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Farm Ponds As Critical Habitats For Native Amphibians:

Field Season 2001 Interim Report

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Executive Summary:

We studied constructed farm ponds in the Driftless Area Ecoregion of southeastern Minnesota during 2000 and 2001. These ponds represent potentially significant breeding, rearing, and over-wintering habitat for amphibians in a landscape where natural wetlands are scarce. We collected amphibian, wildlife, invertebrate, and water quality data from 40 randomly-selected farm ponds, 10 ponds in each of 4 surrounding land use classes: row crop agriculture, grazed grassland, ungrazed grassland, and natural wetlands. This report includes chapters detailing information from the investigations we conducted. Manuscripts are in preparation describing our scientific findings and several management and public information documents are in draft form.

Each of these components will be peer reviewed during winter 2002, with a final report due to LCMR by June 30, 2002.

The USGS has initiated an Amphibian Research and Monitoring Initiative (ARMI) over the last 2 years. We obtained additional funding ($98K) in 2000 and 2001 for the radiotelemetry component of the project via a competitive USGS ARMI grant. Field work will be ongoing in 2002 for this component. USGS Water Resources (John Elder, Middleton, WI) ran pesticide analyses on water samples collected June 2001 from seven of the study ponds. The USGS Upper Midwest Environmental Sciences Center hired a herpetologist, Dr. Water Sadinski, to serve as the Midwest Coordinator for the ARMI monitoring initiative. He is coordinating and overseeing amphibian monitoring on federal land as well as initiating new amphibian research to address environmental stressors and causes of amphibian population declines.

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6. The role of *Ribeiroia ondatrae* (Platyhelminthes: Trematoda) metacercariae in the development of malformed frogs in Minnesota and Wisconsin (summary) (Page 68)
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8. Managing farm ponds as wildlife habitat: a technical guide (abstract) (Page 86)
9. Farm Ponds are Working Wetlands (draft brochure, target audience=landowners)

Key words: Farm pond, amphibian, reptile, bird, mammal, land use, agriculture, toxicology, *Ribeiroia*, *Rana pipiens*,

Website: http://www.umesc.er.usgs.gov/terrestrial/amphibians.html

Summary of Amphibian, Reptile, Mammal, Bird, Fish, and Invertebrate Findings for 2000-2001

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We studied constructed farm ponds and natural wetlands in southeastern Minnesota during spring and summer 2000 and 2001 (Fig. 1). We collected amphibian and habitat data from 40 randomly-selected ponds, 10 ponds in each of 4 surrounding land use classes: row crop agriculture, grazed grassland, ungrazed grassland, and natural wetlands. Our study addresses the following questions: (1) How are measures of amphibian individual, population, and community health associated with land uses surrounding the pond, such as row crops, grassland, and grazed grassland? (2) Are measures of amphibian individual, population, and community health more associated with surrounding landscape features or within-pond characteristics? (Fig. 2) (3) What design features associated with a pond (size, depth, vegetation) will maximize wildlife benefits?

Weather conditions during 2001 contrasted with 2000 in that steady April and May rains led to very wet conditions early in the season, followed by a summer drought. (The 2000 season was dry in May, followed by followed by heavy rainfall in late May through July.) We were very fortunate to be able to study the same 40 ponds in both seasons; one of the ponds dried up in July 2001.

Methods for collecting information on amphibians, reptiles, mammals, birds, fish, and invertebrates were described in the Field Season 2000 Report (Knutson et al. 2001). Over the 2 years of the study we identified 10 species of amphibians at the ponds (Fig. 3, Appendix A), including the tiger salamander (*Ambystoma triginum)*, American toad (*Bufo americanus)*, eastern gray treefrog (*Hyla versicolor)*, chorus frog (*Pseudacris triseriata)*, spring peeper (*Pseudacris crucifer)*, green frog (*Rana clamitans)*, wood frog (*Rana sylvatica)*, leopard frog (*Rana pipiens)*, and pickerel frog (*Rana palustris)*. The blue-spotted salamander (*Ambystoma laterale)* was identified at a single natural wetland. We observed high abundances of the American toad, eastern gray treefrog, and green frog at many ponds.

Six species of snakes and two turtle species were observed at the ponds over the two years of the study (Fig. 4, Appendix A). The common garter snake (*Thamnophis sirtalis*) was the most frequently encountered reptile (18 ponds), followed by painted turtles (*Chrysemys picta*) (11 ponds). One hundred species of birds were observed at the ponds (Fig. 5A-D). The song sparrow (*Melospiza melodia*) was the most frequently observed bird species (40 ponds), followed by the red-winged blackbird (*Agelaius phoeniceus*) (34 ponds), common yellowthroat (*Geothlypis trichas*) (30 ponds), and the American robin (*Turdus migratorius*) (25 ponds). Eighteen species of mammals were observed, with the raccoon (*Procyon lotor*) as the most commonly observed mammal (34 ponds), followed closely by the white-tailed deer (*Odocoileus virginianus*) (33 ponds) (Fig. 6). Five species of fish were identified from the ponds, with brook stickleback (*Culaea inconstans*) the most frequently observed (6 ponds) (Fig. 7). A wide variety of invertebrate taxa were observed in the ponds (Fig 8). Midge larvae (Order=Diptera), crawling water beetles (Order=Coleoptera), and water boatmen (Order=Hemiptera) were the most common invertebrate taxa observed. Our invertebrate sampling concentrated on potential amphibian larval predators and snails and was not a comprehensive inventory of all invertebrates in the ponds.

Literature cited

Knutson, M. G. 2001. Amphibian Research and Monitoring Initiative FY2001 Annual Report of the Upper Mississippi River Region. USGS Upper Midwest Environmental Sciences Center. La Crosse, Wisconsin.

Outreach activities, seminars, posters, and products through December 2001

7 December 1999. Conference symposium presentation: Knutson, M. G. 1999. Riparian zones as breeding habitat for birds, reptiles, and amphibians (paper). Riparian Management Symposium, 61st Midwest Fish and Wildlife Conference, Chicago, IL. 7 December 1999.

15 March 2000. Knutson, M. G. 2000. Declining amphibians: whats the big picture? (briefing). Upper Midwest Environmental Sciences Center Partnership Coordination Meeting, La Crosse, WI. 15 March 2000.

29 March, 2000. USGS News Release on Amphibian Research, including UMESC research on frog habitat on farms (Upper Midwest Environmental Sciences Center) 30 March, 2000: Melinda Knutson was interviewed by Tim Krohn, Reporter with the Mankato Free Press (Mankato, MN), regarding the USGS news release on amphibians.

12 May 2000: The Dakota Elementary School (Dakota, Minnesota) hosted an interactive, outdoor classroom for their entire K-5 school (over 50 students) on May 12, 2000 at the Dresbach, Minnesota US Fish and Wildlife Service boat landing. Participating educators for the day included Melinda Knutson (USGS Upper Midwest Environmental Sciences Center) with a live frog and turtle exhibit. (Specimens were native frogs captured by Shawn Weick, Ben Campbell, Sam Bourassa, and Joel Jahimiak, all of the USGS UMESC). The children squealed with delight as they witnessed live eastern gray treefrogs singing their mating songs. They eagerly contributed their own observations of frogs near their homes as they learned about conserving frog habitat and pondered scientific mysteries surrounding amphibian declines and deformities.

20 June 2000. USGS Biological Resources Division national staff, including Bill Walker, Norita Chaney, Dan Petit, and Colleen Charles toured the region surrounding the USGS Upper Midwest Environmental Sciences Center on 20 June 2000 to learn more

about the Center science program. The tour included a visit to the farm of Art and Jean Thicke, who practice intensive rotational grazing of their dairy herd. The Thicke farm pond was found to be teeming with tadpoles. The pond is part of a USGS UMESC study examining the value of farm ponds for amphibians. Study directors are Melinda Knutson and William Richardson. Tex Hawkins, US Fish and Wildlife Services Private Lands Program, Winona, MN and Fred Kollmann, USDA Natural Resources Conservation Service, Onalaska, WI contributed to a discussion of how farming practices in this region of the Midwest are related to clean water, widlife populations, and national agricultural policy.

16 October 2000; USGS Upper Midwest Environmental Sciences Center hosts planning session to discuss Minnesota amphibian deformities and results of field season 2000. Representatives of the USGS Water Resources Division, Mounds View, MN (Perry Jones, Joe Magner), USDA Natural Resources Conservation Service (Fred Kollmann), Minnesota Pollution Control Agency (Jeff Canfield), and parasitologist Dr. Dan Sutherland (University of Wisconsin, La Crosse) met with Melinda Knutson, Bill Richardson, and Shawn Weick (USGS UMESC) to discuss findings from amphibian research conducted during summer 2000 and develop hypotheses regarding causes of amphibian deformities in Minnesota. We agreed to develop a proposal to pursue ecological factors causing the parasite Riberoiria to proliferate in ponds. Riberoiria infects tadpoles and causes deformities in metamorphs, as demonstrated in field and laboratory studies. However, we also noted that Riberoiria is not present at all sites where high deformities have been observed.

6 December 2000. The USGS Amphibian Research and Monitoring Initiative (ARMI) held its first annual workshop at USGS headquarters in Reston, VA. ARMI is a collaborative effort between USGS Biological Resources, Water Resources and Mapping to undertake the first nationwide assessment of the distribution and status of amphibian populations, to understand the scope and severity of amphibian declines, and provide scientific information for management. Melinda Knutson (USGS Upper Midwest Environmental Sciences Center) presented information on Center activities related to ARMI, including an update on the "Farm Ponds as Critical Habitats for Amphibians" project. One component of this project, tracking post-breeding movements of leopard frogs (Rana pipiens) using radio transmitters, was funded under the research competitive grants component of ARMI. We worked on methods development this year, with full-scale field research planned in 2001.

17 January 2001. Scientists studying frog malformations converged on Minnesota this week to share findings and approaches to solving one of the most puzzling biological problems of the last decade. Ecologists, herpetologists, hydrologists, and toxicologists have all been working on various pieces of the puzzle. Several study sites in Minnesota have deformity rates > 60% for some species. Melinda Knutson, USGS Upper Midwest Environmental Sciences Center, presented information about the USGS Amphibian Research and Monitoring Initiative and presented findings on deformity rates from study sites in southeastern Minnesota, which are part of a 2-year ecological study of constructed farm ponds. A controversy about the involvement of the Minnesota Pollution

Control Agency was the subject of a Minneapolis Star Tribune article on the meeting: http://www.startribune.com/viewers/qview/cgi/qview.cgi?template=metro_a_cache&slug=frog19.

28 February - 1 March 2001. The Wisconsin Chapter of the Wildlife Society focused on wildlife issues associated with several Wisconsin ecosystems during its 2001 Winter Meeting, held in Eau Claire, Wisconsin. Oak ecosystems, forests, and grasslands/wetlands were three key systems addressed. Melinda Knutson, USGS Upper Midwest Environmental Sciences Center, gave a seminar entitled, "Managing farm ponds as amphibian breeding sites in the Driftless Area Ecoregion", which provided an overview of a recent study of farm ponds in southeastern Minnesota. The study is identifying agricultural management practices that support healthy populations of amphibians in an ecoregion characterized by karst geology, high topographic relief, and a mix of agricultural land uses, including row crops, pastures, and forests.

21 March 2001. A study of farm ponds in southeastern Minnesota found 10 amphibian species inhabiting the ponds, including 2 salamander species. Few deformities were found, in contrast to other sites in Minnesota where deformity rates have been as high as 60%. Speaking to the Coulee Region Audubon Society, Melinda Knutson (USGS Upper Midwest Environmental Sciences Center), reported preliminary findings from the first year of the multi-disciplinary USGS study. The presentation was reported in the La Crosse Tribune 26 March 2001 (http://www.lacrossetribune.com/environment/E2.php3). Other scientists working on the study include Bill Richardson, Brent Knights and Shawn Weick (USGS Upper Midwest Environmental Sciences Center), and University of Wisconsin scientists Mark Sandheinrich, Dan Sutherland, Rick Gillis, Josh Kapfer, and Brian Pember.

10 April 2001. A poster presentation, "Effects of wetland type and land use practices on movement and habitat selection by northern leopard frogs (Rana pipiens)" was given at the USGS Upper Midwest Environmental Sciences Center Science Review. Authors were B. Pember, B. Knights, M. Knutson, S. Weick, and D. Sutherland. We are attaching radio transmitters to leopard frogs to study post-breeding habitat use. Field and laboratory work is planned.

12 April 2001. The USGS Amphibian Research and Monitoring Initiative, ARMI, was launched with the primary objective of deriving status and trends assessments of amphibian populations across North America. The Upper Midwest Environmental Sciences Center is leading the ARMI initiative in the Midwest. Melinda Knutson,USGS Upper Midwest Environmental Sciences Center, along with Bill Richardson, Brent Knights and Shawn Weick , USGS UMESC, and University of Wisconsin scientists Mark Sandheinrich, Dan Sutherland, Rick Gillis, Josh Kapfer, and Brian Pember have been studying farm ponds in southeastern Minnesota in a directed research study which will establish standard methods for monitoring midwestern amphibians and answer questions about habitat selection. Melinda Knutson gave a seminar on the ARMI initiative and Center amphibian research at the UMESC Center Science Review.

25 April 2001. Private landowners are key partners in landscape-level research. Conservation actions by private landowners are especially important in parts of North America where public lands are small and scattered. Melinda Knutson (USGS Upper Midwest Environmental Sciences Center) is leading an interdisciplinary study of small farm ponds in southeastern Minnesota to better understand the habitat values of these small wetlands for amphibians and other wildlife species. Preliminary results of the study were discussed recently (25 April 2001) at a meeting of rural women in Houston County, Minnesota, one of the study areas. The women were members of the Black Hammer Lutheran Church Ladies' Aid Society. Many of the women own farm ponds or have neighbors and friends who own farm ponds. Fred Kollmann, USDA Natural Resources Conservation Service, is a collaborator in the study.

26 April 2001. A poster presentation, "Effects of agricultural pond water on the development and metamorphosis of anurans native to the Upper Midwest" was given at the Midwest Chapter of the Society of Environmental Toxicology and Chemistry in Racine, Wisconsin. Authors were J. M. Kapfer, M. B. Sandheinrich, M. G. Knutson, and D. R. Sutherland. Laboratory work was undertaken to assess the toxicity of pond water to amphibians during 2000. In 2001 both laboratory and field work are planned.

14 May 2001. The USGS Amphibian Research and Monitoring Initiative (ARMI) was launched in 2000 to address concerns over amphibian population declines and health risks. A task force has been working for a year to develop guidelines for implementation of the program. The draft document, Conceptual Design and Implementation Guidance for the Amphibian Research and Monitoring Initiative was presented to USGS headquarters and the ARMI Steering Committee in May. Implementation is expected to proceed, with guidance from the steering committee, in 2001. Authors are: Langtimm, C., A. Gallant, W. A. Battaglin, S. Corn, J. R. Sauer, M. G. Knutson, M. Adams, and D. James. 2001. Conceptual design and implementation guidance for the amphibian research and monitoring initiative (Draft). USGS. Denver, CO.

8 June 2001. USGS Director Groat visited a grazed farm pond in Minnesota to see how land use practices affect amphibian populations, as part of a visit to the Upper Midwest Environmental Sciences Center 8 June 2001. A collaborative research study, headed by USGS, with University of Wisconsin-La Crosse, USDA Natural Resource Conservation Service, and US Fish and Wildlife Service partners, is examining the wildlife values of small farm ponds. Dairy farmers Art and Jean Thicke hosted the tour and described how intensive rotational grazing has transformed their hilly farmland from row crops to grazed pastures. This change has improved the quality of thier lives by decreasing their dependence on mechanization and chemical inputs, as well as improving rainfall infiltration and biodiversity. Several species of frogs were present in the ponds, indicating that small farm ponds provide habitat for amphibians in an ecoregion where wetlands are scarce.

1 July 2001. Amphibian Farm Pond Study/Winona Daily News - Reporter Jeff Dankert of the Winona Daily News interviewed USGS ecologist Melinda Knutson and Brian Pember of the US Fish and Wildlife Service about amphibian research they are

conducting on farm ponds. Pember, also a graduate student at the University of Wisconsin-La Crosse, received funding from the USGS Amphibian Research and Monitoring Initiative (ARMI) to conduct master thesis work using radio-telemetry to determine post-breeding habitat use by leopard frogs in an agricultural landscape. Dankert's article is scheduled to appear in the outdoor section of the Sunday, July 1, 2001 edition of the paper. (Melinda Knutson, La Crosse, 608-893-7550 x 68, melinda_knutson@usgs.gov) (Reporter information: Jeff Dankert, Winona Daily News, phone (507)453-3513 or (800)328-2182, jdankert@winonadailynews.com)

31 July 2001. Melinda Knutson (USGS Upper Midwest Environmental Sciences Center) participated in the Minnesota Department of Natural Resources (DNR) Nongame Wildlife Stakeholders Meeting at Camp Ripley (near Little Falls), Minnesota. The 1-day meeting was designed to give partner agencies an opportunity to identify important trends affecting nongame wildlife in Minnesota and envision desireable outcomes of work undertaken by the Minnesota DNR Nongame Wildlife Program over the next 10 years. Partners were also invited to identify program areas where they could contribute to the goals of the Nongame Program. The USGS Upper Midwest Environmental Sciences Center Farm Pond study and the Long Term Resource Monitoring Program are examples of collaborative work that benefit nongame resources in Minnesota.

15 November 2001. Scientists at the USGS Upper Midwest Environmental Sciences Center and the University of Wisconsin, La Crosse River Studies Center found no differences in the survival of leopard frog (Rana pipiens) tadpoles between agricultural farm ponds and natural wetlands in southeastern Minnesota. The tadpoles were raised in mesocosms placed in the ponds and were followed from the egg stage through the metamorph (froglet) stage. In addition, only 1 deformity (N=1000) was observed. Findings were similar for laboratory studies (FETAX assay) conducted on African frog (Xenopus laevis) eggs exposed to water from farm ponds and natural wetlands. Josh Kapfer, Mark Sandheinrich (University of Wisconsin, La Crosse) and Melinda Knutson (USGS UMESC) are reporting their findings in a poster at the National Society of Environmental Toxicology and Chemistry 22nd Annual Meeting in Baltimore 15 November 2001.

29 November 2001. Leopard frogs as environmental bioindicators in southeastern Minnesota (radio interview). Brian Pember, University of Wisconsin-La Crosse graduate student working on a USGS Amphibian Research and Monitoring Initiative (ARMI) - funded research study, was interviewed by Bob Seebo of KWNO Radio, AM 1230, Winona, Minnesota. During the half-hour interview Brian described the life history requirements of northern leopard frogs and the implications of his radio telemetry research for management of agricultural landscapes. Brian has found that leopard frogs can move more than 1000 meters from one pond to another and they will move across many different habitat types, including roads and urban parking lots. His study is one component of research on farm ponds in southeastern Minnesota conducted by the USGS Upper Midwest Environmental Sciences Center, funded by the Legislative Commission on Minnesota Resources.

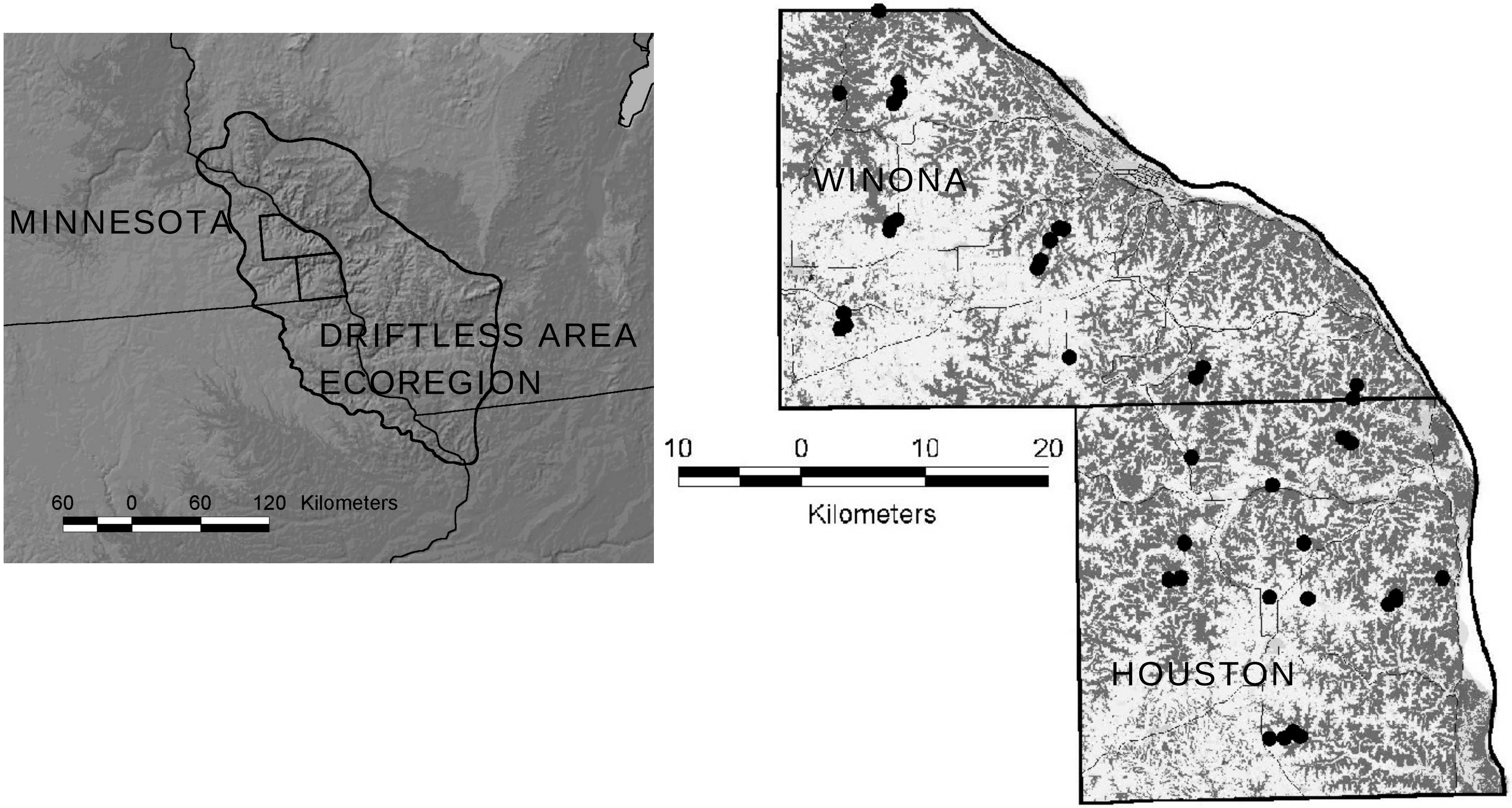

Figure 1. Farm pond study sites in southeastern Minnesota, Houston and Winona counties.

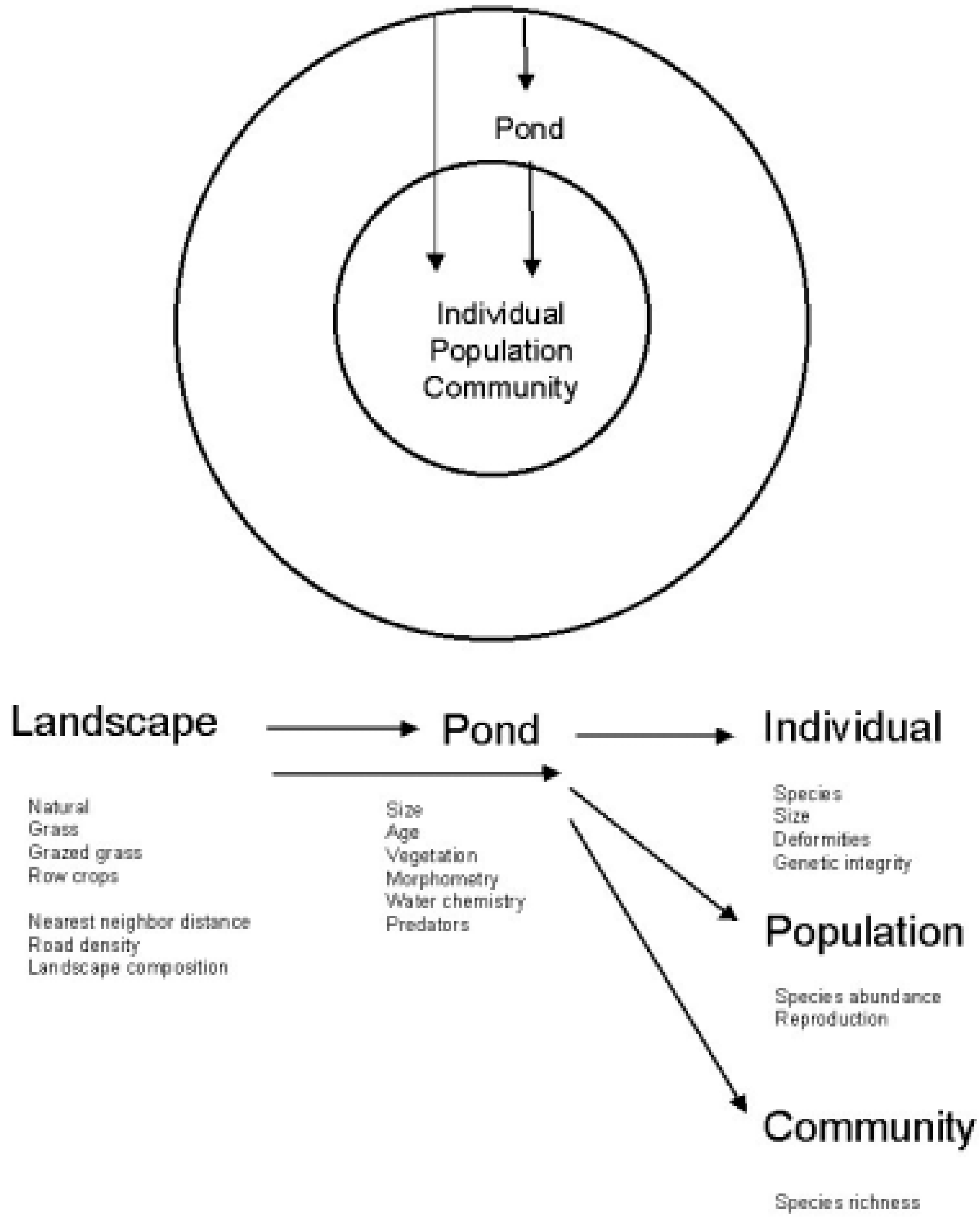

Figure 2. Hypothesized relationships between habitat variables (landscape and pond) with amphibian responses at Individual, population, and community levels

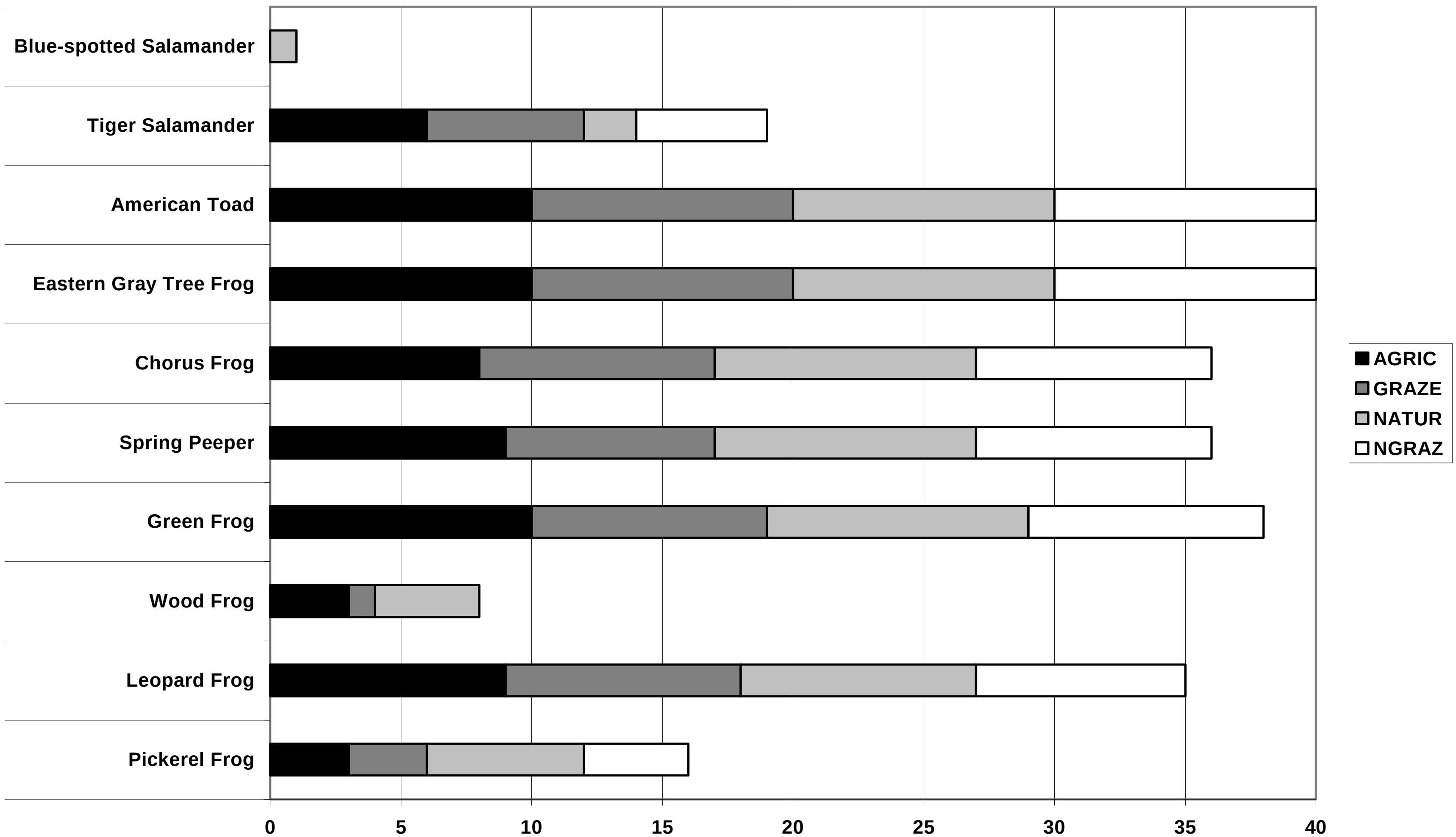

Figure 3. Amphibian species present within four types of surrounding land uses, based on detection by all survey methods, for farm ponds in Houston and Winona Counties, Minnesota, 2000-2001.

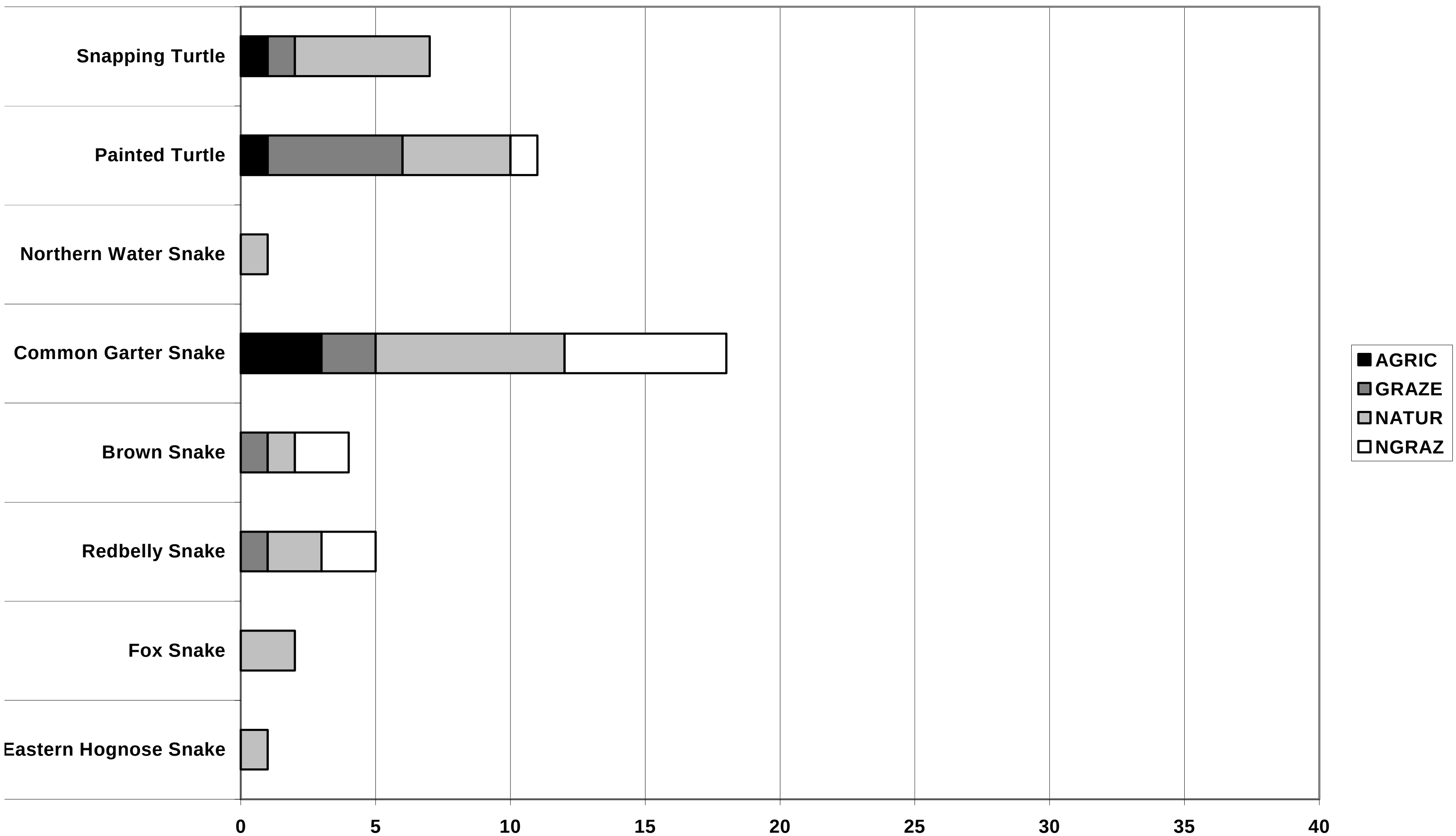

Figure 4. Reptile species present within four types of surrounding land uses, based on detection by all survey methods, for farm ponds in Houston and Winona Counties, Minnesota, 2000-2001.

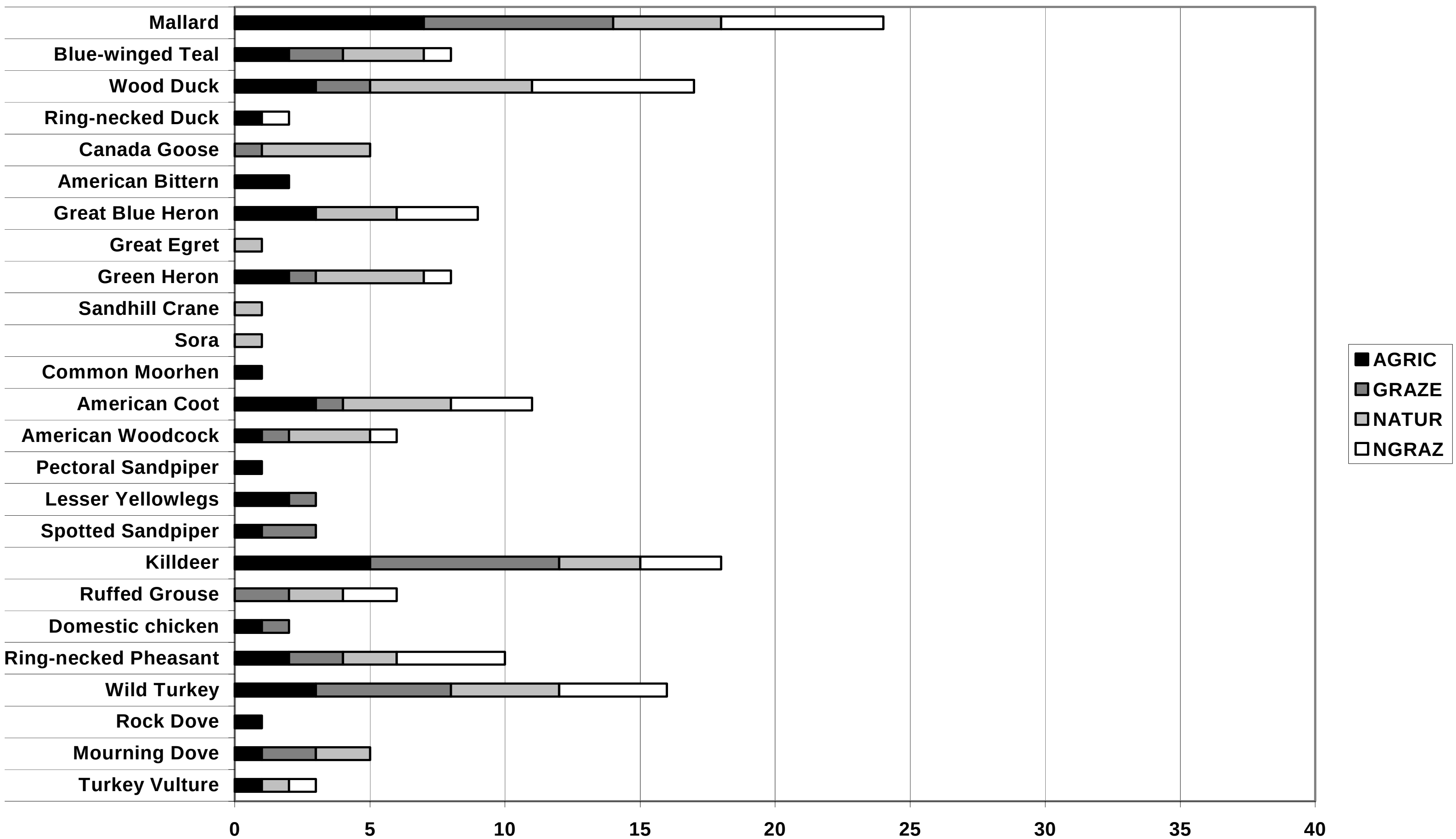

Figure 5A. Bird species present within four types of surrounding land uses, based on detection by point counts, for farm ponds in Houston and Winona Counties, Minnesota, 2000-2001.

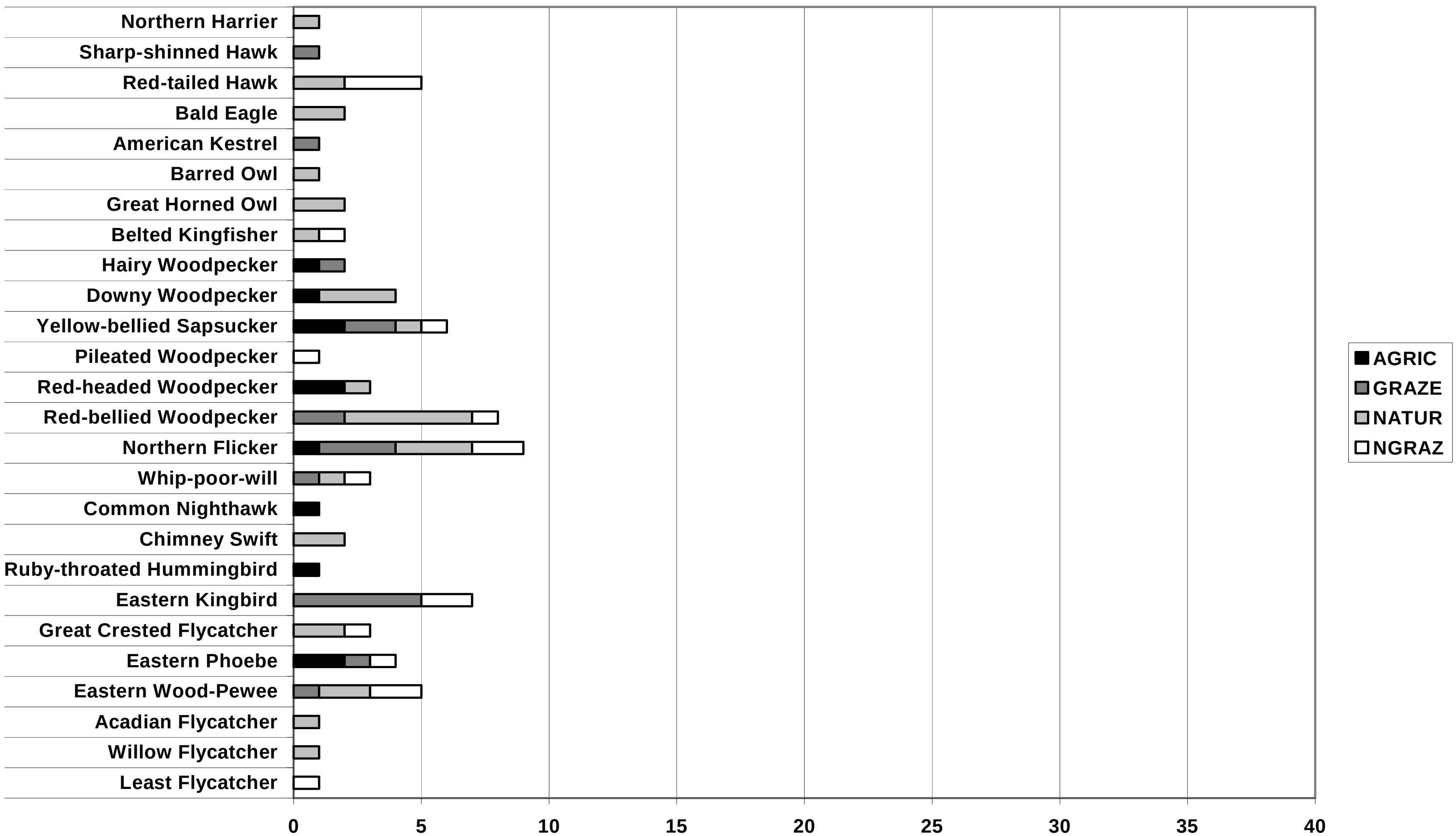

Figure 5B. Bird species present within four types of surrounding land uses, based on detection by point counts, for farm ponds in Houston and Winona Counties, Minnesota, 2000-2001.

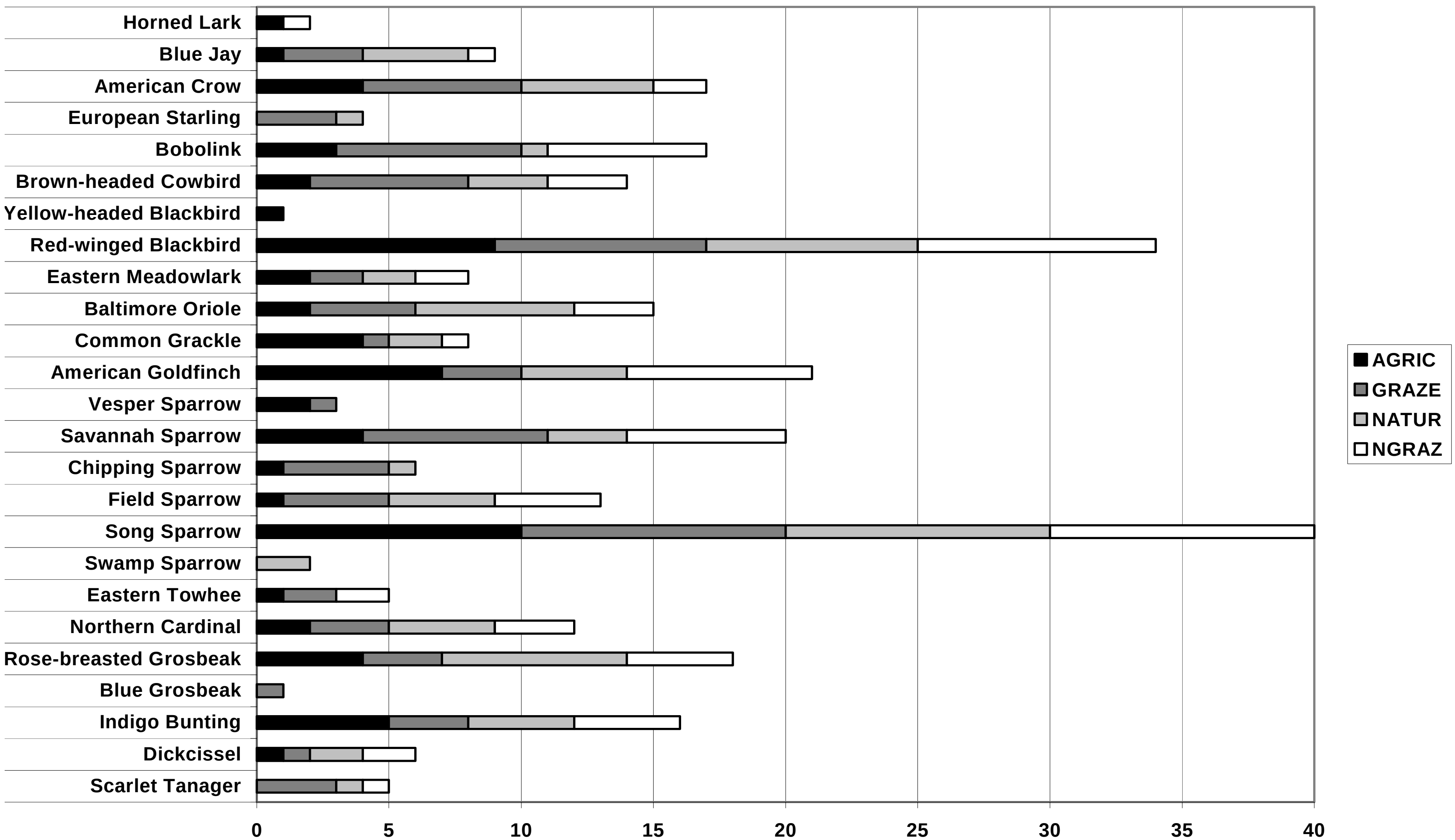

Figure 5C. Bird species present within four types of surrounding land uses, based on detection by point counts, for farm ponds in Houston and Winona Counties, Minnesota, 2000-2001.

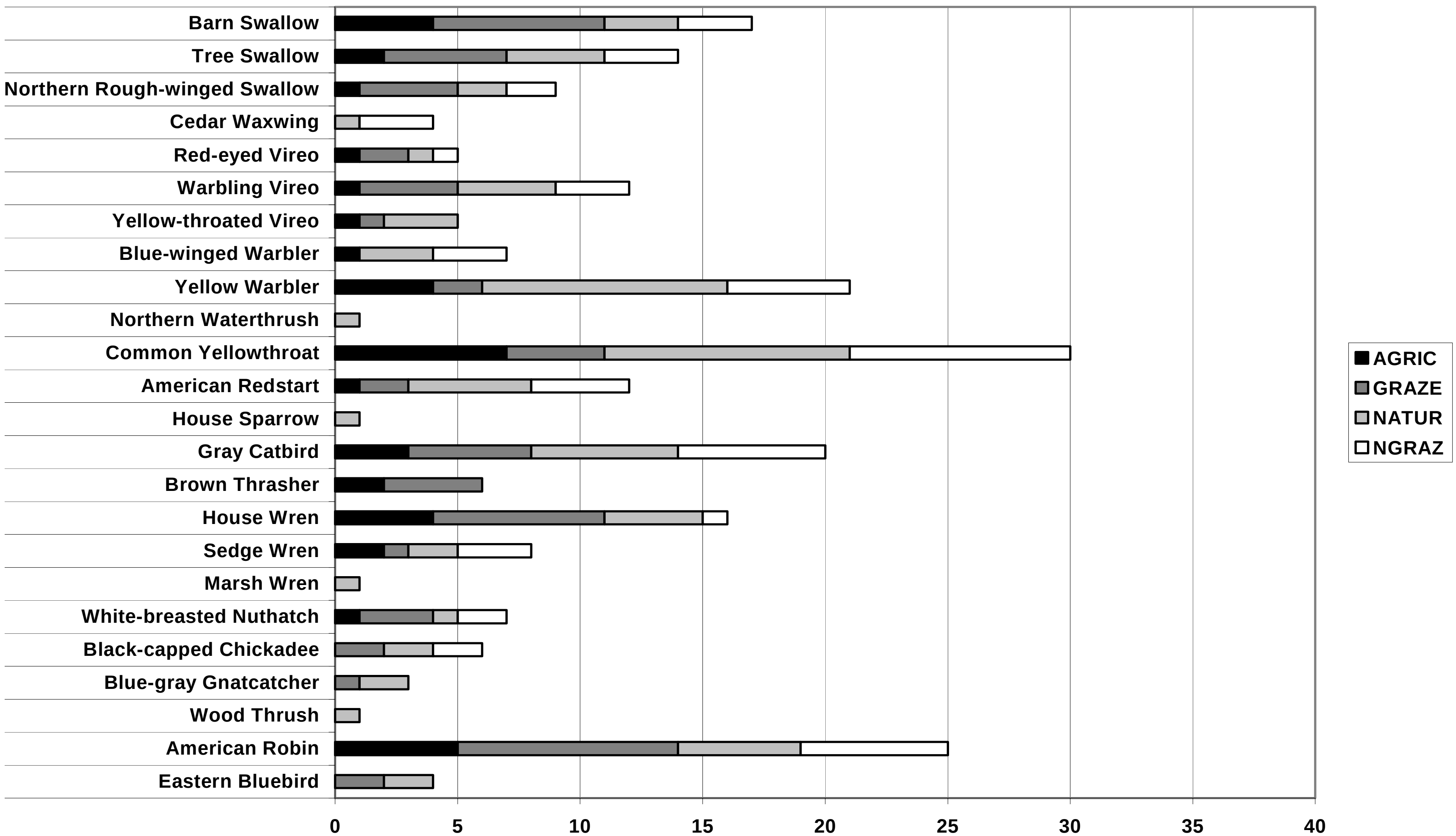

Figure 5D. Bird species present within four types of surrounding land uses, based on detection by point counts, for farm ponds in Houston and Winona Counties, Minnesota, 2000-2001.

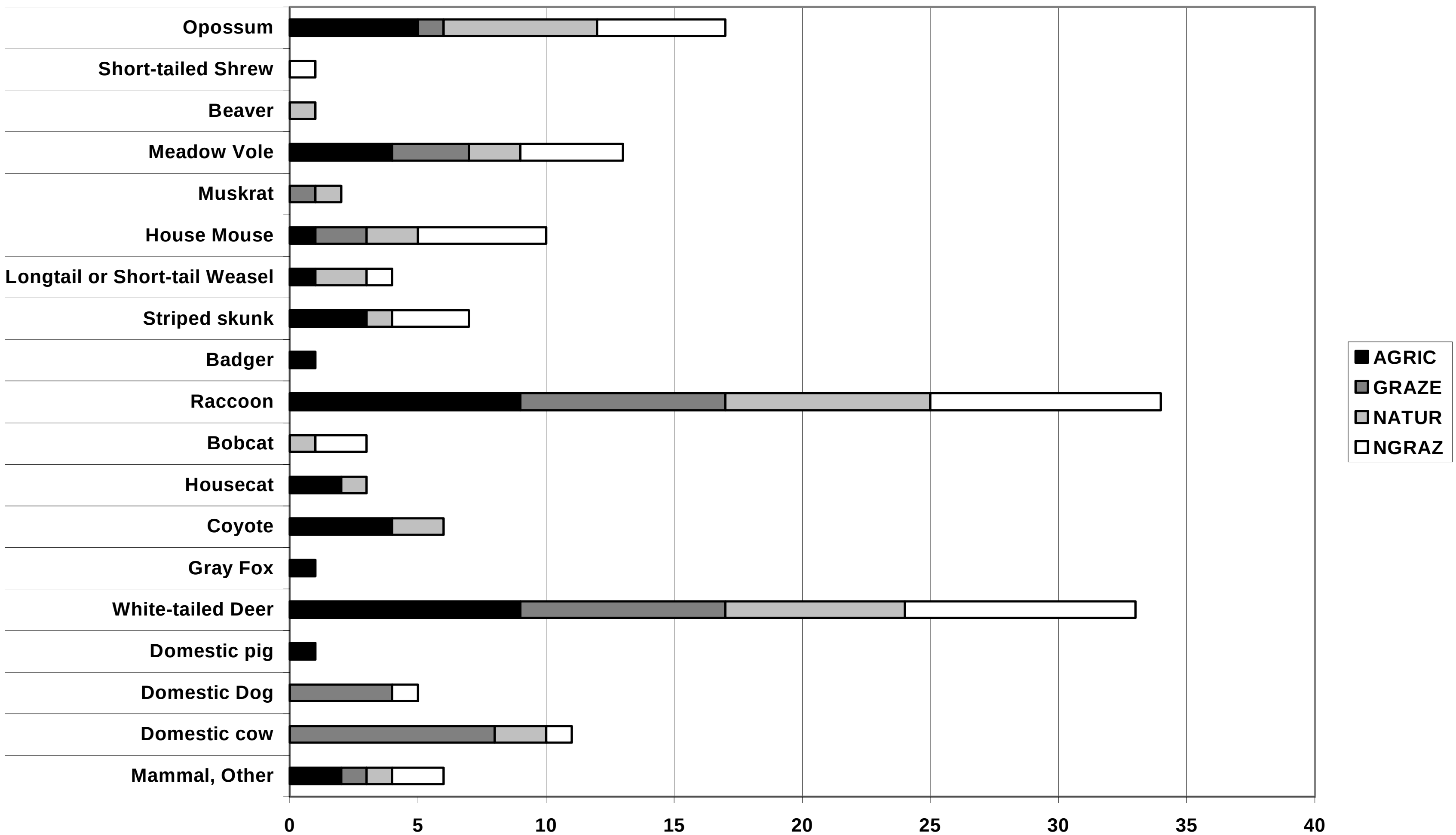

Figure 6. Mammal species present within four types of surrounding land uses, based on detection by all survey methods, for farm ponds in Houston and Winona Counties, Minnesota, 2000-2001 (scent stations excluded grazed ponds, but incidental observations included all ponds.

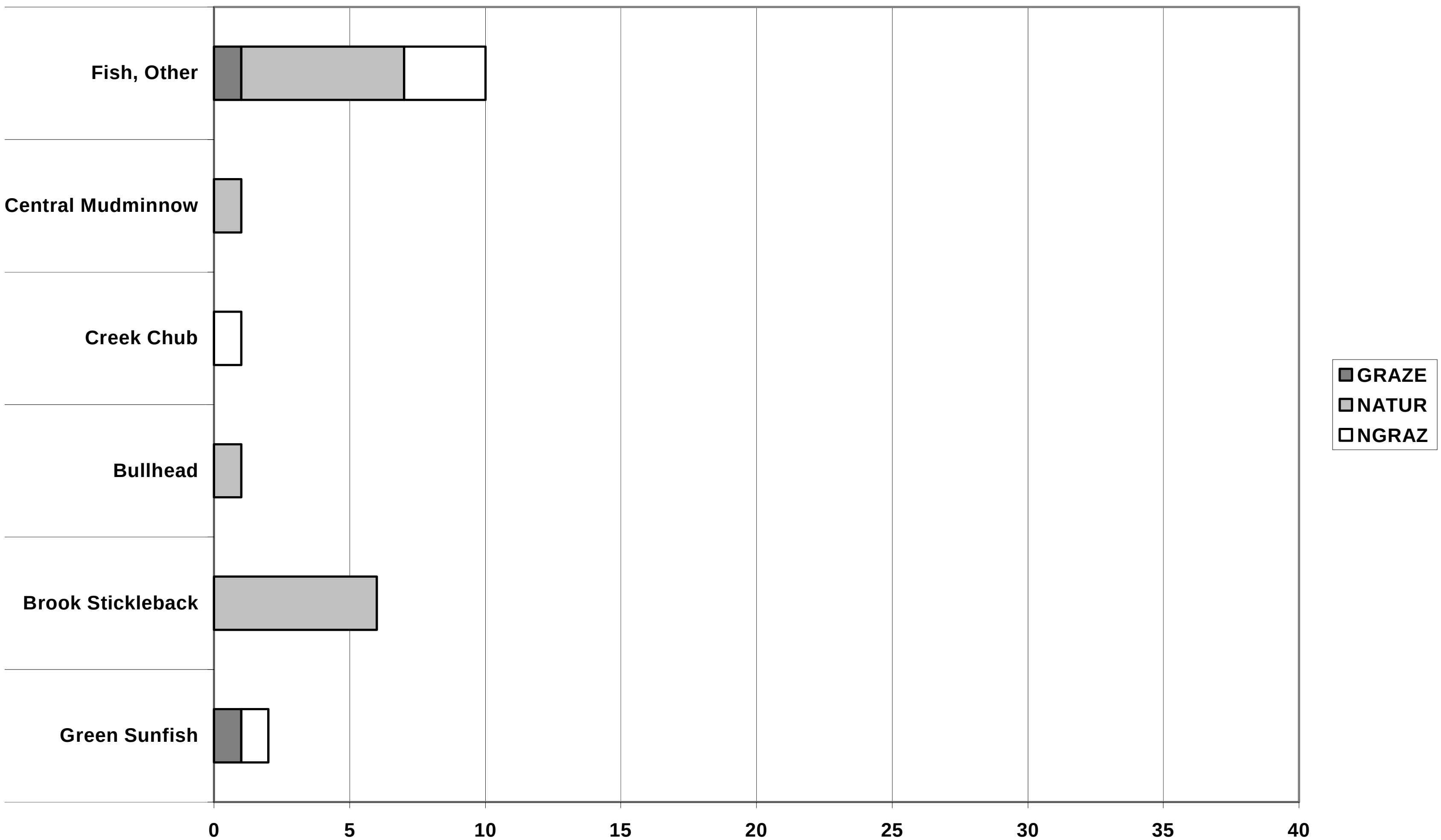

Figure 7. Fish species present within four types of surrounding land uses, based on detection by all survey methods, for farm ponds in Houston and Winona Counties, Minnesota, 2000-2001.

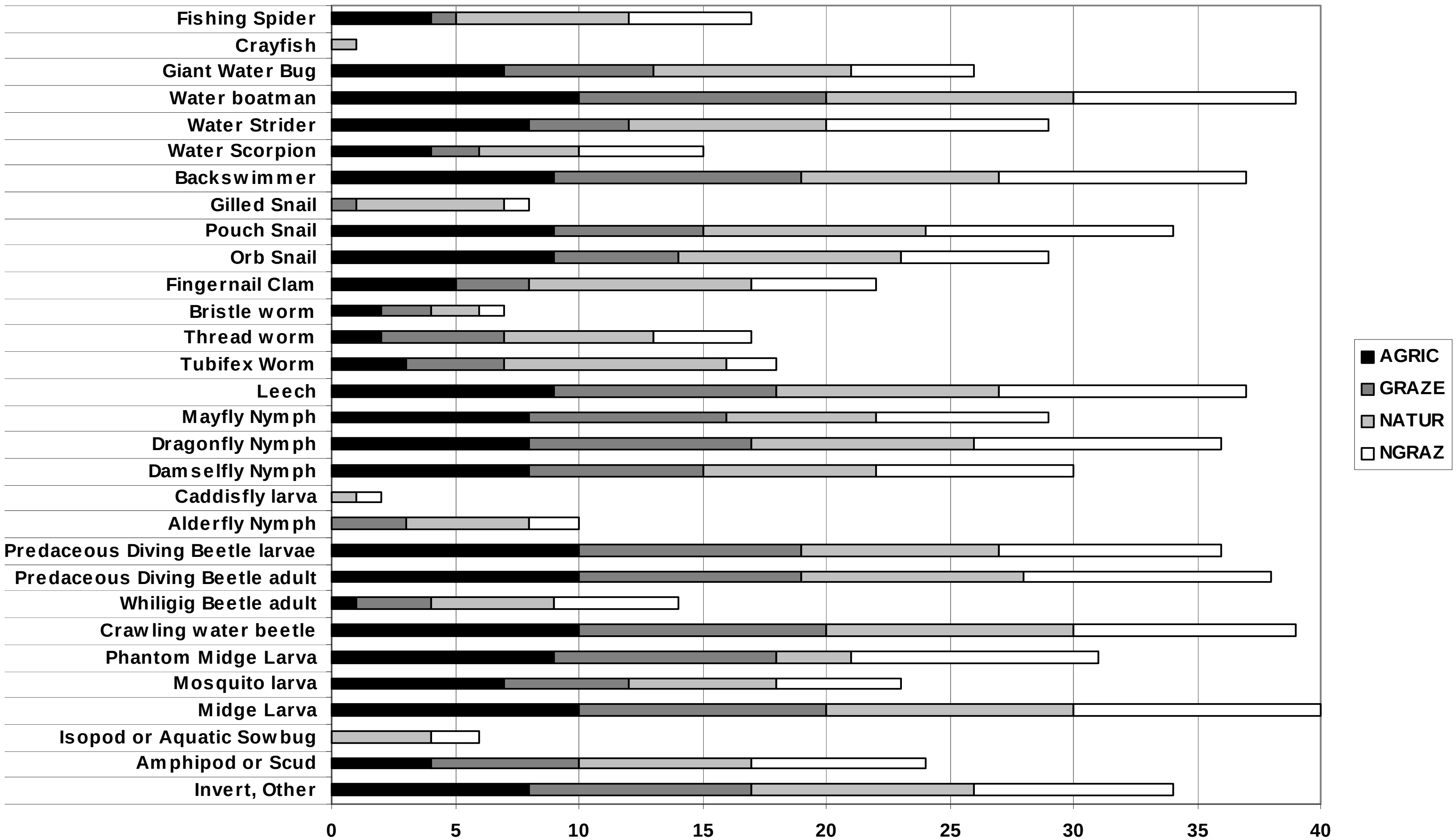

Figure 8. Invertebrate species present within four types of surrounding land uses, based on detection by all survey methods, for farm ponds in Houston and Winona Counties, Minnesota, 2000-2001.

Appendix A. List of common and scientific names for species identified on farm ponds in Houston and Winona Counties, Minnesota, 2000-2001.

Taxa	Common name	Scientific name	Order	Family
Birds	Mallard	*Anas platyrhynchos*	ANSERIFORMES	ANATIDAE
	Wood duck	*Aix sponsa*	ANSERIFORMES	ANATIDAE
	Canada goose	*Branta canadensis*	ANSERIFORMES	ANATIDAE
	Green heron	*Butorides virescens*	CICONIIFORMES	ARDEIDAE
	Common moorhen	*Gallinula chloropus*	GRUIFORMES	RALLIDAE
	Pectoral sandpiper	*Calidris melanotos*	CHARADRIIFORMES	SCOLOPACIDAE
	Killdeer	*Charadrius vociferus*	CHARADRIIFORMES	CHARADRIIDAE
	Ruffed grouse	*Bonasa umbellus*	GALLIFORMES	PHASIANIDAE
	Domestic chicken	*Gallus gallus*	GALLIFORMES	PHASIANIDAE
	Ring-necked pheasant	*Phasianus colchicus*	GALLIFORMES	PHASIANIDAE
	Wild turkey	*Meleagris gallopavo*	GALLIFORMES	PHASIANIDAE
	Mourning dove	*Zenaida macroura*	COLUMBIFORMES	COLUMBIDAE
	Turkey vulture	*Cathartes aura*	CICONIIFORMES	CATHARTIDAE
	Hairy woodpecker	*Picoides villosus*	PICIFORMES	PICIDAE
	Downy woodpecker	*Picoides pubescens*	PICIFORMES	PICIDAE
	Yellow-bellied sapsucker	*Sphyrapicus varius*	PICIFORMES	PICIDAE
	Red-headed woodpecker	*Melanerpes erythrocephalus*	PICIFORMES	PICIDAE
	Red-bellied woodpecker	*Melanerpes carolinus*	PICIFORMES	PICIDAE
	Northern flicker	*Colaptes auratus*	PICIFORMES	PICIDAE
	Chimney swift	*Chaetura pelagica*	APODIFORMES	APODIDAE
	Ruby-throated hummingbird	*Archilochus colubris*	APODIFORMES	TROCHILIDAE
	Eastern kingbird	*Tyrannus tyrannus*	PASSERIFORMES	TYRANNIDAE
	Great crested flycatcher	*Myiarchus crinitus*	PASSERIFORMES	TYRANNIDAE
	Eastern phoebe	*Sayornis phoebe*	PASSERIFORMES	TYRANNIDAE
	Eastern wood-pewee	*Contopus virens*	PASSERIFORMES	TYRANNIDAE

Taxa	Common name	Scientific name	Order	Family
	Acadian flycatcher	*Empidonax virescens*	PASSERIFORMES	TYRANNIDAE
	Willow flycatcher	*Empidonax traillii*	PASSERIFORMES	TYRANNIDAE
	Horned lark	*Eremophila alpestris*	PASSERIFORMES	ALAUDIDAE
	Blue jay	*Cyanocitta cristata*	PASSERIFORMES	CORVIDAE
	American crow	*Corvus brachyrhynchos*	PASSERIFORMES	CORVIDAE
	European starling	*Sturnus vulgaris*	PASSERIFORMES	STURNIDAE
	Bobolink	*Dolichonyx oryzivorus*	PASSERIFORMES	ICTERIDAE
	Brown-headed cowbird	*Molothrus ater*	PASSERIFORMES	ICTERIDAE
	Red-winged blackbird	*Agelaius phoeniceus*	PASSERIFORMES	ICTERIDAE
	Eastern meadowlark	*Sturnella magna*	PASSERIFORMES	ICTERIDAE
	Baltimore oriole	*Icterus galbula*	PASSERIFORMES	ICTERIDAE
	Common grackle	*Quiscalus quiscula*	PASSERIFORMES	ICTERIDAE
	American goldfinch	*Carduelis tristis*	PASSERIFORMES	FRINGILLIDAE
	Savannah sparrow	*Passerculus sandwichensis*	PASSERIFORMES	EMBERIZIDAE
	Chipping sparrow	*Spizella passerina*	PASSERIFORMES	EMBERIZIDAE
	Field sparrow	*Spizella pusilla*	PASSERIFORMES	EMBERIZIDAE
	Song sparrow	*Melospiza melodia*	PASSERIFORMES	EMBERIZIDAE
	Swamp sparrow	*Melospiza georgiana*	PASSERIFORMES	EMBERIZIDAE
	Eastern towhee	*Pipilo erythrophthalmus*	PASSERIFORMES	EMBERIZIDAE
	Northern cardinal	*Cardinalis cardinalis*	PASSERIFORMES	CARDINALIDAE
	Rose-breasted grosbeak	*Pheucticus ludovicianus*	PASSERIFORMES	CARDINALIDAE
	Blue grosbeak	*Guiraca caerulea*	PASSERIFORMES	CARDINALIDAE
	Indigo bunting	*Passerina cyanea*	PASSERIFORMES	CARDINALIDAE
	Dickcissel	*Spiza americana*	PASSERIFORMES	CARDINALIDAE
	Scarlet tanager	*Piranga olivacea*	PASSERIFORMES	THRAUPIDAE
	Barn swallow	*Hirundo rustica*	PASSERIFORMES	HIRUNDINIDAE
	Tree swallow	*Tachycineta bicolor*	PASSERIFORMES	HIRUNDINIDAE

Taxa	Common name	Scientific name	Order	Family
	Northern rough-winged swallow	*Stelgidopteryx serripennis*	PASSERIFORMES	HIRUNDINIDAE
	Cedar waxwing	*Bombycilla cedrorum*	PASSERIFORMES	BOMBYCILLIDAE
	Red-eyed vireo	*Vireo olivaceus*	PASSERIFORMES	VIREONIDAE
	Warbling vireo	*Vireo gilvus*	PASSERIFORMES	VIREONIDAE
	Yellow-throated vireo	*Vireo flavifrons*	PASSERIFORMES	VIREONIDAE
	Blue-winged warbler	*Vermivora pinus*	PASSERIFORMES	PARULIDAE
	Yellow warbler	*Dendroica petechia*	PASSERIFORMES	PARULIDAE
	Common yellowthroat	*Geothlypis trichas*	PASSERIFORMES	PARULIDAE
	Hooded warbler	*Wilsonia citrina*	PASSERIFORMES	PARULIDAE
	American redstart	*Setophaga ruticilla*	PASSERIFORMES	PARULIDAE
	Gray catbird	*Dumetella carolinensis*	PASSERIFORMES	MIMIDAE
	Brown thrasher	*Toxostoma rufum*	PASSERIFORMES	MIMIDAE
	House wren	*Troglodytes aedon*	PASSERIFORMES	TROGLODYTIDAE
	Sedge wren	*Cistothorus platensis*	PASSERIFORMES	TROGLODYTIDAE
	White-breasted nuthatch	*Sitta carolinensis*	PASSERIFORMES	SITTIDAE
	Black-capped chickadee	*Poecile atricapillus*	PASSERIFORMES	PARIDAE
	Blue-gray gnatcatcher	*Polioptila caerulea*	PASSERIFORMES	SYLVIIDAE
	Wood thrush	*Hylocichla mustelina*	PASSERIFORMES	TURDIDAE
	American robin	*Turdus migratorius*	PASSERIFORMES	TURDIDAE
	Eastern bluebird	*Sialia sialis*	PASSERIFORMES	TURDIDAE
Amphibians	Blue-spotted salamander	*Ambystoma laterale*	CAUDATA	AMBYSTOMATIDAE
	Tiger salamander	*Ambystoma tigrinum*	CAUDATA	AMBYSTOMATIDAE
	American toad	*Bufo americanus*	ANURA	BUFONIDAE
	Eastern gray tree frog	*Hyla versicolor*	ANURA	HYLIDAE
	Chorus frog	*Pseudacris triseriata*	ANURA	HYLIDAE
	Spring peeper	*Pseudacris crucifer*	ANURA	HYLIDAE
	Green frog	*Rana clamitans*	ANURA	RANIDAE

Taxa	Common name	Scientific name	Order	Family
	Wood frog	*Rana sylvatica*	ANURA	RANIDAE
	Leopard frog	*Rana pipiens*	ANURA	RANIDAE
	Pickerel frog	*Rana palustris*	ANURA	RANIDAE
Reptiles	Snapping turtle	*Chelydra serpentina*	TESTUDINES	CHELYDRIDAE
	Painted turtle	*Chrysemys picta*	TESTUDINES	EMYDIDAE
	Common garter snake	*Thamnophis sirtalis*	SERPENTES	COLUBRIDAE
	Brown snake	*Storeria dekayi*	SERPENTES	COLUBRIDAE
	Redbelly snake	*Storeria occipitomaculata*	SERPENTES	COLUBRIDAE
	Fox snake	*Elaphe vulpina*	SERPENTES	COLUBRIDAE
Invertebrates	Fishing spider		ARANEAE	LYCOSIDAE
	Giant water bug		HEMIPTERA	BELASTOMATIDAE
	Water boatman		HEMIPTERA	CORIXIDAE
	Water strider		HEMIPTERA	GERRIDAE
	Water scorpion		HEMIPTERA	NEPIDAE
	Backswimmer		HEMIPTERA	NOTONECTIDAE
	Gilled snail		GASTROPODA (CLASS)	LYMNAEIDAE
	Pouch snail		GASTROPODA (CLASS)	PHYSIDAE
	Orb snail		GASTROPODA (CLASS)	PLANORBIDAE (HELISOMA)
	Fingernail clam		PELECYPODA (CLASS)	SPHAERIIDAE
	Bristle worm		OLIGOCHAETA (CLASS)	MANY
	Thread worm		OLIGOCHAETA (CLASS)	MANY
	Tubifex worm		OLIGOCHAETA (CLASS)	TUBIFICIDAE
	Leech		HIRUDINEA (CLASS)	HIRUNDINEA, GLOSSIPHONIIDAE, ERPOBDELLIDAE
	Mayfly nymph		EPHEMEROPTERA	EPHEMERIDAE, HEPTAGENIIDAE, BAETIDAE
	Dragonfly nymph		ODONATA	ANISOPTERA (SUBORDER)
	Damselfly nymph		ODONATA	ZYGOPTERA (SUBORDER)

Taxa	Common name	Scientific name	Order	Family
	Caddisfly larva		TRICHOPTERA	MANY
	Alderfly nymph		MEGALOPTERA	SIALIDAE
	Predaceous diving beetle larva		COLEOPTERA	DYTISCIDAE
	Predaceous diving beetle adult		COLEOPTERA	DYTISCIDAE
	Whiligig beetle adult		COLEOPTERA	GYRINIDAE
	Crawling water beetle		COLEOPTERA	HALIPLIDAE
	Phantom midge larva		DIPTERA	CHAOBORIDAE
	Mosquito larva		DIPTERA	CULICIDAE
	Midge larva		DIPTERA	TENDIPEDIDAE (CHIRONOMIDAE)
	Isopod or aquatic sowbug		ISOPODA	ASELLIDAE
	Amphipod or scud		AMPHIPODA	TALITRIDAE, GAMMARIDAE
Fish	Central mudminnow	*Umbra limi*	ESOCIFORMES	UMBRIDAE
	Creek chub	*Semotilus atromaculatus*	CYPRINIFORMES	CYPRINIDAE
	Brook stickleback	*Culaea inconstans*	GASTEROSTEIFORMES	GASTEROSTEIDAE
	Green sunfish	*Lepomis cyanellus*	PERCIFORMES	CENTRARCHIDAE
Mammals	Opossum	*Didelphis marsupialis*	DIDELPHIMORPHIA	DIDELPHIDAE
	Gray fox	*Vulpes cinegeoargenteus*	CARNIVORA	CANIDAE
	Coyote	*Canis latrans*	CARNIVORA	CANIDAE
	Domestic dog	*Canis familiaris*	CARNIVORA	CANIDAE
	Raccoon	*Procyon lotor*	CARNIVORA	PROCYONIDAE
	Badger	*Taxidea taxus*	CARNIVORA	MUSTELIDAE
	Striped skunk	*Mephitis mephitis*	CARNIVORA	MEPHITIDAE
	Longtail or short-tail weasel	*Mustela frenata or Mustela erminea*	CARNIVORA	MUSTELIDAE
	Housecat	*Felis catus*	CARNIVORA	FELIDAE
	Bobcat	*Felis rufus*	CARNIVORA	FELIDAE
	Beaver	*Castor canadensis*	RODENTIA	CASTORIDAE
	Meadow vole	*Microtus pennsylvanicus*	RODENTIA	MURIDAE

Taxa	Common name	Scientific name	Order	Family
	White-tailed deer	*Odocoileus virginianus*	ARTIODACTYLA	CERVIDAE
	Domestic cow	*Bos taurus*	ARTIODACTYLA	BOVIDAE

Habitat Associations with Amphibian Reproductive Success in Farm Ponds

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We studied constructed farm ponds and natural wetlands in southeastern Minnesota during spring and summer 2000 and 2001. We collected amphibian and habitat data from 40 randomly-selected ponds, 10 ponds in each of 4 surrounding land use classes: row crop agriculture, grazed grassland, ungrazed grassland, and natural wetlands.

The first in-depth analysis we are undertaking of the amphibian data addresses the main research questions for the project at the amphibian population and community levels (1) How are measures of amphibian population and community health associated with land uses surrounding the pond, such as row crops, grassland, and grazed grassland? (2) Are measures of amphibian population and community health more associated with surrounding landscape features or within-pond characteristics? (Fig. 2) and, (3) What design features associated with a pond (size, depth, vegetation) will maximize wildlife (amphibian) benefits?

Methods We ranked the quality of amphibian breeding habitat as high, medium, or low at each pond for each species by combining observations across the breeding season. Ponds where the abundance class of larvae or metamorphs was $\geq$2 on at least 3 visits were ranked as high quality breeding sites. Ponds were ranked as medium quality when the abundance class of larvae or metamophs was $\geq$2 on 2 or fewer visits, or the abundance class of larvae or metamorphs was =1 on at least 3 visits, or egg masses were detected. Low quality sites were all other sites not meeting the above criteria. Calling data were not used to rank breeding habitat quality; we observed amphibian species calling at several sites where no evidence of reproductive success (larvae or metamorphs) for that species was ever observed. We assigned each pond to a class of overall habitat quality. Ponds classed as ‘overall high’ for amphibian habitat quality had 2 or more species with high quality rankings, ponds classed as ‘overall low’ had 0-1 species with high quality rankings.

We ran regression analyses to examine associations between the amphibian response variables and habitat variables. We grouped the habitat variables into categories of landscape, water quality, pond vegetation, pond morphometry, and predator variables and tested these groups of variables as explanatory models. We then ran regression analyses on individual variables within these groups. Model selection methods included step-wise regression and AIC.

Preliminary results We have run regression analyses on the 2000 data. We found that water quality variables as a group had the highest association with overall multi-species reproductive success in the ponds, followed by landscape variables within 500 m of the pond and pond vegetation. Water quality, pond vegetation, and landscape variables at 500 m were also highly associated with reproductive success of individual species, but the specific models varied by species. We found that the diversity of habitat types within 500 m of the pond was positively associated with amphibian reproductive success, while total nitrogen, conductivity, and emergent vegetation were negatively associated.

Data analysis is continuing and we will be writing a scientific paper for publication on the results of this work.

Effects of Agricultural Pond Water on the Survival of Anurans in the Unglaciated Region of Minnesota

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Introduction

Globally declining amphibian populations are due, in part, to habitat loss. Consequently, constructed habitats, such as farm ponds, may be important for maintaining regional populations of amphibians. However, relatively little is known about the suitability and importance of farm ponds for amphibians in Minnesota due to (1) the uncertain relationship between amphibian survival and agriculture reported in previous studies (e.g., Knutson et al. 1999), (2) reports of amphibian malformation and mortality from ponds in portions of Minnesota (Souder 2000, Burkhart et al. 1998), and (3) a limited number of *in situ* field studies involving amphibian toxicology (Rowe et al. 1994). Therefore, this project was designed to determine if agricultural pond water alters amphibian survival. Through laboratory and field studies, we incorporated standardized amphibian toxicology assays, water quality analyses, and field enclosures (mesocosms) to compare anuran survival in agricultural ponds and natural wetlands. We hypothesized that pond type (i.e., agricultural or natural) and pond-water chemistry (e.g., nitrogen, phosphorous) or pesticides would affect anuran survival.

Methods

Laboratory Study—From April 15 to July 20, 2001, water from 19 ponds (10 natural and 9 agricultural) was collected every two weeks for assessment of potential toxicity to amphibians with the Frog Embryo Teratogenesis Assay *Xenopus* (FETAX). The FETAX assay is a standardized static renewal test used to determine if water samples or the constituents of water samples can elicit malformations or mortality in *Xenopus laevis* (African clawed frog). FETAX was performed with standard techniques (ASTM 1998); however, instead of known compounds as test solutions, whole-water samples collected directly from ponds were used. Differences in the survival of larval *X. laevis* between agricultural and natural ponds were compared to water quality data as well as to survival of larval *Rana pipiens* (Northern leopard frog) from our field study.

Water quality characteristics— Pond-water samples were collected and analyzed for several nutrients (i.e., ammonia, total nitrogen, and total phosphorus) during summer 2001 by personnel of the Upper Midwest Environmental Sciences Center, USGS, La Crosse, WI , by standard methods (see water quality section of this report for detailed methods; American Public Health Association et al. 1995). Water samples were collected for nutrient analyses and for the FETAX assay from each pond on the same day. Water chemistry data were compared with survival of *Xenopus laevis* from FETAX assays. In addition, water collected from 7 ponds on June 11-13 was analyzed for pesticides by the U.S. Geological Survey (Middleton, WI).

Field Study--During summer 2001, differences in survival of developing *Rana pipiens* between agricultural (6 ponds) and natural ponds (4 ponds) was assessed with field enclosures (mesocosms) in which *R. pipiens* tadpoles were held and “cultured” through metamorphosis (Figs 1, 2). Mesocosms were 3.65 m long X 0.92 m wide X 1.21 m high. We designed our enclosures with four sides and no bottom to allow developing tadpoles access to desired microhabitats in the sediments. Mesocosms were constructed of a welded aluminum framework and two layers of screening--an outer layer of rigid plastic mesh with 1.3 cm openings, and an inner layer of window screen. The double screen kept out aquatic predators yet allowed water to flow into the mescosm, which supplied a natural food source of algae for developing tadpoles. Screens were held in place with silicone sealant and aluminum strips secured with screws to the aluminum framework (Fig 1). Aluminum flashing was attached with screws to the bottom 30.6 cm of each frame and sides were bolted together with stainless steel stakes that extended an extra 45.8 cm below the bottom edge of the enclosures (Fig 1).

Mesocosms were placed in each pond as soon as tadpoles of *Rana pipiens* were collected. Enclosures were dropped into the water and driven into the sediments by hand so that at least 15 cm of the aluminum flashing was buried. This helped insure that tadpoles could not escape through gaps between the bottom edges of enclosures and sediments. Care was also taken to avoid including large emergent macrophytes within enclosures as they often contain over-wintering eggs of several odonate species that may prey upon young tadpoles. After mesocosms were placed into each pond, a dip net was used to remove potential predators and tadpoles already present. Avian and terrestrial predators were excluded from the mesocosms by mist nets (1.5 cm openings) attached to the top of the mesocosms with plastic ties

Each mesocosm was stocked with 100 tadpoles (approximately Gosner stage 25; Gosner 1960) from the same pond (2 ponds) or, if adequate numbers of tadpoles were not found in the pond, from two reference sites (8 ponds). All enclosures were in place and stocked with tadpoles by June 1, 2001.

Tadpoles in the mesocosms were examined weekly. After significant development of hind limbs occurred in the tadpoles (Gosner Stages 39-41), two sheets of styrofoam (30.6 cm x 30.6 cm) were placed into each enclosure to provide a substrate on which metamorphosing frogs could leave the water. As newly metamorphosed frogs and tadpoles with four limbs (Gosner stage 42 to 46) were observed, they were removed from enclosures, checked for malformations, and counted. Larval survival within a pond was assessed after it was certain that all surviving tadpoles had metamorphosed (as ascertained by dip netting) and was defined as the proportion of tadpoles that reached the sub-adult stage (Gosner stage 42 to 46). Survival of tadpole *Rana pipiens* was compared to that of *Xenopus laevis* from the FETAX assays.

Data Analysis—Differences in the survival of *Xenopus laevis* and *Rana pipiens* in agricultural and natural ponds were analyzed with repeated-measures analysis of variance (ANOVA). A Type I error (α) of $p < 0.05$ was used to reject the null hypothesis of no significance in survival of frogs between ponds. Multiple regression was used to assess the relationship between nutrient levels (ammonia, total nitrogen and total phosphorous) and amphibian survival. Data used in both procedures (regression and ANOVA) were rank transformed due to violations of model assumptions (normality and homogeneity of variances) present in the untransformed data.

Results

There was no significant difference in survival of *Xenopus laevis* and *Rana pipiens* between agricultural and natural ponds ($F_{1, 8}$ = 0.645, P = 0.445). Mean survival of Xenopus laevis was 5% greater in water from agricultural ponds than in water from natural ponds. Mean survival of *Rana pipiens* was 6% lower in agricultural ponds than in natural ponds; however, in 2 agricultural ponds no tadpoles survived (Fig. 3).

Only one malformed *Rana pipiens* tadpole was found from a mesocosm in an agricultural pond. Three of its appendages (both hind limbs and one front limb) were stunted. Data on malformations in *Xenopus laevis* were not assessed because of the subjective nature and discrepancy among individuals inherent in determining what is, in fact, a malformed individual from the FETAX assay.

There was a weak negative relationship between water chemistry and anuran survival (R^2 = 0.067). Of the three nutrients (ammonia, total nitrogen, and total phosphorous), only total phosphorous contributed significantly to the relationship (P=0.047). However, this relationship is so weak that its biological significance is questionable.

Relatively small concentrations of pesticides were measured in the single water sample from the ponds. Of those chemicals measured, atrazine was detected in the highest concentration (0.381 ug/l to 0.551 ug/l). However, these levels are well below those acutely toxic to anurans (Allran and Karasov 2000, Howe et al. 1998). Concentrations of all other chemicals detected were appreciably lower than that of atrazine (Fig 4).

Discussion

We were unable to detect any significant difference in survival of tadpoles in water from agricultural or natural ponds. Therefore, from a water-quality perspective, agricultural ponds seem to be suitable habitats for developing larval anurans within the unglaciated area of Minnesota. However, the effects of other factors on larval survival (i.e., predation, pond desiccation, infectious diseases) within agricultural ponds may be drastically different from those in natural ponds; these variables were not assessed in our study. For example, many of the agricultural ponds contained high densities of the eastern tiger salamander larvae (*Ambystoma tigrinum tigrinum*). Larval tiger salamanders are voracious predators that consume many types of aquatic fauna (including anuran tadpoles; Lannoo and Bachmann 1984). It is possible that if tiger salamander larvae are more abundant in agricultural ponds than in natural ponds, their presence may negatively affect survival of larval anurans.

In addition, from this portion of the project we cannot make any conclusions about potential differences in adult amphibian survival between ponds. However, many adult amphibians were also observed at the agricultural and natural ponds throughout the duration of the study (see reports on amphibian species abundances). Therefore, it is probable that agricultural ponds are able to sustain adequate populations of amphibians and offer suitable refuges and breeding habitats.

Finally, we note the importance of performing field experiments as well as laboratory experiments in studies involving amphibian toxicology. Due to time and resource constraints, we were only able to incorporate a small number of mesocosms in our study. However, to our knowledge this is the first time that enclosures of this size have been used in field experiments to study amphibian toxicology. With greater resources and more experience in utilizing these mesocosms, further studies could potentially yield ecologically important information about the survival of amphibians in natural and artificial habitats.

Acknowledgements

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Figure 1. Mesocosm being constructed near an agricultural pond. Note the aluminum frames, as well as the aluminum flashing, and stainless steel stakes extending out from the bottom edge of the enclosure.

Figure 2. Constructed mesocosm after placement within an agricultural pond.

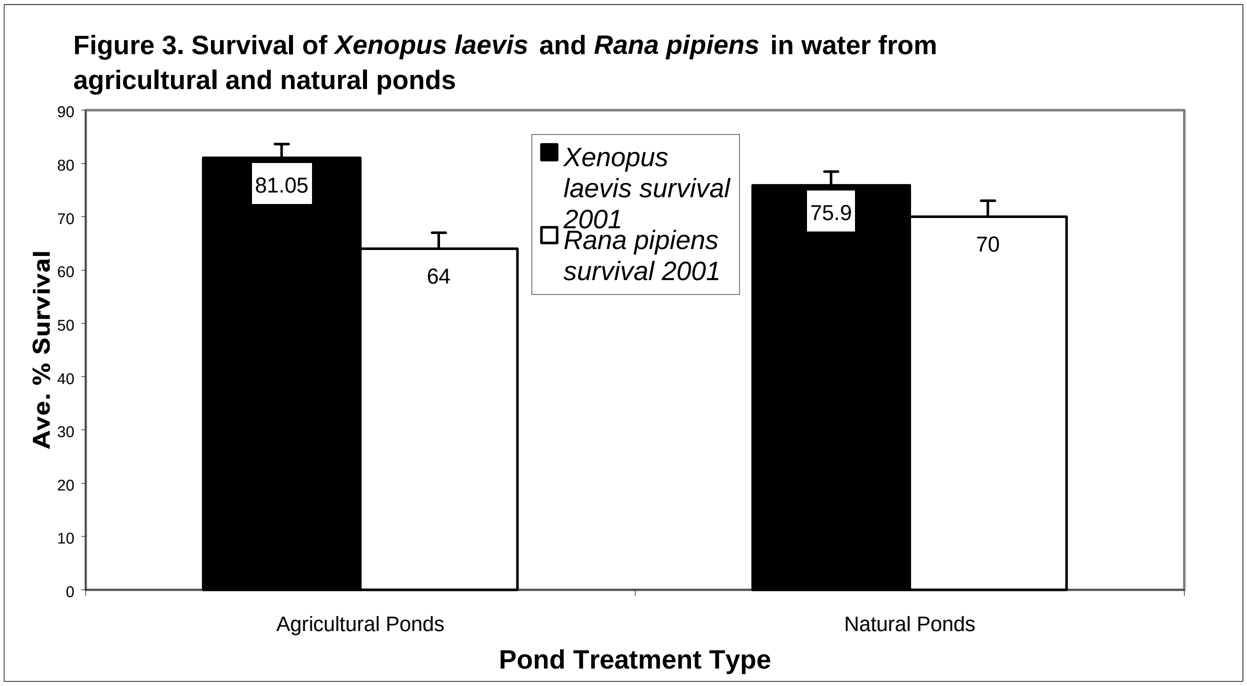
Figure 3. Survival of Xenopus laevis and Rana pipiens in water from agricultural and natural ponds
Ave. % Survival
0
10
20
30
40
50
60
70
80
90
81.05
64
75.9
70
Xenopus laevis survival 2001
Rana pipiens survival 2001
Agricultural Ponds
Natural Ponds
Pond Treatment Type

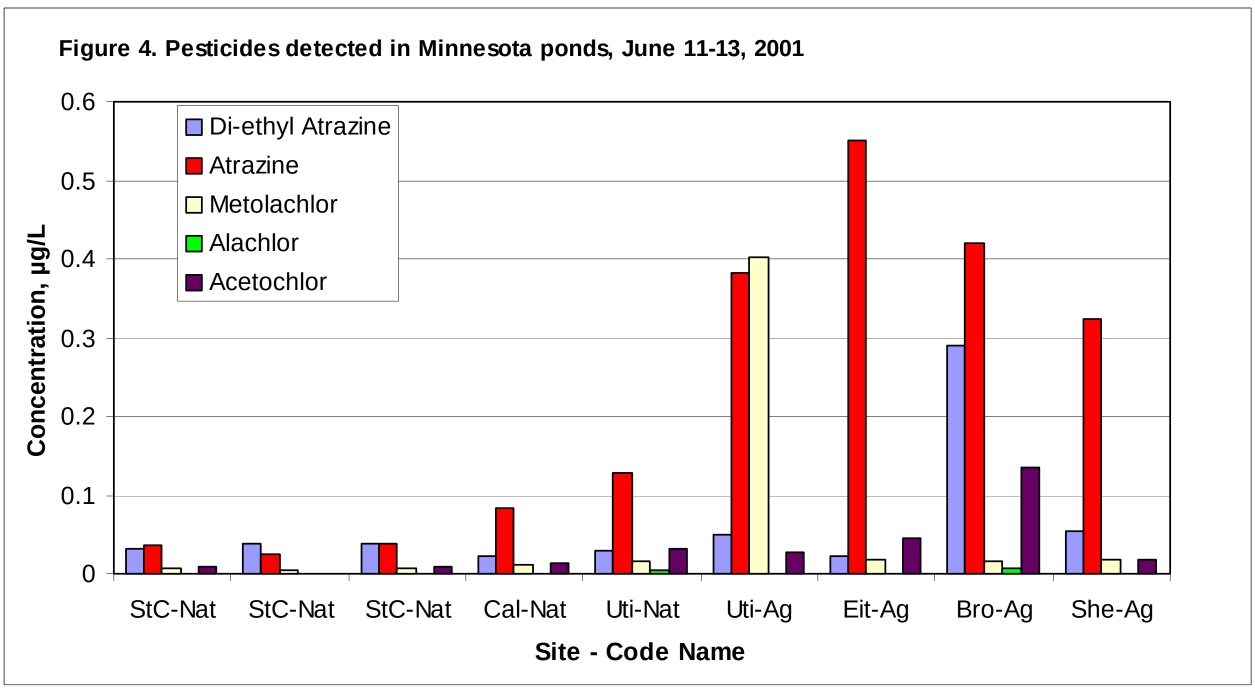
Figure 4. Pesticides detected in Minnesota ponds, June 11-13, 2001
Concentration, µg/L
0
0.1
0.2
0.3
0.4
0.5
0.6
Di-ethyl Atrazine
Atrazine
Metolachlor
Alachlor
Acetochlor
StC-Nat
StC-Nat
StC-Nat
Cal-Nat
Uti-Nat
Uti-Ag
Eit-Ag
Bro-Ag
She-Ag
Site - Code Name

Flow Cytometry as a Tool for Detecting Genotoxic Effects in Amphibians Breeding in Southeastern Minnesota Farm Ponds

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Abstract

High rates of amphibian malformations have been observed in Minnesota and several other states. Flow cytometry is a laboratory test that measures genetic damage based on blood samples and may be useful in evaluating sites with amphibian malformations. We conducted a pilot study of farm ponds in southeastern Minnesota to assess the feasibility of using flow cytometry to assess genetic damage in native amphibians. We tested associations between the type of agricultural land use surrounding a pond and DNA integrity. Amphibians from four reference (one natural wetland, three non-grazed ponds), and five exposed (three grazed ponds, and two agricultural ponds) were examined in the field for deformities and blood samples were collected for testing with flow cytometry. We assumed that the exposed ponds had higher inputs of agricultural contaminants (fertilizers, pesticides, and animal wastes) than the reference ponds. We found no significant differences in DNA profiles between the reference and exposed ponds. However, several specimens from three different ponds produced aneuploid peaks, which is indicative of DNA damage. We found low correlation between amphibian deformities and the coefficient of variation of DNA; deformities were rarely observed and they occurred in both the reference and exposed ponds. We found no evidence that surrounding agricultural land use affected amphibian DNA integrity or malformation rates in southeastern Minnesota. We demonstrated that flow cytometry could be used to detect and quantify DNA damage in native amphibian communities. It may be useful for evaluating amphibian breeding sites exposed to contaminants or other potential genetic stressors.

Introduction

Malformations within amphibian populations have been observed for hundreds of years (de Superville 1740, Valen 1974). Recently, reports of amphibian malformations across North America have increased (Meteyer et al. 2000). In the U.S., frogs with malformations have been observed in 24 states, with most occurring in the mid-northern United States, including Minnesota (Reaser and Johnson 1999). Malformations in amphibians generally involve the hind limbs and are usually expressed as missing limbs or digits, multiple limbs or limb elements, reduced limbs, or morphologically complete but deformed limbs (Sessions and Ruth 1990, Ouellet et al. 1997, Gardiner and Hoppe 1999). Amphibians are vulnerable to water-borne environmental contaminants because of their biphasic life cycle (larva and adult) and semi-permeable skin (Harfenist et al. 1989, Mahaney 1994). Therefore, amphibians that breed in farm ponds exposed to agricultural chemicals may function as environmental indicators of contaminants in agricultural landscapes.

Recent studies have attributed amphibian deformities to several environmental agents. Several studies suggest that high concentrations of retinoids (Gardiner and Hoppe 1999, Sessions et al. 1999), or pesticides and fertilizers from field run-off (Berrill et al. 1997, Berrill et al. 1998) may be responsible for inducing malformations. For example, laboratory exposure to many known toxins has been demonstrated to have mutagenic effects on amphibians (Harfenist et al. 1989). Toxins can induce clastogenic formation of micronuclei, leading to abnormal DNA content (Fernandez et al. 1993, Krauter 1993). However, Sessions and Johnson (1990, 1999) have demonstrated that both experimental and natural infection of frogs with the trematode *Ribeiroia* can lead to limb malformations and decreased survival rates of infected tadpoles. Blaustein (1997) has suggested that exposure to UV-B radiation may also be a contributing factor in amphibian deformities.

Flow cytometry (FCM) is a laboratory procedure that measures genetic damage in blood samples and may be useful in evaluating sites with high rates of amphibian malformations or sites with a history of exposure to contaminants or other stressors. FCM has been used to detect aneugenic and clastogenic effects induced by environmental contaminants and other stressors on the vertebrate genome (Bickham et al. 1988, Lamb et al. 1991, Fernandez et al. 1993). FCM monitors multiple cellular characteristics (Dallas and Evans 1990), accurately estimates cellular DNA content, and detects small changes in DNA caused by exposure to environmental contaminants. Recently, FCM has been used to identify genetic alterations within normal and deformed amphibian populations, and provide complementary evidence of damage when deformities are present (Lowcock et al. 1997). FCM can also be used to screen for abnormal DNA profiles, such as aneuploid mosaicism, a chromosomal condition associated with exposure to pesticides (Lowcock et al. 1997).

We conducted a pilot study of farm ponds in southeastern Minnesota to assess the feasibility of using flow cytometry to assess genetic damage in native amphibians. We present the details of the laboratory method in this paper, but blood samples can also be sent to contract laboratories for testing. We tested whether the type of agricultural land use surrounding a pond was associated with differences in DNA integrity. We expected that amphibians from ponds with higher exposure to agricultural run-off would exhibit more DNA alterations and higher variances in DNA weights than amphibians collected from ponds with lower agricultural inputs.

Materials and Methods

Pond Types Reference ponds were marshes and oxbows of river floodplains (natural wetlands) and constructed ponds surrounded by ungrazed grassland (non-grazed ponds). Exposed ponds were constructed ponds surrounded by row crops (agricultural ponds) or pastures grazed by domestic livestock (grazed ponds). We assumed that the exposed ponds had higher inputs of agricultural contaminants (fertilizers, pesticides, and animal wastes) than the reference ponds. Amphibians from four reference (one natural wetland, three non-grazed ponds), and five exposed (three grazed ponds, and two agricultural ponds) were examined in the field for deformities and blood samples were collected for testing with flow cytometry.

Collection of Specimens and Deformity Assessments Fifty-eight specimens from three species; *Rana clamitans* (N=40), *Rana pipiens* (N=13), and *Rana palustris* (N=5), were randomly collected between May and July 2000 from the ponds. Deformity assessments were conducted in the field when amphibians were late stage metamorphs. During the deformity assessment, the objective was to collect 100 random metamorphs from a single species at a site and examine them for leg, toe, and ocular malformations. Deformed individuals, plus healthy metamorphs (up to 10 individuals) from each pond were collected for necropsy and blood collection for FCM analysis.

Necropsy and Blood Collection Specimens were euthanized with methane tricaine sulfonate (MS-222) (Argent Laboratories, Redmond, WA), and two 10 μl samples of auricular blood were collected with 40 μl heparinized capillary tubes. Each sample was resuspended in 1.5 ml cryovials containing 200 μl of freezing solution (0.25M sucrose, 0.04M trisodium citrate, 5% dimethyl sulfoxide, pH 7.61) and manually agitated. Blood specimens were flash frozen in liquid nitrogen and stored at -70^{o} C until analysis. In addition, individual metamorphs were necropsied and examined for *Ribeiroia* and other parasites to determine their overall health.

Reference Blood Accurate calculation of DNA content for the target species requires knowledge of the DNA content of an internal reference (Tiersch et al. 1989). Therefore, a reference specimen of known DNA content, obtained from *Xenopus laevis* (6.3 pg of DNA/ haploid nucleus), certified as free of potential mutagens was used as an internal control in every sample (Xenopus Express, Homosassa, FL). A healthy *X. laevis* was euthanized as previously described and 4 ml of auricular blood were gathered with a 10 ml syringe and transferred to 80 ml of freezing solution. After manual agitation, the solution was allowed to incubate at room temperature for one minute to allow tissue and clotted blood to settle out of solution. Following incubation, 210 μl aliquots of reference blood were transferred into 1.5 ml cryovials and flash frozen in liquid nitrogen. Reference blood was stored at –70 ^{o}C until needed.

Sample Preparation Prior to sample preparation, ribonuclease stock solution (2 mg/ml) was prepared by adding 20 mg of Ribonuclease A (Sigma Chemical, St. Louis, MO) to 10 ml of autoclaved deionized water. One hundred milliliters of stock staining solution (API) (0.01 M Trizma Base, 0.01 M NaCl, 0.1% NP-40, pH 7.62) was combined with 300 µl of ribonuclease solution, and 5 mg of propidium iodide (Sigma Chemical). The solution was brought to a final volume of 140 ml using distilled water. The API staining solution was covered to prevent exposure to light and was stored at 4^{o} C until needed. Before each run of samples, 300 µl of fresh ribonuclease stock solution was added to the API staining solution.

Samples for FCM analysis were thawed and 200 µl of X. laevis reference blood was added to each target sample. Samples were gently vortexed and immediately placed in an ice bath. Seven hundred and fifty microliters of API staining solution were added to each sample, gently mixed, returned to the ice bath, and reincubated in the dark for two hours. Following incubation, each sample was transferred to a 12x75 mm culture tube by passage through 53 µm nylon mesh to remove clumped cells or tissue debris.

Flow Cytometry Following incubation and filtration, samples were analyzed for DNA content using a FACScan flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, CA). Prior to data acquisition, the linearity and alignment were calibrated with DNA Quality Control Particles (Becton-Dickinson). For each target sample, 20,000 stained nuclei were collected. Propidium iodide, when excited by the argon laser (488 nm), emits fluorescent light over the range of 550 - 650 nm, which is detected by a photomultiplier tube (FL-2) within the flow cytometer. FL2-Width vs. FL2-Area dot plots were used to detect and differentiate erythrocytes from debris, and FL2-A histograms were used to determine DNA content. Data were analyzed using CellQuest software (Becton-Dickinson). Triplicate analysis was performed on 10% of the samples to assess intra-assay variation; the relative SD, defined as the SD divided by the mean, of the triplicate sample runs was 1.63%.

The C-value (pg of DNA/ haploid nucleus) and an estimated sample CV were calculated for every sample. The C-value was calculated from the following equation (Lowcock et al. 1996):

$$\text{Target Species C-value} = \frac{\text{Known C-value of internal reference X Target species peak channel}}{\text{Internal reference peak channel}}$$

The estimated sample CV is defined as the SD of the target peak divided by the mean channel of the target peak, multiplied by 100, and was calculated by the analysis software. C-values and CVs were averaged between replicate FCM analyses of the same individual to obtain a mean value for every specimen. Histograms with the target species DNA profile were analyzed for aneuploid mosaicism.

Statistical Methods All statistical comparisons were performed using the CV value. The association CV and pond exposure status was estimated using a general linear mixed model and restricted maximum likelihood (Wolfinger 1993, Littell et al. 1996). Ponds, as the experimental units, were treated as random effects. This method was also used to estimate the correlation between CVs measured on frogs from the same pond. Adequate replication for these analyses was available for *Rana clamitans* only.

Results

Pond averaged C-values ranged from 6.53 (*Rana pipiens*) to 7.08 (*Rana pipiens*). Mean CVs within the ponds ranged from 1.91 (*R. palustris*) to 6.31 (s^2 = 0.030) (*R. pipiens*). For *R. clamitans*, the association between pond category and CV was small and non-significant (β=0.01, SE=0.24, p=0.98, *t* test, df=3), as was the estimated within-pond correlation among CV values (r=0.08, restricted likelihood ratio test, p=0.48) (Table 1). Residuals appeared approximately normal (Anderson-Darling test, p>0.25).

There was low correlation between deformities and mean CV (Table 1); deformities were rarely observed and they occurred in both the reference and exposed pond categories. During the necropsy, *Rana clamitans* specimens from three ponds, two exposed and one reference pond, were found to contain the parasite *Ribeiroia ondatrae* (Table 1). *Ribeiroia* was found in the limb bud region, indicating that deformities were due to parasite loads instead of DNA damage (Sessions and Ruth 1990, Johnson et al. 1999). The CV of the deformed frog from the exposed pond was 2.15, and the CV of the deformed frog from the reference pond was 3.85. *Rana pipiens* from one of the exposed ponds had a high number of aneuploid peaks and a large CV, indicating genetic damage at that site, even though no deformities were found (Table 1).

Aneuploid mosaicism (Figure 1) was observed in 10% of the specimens analyzed, the highest number of abnormal profiles occurring in *Rana pipiens* (Table 1). Abnormal profiles were most commonly observed in exposed ponds. In particular, aneuploid mosaicism was discovered in all three specimens analyzed from the exposed pond where *Rana pipiens* exhibited a high mean CV of 6.31 (Table 1).

Discussion

We demonstrated that FCM could be used to detect and quantify DNA damage in native amphibian communities, and that it may be useful in evaluating sites with high rates of amphibian malformations or other environmental risk factors. It may be possible to collect the small amount of blood (20 μl) needed for FCM from a toe clip from larger amphibian species (Ranids). For smaller amphibians, the large numbers of metamorphs available at breeding sites during summer allow replicate specimens to be collected without risk to the population. We had access to a flow cytometer and technical assistance (Gundersen Lutheran Medical Center), so we conducted the laboratory testing ourselves. However, contract laboratories are available to conduct the flow cytometry testing. Costs of analysis by contract laboratories vary based on the number of specimens, but for planning purposes estimate at least $100 per sample.

We found no evidence that surrounding agricultural land use affected *Rana clamitans* DNA integrity or malformation rates in southeastern Minnesota. The small within-pond correlation estimates suggest that *Rana clamitans* individuals randomly collected from a single pond may be considered independent observations. However, our sample sizes in this pilot study were low; our findings regarding land use associations should be substantiated using larger sample sizes. Lack of replication within species and among ponds in our pilot study also precluded examination of inter-species effects or pond type effects.

The row crops surrounding exposed ponds in our study area were corn and soybeans, crops not generally associated with heavy pesticide use. We did obtain information on pesticide levels for four exposed and three reference ponds in the same counties as our study ponds during June of 2001. (Two of the exposed ponds were also in our study.) Levels of most of the 47 toxicants measured were below detection levels. However, levels of atrazine ranged from 0.04 - 0.13 μg/l in reference ponds and from 0.32 - 0.55 μg/l in exposed ponds (J. Elder, USGS Water Resources, unpublished data). Levels of di-ethyl atrazine, metolachor, alachlor, and acetochlor during June 2001 were all <0.20 μg/l, except for one exposed pond that had 0.40 μg/l of metolochlor and one exposed pond with 0.29 μg/l of di-ethyl atrazine (J. Elder, USGS Water Resources, unpublished data). In a study of *Rana clamitans* in Quebec, Canada, researchers using FCM demonstrated that frogs exposed to chemical run-off from potato fields had more deformities and DNA alterations than frogs from ponds adjacent to cornfields and control ponds (Bonin et al. 1997). Our C-values for *Rana clamitans* ranged from 6.55 to 6.60 pg of DNA/ haploid nucleus (Table 1), and were very similar to those values calculated in the Canadian study (Bonin et al. 1997).

Power analysis should be used to plan future studies of genotoxic effects on amphibians. Power depends on the effect size (a function of differences between reference and exposed ponds), the variance between ponds, the number of pond types (treatment levels), the alpha level, and the number of replicates (here ponds). Power and sample size requirements may be calculated using formulae and tables provided in standard statistical texts and using commercial and public domain statistical packages (Thomas and Krebs 1997, Zar 1999).

For *R. calamitans,* we found high power ($\geq$90%) to detect an increase of $\geq$0.5 CV units from a reference mean of 3.0 CV using as few as three ponds in each of two pond types (reference and exposed). If, instead, we assume four types of ponds, with means of

3, 3.25, 3.25, and 3.5 CV, we estimate >80% power to detect an overall pond type effect at sample sizes as low as four ponds. These power estimates will likely vary by species and possibly by location, as population variances may vary by species and across space. Inferences on pond (rather than on pond type) will depend on the number of metamorphs or adults obtained per pond. The estimation of power or sample sizes for these inferences will differ somewhat from that used for pond types because inferences on pond should be treated as random effects while inferences on pond type will likely be treated as fixed effects. Power to detect a random effect may be estimated after Zar (1999) or using statistical software (Thomas and Krebs 1997).

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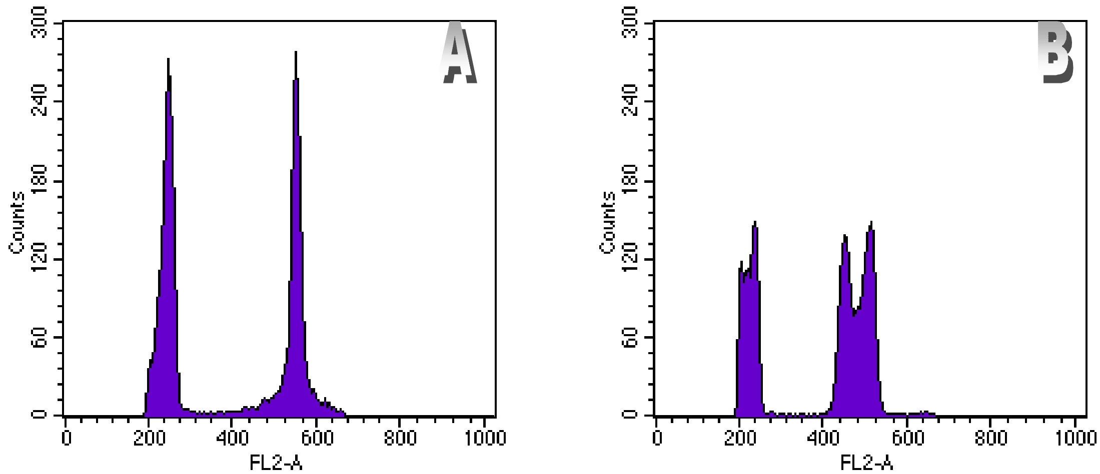

Figure 1. Histogram A shows a normal DNA peak for the reference specimen (*X. laevis*) on the left, and a normal target species (*R. pipiens*) peak on the right. Histogram B shows a normal reference peak on the left, and an aneuploid mosaic target species (*R. pipiens*) peak on the right. Histogram B was generated with a specimen collected from a non-grazed (reference) pond in southeastern Minnesota, 2000.

Table 1. Amphibian deformity, DNA, and parasite statistics for exposed and reference ponds in southeastern Minnesota, 2000.

					Deformity assessment^{c}		DNA analysis and presence of *Ribeiroia*					
Species	Collection date	Minnesota township	Pond^{a} type	Exp.^{b} group	Deform..	N	Mean C-value^{d}	Mean CV^{e}	SE	Aneuploid peaks^{f}	*Rib.* present^{g}	N^{h}
R. clamitans	6-15-2000	Altura	Graze	Exp	1	25	6.55	3.02	0.30	1	1	9
R. clamitans	6-28-2000	Brownsville	Graze	Exp	0	30	6.57	2.87	0.33	0	2	4
R. clamitans	5-25-2000	Utica	Agric	Exp	0	276	6.57	3.28	0.14	0	0	8
R. clamitans	6-15-2000	Utica	Ngraz	Ref	1	102	6.57	2.83	0.15	0	1	10
R. clamitans	5-25-2000	Lewiston	Natur	Ref	0	42	6.60	3.32	0.14	0	0	9
R. palustris	7-19-2000	Eitzen	Ngraz	Ref	0	37	6.74	1.91	0.07	0	0	5
R. pipiens	7-12-2000	Houston	Ngraz	Ref	0	36	7.08	2.88	0.28	2	0	7
R. pipiens	7-06-2000	Sheldon	Agric	Exp	0	107	6.53	2.83	0.37	0	0	3
R. pipiens	7-05-2000	Caledonia	Graze	Exp	0	91	6.91	6.31	0.23	3	0	3

^{a} Graze=grazed pond; Agric=agricultural pond; Ngraz=non-grazed pond; Natur=natural wetland.

^{b} Exp=exposed (i.e. grazed and agricultural) and Ref=reference (i.e. non-grazed and natural) for pond categories.

^{c} Number of frogs deformed and total examined in the field deformity assessment.

^{d} Average DNA weight (pg of DNA/ haploid nucleus).

[e] Average CV (SD of the target peak, divided by the mean target peak channel, multiplied by 100).

[f] Number specimens showing an aneuploid peak (Figure 1) with FCM analysis.

[g] Number of specimens with the parasite *Ribeiroia ondatrae.*

[h] Number of specimens used in FCM analysis and examined for parasites.

Effects of agricultural and urban land use on movement and habitat selection by northern leopard frogs (*Rana pipiens*)

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We studied post-breeding habitat use by northern leopard frogs (*Rana pipiens*) in ponds surrounded by 3 different types of land use in southeastern Minnesota. Pond types included a natural wetland in an agricultural setting, a constructed farm pond in an agricultural setting, and a large natural wetland complex adjacent to an urban industrial park. The urban site is federal land owned by the U. S. Fish and Wildlife Service Upper Mississippi River National Wildlife and Fish Refuge. We planned to fit 15 frogs at each site with radio transmitters during the breeding season and track them until they moved to over-wintering sites. Our objectives were 1) methods development using radio transmitters on northern leopard frogs and 2) quantify post-breeding habitat use by northern leopard frogs in southeastern Minnesota. We refined the radio-telemetry methods for our purposes and applied them to learn about post-breeding habitat use.

Our work is important because the specific risks to amphibians from agriculture and urbanization during the post-breeding period are poorly understood. Row crop agriculture is the dominant land use in much of the Midwestern U.S. Amphibian mortality associated with roads and other urban hazards has been previously documented, but little is known about hazards to amphibians in agricultural settings. Agriculture is suspected as a factor in possible recent declines in amphibian populations in many parts of the USA. Agricultural chemicals have been studied in the lab and in the field, but other agricultural hazards affecting frogs after they leave the breeding pond are poorly understood.

Results

During August – October 2000 we worked to refine radio transmitter attachment methods as applied to leopard frogs in our agricultural settings. Twenty-six frogs were captured and fitted with transmitters attached to external belts fastened around the frog's waist. Four belt materials were examined; nickel bead chain, aluminum bead chain, plastic cable ties and sewn elastic belts. These belt materials did not meet our needs due to the frog's ability to slip out of the harness and the formation of sores around the belt.

We investigated internal transmitter implantation in the laboratory from February – July 2001. Surgical techniques and the frog's tolerance to the surgery were studied. Nineteen frogs, obtained from a biological supply company, were used in the laboratory testing. We tested 2 implantation procedures: subcutaneous and peritoneal transmitter placement. We found internal transmitters to be the method of choice. Internal transmitters lodged in body cavity away from the suture site and did not create a lump on the frog's belly. Subcutaneous transmitters pressed against the sutures, internally rubbed against the skin and created a lump on the frog's belly and did not prove to be any less stressful to the frogs. Two weeks after the surgery the skin at the suture sites had fused. Many of the frogs had started feeding within days of the surgery and had all had increased in weight one week after surgery. Some sores were observed approximately one month after the surgery, but did not affect the frog's ability to feed or move. Two frogs passed the transmitter through their digestive system (gastrointestinal capture). We determined that internal implantation allowed frogs to carry the transmitter for a longer period of time and did not adversely affect the majority of the frogs.

Based on successful laboratory experiments, we used transmitters implanted into the peritoneal cavity to study post-breeding habitat use during 2001. Captured frogs weighing more than 37g received a Holohil BD-2GHX transmitter weighing 1.85g. Transmitters did not exceed 5% of the frog's total weight. Forty-four field surgeries were conducted at the three study sites from May – July 2001. Ten frogs did not recover from the surgeries. Initial problems with the temperature of the MS-222 solution accounted for 8 of the deaths. Only 2 frogs out of 36 surgeries died due to surgical complications after correcting the temperature of the MS-222. Implanted frogs recovered quickly from the surgeries and were released several hours after the procedure. Study frogs at the natural site had an average weight of 47.24g and increased their weight by 15% over the course of the summer. Study frogs at the agricultural site averaged 56.07g and increased their weight by 25%. Study frogs at the urban site weighed an average 41.46g and increased their weight by 30%. Only three frogs developed small sores at the suture site. Mean tracking time for frogs in this study was 52 days; 5 frogs were still located as of 10 November 2001.

Movement distances and rates varied between the sites. The greatest distance traveled from the release site was 1006m at the natural site. Frogs at the agricultural site had the largest mean distance traveled while urban frogs recorded the smallest mean distance. Agricultural frogs also had the highest mean daily speed and total distance traveled. Four frogs crossed roads while migrating from the breeding pond. In three instances frogs migrated to new ponds for over-wintering. Movement paths utilized by these three frogs were direct and did not follow any apparent physical corridors. Figure 1 plots the movement of two frogs at the agricultural site exhibiting a direct movement behavior. Rain gauge data supports the general consensus of previous studies that movement is triggered by rainfall events. We suspect that contact with 10 frogs was lost due to increased movement distances after rain events.

Habitat selection varied between the ponds. At the natural site 39% of the observations were in natural grass followed by aquatic emergents (29%) and wet meadow (15%). Agricultural crops used by the frogs included alfalfa (11%) and soybeans (1%). A mower struck 4 of 8 frogs using alfalfa during routine farming practices. Figure 2 shows two frogs using the alfalfa field. Habitats used at the agricultural site were natural grass (35%), clover (21%) and trees/shrubs (24%). Crops utilized included alfalfa (8%), corn (4%), soybeans (2%) and oats (1%). Aquatic emergents (57%) were the most observed habitat at the urban site followed by natural grass (16%) and roadside grass (15%). Three frogs utilized the natural grass located across a road within an urban setting. Figure 3 shows the movement of frog 6232 into the urban setting.

Over the last 2 years we successfully tested various attachment methods for conducting radiotelemetry studies on *Rana pipiens* in our habitats. We have tested and implemented surgical implantation as the best method for tracking frogs in this study. We have information from one field season on post-breeding habitat use in agricultural, urban, and natural wetland settings. Preliminary data indicates that agricultural settings do present hazards from crop harvest (alfalfa) and frogs cross hazardous barriers such as roads, industrial parking lots, and tilled agricultural fields. We observed that different habitat types are used at different times of the year. We have a draft SOP on the surgical implantation techniques and an SOP on a simple method for holding adult frogs in a laboratory setting.

Plans for 2002

We will continue analyses of the 2001 data, calculating home range sizes and habitat use versus availability using ArcView vegetation maps of the study areas and available computer programs. We will conduct a second and final field season in 2002, with USGS funding, to increase our sample size of habitat use data. We plan a methods paper on our surgical procedures and a paper on home range size, habitat use, and evaluation of urban and agricultural hazards post-breeding for leopard frogs in the Midwestern USA.

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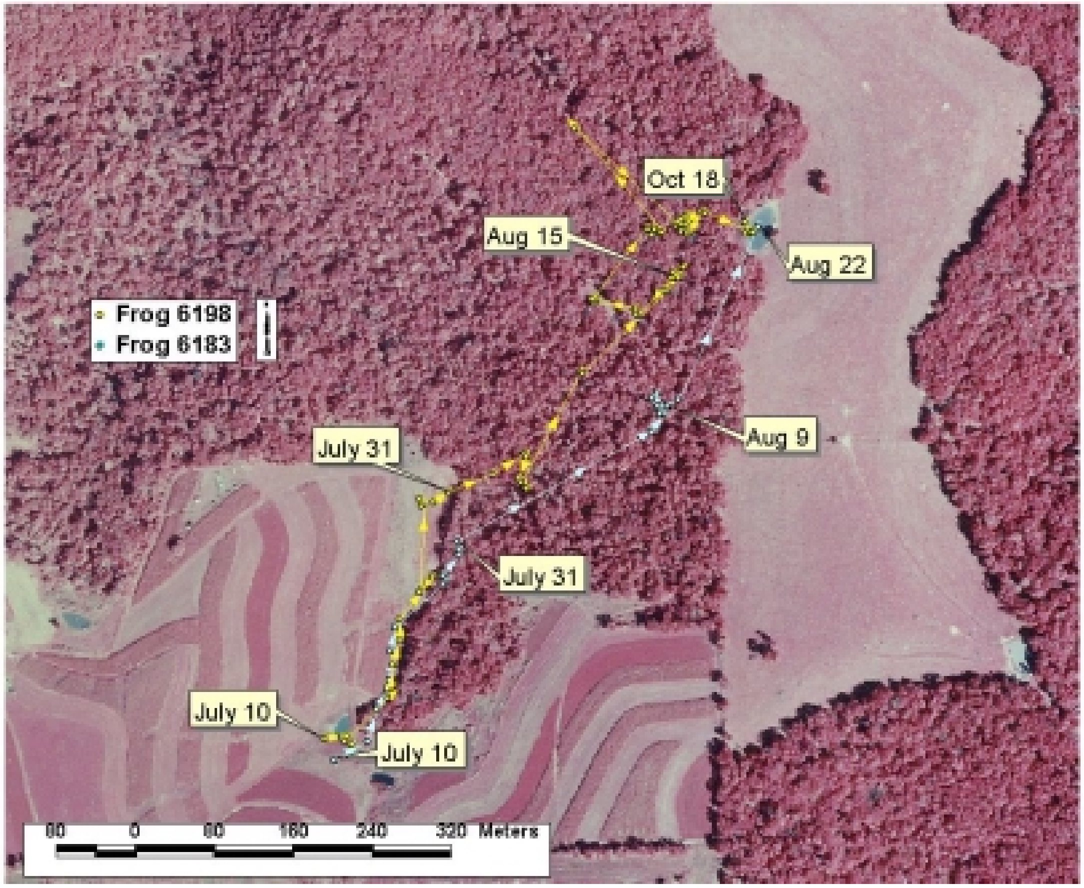

Figure 1. Movement patterns of *Rana pipiens* post-breeding in an agricultural landscape of Houston County, Minnesota, 2001. The movement is from one agricultural farm pond to another pond, requiring movement downslope, then upslope through forest to the second pond. Indications are that frog 6198 will be overwintering at the second pond.

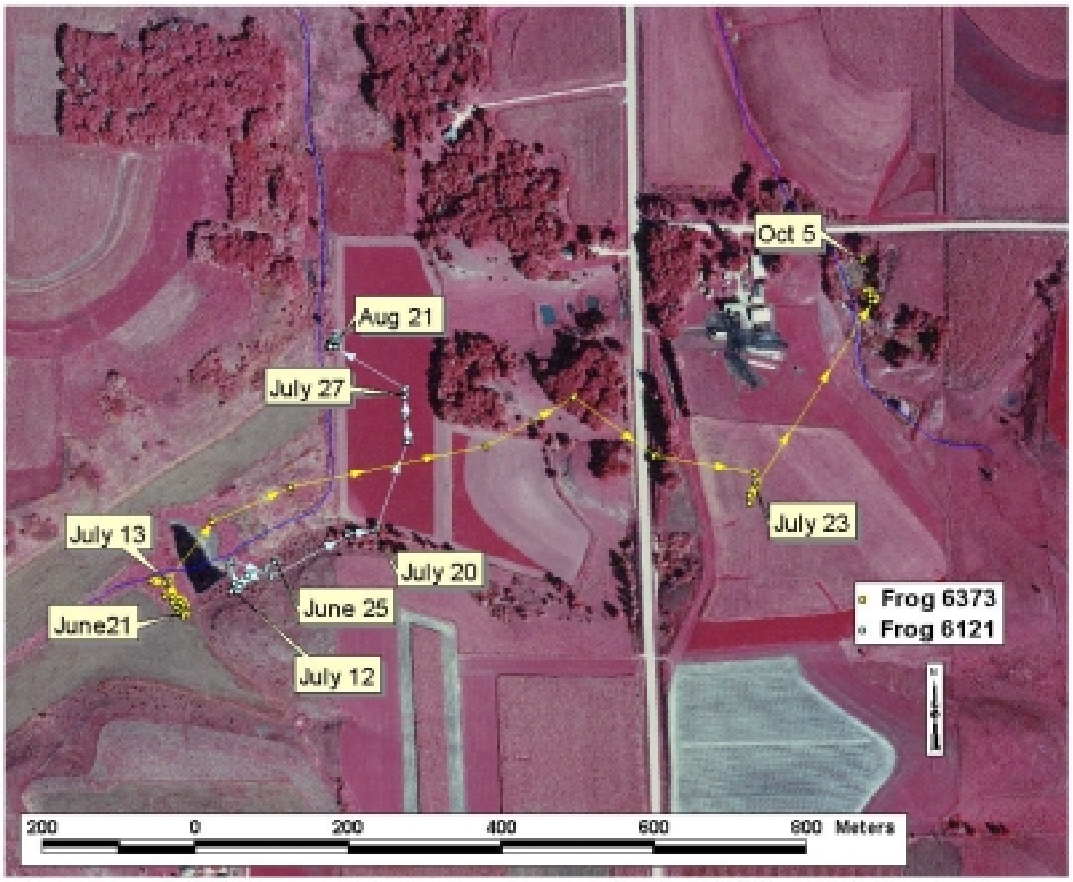

Figure 2. Movement patterns of *Rana pipiens* post-breeding in an agricultural landscape of Winona County, Minnesota, 2001. The movement is from a diked natural wetland (lower left of photo) and through various types of cropland (harvest of alfalfa proved fatal for 4 frogs). Frog 6373 crosses a 2-lane pavement road and ends in a small patch of trees near a stream.

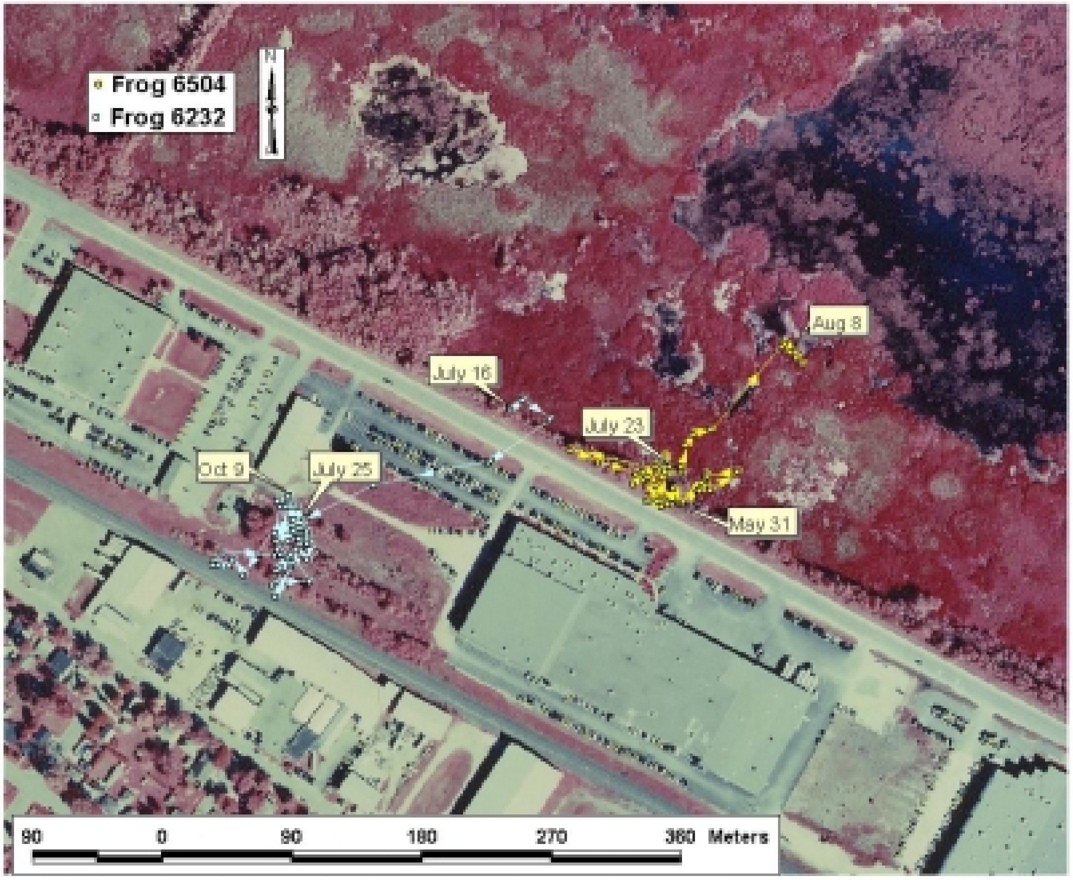

Figure 3. Movement patterns of *Rana pipiens* post-breeding in an urban landscape of Winona County, Minnesota, 2001. Movements of frog 6504 are all within backwaters of the Mississippi River, federal land owned by the U. S. Fish and Wildlife Service Upper Mississippi River National Wildlife and Fish Refuge. Frog 6232 moved out of the Refuge (frog heaven) and into scrub shrub urban land, crossing urban industrial parking lots.

The Role of *Ribeiroia ondatrae* (Platyhelminthes: Trematoda) Metacercariae in the Development of Malformed Frogs in Minnesota and Wisconsin

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The metacercariae of *Ribeiroia ondatrae* have now been shown to be capable of causing the development of abnormal limbs in amphibians. Severe limb abnormalities were induced at high frequencies in Pacific treefrogs (*Hyla regilla*) exposed to cercariae of *R. ondatrae* (Johnson et al 1999). The abnormalities closely matched those observed at field sites, and an increase in parasite intensity caused an increase in abnormality frequency and a decline in tadpole survivorship. The types of malformations observed in experimental tree frogs included ectromely (missing limbs), ectrodactyly (missing digits), cutaneous fusion, taumely (bony triangle), polydactyly (extra digits), polymely (extra limbs) and femoral projections. Less abundant malformations included brachymely (shortened limbs), permanent extension of limbs, brachydactyly (shorted digits) and syndactyly (fusion of digits).

Similarly, laboratory *R. ondatrae* infections induced high frequencies (40-80%) of severe limb malformations in surviving western toads (*Bufo boreas*)(Johnson et al. 2001a). Survivorship declined significantly with increasing parasite exposure, falling to 42% in the heaviest treatment. In contrast to previous experiments with *H. regilla*, cutaneous fusion was the predominant malformation among infected toads in all treatments. Infection also caused polymely (fore and hind), ectromely, polydactyly and various other limb deformities. Taken together these results demonstrated that (i) teratogenic effects of *R. ondatrae* are not limited to tree frogs, (ii) the spectrum of *R. ondatrae*-induced malformations is not confined to hind limbs and (iii) frequency and composition of malformations resulting from infections may vary among amphibian species.

Laboratory studies conducted on northern leopard frogs (*Rana pipiens*) by Rebecca Cole (personal communication, National Wildlife Health Center, Madison, WI, U.S.G.S.) have also supported the hypothesis that *Ribeiroia* can induce developmental abnormalities and decrease survivorship of infected tadpoles and metamorphs.

Field studies also implicate *Ribeiroia* as a cause of amphibian malformations. Over a two-year period, Johnson et al (2001b) monitored the frequency and severity of abnormalities in *H. regilla*, *B. boreas*, *Rana catesbeiana* (American bullfrog) and *Taricha torosa* (California newt) from two northern California ponds. Both ponds were highly eutrophic spring-fed stock ponds constructed in the 1960s. The frequency of abnormalities differed by species, life-history stage, pond and season. The frequency and severity of abnormalities were greater in amphibians from Frog Pond over those from Hidden Pond, and in larval stages over emerging and adult amphibians. Larvae of *T. torosa* exhibited the highest rate of abnormalities, ranging from 15-50%, followed by larval and metamorphic *H. regilla* (10-25%), and finally by metamorphs of *B. boreas* and *R. catesbeiana*, both of which had rates of less than five percent. Within each species, composition of abnormalities was strongly consistent between years, ponds and early life-history stages. The most severe malformations were observed in *H. regilla*, and more than 60% of the abnormalites in treefrogs involved extra hindlimbs, femoral projections and skin webbings. Similarly severe, malformations of *R. catesbeiana* were dominated by extra and missing hind- and forelimbs. In *B. boreas* and *T. torosa*, the most common deformities were missing limbs and digits, which accounted for approximately 75% and 95%, respectively, of the total abnormalities.

During the summer of 1998, Johnson and Lunde collected frogs, toads and salamanders from 103 ponds in six northwestern states (CA, OR, WA, ID, MT, CO). At 42 ponds, deformities were found in six amphibian species at rates ranging from 5% to 90%, and *Ribeiroia* was found at 40 of the 42 ponds and was almost never found at ponds with low deformity rates (Johnson et al 2002).

Both laboratory and field studies have therefore supported the hypothesis of Sessions and Ruth (1990) that amphibian malformations might be caused by trematode metacercariae. Not all investigators, however, have been able to correlate the presence of *Ribeiroia* at amphibian malformation "hotspots." For example, Ouellet et al. (1997) observed a high prevalence of deformities in wild-caught *Rana clamitans* (green frogs), *R. pipiens, Bufo americanus* (American toads) and *R. catesbeiana* from agricultural sites exposed to pesticide runoff in the St. Lawrence River Valley of Quebec, Canada in 1992 and 1993. According to Ouellet, parasites "were not encountered in relation to limb structures." In 1999, David Gardner, University of California-Irvine (personal communication) identified a pond in the foothills of the San Bernadino Mountains near Irvine where *H. regilla* have high rates of missing limbs and eyes. Sutherland (unpublished data) found no *Ribeiroia* in 25 malfomed frogs from the Irvine site. Similarly, we know of a farm pond in Trempealeau Co., WI where high rates of ectromely occurred in metamorphs of *R. clamitans* during 1997, 1998 and 2000. Again no *Ribeiroia* and no other trematode metacercariae were found at the Trempealeau site (Sutherland and Kapfer, unpublished data).

Recently, the status of malformed frog investigations in Minnesota was updated by Rosenberry (2001). Suspected causes for malformed frogs in Minnesota included parasites, chemicals (including pesticides and endocrine disruptors) and ultraviolet light. It was concluded that one or more combinations of chemicals, biological and physical factors are responsible for causing malformations in Minnesota frogs.

In an effort to determine if parasites might be playing a role in the development of deformed frogs, we began investigating the frog-parasite communities of various water bodies in Minnesota and Wisconsin during summer, 1997. This report summarizes the findings of those investigations conducted during 1997-2001. During 1997 and 1998, 80 and 65 frogs, respectively, were collected from west central Wisconsin and necropsied for parasites. Collection sites along the Mississippi River that were thought to be relatively insulated from surrounding agricultural and urban influences were emphasized during these first two years. In 1999, the majority (42/68) of frogs examined for parasites were provided by the Minnesota Pollution Control Agency (MPCA). Samples from these sites (CWB, TRD, NEY, ROI, DOR, ZAG, CBA) typically consisted of three normal and three abnormal individuals; all seven sites represented “hotspots” that were being monitored by MPCA. In 2000, we necropsied a total of 245 frogs. 149 of these frogs were provided by Dr. Melinda Knutson, Upper Midwest Environmental Sciences Center, USGS, as part of an ongoing study of 40 constructed farm ponds in southeastern Minnesota (Winona and Houston counties). Additionally, we examined 18 frogs from several eastern U.S. national wildlife refuges; these frogs were provided by biologists from the U.S. Fish and Wildlife Service. Of the 446 amphibians necropsied for parasites during the 2001 field season, 111 came from the USGS farm pond study, 260 from Minnesota frog deformity “hotspots,” 40 from northwestern Iowa and 35 from eastern U.S. national wildlife refuges.

Frog deformity “hotspots” seem to be rare in southeastern Minnesota and west central Wisconsin. Of the forty farm ponds monitored by USGS during 2000 and 2001 no malformation rates exceeding 5% were observed among six species of amphibians. Along the Mississippi River, malformation rates never exceeded 3% and the only site in Wisconsin examined by us that could be construed as a deformity hotspot was the Trempealeau County farm pond described above where high rates of deformities were observed in 1997, 1998, and 2000 for green frog metamorphs (no deformities were observed in 1999). This pond is surrounded by corn and soybean fields that are routinely spread with manure from a large poultry rearing facility.

Ribeiroia was not found in any frogs from Wisconsin during 1997 and 1998. At this time we were operating under the assumption that the metacercaria responsible for causing limb malformations in frogs was *Manodistomum* (see Sessions and Ruth 1990). Frogs from the Upper Mississippi River (UMR) Valley harbor a diverse fauna of metacercariae that may occur in astounding numbers (hundreds to thousands of trematodes per individual frog). *Alaria*, *Fibricola*, *Manodistomum*, *Euryhelmis*, *Clinostomum*, *Apharyngostrigea pipientis*, Meta A, Meta B, other globbies (including *Aurodistomum chelydrae*) and echinostomes are all frequent and often abundant parasites of frogs from the UMR. It is entirely possible that the presence of *Ribeiroia* was overlooked in frogs from the UMR during 1997 and 1998.

We first saw *Ribeiroia* in frogs from the Upper Midwest in 1999 (see discussion of mink frogs from CWB in Minnesota below). During 1999 we also recovered *Ribeiroia* from Wisconsin for the first time. Two of nine normal *R. pipiens* from Myrick Park marsh in La Crosse were found to harbor 10 and 3 *Ribeiroia*. In 2000, one of seven normal leopard frogs from Myrick Park marsh had 16 *Ribeiroia* and one of four normal leopard frogs and two of eleven normal green frogs from ponds at Walsh Golf Center near La Crosse harbored 19, 32 and 19 *Ribeirioa*, respectively. The *Ribeiroia*-positive frogs from Walsh Golf Center were adult frogs (snout to rump lengths between 7.4 and 7.65 cm). One of the green frogs had melanized most of the *Ribeiroia*, killing many of them and making it difficult to positively identify all of the individuals that were thought to be *Ribeiroia*. Adult leopard, green and bull frogs are frequently able to melanize metacercarial cysts of most trematode species to some degree. The contents of melanized metacercariae are occasionally nondescript and apparently in the process of decomposing; this makes metacercarial species identification difficult or impossible. More often, however, melanized metacercarial cysts contain live and active metacercariae. We have been able to successfully infect ducklings, chicks and pigeons with *Ribeiroia* metacercariae obtained from melanized cysts and to obtain gravid *R. ondatrae* as a result of these experimental infections. (The frog in question was a naturally infected California bull that had overwintered in Pieter Johnson's laboratory.) Apparently melanization does not immediately decrease the viability of all *Ribeiroia* metacercariae.

During 2000, *Ribeiroia* was found at 3 of 16 farm ponds examined (BRO-graz, ALT-graz and UTI-nongraz) in Houston and Winona counties. Prevalence and mean intensity of infection were 31% and 33.2 worms per infected host for BRO-graz, 46.7% and 2.0 worms for ALT-graz and 30% and 2.3 worms for UTI-nongraz. The only two deformed frogs observed at these three *Ribeiroia*-positive sites were a green frog from UTI-nongraz that had a cutaneous fusion on the limb opposite of where a single *Ribeiroia* metacercaria was found and a green frog from ALT-graz with a femur that was distinctly inflated at its distal end and contained two *Ribeiroia* metacercariae encysted near the anus. Two deformed frogs were found at *Ribeiroia*-negative sites.

During 2001, *Ribeiroia* was found at 6 of 13 farm pond sites examined (BRO-ag, HOU-ag, LEW-ag, SHE-ag, LEW-nat and BRO-graz). Again prevalence and mean intensities were relatively low, except for HOU-ag where all ten *R. pipiens* were infected with *Ribeiroia*; the mean intensity of infection at HOU-ag was 33.6 worms (range 19-68). The lone *Ribeiroia* infection at BRO-ag was 68 metacercariae in a normal green frog. The only frog necropsied from LEW-ag was a severely deformed leopard frog with 5 *Ribeiroia*. The remaining three *Ribeiroia*-positive sites had prevalences ranging between 10 and 40 percent and mean intensities ranging between 2.5 and 4.0 worms per infected host. Only seven of the 111 amphibians from farm ponds in Houston and Winona counties examined for parasites during 2001 were deformed and of those seven only three harbored *Ribeiroia*.

In addition to *Ribeiroia*, the farm ponds in Houston and Winona counties also harbored *Fibricola*, “globbies,” *Manodistomum* and echinostomes metacercariae in 2001 and *Fibricola*, “globbies,” an unknown neascus, echinostome and *Clinostomum* metacercariae in 2000.

During 2000, frogs, were received from national wildlife refuges located in the eastern United States. Seventeen out of the 18 frogs were considered to be malformed, and only one harbored *Ribeiroia* metacercariae. The *Ribeiroia*-positive specimen was an extra-legged leopard frog from Iroquois National Wildlife Refuge (NWR) in New York that had 37 *Ribeiroia*. Three southern leopard frogs (*R. autumnalis*) and three *H. versicolor* from Patuxent NWR, three green frogs from Walkill NWR, six northern leopard frogs from Missiquoi NWR, one bull frog from Sunkhaze Meadows, Maine and one bullfrog from Cattail ? in Alstead, New Hampshire lacked *Ribeiroia*. Other metacercariae found from the 18 frogs included *Fibricola* at Missisquoi, *Alaria* from Missisquoi and Sunkhaze Meadows, globbies from Missisquoi and Walkill, *Manodistomum* from Walkill, neascus from Walkill and Missisquoi, an unknown metacercaria ("Meta E") from Walkill, Missisquoi and Iroquois, a progenetic metacercaria ("Meta P") from Sunkhaze Meadows, echinostome metacercariae from Walkill, Iroquois and Missisquoi and an unknown (possibly gorgoderid) immature digene from the kidneys of one leopard frog from Missisquoi.

In 2001 frogs were received from only three eastern national wildlife refuges (11 green frogs from Iroquois NWR, 12 green frogs from Great Bay NWR in New Hampshire and 12 northern leopard frogs from Missisquoi NWR). Nine of the Iroquois frogs were infected with *Ribeiroia* (Mean intensity = 18.8) and two of these (with 5 and 9 *Ribeiroia*) had deformities in one of the hind feet. None of the Great Bay frogs harbored *Ribeiroia* and two of these frogs had deformities (one with bony triangle in the radioulna and expanded width of a single digit of the front limb and the second missing a digit on the left front limb). Two of the frogs from Missisquoi were malformed and one of these harbored a single *Ribeiroia*. In addition to *Ribeiroia*, frogs from Iroquois also harbored globbies, *Manodistomum*, Meta E and echinostome metacercariae and frogs from Missisquoi also harbored *Fibricola*, *Alaria*, globbies, *Manodistomum*, *Clinostomum* and echinostome metacercariae. Great Bay frogs were infected with *Fibricola*, *Alaria*, globbies, *Manodistomum*-like and echinostome metacercariae.

One particularly interesting structure that was found in frogs from the eastern United States (as well as from Minnesota) was a large thick walled cyst that contained amorphous material. These "masses" were located most frequently in the tail resorption site. Masses can range in size up to five times the diameter of a typical metacercarial cyst. Several of the smaller masses appeared to contain remains of dead metacercariae that were apparently in the process of being resorbed. These masses are not anything like the melanized structures seen in many frogs from many North American sites that definitely contain dead, decomposing metacercariae.

In addition to trematode metacercariae, eastern frogs harbored a diverse fauna of other larval and adult parasites. These included adult trematodes (*Haematoloechus* from the lungs, *Megalodiscus* from the rectum, gorgoderids from the urinary bladder), immature intestinal digenes, encysted larval tapeworms, nematodes (adult *Rhabdias* in the lungs and juvenile *Rhabdias* in the coelom, gravid *Ozwaldocruzia* in the intestine, gravid pinworms in rectum and distal end of the intestine, and encysted juvenile nematodes (perhaps spirurids) in the mesenteries. Protozoans included opalinids, *Nyctotherus*, *Entamoeba*, *Tritrichomonas*, a *Hexamita*-like flagellate and an unknown flagellate from the recturm and less frequently the intestine.

During 1999, we came into possession of four severely deformed mink frogs (*Rana septentrionalis*) from CWB in north central Minnesota (courtesy, D. Hoppe). CWB is a notorious and possibly unique frog malformation hotspot that is characterized by extremely high malformation and mortality rates for virtually all amphibian species reproducing in this habitat. Rather than being a pond or marsh, CWB is actually a lake. Amphibian populations have crashed at CWB over the past few years along with most fish and several free-living invertebrate populations. All four of our deformed mink frogs had various degrees of satellite hind limb formation ranging from severe to minor; one frog also had a mandible for which the medial skeletal elements had not formed and which resulted in the frog having a pronounced overbite. Satellite limb formation and incomplete mandible formation were typical of the malformations seen at CWB during 1999 (Personal Communication, David Hoppe, University of Minnesota-Morris). Satellite limbs originate in the region of the pelvis, but do not articulate with the pelvis; rather these limbs arise superficially (i.e., just under the skin) and occur in the presence of a normal right and left hind limb). Our four mink frogs were found to harbor an average of 110 *Ribeiroia* (range 96-125). The levels of *Ribeiroia* metacercariae were among the highest known from naturally infected frogs (Personal Communication, Pieter Johnson, University of Wisconsin-Madison). The metacercariae occurred primarily in the tail resorption site and in subdermal tissues surrounding the anus. The *Ribeiroia* were so numerous that they literally fell away from the frog in packets of 25-50 when the skin was removed. In addition, each mink frog harbored high numbers (average of 11.25) of metacercariae encysted along the margin of the mandible. At CWB, the only two locations that *Ribeiroia* were found were near the anus, the tail resorption site and along the margin of the mandible.

During 1999, *Ribeiroia* metacercariae were also found at the NEY site in south central Minnesota. NEY is a constructed farm pond located near Henderson, MN just outside of the Minnesota River Valley where in 1995 middle school students identified high levels of malformed frogs for the first time from Minnesota. When we visited the NEY site in mid-August, malformation rates were low. Two of the three malformed *R. pipiens* we were allowed to examine from NEY were infected with *Ribeiroia* (1 and 2 metacercariae) and one of three normal leopard frogs was infected with *Ribeiroia*. *Ribeiroia* was not found in six frog samples (3 normal, 3 malformed) from ROI, DOR, ZAG or CBA. All five sites were being monitored by MPCA because they were known to be malformation hotspots in previous years.

In August, 1999, David Hoppe, identified a frog malformation hot spot (designated as TRD) just east of Morris, MN that had malformation rates of metamorphosing *R. pipiens* exceeding 60% (Dave Hoppe, University of Minnesota-Morris). None of six severely malformed and three normal leopard frogs that we examined harbored *Ribeiroia*. In 2000 we did not necropsy frogs from TRD, but Hoppe again recorded high malformation (based on biweekly surveys). In 2001, Hoppe found that malformation rates had declined significantly at TRD. We visited TRD in early August, 2001 and were able to collect only one malformed leopard frog (limbs seemed to not flex at foot joints) out of 14 total frogs collected. None of the fourteen frogs harbored *Ribeiroia*. *Manodistomum* and *Fibricola* were common parasites at TRD in both 1999 and 2001.

In 2000, a new malformation hotspot (designated as HIB) was discovered near Hibbing in northeastern Minnesota. This small oval pond was constructed in 1996 by pushing soil toward the middle of the depression to create a donut shaped pond with a central island. Malformation rates at HIB approached 96% in 2000. Twelve R. pipiens sent to us from HIB were all infected with *Ribeiroia* (mean intensity of 155.5 worms; range 51-266). Ten of the twelve frogs were identified as being abnormal. Malformations included severe cutaneous fusions, satellite limb formation, brachydactyly, polydactyly and unusual soft tissue growths--some of which project externally and some internally from the skin. Many of these growths have encysted *Ribeiroia* metacercariae located at their proximal ends. As with the CWB malformation hotspot, *Ribeiroia* are found primarily in the tail resorption site (76.7% of all worms located in this site) or attached to the inside of the skin ("pants")(18% of all *Ribeiroia*). *Ribeiroia* were also found along the margin of the mandible (2.4%). Unlike, CWB, however, the locations of *Ribeiroia* metacercariae were more widely disseminated. *Ribeiroia* were found extending down to the knee and even out onto the foot in several of the more heavily infected frogs from HIB and five of the frogs had *Ribeiroia* metarcercariae (2.5% of total) located within the coelomic cavity near the urinary bladder.

By 2001 the malformation rates at many of the Minnesota hotspots being monitored by various resource agencies seemed to be on the decline. At HIB malformation rates had declined to low levels (Personal Communication, Perry Jones, USGS)). None of eleven leopard frogs and one of seven wood frogs collected by us at HIB in 2001 were deformed. Six of the *R. pipiens* and 100% of the wood frogs were infected with *Ribeiroia* (mean intensities equal 14.5 and 17.4, respectively). Even the single American toadlet taken at HIB was infected with *Ribeiroia* (3 metacercariae).

At CWB in 2001 the die off of vertebrate and invertebrate populations seemed to have tapered off. Malformation rates were still relatively high, and six of ten mink frogs collected from CWB by Dave Hoppe were deformed. 100% of these frogs were infected with *Ribeioria*, but the mean intensity was 35.4 worms as compared to 110 worms per infected host in 1999 at CWB. A normal green frog from CWB harbored 12 metacercariae of *Ribeiroia*. Likewise, four of eleven R. pipiens obtained from NEY in 2001 harbored *Ribeiroia* (mean intensity of 2.75).

In August, 2001 another new malformation hotspot (CTG) was identified by personnel of MPCA. This site is located in Cottage Grove just southeast of St. Paul, MN. A nine-year old boy found numerous malformed toadlets emerging from a storm water retention reservoir located in a city park. We found all malformed and normal toads necropsied to harbor heavy infections of *Ribeiroia* (18.8 and 13 worms per infected malformed and normal host, respectively).

The other Minnesota frog sites examined for parasites during 2001 were divided amongst sites which were designated as malformation hotspots by MPCA while others were considered “reference sites” where frog abnormalities were not present when MPCA surveyed the sites. In 2001 we found *Ribeiroia* to be present at some hotspots (CWB, NEY, ROI, HIB and CTG), but were unable to find *Ribeiroia* at other hotspots (TRD, GEL, CBA, HYD and ZAG). CWB, HIB, CTG and TRD are the hottest of the hotspots from which we necropsied frogs for parasites. High prevalence and mean intensities of *Ribeiroia* occurred at CWB, HIB and CTG, but *Ribeiroia* has never been found at TRD. Four of eleven leopard frogs from ROI harbored *Ribeiroia* (mean intensity 5.75 worms). *Ribeiroia* was found at two reference sites during 2001. 58% (7/12) of the leopard frogs from BUR were infected with *Ribeiroia* (mean intensity 5.29 worms). The malformation rate at BUR was 5.0% (4/86). A single mink frog (1/4) from the reference site MHL harbored 20 *Ribeiroia* metacercariae, while none of nine normal green frogs from MHL were infected with *Ribeiroia*.

Because we were unable to locate a designated reference site (JWPA), we chose to sample frogs from a nearby location. At this site (given name of FOL), 12% (6/50) of the R. pipiens collected were malformed. None of the twelve leopard frogs necropsied (including five malformed) had *Ribeiroia*.

In conclusion, high burdens of *Ribeiroia* have now been found at several frog malformation hotspots in Minnesota. CWB and HIB are recognized as sites where high levels of severe malformations have been reported previously. It was at CWB and HIB that we found the highest mean intensities (x > 100 worms) of *Ribeiroia* during the same years that these two sites had extremely high malformation rates. Likewise, toads from CTG harbored comparatively heavy infections of *Ribeiroia* (17.8 worms per infected host). Considering the extremely small size of B. americanus metamorphs in comparison to leopard, green and mink frog metamorphs, it is no wonder that they are severely impacted by relatively high levels of *Ribeiroia* metacercariae.

The finding of *Ribeiroia* at reference sites and at USGS farm pond sites during the past several years, all of which have not been documented to have high rates of malformations in metamorphosing amphibians, indicates, when combined with the presence of *Ribeiroia* at many deformity hotspots that *Ribeiroia* is more widely dispersed in Minnesota than was previously thought. In 1999, MPCA was stating that parasites could not be a cause of amphibian malformations in Minnesota because the causative parasite had never been reported from the state. In 2001, we now know that *Ribeiroia* is widely distributed in Minnesota. At some ponds, *Ribeiroia* occurs at low levels (both prevalence and mean intensity of infection) and therefore may not be present in sufficient numbers to infect tadpoles at the crucial moment when limb buds are most susceptible to development of malformation. Likewise, there may be insufficient *Ribeiroia* cercariae penetrating tadpoles and encysting at critical locations where deformities can be effected.

At the same time it is equally obvious that there are malformation hot spots in Minnesota where we have not been able to find *Ribeiroia*. While it may be possible that our sample sizes of frogs are too small to identify all rare parasites, we feel that the small size of many of the ponds examined and the resultant uniformity of parasites found in most of the frogs from a given site, reduce the chances that rare *Ribeiroia* infections are being missed during our surveys. We also do not believe that sites where *Ribeiroia* has not been recovered, are the result of the frog host immunologically eliminating all traces of the parasites prior to our examining the frogs for parasites (a favorite hypothesis of Stanley Sessions). Our experience is that while frogs do in fact react to parasites (melaninzation perhaps being a primary mechanism for elimination) and eventually kill, digest and absorb dead parasites that it is impossible that they eliminate all traces of the parasites within just a couple of weeks or less of metamorphosis. Most parasite metacercariae are still discernible as trematodes after they are dead and even those for which the contents of a cyst are not positively identifiable as trematodes there are usually some in intermediate stages of decompostion for which identification back to live metacercarial cysts can be extrapolated.

Therefore, we conclude that *Ribeiroia* is one cause of the development of malformed frogs in Minnesota, especially at hotspots such as CWB, HIB and CTG where extremely high burdens of *Ribeiroia* are found in the frogs. High malformation rates at other ponds (such as TRD) where *Ribeiroia* have not been found must have occurred as a result of other causes.

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Monitoring Amphibians in Farm Ponds: A Resource Manual

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Land managers are interested in monitoring amphibians in wetlands but often lack the resources to implement it. We are developing a monitoring manual as a guide for conducting amphibian calling, egg mass, and larval surveys in farm ponds in the Upper Midwest. The target user groups for the monitoring manual and key are biologists and conservation professionals. The purpose of the manual is to provide land managers with guidelines for performing amphibian monitoring in farm ponds. We include links to important information resources for identification, life history information, and reporting of amphibian morbidity and mortality. A field key to selected Midwestern amphibian eggs and larvae will also be included.

Managing Farm Ponds as Wildlife Habitat: A Technical Guide

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In some regions of the U.S. where agriculture is the dominant land use, farm ponds are a familiar component of the landscape. Appropriately managed, farm ponds can provide agricultural benefits such as erosion control and water for livestock, as well as high quality wildlife habitat. Farm ponds can be important breeding sites for amphibians because they are isolated from rivers and streams and are frequently lacking fish populations. Fish can depress amphibians populations by preying on tadpoles.

We are developing a technical guide for wildlife managers and conservation professionals to use as a reference on managing farm ponds as wildlife habitat. The scope of the guide is national, covering seven major regions of the United States. The guide is being developed in cooperation with the Wildlife Habitat Management Institute (WHMI) and Natural Resources Conservation Service (NRCS). We plan to post the guide on the Wildlife Habitat Management Institute's website (http://www.ms.nrcs.usda.gov/whmi/) for use by NRCS district conservationists and other staff in working with landowners to enhance conservation values on private land. The guide will contain general information about farm pond ecosystems, and the wildlife habitat values of farm ponds. There will be a section listing selected amphibians, plants, mammals, and other organisms using farm ponds in different ARMI regions. The guide will also address management issues of farm ponds. Some of the management practices addressed are the creation of grass buffers, alternative cattle watering systems, and habitat creation and protection methods. The technical guide will integrate information from research conducted at the Upper Midwest Environmental Sciences Center on farm ponds with information from previous research.