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Use of Artificial Enclosures to Determine the Survival of *Rana pipiens* Larvae in Upper Midwestern Agricultural Ponds

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Amphibians in the upper Midwest use agricultural ponds for breeding. Unfortunately, the risks (both direct and indirect) associated with using these ponds are poorly understood in both amphibian adults and larvae. In order to quantify these risks, we performed a comparison of larval survival rates between agricultural and natural ponds in southeastern Minnesota during the spring and summer of 2001. During this time, larval survival was observed in *Rana pipiens* tadpoles raised through metamorphosis in enclosures placed in agricultural and natural ponds. In addition, we measured the levels of nutrients commonly linked with agricultural ponds (i.e., ammonia, total phosphorous, and total nitrogen), and whether or not nutrient concentration was associated with larval survival. No differences were detected in nutrient levels or survival of larvae reared in agricultural and natural ponds. Furthermore, neither nutrient levels nor pond type significantly predicted larval survival. Our data were highly variable, making the interpretation of our results difficult. The enclosures used to rear tadpoles were highly effective and can be easily incorporated into future studies.

INDEX DESCRIPTORS: larval *Rana pipiens*, survival, agriculture, field study, enclosures.

Global declines in amphibian populations and observations of malformed anurans have increased concerns about the status of amphibians worldwide (Houlahan et al. 2000). Although no single cause for these phenomena has been identified, several factors, including natural (e.g., parasitic infections or disease) and anthropogenic (e.g., toxicants and habitat destruction) causes have been implicated (Carey and Bryant 1995, Rosenberry 2001, Blaustein and Kiesecker 2002, Blaustein and Johnson 2003, Johnson and Sutherland 2004). Amphibians possess a highly permeable epidermis and must regularly absorb water to avoid desiccation, making them particularly susceptible to toxic substances present in certain water bodies (Duellman and Trueb 1994, Pough et al. 1998). Therefore, exposure to environmental contaminants during critical periods of growth, such as larval development and metamorphosis, may greatly influence their survival.

Agricultural practices generate toxicants, in the form of fertilizer or pesticide run-off, that may enter water bodies where amphibians breed, with negative results (Bishop et al. 1997, Fallon et al. 1997, Kroening and Andrews 1997, Stamer et al. 1998, Jofre and Karasov 1999, Marco et al. 1999, Rouse et al. 1999, Allran and Karasov 2000, Allran and Karasov 2001, Wijer et al. 2003). Yet, regional differences in the level of effect that agriculture has on amphibians have been reported (Knutson et al. 1999). Such dissimilarity, perhaps attributable to variation in land-use practices, can result in amphibians making use of agricultural water bodies for breeding, despite the potential risks (e.g., Knutson et al. 1999, Knutson et al. 2004). However, only a small amount of field-oriented research has been conducted on the survival of amphibian larvae through metamorphosis in agricultural ponds.

Field studies that use enclosures to house test subjects *in situ* have greater ecological relevance than laboratory studies alone (Odum 1984, Rowe and Dunson 1994, but see Carpenter 1996). However, as enclosure size increases, the complexity of the system increases and replication within a single pond or wetland becomes harder to achieve (Crossland and La Point 1992, Caquet et al. 1996). Yet, there are many potential benefits associated with using enclosures during amphibian field studies. For example, they allow many of the ecological processes (e.g., gas exchange and nutrient flow) that operate in small water bodies to continue while still allowing researchers to create treatments and controls (Williams et al. 2002). Furthermore, amphibians that hibernate or aestivate in contaminated sediments may be susceptible to toxicant induced mortality (Rowe and Dunson 1994, Fort et al. 1999). Thus, in field studies that incorporate enclosures to rear developing anuran larvae, exposing larvae to sediments and allowing them to feed on periphyton in contact with sediments may better mimic natural conditions. Unfortunately, the enclosures used in most previous studies of this nature were small (< 1 m in diameter) and did not allow test subjects access to pond sediments, or were modified cattle tanks that created a completely artificial environment (Cooke 1981, Morin 1986, Rowe and Dunson 1994, Harris and Bogart 1997, Bishop et al. 2000, T. Edblom, pers. comm., N. Shappell, pers. comm.).

The objective of our research was to test the hypothesis that both anuran larval survival and water nutrient levels would differ between agricultural and natural ponds, and that nutrient levels and pond type would be significant predictors of larval survival. To this end, we assessed the survival of *Rana pipiens* tadpoles reared through metamorphosis in large field enclosures that

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allowed larvae access to the sediments, placed in natural (i.e., not man-made) and agricultural ponds. We predicted that survival of *R. pipiens* larvae would be lower and levels of nutrients (i.e., ammonia, total nitrogen, total phosphorus) higher in agricultural versus natural ponds.

METHODS

Study Area and Pond Selection

Our study was conducted in southeastern Minnesota (Houston and Winona Counties), part of the Driftless Area Ecoregion of the Upper Midwest, an area approximately 41,986 km^2 that was not glaciated during the Wisconsin glaciation period (McNab and Avers 1994, Albert 1995). The region has relatively large relief with bedrock composed mostly of limestone, sandstone and dolomite. Prior to European settlement, the vegetation of the area was dominated by oak (*Quercus* spp.) savanna of mixed grasslands and forests in areas protected from fire. Forests today are comprised of mixed oak and maple (*Acer* spp.) hardwoods interspersed with pastures, hay fields, small towns and cities. Complex topography and erosive soils in the region support less intensive agriculture than in other regions of the Midwest.

Natural wetlands and ponds in this region are scarce and found mainly in the floodplains of rivers and streams, while many remaining natural wetlands have been converted to suit agricultural purposes. There are, however, thousands of artificial agricultural ponds in the region constructed by farmers to prevent soil erosion and to provide water for livestock. These agricultural ponds represent a large portion of the available lentic aquatic habitat in this region, making them important breeding sites for amphibians (Knutson et al. 2004). Most farm ponds are privately owned; adjacent land uses include row crops, livestock (primarily cattle) grazing and forestry. Some ponds are surrounded by fallow grasslands enrolled in the U.S. Department of Agriculture's Conservation Reserve Program (CRP).

Test ponds were classified as natural (of natural origin) or agricultural (artificially created in agricultural landscapes), and were chosen from a sample of 40 randomly selected ponds used in a concurrent study (Knutson et al. 2002, Knutson et al. 2004). Because of the scarcity of natural ponds, we were unable to control for land uses associated with them, though surrounding lands were primarily fallow grassland and upland forests, with an occasional minor component of agriculture (i.e., alfalfa fields or row crops consisting of corn and soybeans).

Water Collection and Water Quality Analysis

Water samples were collected from test ponds every other week from April 15 to July 20 in 2001. This time period was representative of when anuran larvae (in species that do not over winter as tadpoles) were present in test ponds. Each composite sample was comprised of separate water samples collected from four equidistant locations along the pond perimeter that were mixed. Water samples were collected approximately 1 m from the shoreline at mid depth. All samples were refrigerated prior to analyses, which occurred within 30 d of sample collection at the Upper Midwest Environmental Sciences Center (UMESC) Water Quality Laboratory, La Crosse, Wisconsin. Unfiltered samples were examined for total nitrogen, total phosphorus, and ammonia following standard methods after digestion (persulfate method, APHA, 1998). These nutrient analyses were completed on a TrAAcs 800 Continuous Flow Analysis System (Bran and Luebbe, Delavan, Wisconsin). Quality assurance for analyses included blind testing, sample splits, spike recovery and routine evaluation of external standards. We also measured pond area and identified the dominant land cover within 2500 m of the study ponds via Geographical Information Systems, and averaged maximum pond depth over the duration of the study (Table 1).

Field Study

We attempted to assess the survival of *Rana pipiens* larvae in the field under semi-controlled conditions. *Rana pipiens* was chosen as the test organism because it occurs in many of the ponds in this region, but it is a species of conservation concern (Helgen et al. 1998). Therefore, larval *R. pipiens* were held though metamorphosis in field enclosures placed in six agricultural and four natural ponds (one enclosure per pond; Table 1). Logistics prevented us from including an equal number of test ponds in each category.

Enclosures were constructed of a welded aluminum frame (3.7 m long, 0.9 m wide, 1.2 m high) with sides made from two layers of screening: an outer rigid-plastic mesh with 1.3 cm openings and an inner layer of plastic window screen with 1 mm

Table 1. Characteristics of agricultural and natural ponds and source of tadpoles for field study in Houston and Winona Counties, Minnesota, 2001.

Pond Name/Type	Source of tadpoles in field study[a]	Dominant land use in 2500 m	Pond area (ha)	Mean max depth (m)	Survival Rate (%)
Alt/agricul.	StCh/natural	Corn	0.5	1.0	97
Uti/agricult.	StCh/natural	Corn/Soy	0.1	2.0	91
Lew/agricult.	In situ	Corn	0.3	0.5	95
She/agricult.	In situ	Corn/Soy	0.1	0.9	0
Bro/agricult.	Cal/natural	Corn	0.1	1.5	0
Eit/agricult.	In situ	Corn/Soy	0.2	0.6	100
Uti/natural	StCh/natural	Forest	0.3	0.8	78
StCh/natural	In situ	Grassland	1.6	1.4	80
Hou/natural	Cal/natural	Forest	0.3	0.2	32
Bro/natural	Cal/natural	Forest	0.4	0.5	90

[a]Tadpoles from nearby natural ponds within the study area were used to stock the enclosure because the study pond lacked *R. pipiens* egg masses

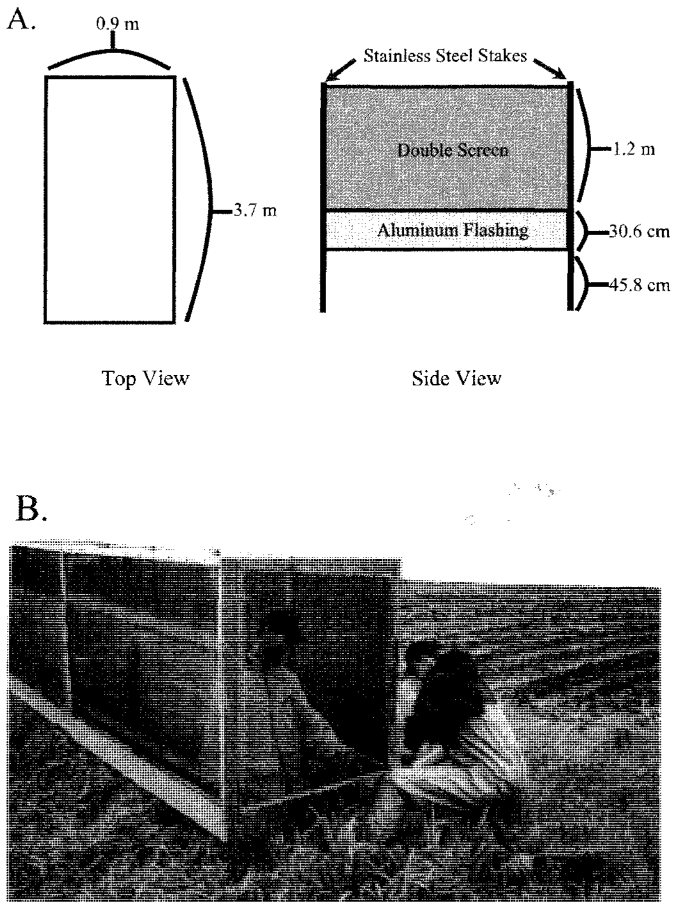

Fig. 1. Diagram of (A.) dimensions and (B.) construction of enclosures used to rear *Rana pipiens* tadpoles through metamorphosis in Houston and Winona Counties, Minnesota, 2001.

openings. This double screen covered the four sides of the enclosure, and was sufficient to keep out aquatic predators (vertebrate and invertebrate), but allowed water flow that supplied a natural source of algae as food for developing tadpoles. Screens were held in place with silicone sealant and aluminum strips secured to the frame with screws. Aluminum flashing, approximately 30.6 cm in width, was attached to the lower part of each enclosure side with screws, and sides were bolted together with stainless steel stakes that extended 45.8 cm below the bottom edge of enclosure sides (Fig. 1). Enclosures had no material lining the bottom, so that developing tadpoles were able to access sediments. Although consideration was given to incorporating several smaller enclosures per pond, rather than one large enclosure, it was determined that, because in this study each pond represented a single experimental unit, doing so would increase the risk of pseudoreplication (Hurlbert 1984).

Enclosures were pressed into the sediment so that at least 15 cm of the aluminum flashing along the bottom of the enclosure's sides was buried. This helped ensure that tadpoles had access to sediment but could not escape through gaps between the bottom edge of the enclosure and sediment. Each enclosure was placed perpendicular to the pond edge, with one end near the deepest point of the pond. This placement allowed tadpoles to adjust to the varying water depths we expected over the course of the experiment and helped prevent complete dessication inside the enclosure if dry weather prevailed. We avoided including large emergent macrophytes within enclosures as they may contain over wintering eggs of several odonate species that might prey upon young tadpoles. Where available, enclosures were placed near emergent vegetation, which offered some shade. The sides and top of each enclosure also provided some shade. The top of each enclosure was covered with a mist net (1.5 cm mesh) to exclude mammalian and avian predators. Netting was cut into 3.6 m long by 0.9 m wide segments that were stretched tight and held in place with plastic ties. After placement in the pond, dip nets were used to remove potential predators or other tadpoles from the enclosure. Two sheets of styrofoam (30.6 cm × 30.6 cm) were also placed into each enclosure by mid-June to act as a substrate for metamorphosing individuals to leave the water (Stages 39 to 41).

Each enclosure was stocked with 100 *Rana pipiens* tadpoles (Stage 22; Gosner 1960) on or before June 1, 2001. When initial plans to collect all larvae *in situ* (i.e., within the pond containing the enclosure) were hindered due to lack of available *R. pipiens* eggs at test ponds, enclosures were stocked from nearby natural ponds (Table 1). In four ponds (three agricultural and one natural), the desired number of tadpoles were collected *in situ* and released into the enclosure. The remaining six ponds (three natural and three agricultural) had to be stocked from nearby ponds.

We monitored the development of tadpoles twice weekly from the date of their release into enclosures through mid July (when most test subjects had metamorphosed). As newly metamorphosed frogs and tadpoles with four limbs (Stage 42 to 46) were observed, they were removed from enclosures and counted to determine survival (approximately 75–90 d). Survival was defined as the percentage of individuals surviving to metamorphosis in each enclosure.

Statistical Analysis

Multivariate analysis of variance (MANOVA) was used to determine if differences existed between levels of nutrients recorded in natural and agricultural ponds. MANOVA was chosen because it simultaneously considers the effects of independent variables (e.g., pond type) on several dependent variables (e.g., ammonia, total phosphorus, and total nitrogen; Zar 1984).

Differences in the survival of larval *Rana pipiens* between enclosures in agricultural and natural ponds were analyzed with an independent sample t-test (Zar 1984). An independent sample t-test was used because our data violated the equal variances assumption of t-tests (Levene's test, $F = 5.42$, $P = 0.48$) (Wielkiewicz 2000). We recognized post-hoc that we would have relatively low power to detect small differences in survival between treatments in the field study, but lacked the resources to increase the number of enclosures. A linear regression was also conducted to determine if ammonia, total nitrogen, total phosphorus levels, and pond type were significant predictors of *R. pipiens* survival (Zar 1984). All statistical analyses were conducted in SPSS v. 11.5 (Chicago, IL).

RESULTS

Water Quality

A total of 12 water samples from natural wetlands and 16 water quality samples from agricultural ponds were taken for analyses. Mean levels of total nitrogen detected ranged from 2.74 (SD = 2.53) mg/l in natural wetlands to 2.42 (SD = 1.24) mg/l in agricultural ponds. Mean levels of ammonia detected ranged from 0.05 (SD = 0.09) mg/l in natural ponds to 0.28 (SD = 0.77) in agricultural ponds. While mean levels of total phosphorus detected ranged from 0.22 (SD = 0.22) mg/l in natural ponds to 0.35 (SD = 0.26) mg/l in agricultural ponds. MANOVA analysis revealed no difference in nutrient levels between agricultural and natural ponds sampled (P = 0.53, Wilks' lambda).

Field Study

No difference in mean survival of *Rana pipiens* larvae was detected between agricultural (63.8%; SD = 49.5) and natural (70%; SD = 25.9) ponds ($t = -0.26$, df = 7.76, $P = 0.80$). The range of mean survival in agricultural ponds was 0–100%, while the range of mean survival in natural ponds was 32–90% (Table 1). Rapidly receding water levels in one agricultural pond required us to determine tadpole survival before some individuals had completed metamorphosis. We included these individuals in the data analyses because they were in late stages of development (Stage 41 to 44). No tadpoles survived in two of the six enclosures constructed in agricultural ponds. There was no evidence of mammalian or avian predation on tadpoles in enclosures.

Linear regression analysis determined that none of the independent variables were related to larvae survival ($F = 0.614$, df = 4, $P = 0.67$, $R^2 = 0.33$).

Therefore, our predictions were incorrect, and we reject the hypothesis that survival would differ in natural and agricultural ponds. However, our results are highly variable and should be viewed cautiously.

DISCUSSION

Based on work by Jofre and Karasov (1999), the levels of ammonia detected in our study were high enough to affect tadpole survival. Ammonia, however, did not seem to greatly affect the survival of our test subjects. The low intensity of agricultural practices in our region, compared with other parts of the Midwestern corn belt, may partially explain the comparably low levels of nutrients detected and why they did not seem to affect test subjects (Stamer et al. 1998, Hunst and Howse 2001). Details regarding water quality from a larger study of 40 ponds, including those described here, are available in Knutson et al. (2002), and Knutson et al. (2004).

In one of the two enclosures where 100% mortality occurred (Bro/Agricultural; Table 1), tadpoles were heavily parasitized by leeches (Hirudinea). The effect of leeches on anurans has been documented for wood frog (*Rana sylvatica*) tadpoles, which are much smaller than *R. pipiens* tadpoles (Berven and Boltz 2001). In the other enclosure in which 100% mortality was witnessed (She/Agricultural; Table 1), many free-living *R. pipiens* tadpoles in the same pond survived through metamorphosis although the larvae within the enclosure died; the cause of these enclosure mortalities was not determined.

In addition to mortality, there may be other biological endpoints (and synergistic interactions among them) relevant to amphibian conservation that were beyond the scope of this project (i.e., sexual development and sex ratios; Blaustein and Kiesecker 2002, Hayes et al. 2002, Tavera-Mendoza et al. 2002). Also, Knutson et al. (2002) reported that excessive use of ponds by cattle lead to a lower number of amphibians and larvae present in certain instances.

Despite the potential risks (toxicological and otherwise) for amphibians present in the agricultural ponds examined in this study, mean survival of larvae was high for both pond types. Yet, it is difficult to determine the biological significance of our results based on the level of variability witnessed in tadpole survival, in particular as it pertains to survival in agricultural ponds. There are a number of direct (e.g., water quality) and indirect effects (e.g., associated land use) that can influence the survival of *R. pipiens* tadpoles in water bodies associated with agriculture. Because we tested the survival of larvae against direct effects, it is possible that the range of survival observed is because indirect effects have greater influence in agricultural settings. Further research on this topic is needed, but for more information on characteristics that affect anuran survival in farm ponds see Knutson et al. (2002) and Knutson et al. (2004).

Despite variable results, the enclosure design used during this study was largely successful (i.e., a high rate of survival to metamorphosis was witnessed in larvae reared in many of the enclosures), and could be incorporated in future studies. Some studies have used similar techniques, but the enclosures incorporated during these past research projects were either: (1) used to create a completely artificial environment (i.e., cattle tanks), (2) small, or (3) did not allow test subjects access to sediments. The enclosures used in our study mitigate all of these problems, and are also particularly robust, meaning they can remain in place for multi-year research projects with only a modicum of maintenance. Implementation of such enclosures, however, must be given consideration. As we found, sample size can be limited by the cost of construction and resources available for assembly. The bulky, heavy nature of the enclosures used in this study makes them difficult to assemble on-site, particularly if researchers must walk large distances from vehicles to study locations. Furthermore, although we chose to place only one enclosure in each test pond, more could be added so long as this is not done *in lieu* of sampling a wide range of ponds and that any potentially confounding effects are considered.

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