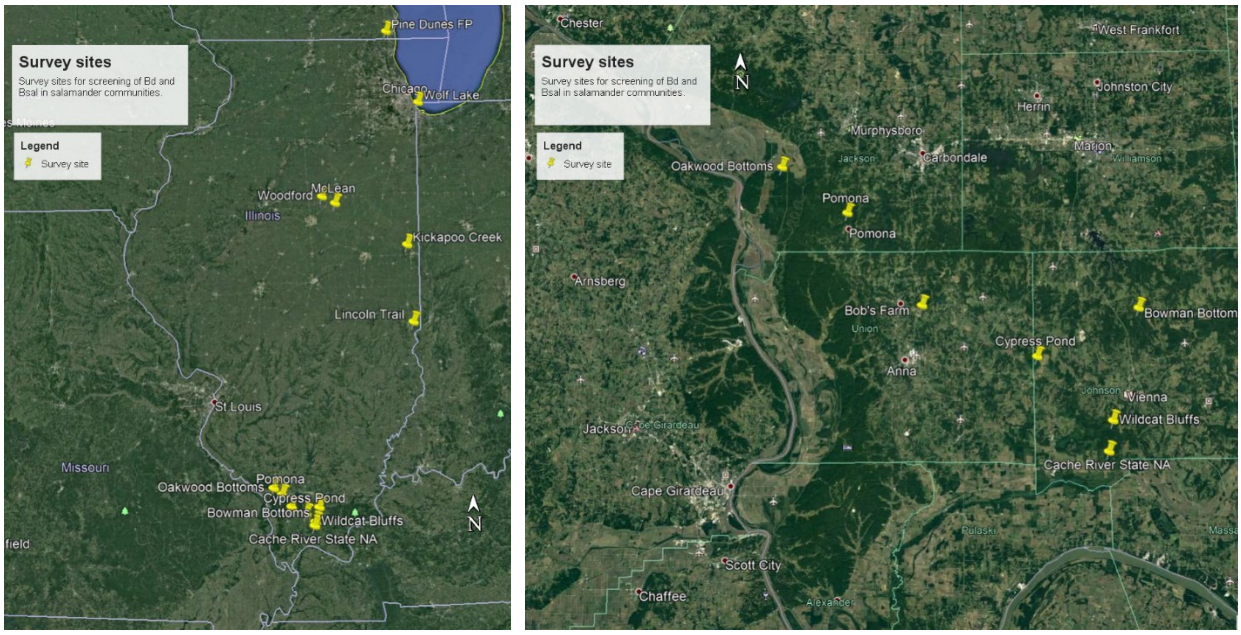


Figure 1. Survey sites for screening of live salamanders in (*left*) Illinois, and (*right*) southern Illinois only.

techniques as described for live salamanders. We obtained collecting information (locality name, date) from the labels attached to specimens and/or jars containing each specimen. We then used Google Maps and Google Earth to georeference localities to most likely coordinates. Unless precisely described on labels (i.e., 2 miles along x road south of y town), most coordinates likely have a margin of error of up to ~2 km.

Skin swabbing

We captured salamanders using powder-free gloves and placed them in individual plastic bags. We examined salamanders visually for signs of chytridiomycosis, such as skin ulcerations, abnormal shedding, poor body condition, and lethargy (Martel et al. 2013; Sabino-Pinto et al. 2015). We then swabbed each salamander once with a dry swab (Medical Wire and Equipment Co. Ltd.). We stroked swabs across the skin on the ventral side of abdomen, legs, and tail a

total of 30 times. We also weighed, measured (snout-vent and tail length), and noted location of capture for each salamander. We dry-stored swabs individually in sterile 1.5 ml centrifuge vials.

Molecular analyses

We extracted DNA from swabs by using Prepman Ultra® (Life Technologies), and analyzed extracts with a probe-based (Taqman) real-time PCR (qPCR) assay on a StepOnePlus™ Real-Time PCR System (Life Technologies) to quantify the amount of genomic material (Boyle et al. 2004). The assay uses genetic markers specific for Bd and Bsal (primers and probe specific to sequence of ITS gene; **Table 1**); and compares each sample to a set of standards (four serial dilutions at concentrations from 100 to 0.1 zoospore genomic equivalents, each in triplicate) to calculate a genomic equivalent. Each plate also includes four negative controls. We converted genomic equivalence values to account for dilution during DNA extraction. For example, to calculate Bd infection intensity we multiplied the qPCR score by 80 to account for subsampling and dilution that occurred during the DNA extraction, resulting in a zoospore equivalent (Z_e) estimate for each frog (Briggs et al. 2010; Catenazzi et al. 2011). This duplex protocol uses a sequence of synthetic, double-stranded DNA that allows simultaneous quantification of infection by Bd and Bsal in a single PCR reaction (**Appendix 1**). We used the synthetic double-stranded DNA (gBlocks synthetic DNA from IDT, Skokie, IL) as standard during real time PCR. The following gene sequences are used, for Bsal: ITS-1 & 5.8S (Blooi et al. 2013, Genbank KC762295); and for Bd: ITS-1 & 5.8S (Boyle et al. 2004, Genbank AY598034).

Table 1. Primers and Taqman probe of ITS gene used for duplex qPCR assay for simultaneous detection of *Batrachochytrium dendrobatidis* (Bd) and *B. salamandrivorans* (Bsal) used in this study. Adapted from Boyle et al. (2004), Blooi et al (2015).

Type	Sequence
Batrachochytrium dendrobatidis	
Forward primer (ITS)	CCTTGATATAATACAGTGTGCCATATGTC
Reverse primer (5.8)	AGCCAAGAGATCCGTTGTCAAA
MGB probe (FAM dye)	CGAGTCGAACAAAAT
Batrachochytrium salamandrivorans	
Forward primer (Ster-F)	TGCTCCATCTCCCCCTCT TCA
Reverse primer (Ster-R)	TGAACGCACATTGCACTCTAC
MGB probe (VIC dye)	ACAAGAAAATACTATTGATTCTCAAACAGG CA

Statistical analyses and mapping

We define infection status as positive when Ze > 0. We calculated the Bd and Bsal prevalence of infection for all locations by dividing the number of infected salamanders by the total number surveyed. Confidence intervals were calculated using Bayesian inference and Jeffrey's non-informative priors in the R package binom. For preliminary analyses of prevalence of Bd (all Bsal assays were negative) we used the statistical program R. Specifically, we used the lme4 package for mixed effects generalized linear models for binomial distribution (glmer command), allowing us to nest factors within Survey sites. We examined the effects of latitude, season and number of co-occurring species. Latitude is a rough proxy for climate in Illinois, because of the strong gradient in temperature between northern and southern Illinois. The metabolism and growth of salamanders and their fungal pathogens are temperature-dependent, and thus we hypothesize that temperature could be an important factor driving Bd distribution in

Illinois. Similarly, temperatures vary across seasons. Furthermore, a seasonal effect could also reflect the consequences of behavioral, physiological and ecological changes of salamanders, such as aggregations during reproduction which favor pathogen transmission. Lastly, the number of co-occurring species is relevant to potential amplifying (reservoir species) or dilution effects playing a role in disease dynamics in salamander communities. We used the number of species we captured during our surveys. For analyses of infection intensities we used generalized linear models on log-transformed Z_e data.

Data access

All information regarding Bd infection data collected during field surveys, and historic data from museum specimens, is available (open access) at http://www.amphibiandisease.org (ark: #will be updated with DOI upon submission#).

Results

Is Bsal present in native communities of salamanders in Illinois?

We did not find Bsal in wild salamanders in Illinois. All salamanders we swabbed from 2014 to 2018, and which we assayed with our duplex protocol tested negative for Bsal (n = 791, 14 species; **Figure 2**; **Table 2**). We screened 14 species of salamanders, including four species in Greatest Need of Conservation (*Ambystoma jeffersonianum*, *A. platineum*, *A. talpoideum*, and *Necturus maculosus*).

Table 2. Salamanders screened for infection by *Batrachochytrium salamandrivorans* (Bsal) in Illinois from 2014 to 2018 (n = 791). In bold species of Greatest Need of Conservation.

Species	# sites	# points	Sample size	Bsal +
Ambystoma jeffersonianum	1	5	68	0
Ambystoma laterale	1	1	29	0
Ambystoma maculatum	4	8	110	0
Ambystoma opacum	2	5	46	0
Ambystoma platineum	1	3	33	0
Ambystoma talpoideum	4	8	114	0
Ambystoma texanum	5	8	114	0
Ambystoma tigrinum	2	3	7	0
Eurycea longicauda	2	3	14	0
Eurycea lucifuga	1	1	2	0
Necturus maculosus	1	2	103	0
Notophthalmus viridescens	4	8	120	0
Plethodon glutinosus	1	3	30	0
Siren intermedia	1	1	1	0
Total	**30**	**59**	**791**	**0**

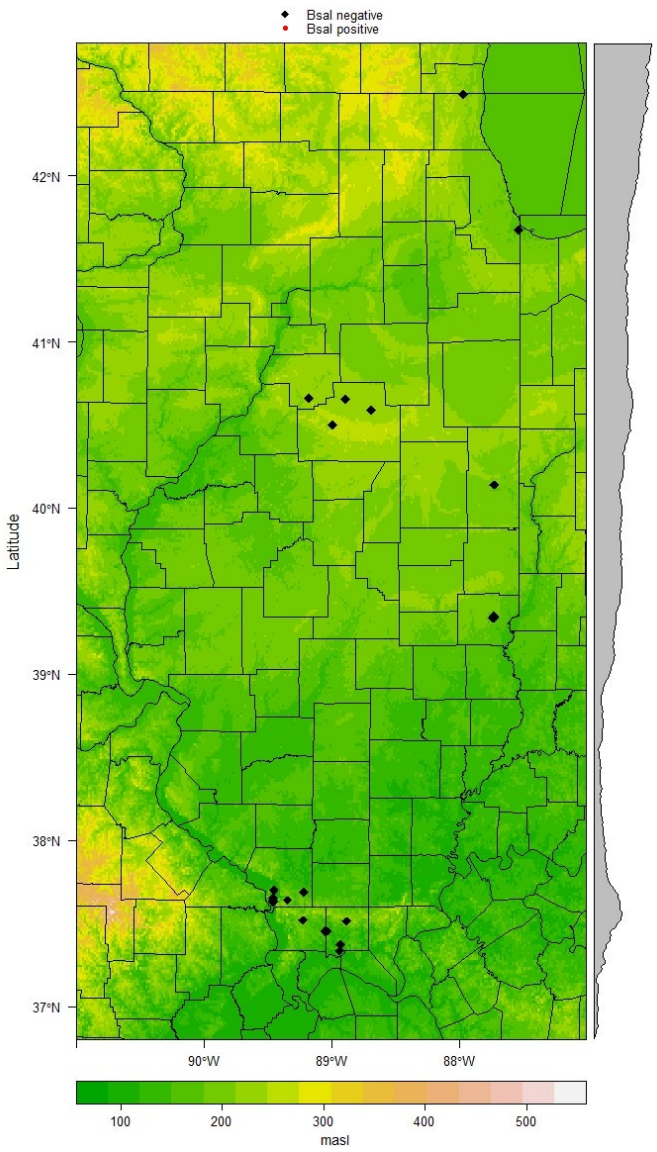


Figure 2. Distribution of *Batrachochytrium salamandrivorans* (Bsal) in live salamanders in Illinois from 2014 to 2018 (n = 791 salamanders). None of the sampled salamanders tested positive for Bsal.

Was Bsal present in Illinois salamanders prior to 2014?

We screened 37 museum specimens of four species previously preserved in the herpetological collection at Southern Illinois University Carbondale (now housed at the Illinois Natural History Survey). All historical samples ranging from 1905–2013 tested negative for Bsal (n= 37; **Figure 3**, **Table 3**).

Table 3. Salamanders screened for infection by *Batrachochytrium salamandrivorans* (Bsal) in Illinois collected from 1905 to 2003 (n = 37 museum specimens). In bold species of Greatest Need of Conservation.

Species	# sites	# points	Sample size	Bsal +
Ambystoma jeffersonianum	1	1	2	0
Ambystoma laterale	3	3	7	0
Ambystoma talpoideum	9	9	18	0
Ambystoma texanum	7	7	10	0
Total	**19**	**19**	**37**	**0**

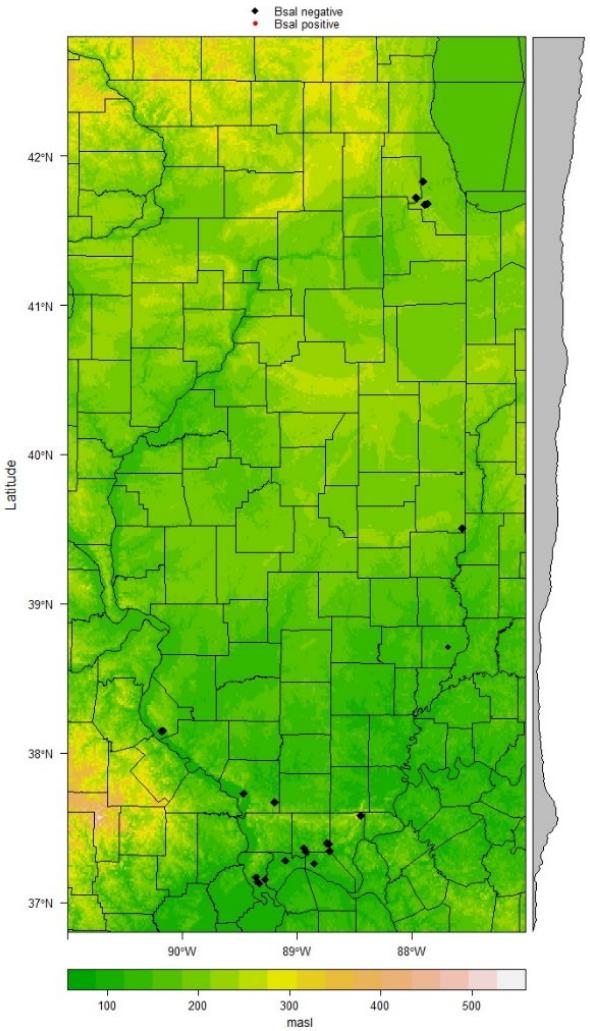


Figure 3. Distribution of *Batrachochytrium salamandrivorans* (Bsal) in preserved salamanders in Illinois from 1905 to 2003 (n = 37 salamanders). None of the sampled salamanders tested positive for Bsal.

Is Bsal present in captive populations of salamanders in Illinois?

We sampled 10 *Hynobius retardatus* and two *Ichthyosaura alpestris* from local pet owners in southern Illinois. All sampled salamanders were negative for both Bd and Bsal. The original plan of sampling a larger number of pet owners, pet stores and herpetological fairs was not carried out for several reasons. First, the budgetary constraints limited travel funding; we prioritized sampling of live salamanders over captive salamanders. Second, personal communications with a team sampling a large number of captive salamanders across the US suggested no Bsal positive captive salamander was detected during the period of the contract. The study, reporting no Bsal positives in 639 U.S. captive salamanders, was recently published (Klocke et al. 2017). Third, regulation proposed by the U.S. Fish and Wildlife Service in January 2016 (but since then no longer prohibiting interstate transport of salamanders) had a strong impact on the salamander pet trade

Specifically, the last issue temporarily reduced the number of available captive salamanders. Importation and interstate transport of 201 salamander species which are hosts for Bsal and injurious to U.S. salamanders was restricted by the U.S. federal government under the Lacey Act (1990; 16U.S.C. §§ 3371–3378 and 18 U.S.C. §§ 42–43; http://www.fws.gov/policy/library/2016/2016-00452.pdf). The ruling effectively reduced the number of imports by 98.4% (Klocke et al. 2017). The Bsal positives detected in the amphibian trade have been associated with shipments from Asia (Martel et al. 2014) and private collections in Germany (Sabino-Pinto et al. 2015) and the U.K (Cunningham et al. 2015).

What is the geographic distribution of Bsal in Illinois?

The pathogen has not been detected in wild or captive specimens within the state of Illinois. There currently are no known records of Bsal within the U.S. On the basis of known occurrences of Bsal in western Europe (Stegen et al. 2017), and in light of Bd's wide distribution, it is likely Bsal could spread throughout the state of Illinois following an eventual introduction. Preventing the introduction of Bsal into the U.S. and the state of Illinois is a conservation priority. It is possible infected captive amphibians, or their inoculated fomite could contaminate wild populations of salamanders, however, this risk may have been mitigated with the decrease in salamander imports into North America. Enforcement of existing import regulations (for salamander species only) and periodic screening of salamanders in potential routes of pathogen introduction are recommended to avoid the introduction and spread of disease. Unfortunately, wild populations of anurans harbor the pathogen in Europe (Stegen et al. 2017) and the import of these amphibians is not regulated under the Lacey Act. It is possible that additional species of anurans, including species traded for frog legs, could also harbor Bsal. The threat of anthropomorphically-mediated introduction of the pathogen seems the most likely threat to date as surveys have yet to find it in North American amphibian communities.

It is possible we have missed Bsal due to mistiming of our surveys, missing infected communities/populations/individuals, or not surveying an area long enough to detect sick individuals before they die. Bsal could also be in the early stages of establishment and could easily be missed if it only exists in small patches or if it kills the infected salamanders before they are screened for the pathogen. To confirm the absence of Bsal, we recommend period screening of screening of salamander populations throughout Illinois. Because Bsal seems to

have poor dispersal capabilities in Europe (Spitzen-van der Sluijs et al. 2018), this strategy could stop the potential spread if introduced to Illinois amphibians.

What is the susceptibility to Bsal infection for native Ambystoma salamander species of Greatest Need of Conservation in Illinois?

We did not complete this component of the project due to the consequences of budgetary constraints effectively freezing the project during the period from May 2016 to August 2017 (see comments at beginning of report). The original plan was to compare the survivorship of experimentally infected salamanders with control (non-infected) salamanders in laboratory infection trials for the four species of *Ambystoma* salamander of Greatest Need of Conservation (*A. jeffersonianum*, *A. laterale*, *A. platineum*, *A. talpoideum*).

Prior to his resignation, Mr. Parrott collected eggs of at least one species (*A. talpoideum*) and hatched Bsal-naïve larvae to be used for susceptibility experiments. Development of these larvae in captivity proceeded at a much slower rate than expected and this unexpected pattern initially delayed the start of experimental infection. The proposed method was to infect freshly metamorphosized young salamanders, because this is the most critical life stage to infection. Rearing larvae in laboratory settings ensured that they developed without being challenged by Bsal or by Bd. Furthermore, egg collection as opposed to larval collection was easier to justify when applying for collecting permits (survivorship increases as larvae age). Collection of eggs of additional species was hindered by the project freeze from May 2016 to August 2017. By the time the funding was resumed in August 2017, and funds again became available for travel outside of southern Illinois for sampling of *A. jeffersonianum*, *A. laterale*, and *A. platineum*, the timing was not ideal for egg/larval collection. Because these species are of Greatest Need of

Conservation, permits only allowed collection of small number of eggs, and thus we could not collect more developed larvae as a replacement (assumed to be Bsal-naïve since Bsal appears to be absent in Illinois).

Previous studies (Martel et al. 2013) and an ongoing project funded by NSF (Matt Gray and collaborators) however suggest that species of *Ambystoma* salamanders are not or weakly susceptible to infection by Bsal, meaning they might become infected but rarely exhibit symptoms of chytridiomycosis and/or die because of this fungal infection. Therefore, it is possible that Bsal will not threaten the four species of *Ambystoma* in greatest need of conservation in Illinois. Salamander species in other families, and especially in the Salamandridae family, are much more susceptible to chytridiomycosis caused by Bsal.

What is the distribution of Bd in salamander communities in Illinois?

In addition to Bsal, we screened 14 species of salamanders, including four species in Greatest Need of Conservation (*Ambystoma jeffersonianum*, *A. platineum*, *A. talpoideum*, and *Necturus maculosus*) for its relative fungal pathogen Bd. Bd is known to infect a large number of amphibian species in all orders (cecilians, anurans and salamanders). Bd is known to occur widely in Illinois (Talley 2014), where is has been present since 1888 at least (Talley et al. 2013).

The overall prevalence of Bd (proportion of infected animals) across all sampled salamanders was 13% (95% CI range = 11–16%; **Table 4, Figure 4**). We detected Bd in nine of 14 sampled species of salamanders (**Table 4**). Prevalence of Bd varied from 3 to 38%, and was highest for *N. viridescens* and species of *Ambystoma* (**Figure 5**), with associated confidence interval ranges smaller than 25% across eight species. Sample size for the ninth species,

Ambystoma tigrinum, was too small to provide robust estimates of prevalence (estimated prevalence is 43% with 95% CI range of 13–75%).

Table 4. Salamanders screened for infection by *Batrachochytrium dendrobatidis* (Bsal) in Illinois from 2014 to 2018 (n = 791). Z_e = number of zoospore equivalents (genomic equivalents). In bold species of Greatest Need of Conservation.

Species	# sites	# points	Sample size	Bd +	Prevalence	95% CI	Ze	95% CI	range
A. jeffersonianum	1	5	68	8	0.12	(0.05-0.20)	100.7	52.1	(0.3-358
A. laterale	1	1	29	1	0.03	(0.00-0.13)	7.0	-	
A. maculatum	4	8	110	15	0.14	(0.08-0.20)	261.4	140.3	(0.1-1975
A. opacum	2	5	46	7	0.15	(0.06-0.26)	1532.2	1362.2	(3.4-9676
A. platineum	1	3	33	0	0.00	(0-0.06)	-	-	
A. talpoideum	4	8	114	7	0.06	(0.02-0.11)	53.0	24.4	6.1-165
A. texanum	5	8	114	18	0.16	(0.10-0.23)	543.9	384.0	(0.9-6873
A. tigrinum	2	3	7	3	0.43	(0.13-0.75)	1164.8	916.8	(17 2977
Eurycea longicauda	2	3	14	1	0.07	(0.00-0.25)	15.1	-	
Eurycea lucifuga	1	1	2	0	0.00	(0-0.57)	-	-	
Necturus maculosus	1	2	103	0	0.00	(0-0.02)	-	-	
No. viridescens	4	8	120	46	0.38	(0.30-0.47)	822.1	210.1	(16 5836
P. glutinosus	1	3	30	0	0.00	(0-0.06)	-	-	
Siren intermedia	1	1	1	0	0.00	(0-0.77)	-	-	
Total	**30**	**59**	**791**	**106**	**0.13**	**(0.11-0.16)**	**631.6**	**146.7**	**(0.1-9676**

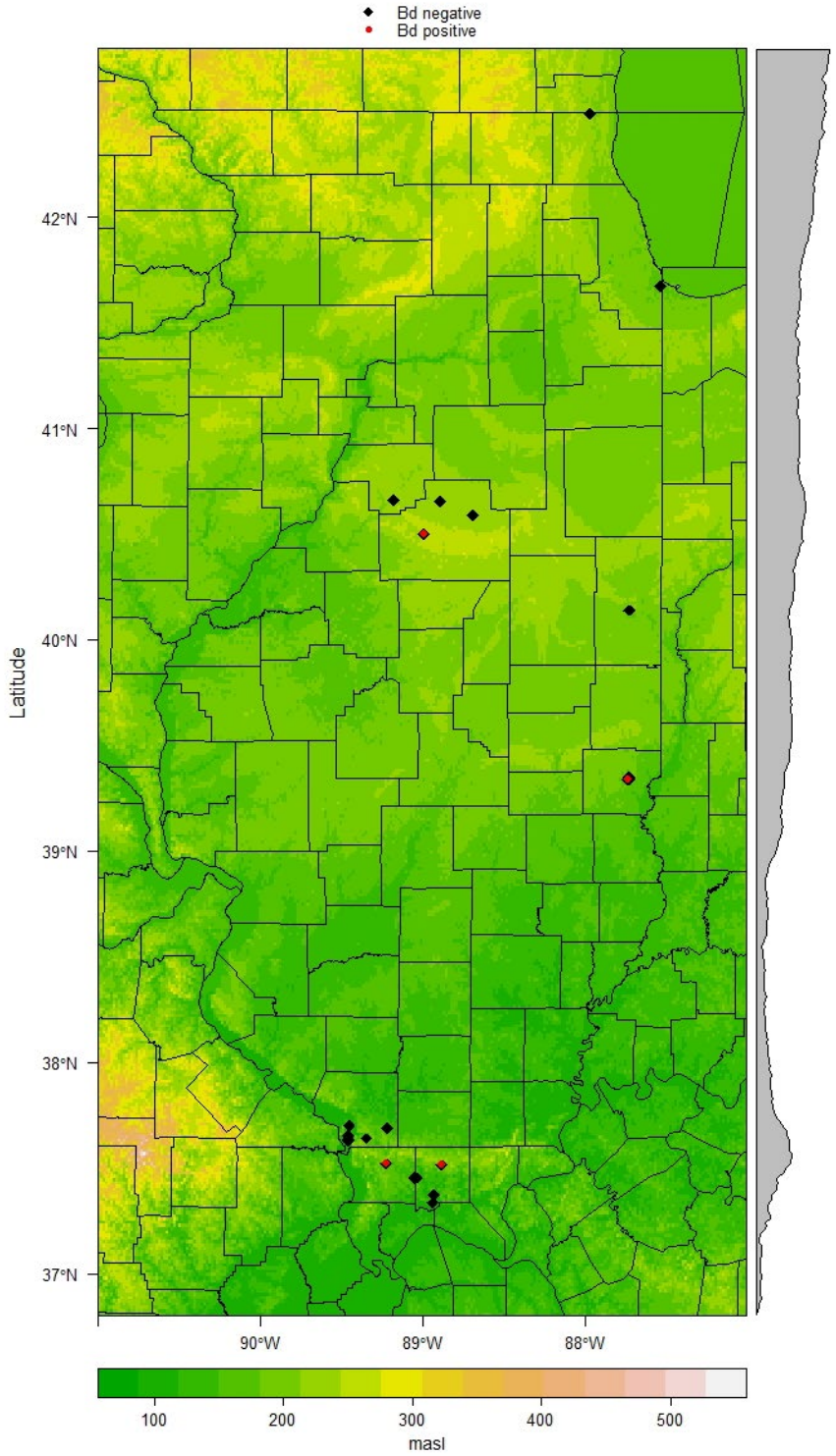


Figure 4. Distribution of *Batrachochytrium dendrobatidis* (Bd) in live salamanders in Illinois from 2014 to 2018 (n = 791 salamanders, 14 sites). None of the sampled salamanders tested positive for Bsal.

Figure 5. Prevalence of Bd across salamander taxa * *S. intermedia*, *A. tigrinum* and *E. lucifuga* are omitted due to low sample size. Numbers refer to sample size.

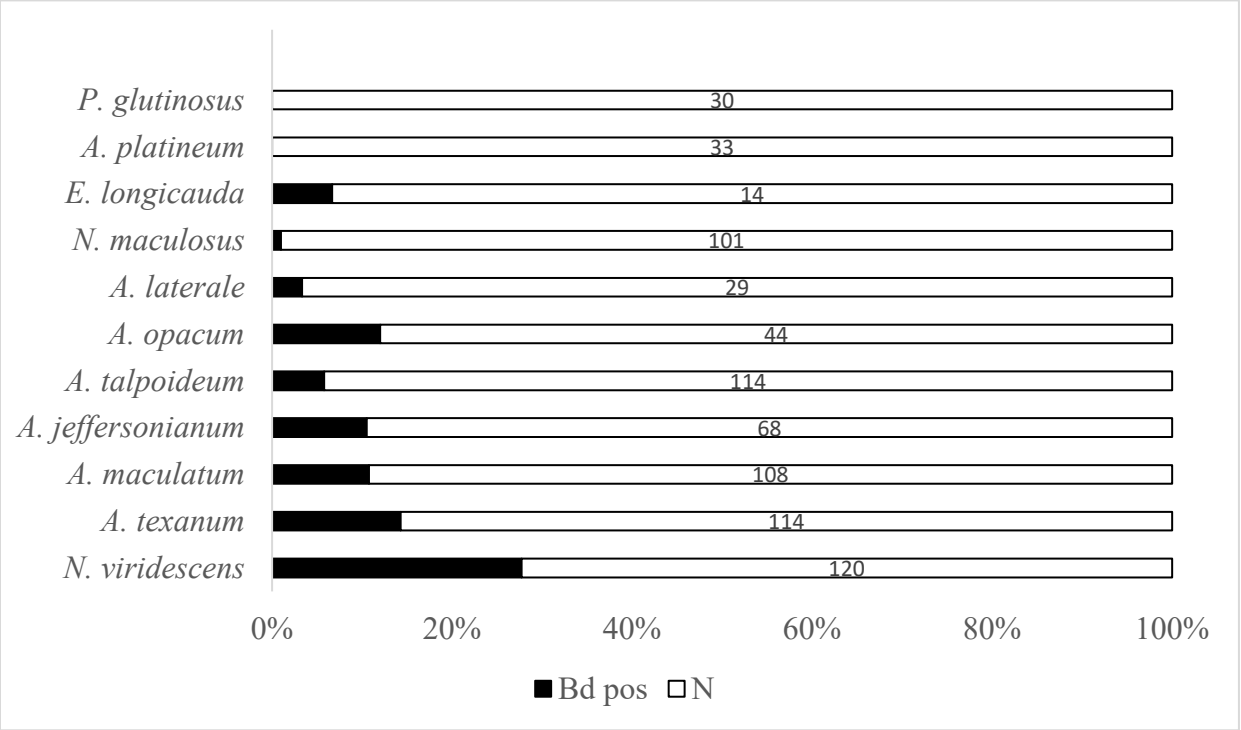


Table 5. Survey sites sampled for presence of *Batrachochytrium dendrobatidis* (Bsal) in Illinois from 2014 to 2018 (n = 790). Z_e = number of zoospore equivalents (genomic equivalents).

Site	# species	Sample size	Bd +	Prevalence	95% CI	Ze	95% CI	range
McLean	1	28	6	0.21429	(0.08-0.35)	24.7	12.66799	(1.1-65.9)
Pomona	4	75	10	0.13333	(0.06-0.22)	316.8	143.2373	(4.2-1263.7)
Woodford	2	5	2	0.4	(0.08-0.77)	10.8	6.93212	(3.9-17.7)
Oakwood Bottoms	5	110	19	0.17273	(0.11-0.25)	641.0	305.3708	(0.1-5473.6)
Wildcat Bluffs	5	45	0	0.00	(0-0.42)	-	-	-
Cache River State NA	8	178	40	0.22472	(0.17-0.29)	648.8	204.7958	(8.4-5836.8)
Lincoln Trail	1	68	8	0.11765	(0.05-0.20)	100.7	52.08788	(0.3-358.3)
Bowman Bottoms	5	34	6	0.17647	(0.07-0.31)	1781.6	1584.54	(3.4-9676.4)
Cypress Pond	3	75	10	0.13333	(0.06-0.22)	828.9	682.8559	(0.9-6873.5)
Bob's Farm ponds	2	7	4	0.57143	(0.25-0.87)	1422.0	677.8906	(94.7-2977.2)
Kickapoo Creek	1	33	0	0.00	(0-0.06)	-	-	-
Pine Dunes FP	1	29	1	0.03448	(0.00-0.13)	7.0	-	-
Wolf Lake	1	103	0	0.00	(0-0.02)	-		-
Total	**39**	**790**	**106**	**0.13**	**(0.11-0.16)**	**631.6**	**146.7**	**(0.08-9676-4)**

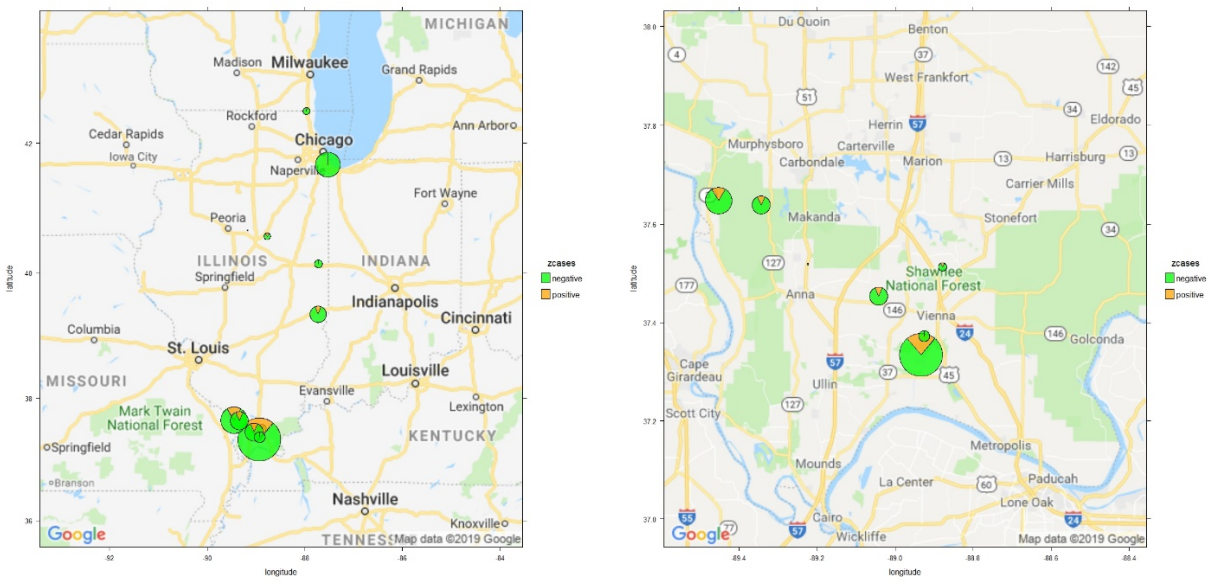


Figure 6. (*left*) Prevalence of Bd infection in salamander communities at sampled sites from 2016 to 2018. Size of pie chart is proportional to sample size. (*right*) Detail for southern Illinois.

We detected Bd on salamanders at all sampled site except Wolf Lake (where mudpuppies were trapped underwater), Wildcat Bluffs, and Kickapoo Creek (**Table 5, Figure 6**). Prevalence at sites with infected individuals ranged from 3 to 22%. At Bob's Farm ponds, small sample size prevented us to calculate a robust estimate of prevalence (estimate 57% with CI range from 17 to 57%). A preliminary analysis of the effect of latitude, a proxy for climatic conditions across Illinois, did not detect any latitudinal effect on prevalence of Bd at our survey sites. A mixed effects models (binomial distribution) incorporating season and number of co-occurring salamander species at each site found an important seasonal effect, particularly for spring when prevalence of Bd is highest and summer when prevalence is lowest (**Table 6,** see **Table 7** for data from Southern Illinois where sampling was repeated across several seasons). The seasonal effect is pronounces for newts (*N. viridescens*, **Figure 7**) and species of *Ambystoma* (**Figure 8**).

Infection intensities (Z_e) are generally below the 10,000 zoospore equivalents threshold usually associated with the onset of lethal chytridiomycosis (**Figure 9**). Only one *A. opacum*

from Bowman Bottoms came close to 10,000 zoospore equivalents. Four other salamanders (three *N. viridescens* and one *A. texanum*) from Oakwood Bottoms, Cache River and Cypress Pond also had relatively high $Z_e > 4{,}000$. These findings suggests that Bd might be more problematic for salamanders living in swamp habitats. Our preliminary statistical analyses did not find any significant effects of latitude, season, site and number of co-occurring species on intensity of infection (model results not presented).

Figure 7. Prevalence of Bd infection in eastern newts (*N. viridescens*) across seasons. Data from survey sites Oakwood Bottoms and Cache River State Natural Area (these sites included three surveyed ponds each).

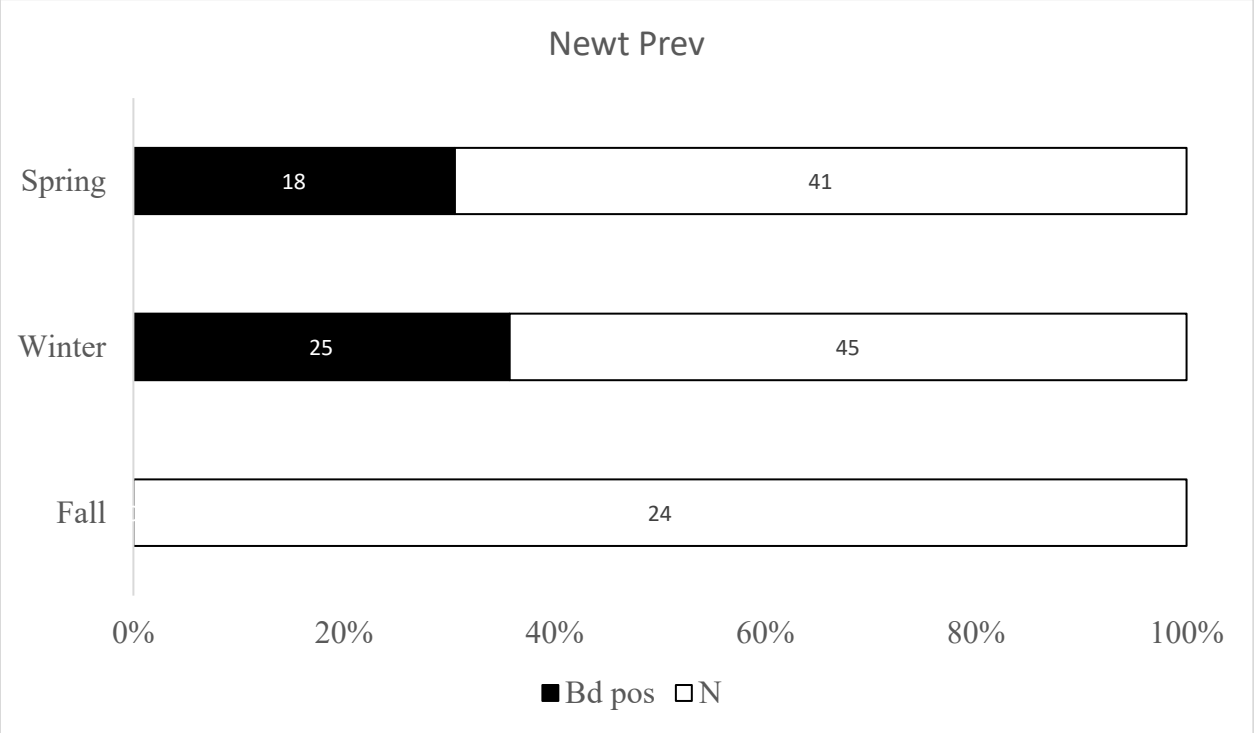


Figure 8. Prevalence of Bd infection in three ambystomatid species (*A. talpoideum, A. maculatum* and *A. texanum*) across seasons. Data from survey sites Oakwood Bottoms, Cypress Pond and Cache River State Natural Area (these sites included three surveyed ponds each).

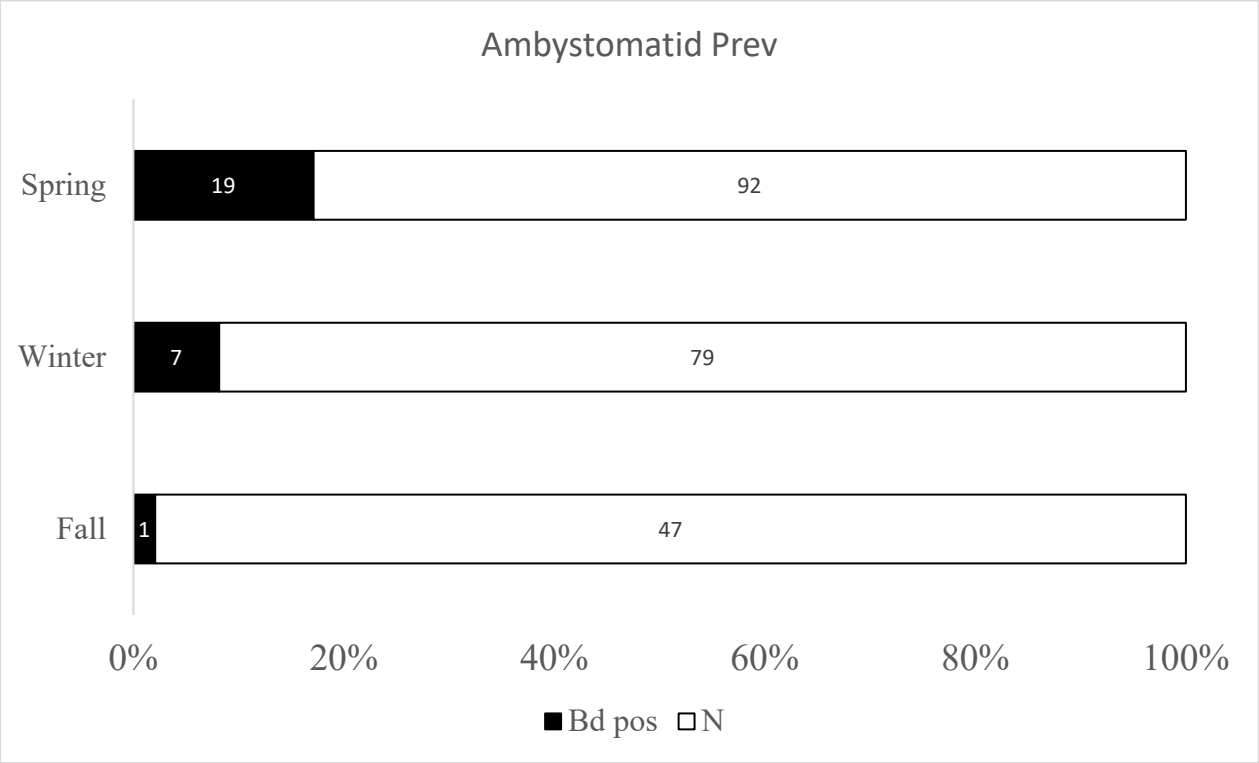


Table 6. Mixed effects model evaluating the effects of season and number of species per site on prevalence of infection with Bd.

	Estimate	Std. Error	z value	P
(Intercept)	-5.90	1.11	-5.30	**<0.001**
Season[T.spring]	3.77	1.05	3.58	**<0.001**
Season[T.summer]	4.08	1.13	3.60	**<0.001**
Season[T.winter]	3.49	1.05	3.33	**<0.001**
Number of species	0.12	0.13	0.98	0.328

Table 7. Prevalence of Bd infection in salamanders (southern Illinois data only, south of latitude 38°) across seasons.

Season	Sample size	Bd +	Prevalence	95% CI	Ze	95% CI	range

spring	168	36	0.21	(0.15-0.28)	224.3	181.2	(0.9-9676.4)
summer	22	2	0.09	(0.01-0.24)	0.8	2.3	(3.4-15.1)
fall	95	1	0.01	(0.00-0.04)	0.1	-	(9.6-9.6)
winter	240	50	0.21	(0.16-0.26)	117.7	102.1	(0.1-5836.8)
Grand Total	**525**	**89**	**0.17**	**(0.14-0.20)**	**125.6**	**0.0**	**(0.1-9676.4)**

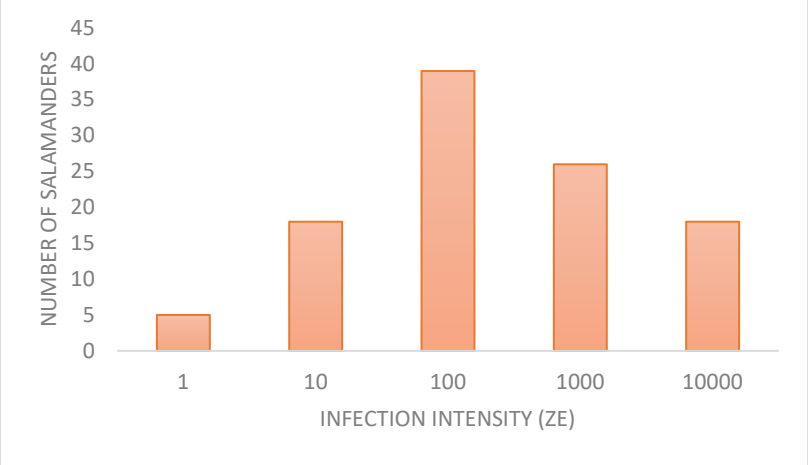


Figure 9. Frequency distribution of infection intensities (Ze) in live salamanders in Illinois (n = 791) sampled from 2014 to 2018.

Was Bd present in Illinois salamanders prior to 2014?

Possibly. We screened 37 museum specimens of four species previously preserved in the herpetological collection at Southern Illinois University Carbondale (now housed at the Illinois Natural History Survey). All but one salamanders tested negative for Bd (n= 37; **Figure 10**). The only Bd-positive sample is an *A. texanum,* collected in 2003 near Embarras river (Lawrence Co.), with Z_e = 85.5 zoospore equivalents. Values of Z_e below 100 are generally not associated with cases of lethal chytridiomycosis, but quantification of Bd infection in preserved specimens is problematic because DNA could have degraded over time.

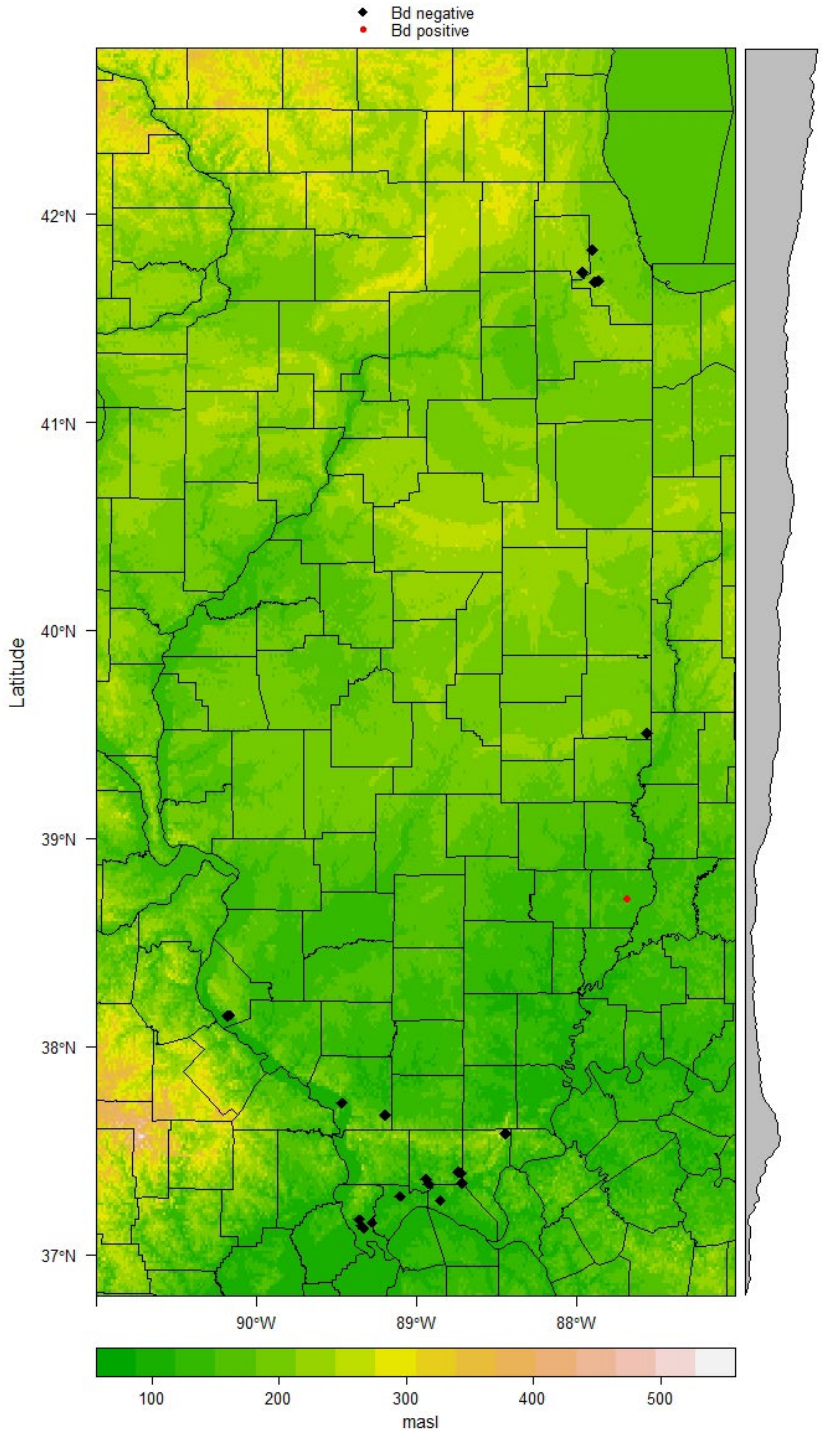
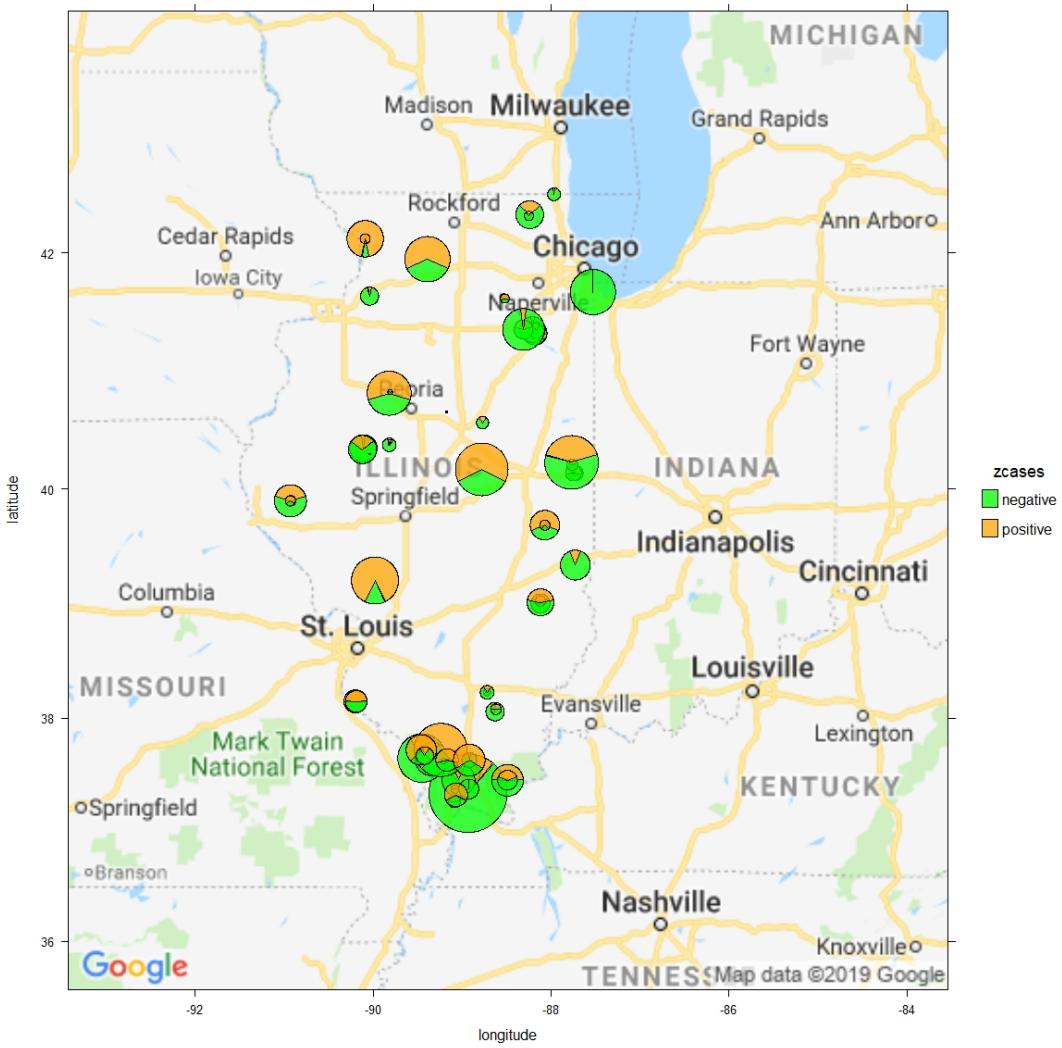


Figure 10. Distribution of *Batrachochytrium dendrobatidis* (Bd) in preserved salamanders in Illinois from 1905 to 2003 (n = 37 salamanders, 19 sites). None of the sampled salamanders tested positive for Bsal.

What is the overall distribution of Bd in Illinois frogs and salamanders?

Our data on Bd infection of Illinois salamanders complement data collected by Brook Talley and Karen Lips in 2008 and 2009 (Talley 2014). In support of their findings, Bd continue to be widespread in Illinois amphibians (**Figure 11**). Although the prevalence of Bd is high in many populations (>50%, **Figure 11**), the intensities of infection are generally below the critical threshold associated with high mortality and mass die-offs.

Figure 11. Prevalence of infection by *Batrachochytrium dendrobatidis* (Bd) in live frogs from 2008 to 2009 (n = 2686, 76 sites, Talley 2014) and live salamanders from 2014 to 2018 (n = 791 salamanders, 14 sites).



Products of this project

Scientific publications

Parrott, J., A. Shepack, D. Burkart, B. LaBumbard, P. Scimè, E. Baruch, A. Catenazzi. 2017. Survey of pathogenic chytrid fungi in salamanders from three mountain ranges in Europe and the Americas. *EcoHealth* DOI: 10.1007/s10393-016-1188-7

White, C. L., A. P. Pessier, M. J. Forzán, M. C. Allender, J. R. Ballard, A. Catenazzi, H. Fenton, A. Martel, F. Pasmans, D. L. Miller, R. J. Ossiboff, K. L. D. Richgels, J. L. Kerby. Amphibian:

Batrachochytrium salamandrivorans chytridiomycosis case definition. *Herpetological Review* 47: 207-209.

Scientific datasets (open access)

Data from Bd and Bsal surveys: http://www.amphibiandisease.org (ark: #will be updated#)

Training of students

Seven SIUC undergraduate students (Chris Smaga, Dan Sears, Nathan Hooven, Megan Colburn, Shannon McQueen, Isaac Harris) and four SIUC graduate students (Josh Parrott, Andrew Rubio, Alex Shepack, Kenny Anderson) were trained in lab safety, standard lab routines and techniques, and qPCR assays during this study.

Public presentations

- Alessandro Catenazzi, Invited seminar at Chicago Herpetological Society Meeting (August 2015)
- Alex Shepack, oral contribution at Amphibian Diseases meeting at Arizona State University, November 2016
- Josh Parrott, oral presentation at Southern Illinois University Ecology Symposium (October 2015)
- Josh, Parrott, poster presentation at Southern Illinois University Ecology Symposium (October 2016)
- Josh Parrott, oral presentation at University of Maine Ecology Symposium (September 2018)

Appendix 1. Sequence of synthetic standards for multiplex qPCR protocol

The following sequence was used to order synthetic double-stranded DNA which we used as positive control and to prepare standards (serial dilutions) in duplex (Bd and Bsal) qPCR protocols for simultaneous detection of chytrid fungi Bd and Bsal. gBlock® sequences produced by IDT DNA.

Sequence length: 31+84+146+5+180+5+435+85+29
Color coding:
Bd ITS-1 & 5.8S, Boyle et al. 2004, Genbank AY598034
Bs ITS-1 & 5.8S, Blooi et al. 2013, Genbank KC762295
Ranavirus Frog Virus 3, Mao et al. 1996, GenBank: DQ906049.1
Random sequence
Flanking sequence

CCCATGAGACATACAAAAAGGTAATGCCGCCAGTTACTACACCCCAGGGGCAACGTTGATGCTCCTAAAAAACTCT
GGCTGGACGCAAGCCGTAACACCCGTGTCACTTCATAATCCTTGATATAATACAGTGTGCCATATGTCACGAGTCG
AACAAAATTTATTTATTTTTTCGACAAATTAATTGGAAATTGAATAATTTAATTGAAAAAAATTGAAAATAAATATT
AAAAACAACTTTTGACAACGGATCTCTTGGCTttttTTTGCAGCAAACGGACACTTCATGTTTTATTTCCATCATGG
AGGACCCATGACGGAAAAGACTTTGCGCTGAAAAATACTCTTTACAAGATTGGGAATCCCATCGAGCCGTTCATGA
TGCGGATAATGTTGTGGTTGATGGCCAGAACGATGAGGGCGTACTTTTGGGCGGTGGTGTACCCAGAGTTGTTAC
CTCCACCTCCTGCGGCGGCCGTGGTGGCCTCAGCGGACAGGGTGACGTTAAGGCTGGCGTTGGTCAGTCTACCGT
AATTGGTGGATCCGGATGGGTGGGGGTCCTGCAGGCTGAGGGCATAAGAGTAGAGGTGGTGCCCGGTGCTGACT
GGGATGGAGGTGGCATAGTACCAGGGCTCCACCAGCGAGTAGTACTCGACTCCCATGTCGGGGAGCCTTGTGGT
GTTTTCGTACACCAGCGTGttttt CATTATCTGCTCCATCTCCCCCTCTTCATCCCTAACCCTATTTTTATATCACTTTTTA
GATGATATAAAAAGACAAGGAAATGAATTAAAAAAAGAAAAATAGAACAAGAAAATACTATTGATTCTCAAACAG
GCATACTCTACAAAGTAGAGTGCAATGTGCGTTCAAAGATTCGATGACGTTTGCAATTCAGGGCTTGATCTACACT
GGATTGCCATTCTCTCAAAGTATTATGCAGGACGGCGTGCGCGTTCCATGTAAACCGGTCTCGACTATACGCCCGT
TTTCGGATC

1 to 31: recommended flanking region
32 to 115: spacer sequence (random, G:C = 0.5)
116 to 261: target sequence Bd
262 to 266: spacer between target sequences (5 Ts)
267 to 701: target sequence Ranavirus
702 to 706: spacer between target sequences (5 Ts)
707 to 886: target sequence Bs
887 to 971: spacer sequence (random, G:C = 0.5)
972 to 1000: recommended flanking sequence

References

AmphibiaWeb, 2016. Information on amphibian biology and conservation. , Berkeley, California.
Blooi, M., Martel, A., Haesebrouck, F., Vercammen, F., Bonte, D., Pasmans, F., 2015. Treatment of urodelans based on temperature dependent infection dynamics of *Batrachochytrium salamandrivorans*. Sci Rep 5, 8037.
Boyle, D.G., Boyle, D.B., Olsen, V., Morgan, J.A.T., Hyatt, A.D., 2004. Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Diseases of Aquatic Organisms 60, 141-148.
Briggs, C.J., Knapp, R.A., Vredenburg, V.T., 2010. Enzootic and epizootic dynamics of the chytrid fungal pathogen of amphibians. Proceedings of the National Academy of Sciences of the United States of America 107, 9695-9700.
Catenazzi, A., Lehr, E., Rodriguez, L.O., Vredenburg, V.T., 2011. *Batrachochytrium dendrobatidis* and the collapse of anuran species richness and abundance in the upper Manu National Park, southeastern Peru. Conservation Biology 25, 382-391.
Crawford, A.J., Lips, K.R., Bermingham, E., 2010. Epidemic disease decimates amphibian abundance, species diversity, and evolutionary history in the highlands of central Panama. Proceedings of the National Academy of Sciences of the United States of America 107, 13777-13782.
Cunningham, A.A., Beckmann, K., Perkins, M., Fitzpatrick, L., Cromie, R., Redbond, J., O'Brien, M.F., Ghosh, P., Shelton, J., Fisher, M.C., 2015. Emerging disease in UK amphibians. Vet Rec 176, 468.
Daszak, P., Cunningham, A.A., Hyatt, A.D., 2003. Infectious disease and amphibian population declines. Diversity and Distributions 9, 141-150.
Fisher, M.C., Henk, D.A., Briggs, C.J., Brownstein, J.S., Madoff, L.C., McCraw, S.L., Gurr, S.J., 2012. Emerging fungal threats to animal, plant and ecosystem health. Nature 484, 186-194.
Grant, E.H.C., Muths, E.L., Katz, R.A., Canessa, S., Adams, M.J., Ballard, J.R., Berger, L., Briggs, C.J., Coleman, J., Gray, M.J., Harris, M.C., Harris, R.N., Hossack, B.R., Huyvaert, K.P., Kolby, J.E., Lips, K.R., Lovich, R.E., McCallum, H.I., Mendelson Iii, J.R., Nanjappa, P., Olson, D.H., Powers, J.G., Richgels, K.L.D., Russell, R.E., Schmidt, B.R., Spitzen-van der Sluijs, A., Watry, M.K., Woodhams, D.C., White, C.L., 2015. Salamander chytrid fungus (*Batrachochytrium salamandrivorans*) in the United States—Developing research, monitoring, and management strategies, In Open-File Report. p. 26, Reston, VA.
Gray, M.J., Lewis, J.P., Nanjappa, P., Klocke, B., Pasmans, F., Martel, A., Stephen, C., Parra Olea, G., Smith, S.A., Sacerdote-Velat, A., Christman, M.R., Williams, J.M., Olson, D.H., 2015. *Batrachochytrium salamandrivorans*: The North American Response and a Call for Action. Plos Pathogens 11.
IUCN, 2014. IUCN Red List of Threatened Species. Version 2014.3. www.iucnredlist.org.
James, T.Y., Toledo, L.F., Rodder, D., da Silva Leite, D., Belasen, A.M., Betancourt-Roman, C.M., Jenkinson, T.S., Soto-Azat, C., Lambertini, C., Longo, A.V., Ruggeri, J., Collins, J.P., Burrowes, P.A., Lips, K.R., Zamudio, K.R., Longcore, J.E., 2015. Disentangling host, pathogen, and environmental determinants of a recently emerged wildlife disease: lessons from the first 15 years of amphibian chytridiomycosis research. Ecol Evol 5, 4079-4097.
Klocke, B., Becker, M., Lewis, J., Fleischer, R.C., Muletz-Wolz, C.R., Rockwood, L., Aguirre, A.A., Gratwicke, B., 2017. Batrachochytrium salamandrivorans not detected in US survey of pet salamanders. Scientific Reports 7.
Longcore, J.E., Pessier, A.P., Nichols, D.K., 1999. *Batrachochytrium dendrobatidis* gen et sp nov, a chytrid pathogenic to amphibians. Mycologia 91, 219-227.
Martel, A., Blooi, M., Adriaensen, C., Van Rooij, P., Beukema, W., Fisher, M.C., Farrer, R.A., Schmidt, B.R., Tobler, U., Goka, K., Lips, K.R., Muletz, C., Zamudio, K.R., Bosch, J., Loetters, S., Wombwell, E., Garner, T.W.J., Cunningham, A.A., Spitzen-van der Sluijs, A., Salvidio, S.,

Ducatelle, R., Nishikawa, K., Nguyen, T.T., Kolby, J.E., Van Bocxlaer, I., Bossuyt, F., Pasmans, F., 2014. Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. Science 346, 630-631.
Martel, A., Spitzen-van der Sluijs, A., Blooi, M., Bert, W., Ducatelle, R., Fisher, M.C., Woeltjes, A., Bosman, W., Chiers, K., Bossuyt, F., Pasmans, F., 2013. *Batrachochytrium salamandrivorans* sp. nov. causes lethal chytridiomycosis in amphibians. Proc Natl Acad Sci U S A 110, 15325-15329.
Sabino-Pinto, J., Bletz, M., Perl, R.G.B., Schmeller, D.S., Mutschmann, F., Wagner, N., Veith, M., Schmidt, B.R., Steinfartz, S., Vences, M., Lötters, S., Pasmans, F., Martel, A., Hendrix, R., 2015. First detection of the emerging fungal pathogen Batrachochytrium salamandrivoransin Germany. Amphibia-Reptilia 36, 411-416.
Schmidt, B.R., 2016. Switzerland bans the importation of all salamander species because of the salamander chytrid. FrogLog 24.
Skerratt, L.F., Berger, L., Speare, R., Cashins, S., McDonald, K.R., Phillott, A.D., Hines, H.B., Kenyon, N., 2007. Spread of Chytridiomycosis Has Caused the Rapid Global Decline and Extinction of Frogs. EcoHealth 4, 125-134.
Sleeman, J.M., 2013. Has the time come for big science in wildlife health? EcoHealth 10, 335-338.
Spitzen-van der Sluijs, A., An, M., Johan, A., Emma, K.B., Wouter, B., Molly, C.B., Lutz, D., Edo, G., Alexander, K., Thierry, K., Kai, K., Arnaud, L., Luis, F.M.d.F., Andreas, N., Dagmar, O., Joana, S.-P., Benedikt, R.S., Jeroen, S., Frank, S., Sebastian, S., Michael, V., Miguel, V., Norman, W., Frank, P., Stefan, L., 2016. Expanding Distribution of Lethal Amphibian Fungus *Batrachochytrium salamandrivorans* in Europe. Emerging Infectious Disease journal 22.
Spitzen-van der Sluijs, A., Spikmans, F., Bosman, W., de Zeeuw, M., van der Meij, T., Goverse, E., Kik, M., Pasmans, F., Martel, A., 2013. Rapid enigmatic decline drives the fire salamander (*Salamandra salamandra*) to the edge of extinction in the Netherlands. Amphibia-Reptilia 34, 233-239.
Spitzen-van der Sluijs, A., Stegen, G., Bogaerts, S., Canessa, S., Steinfartz, S., Janssen, N., Bosman, W., Pasmans, F., Martel, A., 2018. Post-epizootic salamander persistence in a disease-free refugium suggests poor dispersal ability of Batrachochytrium salamandrivorans. Scientific Reports 8.
Stegen, G., Pasmans, F., Schmidt, B.R., Rouffaer, L.O., Van Praet, S., Schaub, M., Canessa, S., Laudelout, A., Kinet, T., Adriaensen, C., Haesebrouck, F., Bert, W., Bossuyt, F., Martel, A., 2017. Drivers of salamander extirpation mediated by Batrachochytrium salamandrivorans. Nature 544, 353-+.
Wake, D.B., Vredenburg, V.T., 2008. Are we in the midst of the sixth mass extinction? A view from the world of amphibians. Proceedings of the National Academy of Sciences of the United States of America 105, 11466-11473.