State Wildlife Grant Program

State of Illinois

Annual Report

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Project Number: T-56-R-1

Project Title: Survey for the pathogen, *Batrachochytrium dendrobatidis*, in Illinois

Project Leader: Jody Shimp, Regional Administrator, Illinois Department of Natural Resources, Region V

Approach: The following is broken down into four objectives. They are as follows:

Objective 1: Survey protected areas of Illinois for the presence of *Bd*
Objective 2: Analyze (PCR) field samples
Objective 3: Survey for *Bd* in museum specimens (histology)
Objective 4: Produce Reports and Maps, give presentations at meetings

A timeline for completion of all objectives is included (Table 1). We are on schedule for all our Objectives, and expect to complete the bulk of field and lab work by early 2010.

Objective 1: COMPLETED
Survey protected areas of the State of Illinois for the presence of *Bd*, focusing on protected areas located within Natural Divisions (Northeastern Morain, Rock River Hill Country, Grand Prairie, Southern Till, Illinois River Bottomlands, Wabash Border, Shawnee Hills). (Spring 2008 & 2009, 31% of budget).

1.1) Determine sites to be surveyed, choosing 2 in the north, 2 in the center, and 2 in the south in year 1 and 6 additional sites in year two.

> **COMPLETED**: We compiled species lists of study sites by consulting with IDNR, FWS and INHS staff and site managers and by conducting library and internet research. We arranged site visits to speak with managers and determine whether appropriate habitat and sufficient abundance of focal species existed at each site and assessed feasibility of each site.

1.2) Capture visually located amphibians. Swab captured amphibians to collect samples for Bd testing. Record individual data. Release all amphibians once swabbing completed.

> **COMPLETED**: A field crew of 2-3 SIUC students conducted surveys to establish the distribution of *Bd* among wetlands in 9 Natural Divisions (Coastal Plain, Middle Mississippi Border, Northeastern Morain, Rock River Hill Country, Grand Prairie, Southern Till, Upper Mississippi / Illinois River Bottomlands, Wabash Border, Shawnee Hills). Multiple protected areas, and most species, including those of conservation priority (e.g., Bird-voiced treefrog,

Crayfish frogs, Illinois chorus frogs) were included. We sampled during spring and early summer when amphibians are most abundant and when environmental conditions are within the optimal range for growth and survival of *Bd* and it is likely to be widely distributed among species and habitats.

We sampled a minimum of 35 individuals per species at each site to ensure collection of 366 swabs per site in 2008, and 100 swabs per site in 2009. In 2009, the number of sites surveyed was increased to 18 sites for better resolution of prevalence estimates throughout Illinois. These are sufficient sample sizes according to calculations to detect disease in natural populations, even if present at low levels (DiGiacomo and Koepsell 1986). We maximized our chances of finding *Bd* when present, by sampling species that have been shown to be infected elsewhere (e.g., most species of *Rana* and *Bufo*). We targeted two types of amphibians: widespread species and those of special interest. At each site, we sampled species that are distributed state-wide (e.g., *Acris crepitans, Bufo americanus, B. fowleri, Hyla chrysoscelis/versicolor, Pseudacris crucifer, P. triseriata, Rana catesbeiana, R. pipiens,* and *R. sphenocephala)* to determine how local environments influence infection. We also sampled species that have previously declined (e.g., *A. crepitans*, *R. areolata*), are restricted in range (e.g., *D. fuscus, Eurycea lucifuga*) or are Threatened or Endangered (e.g., *P. triseriata, H. avivoca*).

We used standard field techniques to survey adult amphibians (Heyer *et al.* 1994). Teams of 2-3 researches walked along wetland edges and in emergent vegetation to visually search for frogs. Individuals were captured by hand, swabbed for *Bd* (Hyatt et al. 2006), toe-clipped for individual identification when necessary (i.e., when a single wetland was sampled on multiple nights), measured (SVL, mass) and then released. Each cotton tip used for swabbing were stored in ethanol (Lips *et al.* 2006). Additional information such as age, sex, perch substrate, reproductive behavior, etc, were recorded when possible. We determined GPS coordinates at the edge of wetlands with a Garmin™ GPS 12 personal navigator.

1.3) Measure microenvironmental and habitat variables, and obtain GPS location at each capture.

COMPLETED: At each sampling location we obtained a GPS reading and recorded relevant environmental variables including: air temperature, water temperature, cloud cover, and precipitation.

Objective 2: PARTIALLY COMPLETED
Use histology to survey for *Bd* in museum specimens collected prior to 1990 from each of the Natural Divisions of Illinois (Summer 2008-Fall 2010; <3% budget).

2.1) Obtain list of all Illinois specimens (including species, collection date and locality data) deposited at SIUC INHS, UIUC, FMNH from curators.

COMPLETED: We requested information from curators of SIUC, INHS, UIUC, and FMNH, and obtained listings of all Illinois specimens collected between 1950-1990. We summarized those lists to determine how many individuals of which species were available statewide in each decade from 1950-1990. We determined which species to sample by identifying those with widespread distribution (Figure 1) and equal effort on sampling from members of the families Bufonida, Hylidae, and Ranidae. Those species were *Bufo americanus*, *B. fowleri*, *Acris crepitans*, *Hyla chrysoscelis/versicolor*, *Rana catesbeiana*, *R. pipiens*, and *R. sphenocephala*.

2.2) Remove a 4x4 mm snip of pelvic patch skin from at least 366 historical specimens collected prior to

1990 (the earliest record of Bd in the state) in each of the 9 natural divisions in the study area, choosing approximately 90 individuals from each decade (1950, 1960, 1970, 1980). Place collected tissue samples in alcohol to be transported to the SIUC Histology lab.

PARTIALLY COMPLETED: We visited SIUC, INHS, and UIMNH in 2008 and 2009 and completed sampling of the SIUC and UIMNH material. All adult specimens collected in Illinois between 1950 & 1989, were identified and selected for tissue samples to be taken. We removed a small (~4x 4 mm) piece of skin from the ventral side of the animal in the area of the pelvic patch for all animals fitting our criteria. The FMNH will be visited in 2010, and we will complete sampling of the remaining 25% of the INHS material. In 2010, we will sample approximately 1,000 specimens collected during the 1990s will be sampled based on species, collection county, and time of year.

2.3) Prepare histological slides of skin, examine under microscope for the presence of Bd. Record dates and locations of infection.

PARTIALLY COMPLETED: We have been working under the guidance of Maureen Dornan in the SIUC Histology lab to prepare slides for standard H&E histology (Puschendorf and Bolanos, 2006). This approach involves fixing the tissue in formalin, dehydrating the tissue in ethanol baths, embedding the tissue into paraffin, sectioning the skin to make very thin slices that are placed on a glass slide for staining. Hematoxylin and eosin (H&E) are the most commonly used stains in histology and histopathology. Hematoxylin colors nuclei blue; eosin colors the cytoplasm pink. To see the tissue under a microscope, the sections are stained with one or more pigments. To date, approximately 230 specimens from the SIUC collections have been fixed onto microscope slides. Fixing slides from the remaining specimens will continue into 2010, and we will begin examining all slides for evidence of *Bd* in museum specimens.

2.4) Enter date and GPS locality of each positive and negative record of Bd into database. Quantify degree and prevalence of infection by Bd among decades in each Natural Division.

TO BE COMPLETED IN 2010: We will use results of the histology surveys to calculate *Bd* infection prevalence and 95% Clopper-Pearson binomial confidence intervals for each Natural Division and for each decade. We will plot locations of all sampled amphibians with ArcView to plot historic distributions of *Bd* in the state, and if possible, to search for environmental cofactors (e.g. Witte 2005; Lips et al. 2003; Brem 2006) of infection.

Objective 3. PARTIALLY COMPLETED
Organize and analyze survey data. (Summer 2008 – Spring 2009; 65% of budget)
3.1) Analyze amphibian swabs to determine the presence of Bd using PCR analysis.

PARTIALLY COMPLETED: Each swabbed sample will be tested for *Bd* in the lab using PCR primers (Annis *et al.* 2004; Boyle et al. 2004; Hyatt et al. 2006). Molecular methodology was learned under the guidance of Dr. Vance Vredenburg, at his lab in San Francisco State University, since the sensitivity of his qPCR test for *Bd* has been proven accurate and precise. Approximately 500 swabs were analyzed under Dr. Vredenburg's guidance in January 2009, including DNA extraction from the swab and qPCR analysis (see Appendix 2 for preliminary results). In Summer 2009, approximately 1,050 swabs were processed for DNA extraction in the laboratory of Dr. Ed Heist at SIUC. DNA extraction will continue into Winter 2009 and Spring 2010, when these samples will be assessed with qPCR in Dr. Vredenburg's lab.

3.2) Enter site data and GPS location into database

PARTIALLY COMPLETED: All microenvironmental, habitat variables, and GPS locations of current sample sites were entered into a database, and have been plotted into ArcView. GPS locations of museum specimens is underway, and will continue into 2010. We will plot *Bd* distribution in the state once Objectives 2 and 3.1 have been completed in ArcView.

3.3) Quantify degree and prevalence of infection by Bd among amphibian species, sites, and habitat types. Determine current Bd distribution: ubiquitous, patchily distributed (by region, habitat, or species), or not present.

PARTIALLY COMPLETED: We will use results of the PCR assay to calculate *Bd* infection prevalence and 95% Clopper-Pearson binomial confidence intervals for each species at each site. This has been completed for approximately 500 swabs collected in 2008 (see Appendix 2). Using data entered in Obj. 3.2, we will attempt to search for environmental cofactors (e.g. Witte 2005) of infection. We will relate infection status to differences in species ecology and microhabitat characteristics (Lips et al. 2003; Brem 2006).

We will summarize and analyze distribution patterns of current and historical *Bd* presence in the state using spatial analysis software. We will determine whether infection is associated with particular landscape features, seasons, or species. We will assess whether amphibian populations are likely to have declined as a result of historic introductions of *Bd* to the state.

Objective 4. TO BE COMPLETED ONCE OBJECTIVES 2 AND 3 HAVE BEEN COMPLETED
Produce Reports and Maps (Fall 2010-Spring 2011; <1% budget)
4.1) Produce maps of current and historic dates and locations of Bd distribution.

We will use spatial analysis and mapping software to create maps of the current and historic distribution in the state.

4.2) Develop management plans to assist land managers based on presence/absence of Bd across state, in particular habitats, and in particular species.

We will use results from the statistical analysis and spatial mapping to recommend potential management options to minimize or control the spread of *Bd*, to evaluate feasibility of reintroduction programs, and to conduct disease surveillance in particular regions or species. We will identify specific management needs depending on whether a region, a site, a habitat, or a particular species is infected or not, and to what degree. These would include ideas for active prevention of spread (e.g., management of human activities, placing bleaching stations at key sites, designing informational signs and brochures, etc), monitoring of wild amphibian populations, management of captive and reintroduced populations, and assessing possibility of future spread (e.g., routes, direction, rates, vectors).

Some examples of management recommendations resulting from information gathered through this study are as follows:

<u>*Ubiquitous:*</u> (A) If we determine that *Bd* is ubiquitous throughout Illinois, managers could use this information to take actions to minimize the spread of *Bd* (e.g., educating site users, providing bleaching stations). There are multiple strains of *Bd* known to date, some more lethal

than others, so even though a site is infected we recommend minimizing additional introductions. We would also recommend molecular analyses to identify the distribution of different strains throughout the state to better manage amphibian populations. (B) In addition, we would not recommend reintroduction of threatened and endangered species anywhere in Illinois until methods are developed to eradicate *Bd* from the wild. (C) Furthermore, we would recommend determination of the population status and changes thereof for all wild populations of threatened and endangered species, as well as those species with restricted ranges. We would use results of the historic survey to determine the likely date and location of the earliest introductions of *Bd* into Illinois and also to evaluate when each region or population was likely to have first been exposed to *Bd* and whether the timing was associated with any information regarding change in populations status.

Patchily distributed: (A) If we determine that *Bd* is patchily distributed (either by region, habitat, or species), then managers could implement activities to minimize the spread of *Bd* (see above). (B) Since *Bd* is easily transported through many types of moist substrates (e.g., water, soil, plants), managers could contain or minimize the spread of *Bd* into healthy amphibian populations by promoting the cleaning of recreational items, footwear and vehicle tires. For example, anglers fishing in infected water bodies could be encouraged to clean bait buckets and fishing gear before leaving the site. (C) We will compare the current distribution to the distribution determined by histological surveys of museum specimens to determine whether currently uninfected areas have always been uninfected and when they are likely to be invaded, and from which direction. We will use GIS maps of current and historic infection to identify potential routes, vectors, and rates of spread of *Bd* to help managers block routes, slow rates or manage vectors. (D) If *Bd* is present in specific species or regions, management strategies could be developed to monitor the population status of both healthy and infected populations, and to determine whether additional protection is needed (e.g., captive breeding, translocations, introductions) under the Endangered Species Act.

Not present: In the unlikely event that *Bd* is currently absent from IL and the historic survey also produces no infections, then state agencies should focus on preventing any future introductions by communicating with members of the pet industry, the horticultural industry and the fisheries industry about the threat of *Bd* introduction, and steps that could be taken to prevent its accidental introduction. Managers could evaluate particular areas that are likely to be infected in the future by either monitoring the population status of focal amphibian species or the disease prevalence of amphibians in regions or habitats of interests. Since surrounding states have documented cases of *Bd*, management might focus on water bodies flowing between two states, or industries and activities that facilitate spread of *Bd* into Illinois. For example, the bait industry, pet industry, anglers and reserve managers may need to be advised of potential contamination of healthy areas from contaminated sources.

4.3) Produce progress reports for IDNR and FWS.

We will provide yearly written progress reports and a final written report from our work to the IDNR, the FWS, and all managers of sites where we conducted the study. We will give presentations at scientific meetings and to the general public.

APPENDIX 1

Table 1: Project schedule for all objectives to be completed. We are on schedule and are making progress on all tasks.

Objective	Sp 08	Su 08	F 0 8	W 08-09	Sp 09	Su 09	F 0 9	W 09-10	Sp 10	Su 10	F 10	W 10-11
Objective 1: Survey protected areas of Illinois for the presence of *Bd*	**X**	**X**			**X**	**X**						
Objective 2: Analyze (PCR) field samples			**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	
Objective 3: Survey for *Bd* in museum specimens (histology)		**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	
Objective 4: Produce Reports and Maps, give presentations at meetings				**X**		**X**		**X**		**X**	**X**	**X**

Table 2: List of specimen holdings for INHS, UIMNH, FMNH, and SIUC by decade for widespread species to be assessed for *Bd* using histologic procedures.

	Specimen Collection Period					
Species	**1950-59**	**1960-69**	**1970-79**	**1980-89**	**1990-99**	**Total**
A. crepitans	283	168	34	9	369	**863**
B. americanus	125	147	52	32	430	**786**
B. fowleri	160	192	32	9	382	**775**
H. chrysoscelis / versicolor	141	25	0	21	151	**338**
R. catesbeiana	86	67	10	14	287	**464**
R. pipiens	23	5	9	7	81	**125**
R. sphenocephala	128	127	7	19	343	**624**
Total	**946**	**731**	**144**	**111**	**2043**	**3975**

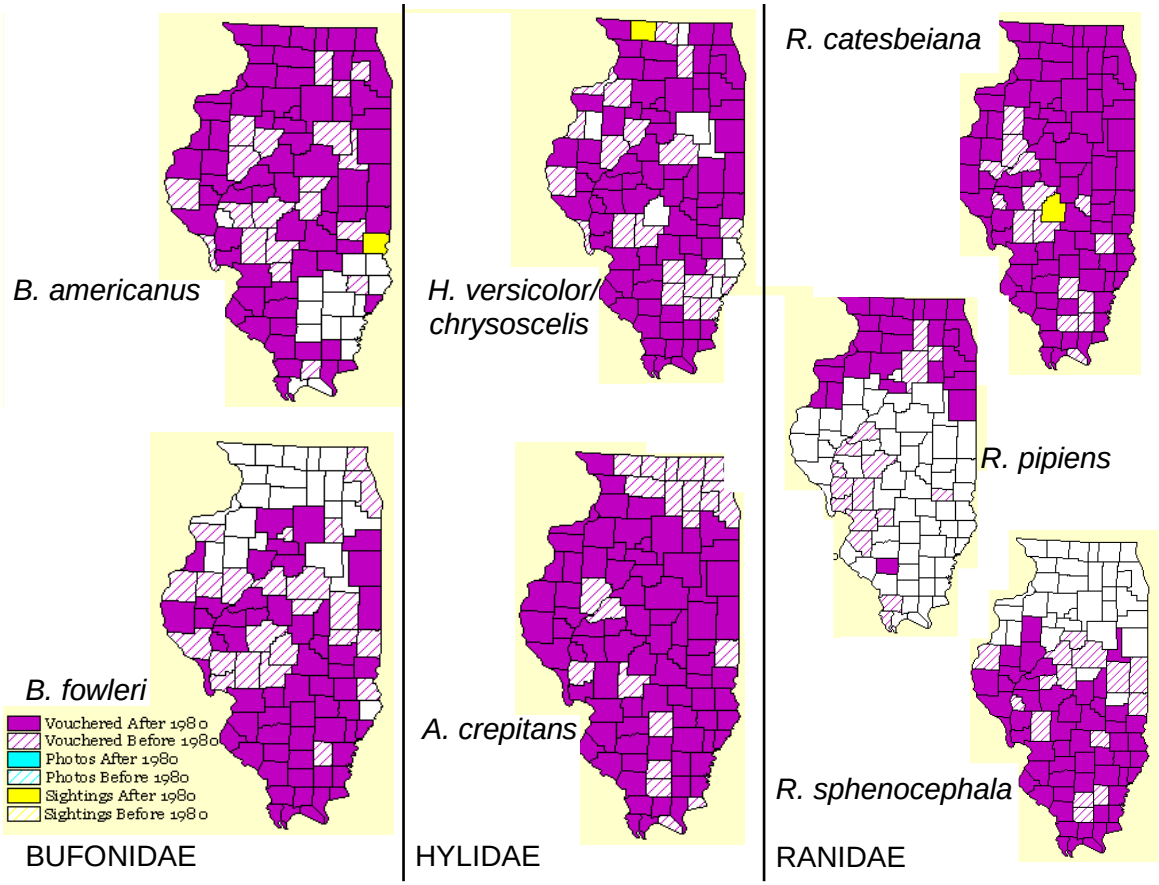

Figure 1: Geographic distributions of focal species for 2009 sampling and histology. Range maps from Illinois Natural History Survey (http://www.inhs.uiuc.edu/animals_plants/herps/ilspecies.html), accessed January 2009, website last updated November 2008.

APPENDIX 2

Preliminary Results from Year 1 (2008):

In January, 2009, I processed 534 swabs collected in the 2008 field season. These swabs came from three large-scale sites ("Midewin", "Kickapoo", and "Cache"), although Midewin was the only site where all swabs collected in 2008 were analyzed (see Figure 3). Midewin swabs were all analyzed since it was hypothesized to be the worst-case scenario site, since it is at the greatest latitude (coolest ambient air temperatures during breeding season) and has had recorded *Bd* presence in previous studies within the same county. Kickapoo is a mid-state site that lies on the eastern boundary of the state. Cache is a southern site; the only samples assessed from the Cache were taken mid-summer (hot ambient air temperature), from two wetlands, and of only two species (*H. avivoca* – a state-listed species, and *H. cinerea*), both of which have restricted ranges. The overall prevalences vary between 0 and 54.1% (Table 3).

Table 3: Overall prevalence values for Preliminary Results from Year 1 (2008) sampling season.

Site	N	*Bd* positive	*Bd* negative	Prevalence (%)	Lower 95% CI	Upper 95% CI
Midewin	355	95	260	26.8	22.2	31.7
Kickapoo	111	60	51	54.1	44.3	63.6
Cache	68	0	60	0	0	5.3

Midewin: Prevalance results from Midewin have been reported by species to look for variation between species. In the geographic area where Midewin lies, *A. crepitans* populations have been reported with *Bd*

in previous studies. Additionally, this seems to be the northeastern-most extent of *A. crepitans* in the state, although its historical range is further north. *A. crepitans* has the largest prevalence of *Bd* infection (62%), while the other historic declining species in the state (*R. pipiens*) has low prevalence (4%) (Table 4). By looking at the prevalence results by wetland, we can see that all wetlands that had frogs to be tested for *Bd* yielded some positive results (Table 5).

Table 4: *Bd* prevalences for Midewin by Species for Preliminary Results from Year 1 (2008) sampling season indicates variation between species.

Species	**N**	***Bd* positive**	***Bd* negative**	**Prevalence (%)**	**Lower 95% CI**	**Upper 95% CI**
Accr	92	57	35	62	51.2	71.9
Buam	20	0	20	0	0	16.8
Hych	33	6	27	18.1	7	35.5
Raca	90	24	66	26.7	17.9	37.0
Racl	41	4	37	9.8	2.7	23.1
Rapi	75	3	72	4	0.8	11.2
Pstr	3	0	3	0	0	70.8
Rabl	2	1	1	50	1.3	98.7

Table 5: *Bd* prevalence for Midewin by Wetland for Preliminary Results from Year 1 (2008) sampling season indicates minimal variation between wetlands, with all wetlands having some positive *Bd* results.

Wetland	N	*Bd* positive	*Bd* negative	Prevalence (%)	Lower 95% CI	Upper 95% CI
A	42	19	23	45.2	29.8	61.3
B	63	38	25	60.3	47.2	72.4
C	61	3	58	4.9	1.0	13.7
D	13	3	10	23.1	5.0	53.8
E	34	6	28	17.6	6.8	34.5
F	49	19	30	38.8	25.2	53.8
H	93	7	86	7.5	3.1	14.9

Kickapoo: Prevalance results from Kickapoo have been reported by species to look for variation between species. Kickapoo has greater *Bd* prevalence values than Midewin, with all species and wetlands tested resulting positive. *R. clamitans* has the greatest prevalence of *Bd* (96%), while *A. crepitans* has the second largest prevalence of *Bd* infection (53%) (Table 6). By looking at the prevalence results by wetland, we can see that all wetlands that had frogs to be tested for *Bd* yielded some positive results (Table 7).

Table 6: *Bd* prevalences for Kickapoo by Species for Preliminary Results from Year 1 (2008) sampling season indicates variation between species, although all have greater prevalence values than expected.

Species	**N**	***Bd* positive**	***Bd* negative**	**Prevalence (%)**	**Lower 95% CI**	**Upper 95% CI**
Accr	19	10	9	52.6	28.9	75.6
Hych	39	11	28	28.2	15	44.9
Pscr	18	9	9	50	26	74
Racl	23	22	1	95.6	78.1	99.9
Buam	8	4	4	50	15.7	84.3
Raca	3	3	0	100	29.2	n/a

Table 7: *Bd* prevalence for Kickapoo by Wetland for Preliminary Results from Year 1 (2008) sampling season indicates minimal variation between wetlands, with all wetlands having positive *Bd* results.

Wetland	N	*Bd* positive	*Bd* negative	Prevalence (%)	Lower 95% CI	Upper 95% CI
D	13	6	7	46.2	19.2	74.9
E	11	7	4	63.6	30.8	89.1
F	4	2	2	50	6.8	93.2
G	1	1	0	100	2.5	n/a
L	81	43	38	53.1	41.7	64.3

Cache: The swabs analyzed for the Cache site are not representative of the entire species assemblage or wetlands sampled. However, none of the 68 samples collected from two species were positive for Bd, for a <15% chance of it being present. Additional analyses of the remaining samples are needed before we can determine whether the southern portion of the state is negative for *Bd*, or whether this simply indicates that that animals collected in mid-summer (when ambient air temperatures are greatest) do not have *Bd* (Table 8), or that these two wetlands do not have *Bd* (Table 9). These are incomplete, but encouraging results, as, *H. avivoca* is a state-listed species, and both *H. avivoca* and *H. cinerea* have extremely limited ranges (Figure 2).

Table 8: *Bd* prevalences for Cache by Species for Preliminary Results from Year 1 (2008) sampling season indicate no *Bd*.

Species	N	*Bd* positive	*Bd* negative	Prevalence (%)	Lower 95% CI	Upper 95% CI
Hyav	44	0	44	0	0	8.0
Hyci	24	0	24	0	0	14.2

Table 9: *Bd* prevalence for Cache by Wetland for Preliminary Results from Year 1 (2008) sampling season indicates no *Bd*

Wetland	N	*Bd* positive	*Bd* negative	Prevalence (%)	Lower 95% CI	Upper 95% CI
F	50	0	50	0	0	7.1
G	18	0	18	0	0	18.5

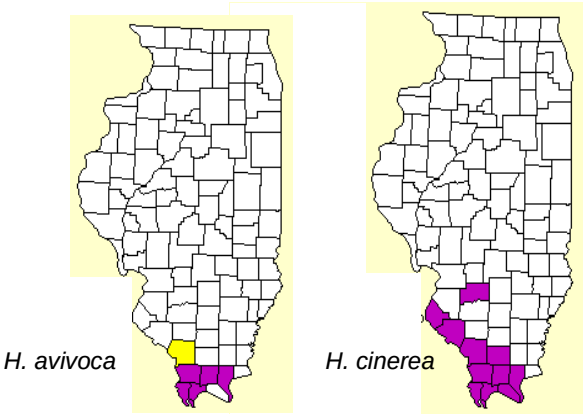

Figure 2: Geographic distribution of vouchered specimens after 1980 (purple) and sightings after 1980 (yellow) for *H. avivoca* and *H. cinerea*. Range maps from Illinois Natural History Survey (http://www.inhs.uiuc.edu/animals_plants/herps/ilspecies.html), accessed January 2009, website last updated November 2008.

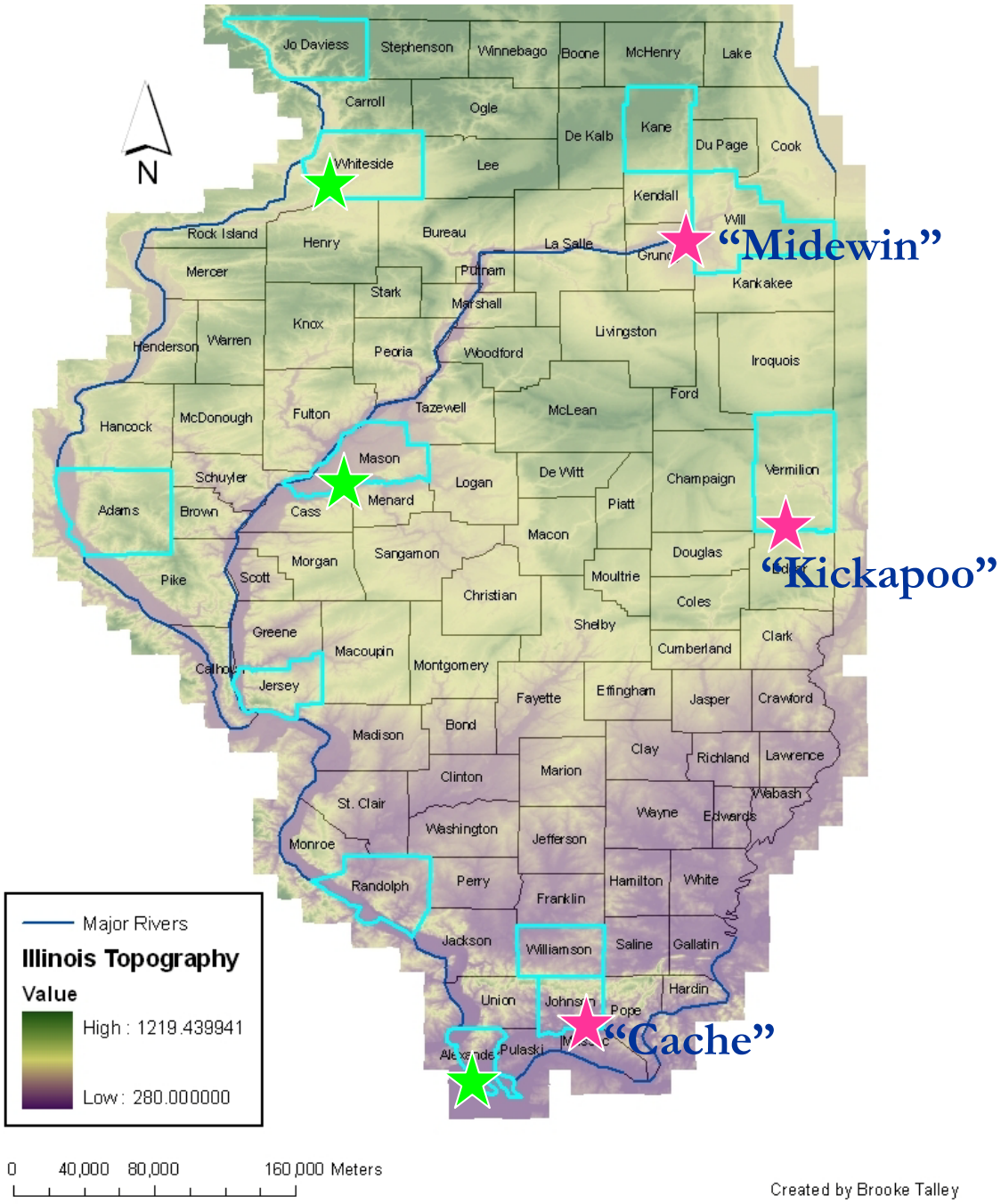

Figure 3: Sample sites from 2008 denoted in Preliminary Results section above are represented as pink stars, while those sampled in 2008 without yet being analyzed are green stars.