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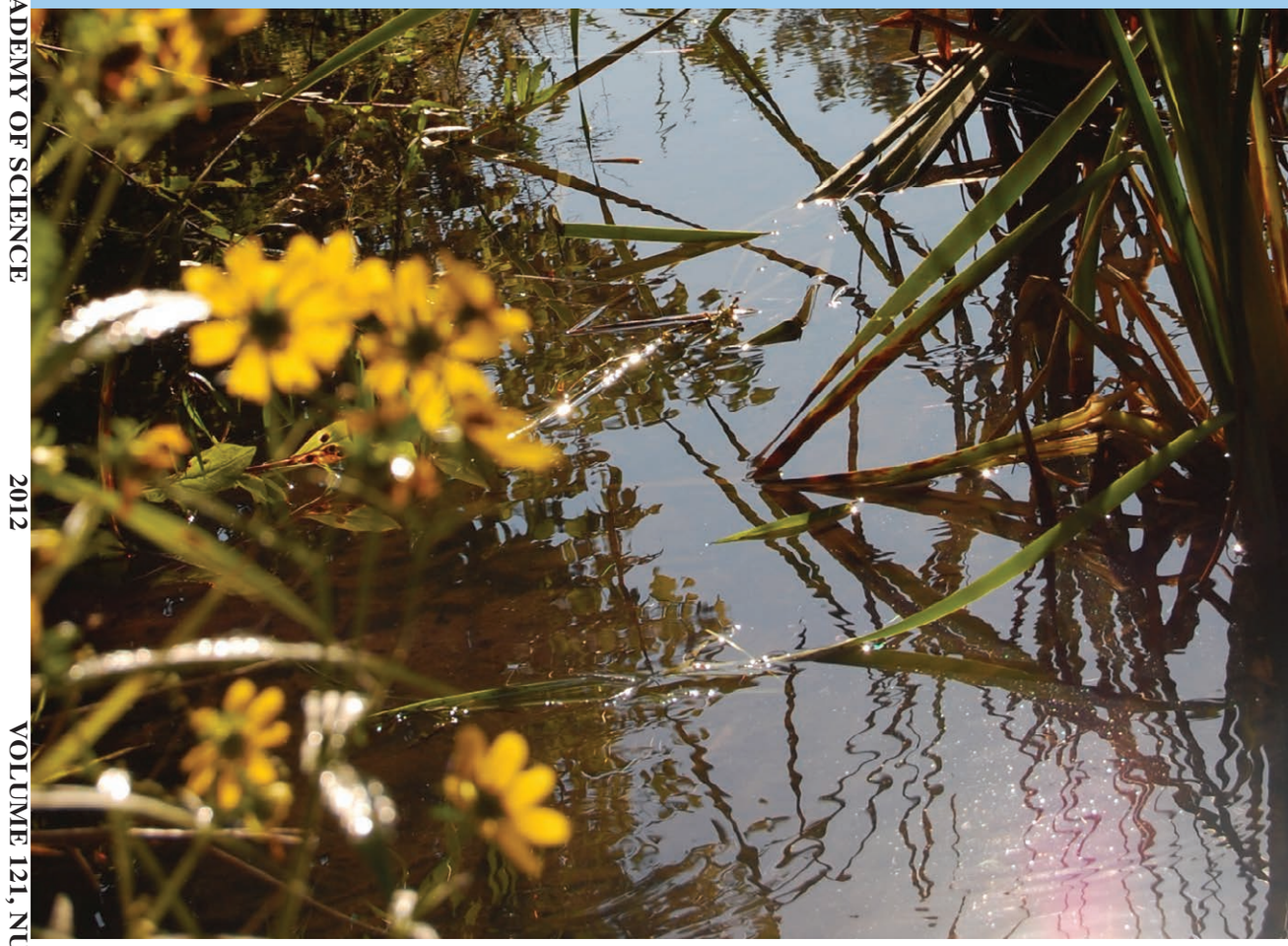

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Cover: Pictured is a beaver-dredged canal in the Nina Mason Pulliam EcoLab on the campus of Marian University in Indianapolis. Canals average a meter wide, about a third of a meter deep, and can be several hundred meters in length. Beaver feeding behavior differs in areas where canals are present because beavers apparently use their canals as transportation networks within areas where they are actively feeding.

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BEAVER-DREDGED CANALS AND THEIR SPATIAL RELATIONSIHP TO BEAVER-CUT STUMPS

Matthew J. Abbott, Brandon Fultz, Jon Wilson, Jody Nicholson, Matt Black, Adam Thomas, Amanda Kot, Mallory Burrows, Benton Schafer and **David P. Benson***: School of Mathematics and Sciences, Marian University, Indianapolis, IN 46222

ABSTRACT. *Castor canadensis* Kuhl (North American beavers) are central place foragers who collect woody plants and building materials from their surroundings and return to a main body of water containing a lodge or food cache. It has been suggested that beavers dredge water-filled canals to extend access to foraging areas; however, the possibility that these engineered transportation routes function as extensions to the beavers' "central place" has yet to be considered. Our objective in this study was to gain a better understanding of the formation and utilization of canals by beavers and thus further elucidate the complex foraging behavior of these ecosystem engineers. During 2004–2011, we mapped beaver ponds, canals, and cut stumps in eight groundwater-fed wetlands, from at least four separate colonies, in Indianapolis, IN. We found that the mean length, depth, and width of the beaver-dredged canals were 604.3 ± 493.1 m, 28.0 ± 22.2 cm, and 107.7 ± 107.1 cm respectively. Two of the canal systems were mapped for multiple years and their length, depth, and width increased over time and supported the prediction that beavers continuously "engineer" these canal systems to extend their foraging area into new locations. In addition, and in contrast to previous studies, we found that the number of beaver-cut stumps was negatively related to distance from canals, but not from the body of water containing their lodges. We recommend that studies of optimal foraging in beavers take canals into account, where applicable, when relating foraging to distance from the "central place."

Keywords: North American beaver, canal, *Castor canadensis*, foraging

INTRODUCTION

As central place foragers, *Castor canadensis* Kuhl (North American beavers) gather food and return to a central location, usually a water body with a lodge, dam, and/or food cache (Aldous 1938, Brenner 1962, Jenkins 1980, Belovsky 1984, Raffel et al. 2009). They feed on herbaceous vegetation as well as the bark and cambium of woody plants including Aspen (*Populus tremuloides*), Willows (*Salix* spp.), Cottonwood (*Populus deltoides*), Ashes (*Fraxinus* spp.), and Maples (*Acer* spp.) (Denney 1952, Hall 1960, Brenner 1962, Belovsky 1984, Roberts and Arner 1984, Baker and Hill 2003). Woody vegetation is either eaten where it is cut, or the stems are transported to a food cache near the lodge or to the lodge itself for construction (Busher 1996).

Beavers usually forage within 100 m of the main water body containing the lodge (Hall 1960, Jenkins 1980, Howard and Larson 1985). Hall (1960) for example, found that 90% of cut stumps were within 35 m of the water body with the lodge. Optimal foraging studies of beavers have found that beavers forage less, and more selectively, the farther they are from the main water body containing the lodge (Jenkins 1980, Belovsky 1984, McGinley and Whitham 1985, Fryxell and Doucet 1991, Raffel et al. 2009). However in some locations, beavers dredge canals apparently to increase accessibility to foraging areas (Berry 1923, Warren 1927, Townsend 1953, Naiman et al. 1986, Rebertus 1986, Johnson and Naiman 1987, Mitchell and Nierring 1993, Butler and Malanson 1994, Gurnell 1998, Rosell et al. 2005). Canals are often flooded by groundwater seeps and can be up to 1 m wide and 100 m long (Berry 1923, Rebertus 1986, Butler and Malanson 1994, Gurnell 1998). How these canals affect their central place foraging behavior has not been studied.

In this study we examine the geomorphology of beaver-dredged canals by measuring and mapping eight canal systems in the Indianapolis,

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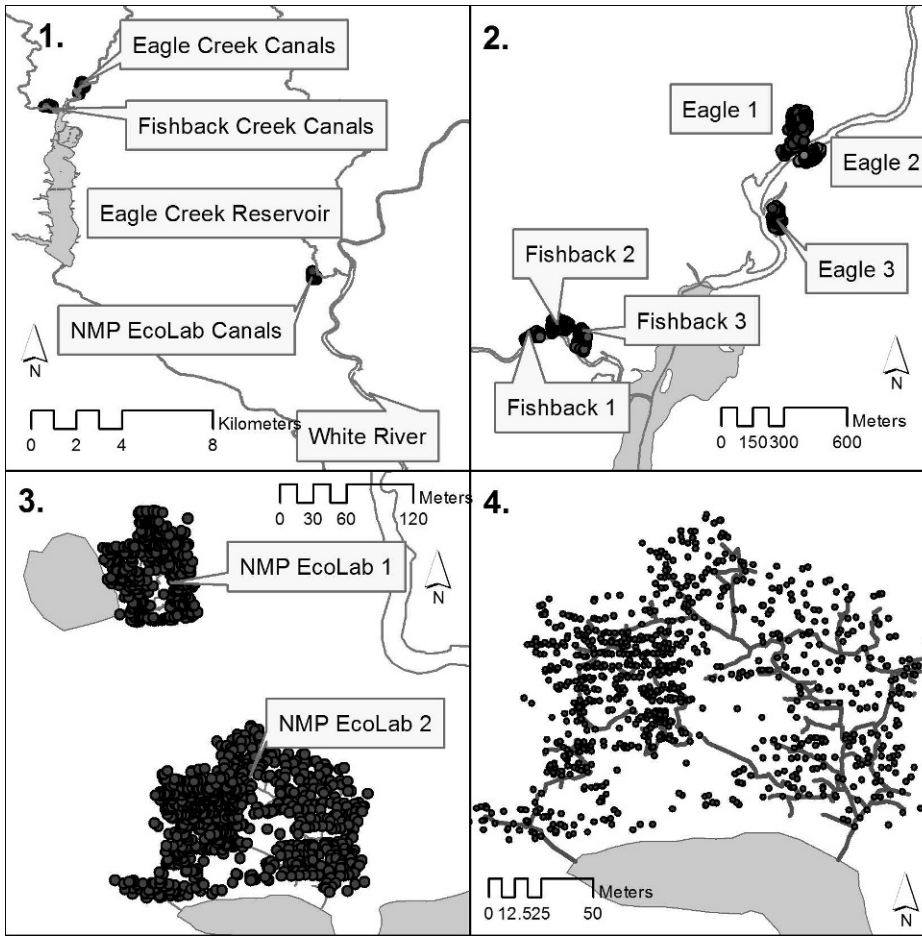

Figure 1.—Beaver-dredged canals and cut stumps in Indianapolis, IN.; (1.) Shows location of Eagle Creek, Fishback Creek, and Nina Mason Pulliam EcoLab beaver-dredged canals in the northwest quarter of Marion County; (2.) Shows the distribution of the Eagle and Fishback Creeks canal systems; (3.) Shows the relationship between the canal systems in the NMP EcoLab; and (4.) The 2009 map of canal system NMP EcoLab 2 showing the relationship between beaver-cut stumps, beaver-dredged canals, and water nbody containing beaver lodges.

IN area. Two of these canal systems were mapped over two consecutive years to examine changes over time. In addition, to assess how canals affect central place foraging behavior, we mapped the distribution of beaver-cut stumps in each of these canal systems.

METHODS

During autumn seasons in 2004–2011 we mapped beaver canals in eight canal systems in Indianapolis, Marion County, IN. Two canal systems were associated with at least one beaver colony on Fishback Creek (FB) (39.884779N, 86.308443W; WGS84), two with at least one colony on Eagle Creek (EC) (39.893472N, 86.297650W; WGS84), and one canal system for each of two colonies at the Nina Mason Pulliam (NMP) EcoLab at Marian University on Crooked Creek (39.818161N, 86.205897W; WGS84; Figure 1). In both the Fishback Creek and Eagle Creek areas, only one lodge was found nearby each, so we assume one colony created the canals in each of the two areas respectively. These canal systems contained primarily groundwater-fed wetlands that were dominated by Willows, Green Ash (*F. pennsylvanica*), Dogwoods (*Cornus racemosa* and *C. amomum*) and American Elm (*Ulmus americana*).

The Fishback Creek canal systems were 1.5km from the Eagle Creek canal systems and both were 11km from the NMP EcoLab canals along Crooked Creek (Figure 1). Canal systems often contain dry segments, segments containing water, and check dams. The Fishback Creek and the Eagle Creek canal systems were connected to their respective creeks by dry segments – essentially a deep-cut trail. The canal systems at the NMP EcoLab each had a check dam separating the canals from the water bodies upon which the lodges were sited. Although the canal systems at the NMP EcoLab were only separated by 120m, we considered them to be created by two different colonies of beaver for the following reasons: 1. there were no signs of movement (trails, tracks, or sightings) between the two areas. 2. there were no cut stumps in the 120m gap between the two areas; and 3. there were two lodges in each of the two areas.

Beaver canals were mapped by walking their length and all tributaries with a hand held Global Positioning System (GPS) unit (accurate to within ±1 m). The canals were defined as gutter-like trails or paths that were dredged lower than the adjacent ground and that were connected to the larger water body containing the lodge. Canals often contained beaver-cut roots, obvious signs of dredging (e.g. pushed/packed mud and debris on the edges of the canal ways), beaver-chewed sticks, and/or beaver-cut stumps. In contrast to a stream or creek, most active canals also contained non-moving water (Berry 1923). Using measuring tape, we measured canal depth at its center, canal width, and water level at the center of each canal at approximately every 10 m along its length. We repeated this mapping procedure for two of the canal systems in 2008 and 2009 to detect changes in canals over time.

To map beaver-cut stumps we walked transects that paralleled the water bodies containing the lodges and were at approximately 10 m intervals and extended to 10 m beyond the farthest canal. We recorded all beaver-cut stumps within 1 m of the transect using GPS. Cut stumps of all sizes were mapped and shrubs with multiple cut stems were considered a single stump. Trees that were girdled without felling were not included. The age of the cut was categorized as ''fresh'' (i.e. youngest) if the surface of the cut was whitish and unblemished, ''old'' if mottled with various shades of gray, and ''rotten and old'' (i.e. oldest) if the cut stump was losing its form. Cut stumps were not mapped in 2008 for the two canal systems that were re-mapped in 2009. In these systems, stumps recorded as ''fresh'' were assumed to be cut in 2009, because they tend to become gray, and thus would be recorded as ''old,'' in less than a year. Minimum distances of cut stumps from canals and water bodies with lodges were calculated using ArcView GIS 9.2 (Environmental Systems Research Institute, Inc., Redlands, CA) and Kendall's rank correlation coefficient ((T) (Kendall 1938)) was used to determine statistical dependence between cut stumps and distance to main water body with lodge or canals. Means are given ± standard deviation.

RESULTS

Geomorphology of beaver-dredged canals.—We mapped a total of 4834 m of beaver-dredged canals in the Indianapolis area (Fig. 1). Collectively, these canals had a mean depth of 28.0 ± 22.2 cm, a mean width of 107.7 ± 107.1 cm., and they contained an average of 13.2 ± 16.2 cm standing water. We found 2662 beaver-cut stumps along the transects with 16% of them being freshly cut, 60% old, and 24% rotten and old.

Between 2008 and 2009 there was a 10% increase in canal length in canal system NMP EcoLab 2 (Figure 1; Table 1). In addition, the average width of the canals increased 21% and the average depth of the canals increased 27% within that same year. In canal system NMP Ecolab 1, there was a 2.5% total increase in canal length, a 6.7% increase in canal width, and an 8.3% increase in canal depth between 2008 and 2009 (Table 1). Evidence of deliberate modification or dredging in both of the canal systems was present in the form of pushed/packed mud and debris on the edges of the canal ways. Of the 932 stumps found in NMP Ecolab 2 in 2009, 26% were freshly cut; while in NMP Ecolab 1, only 2% of the reported 421 stumps were considered fresh (Table 1)

Spatial relationship of beaver-cut stumps to beaver-dredged canals.—In all eight canal systems the number of beaver-cut stumps had a strong inverse relationship with distance from canals ($T = -0.9818$; 2-tailed $p < 0.001$), but not distance from the water bodies containing the lodge ($T = 0.036$; 2-tailed $p = 0.737$) (Table 2 and Fig. 2). Furthermore, 90% of the

Table 1.—Characteristics of eight beaver-dredged canal systems in Indianapolis, IN, 2004–2011. "Main Water 90%" and "canal 90%" are the distances (m) from the main water body containing the lodge or a canal to 90% of the beaver-cut stumps. Means are ± standard deviation.

Canal system	Total length (m)	Mean width (cm)	Mean canal depth (cm)	Mean water depth (cm)	% Flooded	# Stumps mapped	% Fresh-cut stumps	Main Water Pond 90% (m)	Canal 90% (m)
FB1	215	200.4 ± 88.8	50.0 ± 20.9	0	0.00%	83	6.02%	30	12
FB2	253	75.6 ± 28.4	28.9 ± 20.5	0	0.00%	261	11.49%	58	18
FB3	1152	63.1 ± 27.9	17.0 ± 7.8	3.0 ± 4.1	41.67%	206	23.41%	96	20
EC1	1115	170.6 ± 170.2	34.8 ± 24.4	24.5 ± 17.7	100.00%	451	17.29%	154	20
EC2	254	59.0 ± 20.4	29.2 ± 23.2	1.8 ± 4.5	20.00%	130	1.54%	50	16
EC3	201	88.3 ± 84.6	28.9 ± 25.5	3.0 ± 8.3	33.33%	178	3.37%	90	28
NMP1 (2008)	321	120.7 ± 55.6	25.4 ± 9.2	9.1 ± 7.1	100.00%	N/A	N/A		
NMP1 (2009)	329	128.8 ± 52.5	27.5 ± 10.4	16.3 ± 9.9	100.00%	421	2.14%	54	20
NMP2 (2008)	1193	79.2 ± 48.9	23.3 ± 17.4	11.0 ± 12.8	61.04%	N/A	N/A		
NMP2 (2009)	1315	95.8 ± 52.2	29.6 ± 21.0	14.2 ± 19.1	80.33%	932	26.29%	116	16

Table 2.—Spearman rank correlation coefficients showing a strong negative relationship between (a) canals and beaver-cut stumps, but not between (b) main water bodies containing the lodge and cut stumps for eight canal systems in Indianapolis, IN, 2004–2011.

Canal System	(a) Canal (T)	(b) Main Water (T)
FB1	−0.718	−0.005
FB2	−0.771	0.321
FB3	−0.771	0.341
EC1	−0.863	0.303
EC2	−0.716	0.368
EC3	−0.605	0.382
NMP1	−0.920	−0.096
NMP2	−0.926	−0.151

cut stumps in all canal systems were within a range of 30–154 m from the water body with lodge, while 90% of the stumps had a distal range of only 12–28 m from the canals (Table 1). There were no discernible patterns associated with stump age: 90% of fresh stumps were within 8 m of the canals and within 116 m of the main water; 90% of old stumps were within 16 m of the canals and withion 124 m of main water; and 90% of rotten stumps were within 16 m of canals and within 100 m of the main water.

DISCUSSION

Geomorphology of beaver-dredged canals.—The canals described in this study were dredged by beavers and filled with water primarily from groundwater seeps. Our observations were similar to those in other studies that have described canals or "moats" in areas such as peatlands without a constant surface water inflow (Rebertus 1986, Gurnell 1998). However, the canals described in this study were slightly wider and deeper than those that have been found in other areas (Gurnell 1998).

It is apparent that beavers are continuously modifying and lengthening their canal systems over time. In canal systems NMP EcoLab 1 and 2, changes made to the canals (i.e. lengthening, widening, and deepening) between 2008 and 2009 were associated with increased feeding (i.e. more freshly-cut stumps) in the area where those changes took place. This association suggests that the beavers focus their energy on modifying the canals where current food sources are located.

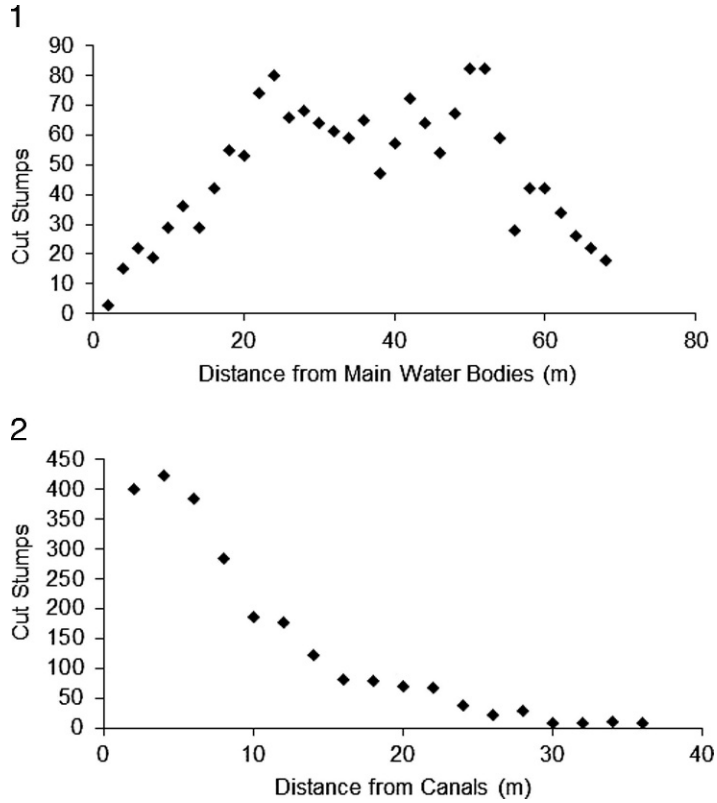

Figure 2.—Distance from main water bodies containing lodges to cut stumps (1.), and from beaver-dredged canals to cut stumps (2.) for beaver-dredged canal systems in Indianapolis, IN, 2004–2011.

In canal system NMP EcoLab 1, the original colony of beavers abandoned the lodge in 2008. The lodge was re-colonized (or at least "visited"), however, by a different colony of beavers just before the canals were re-mapped in 2009 (Pers. Obs.). Consequently, no beaver foraging activity took place for the majority of the time between 2008 and 2009 (Pers. Obs.), this is likely the reason why this canal system did not change as dramatically as the other system and why there was a smaller percentage of fresh (less than one year-old) stumps. When comparing canal systems NMP EcoLab 1 and 2, then, it was evident that the rate of change in canal characteristics was associated with the amount of foraging activity (i.e. fresh stumps) that took place. Canals are likely used as safer and/or easier routes for transporting food back to the lodge or cache (Berry 1923).

Spatial relationship of beaver-cut stumps to beaver-dredged canals.—Most studies of optimal foraging in beavers have found that the distance from a water body with lodge or the "water's edge" is inversely related to the number of woody stems cut (Hall 1960, Jenkins 1980, Belovsky 1984, McGinley and Whitham 1985). We found that the number of beaver-cut stumps was negatively associated with distance from beaver canals, but not from the main water bodies containing the lodges. Therefore, our results suggest that the water body with lidge should not be assumed to be the "water's edge." The beavers in our study utilized their engineered canal systems to forage for 90% of their food sources far beyond the "limited" radius described by Hall (1960) for colonies without canals. Because water filled canals are easier and perhaps safer travel-ways for beavers to use to reach their "central place," when assessing optimal foraging, straight-line distance from a water body with lodge to the lodge or cache may be inadequate to describe the complexity of habitat traversed.

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PHYSICAL AND CHEMICAL LIMNOLOGY OF THREE LAKES WITHIN HOOSIER NATIONAL FOREST

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ABSTRACT. Three reservoirs, Tipsaw, Indian, and Celina lakes, are located in Hoosier National Forest in Perry County, Indiana, have experienced annual fish kills for over a decade. These lakes were evaluated for patterns in their physical and chemical limnology to characterize morphometrics of length, depth, and width and water quality. Chemical variables, including pH, conductivity, major ions, and nutrients, were measured to evaluate water quality differences among lakes. The study lakes were shallow depressions (Z_{mean} = 3.06–5.60 m; Z_{max} = 7.9–17.0 m) and classified as polymictic. The shallow depths typically enabled dissolved oxygen to be distributed throughout the water column and prevented thermal stratification, although pronounced thermoclines developed during 1989, 1991, and 2001. The Carlson trophic index classified these lakes as mesotrophic with the highest index being calculated for Tipsaw (22–52 TI), followed by Indian (12–13 TI), and Celina (5–14 TI). The highest morphoedaphic index (MEI) was observed in Tipsaw (24.3 (mg/L)/ m), followed by Indian (16.8 (mg/L)/m), and Celina (9.1 (mg/L)/m). The MEI values observed in the three lakes translate to catch yields of 20–30 kg/ha. General chemical patterns are towards a stable state or reduction in nitrogen or phosphorus concentrations. No statistically significant trend based on regression of chemical variable by lake over time was observed for any parameter during the study period (ANOVA, $P > 0.05$). The shallow reservoir depths, changing thermocline and oxycline, and reduction in dissolved oxygen can result in increased potential for continuing conditions promoting fish kills.

Keywords: lake morphometrics, bathymetry, water quality, Morphoedaphic index, Carlson trophic index

INTRODUCTION

The National Lake Survey was originally conducted by the U.S. Environmental Protection Agency (EPA) in the 1970's to evaluate the condition of the nation's lakes. A resurgence of interest in cultural eutrophication (Carpenter et al. 1999; Genkai-Kato & Carpenter 2005; Carpenter & Lathrop 2008) and climate change (Soranno et al. 1999; Moore et al. 2009) has resulted in a more rigorous survey design that has been incorporated during the most recent national sampling efforts (US Environmental Protection Agency [EPA] 2010). Limited studies of small reservoirs (< 1000 ha) have previously been conducted nationally and few studies have been conducted in southern Indiana at mostly public lakes (Thornton et al. 1990; Straskrabová & Talling 1994; Wetzel 2001; Clean Lakes Program, unpublished data). The majority of reservoirs (> 20 ha) in southern Indiana are impoundments of streams or rivers (EPA National Lakes Survey, unpublished data). These reservoirs are important for drinking water, recreation, and flood storage capacity. The collection of baseline information for determining patterns in reservoir lake trophic status and for analyzing physical attributes of lentic systems in southern Indiana is needed (Simon et al. 2011).

The morphologic features of lakes are determined by climatic and edaphic factors

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that affect the chemical dynamics of the lake, which in turn, shape the biota within these ecosystems (Ryder et al. 1974; Carlson 1977; Wetzel 2001; Simon & Simon in review). Investigations of the physical and chemical environment of reservoirs in southern Indiana have not been similarly studied as northern Indiana lakes (Blatchley & Ashley 1901, Evermann & Clark 1920), and are unparalleled in similar intensity or duration. Perhaps the best-studied lake in southern Indiana has been Foots Pond, which is a natural oxbow lake of the Wabash River drainage (Hubbs & Lagler 1942; Lagler & Ricker 1942; Ricker & Lagler 1942). No studies of the physical and chemical limnology of reservoirs in southern Indiana ($<$ 1000 ha) have been conducted during either the 2007 or 2012 National Lake Surveys. Only the State sponsored Clean Lakes Program, which is supported by the State of Indiana Department of Environmental Management, conducts annual monitoring. Chemical measures of public lakes have been conducted annually, with additional support of the National Lake Survey during 2007 and 2012. These studies focus primarily on water quality and trophic state determination.

This investigation was conducted with emphasis on determining contributing factors causing annual fish kills. Our objective was to describe the chemical and physical characteristics of three reservoirs in Hoosier National Forest in southern Indiana focusing on eutrophication, hypoxia, and physical morphometrics. These reservoirs have experienced fish kills for the last decade (Simon 2011; Simon et al. 2011). While many aspects of the biota can be determined without knowledge of the physical and chemical characteristics of these lakes, many of the indices of productivity cannot be determined without this information (Ryder et al. 1974). We compare our results from 2005 and 2012 data to Clean Lakes Program data for these same lakes to determine trophic status and morphometric relationships.

MATERIALS AND METHODS

Description of the study area.—The landscape of southern Indiana has few natural lakes (Omernik & Gallant 1988); however, southern Indiana contains 2,729 impounded lakes that were created by damming small streams and moderate sized rivers. Reservoirs are artificial systems that are built when valley ridges were closed and stream channels flooded these areas. Hoosier National Forest occupies an area that is part of the Mitchell Plain (Schneider 1966) and is considered a portion of the Central Corn Belt Plain and Interior Plateau Ecoregion (Omernik & Gallant 1988). The study area incorporates the Corn Belt and Great Plains nutrient ecoregions (Morris & Simon 2012). The study area in Hoosier National Forest is 820.8 km^2 and is managed by the U.S. Forest Service and is primarily composed of natural watersheds that are without heavy anthropogenic impact in the surrounding watersheds (Figure 1). All lake soils were dominated by Deuchars and Markland soils (USDA 2006). Celina, Tipsaw, and Indian lakes are associated with the damming of streams in the Middle Fork Anderson River (Simon 2011) and were investigated for limnological attributes of physical and chemical parameters following the protocols of the National Lake Survey (EPA 2007).

Regional climate and hydrology.—The prevailing climate in southern Indiana is temperate continental, which is modified by the Ohio River so that the climate can take on semi-marine characteristics (National Oceanic and Atmospheric Administration 2003). The mean annual temperature is 13.96° C (National Oceanic and Atmospheric Administration, NCDC 2012). Average annual precipitation at Tell City (the largest nearby city) from 1981–2010 is about 1230 mm; normal seasonal precipitation averages 94.8 mm in the winter, 122.3 mm in the spring, 93.7 mm in the summer, and 100.1 mm in the fall (National Oceanic and Atmospheric Administration, NCDC 2012). Total monthly rainfall is more variable during warm months than during cold months. The total annual precipitation is 1230 mm. The maximum average precipitation occurs in May with an average of 147.75 mm. Annual average snowfall is 112 mm at Tell City, with the predominant snow season from November to March. The coldest month (January) has an average normal monthly temperature of 2.0 °C; the average normal monthly temperature during the warmest month (July–August) is 31 °C.

The reservoirs of Hoosier National Forest (lat 38.206647°N, lon −86.650334°W to lat 38.121382°N, lon −86.645664°W) comprise the only lakes occurring in Perry County greater than 20 ha surface area. Indian, Tipsaw, and

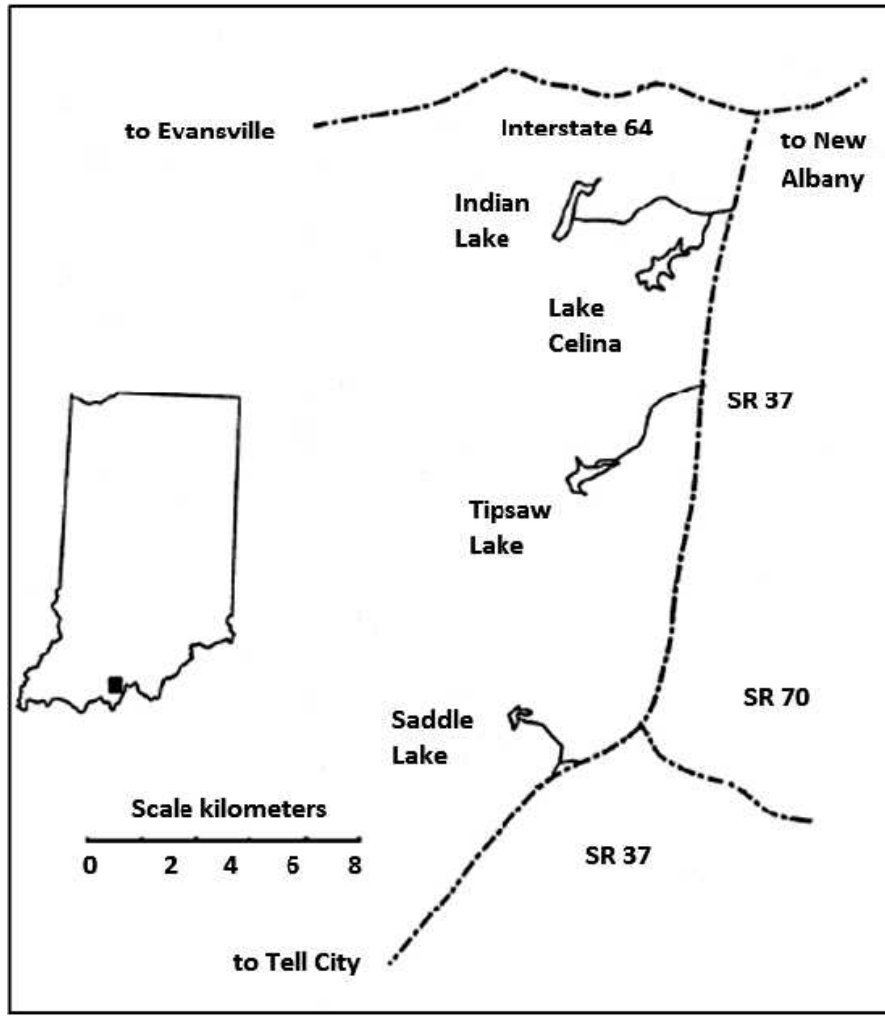

Figure 1.—Study area showing the location of three Hoosier National Forest reservoirs in Perry County.

Celina Lakes are all headwater lakes of the Middle Fork Anderson River drainage. We evaluated three of the largest waterbodies, which were all built during the mid-1960's as recreational lakes.

Bathymetry, Morphometry, and Chemical Limnology.—The bathymetry contour maps of the three lakes (Figures 2–4) were prepared by systematic transect measurement of water column depths using a classical grid design (Wetzel & Likens 1979; Cole 1994). The size and complexity of each lake determined the distance between transects and the number needed to obtain an appropriate bathymetric profile. Tipsaw Lake, the smallest of the three lakes at 53.0 ha, was mapped at 30 m transect intervals. Lake Celina, the largest lake at 66.4 ha, was mapped using a 50 m transect interval as was Indian Lake, which is 61.5 ha. Transect points were established along shore using calibrated measurement tapes to create a square grid profile around the perimeter of each lake. Survey points were marked along shore using flagging at indicated transect distances. Offshore depths were measured at intervals by determining the perpendicular intersection of adjacent shorelines markers and then measuring the distance between points using a calibrated depth finder and Geographic Positioning System (GPS). Transects were measured from a boat under power that was maintained until the latitude and longitude

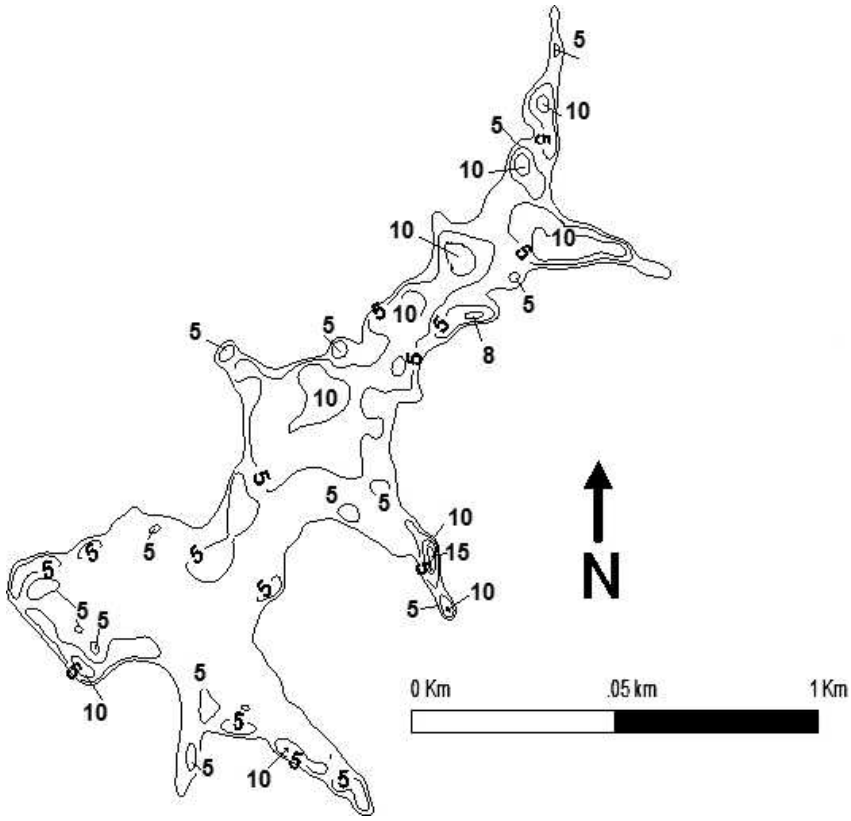

Figure 2.—Bathymetric contour map of Lake Celina based on survey from 16–17 September 2005. Depth contour values in m.

could be recorded and verified along each transect intersection point. A boat mounted Humminbird 3-D depth-finder was used to record depths in non-wadeable areas, while a Philadelphia rod was used to obtain depths in wadeable areas. Depth coordinates were plotted using the GPS reading and a detailed bathymetric map was drawn using Surfer 11.0 (Golden Software 2011). Basin slope was measured using original U.S. Geological Survey 7.5 minute 1:24,000 topographic maps for the length of the reservoir segment. The shoreline perimeter was verified using U.S. Geological Survey 1:24,000 topographic maps and aerial photographs.

Morphometric parameters were calculated from maps following the procedure of Lind (1985) and Wetzel & Likens (1979). Surface morphometric measures included maximum length (l), maximum width (b_{max}), mean width (b_{mean}), surface area (A), shoreline length (L), shoreline breadth and the shoreline development index (D_L). Subsurface morphometrics included volume (V), maximum depth (z_{max}), mean depth (z_{mean}), relative depth (z_r), and basin slope. Edaphic factors are measured by the Morphoedaphic index to evaluate fish productivity, while eutrophication is measured using the Carlson's trophic index based on TP and chlorophyll. Morphoedaphic index calculations followed Ryder et al. (1974) and Carlson's trophic index (TI) followed Carlson (1977). Temperature and dissolved oxygen were measured at the deepest point in each waterbody at 1-m intervals. Chemical measurement, sampling period, and number of samples followed the National Lake Survey protocols with samples completed during a single sample period between June and September (EPA 2007; Clean Lakes Program 2012).

Water samples were taken from the epilimnion and hypolimnion in the deepest portion of the lake using a Kemmerer sampler (Clean Lakes Program 2001; EPA 2007). A digital

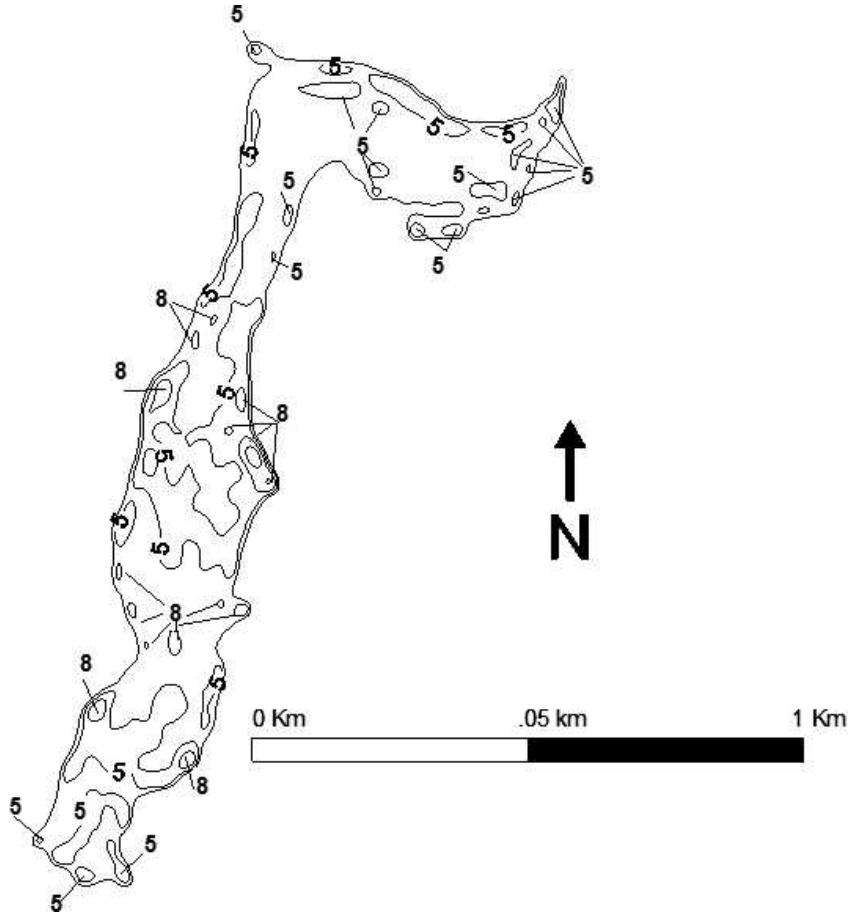

Figure 3.—Bathymetric contour map of Indian Lake based on survey from 16–17 September 2005. Depth contour values in m.

meter (Dow Corning, Inc, Pocket Meter M90) was used to measure dissolved oxygen (DO 0.0–20.00 ± 0.1 mg/L), temperature (−0.5 °C to 100 °C ± 0.1 °C), pH (0 to 14 ± 0.1 SU), specific conductance (0.0 to 1999 ± 1 μS), and total dissolved solids (TDS; 0.0 to 1000 ± 0.1 mg/L). The oxidation-reduction potential (E_h) was measured using a digital meter (LaMotte, Inc, ORPTestr, −200 to 1100 ± 5 mv). Dissolved oxygen was calibrated using a Winkler titration (American Public Health Association 1989) based on Kemmerer sample from the epilimnion and hypolimnion. This parameter was taken from the deepest portion of the lake during all sampling years. During 2005 additional samples were collected from random nearshore areas within each lake as single grab samples. Water samples (n = 10) were taken from within and among each reservoir following the National Lake Survey protocol (EPA 2007). This same protocol is used by the Indiana Clean Lakes Program, which includes a single epilimnion and hypolimnion sample. Laboratory procedures following National Lake Survey procedures (EPA 2007) and quality assurance included field duplicates, blanks, and replicate samples measurement after every 20 samples (Indiana Department of Environmental Management 1986; Clean Lakes Program 2012).

RESULTS AND DISCUSSION

Physical characteristics.—Celina Lake is on the Winding Branch River and is used for recreation and flood control purposes. Its construction was completed in 1969 and it has a normal surface area of 61 ha (153 acres). It is owned by the Department of Agriculture, U.S.

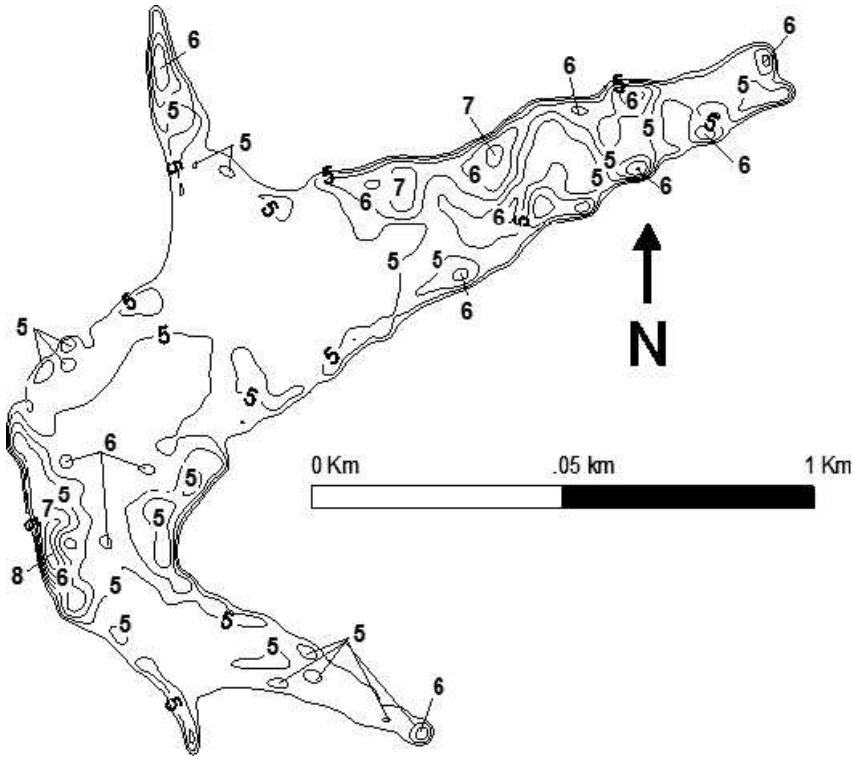

Figure 4.—Bathymetric contour map of Tipsaw Lake based on survey from 16–17 September 2005. Depth contour values in m.

Forest Service. Celina Lake has a dam of earthen construction. The foundation is rock with a soil core. Its dam height is 26.2 m (86 ft) with a length of 310.9 m (1020 ft). Maximum discharge is 2.55 m^3/sec (90 ft^3/sec). Its capacity is 589.97 ha m (4783 acre ft). It drains an area of 7.77 km^2 (3 $miles^2$).

Indian Lake is on the Middle Fork-Anderson River and is used for recreation and flood control purposes. Construction was completed in 1968. It has a normal surface area of 60 ha (149 acres) and is owned by the Department of Agriculture, U.S. Forest Service. The Indian Lake dam is of earthen construction with an earthen core and a rock foundation. Its height is 24.1 m (79 ft) with a length of 289.6 m (950 ft). Maximum discharge is 8.16 m^3/sec (288 ft^3/sec). Its capacity is 471.2 ha m (3820 acre ft). Normal storage is 210.7 ha m (1708 acre ft). It drains an area of 44.03 km^2 (17 $miles^2$).

Tipsaw Lake is used for recreation and flood control purposes. Construction was completed in 1967. Tipsaw Lake has a dam created from earthen construction. The core is earth with a foundation of rock and soil. Its length is 376.4 m (1235 feet). It drains an area of 23.3 km^2 (9 $miles^2$).

Bathymetry maps of the three reservoirs are shown in Figure 2–4, and lake morphometrics are listed in Table 1. All three reservoirs have surface area-to-volume ratios that are very low, a feature uncharacteristic of reservoirs in southern Indiana. The deepest lake was Lake Celina (z_{mean} = 5.6 m), followed by Indian Lake (z_{mean} = 3.50 m), while the shallowest lake was Tipsaw Lake (z_{mean} = 3.06 m). The basin slope (M) of these lakes ranges from 22.4 to 32.1, confirming the shallow depths when compared to the lake surface area. The shoreline-development index (D_L) also shows little variation; the index ranges from 2.07 to 2.91 (Table 1). This index reflects the potential for greater development of littoral communities in proportion to the volume of the lake (Wetzel 2001). The shoreline development index values observed from the three Hoosier National Forest reservoirs are consistent with those from

Table 1.—Morphometric characteristics for three lakes in Hoosier National Forest calculated from bathymetry mapping in September 16–17, 2005. All measurements are in m unless otherwise specified. Shoreline development is unitless.

Measurement	Lake Celina	Indian Lake	Tipsaw Lake
Maximum length (l)	1667	1857	1536
Maximum depth (z_m)	17.0	8.8	7.9
Mean width (b)	341	328	364
Mean depth (z)	5.60	3.46	3.06
Relative depth (z)	2.00	0.78	0.97
Maximum width (b)	1007	688	872
Perimeter (L)	7,776.0	5,982.6	5,548.4
Shoreline Development (D_L)	2.91	2.07	2.18
Surface Area (A) (ha)	66.4	61.5	53.0
Volume development (D_v)	1.29	1.50	1.16
Basin slope (M) (%)	31.0	32.1	22.4
Morphoedaphic index (MEI)	9.1 (mg/L)/m	16.8 (mg/L)/m	24.3 (mg/L)/m

most lakes that develop increased littoral regions (Wetzel 2001). The lake orientation is from east to west in latitudinal profile, possessing elongate basins, with a moderately irregular shoreline.

All three lakes are subject to atmospheric inputs primarily as a result of atmospheric deposition. The majority of the allochthonous input into the lakes comes from runoff from the sloped forested, riparian shoreline. All three lakes exist in a forested landscape, thus leaf litter is received from the adjacent deciduous oak-hickory forest. The shallow littoral zones of all of these lakes contribute to their eutrophic condition because dissolved oxygen and light penetrates to the benthic region despite the presence of suspended solids in the water column.

The three lakes did not thermally stratify during 2005, but did display weak thermocline development in 2011 and 2012 and strongly stratified during 1989, 1991, 1996, and 2001 (Figure 5). Dissolved oxygen was present in the entire water column, although at low levels in the hypolimnion, and a permanent oxidized microzone was present based on nearly 90% of water quality redox measurements (Table 2–4). The mean dissolved oxygen level for Lake Celina and Indian Lake was 6.3 mg/L, while Tipsaw Lake had a mean dissolved oxygen level of 4.7 mg/L. The amount of dissolved oxygen is strongly linked to temperature. The lowest amount of dissolved oxygen was detected in the hypolimnion (0.01 in Lake Celina, and 0.2 mg/L in both Indian and Tipsaw). Supersaturated dissolved oxygen levels occurred in Tipsaw Lake in response to the primary productivity of the large aquatic macrophyte community that was found in the littoral zone. These diel fluctuations occurred because supersaturated diurnal and nocturnal respiration in the large plant beds caused an oxygen deficit, which was observed in both Indian and Tipsaw lakes (Simon et al. 2011).

Chemical characteristics.—The range of pH for each reservoir ranged from 6.4 to 10.9 (Table 2–4). The range of pH values observed during the annual studies had the highest range from Indian Lake (6.4–10.9 SU), followed by Tipsaw (6.9–9.3 SU), and lowest in Celina (6.4–8.3 SU). Extreme pH value shifts were observed in Indian Lake during 2005, which showed the largest range between the epilimnion and hypolimnion (Table 3).

Specific conductance is a measure of the ability of a substance to conduct electricity across a unit length at a specific temperature. Dissolved ions increase the conductivity of water; measurements of specific conductance provide an indication of the amount of dissolved substances in water (Hem 1985). The specific conductance of pure water is low, usually less than 10 μS/cm (Hem 1985). In general, the surface waters of our study lakes had moderate conductance. Conductivity was consistent within lake and ranged from 54.7 to 150.3 μS/cm. The lowest conductivity was observed in Lake Celina (mean=99.5 μS/cm), followed by Indian (116.1 μS/cm), and Tipsaw (150.3 μS/cm) exhibited the highest level (Table 2–4).

Alkalinity measures the capacity of a solution to neutralize acids (Hem 1985). In this study, alkalinities ranged from 64 to 164 mg/L

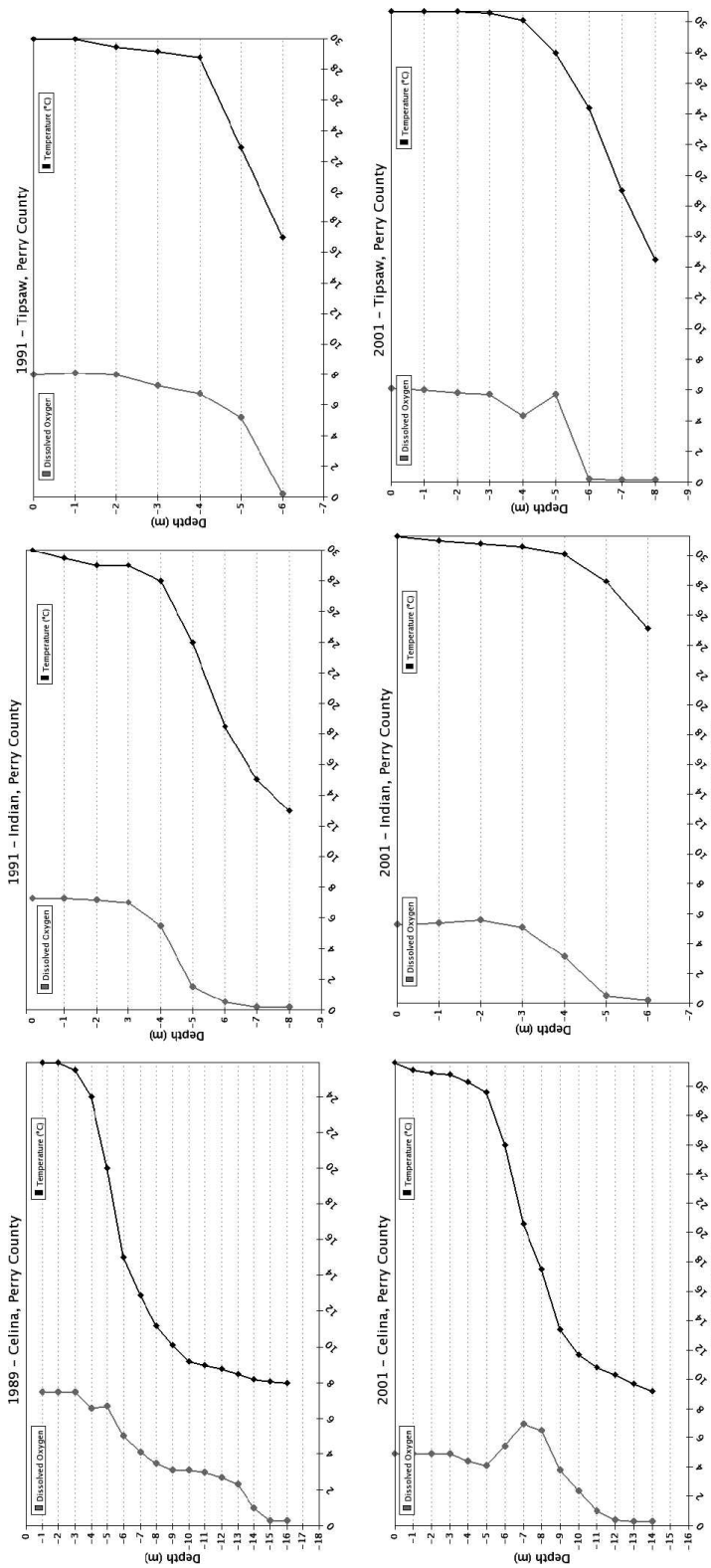

Figure 5.—Depth related dissolved oxygen and temperature thermocline profiles for Lakes Celina, Indian, and Tipsaw for 1989–1991 and 2001 study periods.

Table 2.—Mean, standard deviation, and range in parentheses for field and water chemistry data collected during July 9, 1989, July 22, 1996, August 7, 2001, September 16, 2005, and September 3, 2012 at Lake Celina in Hoosier National Forest. N = samples for each lake collected from the epilimnion and hypolimnion at the deepest portion.

Parameters	1989 (n = 2)	1996 (n = 2)	2001 (n = 2)	2005 (n = 10)	2012 (n = 2)
Alkalinity (mg/L as $CaCO_3$)	39.3 ± 0.92 (38.6–39.9)	36.1 ± 6.08 (31.8–40.4)	30.5 ± 2.12 (29–32)	64 ± 5.65 (60–68)	51 ± 4.2 (48–54)
Hardness (mg/L as $CaCO_3$)	–	–	–	91.5 ± 12.02 (83–100)	89.5 ± 52.1 (83–96)
pH (SU)	6.7 ± 0.42 (6.4–7.0)	7.43 ± 0.88 (6.8–8.1)	7.6 ± 0.99 (6.9–8.3)	7.08 ± 0.29 (6.5–7.6)	7.24 ± 1.11 (6.5–8.0)
Temperature (°C)	13.6 ± 7.2 (8.0–25.9)	17.3 ± 7.2 (10.6–28.1)	19.3 ± 9.4 (9.2–30.9)	25.1 ± 1.9 (22.8–26.8)	21.0 ± 2.7 (9.2–31.6)
Salinity (ppt/L)	–	–	–	0.0 ± 0.0 (0.0–0.0)	0.0 ± 0.0 (0.0–0.0)
Specific Conductance (μS/ cm)	126 ± 22.6 (110–142)	120 ± 15.6 (109–131)	160.5 ± 41.7 (131–190)	102.1 ± 32.6 (75.7–186.3)	143.6 ± 2.12 (98.7–188.5)
Dissolved Oxygen (mg/L)	3.8 ± 2.5 (0.3–7.5)	2.3 ± 3.4 (0.1–7.7)	3.5 ± 2.2 (0.3–7.0)	6.3 ± 1.27 (4.2–8.8)	3.7 ± 2.7 (0.3–6.5)
Dissolved Oxygen (% saturation)	92	97.1	66	79.6 ± 19.0 (50–110%)	87.7
NH_3 (mg/ L)	0.224 ± 0.27 (0.035–0.412)	0.091 ± 0.10 (0.018–0.164)	0.029 ± 0.01 (0.02–0.037)	< 0.0001 ± 0.0 (< 0.0001)	0.022 ± 0.01 (0.015–0.028)
Nitrate + Nitrite (mg/L)	0.36 ± 0.08 (0.303–0.412)	0.10 ± 0.09 (0.039–0.164)	0.08 ± 0.09 (0.013–0.144)	0.30 ± 0.14 (0.20–0.40)	0.30 ± 0.14 (0.20–0.40)
Total Kieldahl Nitrogen (mg/L)	0.58 ± 0.18 (0.447–0.703)	0.31 ± 0.06 (0.27–0.35)	0.45 ± 0.06 (0.41–0.49)	0.55 ± 0.07 (0.5–0.06)	0.42 ± 0.11 (0.34–0.50)
Total Phosphorus (mg/L)	0.02 ± 0.006 (0.015–0.024)	0.03 ± 0.01 (0.023–0.043)	0.02 ± 0.004 (0.016–0.023)	0.045 ± 0.007 (0.039–0.05)	0.038 ± 0.018 (0.025–0.05)
SRP (mg/L)	0.02 ± 0.007 (0.015–0.025)	0.006 ± 0.001 (0.005–0.007)	0.01 ± 0.0 (0.01–0.01)	–	–
Oxidation-Reduction Potential (E_h; mV)	–	–	–	569 ± 255.9 (241–960)	569 ± 255.9 (241–960)
Total Dissolved Solids (mg/L)	–	–	–	50.9 ± 16.5 (37.5–93.1)	50.9 ± 16.5 (37.5–93.1)

as calcium carbonate. Lake Celina had the lowest alkalinity (mean 52.9), followed by Indian Lake (102.9) and Tipsaw Lake (118.5) had the highest alkalinity (Table 2–4).

Water is considered very hard when values exceed 180 mg/L as calcium carbonate. Lake Celina had hard water (mean = 91.5 mg/L calcium carbonate), while Lake Tipsaw (mean = 232 mg/L) and Indian Lake had very hard water (mean = 334 mg/L).

The dissolved solids ranged from 27.7 to 188.9 mg/L, while the mean dissolved solids was lowest for lakes Celina (49.6 mg/L), followed by Indian (58.2 mg/L), and Tipsaw (74.3 mg/L) had the highest levels.

The oxidation-reduction potential (E_h) of water is an index of the exchange activity of electrons among elements in solution. E_h measures the electric potential, using the potential of a hydrogen electrode as a reference point of zero. A positive potential indicates oxidizing conditions in the water; a negative potential indicates reducing conditions, which determines the valence state of metals (Hem 1985). The oxidation-reduction potential of the study lakes ranged in a stepwise progression from −66 to 1020 mv, while the mean for lakes was lowest in Indian (376.9 mv), followed by Celina (417.8 mv), and highest in Tipsaw (558.4 mv). Reducing conditions have been observed

Table 3.—Mean, standard deviation, and range in parentheses for field and water chemistry data collected during July 23, 1991, August 7, 2001, September 16, 2005, and September 3, 2012 at Indian Lake in Hoosier National Forest. N = samples for each lake collected from the epilimnion and hypolimnion at the deepest portion.

Parameters	1991 (n = 2)	2001 (n = 2)	2005 (n = 10)	2012 (n = 2)
Alkalinity (mg/L as $CaCO_3$)	79.8 ± 31.2 (58–102)	62.0 ± 7.08 (57–67)	119 ± 0.00 (119–119)	86 ± 33.9 (62–110)
Hardness (mg/L as $CaCO_3$)	–	–	232 ± 0.0 (232–232)	142.5 ± 62.1 (98–187)
pH (SU)	7.8 ± 0.99 (7.1–8.5)	8.25 ± 1.34 (7.3–9.2)	7.33 ± 0.96 (6.8–10.9)	8.15 ± 1.34 (7.2–9.1)
Temperature (°C)	24.6 ± 6.6 (13.0–30.0)	29.8 ± 2.1 (25.1–31.3)	25.4 ± 0.9 (24.3–27.3)	25.85 ± 8.4 (13.4–31.1)
Salinity (ppt/L)	–	–	0.0 ± 0.0 (0.0–0.0)	0.0 ± 0.0 (0.0–0.0)
Specific Conductance (μS/ cm)	212.5 ± 3.5 (210–215)	232.5 ± 17.7 (220–245)	116.1 ± 46.36 (77–188)	232.5 ± 30.4 (211–254)
Dissolved Oxygen (mg/L)	4.4 ± 3.3 (0.2–7.3)	3.8 ± 2.3 (0.2–5.0)	4.71 ± 2.12 (0.8–6.7)	2.9 ± 2.9 (0.2–5.6)
Dissolved Oxygen (% saturation)	98.3	74	61.6 ± 27.24 (9–94%)	74.2
NH_3 (mg/ L)	0.269 ± 0.35 (0.018–0.52)	0.074 ± 0.072 (0.023–0.125)	< 0.0001 ± 0.0 (< 0.0001)	0.074 ± 0.07 (0.023–0.125)
Nitrate + Nitrite (mg/L)	0.184 ± 0.06 (0.143–0.225)	0.013 ± 0.0 (0.013–0.013)	< 0.001 ± 0.0 (< 0.001)	0.02 ± 0.007 (0.015–0.025)
Total Kieldahl Nitrogen (mg/L)	1.323 ± 0.80 (0.757–1.889)	0.537 ± 0.17 (0.417–0.657)	< 0.001 ± 0.0 (< 0.001)	0.528 ± 0.18 (0.40–0.66)
Total Phosphorus (mg/L)	0.54 ± 0.02 (0.039–0.069)	0.035 ± 0.02 (0.020–0.049)	< 0.001 ± 0.0 (< 0.001)	0.033 ± 0.02 (0.015–0.05)
SRP (mg/L)	0.01 ± 0.0 (0.01–0.01)	0.01 ± 0.0 (0.01–0.01)	–	–
Oxidation-Reduction Potential (E_h; mV)	–	–	644 ± 250.6 (270–1020)	569 ± 255.9 (241–960)
Total Dissolved Solids (mg/L)	–	–	58.2 ± 23.1 (39.2–94.4)	50.9 ± 16.5 (37.5–93.1)

previously during nocturnal periods in Lakes Celina and Indian (Simon et al. 2011), while oxidized conditions were always observed in Tipsaw Lake. In a simultaneously conducted study, both Lakes Celina and Indiana were in an oxidized condition during 90% of the diel measurements (Simon et al. 2011).

The concentration of nitrate plus nitrite, ammonia, and total nitrogen and total phosphorus were determined. Nitrogen concentrations were generally low; NO_3, NO_2, and NH_3 occurred in concentrations of less than 0.3 mg/L. Ammonia concentrations ranged from below detection limits in all three lakes to the maximum measured highest concentrations in Indian (0.269 mg/L), followed by Celina (0.224 mg/L), with the lowest maximum concentrations in Tipsaw (0.057 mg/L). Total Kieldahl Nitrogen was highest between 1989–1991 with the highest concentrations in Indian (mean 1.323), followed by Tipsaw (mean 0.08 mg/L), and lowest concentrations in Celina (0.58 mg/L). Mean total phosphorus concentrations were lowest for Lake Celina (0.04 mg/L), followed by Tipsaw (0.045 mg/L), and highest in Indian (0.54 mg/L). The surface waters of the three study lakes all had low concentrations of nutrients. Ammonia concentrations were less than concentrations that would be toxic to aquatic life. The concentration of nitrate plus nitrite was low, ranging from below detection limits < 0.001 to 0.3 mg/L. Indian Lake had the lowest mean concentration of nitrate plus nitrite (0.184 mg/L), followed by Tipsaw Lake (mean

Table 4.—Mean, standard deviation, and range in parentheses for field and water chemistry data collected during July 23, 1991, August 7, 2001, September 16, 2005, July 18, 2011, and September 3, 2012 at Tipsaw Lake in Hoosier National Forest. N = samples for each lake collected from the epilimnion and hypolimnion at the deepest portion.

Parameters	1991 (n = 2)	2001 (n = 2)	2005 (n = 10)	2011 (n = 2)	2012 (n = 2)
Alkalinity (mg/L as $CaCO_3$)	59.3 ± 0.71 (59–60)	80.0 ± 46.7 (47–113)	164 ± 0.00 (164–164)	45 ± 1.41 (44–46)	62 ± 8.49 (56–68)
Hardness (mg/L as $CaCO_3$)	–	–	334 ± 0.0 (334–334)	–	142.5 ± 62.9 (98–187)
pH (SU)	8.0 ± 1.41 (7.0–9.0)	8.4 ± 1.27 (7.5–9.3)	6.97 ± 0.17 (6.8–7.6)	8.0 ± 1.27 (7.1–8.9)	8.0 ± 1.56 (6.9–9.1)
Temperature (°C)	27.2 ± 4.7 (17.0–30.0)	26.9 ± 5.8 (14.5–30.7)	24.5 ± 1.5 (21.8–26.9)	30.2 ± 3.0 (25.7–32.5)	26.1 ± 8.0 (16.5–32.6)
Salinity (ppt/L)	–	–	0.1 ± 0.0 (0.1–0.1)	–	0.1 ± 0.0 (0.1–0.1)
Specific Conductance (µS/ cm)	200.0 ± 14.1 (190–210)	219.5 ± 16.3 (208–231)	150.3 ± 44.98 (78–189)	129.6 ± 4.17 (127–133)	130.9 ± 13.8 (121–141)
Dissolved Oxygen (mg/L)	6.5 ± 2.7 (0.2–8.1)	4.0 ± 2.7 (0.15–6.1)	6.26 ± 1.76 (4.0–10.2)	5.3 ± 3.6 (0.4–8.4)	4.5 ± 3.9 (0.2–7.9)
Dissolved Oxygen (% saturation)	107.7	78.6	69.6 ± 19.86 (49–102%)	98.3	104.5
NH_3 (mg/ L)	0.023 ± 0.0 (0.023–0.023)	0.057 ± 0.77 (0.018–1.118)	< 0.0001 ± 0.0 (< 0.0001)	0.028 ± 0.01 (0.018–0.036)	0.031 ± 0.01 (0.022–0.040)
Nitrate + Nitrite (mg/L)	0.081 ± 0.08 (0.023–0.139)	0.013 ± 0.0 (0.013–0.013)	0.20 ± 0.0 (0.200–0.200)	0.127 ± 0.13 (0.036–0.217)	0.066 ± 0.013 (0.056–0.075)
Total Kieldahl Nitrogen (mg/L)	0.97 ± 0.55 (0.58–1.365)	1.219 ± 1.23 (0.348–2.09)	0.55 ± 0.07 (0.50–0.60)	0.75 ± 0.03 (0.729–0.773)	0.78 ± 0.06 (0.74–0.82)
Total Phosphorus (mg/L)	0.041 ± 0.02 (0.026–0.056)	0.044 ± 0.03 (0.02–0.068)	0.04 ± 0.008 (0.039–0.050)	0.034 ± 0.02 (0.020–0.048)	0.045 ± 0.02 (0.032–0.058)
SRP (mg/L)	0.019 ± 0.01 (0.01–0.03)	0.011 ± 0.002 (0.01–0.013)	–	–	–
Oxidation-Reduction Potention (E_h; mV)	–	–	571.6 ± 169.9 (320–920)	–	569 ± 255.9
Total Dissolved Solids (mg/L)	–	–	74.3 ± 23.2 (31.1–90.9)	–	50.9 ± 16.5 (37.5–93.1)

0.2 mg/L), and Celina (0.36 mg/L) had the highest level (Table 2–4).

The Carlson trophic index evaluates the three independent variables including chlorophyll, total phosphorus, and secchi disk depth (Carlson 1977). The Carlson trophic index classified these lakes as mesotrophic with the highest Tipsaw (22–52), followed by Indian (12–13), and Celina (5–14) as the lowest and most oligotrophic. The quantities of nitrogen, phosphorus, and the total weight of algal biomass are indicators of water body trophic levels. The EPA recommends that the Carlson index should only be used with lakes that have relatively few rooted plants and non-algal turbidity sources An excessive amount of rooted vascular plants was associated with Lakes Indian and Celina, which may affect TI calculations.

Annual comparison.—General patterns in chemical and physical measures is towards a stable steady state or reduction in nutrients. No statistically significant (ANOVA, $P > 0.05$) trend based on regression of parameter by lake over time was observed for any parameter during the study period. Tipsaw Lake showed the greatest variation in nitrogen and phosphorus levels from the earliest 1991 measured value to current 2012 levels, while Celina and Indian were stable over the same period (Table 2–4).

Over the last two decades, the mean temperature trend is toward increasing levels in Indian

and Celina, while stable in Tipsaw. The trend in dissolved oxygen is declining in Tipsaw and Indian, while stable in Celina. Stable conditions in all lakes were observed in pH. Declining trends in conductivity were observed for Tipsaw, increasing in Celina, and stable for Indian. Alkalinity trend is increasing in Indian, Celina, and stable in Tipsaw. Salinity has increased in Tipsaw, while stable in the other lakes.

Depth profiles showed a strong thermal profile for Lake Celina during 1989, 1996, and 2001 (Figure 5). The thermocline in 1989 was observed at 5 m, 4 m in 1996, and 8 m during 2001. A strong thermocline was observed in Indian lake in 1991, while less conspicuous in 2001 (Figure 5). In Tipsaw Lake, no oxycline was observed in 1991, but was apparent in 2001 (Figure 5), and recently in the 2011 survey. Dissolved oxygen was present in the deepest portion of each lake, which suggests that the sediment-water column microzone is typically in an oxidized condition. Measurement of oxidation-reduction potential substantiates this observation with 90% of the measurements in a positive state.

Trophic status and fish yield.—The Carlson trophic index has increased between 1989–2012. Tipsaw Lake shows the highest increase with an index rise from 23 to 52. Response in both Indian and Celina did not show similar rises; however, the change in littoral aquatic macrophytes is a possible reason for this change. Trophic levels for these lakes should be classified as mesotrophic (Carlson 1977).

The morphoedaphic index (MEI) is a predicator of fish yield that is based on three hierarchial levels including global scales related to area and temperature, regional scales are dependent on nutrient and mean depth as area and temperature are held constant, and within regional level at which either nutrient or depth variable can be a predictor while the remaining variable is constant (Ryder 1982). Higher MEI values are directly correlated with higher fish yield. The highest MEI value was observed in Tipsaw (24.3 (mg/L)/m), followed by Indian (16.8 (mg/L)/m), and lowest in Celina (9.1 (mg/L)/m; Table 1). The MEI values observed in the three lakes translate to catch yields of 20–30 kg/ha (Ryder et al. 1974).

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SEVENTEEN YEARS OF CHANGE IN TWO *SPHAGNUM* BOGS IN NOBLE COUNTY, INDIANA

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ABSTRACT. This investigation continues long-term monitoring of vegetation change in Tamarack and Hickory Bogs at the Merry Lea Environmental Center in Noble County. Tamarack Bog was drained in 1899, accelerating its succession. Over the past 100 years, it has been visited by several notable scientists, who documented aspects of its vegetation, including Charles R. Dryer (1899), Charles C. Deam (1916), Ray C. Friesner (1935), and Alton A. Lindsey (1972). In 1993, A. L. Swinehart conducted the first systematic, quantitative study of Tamarack Bog, as well as Hickory Bog (a tiny *Sphagnum* bog nestled within the crest of an esker). The same quadrats used in 1993 were used in the present study (2010) to examine changes in the peatlands over the past 17 years. Indicator species analysis, multiple response permutation procedure, and non-metric multidimensional scaling were used to analyze changes in frequency, cover, and presence / absence. The flora in undisturbed Hickory Bog is unchanged, whereas, Tamarack Bog has exhibited significant change.

Keywords: Peatland, bog, *Sphagnum*, *Larix laricina*, succession

INTRODUCTION

The Indiana Department of Environmental Management estimates that 85% of Indiana's wetlands have been lost to drainage and filling since the 1780's. These losses included *Sphagnum* bogs and tamarack swamps. Based on the characteristic peatland soil, Houghton Muck, there was at least 62,087 ha (149,009 acres) of peatlands in Indiana (Swinehart 1997). Only a fraction of the associated plant communities has survived to the present, due to human activity.

While there are approximately 70 peatlands (bogs, fens, and forested peatlands) registered with the Indiana Department of Natural Resources, Division of Nature Preserves (IDNR-DNP) (most of which have some form of protection), peatlands with healthy, reproducing tamarack populations are few. Compilation of occurrence data from Purdue University's Kriebel Herbarium (PUL), Indiana University's Deam Herbarium (IND), the IDNR-DNP database, as well as from Blatchley & Ashley (1901), Dryer (1901), Taylor (1907), Lindsey et al. (1969), and Wilcox (1982), show that tamarack was once widespread in northern Indiana, occurring in 18 counties. Tamarack (and associated bog communities) has since disappeared from most of these sites. Tamarack communities at most of the remaining sites are not likely to survive long. Reconnaissance reports in the late 1970's by employees of the IDNR (1996) on the status of tamarack sites in Indiana, include statements such as, "most not vigorous", "four trees, seem to be dying", "Only about a dozen trees, further deterioration likely", and "Being enveloped by hardwoods".

Peatlands in Indiana naturally trend toward development into lowland forests dominated by *Acer rubrum* (Swinehart & Parker 2000). This has been reported by other investigators studying peatlands in the southern Great Lakes Region (Transeau 1905, Crow 1969, Sytsma & Pippen 1982, Crum 1988). While this occurs naturally at a relatively slow rate, drainage of peatlands accelerates the transition from the "bog-conifer type" community to a "hardwood-conifer type" community dominated by red maple (LeBarron & Neetzel 1942). A floating mat, typical of bogs, can rise and fall with changing water-levels, keeping the substrate saturated, but not covered with standing

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water. Drainage can ground floating mats, resulting in flooding of the substrate during water-level fluctuations. These conditions of intermittent flooding (and associated release of N and P from decomposition during dry spells) favor red maple (Moizuk & Livingston 1966). Water-level fluctuations, coupled with the shade created by a red maple canopy, negatively affect tamarack regeneration (Duncan 1954) and ultimately lead to their demise and the demise of the associated understory flora, especially in Indiana and other southern reaches of its natural range where climate may not be optimal.

Investigation of the current condition and rate of succession of Indiana's peatlands, especially tamarack swamps, is necessary to guide conservation management and restoration efforts. While many studies have characterized the standing vegetation of peatlands in the northern hemisphere, few have monitored changes in peatland vegetation by subsequent quantitative sampling (see Backéus 1972; Frankl & Schmeidl 2000; Gunnarsson et al. 2002; Pellerin et al. 2008).

The year 2010 marked the 17th year since Tamarack and Hickory Bogs in Noble County, Indiana, were systematically surveyed (Swinehart 1994). The objectives of the present study are to systematically sample the current flora of Tamarack and Hickory Bogs and compare the current flora with the flora recorded in 1993 to determine the rate and process of plant succession.

STUDY AREA

Tamarack Bog is located in Noble Township, Noble County, Indiana (SWQ, SWQ, Sec 7, T33N, R9E). Hickory Bog is located in Washington Township, Noble County, Indiana (NEQ, NEQ, Sec 12, T33N, R8E). Both bogs are situated within the boundaries of the Merry Lea Environmental Center of Goshen College (Figure 1). Both Hickory Bog (0.6 ha) and Tamarack Bog (14 ha) are surrounded by *Quercus-Carya* forest buffers which separate the bogs from adjacent agricultural fields (Swinehart *et al.* 2001). In 1993, Swinehart (1994) found that invasion by shade-tolerant trees (*Acer rubrum, A. saccharinum,* and *Quercus palustris*) within Tamarack Bog had reduced the remnant bog species to a 10,500 m^2 wet depression in the center of the wetland basin.

Hickory bog.—Hickory Bog was discovered by Swinehart in 1991 and is located on top of an esker which was formed during the retreat of the Saginaw Lobe of Late Wisconsin glaciation. This esker may have been formed by a high, narrow crack or tunnel in the Saginaw ice (Dryer 1901). The roof of this tunnel may have collapsed to create an open ice-walled canyon into which more glacial debris was deposited (Dryer 1901). However, it is more likely that this esker was formed from a tunnel, not a canyon, due to the presence of a layer of glacial till (derived from the roof of the tunnel) on top of the outwash deposit.

Tamarack bog.—Tamarack Bog was probably a floating mat of bog vegetation connected to Old Bear Lake by wetland prior to the lake's drainage *ca.* 1850 (Swinehart 1994). Dryer (1901) was the first scientist to record a visit to Tamarack Bog and noted its location within the same esker lake system as Hickory Bog. Dryer's visit occurred after the first drainage of Old Bear Lake, but prior to the second drainage of the lake system in November, 1899. Blatchley and Ashley (1901) visited High Lake shortly after the drainage in 1899, and took a photograph of the lake showing a mature tamarack forest in the background. A mature tamarack forest would need relatively firm substrate suggesting that the initial grounding of the mat of bog vegetation had occurred prior to the 1899 drainage. Charles Deam noted the presence of *Andromeda glaucophylla* in 1920, and in 1935 and 1938, Ray Friesner reported *Vaccinium oxycoccos* and *Sarracenia purpurea*, respectively (IDNR 1992). These qualitative reports indicate a bog community. Alton Lindsey conducted the first cursory floral survey in 1972 and reported *Cypripedium acaule*, *Lycopodium clavatum*, twenty living tamarack trees, and an open *Acer* canopy forming above the tamarack (Swinehart 1994).

The first quantitative study of the bog was conducted in 1993 (Swinehart 1994, Swinehart & Starks 1994). *A. glaucophylla, V. oxycoccos, L. clavatum*, and *S. purpurea* were absent and the population of *Larix laricina* was reduced to three living specimens. Lindsey visited the bog with Swinehart in 1993 and noted that the *Acer* canopy had closed significantly since 1972. Tree cover has also been shown to increase in drained bogs in Sweden and Switzerland (Freléchoux et al. 2000; Linderholm & Leine 2004). The drainage of Old Bear Lake caused grounding of the vegetative mat which increased

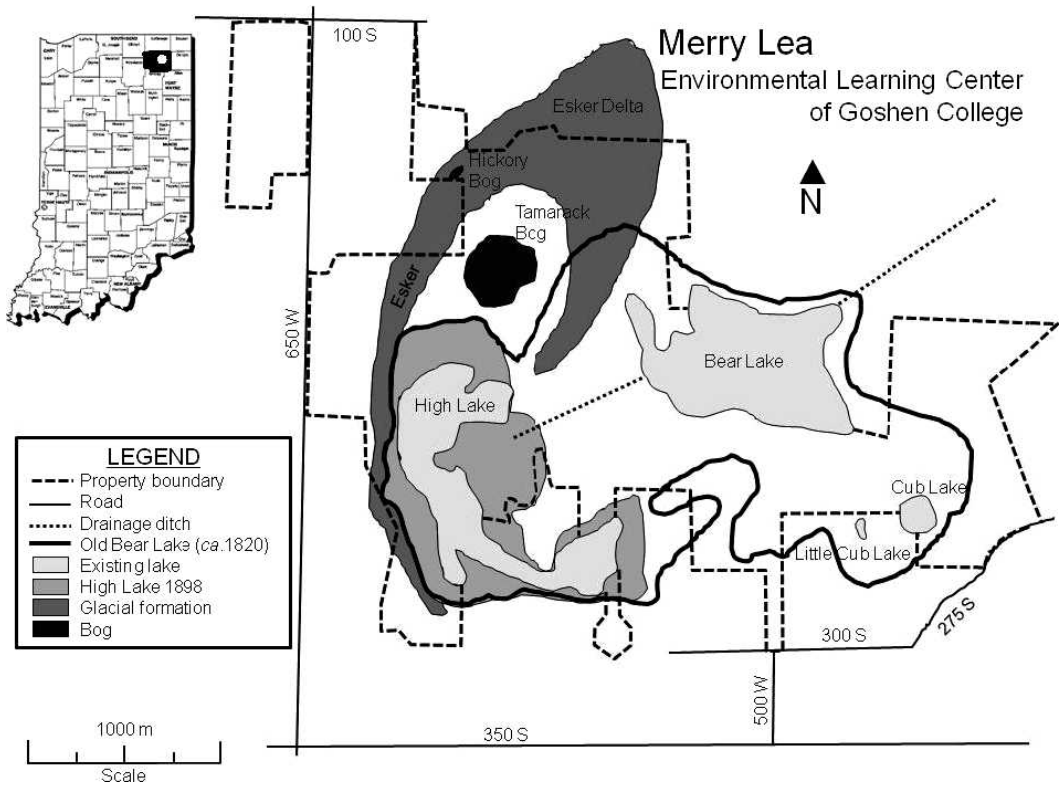

Figure 1.—Map of the study area showing the location of Hickory and Tamarack Bogs at the Merry Lea Environmental Learning Center of Goshen College (Secs. 12, 13 of T33N, R8E and Secs. 7, 17, 18, T33N, R9E).

available oxygen and nutrients for plant growth and accelerated the rate of succession towards a hardwood swamp from that of an open bog (Swinehart 1994).

MATERIALS AND METHODS

Herbaceous and woody vegetation were sampled using nested quadrats arranged systematically. Placement of quadrats was consistent with Swinehart (1994) in both bogs. Remains of permanent markers placed by Swinehart facilitated the re-establishment of the west-east baseline of Tamarack Bog and the southwest-northeast baseline along the long axis of Hickory Bog, with transects placed at twenty meter intervals perpendicular to each baseline. The presence of the permanent markers ensured that the quadrats were consistent with Swinehart's 1993 placement. Quadrats were placed at 15-m intervals along each transect in both bogs. Fourteen quadrats were established in Hickory Bog and thirty-seven in Tamarack Bog. The quadrats were nested. Aquatic, ground, and herbaceous layer vegetation was sampled using 1 m^2 quadrats. Shrubs and trees were sampled using 100 m^2 quadrats. The aquatic layer was comprised of submerged or floating plants; the ground layer was restricted to bryophytes; the herbaceous stratum was characterized as vegetation less than 1 m in height; the shrub layer was characterized as vegetation with diameter at breast height (DBH) $\leq$ 4 cm; the tree layer was characterized as vegetation with living stems $>$ 4 cm DBH. Shrub and tree data was not collected for Hickory Bog due to logistical problems associated with the treacherous floating mat. Percent frequency and percent cover were calculated for the herbaceous, ground, and aquatic layers in each bog. Percent frequency was calculated for each bog by dividing the number of quadrats in which a species was found by the total number of quadrats sampled in that bog. Percent cover was calculated by dividing the sum of observed

cover values for each species in each bog by the total number of quadrats in that bog. Density, rather than percent cover, was calculated for the tree and shrub layers. Basal area was also calculated for the tree layer using a tape measure to determine DBH. Importance values were determined for each species in each bog by calculating the average of the relative frequency and relative cover. Taxonomic nomenclature of gymnosperms and angiosperms, ferns, mosses, and liverworts follows Voss (1980, 1985, 1996), Cobb (1963), Crum & Anderson (1981), and Conard (1956), respectively.

Analysis of the herbaceous layer and ground layer data was conducted using Non-metric multidimensional scaling (NMS), Multiple Response Permutation Procedure (MRPP) and Indicator Species Analysis (ISA). NMS provided a two dimensional graphical representation of the data, showing relative similarity of vegetation in the quadrats based on presence / absence, frequency, and cover. MRPP tested for difference in percent cover, percent frequency, and presence/absence of species between the two samplings. ISA identified the species that account for the greatest difference in percent cover, percent frequency, and presence / absence of species between two samplings.

The sampling of Tamarack Bog, for the present study, was conducted from June 10 – June 13, 2010. The sampling conducted in 1993 began on May 9 and ended on May 12. Because the differing months of collection (representing about 30 days) may have resulted in differences in cover related to early seasonal growth (rather than long-term successional changes), some species were removed from the statistical comparison of the two time periods. Based on observations of changes in spring growth between May and June of the species present, three species seemed likely to have added more significant foliage cover between May and June than the other species in the herbaceous layer. These were *Dryopteris spinulosa*, *Osmunda cinnamomea*, and *Rubus allegheniensis*. The same statistical test was conducted with the inclusion of these species for comparison.

RESULTS AND DISCUSSION

HICKORY BOG

Flora of hickory bog.—Eighteen vascular plant and three bryophyte species were identified in the sampling area in Hickory Bog (Table 1). The bog contains a number of species common to mineral-rich tall-shrub bogs, including *Cephalanthus occidentalis*, *Dulichium arundinaceum*, *Toxicodendron vernix*, *Triadenum fraseri*, and *Sphagnum fimbriatum*. Three species identified in the current study were not present in the previous study of Hickory Bog (Swinehart et al. 2001). These species, *Polygonum sagittatum*, *Carex echinata*, and *Cicuta bulbifera,* are common in wet areas in northern Indiana. Only one species, *Carex crinita*, from Swinehart's previous study of Hickory Bog was not found within the study area. This species may still be present in Hickory Bog because a qualitative survey was not conducted. There were no significant changes in the order of importance (based on importance values) of herbaceous flora in Hickory Bog, and *Sphagnum fimbriatum* remained the most important plant in the ground layer.

Statistical analysis.—Statistical examination of the herbaceous layer provides reliable indication of wetland status because the herbaceous layer changes more quickly than the higher layers in response to altered hydrology (Tiner 1999). MRPP indicated that the herbaceous community of Hickory Bog did not show statistical difference between 1993 and 2010 (p-value 0.275). Graphical confirmation was indicated by NMS with an ordination that did not display distinct groupings of quadrats (Figure 2). The relatively slow rate of succession of Hickory Bog is similar to that of Smith's Bog, another small *Dulichium* dominated bog in Cheboygan Co., MI. Woollett et al. (1926) predicted that the open water in Smith's bog would disappear in 10-20 years and be replaced by a *Carex* meadow followed by high-shrub species. However, Johns (1966) reported that "their predictions have not been justified by the intervening 34 years. The bog pool is scarcely smaller, and the water has certainly not disappeared". The study of another bog in northern Michigan exhibited similar slow rate of change. Following a series of dry years ending in 1927, Jewell and Brown (1929) expected Mud Lake to be completely covered by the sedge meadow of Mud Lake Bog during the next series of dry years. However in 1973, most of Mud Lake was still open water forty-six years later (Schwintzer & Williams 1974). Hickory Bog reflects the relatively slow rate of succession exhibited by these undisturbed bogs in northern Michigan.

Table 1.—Vegetation of Hickory Bog, Noble County, Indiana, June 2010 (14 quadrats). Percent frequency, percent cover, and importance values are listed for the herbaceous, ground, and aquatic layers. Relative values are listed in parentheses. "ND" means that the species was present, but no numerical data is available.

Layer/Species	% Freq.	% Cover	I.V.
HERBACEOUS LAYER			
Dulichiuma arundinaceum	43 (10)	21 (35)	23
Impatiens capensis	50 (12)	17 (28)	20
Cephalanthus occidentalis	36 (8)	7 (12)	10
Leersia oryzoides	57 (13)	4 (7)	10
Triadenum fraseri	29 (7)	3 (5)	6
Thelypteris palustris	36 (8)	1 (2)	5
Boehmeria cylindrica	29 (7)	1 (1)	4
Polygonum sagittatum	29 (7)	1 (1)	4
Bidens sp.	21 (5)	1 (2)	3
Unidentified sedges	21 (5)	1 (1)	3
Eupatorium perfoliatum	21 (5)	1 (1)	3
Galium sp.	21 (5)	0.4 (1)	3
Cicuta bulbifera	7 (2)	0.3 (0.5)	1
Carex echinata	7 (2)	0.2 (0.4)	1
Ilex verticillata	7 (2)	0.2 (0.4)	1
Onoclea sensibilis	7 (2)	0.2 (0.4)	1
Toxicodendron vernix	7 (2)	0.2 (0.4)	1
GROUND LAYER			
Sphagnum fimbriatum	14 (29)	3.1 (56)	42
Aulacomnium palustre	14 (29)	2 (32)	30
Leptodictyum riparium	21 (43)	0.4 (6)	17
AQUATIC LAYER			
Lemna minor	57 (100)	39 (100)	100
Wolffia sp.	ND	ND	ND

TAMARACK BOG

Flora of tamarack bog.—Twenty-six vascular plant and nine bryophyte species were identified within the sampling area in Tamarack Bog (Table 2). Many of these species are remnant bog species. The remnant tamarack bog species in the shrub layer include *Vaccinium corymbosum*, *Ilex verticillata*, *Aronia melanocarpa*, and *Nemopanthus mucronatus*. Remnant tamarack bog species of the herbaceous layer include *Maianthemum canadense*, *Trientalis borealis*, *Osmunda cinnamomea*, *Carex trisperma*, and *Rubus hispidus* (northern raspberry).

Cf. Nyssa sylvatica is the only tree species not previously recorded and may have been overlooked in previous investigations. It is found in other forested peatlands in northern Indiana (Swinehart et al. 2001).

Vascular plant species which were not previously observed in Tamarack Bog include *Viola* sp., *Boehmeria cylindrica*, *Polygonum virginianum*, *Leersia oryzoides*, and *Triadenum fraseri*. Each of these species is adapted to wet conditions. *Triadenum fraseri* is commonly found in bogs (Conway 1949). Swinehart (1994) noted the presence of *Viola* sp. in the wetland surrounding Tamarack Bog, but not in the bog proper.

There were no herbaceous species from the 1993 quadrats that were not found in the 2010 quadrats. However, there were some marked changes in importance. *Osmunda cinnamomea* replaced *Maianthemum canadense* as the most important species in the herbaceous layer (Table 2). The importance value of *O. cinnamomea* went from 8 in 1993 (ranked third; Swinehart & Starks 1994) to 27 in 2010, whereas the importance value of *M. canadense* went from 20 in 1993 (Swinehart & Starks 1994) to 9 in 2010 (ranked third). Likewise, *Trientalis borealis* declined in importance since 1993, from a value of 9 to 7 and from second most important to sixth most important. *Rubus hispidus*, *Rubus allegheniensis*, and *Acer rubrum*

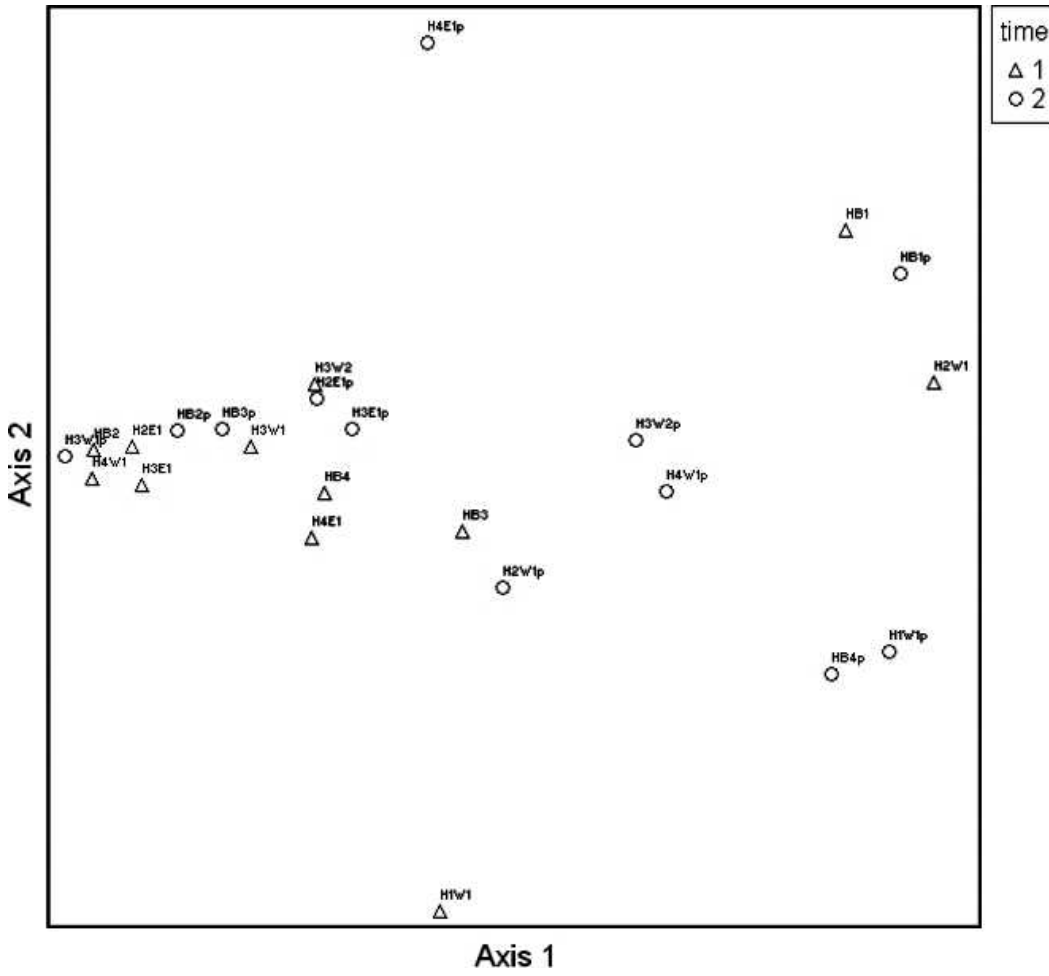

Figure 2.—Non-metric Multidimensional Scaling Ordination for Hickory Bog. Time 1 represents quadrats from 1993; Time 2 represents quadrats from 2010. Notations next to each data point identify individual quadrats.

(as seedlings) have surpassed *T. borealis* in importance since 1993 (Table 2).

Although they occurred outside of the quadrats, some effort was placed on searching for rare and notable species. All but one of the three living *Larix laricina* noted by Swinehart (1994) have died, and the *Cypripedium acaule* noted by Lindsey in 1972 and Swinehart in 1993 were not observed, nor were the *Lycopodium lucidulum* and *Lycopodium obscurum* that Swinehart found in 1993.

Statistical analysis.—MRPP indicates that the herbaceous community in Tamarack Bog from 1993 is statistically different from the herbaceous community in 2010 ($p<0.001$). NMS ordinations display the 1993 and 2010 quadrats as distinct clusters with little overlap regardless if the three late foliage species (*D. spinulosa, O. cinnamomea*, and *R. alleghenien-sis*) are included (Figure 3) or not (Figure 4). Qualitative change observed between Lindsey's 1972 survey and Swinehart's 1993 survey is confirmed by quantitative change between 1993 and 2010. Similar significant changes in bog communities have been observed in anthropogenically disturbed bogs located in Canada over a three decade period (Pellerin et al. 2008).

MRPP and NMS reveal significant change, but do not indicate what species account for the majority of the change. ISA identified *Ilex verticillata*, *Maianthemum canadense*, *Trientalis*

Table 2.—Vegetation of Tamarack Bog, Noble County, Indiana, June 2010 (37 quadrats). Percent frequency, density (D), basal area (BA), and importance values (IV) are listed for the tree layer. Percent frequency, density, and importance values are listed for the shrub layer. Relative values are listed in parentheses.

Layer/Species	% Freq.	D (#/ha)	BA (m^2/ha)	IV
TREE LAYER				
Acer rubrum	92 (54)	2 (69)	0.2 (73)	62
Prunus serotina	27 (16)	0.3 (8)	0.02 (9)	12
Quercus palustris	22 (13)	0.3 (8)	0.04 (17)	11
cf. *Nyssa sylvatica*	22 (13)	0.4 (10)	0.002 (1)	11
Ulmus americana	5 (3)	0.1 (3)	0.0006 (0.3)	3
Sassafras albidum	3 (2)	0.03 (1)	0.0001 (0.1)	1
SHRUB LAYER				
Ilex verticillata	86 (30)	5 (40)		35
Lindera benzoin	84 (29)	3 (19)		24
Rubus allegheniensis	30 (10)	4 (31)		21
Acer rubrum	19 (7)	0.4 (3)		5
Vaccinium corymbosum	19 (7)	0.3 (2)		4
cf. *Nyssa sylvatica*	19 (7)	0.3 (2)		5
Prunus serotina	11 (4)	0.2 (1)		2
Nemopanthus mucronatus	8 (3)	0.1 (1)		2
Aronia melanocarpa	5 (2)	0.08 (1)		1
Amelanchier sp.	3 (1)	0.03 (0.2)		1
Sassafras albidum	3 (1)	0.03 (0.2)		1

Layer/Species	% Freq.	%Cover	I.V.
HERBACEOUS LAYER			
Osmunda cinnamomea	68 (12)	30 (42)	27
Rubus hispidus	59 (10)	11 (15)	13
Rubus allegheniensis	38 (7)	9 (12)	9
Maianthemum canadense	76 (13)	4 (5)	9
Acer rubrum	73 (13)	4 (5)	9
Trientalis borealis	62 (11)	2 (3)	7
Dryopteris spinulosa	43 (8)	3 (5)	6
Ilex verticillata	24 (4)	2 (2)	3
Lindera benzoin	16 (3)	1 (2)	2
Viola sp.	16 (3)	1 (1)	2
Parthenocissus quinquefolia	16 (3)	1 (1)	2
Carex trisperma	11 (2)	1 (2)	2
Polygonum virginianum	11 (2)	1 (1)	1
Leersia oryzoides	11 (2)	1 (1)	1
Boehmeria cylindrica	8 (1)	0.4 (1)	1
Vaccinium corymbosum	8 (1)	0.2 (0.3)	1
Prunus serotina	8 (1)	0.1 (0.2)	1
Quercus palustris	8 (1)	0.1 (0.2)	1
Aronia melanocarpa	5 (1)	0.2 (0.3)	1
Triadenum fraseri	3 (0.5)	0.2 (0.2)	0.4
Bidens sp.	3 (0.5)	0.03 (0.04)	0.3
GROUND LAYER			
Pallavicinia lyellii	86 (43)	6 (46)	44
Aulacomnium palustre	35 (17)	2 (20)	19
Thuidium delicatulum	22 (11)	1 (12)	11
Sphagnum recurvum var. *tenue*	11 (5)	1 (11)	8
Sphagnum palustre	14 (7)	1 (10)	8
Tetraphis pellucida	19 (9)	1 (4)	7
Plagiothecium denticulatum	14 (7)	0.3 (3)	5
Leucobryum sp.	11 (5)	0.3 (2)	4
Lophocolea sp.	3 (1)	0.03 (0.2)	1

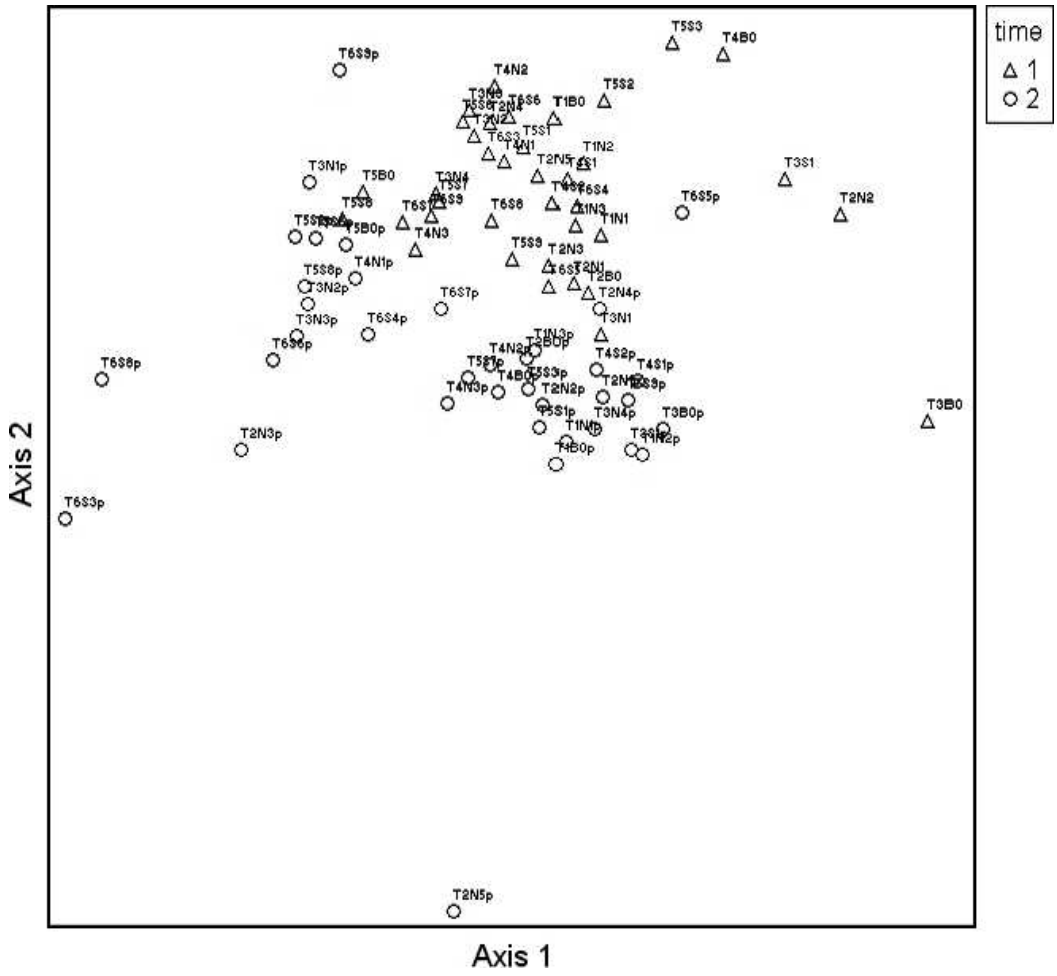

Figure 3.—Non-metric Multidimensional Scaling Ordination for Tamarack Bog including *Dryopteris spinulosa*, *Osmunda cinnamomea*, and *Rubus allegheniensis*. Time 1 represents quadrats from 1993; Time 2 represents quadrats from 2010. Notations next to each data point identify individual quadrats.

borealis, and *Viola* sp. as the indicator species which accounted for the majority of the difference between 1993 and 2010. ISA showed that *M. canadense* and *T. borealis* were more prominent in 1993, whereas *I. verticillata* and *Viola* sp. were more prominent in 2010. In Indiana, *M. canadense* and *T. borealis* are almost always found in tamarack bogs (Deam 1940). Additionally, *I. verticillata* is common in bogs and swamps almost exclusively in northern Indiana (Deam 1940). *Viola* sp. is a recent invader to the bog from the surrounding wetland. Although many communities occur along continuous gradients without definite boundaries (Whittaker 1975), bogs often have distinct species composition and abiotic settings which distinguish them from other surrounding communities (Kintsch & Urban 2002). Currently the boundary between the remnant bog community in Tamarack Bog and the surrounding swamp is beginning to disappear. The presence of *Viola* sp. as an indicator for 2010, coupled with the colonization of this species from the swampy wetland surrounding Tamarack Bog, shows that this community continues to rapidly transition from tamarack bog to hardwood swamp.

Neither Hickory Bog nor Tamarack Bog was directly altered by humans since the previous study in 1993. However, Tamarack Bog has exhibited significant change in the plant community, whereas Hickory Bog has not. The lack of significant change in vegetation in Hickory

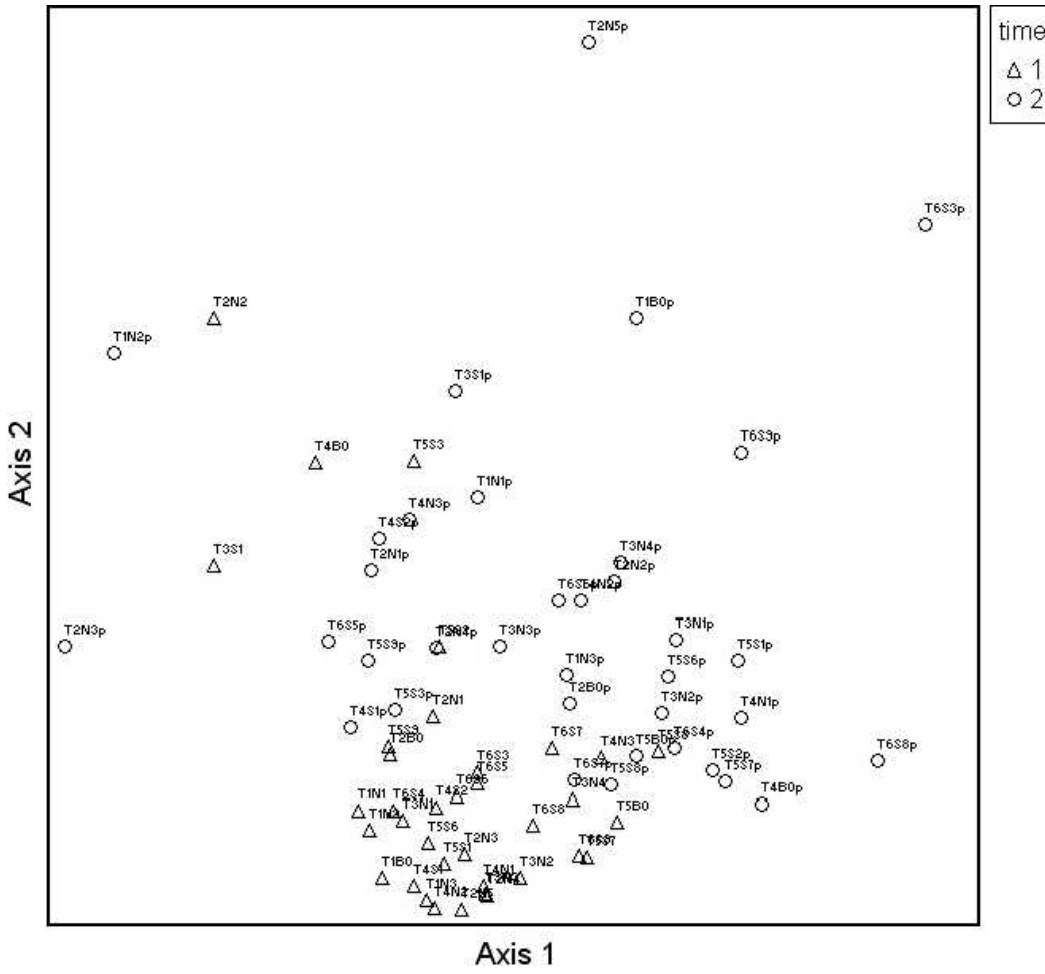

Figure 4.—Non-metric Multidimensional Scaling Ordination for Tamarack Bog excluding *Dryopteris spinulosa*, *Osmunda cinnamomea*, and *Rubus allegheniensis*. Time 1 represents quadrats from 1993; Time 2 represents quadrats from 2010. Notations next to each data point identify individual quadrats.

Bog is likely due to the persistence in edaphic conditions provided by the floating mat. So long as the floating mat persists, it can rise and fall with seasonal water-level changes, thus maintaining saturated conditions most suitable to peatland species. Thus, successional change is likely to be slow until the debris peat below the mat accumulates to the point where the mat becomes grounded. When the mat becomes grounded and can no longer sustain fully saturated conditions during low water levels (especially in the summer), oxidation in the surface of the peatland releases nutrients. This, along with the increased stability of the substrate favors pioneering marsh and swamp species, including trees. The authors speculate that the rate of succession of vegetation after the loss of the floating mat is much faster due to significant changes in edaphic conditions. Evidence for this appears to be provided by the significant changes that have taken place in Tamarack Bog in only 17 years (as well as other major changes over the past 100 years). Although the drainage of the lake, and subsequent grounding of the floating mat of Tamarack Bog, accelerated the initial transition from open bog to swamp, the rate of change after the grounding is probably consistent with what the rate of change would have been if the mat had grounded naturally by accumulation of sedimentary peat.

CONCLUSION

The degraded nature, rapid anthropogenic succession, and geographic isolation of the tamarack bogs in Indiana is such a potential hindrance to the natural establishment of these communities (with their unique genome) on newly exposed lakeshores and bog soils, that tamarack seedlings in the state are already a rare occurrence. Restoration and management of these rare communities in Indiana will be required to prevent their extirpation from the state.

ACKNOWLEDGMENTS

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EFFECTS OF HYPERTHERMIA ON THE ULTRASTRUCTURE OF SPONTANEOUS MOUSE MAMMARY TUMORS WITH REFERENCE TO VIRAL DYSMORPHOGENESIS

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ABSTRACT. The mechanisms of cell death by hyperthermia were investigated in the spontaneous mammary tumors of the Strong A strain of mice. Circulating hot-water in a latex bag was used to apply a local heat dose of 46°C for 1 hour to the tumors in anaesthetized mice. The tumors were surgically removed from the mice under anesthesia at various times after heat treatment and studied by electron microscopy. Cytoplasmic swelling and condensation of nuclear chromatin occurred 5 minutes after heat treatment. Degenerative changes then became progressively more pronounced at 24, 48, and 72 hours after heat treatment. This included disorganization of the cytoplasm and loss of organelles, a marked increase in the number and size of lysosomes, the disruption of plasma membranes, the loss of nuclear membranes, nucleoli, and the fragmentation of condensed chromatin. There was also infiltration by granulocytes, and bundles of collagen fibrils into the tumor tissue. The mouse mammary tumor virus particles in the heated tumor cells were deformed and turned into amorphous masses. Our findings suggest that heat induced degenerative changes in the spontaneous mouse mammary tumors occurs through a combination of mechanisms including mitochondrial damage, rupture of cell membranes, damage to nuclei, and a marked increase in lysosomal activity, the latter playing the primary role in the hyperthermic killing of malignant cells.

Keywords: Hyperthermia, plasma membranes, lysosomes, mitochondria and chromatin

Hyperthermia is a very old form of cancer treatment in man. The first recorded use of hyperthermia in cancer treatment appeared in the writings of Indian physician Ramajama (2000 B.C.) who observed the palliative effects of applying hot irons to superficial tumors (Storm & Morton 1983; Coffey *et al.* 2006; Horseman & Overgaard 2007). Hyperthermia has been used alone or in combination with radiation and chemotherapy for many years (Dewey *et al.* 1973; Horsman *et al.* 2001; Halika *et al.* 2007). However, despite the promising results, it has not been widely accepted because of limitations in clinical application (Hildebrandt *et al.* 2002; Horseman & Overgaard 2007; Pennacchioli *et al.* 2009).

It has been shown that certain tumors in mice, dog, and man can be destroyed by immersing affected areas in a constant temperature water bath at a 42 to 46°C range for 7½ to 60 minutes without damaging the surrounding normal tissue. Destructive effects of heat began at 42°C, at which temperature it took several hours to damage the tumor tissue. Neuroblastoma in an infant immersed in hot water bath at 46–47°C temperature for 67 minutes was completely destroyed (Crile 1961; 1962). Cavaliere *et al.* (1967) found that hyperthermia in the 42–45°C temperature range caused irreversible damage to Novicoff hepatoma cells but not to normal rat liver cells, suggesting that tumor cells were more sensitive to heat than normal cells. Hyperthermia delivered by radiofrequency electromagnetic fields to EM-6 tumors implanted in mice showed an almost 50% cure rate of the tumors heated for 5 minutes at 44°C temperature (Marmor *et al.* 1977). In recent years, new techniques have been used for the application of hyperthermia. Xie and Sun (2006) demonstrated that hyperthermia induced by an

* Our esteemed colleague and co-investigator Dr. Duncan T. Kennedy passed away after completion of this study.

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electrothermal needle in combination with electrochemical therapy was a potentially effective procedure in treating solid malignant tumors in mice. An attractive approach for the treatment of deeply seated malignant tumors with heated magnetic iron particles is receiving considerable attention. In this procedure, magnetic iron nanoparticles are delivered to the tumor and then heated by an external magnetic field set at frequencies to generate the required temperature. However, one of the serious drawbacks of this procedure is the unwanted heating of the surrounding normal tissue (Ito *et al.* 2006; Thiesen & Jordan 2008). More recently, thermal treatment of the lumpectomy cavity with a hot balloon has been developed and tested in the goat mammary gland as an adjunct to surgery of breast malignant tumors (Alvarado *et al*. 2009). Only a few histological and ultrastructural studies have been carried out on the effects of hyperthermia on malignant tumors. Light and electron microscopic studies were carried out on mouse mammary carcinoma HB implanted in the flank of C3H mice treated with high-frequency diathermy at 41–43°C temperature range for 30 minutes (Overgaard & Overgaard 1972; Overgaard 1976). The effects of hyperthermia were investigated by light and electron microscopy in EMT-6 neoplasms implanted in adult C3H strain mice using radiofrequency electromagnetic fields at 44°C temperature for 30 minutes (Fajardo *et al.* 1980). However, the ultrastructural changes in spontaneous mouse mammary tumors exposed to hot-water hyperthermia have not yet been investigated. The mouse mammary tumor virus (MMTV) is known to cause mammary carcinoma in C3H and Strong A strains of mice. It is found in large amounts in lactating mammary tissue and thus readily transferred to suckling mice in which the incidence of developing mammary carcinoma is high (Bishop 1978; Jawetz *et al.* 1987).

The aim of the present study was to elucidate the mechanism by which hyperthermia applied through a hot-water bag induces subcellular destructive changes including MMTV in the spontaneous mammary tumors in the Strong A inbred strain of mice.

METHODS

Adult females of the Strong A strain of mice which have been inbred for many generations and have a high frequency of spontaneous mammary tumors, were used in this study. The animals were obtained from the colony of mice maintained in the animal room facility of the Biology Department, Ball State University, Muncie, IN. The animals were treated according to an approved Ball State University Animal Care and Use Committee protocol. Female mice bearing a spontaneous tumor about 10 mm in diameter were anaesthetized with an intra-peritoneal injection of 40 mg/kg Nembutal. The tumors located in the anterior thoracic wall were then exposed to hyperthermia through a thin latex bag that contained circulating hot-water, according to the method designed by Walker (1980). This method is a form of water heating in which intra-tumormal temperature rises because of conduction, rapidly achieving uniform temperature distribution (Walker, 1980). Tap water in a 2-gallon polycarbonate jar or reservoir was heated to 47°C using a TU-15 TEMPUNIT thermoregulator (Bailey Instruments, Saddle Brook, NJ). Circulating hot water from the reservoir was delivered to the heating unit, through silicon tubing using a bellows-type metering pump (Bailey Instruments, Saddle Brook, NJ). A separatory funnel was used between the metering pump and the heating unit in order to provide regular flow of water to the heating unit. After circulating through the heating unit and the water bag, hot water was returned through silicon tubing to the reservoir. The heating unit proper was constructed from Plexiglas tubing and had an inlet and outlet. To obtain a good temperature distribution, holes were drilled at an angle to direct the flow of water as an eddy around the tumor. Horizon Stimula contraceptive sheaths (Akwell Industries, Skokie, IL) were used as water bags (Fig. 1). Good thermal contact between the conducting bag membrane and the tumor was achieved by using chemically inert K–Y Jelly. The tumors were heated at 46°C temperature for 1 hour. During the experiments, body core temperature was monitored rectally by a Bailey rectal probe for mice (Bailey Instruments, Saddle Brook, NJ). The tumor temperature was measured with a Bailey digital thermocouple thermometer microprobe embedded in the tip of a fine needle for placement within the tumor for measuring intratumoral temperature. The unheated and heated tumors were surgically removed under anesthesia at 5 and 20 minutes, and at 24, 48, and 72 hours after

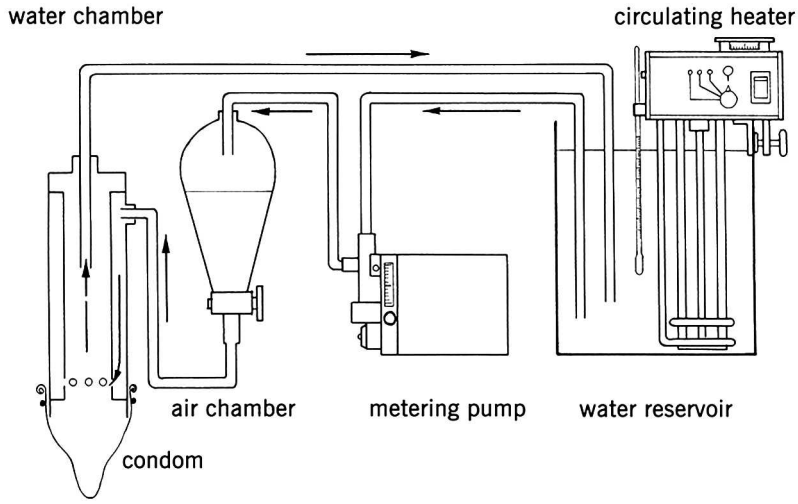

Figure 1.—Diagrammatic representation of the heating apparatus used in this study.

heat treatment. After surgical removal of the tumors, the mice were returned to the colony for further breeding. The experimental animals exhibited no systemic side effects after the heat treatment. After removal, the tumors were processed for electron microscopy. The tumor tissue was fixed at room temperature in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) post-fixed in 1% osmium tetroxide in the same buffer, dehydrated in an ethanol series to propylene oxide, and embedded in poly/BED resin (Polysciences, Warrington, PA). Polymerization was carried out at 60°C overnight. Thin sections were cut on a Porter-Blum MT2 ultra microtome, stained with uranyl acetate and lead citrate and examined with a Hitachi HU 11A transmission electron microscope.

RESULTS

Temperature Measurements.—Tap water was heated in the reservoir to a temperature of 47°C. After circulating through the tubing the temperature recorded was 46°C at the tip of the heating water bag. There was thus a loss of 1°C, as the hot water circulated from the reservoir to the water bag. The surface of the tumors thus received a heat dose of 46°C. The body core temperature of the anaesthetized mice monitored by a rectal probe recorded an average reading of 32°C. The rectal temperature rose 1°C during the initial few minutes of heating, but returned to near normal after 1 hour. During heat application, an average intratumoral temperature of 40°C was recorded. The tumor tissue received a heat dose of 8°C higher than the mouse's body core temperature.

Electron Microscopy.—The electron micrographs, of the untreated mouse mammary tumor cells were bounded by delicate plasma membranes and displayed large nuclei containing uniformly distributed euchromatin and conspicuous marginal heterochromatin abutting the nuclear membrane and a prominent nucleolus (Figs. 2A, 2B). The cytoplasm contained small mitochondria, abundant ribosomes, and a few lysosomes, but rough endoplasmic reticulum and Golgi complexes were sparse (Figs. 2A, 2C). In addition, numerous virus particles were present around vacuoles and dispersed in the cytoplasm of tumor cells. A few virus particles were also present in the lumina of the vacuoles. (Figs. 2A, 2D).

In the 1 hour heat-treated mammary tumor cells degenerative ultrastructural changes occurred in the nuclei and the cytoplasm, which became progressively more pronounced with an increase in time after heat treatment. At 5 minutes following heat treatment, the tumor cells displayed moderate condensation of the nuclear chromatin and swelling of the cytoplasm due to fluid accumulation in the round spaces (Fig. 3A). The plasma membranes of the tumor cells remained intact (Fig. 3B).

Twenty minutes after heat treatment, there was an increase in condensation of the nuclear chromatin accompanied by shrinkage of the

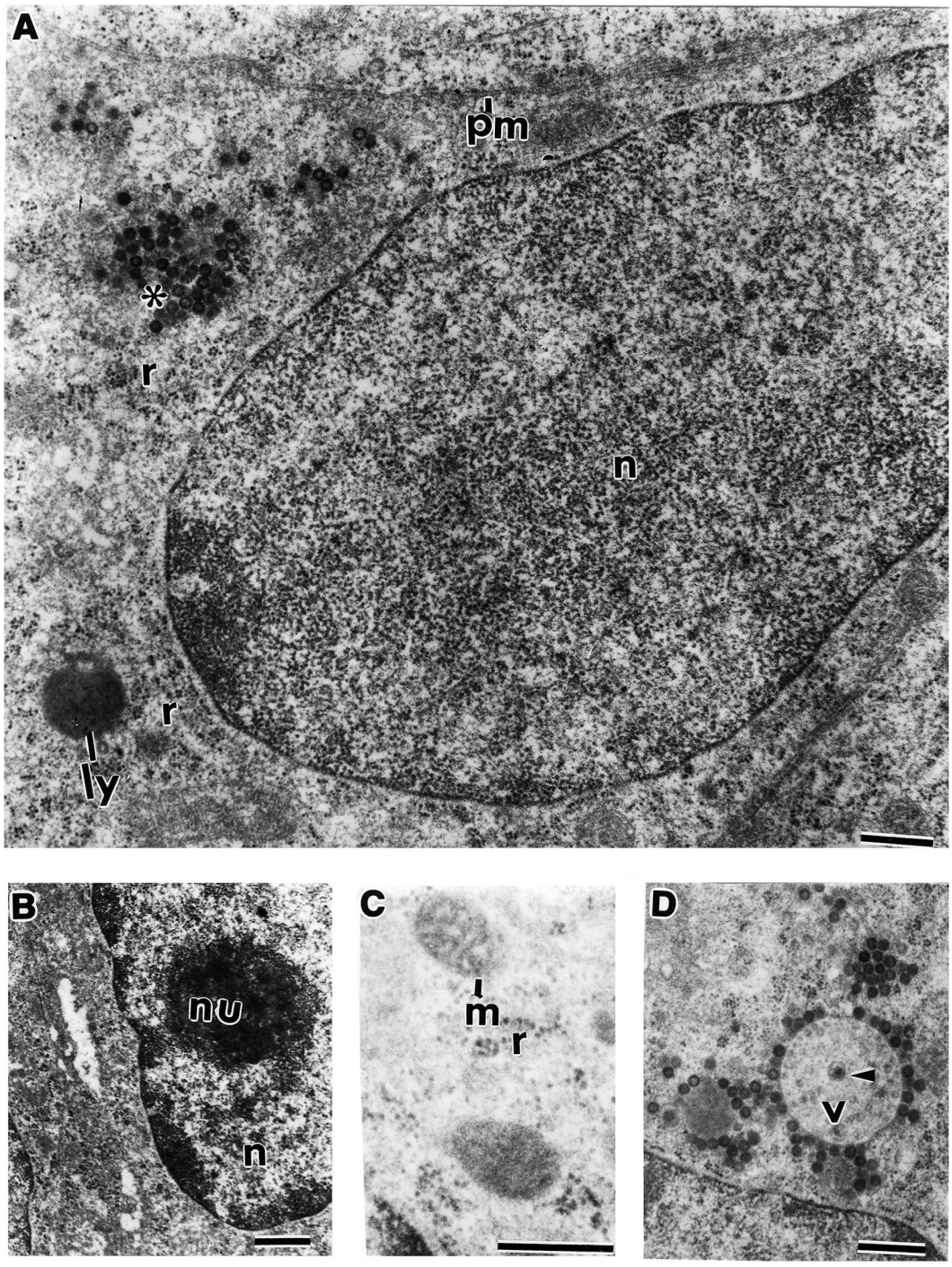

Figure 2.—Electron micrographs of untreated tumor cells. **(A)** A tumor cell showing a delicate plasma membrane (pm) and nucleus (n) containing euchromatin and conspicuous marginal heterochromatin. The cytoplasm contains a few lysosomes (ly), abundant ribosomes (r), and a cluster of virus particles (*). **(B)** Part of an untreated cell nucleus (n) displaying a prominent nucleolus (nu). **(C)** Cytoplasm of an untreated tumor cell showing mitochondria (m) and ribosomes (r). **(D)** Untreated tumor cell cytoplasm displaying many immature virus particles in it and around a vacuole (v). A mature virus particle (arrowhead) is also seen in the lumen of the vacuole (v). Scale bar: 0.5 μm A–D.

cytoplasm. The cytoplasm displayed small electron-opaque mitochondria, large vacuoles (Fig. 3C), and altered electron-lucent virus particles (Fig. inset 3C). Many electron-dense lysosomes were found in the tumor cell's disorganized cytoplasm (Fig. 3D). Clusters of collagen fibrils infiltrated into the tumor mass (Fig. 3E).

The destructive changes in the 1 hour heat-treated tumor cells became progressively more pronounced at 24 and 48 hours after heat treatment. At 24 hours the nuclei of the tumor cells appeared small and shrunken with disrupted nuclear membranes, markedly condensed chromatin and obscured nucleoli. The cytoplasm became disorganized and mitochondria were dilated with a loss of cristae (Fig. 4A). The intracellular virus particles became electron-lucent (Fig. 4A inset). Large lysosomes with heterogeneous contents including virus particles, multivesicular bodies, and many electron-dense lysosomes were found in the cytoplasm of the tumor cells (Figs. 4B, 4C). Large lipid droplets were also seen in the cytoplasm of the tumor cells (Fig. 4D).

At 48 hours after heat treatment the tumor cells displayed small shrunken degenerating nuclei with dark markedly condensed chromatin and a wide clear perinuclear space occasionally containing altered virus particles were found in the tumor cells. (Fig. 5A). The cytoplasm became highly vacuolated with numerous virus particles in it and around the vacuoles. There were also seen a few apparently intact virus particles and remnants of damaged particles in the lumina of vacuoles (Fig. 5B). The cytoplasm also displayed multivesicular bodies and many electron-dense lysosomes but other organelles were not seen (Fig. 5C). The tumor mass was infiltrated by collagen fibrils (Fig. 5C) and eosinophilic granulocytes containing many banded specific granules (Fig. 5D).

At 72 hours after heat treatment, the tumor cells displayed pronounced destructive changes. The plasma membranes were lost, and the cytoplasm was completely disorganized, and most of the organelles were destroyed with the exception of lysosomes. The small, dark pyknotic nuclei had disrupted nuclear membranes and were devoid of nucleoli (Fig. 6A). The cytoplasm contained many electron-dense lysosomes (Fig. 6B) and large lamellar lysosomes (Fig. 6C). A few altered virus particles were found in the cytoplasm (Fig. 6D). The tumor tissue was infiltrated by abundant collagen fibrils (Fig. 6E).

In the untreated tumor cells, the mouse mammary tumor virus (MMTV) occurred in two morphologically distinct forms: A type and B type. The A type 'immature' particles about 87nm in diameter consisted of a ring-shaped nucleoid surrounded by a membrane and a submembranous electron-dense layer or shell. These particles mainly occurred in the cytoplasm and around the vacuoles but a few were found in the lumina of the vacuoles (Figs. 2A, 2D, 7A, inset 7A). The A type particles after acquiring a membranous envelope were budded off into the extracellular space as B type particles (Fig. 7C). The B type 'mature' particles about 98nm in diameter consisted of an eccentric round nucleoid covered by a loose membrane and a thin submembranous layer (Fig. 7C inset). The loose, flaccid membraneous envelopes apparently caused heterogeneous shapes and size of B type particles in the extracellular space of the thin sections (Fig. 7C). The B type particles occurred mainly in the extracellular spaces, but a few particles were also found in the lumina of the vacuoles (Figs. 2D, 7C).

In the 1 hour heat-treated tumor cells 48 hours after heat treatment, at higher magnifications the MMTV particles in the cytoplasm and those in the vacuoles were morphologically altered and converted into amorphous masses. Some particles remained intact (Fig. 7B). Since the plasma membranes of the tumors were disrupted by heat treatment, budding of the virus particles was inhibited. The virus particles seen in the extracellular space were apparently released prior to heat treatment of the tumors (Fig. 7D).

DISCUSSION

This study has shown that destructive ultrastructural changes occur in the spontaneous mouse mammary tumors exposed to hot-water hyperthermia. The changes started as early as 5 minutes after the tumor was heat-treated for 1 hour at 46°C. The tumor morphology became progressively more pronounced within the days after heat treatment.

The heat-treated tumor cells displayed degenerative changes in the cytoplasm, plasma membranes, and the nuclei. The virus particles in the heat-treated tumors were also altered.

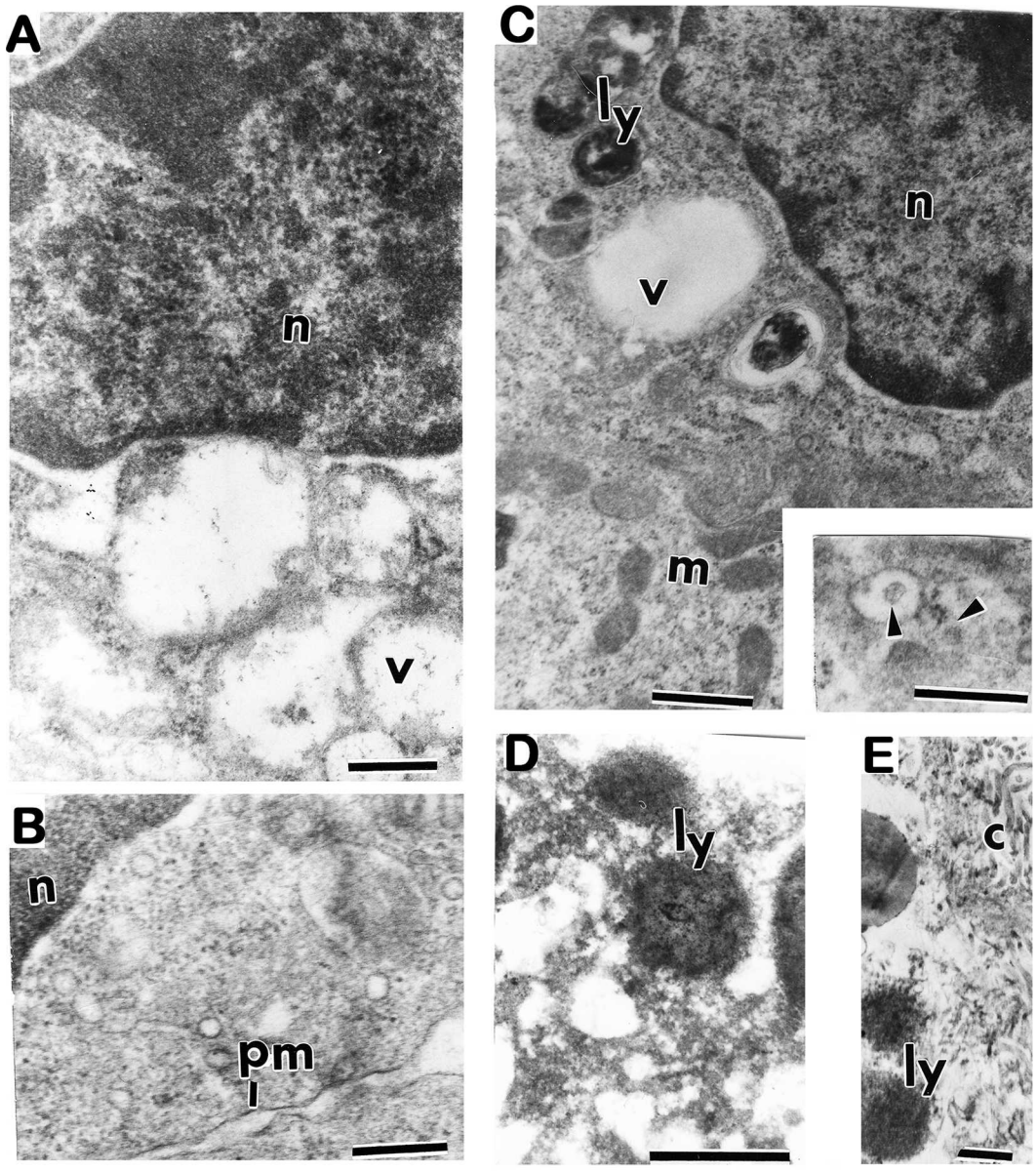

Figure 3.—Electron Micrographs of 1 hour heat-treated tumors 5 minutes and 20 minutes after heat treatment. **(A)** A portion of a heat-treated tumor cell 5 minutes after hear treatment showing swelling of cytoplasm and condensation of nuclear chromatin, a nucleolus (n), and vacuole (v). **(B)** A segment of a tumor cell 5 minutes after heat treatment showing an intact plasma membrane (pm) and nucleus (n). **(C)** Part of a tumor cell 20 minutes after heat treatment showing a nucleus (n) with increased condensation of chromatin. The cytoplasm displays many lysosomes (ly), electron-opaque mitochondria (m) and large vacuoles (v). The inset shows altered electron-lucent virus particles (arrowhead) in the tumor cell cytoplasm. **(D)** Part of a tumor cell 20 minutes after heat treatment showing electron-dense lysosomes (ly) in the disorganized cytoplasm. **(E)** Bundles of collagen fibrils (c) and lysosomes (ly) in the tumor tissue. Scale bar: 0.5 μm A, B, inset C; 1 μm C–E.

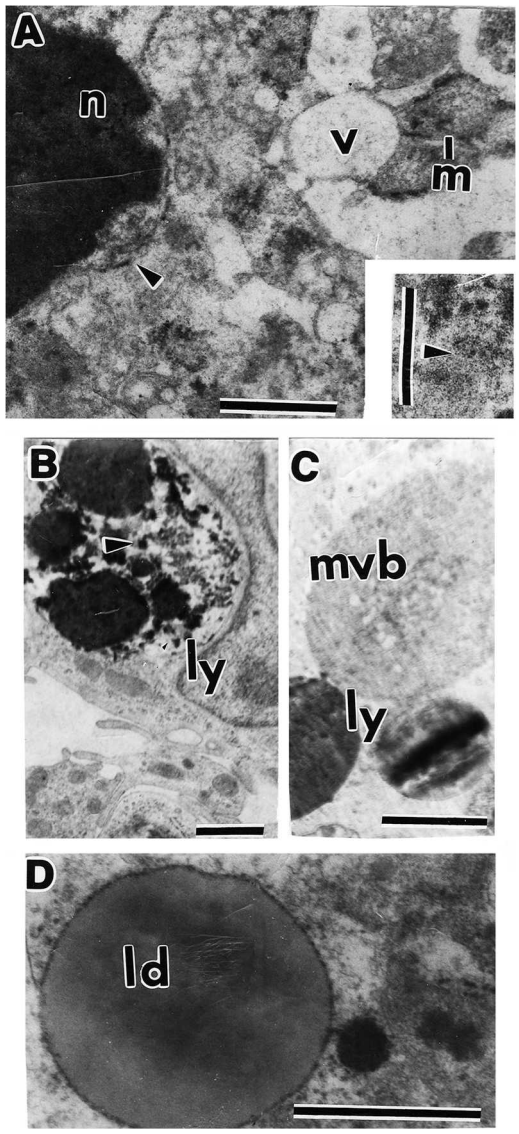

Figure 4.—Electron micrographs of 1 hour heat-treated tumor cells 24 hours after heat treatment. **(A)** Part of a tumor cell showing small pyknotic nucleus (n) with broken nuclear membrane (arrowhead) The cytoplasm contains vacuoles (v) and mitochondria (m) devoid of cristae. Inset shows altered intracytoplasmic virus particles. **(B)** A large lysosome (ly) containing heterogeneous contents including a virus particle (arrowhead) in a tumor cell's cytoplasm. **(C)** A multivesicular body (mvb) and lysosomes (ly) in a tumor cell's cytoplasm. **(D)** A large lipid droplet (ld) in the cytoplasm of a tumor cell. Scale bar: 1μm A–D and A inset.

The first changes observed 5 minutes after heat treatment were cytoplasmic swelling and condensation of the nuclear chromatin followed by increased shrinkage and disorganization of the cytoplasm resulting in the loss of endoplasmic reticulum ribosomes, Golgi complexes, and disruption of mitochondria. After 20 minutes, 24, 48, and 72 hours after heat treatment, observations indicated the continuation of degenerative changes. There was a marked increase in the number and size of lysosomes in the cytoplasm of heat-treated tumor cells, which peaked at 24 hours after heat treatment. Similar findings have been reported by Overgaard (1976) in implanted mouse mammary tumors treated with high-frequency diathermy at 41–43°C temperature range for 30 minutes. Damage to mitochondria by heat apparently inhibits aerobic metabolism. Anaerobic glycolysis, which in turn becomes important, tends to increase acidity in the heated energy-deficient tumor cells, thus making them more susceptible to damage by lysosomal acid hydrolases (Overgaard 1976; Fajardo *et al.* 1980).

The disruption of the plasma membranes of the heat-treated cells observed in this study apparently changes the permeability allowing materials to flow in and out of the cells causing swelling and disorganization of the cytoplasm. It has been proposed that cellular heat injury is associated with a dramatic increase in membrane permeability to Na^+ and K^+ between intracellular milieu and extracellular spaces. A change in the stability of lipoproteins and enzymes, which maintain structural integrity of the plasma membrane, is thought to cause cellular heat injury and ultimately cell death (Fajardo *et al.* 1980; Overgaard 1976).

Loss of the rough endoplasmic reticulum, ribosomes, and nucleoli apparently inhibits cellular protein synthesis and polymerization of RNA and DNA in heat-treated tumor cells (Bowler *et al.* 1973; Fajardo *et al.* 1980). The presence of large lipid droplets in the heat-treated tumors probably represents accumulation of saturated fatty acids (Bowler *et al.* 1973), but their role in heat injury remains unclear.

The nuclear changes in the heat-treated tumor cells include condensation of the chromatin, loss of nucleoli, and disruption of nuclear membranes, followed by fragmentation

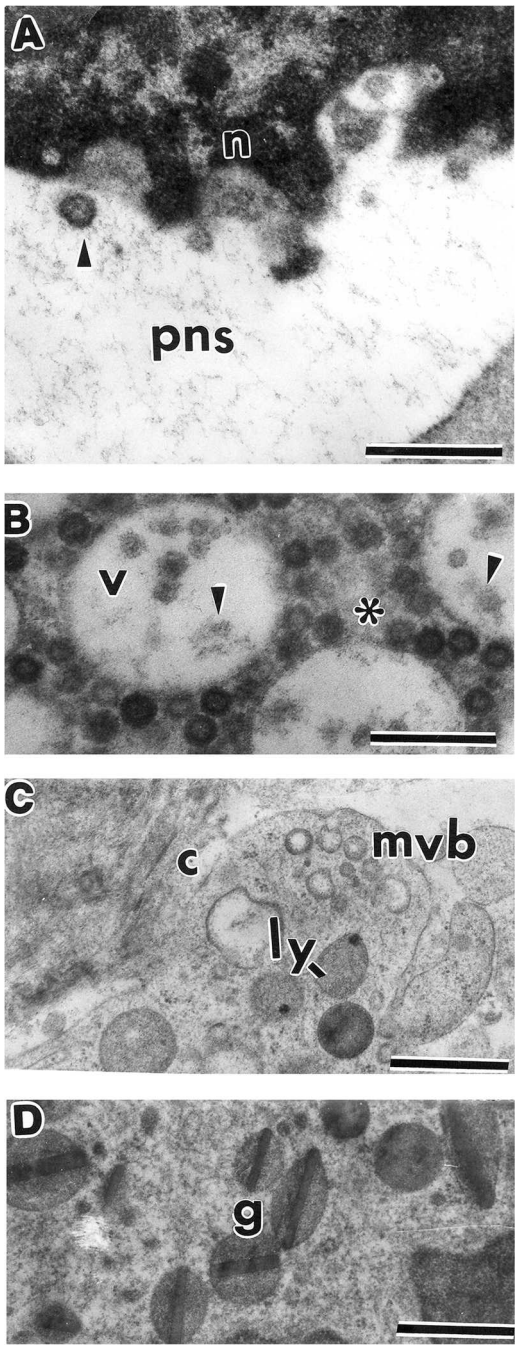

Figure 5.—Electron micrographs of 1 hour heat-treated tumor cells 48 hours after heat treatment. **(A)** Small pyknotic and degenerating nucleus (n) of a tumor cell surrounded by a large perinuclear space (pns) containing an altered virus particle (arrowhead). **(B)** Highly vacuolated cytoplasm of a tumor cell containing numerous immature virus particles (*) around vacuoles (v). A few mature virus particles and remnants of damaged virus particles (arrowheads) are seen in the vacuoles. **(C)** A multivesicular body (mvb), lysosomes (ly) and infiltrated collagen fibrils (c) in the cytoplasm of a tumor cell. **(D)** A portion of an eosinophilic granulocyte that infiltrated into the tumor containing many banded specific granules (g). Scale bar: 0.5μm A, B; 1μm C, D.

of nuclear chromatin. These findings clearly indicate severe heat damage to the nuclei of the tumor cells. It has been demonstrated that hyperthermia induces unfolding of the nuclear matrix and the subsequent changes in the binding of the specific proteins to the matrix, which may play an important role in hyperthermic killing of the tumor cells (Roti Roti *et al*. 1998; Coffey *et al*. 2006) The marked increase in infiltration of granulocytes, and bundles of collagen fibrils into the tumors 48 and 72 hours after heat treatment, suggests that remnants of tumor cells destroyed by heat are being phagocytosed and replaced with fibrosis (Alvarado *et al*. 2009). The heated tumors, if not surgically removed, would most likely become completely filled with fibrous tissue and eventually fall off (Crile, 1961).

The mammary adenocarcinoma in certain strains of mice is caused by mouse mammary tumor virus, (MMTV), which is an RNA retrovirus. It occurs in two forms: A type intracellular particles consisting of a ring-shaped nucleoid surrounded by a membrane and submembranous layer or shell and B type extracellular particles consisting of an eccentric round nucleoid covered by a loose membrane. In our study, MMTV particles resembled those described by other investigators (Bernhard >*et al*. 1955; Jawetz *et al*. 1987 Lyons & Moore 1975) except that A type particles displayed a prominent submembranous shell which resembled that of the immature particles of the human immunodeficiency virus (HIV) (Nermut 1994). The MMTV particles like those of other retroviruses apparently enter the mammary cells by adsorption, and after multiplying within the cells new particles are released by budding into the extracellular spaces. The presence of MMTV particles in vacuoles suggests that in addition to adsorption, the virus particles also enter the mammary cells by phagocytosis

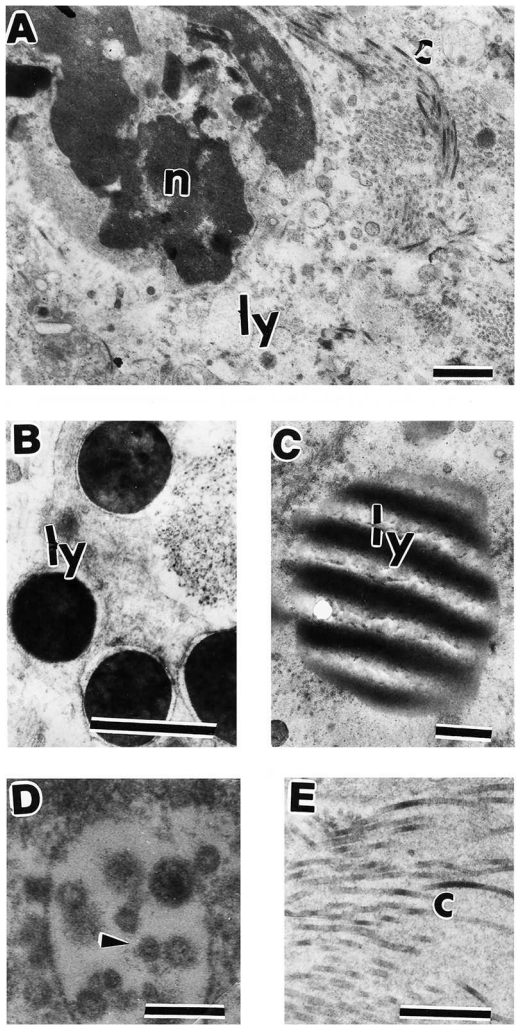

Figure 6.—Electron micrographs of 1 hour heat-treated tumor cells, 72 hours after heat treatment. **(A)** Small shrunken nucleus (n) of a tumor cell with extremely condensed chromatin and lost nuclear membrane. The surrounding degenerated cytoplasm contains small lysosomes (ly) and infiltrated collagen fibrils (c). **(B)** Dense lysosomes (ly) in the cytoplasm of a tumor cell. **(C)** A large lamellar lysosome (ly) in a tumor cell's cytoplasm. **(D)** Altered virus particles (arrowhead) in the cytoplasm of a tumor cell. **(E)** A field of tumor tissue showing abundant collagen fibrils (c). Scale bar: 1μm A–E.

(Morgan *et al*. 1969; Murray *et al*. 2002). It is thought that the vacuoles containing virus particles fuse with lysosomes. The acidity of the lysosomes causes the outer viral membranes to fuse with the membranes of the lysosomes causing the release of the nucleocapsids into the cytoplasm without being hydrolyzed by lysosomal enzymes (Simmons *et al*. 1982). In the heat-treated tumor cells, the structure of MMTV particles was altered. At higher magnifications, 48 hours after heat treatment the virus particles in the cytoplasm and vacuoles were deformed turning into amorphous masses. The viral RNA is synthesized in the nuclei of infected cells (Bishop 1978). The degenerative changes that occurred in the nuclei of heat-treated tumor cells, apparently inhibited RNA synthesis and consequently impeded the formation of new MMTV particles. The budding of the deformed virus particles was further inhibited by disruption of the plasma membranes in the heated tumor cells. Some intracellular virus particles which appeared structurally intact in heated tumor cells, were most likely functionally inactive. This view is supported by the observation that human warts which are circumscribed lesions caused by *Verruca, vulgaris* virus, when exposed to hot-water at 45–48°C temperature range for 1–1.5 hours, were cured and completely disappeared, since the virus apparently became inactive and decomposed following heat treatment (LoCricchio 1962).

This study has shown that hot-water hyperthermia causes profound degenerative changes in the cytoplasmic elements and destruction of MMTV particles, disruption of plasma and nuclear membranes, loss of nucleoli and nuclear membranes, condensation and fragmentation of nuclear chromatin of the mouse mammary tumor cells. Marked increase in the number and size of lysosomes in the heated tumor cells supports the hypothesis of Overgaard (1976) that increased lysosomal activity with the release of hydrolytic enzymes is the primary cellular reaction to hyperthermia. It is likely that all of these mechanisms play a role in hyperthermic killing of malignant tumor cells; but the marked increase in lysosomal activity is of prime importance. Our findings strongly suggest that hyperthermia delivered through a hot-water bag can be safely used to destroy certain human malignant tumors, including cervical cancer and head and neck squamous cell carcinomas.

ACKNOWLEDGMENTS

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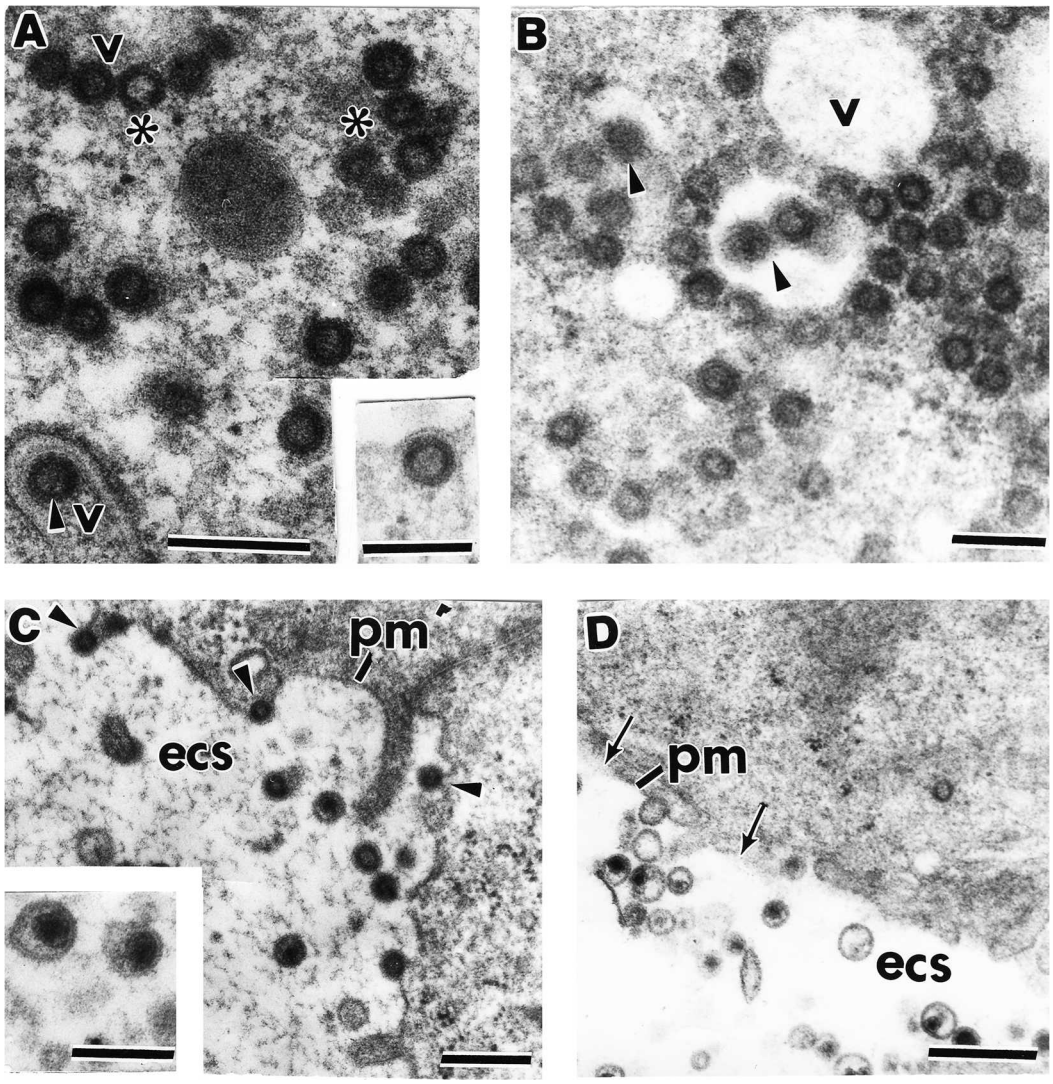

Figure 7.—An electron micrograph showing MMTV particles in the tumor cells. **(A)** Untreated tumor cell showing numerous A type virus particles (*) in the cytoplasm, around vacuoles (v) and a virus particle (arrowhead) in the lumen of a vacuole (v). Inset shows one A type virus particle at higher magnification. **(B)** Tumor cell heat-treated for 1 hour, 48 hours after treatment, showing numerous A type virus particles in the cytoplasm and vacuoles (v) structurally altered and turned into amorphous masses (arrowheads). A few virus particles remain intact. **(C)** The peripheral part of an untreated tumor cell showing virus particles budding (arrowheads) from the plasma membrane (pm) into the extracellular space (ecs). Inset shows mature B type virus particles at higher magnification. **(D)** Tumor cell 1 hour heat-treated, 48 hours after heat treatment displaying the plasma membrane (pm) ruptured (arrows) and absence of virus budding. Virus particles seen in the extracellular space (ecs) were apparently released from the infected tumor cell prior to heating. Scale bar: 0.25μm A–D, and insets A, C.

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SCREENING OF INSECTICIDES IN BATS FROM INDIANA

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ABSTRACT. This study identified insecticides that were detected in bats obtained from Indiana's Lake Michigan watershed. Forty bats collected from Lake, Porter and LaPorte Counties, Indiana, were analyzed for pyrethroid, organochlorine, organophosphate and carbamate insecticides. Additionally, brain cholinesterase activity of 332 bats from throughout Indiana was measured and cholinesterase reactivation tests were performed. Organochlorine pesticides (dieldrin, DDT, DDE, DDD and heptachlor epoxide) were detected in 97.5% of the tested bats; organophosphate compounds (primarily diazinon) were detected in 30%; pyrethroids in 12.5% and carbamates in 2.5% of the bats, respectively. Cholinesterase determination and reactivation tests yielded both false negative and false positive errors, which indicate that reactivation methods are not suitable for analyzing tissues from animals that are not recently dead. These results are among the first reported detections of pyrethroids and carbamates in bat tissues.

Keywords: Insecticides, bats, exposure, sentinels

INTRODUCTION

Bats are the primary predator of night flying insects including many agricultural pests (Lee & McCracken 2005; Whitaker 1995; Whitaker & Hamilton 1998). Whitaker (1995) estimated that a typical Midwestern big brown bat (*Eptesicus fuscus*) colony of 150 bats may consume in a season 600,000 spotted cucumber beetles (*Diabrotica undecimpunctata, Chrysomelidae*), 194,000 scarab beetles (*Scarabaeidae*), 158,000 leafhoppers (*Cicadellidae*) and 335,000 stinkbugs (*Pentatomidae*). All of these are significant pest species. Other bat species feed on moths such as the Turnip moth (*Noctuidae*), the larva of which (cutworm) is an important garden pest (Whitaker & Hamilton 1998). As such, bats are likely to play an important role in reducing damage to crops. Bat populations worldwide are declining (Kunz & Fenton 2003). In Indiana, several of the bat species are also declining (Whitaker et al. 2002). This is of particular concern because Indiana has the largest hibernating populations of the federally endangered Indiana bat (*Myotis sodalis*). During the winter of 2007, 50.8% of the total world population of this bat species hibernated in Indiana (FWS 2008). O'Shea & Clark (2002) suggested that insecticides may be an important contributor to bat declines. Eidels at al. (2007) analyzed bats and guano from Indiana and found insecticide residues in all of the samples. Nevertheless, the role of insecticides in the decline of bats remains unclear, as no recent toxicological studies on bats have been published.

Most bat species in North America feed on large quantities of insects (Whitaker 1996). For example, a single little brown bat (*Myotis lucifugus*) consumes as much as 28–85% its body weight in insects per day (Edythe et al. 1977; Kurta et al. 1989). Feeding on large quantities of insects may bring bats into contact with a wide range of insecticides. Testing of bats for insecticide residues could promote

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detection of insecticides in the environment, including those that have not been documented to bioaccumulate in bats.

The present study was designed to identify which insecticides can be found in free ranging bats in Indiana. Identifying insecticides in free ranging bats is challenging because bats are likely to be exposed to insecticides while foraging away from their roosts which may cause them to be incapacitated and not able to return to their roosts. Therefore, sampling bats using the standard methods, such as netting major fly routes or collecting bats from their roosts, is not likely to provide much information on the more acute and toxic insecticides. To increase the prevalence of affected bats in the sample population, bats that were already distressed (i.e. sick, dead or otherwise incapacitated) were tested. Throughout the years bats from Indiana were collected by the public and submitted to the *Indiana State Department of Health* rabies laboratory for rabies testing because they were distressed. Only about 5% of these bats were found to be rabid (Whitaker & Douglas 2006) leaving the cause for the incapacitation of about 95% of them unexplained. Eidels et al. (2007) examined nine of these bats and found that all nine contained insecticide residues. In the current study, non-rabid bats from the rabies laboratory were tested. Whole body analyses were used to identify insecticide residues in the bats. To complete the residue analyses cholinesterase (ChE) bioassays were performed. These bioassays measured the effect of ChE inhibiting insecticides (organophosphorus compounds and carbamates) on the bats' brains. ChE inhibitors are labile in living tissues and therefore may not be detected in residue analysis (Hill 1989, 1995; O'Shea & Clark 2002). The bioassays were conducted to identify exposed bats in which residues were not detected.

METHODS

Animals.—A total of 332 bats were collected from throughout Indiana by the public between September 2005 and December 2007. Bats were submitted to the *Indiana State Department of Health* rabies laboratory for rabies testing and found non-rabid. Brains were removed and a portion of each brain was used for rabies testing. The remaining brain was kept for cholinesterase (ChE) bioassays. Non-rabid bats (stored in Ziploc® bags) and brains from non-rabid bats (stored in 1.5 ml Eppendorf tubes) were transported frozen to *Indiana State University* where they were kept frozen at −60°C until analysis. 40 of these bats were collected from Lake, Porter and LaPorte Counties in North West Indiana (Figure 1). Lake, Porter and LaPorte Counties cover an area of approximately 3,600 km^2 where the land is in urban, industrial and agricultural use. The species and sex composition of the 40 bats is outlined in Table 1.

Figure 1.—Indiana map with Lake, Porter and LaPorte Counties, Northern Indiana colored grey. Map by the Indiana Business Research Center, January 2004 http://www.stats.indiana.edu/.

Whole body residue analysis.—Whole body chemical analysis of the 40 bats collected from Lake, Porter and LaPorte Counties (Table 1) was conducted to identify 35 insecticides from four groups (Table 2).

Shortly before analysis, bats were weighed and identified (species, sex and age: juvenile or adult, determined by forearm length). Skin and wings were removed and the carcasses sent on dry ice in 60 ml short wide-mouth clear glass jars (I-CHEM Brand) to *Southern Illinois University Carbondale* where they were analyzed for insecticide residues.

Analytical methods: Samples were processed in two batches of 20 bats. The first 10 samples

Table 1.—Species and sex composition of the 40 bats collected from Lake, Porter and LaPorte Counties. One of the bats, whose sex was not determined, was a new born.

Species	Males	Females	Sex not determined	Total
Big brown bats (*Eptesicus fuscus*)	20	13	2	35
Silver-haired bats (*Lasionycteris noctivagans*)	2	1		3
Red bats (*Lasiurus borealis*)	1	1		2
Total	23	15	2	40

in batch 1 were extracted using approximately 5 g of tissue per specimen. Because of the high lipid content of the bats, the remaining 10 samples in batch 1 and the second batch of 20 bats were extracted using approximately 2 g of tissue.

Gas chromatography with electron capture detector (GC-ECD) and nitrogen-phosphate detector (GC-NPD) (Agilent Technologies, Palo Alto, CA, USA) were used for insecticide analysis (Belisel & Swineford 1988; You & Lydy 2004). Analyses of eight pyrethroid, 19 organochlorine, six organophosphate and two carbamate insecticides (Table 2) were performed. After homogenization, bat samples were extracted with a mixture of methylene chloride : acetone (1:1, volume: volume) using an accelerated solvent extractor (ASE 200, Dionex, Sunnyvale, CA, USA). Extracts were cleaned and fractionated by tandem solid phase extraction into different groups for instrumental analysis (You & Lydy 2004). A sub-sample of bat tissue was used to measure lipid content spectrophotometrically with vanillin-H_3PO_4 reagent after acid-digestion (Van Handel 1985). Lipid analyses were performed using three replicates.

The carbamate insecticide carbaryl (1-naphthyl methylcarbamate) could not be quantified in the bat samples due to a matrix enhancement in the response factor seen on the gas chromatograph likely due to the high lipid concentrations in the bats. To offset this situation, standards were made in 0.05% and 0.1% corn oil in an attempt to mimic the matrix enhancement that occurred in the bats.

Quality control measurements including accuracy (percent recoveries) and precision (relative standard deviations) were calculated for each batch of 20 bat samples in addition to the processing of method detection limits (sensitivity), a blank, lab control spike, matrix spike and matrix spike duplicate.

Brain cholinesterase activity and reactivation testing.—Cholinesterase (ChE) activity was measured in the brains of 332 bats from throughout Indiana. Frozen brains were homogenized in the presence of 7.4 pH Tris buffer using laboratory homogenizer (Polytron PT 10/35 with Power Control Unit PCU-11, Kinematica, Inc., Bohemia, New-York, USA). For each sample, brain ChE activity was measured (Fairbrother et al. 1991; Ellman et al. 1961), thereafter, ChE reactivation tests were performed. Cholinesterase activity was measured colorimetrically using a modification of the Ellman assay (Fairbrother et al. 1991; Ellman et al. 1961). Cholinesterase activity was expressed as micromoles of acetylthiocholine iodide (substrate) hydrolyzed per minute (i.e. units) per gram of brain tissue wet weight (units/g).

The ChE reactivation tests measure increase of brain ChE activity in exposed bats after

Table 2.—List of insecticides tested in residue analysis.

Group	Compound
Pyrethroids	bifenthrin, lambda-cyhalothrin, cyfluthrin, cypermethrin, permethrin, esfenvalerate, deltamethrin and fenpropathrin
Organochlorines	alpha-BHC, beta-BHC, gamma-BHC, delta-BHC, p,p'-DDE, p,p'-DDD, p,p'-DDT, aldrin, gamma-chlordane, alpha-chlordane, dieldrin, endrin, endrin ketone, endosulfan I, endosulfan II, endosulfan sulfate, heptachlor, heptachlor epoxide and methoxychlor
Organophosphates	chlorpyrifos, malathion, tebupirimphos, dichlorvos, diazinon, terbufos
Carbamates	carbaryl, carbofuran

removing the insecticide molecules that bonded and neutralized the brain ChE enzymes. Each homogenized brain sample was divided into five aliquots. Presence of cholinesterase inhibition due to organophosphate (OP) exposure was identified using a method described by Martin et al. (1981). Two aliquots from each brain sample were incubated for 30 minutes at 24°C: one (the test aliquot) in the presence of pyridine 2-aldoxime methiodide (2-PAM) and the other (the control aliquot) in a 7.4 pH Tris buffer. Thereafter, ChE activity was measured simultaneously in both aliquots. If OP inhibited enzymes were present in the test aliquot, 2-PAM should remove the bound OP and, by that, increase the brain ChE activity (Fairbrother et al. 1991). To identify inhibition of ChE caused by exposure to a carbamate insecticide, a method described by Hunt & Hooper (1993) was used. Two aliquots from each homogenized brain sample were further diluted with 7.4 pH Tris buffer solution and incubated for 3 hours, one (the test aliquot) at 37°C and the other (the control aliquot) at 4°C. If carbamates were present in the test aliquot, incubation at 37°C should accelerate the diffusion of insecticide molecules to the buffer solution and, therefore, accelerate the rate of spontaneous reactivation of ChE in that aliquot (Hunt & Hooper 1993). For both OP and carbamate reactivation tests, brain ChE reactivation was considered significant if the activity of the test aliquot was at least 5% higher than the activity of the control aliquot; and a one tailed Student's t-test showed a statistically significant difference ($P<0.05$) between the activity level measured in the two aliquots (Hunt & Hooper 1993).

The ChE analyses were performed using three replicates and the mean value of the replicates was used in all calculations. An aliquot with a coefficient of variation greater than 5% among its replicates was rerun. For each batch of samples (30 aliquots), a blank and a known standard were processed. Reagents were made fresh daily.

RESULTS

Bats tested in this study had been submitted to the rabies laboratory throughout the year, with 35% of them submitted during the peak activity period in August. Of the tested bats, 88% were big brown bats (*Eptesicus fuscus*). Big brown bats live mostly around human habitat, therefore they have high potential to be encountered by members of the public as well as exposed to pesticides. Detailed results including frequencies and concentration ranges of insecticide residues in the tested bats are presented in Table 3.

Whole body residue analysis.—The average lipid concentration in the 40 bats tested was 7.07% (range: 1.52–25.5%). The median lipid concentration was 5.76%.

Organochlorine insecticides (OCs) were found in 97.5% of the 40 bats tested (Table 3). They were found in both sexes, across species and throughout the year. The following OCs were detected: Dieldrin ((1a*R*,2*R*,2a*S*,3*S*,6*R*,6a*R*,7*S*,7a*S*)-3,4,5,6,9,9-hexachloro-1a,2,2a,3,6,6a,7,7a-octahydro-2,7:3,6-dimethanonaphtho[2,3-*b*]oxirene), DDT (dichlorodiphenyltrichloroethane 1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane), DDE (Dichlorodiphenyl-dichloroethylene (2,2-*bis*-(4-chlorophenyl)-1,1-dichloroethene)), DDD (Dichlorodiphenyl-dichloroethane 1-chloro-4-[2,2-dichloro-1-(4-chlorophenyl)ethyl]benzene), heptachlor epoxide (Epoxyheptachlor 1,4,5,6,7,8,8-Heptachloro-2,3-epoxy-3a,4,7,7a-tetrahydro-4,7-methanoindan), endosulfan (6,7,8,9,10,10-Hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepine-3-oxide) and alpha-chlordane (α-Octachloro-4,7-methanohydroindane).

Five (12.5%) of the bat samples contained pyrethroid residues and all contained detectable concentrations of OCs. Two of the five also contained organophosphates. All five bats were male *E. fuscus* submitted to the rabies lab between June and September (2005–2007 combined). Two bats were found in Porter County, two in Lake County and one in LaPorte County. Their lipid concentration ranged between 2.42–14.1% (mean = 7.6%, median = 7.72%).

Ten of the bats (25%) had detectible levels of OPs. Of these, seven were found between May and August, one was found in March and two in November–December. Five were females and five males. Five were found in Lake County and five in Porter County. Lipid concentrations of these ten bats ranged between 2.67–10.3% (mean = 9.1%, median = 6.53%). Nine of the ten bats contained diazinon (*O*,*O*-Diethyl *O*-[4-methyl-6-(propan-2-yl)pyrimidin-2-yl] phosphorothioate). Of these nine, eight were *E. fuscus* and one was a red bat (*Lasiurus borealis*). Two of the bats that contained diazinon also contained pyrethroids. The one bat that contained chlorpyrifos (*O*,*O*-Diethyl *O*-(3,5,6-trichloro-2-pyridinyl) phosphorothioate

Table 3.—Frequency and concentration of insecticides in bats (n = 40) from Lake, Porter and LaPorte Counties, Indiana. dnq – detected, not quantified.

Insecticides	Frequency of contaminated samples	% Contaminated samples	Pesticide body residues mg/kg wet weight			Reporting limit mg/kg wet weight
			Range	Average	Median	
Organochlorines	**39**	**97.5**				
dieldrin	36	90	0.01–1.5	0.15	0.09	0.005
DDE	36	90	0.06–5.16	0.94	0.37	0.005
DDD	14	35	0.01–0.31	0.09	0.03	0.005
DDT	15	37.5	0.02–0.53	0.13	0.07	0.005
heptachlor epoxide	9	22.5	0.01–0.78	0.20	0.06	0.005
endosulfan I	3	7.5	0.01–0.02	0.02	0.02	0.005
alpha-chlordane	1	2.5	0.04	0.04	0.04	0.005
Pyrethroid	**5**	**12.5**				
bifenthrin	1	2.5	0.37	0.37	0.37	0.005
lambda isomer	1	2.5	0.01	0.01	0.01	0.005
lambda-cyhalothrin	1	2.5	0.18	0.18	0.18	0.005
permethrin	1	2.5	0.02	0.02	0.02	0.005
cypermethrin	1	2.5	0.00002	0.00	0.00	?
cypermethrin 1	1	2.5	0.01	0.01	0.01	0.005
cypermethrin 3	1	2.5	0.005	0.00	0.00	0.005
cypermethrin 2 (+4)	1	2.5	0.004	0.00	0.00	?
esfenvalerate	2	5	0.01–0.03	0.02	0.02	0.005
esfenvalerate 1	1	2.5	0.01	0.01	0.01	0.005
esfenvalerate 2	1	2.5	0.03	0.03	0.03	0.005
Organophosphates	**12**	**30**				
chlorpyrifos	1	2.5	0.12	0.12	0.12	0.005
diazinon	9	22.5	0.03–0.81	0.43	0.60	0.025
Carbamates	**1**	**2.5**				
carbaryl	1	2.5	dnq			0.025

was *E. fuscus*. All 10 bats also contained OCs. None of the 10 bats showed significant reactivation of ChE activity after incubation with 2-PAM.

Carbaryl (1-naphthyl methylcarbamate) was detected in one bat, though it could not be quantified. In an attempt to measure the quantity of the carbaryl residues, standards were made in corn oil. Adding lipid to the standards did increase the response factor, however, it was not sufficient to account for the enhancement seen in the bat specimen. The exposed individual was a female *L. borealis* found in July in Porter County. Its lipid concentration was 3.12%. In addition to carbaryl, this bat had diazinon and OC residues. In the brain ChE reactivation tests, this bat showed significant spontaneous reactivation of at least 5% of its brain ChE function.

Statistical analysis: Since OC insecticides were found in all of the sampled bats but one, there was no need to conduct χ^2 statistical tests to identify relationships between exposure to OCs and other factors of this sample such as geographic origin of the bats, their sex or species. This sample indicates that all the geographic areas tested had equal OC contamination levels. Similarly both sexes and all species in the sample were equally contaminated. Therefore this insecticide group was not included in the following statistical analysis. There was no significant relationship between insecticide exposure and the county of the bats' origin (χ^2 (2)=0.57, p=0.75); between insecticides and sex (Chi square(1)=0.34, p=0.56) and between insecticides and season (χ^2(3)= 2.39, p=0.5). This implies that insecticide residues were found equally in all three counties; that male and female bats were equally exposed to contamination; and that bat exposure to insecticides was equally distributed throughout the year. The results of these analyses should be interpreted with care because of the small sample size (n=40) of this

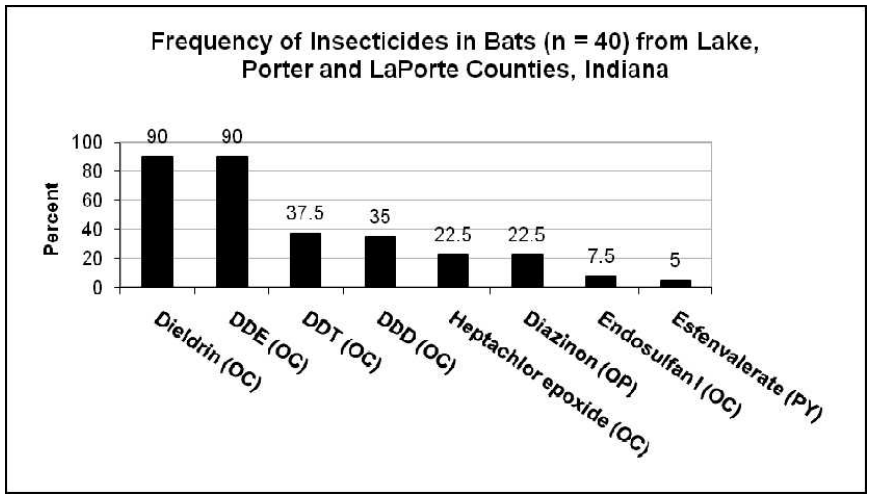

Figure 2.—Frequencies of the main insecticides detected in the 40 bats tested. Bats were collected from Lake, Porter and LaPorte Counties, Indiana by the public and submitted to the *Indiana State Department of Health* rabies laboratory between September 2005 and December 2007. The letters in parentheses represent the insecticide group: OC – organochlorines, OP – organophosphates, PY – pyrethroids.

part of the survey (Table 1). Pyrethroid and cholinesterase insecticide residues were not detected in any of the individuals of *Lasionycteris noctivagans* tested. The ChE inhibitors (diazinon and carbaryl) were found in one of the two *L. borealis* tested.

Bats with insecticide mixtures and heavier residue burdens: 37 of the 40 bats tested (92.5%) had residues of more then one insecticide; of these, 70.3% (26 bats) had residues of three or more insecticides. Thirteen bats (32.5%) had residues of pyrethroids or/and ChE inhibitors; all of them had residues of OC as well. Sixteen (40%) of the bats had residues of three or more insecticides with relatively high residual load of at least one insecticide. The average lipid concentration in these bats was 8.51% (range of 1.77–23.2%) and the median was 6.92%. There was no significant relationship between the distribution of the more heavily contaminated bats and county (χ^2 (2) = 2.4, p = 0.3); sex (χ^2 (1) = 0.002, p = 0.97) or season (χ^2 (3) = 2.69, p = 0.44). The results of these analyses should be interpreted with care because of the small sample size.

Brain ChE activity and reactivation testing.—Of the 332 bat brains tested six (1.8%) showed significant recovery of brain ChE function after incubation with 2-PAM. As part of this survey, 33 of the 332 bats whose brains were tested for ChE activity and reactivation were also analyzed for pesticide body residues. While 10 of these 33 bats contained residues of the OPs diazinon and chlorpyrifos, none of them demonstrated significant ChE reactivation after incubation with 2-PAM. One hundred and fifty five (46.67%) of the samples showed significant spontaneous recovery of 5% or more. No significant relationships were found between spontaneous reactivation and sex (χ^2 (2) = 0.63, p = 0.73), county (for counties with sample size of 7 or more bats, χ^2 (10) = 9.56, p = 0.48) or season (χ^2 (3) = 1.75, p = 0.63).

DISCUSSION

Overall, the majority of the bat samples had detectable concentrations of dieldrin and DDE, while many samples also had detectable concentrations of DDT, DDD, heptachlor epoxide and diazinon (Figure 2). Organochlorines were by far the major insecticides detected and were found in 97.5% of the bat samples. Chronic exposure to insecticides from this group may cause a variety of clinical effects based on the insecticide and the species examined. In mammals, signs of OC exposure include anorexia, muscular weakness, hyper-excitability, muscle twitching, ataxia, visual blurring, loss of consciousness and death (Ecobichon 2001). Chronic exposure to OCs may hamper the ability of bats to enter torpor and to forage effectively and therefore to control their ener-

getic requirement. Energetic deficiency may lead to inability of female bats to support their young. In extreme cases it may cause indirect death of affected bats. Chronic exposure to OCs may also lead to reproductive disorders (Ecobichon 2001) and may cause further reduction of the already low reproductive rate of bats (one to two young per year by most species, three to four in red bats).

Due to the high persistence of OCs and their tendency to bioaccumulate, the U.S. Environmental Protection Agency (EPA) had imposed restrictions on the use of most OCs since the 1970s and early 1980s. By 1988, only four OCs were still used in agriculture in the USA and included dicofol (2,2,2-trichloro-1,1-bis(4-chlorophenyl)ethanol), endosulfan, lindane ((1*r*,2*R*, 3*S*,4*r*,5*R*,6*S*)-1,2,3,4,5,6-hexachlorocyclohexane) and methoxychlor (1,1,1-Trichloro-2,2-bis(4-me thoxyphenyl)ethane). The use of dieldrin, DDT, DDE and DDD was restricted in the 1970s (Ecobichon 2001; Harte 1991; Nowell et al. 1999; USGS 1999). Therefore, bat residues from these insecticides are the result of continued bioaccumulation and persistence of OC pesticides that were applied more than 30 years ago. Because almost all bats in the sampled population contained OCs, it is likely that many of the bats populating the sampled areas, including several species and both sexes are contaminated with these chemicals.

Forty percent of the 40 bats tested had residues of three or more types of insecticides. All these bats contained OCs and 81% of them contained more than one type of organochlorines. Considering the high persistence of OCs it is not surprising that many of these compounds are still common in the environment and become available to bats.

Another prevalent insecticide was diazinon. It was detected in 22.5% of the bats. Diazinon is an organophosphate pesticide used in agricultural applications to control a variety of insects. It is registered to control foliage and soil insects as well as pests of many fruit, nut, vegetable, and field crops. Diazinon is also used in cattle ear tags. Up until the early 2000s, diazinon was one of the most widely used insecticides in the U.S. for household, lawn and garden pest control (up to 3,500 metric tons used each year) as well as agricultural pest control (about 30% of all use). By the end of 2004, EPA phased out and eliminated all residential uses of this insecticide, but approval for agricultural uses continued. The EPA risk assessment conducted in 2002, stated that diazinon posed unacceptable risks to birds and other wildlife species. As a result, the agency announced in 2006 its plan to phase out within 2–5 years certain agricultural crop uses, phase out the use of a granular formulation and aerial applications, and reduce the overall amount and frequency of use (EPA 2006, 2008).Approximately 6,000 metric tons of the active ingredient diazinon were still used annually on agricultural sites in 2008 (EPA 2008).

It may be that finding diazinon residues in such high rates while its use is being significantly reduced, implies that this chemical is more persistent in the environment than thought. Ferrando et al. (1992) tested persistence of various insecticides (organochlorines, OPs and carbamates) in unfiltered lake water (pH = 9.0 ± 0.5) stored at 22°C. The most persistent insecticides in their study were diazinon (half life of 71 h in natural water) and thiobencarb (*S*-[(4-chlorophenyl)methyl] *N,N*-diethylcarbamothioate), which is a carbamate with half life of 74 h in natural water. This implies that ChE inhibitors can persist in surface water for days after application and longer during the colder months (fall and spring). Contaminated water after ChE inhibitor application may therefore become another exposure medium of diazinon to wildlife.

The other ChE inhibitors identified in the sampled bats were chlorpyrifos and carbaryl. Finding ChE inhibitors in bat samples is surprising because they are short-lived and do not persist in the living body. Several authors have suggested that since ChE inhibitors do not tend to bioaccumulate in living tissue, their presence is indicative of recent exposure prior to death (Hill 1989, 1995; O'Shea & Clark 2002). Nevertheless, the presence of ChE inhibitor residues in the absence of demonstrated ChE inhibition supports, but does not confirm causation in a specific mortality (Eidels et al. 2007).

Pyrethroid insecticides entered the marketplace in 1980. Within two years they comprised more than 30% of the worldwide insecticide usage (Ecobichon 2001). These are synthetic chemicals whose structures mimic the natural insecticide pyrethrins. Pyrethrins are produced by the flowers of the Pyrethrum plants (*Chrysanthemum cinerariaefolium* and *C. coccineum*).

They are highly biodegradable and break down easily when exposed to sunlight. Pyrethroids were manufactured to persist longer in the environment than pyrethrins. Pyrethroids bind sodium channels on the axon membrane of nerve cells. As a result, the channels are unable to close normally which leads to continuous nerve stimulation. Clinical signs of pyrethroid exposure include tremors and inability to produce coordinated movement (Valles & Koehler 1997). The EPA (2006) decision to phase out some of the OPs and restrict the use of others led to their gradual replacement with pyrethroids. Pyrethroids were detected in five of the 40 bats tested; all were male *E. fuscus*. Three of these bats contained more than one type of pyrethroid. None of the individual insecticides in this group occurred in more than two bats. Two of the five bats with pyrethroid residues contained diazinon residues as well and all five had OC residues.

Ten (77%) of the 13 bats containing ChE inhibitors and/or pyrethroid residues were collected between May and September which are the active months for the bats and also the seasons in which insecticides are more likely to be applied. Detection of pesticides that do not persist in living tissues is expected to be highest immediately after application.

Only 1.8% of the 332 bats tested for brain ChE reactivation showed significant recovery of brain ChE activity. Furthermore, of the 10 bats that their whole body residue analysis confirmed that they contained OP residues, none showed significant recovery of brain ChE activity after incubation with 2-PAM. This raises the question whether the reactivation tests identified all the bats that were exposed to OP. If the initial dose of OP is too small to affect the brain or the inhibited enzymes become resistant to reactivation, reactivation tests will not identify exposure to OPs (Wilson et al. 1992). OP inhibited ChE may become resistant to reactivation by undergoing 'aging' process. Aging occurs when conformational change in the molecular structure of the organophosphate occurs after the initial organophosphate cholinesterase bonds are formed. This structural change increases the strength of the organophosphate-cholinesterase bond and makes the complex irreversibly bonded (Johnson et al. 2000). Aging is associated with dealkylation (the removal of alkyl group, an organic molecule made up of chains of carbon and hydrogen atoms joined by single bonds) by C-O or P-O fission (Beauregard et al. 1981). This process can occur within hours to days after the initial effect, depending upon the structure of the OP, the pH and the temperature (Johnson et al. 2000).

If aging occurred, incubation with 2-PAM of brains from even severely poisoned animals with significant inhibition of brain ChE will not yield enzyme reactivation. Wilson et al. (1992) measured brain ChE reactivation after incubation with 2-PAM in quail poisoned with parathion. Reactivation persisted for two days at ambient temperature. After four days at ambient temperature, there was no reactivation of brain enzyme in the birds. Bats that were submitted to the rabies laboratory were already dead. Storage conditions prior to submission were unknown. It could be that if the bats were not frozen immediately after death, aging of their brain enzymes occurred. In that case, enzyme reactivation may not be a suitable method to identify OP exposure. Due to potential false negatives no specific conclusions could be made with regard to exposure of bats to OPs in Indiana.

Cholinesterase enzymes inhibited by carbamates regain their full activity as the enzyme-carbamate bond degrades. Hunt & Hooper (1993) developed an assay that identifies this spontaneous reactivation of brain ChE as an indicator of carbamate poisoning. The problem rose when the same authors identified similar spontaneous reactivation of brain ChE activity in brains that were not exposed to carbamates (Hunt & Hooper 1993). They attributed this reactivation to a slow release of divalent cations (Ca^{2+} or Mg^{2+}) from cellular membranes fractured during the brain homogenization process. To capture these cations they added the chelating agent EDTA (ethylenediaminetetraacetic acid, tetrasodium salt) to the brain homogenate. This effectively eliminated the increase in ChE activity in non-contaminated brains of birds and mammals tested by these authors. Hunt & Hooper (1993) also incorporated EDTA in their spontaneous reactivation assay (Hunt & Hooper 1993).

For this work, the Hunt & Hooper's (1993) reactivation assay was used to identify carbamate poisoning and EDTA was added in the concentration recommended by these authors (and later in higher concentrations) to all brain samples. Of the 332 samples tested, 46.67%

showed significant recovery of at least 5% of brain ChE activity. This reactivation occurred equally throughout the year, in both males and females and across the different counties in Indiana (with a sample size of 10 bats or more). Such high exposure rates to pesticides that do not persist in the environment (Hill 1989, 1995; O'Shea & Clark 2002) can only occur shortly after a vast application of large quantities of these insecticides. Because of the unrealistically large percentage of bats that demonstrated significant spontaneous reactivation and the fact that some of these bats were found during winter when insecticides are rarely in use we suspect false positive errors in our testing. We shall describe shortly the measures that may be taken against such bias. Spontaneous increases in ChE activity in bat brains may result from a process other than the one suggested by Hunt & Hooper (1993). If that is the case, the Hunt & Hooper (1993) reactivation assay should not be used without adjustments to identify carbamate poisoning in bats. Nevertheless, while the option of a false positive error exists, no conclusions could be made based on these results regarding the exposure of bats to carbamates in Indiana.

The results of this research suggest that even today, approximately 30 years after OC insecticides were phased out, bats in the Indiana Great Lakes region are still exposed to these insecticides. To fully appreciate the implications, the effects of chronic exposure of bats to OCs should be further explored. The presence of ChE inhibiting insecticides in bats from the Great Lakes region was also demonstrated. Clark (1986) orally dosed bats and mice with the OP methyl parathion and found that although the LD_{50} for bats was 8.5 times the LD_{50} for mice; bats lost coordination after one hour and were still unable to right themselves at the end of the study, 24 hours after dosing. Mice, on the other hand, recovered back to normal within 2–3 hours after the OP administration. Clark (1986) suggested that free ranging bats that are exposed to OPs, may lose coordination, fall to the ground and die. To assess the risk that ChE inhibitors pose to bats, controlled dosing studies as well as field studies testing free ranging bats following insecticide applications are required. This information, combined with statistical data on the use of ChE inhibitors should allow an estimation of the overall impact of these pesticides on bats. Pyrethroids and carbamates were also detected in bats as part of the residue analysis performed in the current study. These results are among the first to reported residue detections of pesticides from these groups in bats.

This work is the first step in investigating the impact of insecticides on bats. Its objective was to identify the major insecticides to which bats are exposed in order to focus further investigation on these specific pesticides. Because the sampling of bats was not random, no assumptions could be made with regards to the extent of exposure in the overall bat population. Nevertheless, testing already distressed bats allowed identification of insecticide residues in these animals without sacrificing healthy free ranging bats.

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A NEW JUNIOR SYNONYM FOR *RAPTOHEPTAGENIA CRUENTATA* (WALSH, 1863) AND REMARKS ABOUT NEARCTIC *HEPTAGENIA* WALSH, 1863 (INSECTA: EPHEMEROPTERA: HEPTAGENIIDAE)

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ABSTRACT. Examination of a rediscovered slide of holotype genitalia of *Heptagenia patoka* Burks, 1946, (Insecta: Ephemeroptera: Heptageniidae) revealed that *H. patoka* is synonymous with *Raptoheptagenia cruentata* (Walsh, 1863) [= *H. patoka*, **new synonym**]. With the synonymy of *H. patoka* under *R. cruentata*, all North American *Heptagenia* Walsh, 1863, species are known in the larval stage. The larvae of *H. dolosa* Traver, 1935, and *H. townesi* Traver, 1935, have not been described yet, but recently they were associated with adults. Both species are part of the *H. marginalis* Banks, 1910, species group. *Heptagenia townesi* differs from *H. marginalis* by having longer apical spines on the segments of the caudal filaments, but further study of *H. dolosa* will be required to elucidate possible diagnostic characters.

Keywords: Mayflies, systematics, taxonomy, aquatic insects

INTRODUCTION

The mayfly genus *Heptagenia* Walsh, 1863, (Insecta: Ephemeroptera: Heptageniidae) is distributed throughout the Holarctic biogeographic realm and part of the Oriental realm, with twelve species currently recognized from North America (Webb et al. 2007; Webb & McCafferty 2008). A thirteenth Holarctic species may be present (Kjærstad et al. 2012).

Heptagenia patoka Burks, 1946, has been considered a species endemic to the Central Lowlands of Illinois and Indiana, USA (Randolph & McCafferty 1998). It was described on the basis of a single male adult collected 19 July 1945 from Patoka, Illinois (Burks 1946), and its larva remains unknown (Webb et al. 2007). The species has been reported from only one other location in Illinois and from two locations in Indiana (Randolph & McCafferty 1998).

Webb et al. (2007) recently restricted the concept of *H. patoka* to the holotype, and they noted that subsequent reports were based on specimens from places where a very similar species, *Raptoheptagenia cruentata* (Walsh, 1863) (Whiting & Lehmkuhl 1987), has been found (Randolph & McCafferty 1998), including the Indiana locale of the *R. cruentata* neotype (McCafferty 1988). *Raptoheptagenia cruentata* is a species of big rivers from much of central North America (Waltz et al. 1998).

Webb et al. (2007) indicated that *H. patoka* and *R. cruentata* might be synonymous but that the synonymy could not be determined because the genitalia of the *H. patoka* holotype had been lost. Burks (1946: 6, 1953: Fig. 369) provided figures of those genitalia. Burks (1953: 181) distinguished *H. patoka* from *R. cruentata* by the narrower apices of the median penes titillators in *H. patoka*. Burks (1953) also indicated a concave shape of the apices of the penes lobes for *H. patoka* (Burks 1953: Fig. 367), compared to a convex shape of the same lobes for *R. cruentata* (Burks 1953: Fig. 369). The species otherwise are similar, including body coloration and wing maculation.

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METHODS

We confirmed that the material reported by Randolph & McCafferty (1998) as *H. patoka* (see materials examined, below) actually is *R. cruentata*.

We located the slide containing the missing holotype genitalia of *H. patoka* at the Illinois

Natural History Survey (see materials examined, below), where the rest of the holotype is housed (Webb 1980). We examined it carefully via compound light microscopy, paying particular attention to details revealed by passing the plane of focus up and down through the slide.

Materials examined are deposited in the Biodiversity Institute of Ontario, Guelph, Ontario, Canada [BIO]; the Clemson University Arthropod Collection, Clemson, South Carolina, USA [CUAC]; the Illinois Natural History Survery, Champaign, Illinois, USA [INHS]; collections of Pennington and Associates, Inc., Cookeville, Tennessee, USA [PAI]; and the Purdue University Entomological Research Collection, West Lafayette, Indiana, USA [PERC].

RESULTS

Examination of the *H. patoka* holotype genitalia revealed that the penes lobes of *H. patoka* are apically convex, unlike the illustration given by Burks (1953: Fig. 367). The median titillators appear to be narrower than those attributed to *R. cruentata* only because of their fixed orientation on the slide. This observation is apparent upon microscopic examination when the plane of focus is passed up and down through the slide; although the genitalia are slide-mounted, they are three-dimensional, and only parts of the genitalia are in focus at any given time while examining them using a standard compound light microscope. Furthermore, dorsolateral and dorsal submedian spines are absent from the penes lobes, which is the case with *R. cruentata* but with none of the other North American *Heptagenia*. On the basis of these observations, we consider *H. patoka* to be synonymous with *R. cruentata* (Walsh, 1863) [= *H. patoka* Burks, 1946, **new synonym**].

DISCUSSION

With the synonymy of *H. patoka* and *R. cruentata*, the larval stages of all North American *Heptagenia* species now are known. Larvae of the southeastern species (McCafferty et al. 2010) *H. dolosa* Traver, 1935, and *H. townesi* Traver, 1935, have not yet been described, but they tentatively have been associated with identified adults through molecular methods (Webb et al 2012). Both *H. dolosa* and *H. townesi* share the longitudinal dark abdominal markings characteristic of larvae in the *H. marginalis* Banks, 1910, species group.

Heptagenia townesi appears to differ from larvae of *H. marginalis* by having longer apical spines on the segments of the caudal filaments (1/3 the length of the segment for *H. townesi* compared to 1/8 the length of the segment for *H. marginalis*).

Only a single larval specimen of *H. dolosa* is known, and no reliable morphological characters for species diagnosis of this stage have been discovered. However, the male and female adults are identifiable on the basis of a combination of characters, including small size, pale caudal filaments, a single median femoral band, a lack of dark mesonotal markings and limited markings on the middles of abdominal terga. The male adult is further distinguishable from related species by the absence of distinct projections on the inner margins of the penes lobes (Traver 1935, Traver 1935: Figs. 94–95).

Webb et al. (2007) flagged *H. dolosa* and the perhaps more widespread (Jacobus & McCafferty 2001: 66) *H. julia* Traver, 1933, as ''doubtfully good species'' at that time. However, recent evidence from DNA barcoding (Webb et al. 2012) suggests that both *H. dolosa* (discussed above) and *H. julia* are highly divergent from other North American *Heptagenia* and thus are likely good species.

Heptagenia julia is most similar to *H. pulla* (Clemens, 1913). Male adults of *H. julia* differ from those of *H. pulla* primarily by having penes with more acute apical projections and median spines that are subequal in size, their smaller size, and their indistinctly colored caudal filaments. Later larval instars of the two species differ mostly in size, with *H. julia* being nearly 3 mm shorter in body length than *H. pulla* (Traver 1935).

MATERIALS EXAMINED

Heptagenia dolosa.—One larva, USA, Georgia, Oconee/Rabun Counties, Chattooga River at State Road 28, 5-V-2010, J Worsham [PAI]. One female adult, USA, North Carolina, Swain County, Oconaluftee River at the Blue Ridge Parkway, 23-VII-2009, CR Parker, A Kumar [BIO].

Heptagenia julia.—Two male subimagos, USA, North Carolina, Swain County, Oconaluftee River at Blue Ridge Parkway, 23-VII-2009, CR Parker, A Kumar [BIO].

***Heptagenia marginalis*.**—One larva, USA, Kentucky, Whitley County, Cumberland Falls State Park, Cumberland River at State Road 90, 21-VI-2005, B Barnd [PERC]. One larva, USA, Pennsylvania, Wyoming County, Susquehanna River, 4 miles SSE of Meshoppen, 25-VII-2005, EB Kratzer, DH Funk [PERC].

***Heptagenia patoka*.**—HOLOTYPE, one male adult, USA, Illinois, Marion County, Patoka, 19-VII-1945, Ross, Sanderson, genitalia on slide [INHS].

***Heptagenia townesi*.**—One female adult, USA, North Carolina, Swain County, Ekeneetlee Creek, 75 m upstream of confluence with Eagle Creek, 10-V-2003, BD Heinold, S Higdon [INHS]. One larva, USA, South Carolina, Pickens County, Estatoe Creek at Laurel Valley Road, 19-IX-1997, S Spichiger [CUAC].

***Raptoheptagenia cruentata*.**—NEOTYPE larva, USA, Indiana, Martin County, East Fork White River at Hindostan Falls Public Fishing Site, 15-VII-1982, AV Provonsha, V VanAllen [PERC]. One male adult, one female adult, USA, Illinois, Pike County, lights at Motel Pike, west side of Pittsfield, 10-VI-1997, MA Harris, DL Adolphson [INHS]. One larva, USA, Indiana, Jefferson County, Ohio River, 26-V-1981 [PERC]. One male adult, two female adults, USA, Indiana, Martin County, East Fork White River at Hindostan Falls Public Fishing Site, 8-VI-1978, M Minno, D Bloodgood [PERC]. One male adult, same locale, 2-VII-1974, Provonsha, Lick [PERC]. One male adult, two female adults, same locale, 2-VII-1974, AV Provonsha, L Dersch, M Lick [PERC]. One adult, same locale, 20,21-VI-1974, AV Provonsha, L Dersch [PERC]. Adults (number unknown), USA, Indiana, Parke County, Racoon Lake, 24-VI-1973, HR Lawson [PERC]. One adult, USA, Indiana, Tippecanoe County, Wabash River at West Lafayette, 13-VII-1973, Provonsha [PERC]. Three adults, same locale, 18-VI-1974, Provonsha [PERC].

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IS THERE HOPE FOR THE HOOSIER FROG? AN UPDATE ON THE STATUS OF CRAWFISH FROGS (*LITHOBATES AREOLATUS*) IN INDIANA, WITH RECOMMENDATIONS FOR THEIR CONSERVATION

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ABSTRACT. Crawfish Frogs (*Lithobates areolatus*) are a State Endangered species that have experienced declines through much of their range in Indiana. We conducted surveys at nine historical sites and detected Crawfish Frogs at only one of them. Data suggest this species has been extirpated from Benton, Fountain, and Vermillion counties in the north, Vanderburgh and Warrick counties in the south, and Morgan and Monroe counties in the east. Robust populations of Crawfish Frogs persist in two areas, at Hillenbrand Fish and Wildlife Area-West in the southwest, and at Big Oaks National Wildlife Refuge in the southeast. One cluster of populations remains in Spencer County, in the south. Our data suggest that there are fewer than 1,000 adult Crawfish Frogs in Indiana: Big Oaks supports about 300 animals, Hillenbrand supports about 200 animals, and remaining animals are scattered among populations that are generally small and located on private lands in southwestern Indiana. Despite these pessimistic data, Crawfish Frogs are resilient and will establish populations at new sites when habitat becomes available and animals are close enough to colonize. If Crawfish Frogs are to persist in Indiana, they must become a component of the management plans on both public and private lands. When this occurs, not only could the precipitous decline of Crawfish Frogs in this state be halted, but Indiana's public grasslands are extensive enough that intervention could lead to the eventual downlisting of the species.

Keywords: Crawfish Frog, *Lithobates areolatus*, status, conservation, management

INTRODUCTION

Crawfish Frogs (*Lithobates areolatus*), formerly known as Hoosier Frogs (Hay 1892; Test 1893), are secretive, burrow-dwelling anurans inhabiting parts of the central and south-central United States (Smith 1950; Parris & Redmer 2005). In Indiana, Crawfish Frogs occur predominately in the western—especially the southwestern—portion of the state, although a presumably isolated series of sites now occurs at Big Oaks National Wildlife Refuge (NWR) in southeastern Indiana (Minton 2001; Haswell 2004; Engbrecht & Lannoo 2010). Once considered "locally plentiful" in Indiana, Crawfish Frog declines in the 1970s and 1980s led to their listing as a State Endangered Species in 1988 (Indiana Department of Natural Resources [IDNR] Technical Advisory Committee [TAC] 1987; Minton 2001).

Two of us recently published the known historic distribution of Crawfish Frogs in Indiana (Engbrecht & Lannoo 2010). We relied on records from the literature, museum specimens, IDNR datasets, and unpublished accounts. The purpose of the present study is to

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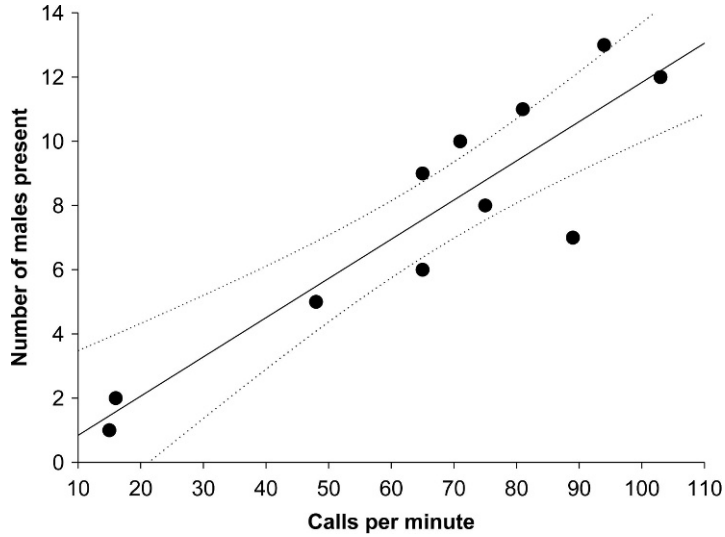

Figure 1.—Graph showing the relationship between call rate (calls/min) and number of *Lithobates areolatus* males present in wetland. Linear regression shows a highly significant correlation between number of males present and maximum call rate ($p = .0001$, $r^2 = 0.83$). Data from Nate's Pond and Cattail Pond at HFWA-W were combined for this analysis.

compare the current known distribution of Crawfish Frogs against this historic baseline and recommend ways in which Crawfish Frogs can be conserved. We provide the results of surveys and give a revised assessment of the species' status in Indiana.

METHODS

We used a number of survey techniques to assess the status of Crawfish Frogs including breeding call surveys (conducted both manually and using automated recording systems [ARS]), egg mass surveys, incidental road kill data, drift fence surveys, aquatic surveys for tadpoles, and terrestrial surveys for newly metamorphosed juveniles (Heyer et al. 1994; Dodd 2010; Heemeyer et al. 2010). Crawfish Frog populations are most easily detected by the loud, distinct calls of breeding males (Swanson 1939; Gerhardt 1975), and thus call surveys offer the best opportunities for detecting populations. Indeed, in this study, the majority of locality data were obtained using call surveys conducted manually or using ARS (Weir & Mossman 2005; Dorcas et al. 2010).

We systematically and opportunistically surveyed for Crawfish Frogs across western and southern Indiana from 2009–2011, visiting previously documented sites and those with the potential to host Crawfish Frog populations. Among these sites we systematically surveyed nine historic localities (most recent records from 1949–1991; Engbrecht & Lannoo 2010) using Song Meter® recording units (Wildlife Acoustics Inc, Concord Massachusetts, USA). Recordings were analyzed using a Dell Latitude™ E6400 Series laptop computer and Song Scope® call recognition software (Wildlife Acoustics Inc, Concord Massachusetts, USA).

To provide a rough estimate of population size at our surveyed wetlands, we used the technique described by Engbrecht (2010). This method couples maximum calling rates to numbers of males present in wetlands (Fig. 1). The association between chorusing intensity and abundance levels has also been noted in other studies of North American anurans (Lepage et al. 1997; Crouch & Paton 2002). For example, Nelson & Graves (2004) found an association between increasing population sizes and increasing call index values in Green Frogs (*Lithobates clamitans*). They state that call rates may provide a more accurate indicator of population density than call index values. We refer the reader to Engbrecht (2010) for a detailed description of this technique.

The overall population size of Hillenbrand Fish and Wildlife Area-West (HFWA-W) was estimated using data collected from drift fences, funnel traps, and chorusing levels. At Big Oaks NWR, population sizes were estimated using egg mass surveys at wetlands where calling

Table 1.—Survey sites where *Lithobates areolatus* was detected in Indiana from 2009–2011. Maximum call rates are given in calls/min. The Hillenbrand FWA-W population estimate is based on pitfall trapping, funnel trapping, and relative chorusing levels. The population estimate for Big Oaks NWR is based on egg mass counts and chorusing levels (see text for details). Maximum call rate at the Ronk Locality was reported by Ron Ronk (pers. comm.).

Site	County	Maximum call rate	Estimated number of calling males	Estimated population size
Daviess County South 1	Daviess	32	3–6	12–24
Daviess County South 2	Daviess	10	1–2	4–8
Daviess County South 3	Daviess	16	2–3	8–12
Klueh Locality	Daviess	–	–	–
Odon 1	Daviess	–	–	–
Odon 2	Daviess	22	2	8
Goose Pond 4	Greene	–	–	–
Goose Pond Private 1	Greene	39	4–7	16–28
Goose Pond Private 2	Greene	21	2–4	8–16
Hillenbrand FWA-W Cluster	Greene	–	–	~200
Hillenbrand Offsite	Greene	–	–	–
Jasonville 1	Greene	6	1	4
Jasonville 2	Greene	–	–	–
Owen County Historic	Owen	84	10–12	40–48
Owen County Recent	Owen	65	7	28
Spencer County Cluster	Spencer	–	–	–
Dugger	Sullivan	56	6	24
Hymera	Sullivan	46	4–6	16–24
Ronk Locality	Sullivan	80	9	36
Stonebraker Locality	Sullivan	36	4	16
Dave's Pond	Vigo	57	6	24
Big Oaks NWR Cluster	Jefferson, Jennings, Ripley	–	–	~300

males were heard, which we assumed equaled the number of females, and a 1:1 sex ratio was applied to estimate population size (Kinney 2011). In cases where distant chorusing was heard at restricted sites and egg masses could not be counted, chorusing levels were used to estimate population counts.

RESULTS

We detected Crawfish Frogs at fewer than 60 sites throughout the state, including 21 localities in southwestern Indiana and > 27 localities at Big Oaks NWR in southeastern Indiana (Table 1; Fig. 2). Crawfish Frogs were detected at only one of nine historic sites (Engbrecht & Lannoo 2010) and were not detected at 25 sites where they had recently been reported (2000—2008; Tables 1, 2). Population estimates ranged from four in the smallest population to 48 in the largest (Table 1). Populations at HFWA-W and Big Oaks National Wildlife Refuge were estimated to be approximately 200 and 300 individuals, respectively (Table 1). To parallel Engbrecht & Lannoo (2010) we present detailed results by county.

Clay County.—We surveyed for Crawfish Frogs near Brazil where IDNR personnel identified chorusing between 2004 and 2008 (Engbrecht & Lannoo 2010), but did not detect them. We also conducted surveys in areas of extensive grassland habitat at Chinook Fish and Wildlife Area (FWA) but did not detect Crawfish Frogs.

Daviess County.—Crawfish Frogs continue to persist in two distinct clusters in Daviess County, one in the south-central region of the county, the other northeast of Odon (Fig. 2). We identified a new breeding site in the south-central cluster in 2009 (Table 1; Fig. 2). Habitat at this site consists of an old cattle pond surrounded by an abandoned pasture. This population may be in jeopardy as the wetland is currently being used to rear game fish. We also detected Crawfish Frogs at two previously identified localities in this cluster in 2009 and 2010 (Table 1; Fig. 2). A fourth breeding site

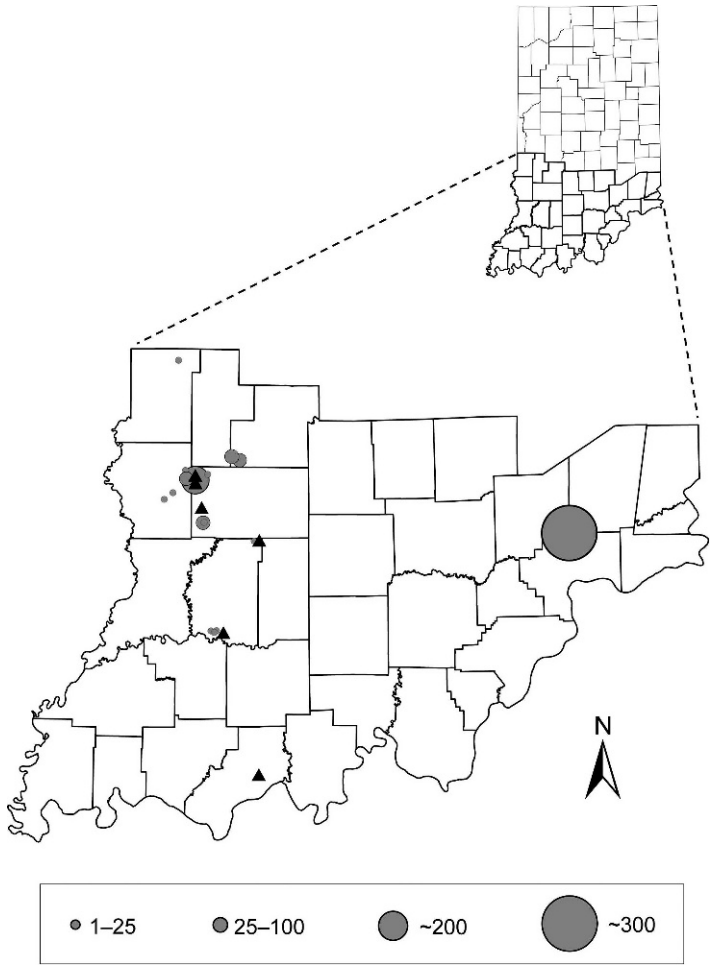

Figure 2.—Population estimates for *Lithobates areolatus* in Indiana. We estimated the overall population size of HFWA-W using data collected from drift fences, funnel traps, and relative chorusing levels. Estimates for Big Oaks are based on egg mass counts and relative chorusing levels. Circle size indicates estimated population size; triangles represent localities that lack data sufficient for calculating population estimates.

located by State Herpetologist Sarabeth Klueh during a March 2011 survey (Klueh, pers. comm.; Table 1; Fig. 2) appears to be a re-confirmation of an IDNR point originally discovered between 2004 and 2008 (Engbrecht & Lannoo 2010). We did not detect Crawfish Frogs at three sites in this cluster identified by IDNR between 2004 and 2008 (Engbrecht & Lannoo 2010; Table 2).

Surveys performed at the cluster northeast of Odon revealed Crawfish Frogs from one new locality (Table 1; Fig. 2). Calling at this site was light in 2009, perhaps representing only one male; we did not detect chorusing here in 2010. Crawfish Frogs were detected at a second, previously identified locality, in 2009 and 2010. Frogs at this site bred in what appeared to be a small livestock pond adjacent to a cattle pasture. We did not detect Crawfish Frogs at one of the sites where IDNR personnel reported hearing them between 2004 and 2008 (Engbrecht & Lannoo 2010; Table 2).

We surveyed for Crawfish Frogs east of Odon where Minton and W. M. Overlease collected a specimen in 1953 (Engbrecht & Lannoo 2010) but heard no calling (Table 2).

Fountain County.—Engbrecht & Lannoo (2010) noted a single Crawfish Frog locality near Kingman based on a specimen collected by Minton in 1951. We surveyed here but heard no calling

Table 2.—Survey sites where *Lithobates areolatus* was not reconfirmed during surveys performed from 2009–2011. Populations where the status is unknown are represented by (U); populations presumed to have been extirpated are represented by (X).

Site	County	Status	Most recent record
Brazil	Clay	(U)	2004–2008
Daviess County Historic	Daviess	(X)	1953
Daviess County South 4	Daviess	(U)	2004–2008
Daviess County South 5	Daviess	(U)	2004–2008
Daviess County South 6	Daviess	(U)	2004–2008
Odon 3	Daviess	(U)	2004–2008
Fountain County Historic	Fountain	(X)	1951
Goose Pond 1	Greene	(U)	2004–2008
Goose Pond 2	Greene	(U)	2004–2008
Goose Pond 3	Greene	(U)	2004–2008
Greene County Historic	Greene	(X)	1949
Jasonville 3	Greene	(U)	2004–2008
Jasonville 4	Greene	(X)	2004–2008
Linton	Greene	(X)	2004–2008
Scotland	Greene	(U)	2004–2008
Big Oaks	Jefferson	(X)	2006
Monroe County Historic	Monroe	(X)	1991
Morgan County Historic	Morgan	(X)	1987
Spencer County 2	Spencer	(U)	2008
Cass	Sullivan	(U)	2004–2008
Glendora	Sullivan	(U)	2004–2008
Glendora East	Sullivan	(U)	2004–2008
Greene/Sullivan County Line	Sullivan	(U)	2004–2008
Hymera	Sullivan	(U)	2004–2008
Morton Pond South	Sullivan	(U)	2004–2008
Shakamak	Sullivan	(U)	2004–2008
Sullivan County Historic	Sullivan	(X)	1952
Timm 1	Sullivan	(X)	2000
Timm 2	Sullivan	(X)	2000
Timm 3	Sullivan	(X)	2000
Vermillion Historic	Vermillion	(X)	1951
Vigo County Historic	Vigo	(X)	1967
Vigo/Parke County Line	Vigo	(X)	2004–2008

(Table 2). This site is dominated by agriculture and forest, and grassland habitat is sparse. We know of no other records of Crawfish Frogs at this site or anywhere else in Fountain County since 1951.

Greene County.—We identified several new localities in Greene County, most notably at HFWA-W (Table 1; Fig. 2). The land that was to become HFWA-W was severely disturbed by surface mining activities (Lannoo et al. 2009), but restoration efforts by IDNR land managers over the past 25 years have provided prairie and semi-permanent wetland habitats that host about 200 breeding adults (Kinney 2011). We detected chorusing at seven wetlands at HFWA-W, and in 2011, calling was heard from a new, currently unidentified location off site. Hillenbrand FWA-W likely supports the densest assemblage of Crawfish Frogs in Indiana.

Crawfish Frogs are well known from the Goose Pond basin south of Linton, and IDNR personnel identified Crawfish Frogs at six sites from 2004–2008 (Engbrecht & Lannoo 2010). Our surveys, however, suggest that Crawfish Frogs have declined at Goose Pond. We sampled five of the six previously known localities and detected Crawfish Frogs at only two (from 2009–2011 at the first site, in 2009 and 2010 at the second; Tables 1, 2; Fig. 2). Both of these sites are on adjacent private property. In March 2011, Lee Sternenburg (pers. comm.) detected Crawfish Frogs from a new site on Goose Pond FWA several kilometers from the two sites previously mentioned.

Shortly after, we confirmed light chorusing (perhaps only one or two males) at this new site. Populations at these three sites appear to be at risk due to changes in habitat (encroachment of woody vegetation at privately owned sites; Williams et al. 2012) and small population sizes (e.g., Goose Pond FWA site). Constructing additional fishless wetlands in grassy upland habitat could help secure the persistence of this species in the greater Goose Pond basin.

We also detected chorusing at two previously known sites near Jasonville in 2009 and 2010 (the locality and chorusing rate at one of these sites could not be determined; Engbrecht & Lannoo 2010; Table 1; Fig. 2). We did not detect Crawfish Frogs at four previously known sites near the towns of Scotland, Linton, and Jasonville (Engbrecht & Lannoo 2010).

We surveyed Minton's 1949 collection site near Worthington (Engbrecht & Lannoo 2010) but did not detect Crawfish Frogs (Table 2).

Monroe County.—Crawfish Frogs were reported from the Beanblossom Creek bottoms as recently as 1991 (Engbrecht & Lannoo 2010). We conducted surveys at this historic site but did not detect them (Table 2). Open, grassy habitat is present, although the large wetlands, particularly those that connect with Beanblossom Creek during high water, may now contain fish and may therefore be unsuitable for Crawfish Frog reproduction (Werschkul & Christensen 1977; Phillips et al. 1999).

A more recent record comes from Brodman (2003), who detected Crawfish Frogs at an undescribed locality during surveys conducted from 1998–2001. This record represents the last published report of Crawfish Frogs in Monroe County.

Morgan County.—Indiana DNR surveys conducted from 2004–2008 at Robert Luker's historic locality (Engbrecht & Lannoo 2010) failed to detect Crawfish Frogs; we also did not detect them (Table 2). Scattered pastures and grassy fields remain but have been reduced by recent construction. This population appears to be extirpated, and we know of no current populations in Morgan County.

Owen County.—We confirmed Crawfish Frogs at the single known extant site in southwestern Owen County from 2009–2011 (Engbrecht & Lannoo 2010; Table 1; Fig. 2). Surveys conducted in 2011 at Minton's 1954 historic site (Engbrecht & Lannoo 2010) identified Crawfish Frogs calling from at least two different wetlands (Table 1; Fig. 2). The first consists of a degraded cattle pond. The second, which could not be definitively located, appears to be situated approximately 1 km away. Both sites are located in a relatively large series of pastures and grassy fields.

Spencer County.—Crawfish Frogs are known from two locations in Spencer County (Fig. 2). The first, discovered in March 1998, consists of several breeding sites located northeast of Newtonville (Lodato & Dugas, In Press; Table 1). This area has been visited each year since, and populations persist. In March 2011, a new, sizeable breeding chorus was heard from a wetland located in open, brushy grassland about 500 m northwest of the primary population. This region consists of rolling grasslands and wetland swales on reclaimed surface mined land, and is in private ownership.

A second record is based on a single male Crawfish Frog taken in March 2008 near Chrisney (Lodato & Dugas In Press; Table 2). This site is approximately 6.5 km southwest of the known breeding population northeast of Newtonville, described above. The animal was found on State Route 70 during a nighttime rainstorm. A photograph of this specimen, catalogued in the Illinois Natural History Survey collection (INHS 2011n), serves as the voucher for Crawfish Frogs in Spencer County. Surveys conducted during the springs of 2009, 2010, and 2011 failed to reveal this population (Table 2).

Sullivan County.—We documented two new breeding sites in 2011, both near a large, reclaimed coal mine in the eastern portion of Sullivan County (Fig. 2). The first site, originally reported by retired IDNR biologist Roger Stonebraker, is located in managed grassland on private property (Table 1). This site was converted from agriculture to managed grassland in 2000, with wetland construction taking place around 2003 (Stonebraker, pers. comm.). Stonebraker has heard chorusing at this site since 2009, and choruses have intensified each year. The second Crawfish Frog site, approximately 4.5 km away, is situated in an agricultural field near Dugger (Table 1). Frogs at both sites are likely using nearby state-owned grasslands as terrestrial habitat.

We re-confirmed two previously known populations in Sullivan County (Engbrecht & Lannoo 2010; Fig. 2). The first came from a cluster of breeding sites near Hymera where we

heard chorusing each year from 2009–2011 (Table 1). The second was reported by retired IDNR Property Manager Ron Ronk (Table 1) and confirmed in 2011.

We surveyed for Crawfish Frogs at Minton's 1951 collection site near Shelburn (Engbrecht & Lannoo 2010) but did not detect them (Table 2). This area is heavily farmed and little natural upland or wetland habitat remains.

We did not hear Crawfish Frogs at 10 sites where they had previously been reported in Sullivan County (Table 2). Many of these sites were originally identified by Timm (2001) and by IDNR personnel at Minnehaha FWA. Our surveys here and at Greene-Sullivan State Forest's Dugger Unit failed to reveal any populations.

Vermillion County.—We performed surveys at a historic site near Perrysville (Engbrecht & Lannoo 2010), but Crawfish Frogs were not detected (Table 2). Most of the area has been converted to agriculture. Crawfish Frogs have not been documented in Vermillion County since 1951.

Vigo County.—David Rubin documented Crawfish Frogs at a locality in northeast Vigo County in 1964 (Rubin 1965). We visited this site (now known as ''Dave's Pond'') and detected Crawfish Frogs each year from 2009–2011 (Table 1; Fig. 2). Dave's Pond may now contain the northernmost population of Crawfish Frogs remaining in Indiana, and currently represents the only known extant population in Vigo County.

We did not detect Crawfish Frogs during surveys at a historic site near Fontanet in northeast Vigo County (Engbrecht & Lannoo 2010; Table 2). This area is characterized by a matrix of agriculture, woods, and grassland habitat. We also failed to detect Crawfish Frogs at a second site located along the Parke/Vigo County line where calling was heard by IDNR personnel between 2004 and 2008.

Jefferson, Jennings, and Ripley counties.—Records for Big Oaks NWR are placed together in this section. We systematically searched portions of Jefferson, Jennings, and Ripley counties contained within Big Oaks NWR, and opportunistically searched areas outside the refuge using call surveys to locate Crawfish Frog breeding choruses.

We confirmed breeding (i.e., found egg masses) at 15 Jefferson County wetlands. Three of these were discovered in 2009, one in 2010, and nine in 2011. Calling at two wetlands had been heard in previous years (one wetland in 2004, one in 2007). We detected between one and 15 Crawfish Frog egg masses at these sites. The size and shape of the breeding ponds varied, ranging from small round bomb craters to large, shallow, flat-bottomed wetlands. All breeding ponds were in grassland habitat, with the exception of one pond located in a late-successional deciduous forest (Williams et al. 2012).

Crawfish Frogs appear to have been locally extirpated at two sites in the Jefferson County portion of Big Oaks NWR. We heard chorusing at the first site in 2008 and trapped a single male in 2009, but did not detect Crawfish Frogs in 2010 or 2011. We have not heard Crawfish Frogs calling from the second site since 2006.

Within the Jennings County portion of Big Oaks, we confirmed four Crawfish Frog breeding wetlands. We first heard calling at two sites in 2008 and two others in 2011. Each wetland had between three and five egg masses. All four wetlands were small (< 50 m^2), shallow (< 1 m), and situated in grassland habitat.

We confirmed seven Crawfish Frog breeding wetlands in the Ripley County portion of Big Oaks. Calling was first heard at two wetlands in 2004, one in 2010, and the other four in 2011. We found from one to five egg masses in each wetland. Wetlands varied in size from 10 m^2 to 150 m^2, and had maximum depths from 0.5–1.5 m. All were located in grassland habitats. Calling was also heard within the restricted area of the Indiana Air National Guard, Jefferson Range, which is surrounded by Big Oaks NWR. Several of these locations supported large choruses.

DISCUSSION

Crawfish Frogs are listed as State Endangered in Indiana and our data indicate that this level of protection is currently warranted. We did not detect Crawfish Frogs at eight of nine historic sites, and they appear to now be extirpated in 11 of 20 Indiana counties (Fig. 3). These losses have been partially offset by recent recolonizations of large restored grasslands on public lands. Undoubtedly Crawfish Frogs are at risk and are in danger of being extirpated in Indiana within the next half century (Fig. 3).

Current distribution.—Remaining Crawfish Frog populations in Indiana are concentrated

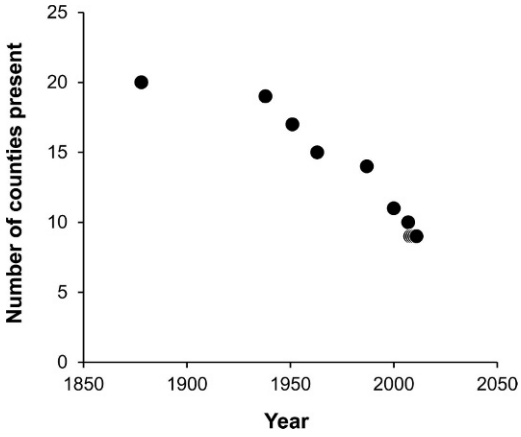

Figure 3.—*Lithobates areolatus* declines in Indiana by county. Extant populations are currently known from only nine Indiana counties. Data are based on last known records of Crawfish Frogs for each county the species occurred in from 1878 through 2011.

in two areas, in Greene and Sullivan counties (especially HFWA-W) in the southwest, and on Big Oaks NWR in the southeast. Hillenbrand FWA-W populations are few (seven), but concentrations of breeding adults are high (> 60 adults at Nate's Pond; > 80 adults at Big Pond), while densities of breeding adults at Big Oaks NWR are low (generally < 30 adults/wetland) but populations are numerous (> 27). Outside of these two areas, with the possible exception of the cluster in Spencer County, populations are smaller and scattered, and generally exist on private property. Our data suggest fewer than 1,000 adult Crawfish Frogs remain in Indiana. Big Oaks NWR supports around 300 breeding animals, HFWA-W supports about 200 breeding animals, and fewer than 500 breeding animals persist in the remaining populations, mostly on private lands (Fig. 2).

While our sampling scheme involved visiting the majority of recent Crawfish Frog localities and many of the historic sites in Indiana, we recognize that undocumented populations may remain. However, even doubling our current estimated number of breeding adults in Indiana places the estimate at only a fraction of the number of eggs contained in a single Crawfish Frog egg mass (2,200–9,900; Trauth et al. 1990; Redmer 2000; Kinney 2011).

Pattern of change in Indiana.—Historically, Crawfish Frogs were known from western Indiana, ranging from the Ohio River north to Fountain and Benton counties (Engbrecht & Lannoo 2010). However, there are currently no data to suggest the few northern populations in Benton, Fountain, and Vermillion counties persist. Further, in the south, all but one or two known populations from Indiana's Ohio River border counties (Vanderburgh, Warrick, and Spencer) have been extirpated; Crawfish Frogs remain only in a small portion of Spencer County. Crawfish Frog populations in Morgan and Monroe counties may also be extirpated. Collectively, these declines seem to have produced a range contraction in the northern, eastern, and southern portions of the historic range of Crawfish Frogs in Indiana.

This pattern of population extirpation outside areas of expansive grasslands with ephemeral wetlands in Indiana is repeated in every state east of the Mississippi River (Engbrecht & Lannoo 2012a). Crawfish Frogs have been reduced to a handful of populations in Mississippi (T. Mann, pers. comm.) and Tennessee (F. Scott, pers. comm.), and are uncommon and declining in parts of Illinois (Phillips et al. 1999). Besides HFWA-W and Big Oaks NWR, perhaps the healthiest Crawfish Frog populations east of the Mississippi River are now located on the coal spoil prairies of western Kentucky (J. MacGregor, pers. comm.) and perhaps in southern Illinois.

Causes of declines.—The pattern of extirpation and colonization of Crawfish Frogs in Indiana is easily understood from their biology. Crawfish Frogs require three habitat features: 1) large grassland complexes, 2) the presence of burrowing crayfish, and 3) fishless seasonal or semipermanent wetlands for breeding. Further, Kinney (2011) has demonstrated low larval and juvenile survivorship in Crawfish Frogs, but a relatively high (43%) annual survivorship in adults. Heemeyer & Lannoo (2012) have shown that Crawfish Frogs will return to the same upland burrow year-after-year (two years in her study, subsequent data show burrow philopatry over three years and suggest frogs will use the same burrow for much of their life [> 5 years]). Heemeyer & Lannoo (2012) also demonstrate that frogs in burrows are 12 times less likely to be preyed upon than frogs undergoing breeding migrations or ranging behaviors. Taken together, these studies show that the persistence of Crawfish Frog populations depends upon the persistence of long-lived adults, and the persistence of adults depends on the persistence of burrows.

Burrowing crayfish are widespread in Indiana, and none require special attention for their conservation status (Thoma & Armitage 2008).

Superimposed on what may have already been a gradual decline of Crawfish Frogs due to habitat loss was a decline in populations beginning around 1970 (Minton 2001). This decline may have been the result of an epidemic caused by the chytrid fungus (*Batrachochytrium dendrobatidis*; *Bd*), spreading like a wave through vulnerable populations (Kinney et al. 2011). Today, we know that *Bd* is acting in an endemic fashion and produces low rates of mortality among post-breeding adults (Kinney et al. 2011).

The need to manage populations on public and private lands.—While the threat to Crawfish Frog populations from *Bd* appears to have diminished, the threat from habitat loss continues, and may be intensifying as remaining populations on private lands become smaller and more isolated. Indeed, the pattern of decline of Crawfish Frog populations in Indiana (Fig. 3) suggests that in perhaps the next half-century most remaining populations will be on public lands managed by state or federal biologists. It is essential that Crawfish Frogs become a component of the management plans in these areas if they are to avoid extirpation in the state (see Appendix 1 for specific management recommendations).

While the relatively large number of frogs on a handful of public lands provides a buffer against threats such as disease, the smaller, scattered populations on private lands may function to preserve genetic diversity (Nunziata et al., In Press). State and federal biologists who regularly work with private landowners, including coal companies, can help secure remaining populations by providing assistance to preserve these small, scattered populations.

Finally, because of the recent work detailed above, the prospect of restoring populations in Indiana is now within reach. Since Crawfish Frogs will colonize new sites where adequate habitat is available (whether through natural dispersal or anthropogenic reintroduction), land managers have the opportunity to expand and establish populations by managing for grassland ecosystems. If Crawfish Frogs are incorporated into both public and private land management plans, the grasslands in the southern portion of the state are extensive enough to at least double the number of populations, which would enable us to downlist this species from Endangered to Special Concern.

APPENDIX 1: HABITAT RECOMMENDATIONS

Three key habitat variables are essential for maintaining healthy Crawfish Frog populations: 1) expansive grassy terrestrial habitat, 2) the presence of burrowing crayfish, and 3) fishless, seasonal or semi-permanent wetlands. Here we offer habitat recommendations for conserving Crawfish Frogs.

Terrestrial habitat.—Crawfish Frogs spend most of the year occupying burrows in open, grassy habitat, and will use the same burrows year after year (Hoffman et al. 2010; Heemeyer & Lannoo 2012; Williams et al. 2012). Because of this fidelity to specific burrows, conserving terrestrial habitat is critical. We recommend that landowners and land managers restore and/or maintain open grassy habitat when possible. In order to preserve the structural integrity of burrows, it is critical that land managers on sites that host Crawfish Frogs avoid plowing and disking. If plowing is required for installing food plots or firebreaks, we recommend that plow strips be located as far from wetlands as possible, and that the same areas be plowed year after year.

In Indiana, extant Crawfish Frog populations are associated with several forms of open habitat including managed prairie, grassy meadow, abandoned field, hayfield, shrub land, and livestock pasture. Prescribed burning is frequently used in grassland management plans to control woody vegetation. Fire will kill exposed Crawfish Frogs, but frogs in burrows will avoid injury by retreating underground (Engbrecht & Lannoo 2012b; Heemeyer et al. 2012). Spring burns can put adults migrating to and from breeding wetlands at risk, while late summer burns may put postmetamorphic juveniles at risk. Controlled burns are currently being used to manage grasslands at both HFWA-W and Big Oaks NWR, sites where Crawfish Frog populations in Indiana are most robust. We do not suggest restrictions on this practice; woody encroachment is probably a more potent threat to Crawfish Frog populations than prescribed burns.

Aquatic habitat.—Crawfish Frogs depend on fishless bodies of water for breeding (Bragg 1953; Phillips et al. 1999; Johnson 2000; Minton 2001; but see Palis 2009). If Crawfish Frog populations are to persist, fish introductions must be avoided. To prevent natural

colonizations of predatory fish, wetlands designed to augment Crawfish Frog populations should not be constructed in areas subjected to riparian flooding. Seasonal drying of wetlands will eliminate established fish populations (Lannoo 1996).

Burrowing crayfish.—Crawfish Frogs have a close association with burrowing crayfish and depend on them for the construction of their subterranean burrows. Thoma and Armitage (2008) note that five species of primary burrowing crayfish occur in Indiana, two of which, the painted-hand mudbug (*Cambarus polychromatus*) and the devil crayfish (*C. diogenes*), occur statewide. Delineating management guidelines for burrowing crayfish is beyond the scope of this paper, however Thoma and Armitage (2008) note that no burrowing crayfish species is currently of conservation concern in Indiana.

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BREEDING FREQUENCY AND SUCCESS OF EASTERN SPADEFOOTS, *SCAPHIOPUS HOLBROOKII*, IN SOUTHERN ILLINOIS

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ABSTRACT. The Eastern Spadefoot, *Scaphiopus holbrookii*, an anuran that ranges widely across eastern North America, exhibits latitudinal variation in breeding activity. In the southern United States, Eastern Spadefoots breed any time of year, whereas further north breeding activity is restricted to the warmer months. In the Midwest, Eastern Spadefoots are described as breeding any time meteorological conditions are favorable between March and September. I examined this commonly held belief at a southern Illinois sinkhole from 1996 to 2012. Eastern Spadefoots bred (oviposited) 26 times, up to three times per year, during 12 of 17 years (70.5%). Breeding occurred from March through July, but most frequently (69.2%) during April and May. Most (92.3%) breeding events occurred during months of above-average precipitation. Recruitment of juveniles followed seven breeding events (in April and May only) in 5 of 17 years and was dependent upon rainfall subsequent to breeding activity. Observations of breeding activity suggest that Eastern Spadefoots in the Midwest breed most often in spring, coincident with favorable meteorological conditions, and that breeding success may be reliant on rainfall subsequent to breeding.

Keywords: Breeding frequency, breeding season, breeding success, Eastern Spadefoot, Illinois, *Scaphiopus holbrookii*

INTRODUCTION

Temperate-zone anuran breeding activity is typically cyclic, occurring annually in late-winter/spring or summer, depending upon the species (Duellman and Trueb 1986; Stebbins and Cohen 1995). Seasonal variation in breeding activity of temperate-zone anurans is strongly influenced by latitude. For example, the breeding season of wide-ranging anurans is typically abbreviated at higher latitudes and prolonged at lower latitudes (Bury and Whelan 1984; Ritke et al. 1990; Redmer and Brandon 2003; Green 2005; Mitchell and Lannoo 2005). The latitudinal variation in length of breeding season may be the result of species-specific responses to temperature and rainfall thresholds.

Eastern Spadefoots (*Scaphiopus holbrookii*) range widely across eastern North America (approx. 26–43° latitude) and exhibit latitudinal variation in breeding activity. They are explosive breeders (Wells 1977) and breeding activity is generally more closely correlated with heavy rainfall than with season (Palis 2005). At the southern end of the range (Florida), Eastern Spadefoots can breed anytime during the year when suitable meteorological conditions occur (Hansen 1958; Greenburg and Tanner 2005). In the northern portion of the range (New England), however, breeding activity is restricted to rainy periods occurring only during warmer months (Hansen 1958; Klemens 1993). In southern Illinois, which is intermediate in latitude between the southern and northern range limits of Eastern Spadefoots, the exogenous cues of heavy rains and warm temperatures that stimulate Eastern Spadefoot breeding activity (Hansen 1958) may occur year-round, and Eastern Spadefoots become surface-active during warm, wet periods, even in winter (pers. obs.). Nonetheless, Eastern Spadefoots are only known to breed between March and September in Illinois (Smith 1961; Phillips et al. 1999), which suggests a temperature threshold below which Eastern Spadefoots will not breed (Bragg 1945; Gosner and Black 1955; Hansen 1958).

The proximal cue that stimulates Eastern Spadefoot emergence from subterranean burrows and movement to breeding sites is thought to be soil moisture. Hansen (1958) suggested that physiological uptake of water activates gametogenesis in Eastern Spadefoots and that soil saturation resulting from heavy rainfall stimulates reproductive behavior. In southern Illinois, soil moisture and rainfall are higher in

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spring than at any other time of year (Hollinger and Isard 1994; NOAA 2002). Thus, combined with rising air and soil temperatures, ample rainfall and soil moisture in spring may provide a greater likelihood of stimulating Eastern Spadefoot breeding activity than at any other time of year. I examined this assumption at a breeding site in southern Illinois. Specifically, my goals were to (1) determine whether Eastern Spadefoots in southern Illinois breed any time suitable meteorological conditions occur from March through September and (2) determine the annual frequency of breeding activity and the frequency of occurrence of successful juvenile recruitment.

METHODS

Study site.—I studied the breeding activity of Eastern Spadefoots at a naturally-formed sinkhole in Union County, Illinois (37°27″N, 89°16″W) annually from March through September 1996–2012. The sinkhole is dry except following heavy rains or extended periods of rain. Ponding of water is possible because the bottom of the sinkhole is overlain with soil and does not have a direct connection to subterranean karst features. During this study, maximum surface area of water in the sinkhole was 0.16 ha, maximum depth was 2 m, and maximum hydroperiod was 53 successive days. The sinkhole is in a residential area bordered by mowed lawn to the east, north, and west, and a 0.15 ha woodland to the south. Predominant tree species of the woodland include loblolly pine (*Pinus taeda*), tuliptree (*Liriodendron tulipifera*), sweetgum (*Liquidambar styraciflua*), black walnut (*Juglans nigra*), white oak (*Quercus alba*), northern red oak (*Quercus rubra*), and hackberry (*Celtis occidentalis*). The central portion of the sinkhole is enclosed by a wood-rail fence. At the initiation of this study, the slopes of the sinkhole were mowed to the perimeter of the fence. Beginning in June 1996, the outside perimeter of the fence was no longer mowed, resulting in a flush of weedy herbaceous growth that was followed by encroachment of woody vegetation dominated by boxelder (*Acer negundo*). The sinkhole is surrounded by well-drained and friable Alford silt loam (USDA 1979).

Data collection.—During or following heavy or extended rain events occurring from March through September each year, I examined the sinkhole for evidence of Eastern Spadefoot breeding activity. Visitation varied considerably, corresponding to annual differences in rainfall. I visited the sinkhole at night to listen and look for the presence of adults and/or examined the sinkhole during the day for the presence of eggs or tadpoles. I recorded air and water temperatures (to the nearest 0.5°C) at the sinkhole using a pocket thermometer when adults were present, and recorded rainfall daily (to the nearest 0.5 mm) throughout the year in a rain gauge. Groundwater and soil moisture, which could affect hydrology, were not measured. Following breeding events, I returned to the sinkhole every 1–3 d to monitor tadpole development through metamorphosis or until tadpole mortality resulted from premature drying of the pond. I considered breeding events successful when they resulted in metamorphosis of tadpoles.

Statistical analyses.—I analyzed data using Statistix 8.0 software (Analytical Software, Tallahassee, Florida, USA). Prior to analyses, I examined data for assumptions of normality using Shapiro-Wilk Tests. I made pairwise comparisons of rainfall and hydroperiod between successful and unsuccessful breeding events using t-tests. I present data as mean $\pm$ 1 SE and consider $P < 0.05$ statistically significant.

RESULTS

Eastern Spadefoots bred (oviposited) 26 times, 1–3 times per year, during 12 of 17 years (70.6%; Table 1). There was no Eastern Spadefoot breeding activity (calling or calling and oviposition) in 5 of the 17 years (Table 1). Of these 5 years, rainfall was below average in 2004, 2005, and 2012; below average during the first half of 2001; and near-normal in 2000.

Breeding activity occurred from 11 March through 27 August, whereas oviposition was more limited, occurring from 11 March through 14 July. Egg-laying occurred most frequently (69.2%) during April and May. Most breeding events (N = 24; 92.3%) occurred during months when precipitation was above the 17-year average (Fig. 1). The two exceptions included a single breeding event each in April 1997 and May 2007 that occurred during below-average monthly rainfall. Above-average monthly precipitation occurred 33 times in spring (March through June), eliciting breeding by Eastern Spadefoots 23 times (69.7%); above-average precipitation occurred 14 times in

Table 1.—Monthly summary of Eastern Spadefoot breeding activity at a Jonesboro, Illinois sinkhole, March–August 1996–2012. B = breeding (oviposition by females following calling by males), C = calling males only, no oviposition, M = newly metamorphed juveniles produced following breeding. The number preceding each letter indicates number of events.

	Mar	Apr	May	Jun	Jul	Aug
1996		2M				
1997		1B	2M			
1998		2B		1B		
1999		1B				
2000						
2001						
2002		1B	1M			
2003			2B	1B		
2004						
2005						
2006	1B				1B	1C
2007			1B	1B		
2008	2B		1M			
2009			1B	1B		
2010		1B	1B			
2011		1M				
2012						

summer (July and August), stimulating breeding activity twice (14.3%) in only one year (2006). Summer 2006 breeding activity included oviposition by females on 14 July but was limited to vocalization by males without response by females (i.e., no females present) on 27 August.

Rainfall totals 24 hours prior to breeding and one week prior to breeding (N = 25 each) averaged 56.5 ± 8.5 mm (range = 3.0–209.0 mm) and 97.0 ± 9.5 mm (range = 3.0–228.0 mm), respectively. Air and water temperatures (N = 22 each) averaged 19.0 ± 1.0°C (range = 13.0–26.0°C) and 17.0 ± 0.5°C (range = 12.0–24.0°C), respectively, during breeding events.

Recruitment of juveniles followed seven breeding events (26.9%), twice in 1996 and 1997, and once each in 2002, 2008, and 2011 (Table 1). Although the sinkhole held water longer (mean = 27 ± 2.5 d, range = 18–39 d) when breeding was successful than when it was not (mean = 19 ± 3 d, range = 4–53 d) the difference was not statistically significant (t = 1.48, df = 24, P = 0.1512). The lack of statistical difference is due to inclusion of the three March breeding events that, despite relatively long hydroperiods of 44, 45, and 53 days, were unsuccessful (i.e., no juvenile recruitment). When limiting the comparison of hydroperiod between successful and unsuccessful (mean = 14 ± 1 d, range = 4–21 d) breeding events to warmer months (i.e., April through July), the difference between the two is highly significant (t = 5.26, df = 21. P < 0.0001). The length of hydroperiod was influenced by amount of rainfall subsequent to breeding. There was no difference in the amount of rainfall received 24hours prior or one week prior to successful or unsuccessful breeding events (Table 2). However, rainfall totals fol-

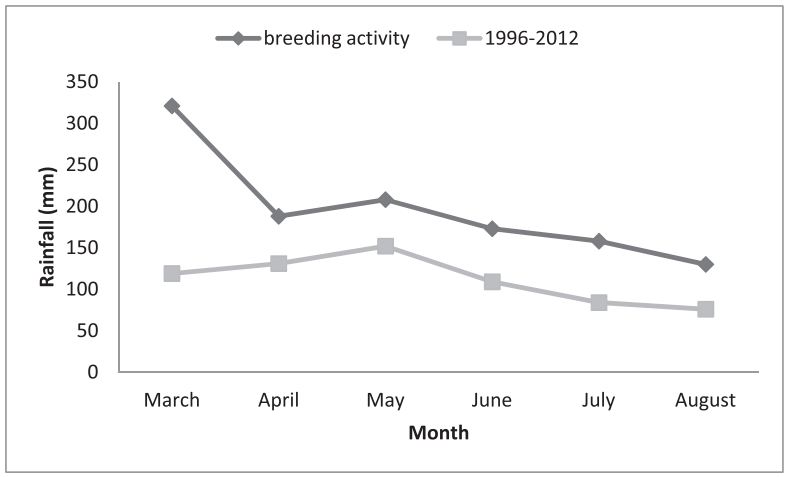

Figure 1.—Monthly mean rainfall and monthly mean rainfall that resulted in breeding activity by Eastern Spadefoots at a Jonesboro, Illinois sinkhole pond, March–August 1996–2012.

Table 2.—Comparison of rainfall (mm) received 24-h before, one week before, and after successful and unsuccessful Eastern Spadefoot breeding activity at a Jonesboro, Illinois sinkhole, March–August 1996–2012. Post-breeding rainfall was recorded until tadpole metamorphosis or until the pond dried and tadpoles died. Data are expressed as mean ± SE and range.

	24 h before breeding	1 week before breeding	Post breeding
Successful breeding	66.5 ± 14.5 (4.0–115.0)	110.0 ± 21.0 (58.0–228.0	199.0 ± 56.0 (28.0–481.0
Unsuccessful breeding	53.0 ± 10.5 (3.0–209.5)	92.0 ± 10.5 (3.0–222.5)	64.5 ± 18.0 (0–290.0)
t-test result	$t = 0.71$, P = 0.4867	$t = 0.86$, P = 0.3972	$t = 3.00$, P = 0.0061

lowing successful breeding events (recorded until metamorphosis) were significantly greater than rainfall totals following unsuccessful breeding events (recorded until the pond dried and tadpoles died [Table 2]).

DISCUSSION

Eastern Spadefoot breeding activity in the Midwestern United States is described as episodic and closely correlated with heavy precipitation ''any time during spring or summer'' (Minton 2001) or ''any time between March and September'' (Smith 1961). The results of my study generally agree with these descriptions: Eastern Spadefoot breeding activity occurred during relatively warm weather (air temperatures $\geq 13^{\circ}C$) during spring and summer and after rainfall sufficiently heavy or of sufficient duration to cause ponding of water in the sinkhole. However, my observations suggest that at the latitude of southern Illinois, Eastern Spadefoots have a definable period when breeding is most likely to occur: spring. All but one breeding event (96.1%) during the 17-year period occurred between 11 March and 14 June. These dates correlate well with calendar spring (20 March–20 June) and meteorological spring (1 March–30 May).

A spring-breeding tendency also occurs in other states at or near the latitude of southern Illinois. In Kentucky, 83.8% of observed Eastern Spadefoot choruses occurred from March through June (J.R. MacGregor, pers. comm.), and in a summary of five mid-latitude states (Indiana, Kentucky, Ohio, Virginia, and West Virginia), 81.1% of observed Eastern Spadefoot breeding choruses were recorded from March through June (Hansen 1958). In addition, 80.0% of published observations of Eastern Spadefoot breeding activity in Indiana made after Hansen's (1958) summary occurred from March through June (Rubin 1968; Minton 2001; Geboy et al. 2008; Engbrecht et al. 2009; Klueh and Mirtl 2011).

Spring is a time of rising air temperatures and plentiful rainfall. In southern Illinois, precipitation and soil moisture is higher in spring than at any other time of year, and rain storms are more frequent in spring than any other season (Hollinger and Isard 1994; Huff and Angel 1989; NOAA 2002). These precipitation patterns correlate well with the Eastern Spadefoot requirement of enough moisture to fill breeding wetlands and stimulate breeding activity. Although Eastern Spadefoots may be capable of breeding ''any time'' from March through September in the Midwest, they appear to be more likely to breed in spring than summer.

Breeding at the southern Illinois sinkhole was successful (i.e., resulted in juvenile recruitment) seven times in 5 of 17 years. Years of successful reproduction were separated by up to five years without successful reproduction and without breeding (Table 1). Breeding success was strongly influenced by amount of precipitation following breeding and month of breeding. Breeding was successful only if enough rainfall followed the breeding event to retain water in the sinkhole long enough for successful metamorphosis of tadpoles. This requirement was best met from March through June. There was risk, however, in breeding too early in the season. For example, despite receiving enough precipitation after three March breeding events to generate three of the longest hydroperiods observed during the study (44, 45 and 53 d), tadpoles were unable to metamorphose before the pond dried. This is likely due to low water temperature, which slows larval growth and development (Richmond 1947; Gosner and Black 1955), prolonging the larval period. Autumn breeding could risk a similar fate.

Abundant rainfall subsequent to breeding is critical to providing the hydroperiod needed to ensure Eastern Spadefoot breeding success. Eastern Spadefoots bred most frequently

(69.5%) in April and May, and tadpole metamorphosis occurred only after April and May breeding events. May is, on average, the wettest month of the year in southern Illinois (NOAA 2002) and was the wettest month, on average, during my 17-year study (Fig 1). Thus, at the latitude of southern Illinois, there appears to be a strong correlation between Eastern Spadefoot breeding activity and breeding success and the wettest time of the year.

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ON THE CONTRIBUTION OF ALLOMETRY TO MORPHOLOGICAL VARIATION IN A FRESHWATER GASTROPOD *ELIMIA LIVESCENS*

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ABSTRACT. Morphological variation attributable to allometry in the freshwater gastropod *Elimia livescens* Menke, 1830 is described. Geometric morphometric analyses were used to examine shape variation and visual ontogenic deformation patterns for a population of *E. livescens* along the West Fork of the White River, Delaware County, Indiana. A strong allometric slope from more globose and robust shelled smaller individuals to increasingly fusiform shell shapes in larger individuals was identified. The relatively high allometric slope was interpreted as evidence of functional importance and maintenance through selection.

Keywords: gastropods, morphology, allometry

Morphological variation in freshwater gastropods has been attributed to allometry, predation, competition, and environmental variation (Vermeij & Covich 1978; Kemp & Bertness 1984; DeWitt 1998; Dunithan et al. 2011). Shape variation of most taxa can be described with a power function that varies with body size (e.g., allometry; Huxley 1932; Peters 1983). Allometric variation can result from competition among and within age groups (Kemp & Bertness 1984), selection for exaggerated secondary characters (Kodric-Brown et al. 2006), environmental associations (Hollander et al. 2006), and can be present in taxa due to ancestral states. Environmental and biotic variation can also influence morphology through the alteration of allometric trajectories (Kemp & Bertness 1984) or through plasticity at a given point along a growth continuum (Hoverman & Relyea 2007; Bourdeau 2009).

Phenotypic plasticity has been attributed to both habitat and community structure (Minton et al. 2011). For example, DeWitt et al. (2000) and Krist (2002) identified shell morphology variation in freshwater gastropods that supported elongate morphology as a functional response to resist crayfish predation. *Elimia livescens* is a freshwater gastropod that occupies a wide range of habitats and has high morphological variation that covaries with abiotic environmental variables (Dunithan et al. 2011). However mechanisms for the direct cause of this morphological variation are mostly unknown. Although morphological variation that covaries with environmental variation appears to be adaptive, this research area is understudied (Callery et al. 2001). Identifying morphological corollaries can provide further information about ecological and evolutionary patterns (Hollander et al. 2006). The objective of this study was to describe morphological variation in *E. livescens* as a function of allometry.

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METHODS

We collected gastropods by hand using visual searches in the West Fork of the White River in Delaware County, Indiana, USA. This reach of the White River is a non-sinuous third order stream with coarse substrate, moderate flow, and mean depth of 1 m. All collected individuals were photographed (Nikon D70 – AF Micro Nikkor Macro Lens) against a scale reference and digitized using tpsDig2 software (Rohlf 2008) with a series of 12 repeated landmarks (Fig. 1), and described using shape regression (tpsRegw; Rohlf 2011) and relative warp analysis (RWA; tpsRelW; Rohlf 2007). Landmark locations were chosen based on previous morphometric analyses of *E. livescens* (see Dunithan et al. 2011). To reduce bias due to landmarks placed along curvatures, landmarks '3' and '5' were considered semi-sliding. Allometry was assessed by regressing relative

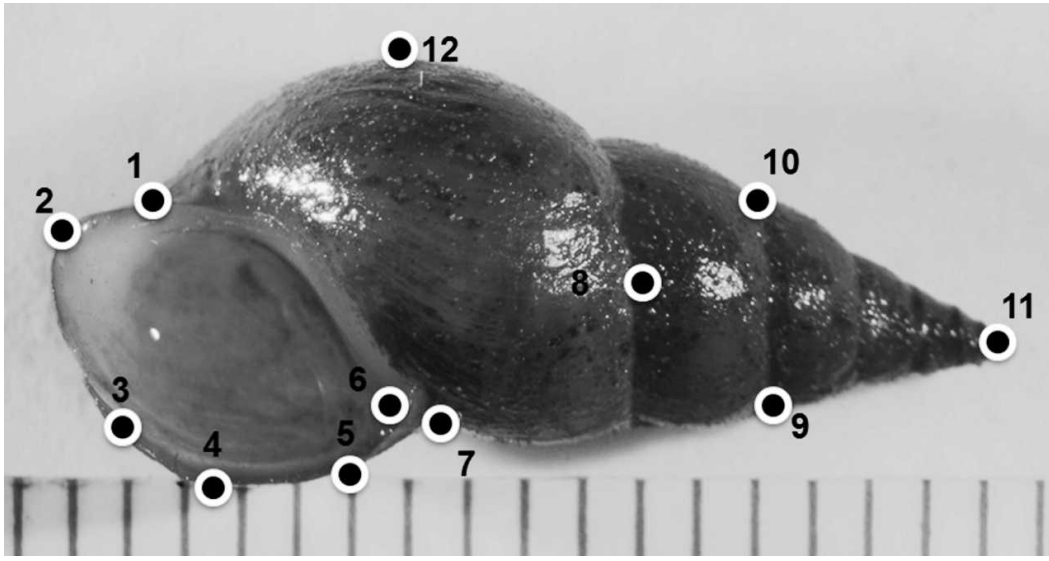

Figure 1.—Landmarks used in morphometric analyses of *Elimia livescens*.

warp scores against body size (e.g. centroid size) for collected individuals that spanned the entire size range present within the White River collecting site. Relative warp axes were used for subsequent description and visualization of the morphological variability present. Visualization of shape change used thin plate splines deformation grids. Following analysis all specimens were vouchered and deposited in the Ball State University invertebrate collection.

RESULTS

Elimia livescens (n=80) with body size ranges 4–17 mm was collected from the White River (Fig. 2). Regression analysis (using tpsRegr; Rohlf 2011) of shape versus size accounted for 22% of the overall allometric variability (Wilks λ 0.14, $F_{20,1560} = 21.6$, $p < 0.001$; Fig. 3). Relative warp analysis resulted in two significant axes that explained 57% of the total shape variation. RWA1 (eigenvalue 180) explained 38% of the total variation among individuals

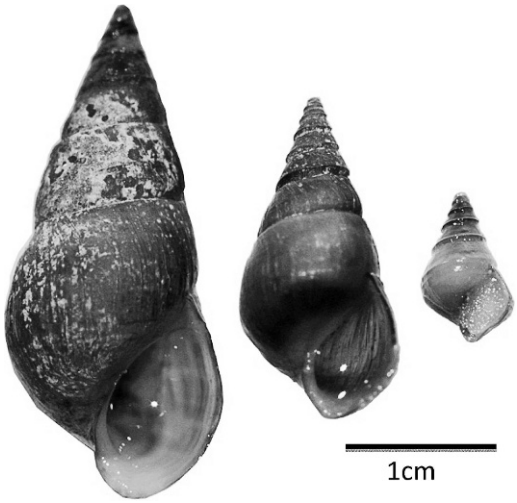

Figure 2.—Three *Elimia livescens* individuals to demonstrate the range of sizes in the White River, Indiana, USA.

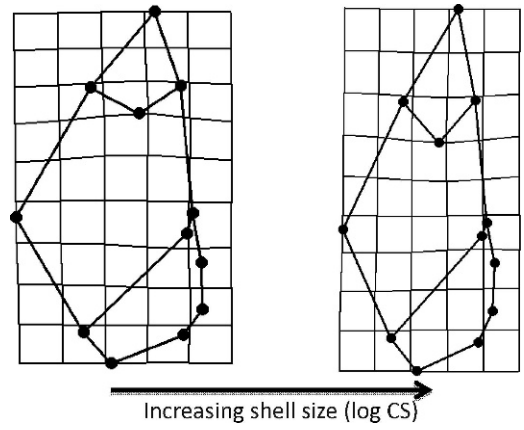

Figure 3.—Thin plate spline deformation grids corresponding to morphology – size (log CS is centroid size) allometry gradient from smaller (left image) to larger (right image) individuals.

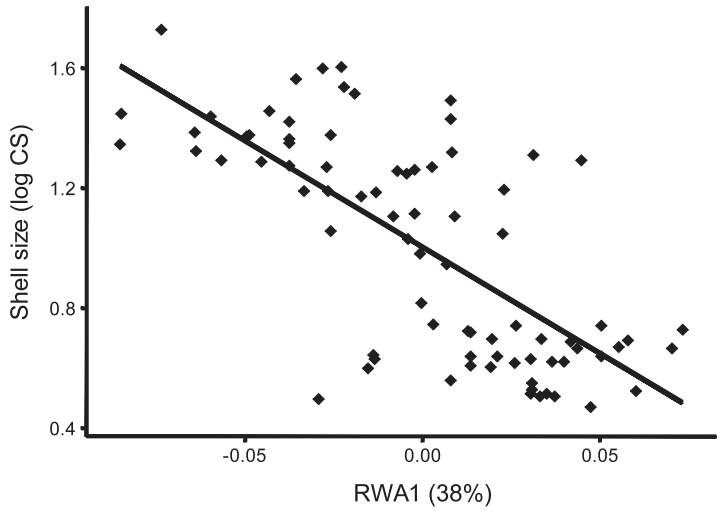

Figure 4.—Scatterplot of *Elimia livescens* shell size (log CS is centroid size) and primary axis morphology.

and had positive loadings for small, robust shape and negative loadings of large, fusiform and elongate shape ($r = -0.72$; $p < 0.001$; Fig. 4). RWA2 (eigenvalue 83) explained 19% of the total variation among individuals and had positive loadings for increased body size and negative loadings for thick spires and large, rectangular apertures ($r = 0.37$; $p < 0.001$).

DISCUSSION

We identified shape change in *E. livescens* concurrent with developmental changes in body size. Although we expected allometry, the high degree of slope with shape implies the potential for maintenance costs (Gould 1968). Allometric slope has been hypothesized to be conserved by phylogeny (Urdy et al. 2010) and influenced by environment (Kemp & Bertness 1984). Thus, it likely varies predictably among populations. We suggest that allometry is a primary source of morphological variation in this taxon and that local habitat (Dunithan et al. 2011), community attributes of predators, competitors, and parasites (Vermeij 1982; Krist 2002, 2009; Covich 2010), and large scale geographic variables (Trussel 1997; Minton et al. 2009) can influence developmental trajectory and adult morphologies through phenotypic plasticity. Functional variation in morphology likely is a response to varying biotic and abiotic conditions to increase survival, dispersal, and population success. Future research should focus on discerning the degree that environment influences morphological allometry and plasticity and to what extent this may influence *E. livescens* ecology.

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2013. Proceedings of the Indiana Academy of Science 121(2):167–168

INDIANA ACADEMY OF SCIENCE COUNCIL

MINUTES OF NOVEMBER 10, 2012 MEETING

Members Present: Ron Richards, Delores Brown, Dale Edwards, Jarka Popovicova, Rich Kjonaas, Marcia Gillette, Mike Finkler, Ed Frazier, Stan Burden, Mike Foos. Guests: Uwe Hansen, Bill McKnight.

Called to order: 9:30 AM.

INTRODUCTIONS

Discussion of minutes of March Council meeting: Minutes had already been approved per the Operating Policy passed November 2011. [The Secretary shall email minutes requiring Council approval following Council meetings. The Council shall respond with any recommended changes to the minutes within 30 days of receipt. After 30 days, barring no changes, the minutes will be considered ''approved'' and published on the website. (Amended November 29, 2011)]

Correspondence from Gene Kritsky: re. possibility of advertising *Periodical Cicadas* when the next generation hatch. This was discussed; no specific action was taken.

President's Report: (Mike Finkler) Reported his activities in office: BioBlitz; Subaru event in August; Council Retreat: increase visibility, participation and membership; Celebrate Science – support at $5K; Talent Search – need to provide information about talent search, is there something we can do to help students make better presentations; IAS Junior Academy – missed most of the conference; Facebook page announcement, perhaps we could create a forum (chat room) for HS students who are interested in science – Delores is working on a proposal to make this work; comment on IAS store – let's look into it; Expand IAS office – the desire to expand office had some comments of support; encourage members to seek new members.

Publications Committee: (Bill McKnight) [report on IAS web site] Bill commented on the IU Press connection with us; IU Press will market all of our books we have contracted to have them sell, including the *Periodical Cicadas;* there was no discussion.

Proceedings: (Uwe Hansen) [report on IAS web site] The next issue (121:1) is ready for press; issue (121:2) should be ready early in the year. There are only 1 or 2 manuscripts for volume 122; comments were made about the possibility of sectional associate editors to assist with specific topics; Uwe will look into revitalizing the editorial board.

Treasurer Report: (Ed Fraizer) [report on IAS web site] Ed covered the high points on the written report.

Executive Director: (Delores Brown) [report on IAS web site] Delores described activities and listed grant requests and other fundraising; reported costs and results of survey of satisfaction; outlined plans for 2013 meeting, preliminary program, exhibitors are invited, family day activities, speakers, schedule, poster schedule. Some discussion about poster scheduling at the meeting followed.

Academy Foundation: (Stan Burden) [report on IAS web site] Stan pointed out that it is important to be aware of our income when budgeting for future years.

Research Grants: (Jarka Popovicova) Jarka noted that PI's of all grant recipients must be members, the committee wants to have proposals with important subject matter and grant recipients to support IAS. There was discussion about the bylaw passed by Council that will come up for a vote of the Membership at the Annual meeting in March. There was discussion about the distribution of grant recipients through various campuses and universities.

Nominations and Elections: (Delores Brown) The election will include president-elect, member at large, 2 research grants members.

Science and Society: (Delores Brown) The Academy supported CSI this year with $5K support, and participation of a number of members. It seems appropriate to support CSI again next year.

Youth Activities: [report on IASb site]

Biodiversity Report: [report on IAS web site]

Webmaster: (Mike Foos) [report on IAS web site] Mike asked for photos for the homepage; announced new web capabilities: student voting, early registration earlier, automatic member payment, find a scientist capability; announced the status of digitizing the *Proceedings* supported by the LSTA grant.

Newsletter: The newsletter is available but not yet uploaded.

New Business: Parliamentarian Ed Frazier made a motion to change section '16. Sections' of the Operating Policy to insert "Botany" and delete "Mycology/Plant Pathology". Passed unanimously.

Discussion of recommendation of members to become Fellows: It was agreed that any member can recommend a person to be nominated as a Fellow.

Announcements of upcoming deadlines were made.

Adjourned: 12:53 PM

Respectfully submitted,

Mike Foos.

INDIANA ACADEMY OF SCIENCE
2012 Year End Financial Report

	Balance 1-Jan-2012	Revenues	Expenses	Balance 31-Dec-2012
OPERATING FUND				
Dues		26,940.00		
Interest		65.64		
Misc. Income		648.00		
Annual Meeting		26,905.00		
Foundation Support		149,921.00		
Officer's Expenses			121,264.12	
Operating Expenses			15,649.50	
Financial Expenses			1,588.07	
Newsletter Expenses			2,000.00	
Annual Meeting			28,826.29	
Program expenses			770.00	
Web Site			20,721.02	
Operating Fund Total	39,578.49	204,479.64	190,819.00	53,239.13
RESTRICTED FUNDS				
Proceedings	17,137.04	14,994.70	16,775.38	15,356.36
Publications	(19,853.89)	10,087.19	21,366.18	(31,132.88)
* Research Grants	22,483.17	74,390.54	77,256.04	19,617.67
Lilly Library	6,578.44	0.00	0.00	6,578.44
Welch Fund	6,916.63	91.93	0.00	7,008.56
Life Members Fund	13,045.25	0.00	0.00	13,045.25
Past Presidents Fund	8,599.17	0.00	0.00	8,599.17
Special Projects	22,239.37	0.00	5,000.00	17,239.37
Transition Office	(1,092.98)	0.00	3,133.88	(4,226.86)
Total Restricted Funds	76,052.20	99,564.36	123,531.48	52,085.08
Prepaid Dues	5,885.00	8,400.00	5,885.00	8,400.00
TOTAL FUNDS	**121,515.69**	**312,444.00**	**320,235.48**	**113,724.21**
FUNDS ON DEPOSIT				
Checking Account	48,326.17	362,376.50	385,325.55	25,377.12
Money Market Savings	60,001.19	70,065.64	55,000.00	75,066.83
Cert. of Deposit	13,188.33	91.93	0.00	13,280.26
TOTAL FUNDS DEPOSITED	**121,515.69**	**432,534.07**	**440,325.55**	**113,724.21**

* Provided 28 senior member grants and 42 high school grants.

ACADEMY FOUNDATION FUNDS		
TOTAL FOUNDATION FUNDS	**7,428,367.00**	**8,050,251.71**

Foundation Funded Used For:

Proceedings	6,111.70
Research Grants	71,696.97
Operating Func	149,921.00
Total	227,729.67

Edward L. Frazier
Treasurer

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