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Historic occurrence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in hellbender *Cryptobranchus alleganiensis* populations from Missouri

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ABSTRACT: The pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*) was recently detected in Missouri hellbender *Cryptobranchus alleganiensis* populations that have declined precipitously for unclear reasons. The objective of this study was to determine whether *Bd* occurred historically in Missouri hellbender populations or is a relatively novel occurrence. Epidermal tissue was removed from 216 archived hellbenders collected from 7 Missouri streams between 1896 and 1994. Histological techniques and an immunoperoxidase stain were used to confirm historic occurrence of *Bd* infection in hellbenders from the North Fork of the White (1969, 1973, 1975), Meramec (1975, 1986), Big Piney (1986), and Current rivers (1988). *Bd* was not detected in hellbenders from the Niangua, Gasconade or Eleven Point rivers. The study detected no evidence for endemism of *Bd* in Missouri hellbender populations prior to 1969, despite the fact that nearly one third of the hellbenders sampled were collected earlier. Our findings are consistent with the hypothesis that *Bd* is a non-endemic pathogen in North America that was introduced in the second half of the twentieth century.

KEY WORDS: *Batrachochytrium dendrobatidis* · Chytrid · Hellbender · *Cryptobranchus alleganiensis* · Missouri · Amphibian decline

INTRODUCTION

Chytridiomycosis, a potentially lethal disease caused by the fungus *Batrachochytrium dendrobatidis* (hereafter *Bd*) (Longcore et al. 1999, Pessier 2007), has been implicated in rapid declines, mass mortalities, and local extinctions of amphibians worldwide (Stuart et al. 2004, Skerratt et al. 2007). First described in 1998 (Longcore et al. 1999), *Bd* is the only member of the family Chytridiales to infect vertebrate hosts by colonizing keratinized mouthparts of larvae and keratinized epidermis in post-metamorphic amphibians (Berger et al. 1998). In the first study to identify pathogenesis of chytridiomycosis, Voyles et al. (2009) observed disrupted cutaneous function followed by cardiac arrest in infected green tree frogs *Litoria caerulea*. It is unclear whether *Bd* is a widespread endemic pathogen with peaks of infection (Rachowicz et al. 2005) or an introduced 'spreading pathogen' (Skerratt et al. 2007). Currently *Bd* has been identified on 6 continents in over 200 species (Skerratt et al. 2007), and genetic evidence supports the hypothesis that *Bd* is a recently spread and novel pathogen for many amphibian species (Fisher et al. 2009, James et al. 2009).

Determining the cause of amphibian declines is often a difficult task (Stuart et al. 2004). Due to the potential virulence of chytridiomycosis (Skerratt et al. 2007) and the broad geographical range of *Bd* (Fisher et al. 2009),

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the disease is often implicated as an explanation for historical amphibian declines in the absence of a more obvious cause (i.e. habitat loss) (Stuart et al. 2004). However, because *Bd* was not described prior to 1998, it is unknown whether the fungus was present in several amphibian populations that began declining as early as the 1960s (Houlahan et al. 2000, Stuart et al. 2004). Additionally, while most amphibian species can become infected with *Bd* (Pessier 2008), severity of infection and lethality can vary with host species (Woodhams et al. 2007), life stage (Garner et al. 2009), habitat (Kriger & Hero 2007a), and climate (Kriger & Hero 2007b); and some species may acquire immunity to *Bd* infection over time (Retallick et al. 2004, Richmond et al. 2009). Therefore, presence of *Bd* does not equate to clinical disease, lethality, or compulsory declines of a population in which it occurs. While detection of *Bd* in a declining population is of interest, implications of its presence are often complex to interpret. Retrospective studies involving archived specimens are useful for determining historical occurrence of *Bd* in a population and can sometimes provide justification for further inquiry into the potential role *Bd* may have played in population declines (Berger et al. 1998, Lips et al. 2006).

Hellbenders *Cryptobranchus alleganiensis* are large (up to 74 cm), fully aquatic, long-lived (25+ yr) salamanders that inhabit cool, highly oxygenated streams in portions of eastern and central North America (Smith 1907, Nickerson & Mays 1973, Taber et al. 1975). Currently, 2 subspecies of hellbender have been described. The eastern hellbender *C. a. alleganiensis* occurs from New York south to Georgia and as far west as Missouri, where a disjunct population occurs in north-flowing streams draining to the Missouri and Mississippi rivers. The Ozark hellbender *C. a. bishopi* is endemic to south-flowing streams within the Black and White River drainages of southern Missouri and northern Arkansas. Currently, there are 7 relatively isolated, extant hellbender populations in Missouri that are identified by river (Routman et al. 1994, Sabatino & Routman 2008). Eastern hellbender populations in Missouri include those from the Niangua, Big Piney, Gasconade, and Meramec Rivers, while the Current, Eleven Point, and North Fork of the White Rivers represent the range of the Ozark subspecies (Fig. 1). Enigmatic range-wide declines of both subspecies, averaging 77%, have occurred in Missouri since the 1980s (Wheeler et al. 2003). The initial detection of *Bd* in Missouri hellbender populations in 2006 and later surveys confirmed presence of the fungus throughout the species' range in the state (Briggler et al. 2007, 2008). The objective of the present study was to determine whether *Bd* occurred historically in Missouri hellbender populations or may be a relatively novel occurrence, through examination of archived hellbenders collected from 7 Missouri populations (i.e. rivers) between 1896 and 1994.

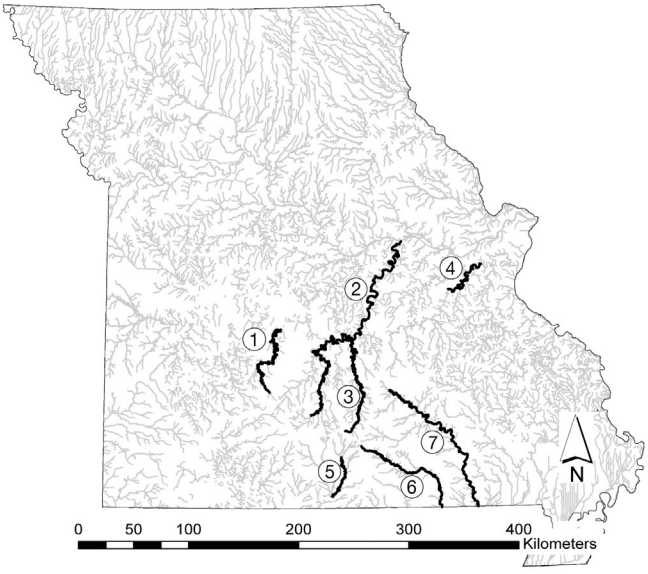

Fig. 1. Map of Missouri rivers, showing the spatial distribution of eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) and Ozark hellbender (*C. a. bishopi*) populations. Eastern hellbenders are restricted to northeasterly flowing streams including the Niangua (1), Gasconade (2), Big Piney (3) and Meramec (4) rivers. Ozark hellbenders occur in south-flowing streams including the North Fork of the White (5), Eleven Point (6), and Current (7) rivers

MATERIALS AND METHODS

Between March 2008 and May 2009, 11 museums were visited and 234 archived *Cryptobranchus alleganiensis* collected from 7 Missouri rivers between 1896 and 1994 were accessed. Collector, date of collection, river, and locality information for each specimen were recorded. Total length (mm) of each hellbender was documented and gender identified when evident without destructive sampling. Hellbenders were categorized as larvae (external gills present), juveniles (total lengths ≤ 300 mm and lacking swollen cloaca) or adults (total length > 300 mm or swollen cloaca). Tissue collection was prohibited for 1 holo- and 5 paratype specimens. Twelve hellbenders were larvae that were hatched in captivity or were too small to prevent destruction of skeletal tissue during sampling and therefore were not included in the study. Tissue was collected from a single digit of each of the remaining 216 hellbenders similar to the methods of Ouellet et al. (2005). Longitudinal cross-sections of epidermis were separated from bone and placed into uniquely labeled

histology cassettes. Cassettes were stored in neutral buffered 10% formalin prior to being embedded in paraffin, sectioned at 4 µm, and stained with haematoxylin and eosin (H&E) (University of Missouri Veterinary Diagnostics Lab) following methods of Berger et al. (1999). Samples were diagnosed as negative, positive, or suspicious (i.e. structures suggestive of *Bd* were observed but confirmation needed) according to methods of Berger et al. (1999).

To remain conservative in our diagnosis of *Bd*-positive samples, especially for a few suspicious samples that contained an Oomycete fungus, an immunoperoxidase (IPX) stain was applied to confirm positive diagnoses and clarify suspicious diagnoses. *Bd* antibodies were obtained from Dr. Alex D. Hyatt (CSIRO Animal Laboratory, Australia) and used to produce the IPX stain (University of Missouri Veterinary Diagnostics Lab) according to methods of Berger et al. (2002) at a dilution of 1:1000. Effective staining with the IPX was confirmed using *Bd* positive and negative controls. The positive control consisted of epidermis from a digit of a hellbender that died in 2008 after displaying lethargy and excessive sloughing of the epidermis. When stained with only the H&E, all life stages of *Bd* were evident in the positive control. Negative controls included additional sections cut from samples collected from a Gasconade River hellbender in 1896, and a Current River hellbender collected in 1930 that appeared entirely normal when stained with the H&E alone. Blocks of positive and suspicious tissue were sectioned at 4 µm and stained with the IPX. Positive samples were confirmed and diagnosis of suspicious tissues was clarified using criteria of Berger et al. (1999) in combination with staining from the IPX (Berger et al. 2002).

RESULTS

Upon examination, 10 of the 216 samples collected lacked keratin and were not included in the study. The remaining 206 hellbenders sampled were comprised of 48 juveniles and 158 adults (90 males, 53 females, and 15 unknown gender). Mean total length of eastern hellbenders was 394 mm (SE = 9 mm, n = 92, range = 170 to 523 mm), and mean total length of Ozark hellbenders was 340 mm (SE = 8 mm, n = 102, range = 82 to 496 mm). Subspecies and location data was lacking for 12 hellbenders included in the study.

Bd was detected in 4 populations and 6.3% (13 of 206) of hellbenders (Fig. 2). All 13 *Bd*-positive hellbenders were collected and deposited in collections prior to or during the period when many hellbenders populations in Missouri declined (Fig. 2). The earliest detections of *Bd* included 5 of 22 Ozark hellbender collected from the North Fork of the White River in 1969. In the Meramec, Big Piney, and Current River populations, the earliest detection of *Bd* occurred in 1975, 1985, 1988, respectively. Simultaneous occurrence of *Bd* infection and saprolegniasis (infection of *Saprolegnia* sp.) occurred in a Current River sample from 1988 and in 3 Meramec River samples from 1975 (Table 1). No sign of *Bd* or saprolegniasis was detected in samples from Niangua, Eleven Point, or Gasconade river hellbenders.

Samples were unevenly distributed both temporally (n = 1 to 43 per year) and spatially (n = 9 to 87 per river) (Fig. 2). Out of 206 hellbenders sampled, 58 (28.1%) were collected prior to the earliest date in which *Bd* was detected (i.e. 1969). The Meramec was the only river not represented prior to 1969.

Although juveniles composed nearly one quarter of the sample, infected hellbenders were mature adults of total lengths ranging 274 to 490 mm (Table 1). Over half (n = 8) of the infected hellbenders were male, one was female, and gender was unknown for 4 *Bd*-positive hellbenders (Table 1). Histology confirmed that *Bd* was restricted to discrete portions of the stratum corneum and upper stratum granulosum of hellbender integument. Morphology of the fungus was consistent with that described by Longcore et al. (1999) and Berger et al. (1999). *Bd* lesions in hellbenders were characterized by mild focal hyperkeratosis and occasional sloughing of the epidermis near area of infection (Fig. 3). Generally, infection appeared light, with 0 to 25 *Bd* zoosporangia typically visible in a given frame (400×) of any positive sample. The heaviest infection was characterized by approximately 100 *Bd* zoosporangia in a single frame of view (400×). All life stages of *Bd* were observed, including immature and mature thalli with internal zoospores and empty zoosporangia sometimes with obvious discharge papillae (Fig. 4). With the IPX, *Bd* stained deep red in contrast to other artifacts (Fig. 4), and rhizoids of *Bd* zoospores were visible, which rarely occurred with the H&E stain. The IPX was useful for confirming presence of *Bd* when occurrence was limited to immature or developing life stages.

In 4 of the 13 *Bd*-positive samples, *Bd* occurred along with the Oomycete fungus *Saprolegnia* sp. (Table 1). Presence of *Saprolegnia* was obvious in tissues stained with H&E alone, largely due to hyphae that extended into dermal tissue. However, no evidence of *Saprolegnia* was detected in *Bd*-negative samples. Because in cross section some fungal hyphae appear similar in shape and size to *Bd* (Berger et al. 1999), application of the IPX was particularly useful for confirming presence of *Bd* when the 2 fungi co-occurred. When *Saprolegnia* and *Bd* co-occurred, *Bd* stained heavily with the IPX, was restricted to within

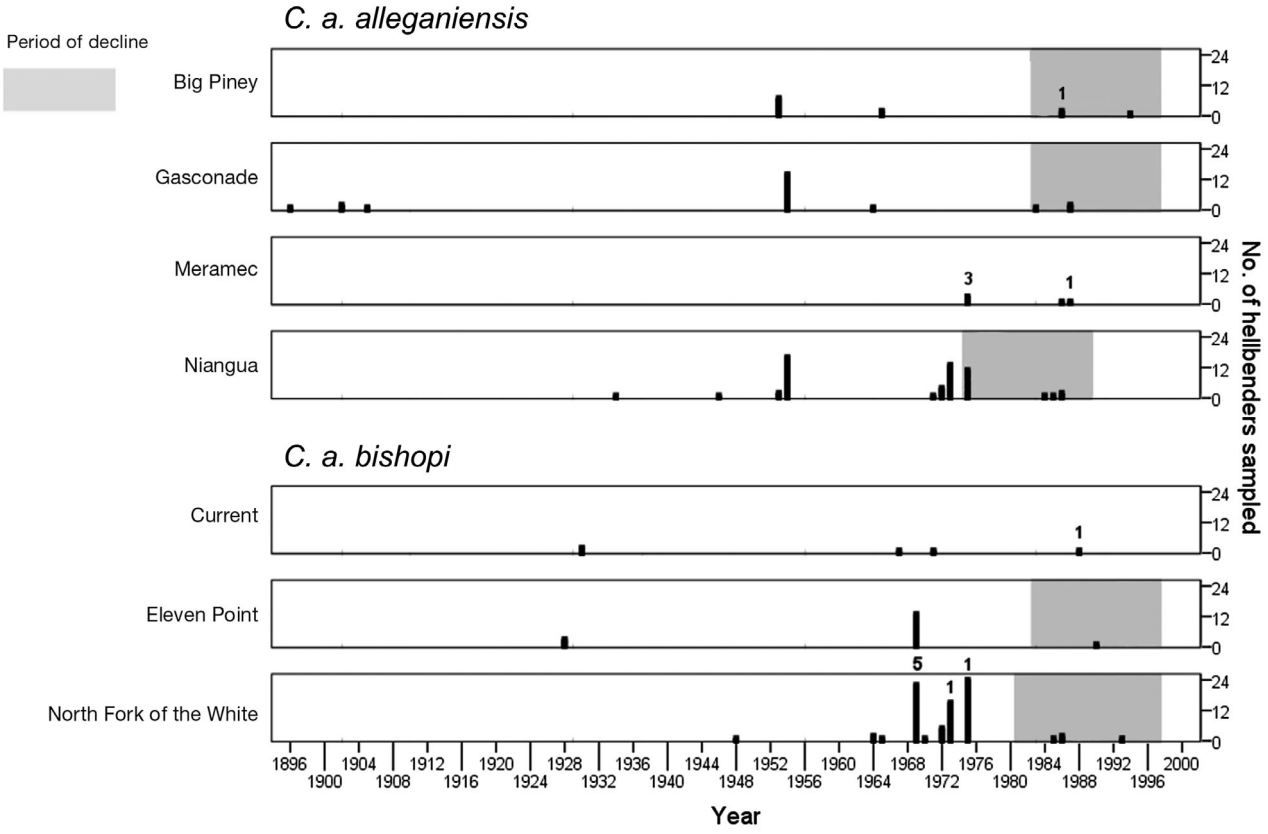

Fig. 2. *Cryptobranchus alleganiensis alleganiensis* and *C. a. bishop.* Spatial and temporal distribution of archived hellbenders that were sampled and number of samples positive for *Bd* (numbers above bars). Data is presented relative to periods of decline (dark gray shading) when eastern hellbender populations regionally declined by about 80%, and Ozark hellbender populations underwent regional declines of about 70% (Wheeler et al. 2003). A lack of historic data from the Meramec and Current Rivers precludes knowledge of the timing or intensity of declines in these populations. Note occurrence of the fungus in the Big Piney and North Fork of the White rivers prior to or during the period of declines in these populations

Table 1. *Cryptobranchus alleganiensis alleganiensis* and *Cryptobranchus alleganiensis bishop.* Archived eastern and Ozark hellbenders collected from 4 Missouri streams between 1969 and 1988 that were confirmed positive for *Bd* infection via histology. Eastern hellbender streams include the Big Piney and Meramec Rivers and Ozark hellbender streams include the Current and North Fork of the White Rivers. Museums: INHS, Illinois Natural History Survey; MPM, Milwaukee Public Museum; MVZ, Berkeley Museum of Vertebrate Zoology; UMMZ, University of Michigan Museum of Zoology. Rivers: BPR, Big Piney River; CR, Current River; MR, Meramec River; NFWR, North Fork of the White River. TL: total length

Museum	Catalog No.	River	Locality	Date	Year	TL (mm)	Gender
MVZ	205728	BPR	Boiling Spring access/Texas County	23 May	1986	383	Unk.
MPM	11309[a]	MR	5 to 6 miles upstream Indian Springs campground	21 Jul	1975	330	M
MPM	11310[a]	MR	5 to 6 miles upstream Indian Springs campground	21 Jul	1975	311	Unk.
MPM	11311[a]	MR	5 to 6 miles upstream Indian Springs campground	21 Jul	1975	350	M
MVZ	205737	MR	Phelps County, where Meramec Spring enters	10 Sep	1986	385	M
INHS	11236[a]	CR	Cave Spring/Shannon County	1 Jan	1988	445	F
UMMZ	139000A	NFWR	Blair Bridge/Althea Spring	13 Sep	1969	456	Unk.
UMMZ	139000B	NFWR	Blair Bridge/Althea Spring	13 Sep	1969	490	Unk.
UMMZ	139000C	NFWR	Blair Bridge/Althea Spring	13 Sep	1969	443	M
UMMZ	139000D	NFWR	Blair Bridge/Althea Spring	13 Sep	1969	411	M
UMMZ	139000F	NFWR	Blair Bridge/Althea Spring	13 Sep	1969	428	M
MPM	7237	NFWR	Blair Bridge/Althea Spring	3 Oct	1973	274	M
MPM	11248	NFWR	Blair Ford	2 Jul	1975	390	M

[a]Denotes co-occurrence of *Bd* and *Saprolegnia* sp. in tissue sampled

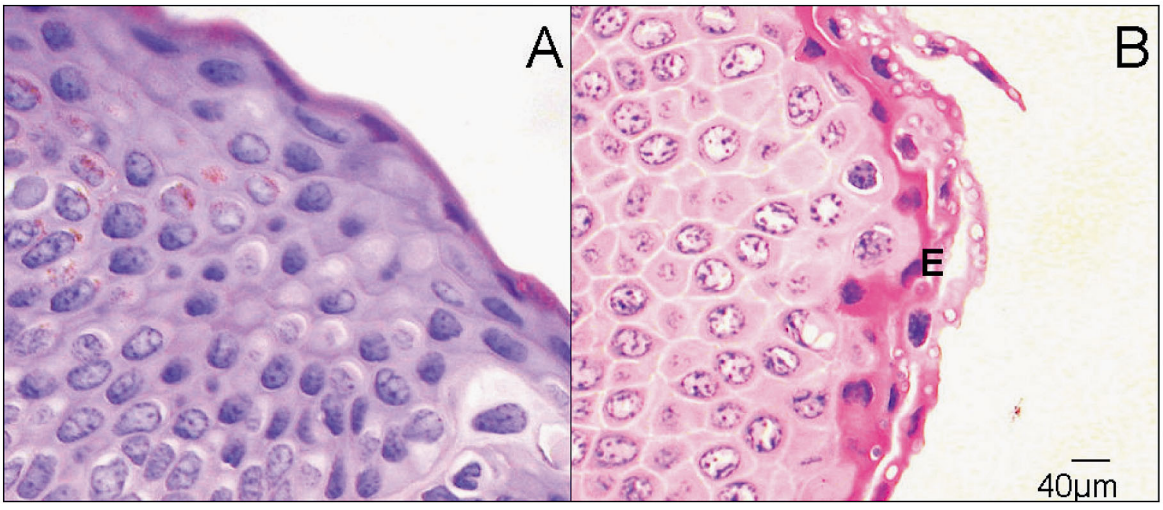

Fig. 3. *Cryptobranchus alleganiensis.* (A) Normal and (B) infected sections of hellbender skin stained with haematoxylin and eosin (H&E). Note that infection is restricted to the epidermis (E), which is thickened and beginning to slough. Empty *Bd* zoosporangia appear as clear circular structures

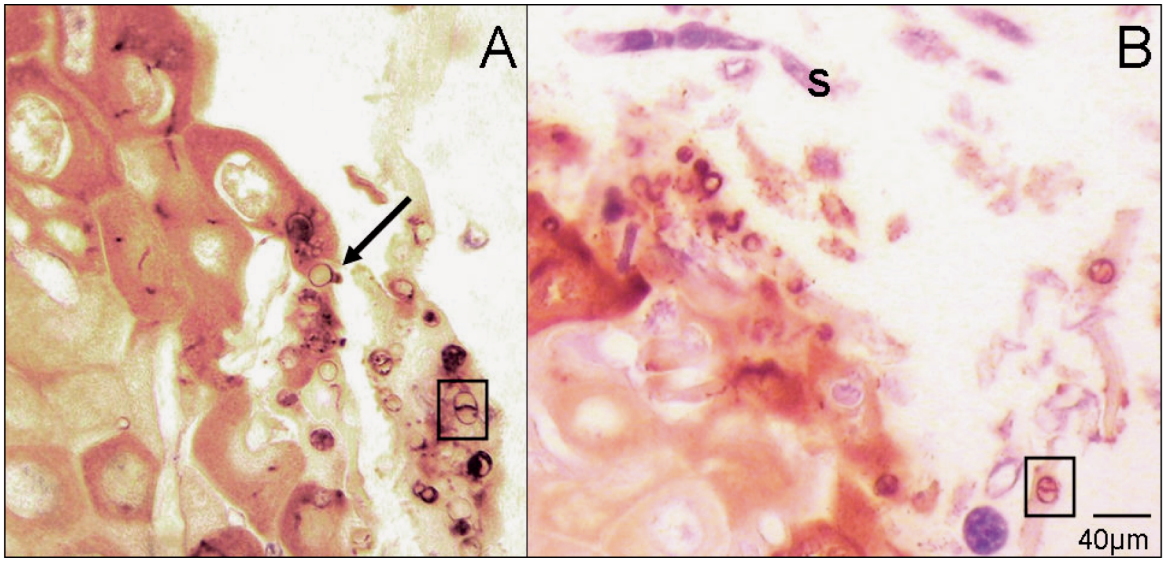

Fig. 4. *Cryptobranchus alleganiensis.* (A) Section of hellbender skin infected only with *Bd* and (B) a section where *Bd* infection co-occurred with *Saprolegnia* sp., both stained with the immunoperoxidase. Chytrids are distinguishable by dark red staining, presence of internal septae (boxes), and discharge tubes (arrow), while *Saprolegnia* (S) exhibits hyphae that stain a lighter purple

epidermal cells of the stratum corneum and lacked hyphae. In contrast, *Saprolegni*a had hyphae that extended into dermal layers and stained light blue or purple (Fig. 4B).

DISCUSSION

The occurrence of *Bd* infection in *Cryptobranchus alleganiensis bishopi* in 1969 is the earliest reported occurrence in *C. alleganiensis* throughout the species' range, the earliest published report of infection from an amphibian in Missouri, and one of the earliest confirmed cases for any amphibian within the USA. In comparison, the earliest report of *Bd* infection in North America is from a green frog *Rana clamitans* collected in 1961 from Quebec, Canada (Ouellet et al. 2005). The earliest published occurrence of *Bd* in the United States is cited as originating in Wyoming between 1960 and 1969 (exact date not reported) (Ouellet et al. 2005).

Findings from the present study are consistent with the hypothesis that *Bd* is a recently spread and novel pathogen in North America where it was first introduced in the latter half of the twentieth century.

The present study detected no evidence for endemism of *Bd* in Missouri hellbender populations prior to 1969, despite the fact that nearly one third of the hellbenders sampled were collected earlier. However, results from the present study should be interpreted within the context of data collected from museum specimens. Collectors did not use a probabilistic sampling design to collect hellbenders. The resulting unequal distribution of samples over time and space may explain the lack of *Bd* detection in all rivers and some years. For example, in the North Fork of the White River, detection and prevalence of *Bd* was correlated with the number of animals collected each year (Fig. 2). Only one Eleven Point River sample existed post 1969, and 4 Gasconade River samples existed post 1954, making it unlikely or impossible to detect *Bd* from either river even if it were introduced around or after the late 1960s. Our inability to detect *Bd* in certain rivers or time periods should not be interpreted as confirmed absence of the fungus, but rather as an inability to confirm presence of *Bd* if it did occur. It is possible that more intense sampling of specimens (e.g. from additional digits) or application of the IPX to samples diagnosed as negative using the H&E alone may identify *Bd* in other populations and time periods. Despite limitations in the data set, the present study contributes novel information concerning the potential length of time *Bd* has occurred in Missouri hellbender populations and the historical range of *Bd* in North America.

Though findings of the present study indicate that *Bd* was historically introduced to Missouri waterways, the mode of introduction remains unsubstantiated. Non-native trout stocking increased dramatically on the North Fork of the White River after 1960 (Alsup 2005), which roughly coincides with the earliest detection in the present study. One possibility is that the fungus may have been introduced and spread anthropogenically through contaminated water sources or recreational traffic.

The naivety of some amphibians to *Bd* can result in clinical disease (i.e. chytridiomycosis), lethality, and population decline (Skerratt et al. 2007). We lack the data to determine what role, if any, *Bd* may have played in Missouri hellbender declines. Multiple studies have indicated that pathogenicity of *Bd* may be less severe for salamanders than for many frog and toad species (Davidson et al. 2003, Harris et al. 2006, Venesky et al. 2010). However, unlike many caudates, hellbenders have an entirely aquatic life history and are restricted to permanent flowing water bodies (Petranka 1998). Many *Bd* infections in semi-aquatic amphibians can be mitigated via behavioral fever (Woodhams et al. 2003), and intensity and duration of infection limited by high summer temperatures and ephemeral drying of ponds. In contrast, the spring-fed streams where hellbenders occur in Missouri often maintain year-round temperatures (see Nickerson & Mays 1973) ideal for *Bd* (Piotrowski et al. 2004), as evidenced by our detection of *Bd* infection in hellbenders during all seasons (Table 1). Persistence of even mild *Bd* infections over a long term may have negative implications for salamanders like hellbenders. For example, *s*aprolegniasis is a common and sometimes lethal secondary infection to cutaneous injury or immunocompromised individuals (Noga 1993, Pessier 2002). The co-occurrence of *Saprolegnia* and *Bd* in multiple hellbenders may indicate that *Bd* is more common in immune-compromised hellbenders, or that *Bd* infection may increase susceptibility of hellbenders to other infection.

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