HABITAT FRAGMENTATION AND WOODLAND AMPHIBIANS: CONSEQUENCES FOR DISTRIBUTION, GENETIC DIVERSITY AND FITNESS RESPONSES TO UV-B RADIATION

DISSERTATION

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By

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ABSTRACT

Recent declines in amphibian populations have heightened the importance of understanding amphibian/habitat relationships. Because of poor dispersal abilities and physiological constraints, amphibians may be especially susceptible to the effects of habitat fragmentation. In this dissertation, I investigate landscape-level influences of fragmentation on woodland amphibian species distributions, as well as population-level impacts on genetic diversity and related fitness consequences for wood frogs (*Rana sylvatica*).

For the first part of my research, I surveyed 25 woodlots and one area of continuous forest in Crawford County, Ohio for amphibians and evaluated 13 models concerning amphibian species richness and the presence of individual species in woodlots. I found 13 species of amphibians within the study plot, indicating that small woodlots within an agricultural matrix are important amphibian refuges. Hydroperiod was the most important habitat characteristic for predicting species richness. Landscape characteristics were relatively unimportant.

Next, I analyzed the genetic diversity of wood frog populations in relation to characteristics of their local habitat and landscape, to determine whether populations within woodlots have become genetically differentiated and/or have

lost genetic diversity. I found genetic distance to be correlated with geographical distance. Populations from breeding ponds with longer hydroperiods were more genetically diverse. I also assessed the genetic diversity of eight wood frog populations, and compared the genetic diversity of each population with the mortality and deformity rates of lab-reared eggs and larvae. Although there were weak negative correlations, my analyses failed to find a significant relationship between genetic diversity and deformity or mortality rates.

The final component of my research was an investigation of a synergism between UV-B radiation and genetic diversity, influencing mortality and deformity rates in wood frogs. I measured the genetic diversity of 12 populations, and exposed eggs/larvae from those populations to three different UV-B treatments. UV-B exposure significantly increased larval mortality and deformity rates. Populations with low genetic diversity suffered greater egg and larval mortality rates and deformity rates. Further, the interaction between UV-B treatment and genetic diversity significantly influenced larval mortality rates. This is the first study to document such an interaction between genetic diversity and resistance to an environmental stressor.

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CHAPTER 1

INTRODUCTION

Within the past two decades, researchers have noted a decline in amphibian populations around the world (Blaustein and Wake 1990; Phillips 1990). These declines, which have occurred in seemingly pristine habitats as well as disturbed areas, have increased the urgency of understanding amphibian/habitat relationships. This dissertation is an investigation of a collection of possible effects of anthropogenic habitat fragmentation on woodland amphibian populations, from landscape-level influences on species distribution, to population-level impacts on genetic diversity and concomitant fitness consequences.

Anthropogenic habitat fragmentation and loss are recognized as major contributors to the general decline in biodiversity worldwide, including that of amphibians (Fellers et al. 1993; Lannoo et al. 1994; Hecnar and M'Closkey 1996). Amphibians may be especially susceptible to the effects of habitat fragmentation. Compared to other vertebrates, many amphibians have relatively poor dispersal capabilities. Amphibian movement is limited by physiological constraints of thermal requirements and water retention. Within habitat patches,

species diversity may decline over time if extirpations are not offset by colonizations. The ability of a species to colonize a patch from which it has been extirpated will depend on several factors, including the vagility of the species, the distance between patches, and the character of the intervening matrix.

Populations that persist within patches may lose genetic diversity over time. Anthropogenic habitat fragmentation can result in decreased gene flow, or even genetic isolation, of once-connected populations. Over time, a lack of connectivity among populations can result in loss of genetic diversity and increased homozygosity (Templeton et al. 1990; Frankham 1996). When populations lose genetic diversity, they may be less capable of adapting to changing environments and may be left more susceptible to new stressors, diseases, and parasites, resulting in an increased probability of extirpation (Krebs and Loeschcke 1994; Vrijenhoek 1994). In addition, increased homozygosity can lead to decreased fitness as measured by growth, fecundity, or developmental stability (Mitton and Grant 1984; Mitton 1993; Vrjenhoek 1994; Reed and Frankham 2003).

An increasing level of ultraviolet-B radiation (UV-B; 280-320 nm wavelength) is one environmental stressor with which amphibians are faced, and which may be leading to global declines in amphibian populations. Increased exposure to UV-B has been widely implicated in amphibian declines, either as a single factor or in conjunction with other factors. Increasing levels of UV-B radiation are reaching the earth's surface due to ozone depletion (Stolarski et al. 1992; Kerr and McElroy 1993). The global nature of increasing levels of UV-B radiation has

made it an attractive hypothesis for world-wide amphibian declines, whether by itself or in combination with other factors. UV-B radiation damages DNA, creating mutations such as cyclobutane pyrimidine dimers (Licht and Grant 1997). Photolyase enzymes can repair the damage, but the level of enzyme activity varies from species to species, and among conspecific individuals (Blaustein 1994a; van de Mortel 1998; Belden and Blaustein 2002). Blaustein and colleagues' (1994a) study was the first to document that current levels of UV-B radiation could be directly increasing mortality in some amphibian species. Although not all studies have found a detrimental effect of natural levels of UV-B radiation on amphibians (e.g. Grant and Licht 1995; Blaustein et al. 1996), considerable evidence now exists that UV-B radiation may be an important factor in at least some declining amphibian populations (e.g. Blaustein et al. 1995; Hays et al. 1996; Anzalone et al. 1998; Lizana and Pedraza 1998).

For this dissertation, I studied an agricultural, fragmented landscape in north-central Ohio in order to answer the following questions: (1) Which habitat characteristics are important in determining amphibian species distributions in an agricultural fragmented landscape? (2) Have wood frog (*Rana sylatica*) populations in this patchy landscape diverged genetically, and which habitat characteristics are associated with greater genetic diversity? (3) Does genetic diversity of wood frog populations influence mortality and deformity rates of embryos and larvae? (4) Are wood frog populations with lower levels of genetic diversity more susceptible to the effects of UV-B radiation?

For the first component of this dissertation, I surveyed for amphibians 25 woodlots and one area of continuous riparian forest in southern Crawford County, Ohio. I used an information-theoretic approach to evaluate the effectiveness of 13 *a priori* models in predicting amphibian species richness and the presence of individual species in woodlots (Chapter 2). I identified 13 species of amphibians within the study plot, and every woodlot contained at least one amphibian species. The most important variable in predicting total amphibian and anuran species richness was hydroperiod. For caudates, woodlot edge-to-area ratio, hydroperiod, pH, and ammonia were important characteristics in predicting species richness. Landscape characteristics, such as distance between woodlots and aggregate length of roads and railways near woodlots, were relatively unimportant in predicting amphibian species richness. This study demonstrated that woodlots within agricultural landscapes are important refuges for a variety of amphibian species.

For the second component of my dissertation, I analyzed the genetic diversity of wood frog (*Rana sylvatica*) populations in relation to characteristics of their local habitat and landscape to determine whether populations within isolated woodlots have become genetically differentiated and/or have lost genetic diversity (Chapter 3). I found genetic distance to be correlated with geographical distance between sampling locations. A UPGMA cluster analysis showed that genetic distances were more different within the same population sampled in consecutive years than between geographically separate populations, providing some evidence for temporal segregation of wood frog populations. Using an

information-theoretic approach, I tested 15 *a priori* models to investigate which habitat variables might determine genetic diversity. A single habitat variable, pond hydroperiod, best explained differences in genetic diversity among woodlot populations. These data suggest that wood frog populations within relatively small, isolated woodlots can be as genetically diverse as those in large, continuously forested habitat as long as the hydroperiod of the breeding pond is sufficiently long.

For the third component of my dissertation, I hypothesized that the genetic diversity of a wood frog population would be inversely related to mortality and deformity rates of eggs and tadpoles from that population reared in the laboratory (Chapter 4). I assessed the genetic diversity of wood frog eggs collected from eight genetically distinct populations. I then compared the genetic diversity of each population with the mortality and deformity rates of lab-reared eggs and tadpoles. I observed an overall mortality rate of 21.7%, and an overall deformity rate of 7.5%. Most mortality occurred at the egg stage, and deformities consisted of axial malformations and edema. Although there were weak negative correlations, MANCOVA analysis failed to find a significant relationship of genetic diversity with either deformity or mortality rate of wood frog eggs and larvae.

The final component of my dissertation research was an investigation of a potential synergism between UV-B radiation and genetic diversity affecting mortality and deformity rates in wood frog embryos and larvae (Chapter 5). I measured the genetic diversity of 12 wood frog populations and exposed wood frog eggs/larvae from those populations to three different UV-B treatments:

direct sunlight, sunlight filtered by a UV-B-blocking filter (Mylar), and sunlight filtered by a UV-B-transmitting filter (acetate). I found a strong effect of UV-B treatment on larval survival and deformity rates, but not on embryonic mortality. I found significant effects of genetic diversity on egg mortality rates, larval survival, and deformity rates. Further, I found that the interaction between UV-B treatment and genetic diversity significantly increased larval mortality rates. This is the first study to document that loss of genetic diversity causes increased vulnerability to an environmental stressor. Differences in genetic diversity between populations may help explain patterns of amphibian decline.

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CHAPTER 2

PATCH AND LANDSCAPE CHARACTERISTICS ASSOCIATED WITH THE DISTRIBUTION OF WOODLAND AMPHIBIANS IN AN AGRICULTURAL FRAGMENTED LANDSCAPE: AN INFORMATION-THEORETIC APPROACH

Weyrauch, S.L. and T.C. Grubb, Jr. 2004. Patch and landscape characteristics associated with the distribution of woodland amphibians in an agricultural fragmented landscape: an information-theoretic approach. Biological Conservation 115:443-450.

ABSTRACT

In the midwestern United States, agricultural landscapes with scattered patches of fragmented forest are common. To investigate the relationship between amphibian distributions and wetland, woodlot, and landscape characteristics, we studied the pond-breeding amphibians within a 15,450-ha plot

in rural north-central Ohio. We surveyed 25 woodlots and one area of continuous riparian forest for amphibians, and each surveyed woodland contained at least one temporary wetland. We used Akaike's Information Criterion (AIC) to evaluate the effectiveness of 13 *a priori* models in predicting total amphibian species richness, anuran richness, caudate richness, and the presence of individual species in woodlots. We identified 13 species of amphibians within the study plot, and every woodlot contained at least one amphibian species. The most important variable in predicting total amphibian and anuran species richness was hydroperiod. For caudates, woodlot edge-to-area ratio, hydroperiod, pH, and ammonia were important characteristics in predicting species richness. Woodlots within agricultural landscapes are important refuges for amphibians.

INTRODUCTION

Concerns for global amphibian declines (Blaustein and Wake 1990; Phillips 1990) have increased the urgency of understanding amphibian-habitat relationships. Anthropogenic habitat fragmentation and loss are recognized as major contributors to the general decline in biodiversity worldwide, including that of amphibians (Fellers et al. 1993; Lannoo et al. 1994; Hecnar and M'Closkey 1996). Natural habitats are increasingly being fragmented by human activities, and the impacts of fragmentation on various species have been the focus of a number of recent studies (e.g., Laurance and Bierregaard 1997; Debinski and

Holt 2000). Patches of remaining habitat may lose species diversity over time if colonization from other patches is not sufficient to offset extirpations. The ability of a species to colonize a patch from which it has been extirpated will depend on several factors, including the vagility of the species, the distance between patches, and the character of the intervening matrix.

Recently, amphibian habitat fragmentation has been the subject of an increasing number of studies (e.g. Gibbs 1998a; Vos and Chardon 1998; Kolozsvary and Swihart 1999; Lehtinen et al. 1999; Vallan 2000). Compared to other vertebrates, many amphibians have relatively poor dispersal capabilities. Amphibian movement is limited by physiological constraints of thermal requirements and water retention. Studies of the effects of habitat fragmentation on amphibians have produced variable results depending on the species and habitats studied.

Some studies have found amphibian species richness to be positively associated with woodlot area (Hecnar and M'Closkey 1997; Knutson et al. 1999; Vallan 2000). Juvenile woodland amphibians such as wood frogs (*Rana sylvatica*) and spotted salamanders (*Ambystoma maculatum*) rarely disperse beyond woodlots into open areas such as clear-cuts and fields (deMaynadier and Hunter 1999). However, even woodland species may cross open areas such as residential lands to access breeding ponds (Gibbs 1998b). Some amphibians, particularly anurans, prefer meadow and prairie habitats to woodlands, and their occurrence may not be related to forest area. Forest edges may provide habitat for such species and some studies have found a positive correlation between

habitat heterogeneity (including high amounts of forest edge) and amphibian species richness (Burbrink et al. 1998; Knutson et al. 1999). Some researchers have found no association between amphibian species richness and wetland area (Hecnar and M'Closkey 1997; Knutson et al. 1999) or number of ponds (Knutson et al. 1999), but other studies have found significant effects of wetland surface area (Vos and Chardon 1998; Laan and Verboom 1999), depth (Laan and Verboom 1999), area of marsh vegetation (Vos and Chardon 1998), and hydroperiod (Kolozsvary and Swihart 1999; Snodgrass et al. 1999).

Some workers studying patterns of amphibian diversity have found significant correlations with such landscape connectivity variables as distance between wetlands (Lehtinen et al. 1999; Snodgrass et al. 1999), road density (Lehtinen et al. 1999), and proximity of woodlands (Laan and Verboom 1999). Roads may be a barrier to amphibian dispersal either through direct mortality (Fahrig et al. 1995; Ashley and Robinson 1996; Gibbs 1998a) or as a psychological barrier (Gibbs 1998a). The probability of a wetland being inhabited by amphibians is reduced when the wetland is located near roadways (Vos and Chardon 1998). Increased traffic intensity has a negative effect on the sizes of amphibian populations that exist near roads (Fahrig et al. 1995).

In the midwestern United States, large tracts of land were cleared for agriculture in the mid-1800s, leaving scattered forest remnants of various sizes as habitat patches. Many of these woodlots contain wetlands, the presence of which may have discouraged their clearing for agriculture or other uses. North-central Ohio represents such a landscape, and is the location of a 15,450-ha

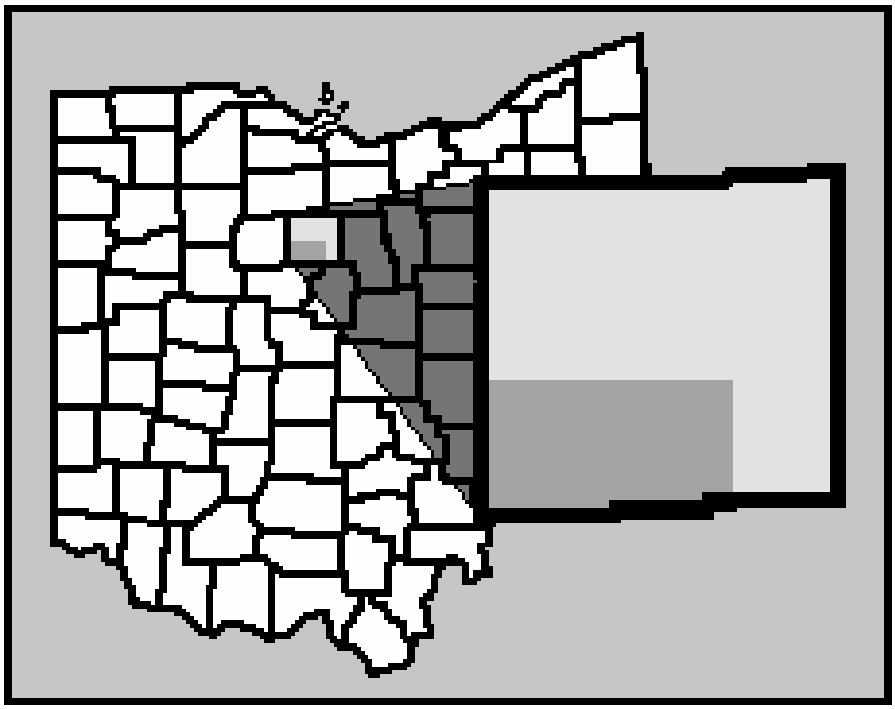
study plot that is currently the focus of fragmentation studies on several species (e.g. Doherty and Grubb 2000, 2002). Here, we report the results of a survey for amphibians in 25 woodlots and one area of continuous riparian forest within the study plot that contain wetlands. Specifically, we investigated whether pond, woodlot, and landscape characteristics can be used to predict amphibian species richness and individual species occurrences. The results of this study are important for understanding amphibian habitat associations in agricultural landscapes, where scattered, isolated woodlots provide the only remaining habitat for amphibians.

METHODS

Study Area and Field Methods -- The study site is a 9.6- X 16-km rural landscape in southern Crawford County, Ohio (Figure 2.1). The primary land use in the area is row-crop agriculture, with soybeans, corn, and wheat the predominant crops. Interspersed throughout the area are over one hundred woodlots of varying sizes. The topography is relatively flat, and two rivers (the Sandusky and the Olentangy) run through the western and eastern ends of the study plot, respectively. Numerous intermittent streams, drainage ditches, and roads also cross the area.

We selected 25 woodlots and one area of continuous riparian forest in the study area for amphibian surveys. The selected woodlots, all containing temporary wetlands, were surveyed at least three times per year for three

Figure 2.1. Location of the 9.6- X 16-km study area in Crawford County, Ohio. The study area is shown in dark gray. The southwestern corner of the study area is located at lat./long. 40°42’10”, 83°5’31”.



consecutive years (1998-2000), with survey periods distributed throughout the breeding seasons of local amphibian species (February through August, depending on local weather conditions). All sites were surveyed using four methods: visual encounter surveys, auditory surveys, egg mass identification, and larval identification. A typical survey period involved systematically walking through a pond, searching for and identifying egg masses, identifying adults found opportunistically, and identifying anuran calls heard during the survey. Transects walked through the ponds were either in the form of concentric ovals to the center of the pond, or in straight lines perpendicular to one shore of the pond. Transects through the ponds were separated from each other by 2-3 m, depending on the vegetation and turbidity of the water. When larvae were present, they were sampled via dipnetting. Identifiable larvae were recorded to species in the field and released immediately. Larvae not identifiable in the field were transported to the laboratory for further examination and/or for rearing through metamorphosis for identification. After identifications were made, animals were returned to their natal ponds. Survey periods were not time constrained; more time was spent at woodlots with larger or more wetlands. Surveys were conducted during the daytime, to facilitate location of egg masses.

From February to September 2000, the deepest wetland in each woodlot was visited at least once a month, until the pond dried, to monitor pond depth and hydroperiod. During the spring (April-June) of 2000, the perimeters of the wetlands in each woodlot were measured by walking the edge of each pond when it was judged to be full and converting paces to meters. Wetland habitat

was characterized by estimating the percent wetland area dominated by emergent aquatic vegetation (such as cattails and sedges), buttonbush (*Cephalanthus occidentalis*), or lacking emergent aquatic vegetation. Samples for water chemistry analysis were taken from each pond in May 2000. Samples from the center of each pond were collected in 125 mL polyethylene bottles, placed in a cooler and transported to the laboratory. Samples were refrigerated at 4°C until analysis could be completed, and all analyses were completed within 48 hours. Ammonia was analyzed using a Hach test kit, and pH was measured in the field using an Orion QuiKcheK Model 106 pH meter.

Woodlot and Landscape Characterization -- Woodlot and landscape characters were determined from aerial photographs and U.S.G.S. topographic maps (7.5 minute series). A woodlot was defined as a continuous stand of trees undivided by roads, rivers, or fields. The six woodlot and landscape parameters measured using digitized topographic maps and aerial photographs were: woodlot area (ha), woodlot edge-to-area ratio (m/ha), distance to nearest woodlot (m), percent woodlot edge bordering cropland, length of all roads and railroads within 1 km of woodlot (m), and distance to nearest watercourse (m).

Statistical Analyses -- We used an information-theoretic approach to investigate the relationships between habitat models and amphibian species richness (Burnham and Anderson 1998). The information-theoretic approach allows one to select a “best” model and to rank the remaining models. The “best” model is

based on the Kullback-Liebler distance, a measure of the information lost when a model is used to estimate full reality (Burnham and Anderson 1998). Burnham and Anderson suggest the information-theoretic approach should be used in observational studies, where other hypothesis testing methods may lead to “data dredging” and over-fitted models.

Only the 25 isolated woodlots were included in the Akaike Information Criterion (AIC) analysis. Data for the area of continuous riparian forest is presented for comparison. Using SAS release 8.1 software, we calculated correlation coefficients for all pairwise combinations of explanatory variables, and if pairs were highly correlated (>0.80), we eliminated the less significant variable in relationship to species richness. We constructed plots of residuals versus predicted values to test assumptions of constant variance, and no major violations were found.

Using the 12 habitat variables, we built 13 *a priori* models to test in predicting anuran, caudate, and total amphibian species richness. These models are (1) Global (variables include pH, ammonia, number of ponds in woodlot, hydroperiod of longest-lasting pond, perimeter of all wetlands in woodlot, % wetland as marsh, woodlot area, woodlot edge:area ratio, distance to nearest woodlot, % adjacent land as crops, length of roads & railways within 1km, and distance to nearest watercourse); (2) Wetland (variables include pH, ammonia, number of ponds in woodlot, hydroperiod of longest-lasting pond, perimeter of all wetlands in woodlot, and % wetland as marsh); (3) Water Chemistry (variables include pH and ammonia); (4) Wetland Size (the variable is wetland size); (5) Wetland

Diversity (variables include number of ponds in woodlot and % wetland as marsh); (6) Wetland Hydroperiod (the variable is wetland hydroperiod); (7) Woodlot (variables include woodlot size and woodlot edge:area ratio); (8) Woodlot Size (the variable is woodlot size); (9) Woodlot Edge (the variable is woodlot edge); (10) Patch (variables include pH, ammonia, number of ponds in woodlot, hydroperiod of longest-lasting pond, perimeter of all wetlands in woodlot, % wetland as marsh, woodlot size, and woodlot edge:area ratio); (11) Landscape (variables include distance to nearest woodlot, % adjacent land as crops, length of roads and railways within 1km, and distance to nearest watercourse); (12) Landscape Hostility (variables include % adjacent land as crops and length of roads and railways within 1km); and (13) Landscape Connectivity (variables include distance to nearest woodlot and distance to nearest watercourse).

Using AIC_C values derived from multiple linear regression, we calculated Akaike model weights (ω_m) to rank models and Akaike parameter weights (ω_p) to rank individual habitat variables for their usefulness as predictors of anuran, caudate, and total amphibian species richness. To evaluate *a priori* models for each species, we used stepwise logistic regression to obtain AIC_C values and calculated Akaike model and parameter weights as above. When species were either very common (present in $\geq$ 23 woodlots) or very rare (present in $\leq$ 3 woodlots), we did not compute Akaike values. This rule resulted in the elimination of smallmouth salamanders, cricket frogs, and Fowler's toads. In some cases, our models were overparameterized leading to an inability to

identify a unique maximum likelihood estimate. If the logistic regression procedure could not determine a maximum likelihood estimate for a given model, no Akaike value was calculated for that model and species.

We thought edge-to-area ratio might become a more important factor as woodlot size decreased. We tested this *post hoc* hypothesis by conducting a regression analysis to determine the effect of the interaction term between woodlot size and edge-to-area ratio on caudate richness. For this analysis, we converted edge-to-area ratio to area-to-edge ratio and multiplied it by woodlot size to obtain the interaction term. We then ran a linear regression of woodlot size, woodlot area-to-edge ratio, and the interaction term against caudate richness, and used an F-test to examine the significance of the interaction term.

RESULTS

Study woodlots varied in size from 0.73 to 31.10 ha, and the distance between woodlots ranged from 5 to 958 m. All woodlots contained at least one wetland and the maximum number of wetlands in a woodlot was seven. The pH of all ponds studied was circumneutral when measured during May 2000. Further descriptions of variable data are shown in Table 2.1.

Thirteen species of amphibians were identified in the Crawford County study site (Table 2.2). While every woodlot surveyed contained at least one amphibian species, the mean number of amphibian species per woodlot was 5.3 $\pm$ 2.8. Five

Table 2.1. Habitat and landscape variables for 25 woodlots, and their Akaike parameter weights (ω_p) for anuran, caudate, and total amphibian species richness, as well as individual species. Rankings of the three most important variables are given in parentheses. The sign of the relationship is also given in parentheses. The landscape variables "distance to nearest woodlot" and "distance to nearest watercourse" have been omitted due to lack of explanatory value.

Variable	Mean ± StDev	Range
pH	7.364 ± 0.291	7.000 – 7.900
Ammonia (mg/L NH_3)	1.554 ± 0.912	0.400 – 3.600
Number of ponds in woodlot	2.360 ± 1.680	1 – 7
Hydroperiod (months)	5.560 ± 1.917	2 – 8
Perimeter of all wetlands in woodlot (m)	232.1 ± 167.4	32.8 – 610.0
% wetland as marsh	22.60 ± 24.20	0 – 80
Woodlot area (ha)	9.88 ± 7.09	0.73 – 31.30
Woodlot edge (m):area (ha) ratio	185.2 ± 76.9	85.0 – 449.0
% adjacent land as crops	87.92 ± 24.04	0 – 100
Length of roads & railways within 1km (m)	3682 ± 1062	988 – 5650

continued

Table 2.1 continued

ω_p *Anuran*	ω_p *Caudate*	ω_p *Amphibian*	ω_p *A. jeffersonianum*	ω_p *A. tigrinum*
(+) 0.006	(+) 0.239 (3)	(+) 0.008	(+) 0.019	(+) 0.014
(-) 0.006	(-) 0.239 (3)	(-) 0.008	(-) 0.019	(+) 0.014
(+) 0.005	(+) 0.004	(+) 0.014	(+) 0.000	(-) 0.027
(+) 0.782 (1)	(+) 0.324 (2)	(+) 0.853 (1)	(+) 0.050	(+) 0.065 (3)
(+) 0.163 (2)	(+) 0.036	(+) 0.069 (2)	(+) 0.768 (1)	(-) 0.047
(+) 0.005	(-) 0.004	(+) 0.013	(-) 0.000	(+) 0.027
(+) 0.035 (3)	(+) 0.107	(+) 0.027	(+) 0.084 (3)	(+) 0.334 (2)
(-) 0.027	(-) 0.356 (1)	(-) 0.043 (3)	(-) 0.098 (2)	(-) 0.708 (1)
(-) 0.000	(-) 0.008	(-) 0.000	(-) 0.003	(+) 0.037
(-) 0.000	(-) 0.008	(-) 0.000	(-) 0.003	(-) 0.037

continued

Table 2.1 continued

ω_p *B. americanus*	ω_p *H. versicolor*	ω_p *P. crucifer*	ω_p *P. triseriata*	ω_p *R. c. melanota*
(+) 0.008	(+) 0.070	(+) 0.001	(+) 0.010	(+) 0.008
(-) 0.008	(-) 0.070	(-) 0.001	(-) 0.010	(-) 0.008
(+) 0.332 (1)	(+) 0.124	(+) 0.008	(+) 0.093 (2)	(+) 0.010
(+) 0.066	(+) 0.127 (3)	(+) 0.798 (1)	(+) 0.019	(+) 0.845 (1)
(+) 0.142	(+) 0.118	(+) 0.013	(+) 0.880 (1)	(+) 0.023
(+) 0.332 (1)	(+) 0.124	(+) 0.008	(+) 0.093 (2)	(+) 0.010
(+) 0.166	(+) 0.430 (1)	(+) 0.068 (2)	(+) 0.011	(+) 0.116 (3)
(-) 0.213 (3)	(-) 0.225 (2)	(-) 0.062	(-) 0.012	(-) 0.124 (2)
(-) 0.014	(-) 0.044	(+) 0.066 (3)	(-) 0.001	(-) 0.008
(-) 0.014	(-) 0.044	(-) 0.066 (3)	(-) 0.001	(+) 0.008

continued

Table 2.1 continued

ω_p *R. pipiens*	ω_p *R. sylvatica*
(+) 0.029	(+) 0.004
(+) 0.029	(-) 0.004
(+) 0.039 (3)	(+) 0.000
(+) 0.853 (1)	(+) 0.012
(+) 0.107 (2)	(+) 0.699 (1)
(+) 0.039 (3)	(+) 0.000
(-) 0.014	(+) 0.205 (2)
(-) 0.014	(-) 0.118 (3)
(-) 0.010	(+) 0.004
(+) 0.010	(-) 0.004

Table 2.2. Species found in 25 woodlots and one area of continuous riparian forest within the Crawford County study site, and their frequencies of occurrence (*n* = 26).

Species	Common Name	Number of Locations
Ambystoma texanum	Smallmouth salamander	24
Rana sylvatica	Wood frog	22
Ambystoma jeffersonianum	Jefferson-complex salamanders	18
Pseudacris triseriata	Western chorus frog	18
Hyla versicolor	Gray treefrog	17
Bufo americanus americanus	Eastern American toad	10
Pseudacris crucifer	Spring peeper	9
Rana pipiens	Northern leopard frog	7
Ambystoma tigrinum tigrinum	Eastern tiger salamander	5
Rana clamitans melanota	Green frog	5
Ambystoma maculatum	Spotted salamander	4
Acris crepitans blanchardi	Blanchard's cricket frog	1
Bufo fowleri	Fowler's toad	1

woodlots supported nine species, the maximum number recorded in a woodlot. The area of riparian continuous forest we surveyed measured 89.95 ha and supported eight species of amphibians. The most common species encountered were wood frogs (*Rana sylvatica*) and smallmouth salamanders *(Ambystoma texanum*). Two species, Blanchard's cricket frog (*Acris crepitans blanchardi*) and Fowler's toad (*Bufo fowleri*), were found at only one site each.

The Wetland Hydroperiod Model was the best model for predicting anuran, caudate, and total amphibian species richness (Table 2.3). To illustrate the strength of the relationship between hydroperiod and anuran, caudate, and total amphibian species richness, the regression lines are shown in Figure 2.2. In each case, the relationship between species richness and hydroperiod was highly significant (p=0.001 for anuran richness, p=0.011 for caudate richness, and p=0.000 for total amphibian species richness). Evidence that this model was the best model was much greater for anurans than for caudates. For caudates, the ΔAIC_c values of two other models (the Water Chemistry and Woodlot Edge models) fell within 2 of the best (Hydroperiod) model, making them equally likely to be the best model (Burnham and Anderson 1998).

Akaike parameter weights (Table 2.1) indicated that hydroperiod was the most influential habitat variable for anuran and total amphibian species richness; that is, hydroperiod had the highest probability of being included in the best model. For caudates, woodlot edge-to-area ratio was the most important habitat character, followed by hydroperiod. For all groups of amphibians, landscape variables were the least influential.

Table 2.3. Akaike model weights (ω_m) for anuran, caudate, and total amphibian species richness, as well as individual amphibian species found in the study woodlots. Boldfaced values indicate models included in the 95% confidence interval for the best model. * indicates cases where no maximum likelihood estimate, and therefore no Akaike values, could be calculated due to overparameterization of models. Models that did not fall within the 95% confidence interval for at least one group or species (Global, Patch, and Landscape models) have been omitted.

Model	ω_m *Anuran*	ω_m *Caudate*	ω_m *Amhibian*	ω_m *A. jeffersonianum*
Wetlands	0.003	0.006	0.001	*
Water Chemistry	0.002	**0.238**	0.006	0.019
Wetland Size	**0.160**	**0.034**	**0.067**	**0.768**
Wetland Diversity	0.002	0.003	0.012	*
Wetland Hydroperiod	**0.779**	**0.323**	**0.852**	**0.050**
Woodlot	0.009	**0.080**	0.010	0.022
Woodlot Size	**0.026**	0.028	0.017	**0.062**
Woodlot Edge	0.018	**0.278**	**0.033**	**0.076**
Landscape Hostility	0.000	0.007	0.000	0.001
Landscape Connectivity	0.001	0.007	0.000	0.003

continued

Table 2.3 continued

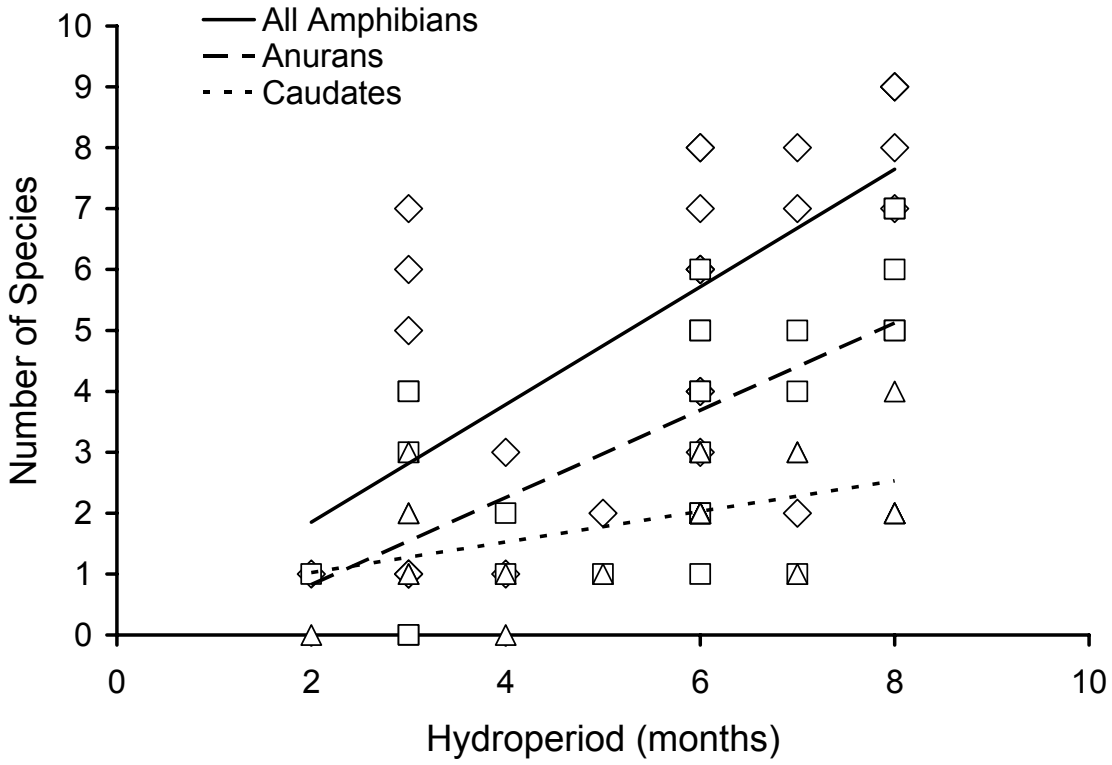
ω_m *A. tigrinum*	ω_m *B. americanus*	ω_m *H. versicolor*	ω_m *P. crucifer*	ω_m *P. triseriata*
0.000	0.001	0.001	0.000	0.008
0.014	0.007	**0.068**	0.001	0.002
0.046	**0.142**	**0.117**	0.013	**0.872**
0.027	**0.331**	**0.122**	0.007	**0.086**
0.065	**0.065**	**0.126**	**0.798**	0.012
0.243	**0.043**	**0.160**	0.016	0.003
0.091	**0.123**	**0.270**	**0.052**	0.008
0.465	**0.170**	**0.065**	**0.046**	0.009
0.012	**0.104**	0.026	0.001	0.000
0.034	0.009	**0.040**	**0.066**	0.001

continued

Table 2.3 continued

ω_m *R. c. melanota*	ω_m *R. pipiens*	ω_m *R. sylvatica*
*	**0.022**	*
0.008	0.007	0.004
0.023	**0.085**	**0.699**
0.010	**0.017**	*
0.845	**0.831**	0.012
0.075	0.004	**0.039**
0.040	0.010	**0.162**
0.048	0.011	**0.079**
0.006	0.004	0.001
0.008	0.010	0.004

Figure 2.2. Fitted line plot of total amphibian (diamonds), anuran (squares), and caudate (triangles) species richness versus wetland hydroperiod for 25 Crawford County woodlots. Total Species Richness = -0.086 + 0.96 Hydroperiod; *S* = 2.22, *R-Sq(adj)* = 39.6%. Anurans = -0.62 + 0.71 Hydroperiod; *S* = 1.68, *R-Sq(adj)* = 37.9%. Caudates = 0.54 + 0.26 Hydroperiod; *S* = 0.86, *R-Sq(adj)* = 21.9%.


All Amphibians
Anurans
Caudates
Number of Species
Hydroperiod (months)
0
1
2
3
4
5
6
7
8
9
10
0
2
4
6
8
10

Akaike model weights for each of the nine species evaluated are shown in Table 2.3. Again, landscape models were relatively unimportant. However, the Landscape Connectivity model fit within the 95% confidence interval for the best model for three species (*A. tigrinum*, *Hyla versicolor*, and *Pseudacris crucifer*). The Landscape Hostility model was contained in the 95% confidence interval for the American toad. For most species, one of the wetland-centered models was the best Kullback-Liebler model. The two exceptions were the tiger salamander (*A. tigrinum*) and gray treefrog (*Hyla versicolor*). For these species, woodlot models had higher Akaike weights. For tiger salamanders, the Woodlot and Woodlot Edge models were essentially equivalent, having ΔAIC_C values within two points of each other. For the gray treefrog, the Woodlot Size model was the best model, but Wetland Size, Wetland Diversity, Wetland Hydroperiod, and Woodlot Models had ΔAIC_C values less than 2.

Akaike parameter weights for the nine species evaluated again highlight the importance of wetland variables and the relative lack of importance of landscape variables in predicting the occurrence of individual species (Table 2.1). Hydroperiod or perimeter of all wetlands was the most important variable for nearly all anurans. Woodlot area was the most important variable for gray treefrogs. For American toads, the number of ponds in a woodlot and the percent of wetland characterized as marsh tied as the variables most important in predicting occurrence. For the two caudate species analyzed, variables characterizing the woodlot and landscape were more important in predicting occurrence than they were for anurans. Woodlot edge-to-area ratio was the

most important variable for tiger salamanders, and the second-most-important variable for Jefferson-complex salamanders (*A. jeffersonianum*).

DISCUSSION

In general, we found that within this agricultural landscape habitat variables characterizing wetlands were the most important variables in determining amphibian species richness. Woodlot and landscape variables were less and least important, respectively. The Wetland Hydroperiod Model was the best Kullback-Liebler model for our data on anuran, caudate, and total amphibian species richness. The longer the hydroperiod, the greater the number of species supported by a wetland. This relationship is likely a function of the distribution of local amphibian breeding seasons. Species encountered in this study breed from late winter (e.g., most *Ambystoma* spp. in late February-early April) to summer (e.g., *A. crepitans blanchardi* and *R. clamitans melanota*). It is important to note that no permanent wetlands were included in this survey, so fish depredation in permanent ponds was not a factor limiting species richness. It should also be noted that the hydroperiods we recorded from February to September 2000 are only representative of that particular year. The hydroperiods of wetlands within the study woodlots varied significantly from year

to year, but the data from 2000 serve as a useful relative measure of the hydrological characteristics of the breeding ponds in this study.

Anuran species that tend to spend much of the non-breeding period within and near wetlands (*R. pipiens* and *R. clamitans melanota*) were essentially limited to long-lasting ponds (Tables 2.1 and 2.3). For species known to exhibit greater dispersal capabilities (Duellman and Trueb 1994) and inhabit more open areas, woodlot and landscape variables were important (e.g., *A. tigrinum* and *B. americanus*). Wetlands characterized as marshes were particularly important for the American toad, gray treefrog, western chorus frog, green frog, and northern leopard frog (Table 2.1). At least three of these species (the American toad, green frog, and northern leopard frog) are generally associated with grasslands and open areas (Knutson et al. 2000). Grassland habitats are rare within the Crawford County landscape, but it is possible that the margins of marshes within woodlots, and the marshes themselves once they dry, can serve as "grassland" habitat.

Wetland Hydroperiod was clearly the best model for anurans, but several other competing models also ranked high for caudates (Table 2.3). This is likely due to their breeding seasons being more broadly distributed. For caudates, a greater number of factors had large Akaike weights, including water chemistry and woodlot characteristics. Water chemistry measurements for this study were only taken once (May 2000) and may not be representative of the water chemistry characteristics of these ponds at other times of the year. However, water chemistry sampling was timed to coincide with the larval periods of most

species in the study area, and serves as a relative measure of the water quality characteristics of the breeding ponds surveyed.

The most important single variable in determining the number of salamander species inhabiting a woodlot was its edge-to-area ratio. Salamanders generally have a larger surface-to-volume ratio than frogs and toads, making them more vulnerable to evaporative water loss. Woodlots with high edge-to-area ratios may experience greater penetration of wind and sunlight, leaving their substrates drier. This may explain the negative relationship between edge-to-area ratios and caudate presence.

One might hypothesize that the edge-to-area ratio would become important only for small woodlots. To investigate this hypothesis we conducted a *post hoc* analysis of the interaction term between woodlot edge-to-area ratio and woodlot size. This analysis failed to support the hypothesis ($p = 0.0865$). However, because a trend was evident even if non-significant, additional research into the relationship between edge-to-area ratios and ambystomatid salamander distributions would be useful in assessing habitat quality for these species.

Landscape variables in this study had a relatively weak influence on amphibian species richness. However, landscape characters tended to be more important for caudates than for anurans (Table 2.3). The relationships between landscape variables and amphibian distributions in general may be underestimated in this study due to the relative homogeneity of the landscape. If there were greater variation in landscape characters (i.e., if the study area

encompassed a range of habitats, from urban to rural), landscape variables may have had a greater effect.

Other researchers have noted the importance of maintaining wetlands with a diversity of hydroperiods to support amphibian populations over the long term (Lannoo 1998; Semlitsch 2000). During wet years, semi-permanent ponds can support predatory fishes, making them unsuitable for amphibian larvae. More ephemeral ponds are important breeding sites during these times. During drought years, however, ephemeral ponds with short hydroperiods may dry too quickly, making them unsuitable (Lannoo 1998). Semipermanent or permanent ponds may then favor amphibians over fishes. We found that wetlands with longer hydroperiods (up to 8 months) supported a greater number of amphibian species. If global warming increases the frequency of droughts, wetlands with relatively long hydroperiods will be increasingly important to amphibians. It may be beneficial to create additional wetlands of varying hydroperiods adjacent to existing wetlands, particularly where the existing amphibian populations are dependent solely on a short-hydroperiod pond. New wetlands are readily colonized by amphibian species when located adjacent to existing ponds (Weyrauch 2002). Supplementary breeding ponds could provide alternative breeding sites during varying climatic conditions.

The study woodlots supported a total of 13 species of pond-breeding amphibians. This represents approximately 70% of the pond-breeding amphibian species known to occur in the north-central region of Ohio, including 100% of the ambystomatid salamanders. Even in landscapes dominated by

industrialized agriculture, woodlots containing fishless wetlands are important refuges for Midwestern amphibians. Management for pond-breeding amphibians should include preserving woodlots with a variety of wetland types (from open-canopy marshes to closed-canopy pools), but especially those with long hydroperiods. Management for ambystomatid salamanders, in particular, should include maintaining large tracts of forest with low edge-to-area ratios.

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CHAPTER 3

GENETIC STRUCTURE OF A WOOD FROG METAPOPULATION IN AN AGRICULTURALLY FRAGMENTED LANDSCAPE

To be submitted to the Journal of Herpetology by S.L. Weyrauch and T.C. Grubb, Jr.

ABSTRACT

Anthropogenic habitat fragmentation can lead to a reduction in gene flow among once-connected populations. Over time, a lack of connectivity can result in reduced genetic diversity within populations and genetic divergence among populations. We studied wood frog (*Rana sylvatica*) populations of woodlots within an agriculturally fragmented landscape in north central Ohio to determine whether these populations have become genetically differentiated and/or have lost genetic diversity. We tested one embryo from each of 108 wood frog egg

masses from seven populations in 1999, 151 egg masses from eight populations in 2000, and 351 egg masses from 17 populations in 2004, using RAPD markers to assess genetic diversity and divergence. Nei's (1978) unbiased genetic distance was correlated with geographical distance between sampling locations. In our study area, a UPGMA cluster analysis showed that genetic distances were more different within the same population sampled in consecutive years than between geographically separate populations, providing some evidence for temporal segregation of wood frog populations. Using an information-theoretic approach, we investigated which habitat variables might be related to genetic diversity. Analysis of 15 models with Akaike's Information Criterion (AIC) found that one variable, pond hydroperiod, best explained differences in genetic diversity among woodlot populations. Our data suggest that wood frog populations within relatively small, isolated woodlots can be as genetically diverse as those in large, continuously forested habitat as long as the hydroperiod of the breeding pond is sufficiently long.

INTRODUCTION

Anthropogenic habitat fragmentation can result in decreased gene flow, or even genetic isolation, among once-connected populations. Over time, a lack of connectivity among populations can result in loss of genetic diversity and increased homozygosity (Templeton et al. 1990; Frankham 1996). When populations lose genetic diversity, they may be less capable of adapting to

changing environments and may be left more susceptible to new stressors, diseases, and parasites, resulting in an increased probability of extirpation (Krebs and Loeschcke 1994; Vrijenhoek 1994). In addition, increased homozygosity can lead to decreased fitness as measured by growth, fecundity, or developmental stability (Mitton and Grant 1984; Mitton 1993; Vrjenhoek 1994; Reed and Frankham 2003).

When populations are subdivided due to habitat fragmentation, several factors determine whether the resulting subpopulations experience a reduction in inter-subpopulation dispersal, and thus gene flow, and how quickly the effects of such a reduction are felt. These factors include characteristics specific to the species under consideration as well as characteristics of the particular landscape. Species-centered factors include the vagility and perceptive resolution of the species (With 1994; With and Crist 1995). Population-centered factors include fecundity (Söndgerath and Schröder 2002) and effective population size (Hedrick and Miller 1992; Templeton and Read 1994).

Landscape-centered characters pertinent to inter-patch dispersal include the distance between habitat patches and the character of the intervening matrix (Merriam et al. 1989; Thomas and Hanski 1997; Fahrig and Merriam 1999). Rather than a landscape of discrete patches of suitable habitat dispersed within an unsuitable matrix, an actual environment consists of areas of varying quality for a given species. In other words, all matrices are not equivalent, just as all patches are not (Thomas and Hanski 1997). Matrices that are less-disturbed, less chemically or physically hostile, or otherwise more amenable to the

movement of a species in question will be more likely to facilitate dispersal between patches. "Stepping stone" patches and corridors may also improve the probability of movement between populations (Weins 1996; Haddad 1999; Söndgerath and Schröder 2002).

Higher rates of migration are needed to maintain genetic diversity when population sizes are small. If dispersal between patches is low or nonexistent, genetic diversity will decline over time through genetic drift, and the rate of decline will increase as population size decreases (Templeton and Read 1994). Therefore, whether populations existing within a fragmented habitat experience a loss of genetic diversity depends on a variety of factors and these factors can interact and change over time.

Amphibians may be especially susceptible to genetic isolation due to their relatively low vagility, their philopatric nature, and the need of most species for a moist microhabitat. We studied wood frog (*Rana sylvatica*) populations of woodlots within an agriculturally fragmented landscape in north central Ohio to determine whether these populations have become genetically differentiated and/or have lost genetic diversity since the mid-1800s when much of the landscape was converted to agricultural use. Wood frogs typically live in wooded habitats and breed in ephemeral ponds. Because of its low rates of inter-population dispersal even within undisturbed habitat (Berven and Grudzien 1990) and its strong preference for forested habitats (DeMaynadier and Hunter 1999), we expected this species to be prone to genetic isolation due to forest fragmentation.

We predicted that levels of genetic diversity in wood frogs would be correlated with factors that determine the size of the population and the level of inter-population dispersal. Woodlot size, number of wetlands within a woodlot, and wetland hydroperiod were expected to be positively associated with size of wood frog populations. Larger woodlots would likely provide a moister, more hospitable terrestrial habitat promoting wood frog survivorship. Temporary wetlands with longer hydroperiods would be more reliable breeding sites, especially in dry years when recruitment at ponds with lesser hydroperiods could be drastically reduced. We expected wood frog populations in woodlots separated from other woodlots by a smaller distance to be more likely to experience immigration, and thus likely to have higher genetic diversity. The presence of more roads and railways near woodlots was predicted to be associated with less successful inter-population dispersal and, thus, lower within-population genetic diversity. We also expected genetic distance between populations to be correlated with the geographical distance between them.

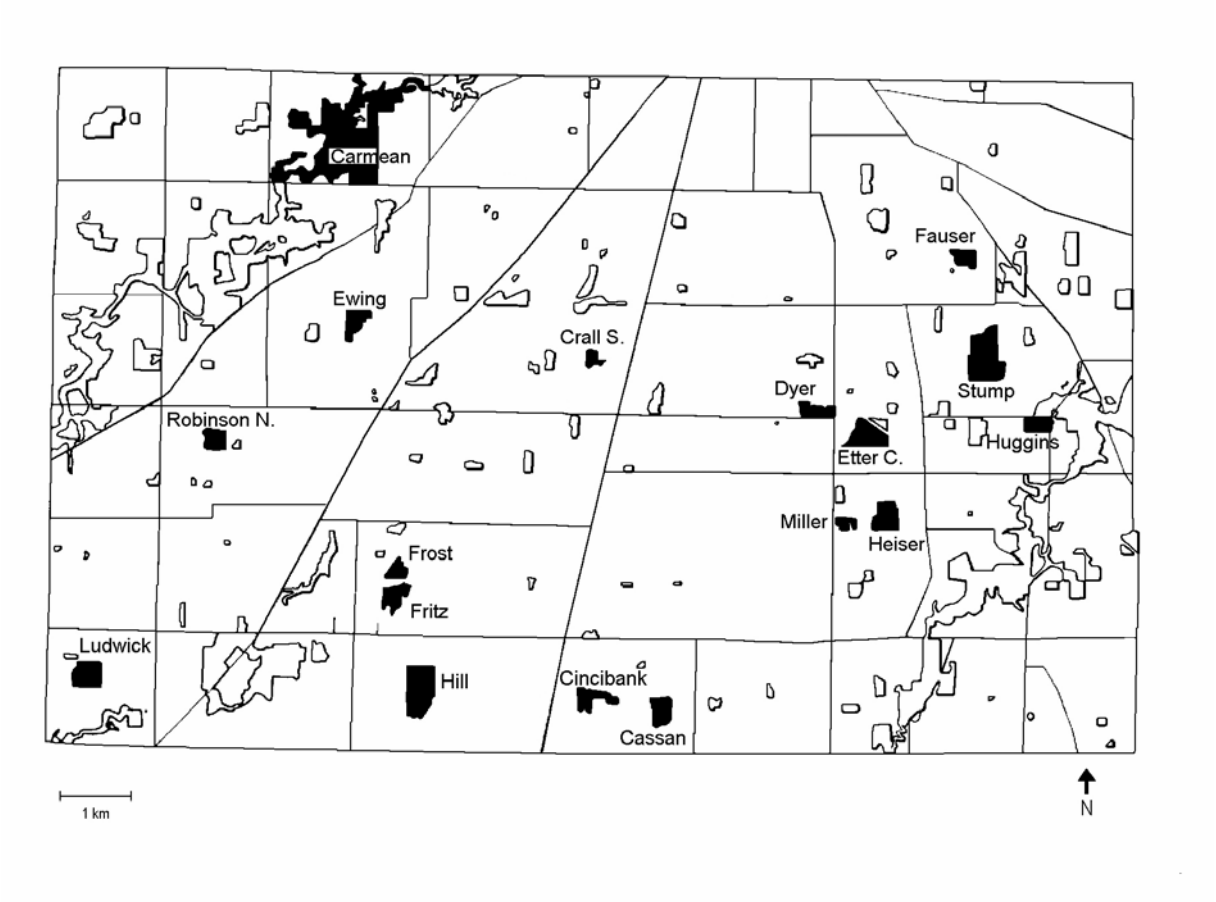
METHODS

Sample Collection. -- Wood frog eggs were collected from isolated woodlots and continuous forest in 1999, 2000 and 2004. A woodlot was defined as a continuous stand of trees undivided by roads, rivers, or fields. In 1999, wood frog eggs were collected from six woodlots in Crawford County, Ohio, (Figure 3.1), with one area of continuous forest in Warren County, Ohio serving as an

outgroup. The Warren County outgroup is located approximately 170 km south of the Crawford County study landscape. In 2000, wood frog eggs were again collected from these same sites, plus one additional area of continuous forest in Crawford County, “Carmean,” where eggs were unavailable in 1999 due to drought. As both 1999 and 2000 were relatively dry years and wood frogs did not breed at many sites, we collected samples again in 2004, a relatively wet spring conducive to wood frog breeding. We were able to collect wood frog eggs from 17 woodlots in Crawford County in 2004, including those sampled in 1999 and 2000.

Within each pond, approximately 30 eggs were collected per egg mass, with a maximum of 30 egg masses sampled per pond. If 50 or fewer egg masses were present in a pond, the number of egg masses was counted. If more than 50 egg masses were present, the number of egg masses was visually estimated. Only one pond was sampled per woodlot. Each sample of eggs was placed in a separate plastic vial of pond water and transported to the laboratory in a cooler. Eggs were maintained in the laboratory in aged, aerated tap water until the embryos were near hatching [Gosner stages 18-20 (Gosner 1960)]. Three randomly selected embryos from each egg mass were then removed from the egg capsules and each was placed in an individual autoclaved 1.5-mL Eppendorf tube. Five hundred µL of Longmire’s solution (100 mM Tris pH 8.0, 100 mM EDTA, 10 mM NaCl, and 0.5% SDS) were added to each tube, and the embryos were carefully crushed using a pestle to avoid damaging the DNA. The samples were labeled and stored at 4ºC until DNA could be extracted.

Figure 3.1. The study area in southern Crawford County, Ohio. Woodlots from which wood frog populations were genetically tested are filled and named.


Carmean
Ewing
Crall S.
Fauser
Stump
Dyer
Robinson N.
Etter C.
Huggins
Miller
Heiser
Frost
Fritz
Ludwick
Hill
Cincibank
Cassan
1 km
N

DNA Extraction and PCR. -- Using phenol-chloroform extraction, DNA was extracted from one randomly selected embryo of the three embryos preserved per egg mass. To digest the tissue, 30 µL of 20 mg/mL proteinase K solution were added to each preserved embryo and the samples were shaken periodically while incubated at 65°C for 4 hours. DNA was then extracted twice in 500 µL of phenol, once in a 25:24:1 solution of phenol:chloroform:isoamyl alcohol, and once in a 24:1 solution of chloroform:isoamyl alcohol. Samples were then purified via dialysis in a solution of 50 mL of 40X TNE_2 (400 mM Tris, pH 8.0, 4M NaCl, 40 mM EDTA) and 1950 mL dH_2O overnight. Finally, after samples had been transferred to autoclaved 1.5-mL Eppendorf tubes, DNA concentration was measured using a Spectronic Model 21 D spectrophotometer.

We used Randomly Amplified Polymorphic DNA (RAPD) markers to analyze the genetic structure of the wood frog populations. Amplification products are segments of the genome flanked by "inward-oriented" sequences complementary to a short (typically 10 bp), arbitrary primer (Williams et al. 1990). Although the resulting markers are dominant and heterozygotes cannot be directly detected, RAPDs are useful in studies of population structure (Parker et al. 1998). RAPDs have been successfully used in studies of population structure in a wide range of animals, including insects (Zhou et al. 2000), mammals (Fowler et al. 1999; Antolin et al. 2001; Vucetich et al. 2001; Cooper 2000), birds (Giesel et al. 1997; Haig et al. 1996), reptiles (Jäggi et al. 2000; Gibbs et al. 1994) and amphibians (Gibbs 1998).

Our methods for obtaining RAPD markers by PCR were adapted from Williams et al. (1990). DNA samples were amplified using a Biometra Uno-Thermoblock thermocycler with the following program: one cycle of 1 minute at 94°C, 2 minutes at 37°C, and 1.5 minutes at 72°C, followed by 37 cycles of 1 minute at 94°C, 1 minute at 37°C, and 0.17°C/s ramp to 72°C for 3 minutes. Each approximately 21.5-µL reaction volume consisted of 9.6 µL autoclaved dH_2O, 0.1 ng of template DNA (approximately 1 µL), 1.9 µL buffer (containing 200 mM Tris-HCl and 500 mM KCl), 2.6 µL of 10 mM dNTPs, 1.5 µL of RAPD primer (10µM), 0.17 µL Taq polymerase (5 U/µL), and 4.5 µL of 50 mM $MgCl_2$. A negative control, substituting autoclaved dH_2O for DNA, was included for each set of reactions. Upon completion of the PCR cycle, the samples were refrigerated at 4°C for up to one day. Four µL of xylene cyanol, bromphenol blue loading buffer were then added to each reaction tube and mixed, and the samples were loaded into the 1.4% agarose gels in 1 X TBE. In the last lane of each gel, we loaded a 123 bp ladder. After having been run at 31V for 19 hours, gels were stained in ethidium bromide for 20 minutes, destained in water for 5 minutes, visualized under UV light, and digitally photographed.

We screened 18 RAPD primers on 24 wood frog embryos from four different populations. Twelve of the 18 screened primers were from standard Operon Technology, Inc. oligonucleotide kits. The remaining 6 primers were also obtained from Operon, but were custom primers described by Kimberling and co-workers (1996) as being successful in a study of northern leopard frog (*Rana pipiens*) populations. Three primers (K-01, K-02, and 1-1) were selected for use

based on the crispness of the banding patterns. Primers K-01 (5' to 3' sequence CATTCGAGCC) and K-02 (5' to 3' sequence GTCTCCGCAA) were from Operon oligonucleotide kit K, while Primer 1-1 was a custom oligonucleotide (5' to 3' sequence GAAGCGCGAT). Strong, clear bands were scored as either present (1) or absent (2) using Kodak I.D. 2.0.2 software. A total of 32 loci were scored using these methods.

Because repeatability is sometimes an issue with the RAPD method, we assessed the repeatability of our banding patterns for each primer. We did this by blindly scoring a gel of 20 randomly selected individuals (adapted from Gibbs et al. 1994), and comparing those banding profiles with the profiles on the original gels. For each locus, a repeatability index was calculated as the proportion of those fragments that were identical in the original and the repeatability gel.

Data Analysis. -- Genetic distance between populations was analyzed by cluster analysis using TFPGA version 1.3 software (available from author Mark P. Miller, Mark.Miller@cnr.usu.edu, or by download from http://bioweb.usu.edu/mpmbio/). Nei's unbiased distances (Nei 1978) were calculated and a UPGMA cluster analysis was performed, generating a dendrogram for the combined 1999 and 2000 data. Mantel tests with 999 permutations were also performed separately on 1999 and 2000 data to examine any relationships between genetic and geographical distances.

We used the average Gini-Simpson Index (GSI) (Gibbs 1998) to estimate genetic diversity within each wood frog population. GSI was calculated as $\sum [1-(q_1^2 + q_2^2)]$, where q_1 and q_2 are the proportions of individuals in a population possessing and lacking a RAPD marker, respectively. GSI, and thus genetic diversity, is maximized when half of the individuals in a population possess, and half lack, each marker.

Because of the relatively small number of populations sampled in 1999 and 2000, we used genetic diversity data from these years to perform an exploratory analysis of the relationships between GSI and the following five habitat variables: woodlot size (ha), wetland hydroperiod (months), number of ponds in the woodlot, aggregate length (m) of roads and railways within 1km of the woodlot, and distance (m) to the nearest woodlot containing at least one wetland. Woodlot and landscape characters were measured from aerial photographs and U.S.G.S. topographic maps (7.5 minute series). A woodlot was defined as a continuous stand of trees undivided by roads, rivers, or fields. Pond hydroperiod was measured by visiting woodlots at least once a month from February to September 2000 and monitoring the depth of the breeding ponds.

For our exploratory analysis, we used Minitab Release 13 software to perform regression analyses of each habitat variable on GSI. Habitat variables that had adjusted R-square values less than 5% were eliminated from the set of variables included in the global model used to analyze the 2004 data. Because GSI was most strongly related to hydroperiod, we also used linear regression to test a

post-hoc hypothesis that population size (as estimated by the number of egg masses deposited at each pond) was correlated with hydroperiod.

We then employed an information-theoretic approach to investigate the relationships between habitat models and wood frog genetic diversity (Burnham and Anderson 1998). Because we eliminated the number of ponds in a woodlot as a variable in our exploratory analysis, our set of variables for building predictive models of genetic diversity included woodlot size, pond hydroperiod, distance to nearest wetland-containing woodlot, and aggregate length of roads and railroads within 1 km of the woodlot. We created models composed of all combinations of the habitat variables and tested them using Akaike's Information Criterion (AIC), adjusted for small sample sizes (AIC_C). Using AIC_C values derived from multiple linear regression, we calculated Akaike model weights (ω_m) to rank models and Akaike parameter weights (ω_p) to rank individual habitat variables for their usefulness as predictors of wood frog genetic diversity.

RESULTS

Of the 32 markers scored, seven had a repeatability index of less than 80% and were therefore eliminated from the analysis. Of the remaining 25 markers, the average repeatability score was 89%, and seven markers had a repeatability score of 100%.

A dendrogram produced by UPGMA cluster analysis of 1999 and 2000 data is shown in Figure 3.2. Wood frog populations sampled from the same woodlot in

both years did not necessarily cluster together. In some cases, populations from a collection site clustered relatively close together (e.g. Caesar Creek, Huggins, Ewing, and Etter C.). However, in all cases, populations sampled from the same location in different years were more genetically distinct from each other than they were from one or more other sampling sites. The Mantel test found a significant correlation between genetic distance and geographic distance in populations sampled in 2000 ($p=0.040$), but not in 1999 ($p=0.266$). Figure 3.3 shows the relationship between geographical and genetic distances for populations in 1999 and 2000.

Table 3.1 lists populations sampled in 1999, 2000 and 2004, ranked by their Gini-Simpson Indices. In all three years, Huggins woodlot had the highest GSI. The GSI for the outgroup from Caesar Creek remained relatively stable between years, but in 1999 this population fell in the middle of the range for genetic diversity, while in 2000 it had the second-highest GSI.

We removed the data from Carmean woodlot from the AIC analysis because exploratory analysis found it to be an outlier (as a continuously forested riparian area, it had a much larger woodlot area than the other, isolated woodlots). AIC_C values and model weights (ω_m) for the 15 models analyzed are given in Table 3.2. AIC parameter weights (ω_p), which give the likelihood that a given variable will be included in the best model, are shown in Table 3.3. The hydroperiod model best explained differences in genetic diversity, with ponds having longer hydroperiods supporting wood frog populations with greater genetic diversity.

Figure 3.2. UPGMA cluster analysis using Nei's unbiased distance (Nei 1978) for wood frog populations sampled in 1999 and 2000.

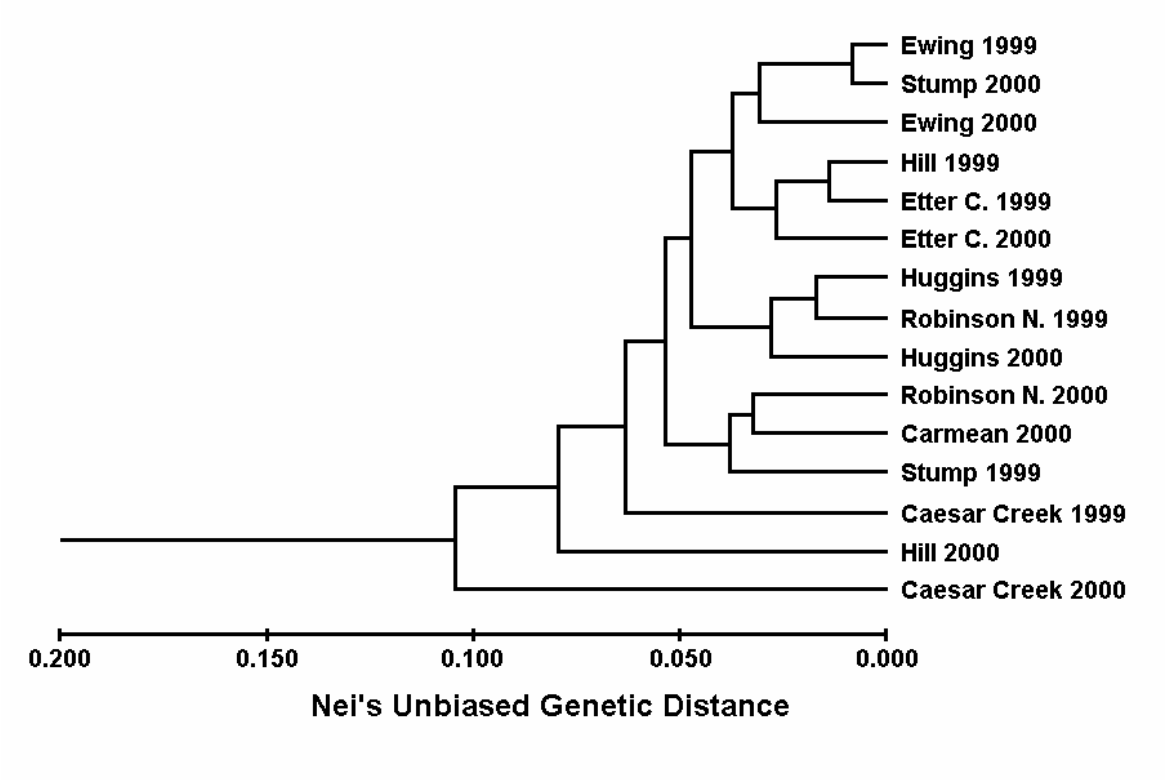

Ewing 1999
Stump 2000
Ewing 2000
Hill 1999
Etter C. 1999
Etter C. 2000
Huggins 1999
Robinson N. 1999
Huggins 2000
Robinson N. 2000
Carmean 2000
Stump 1999
Caesar Creek 1999
Hill 2000
Caesar Creek 2000
0.200
0.150
0.100
0.050
0.000
Nei's Unbiased Genetic Distance

Figure 3.3. The relationship between Nei's (1978) unbiased distance and the log of geographical distances between populations. Data from 1999 and 2000 are presented as circles and squares, and trendlines as dashed and solid lines, respectively.

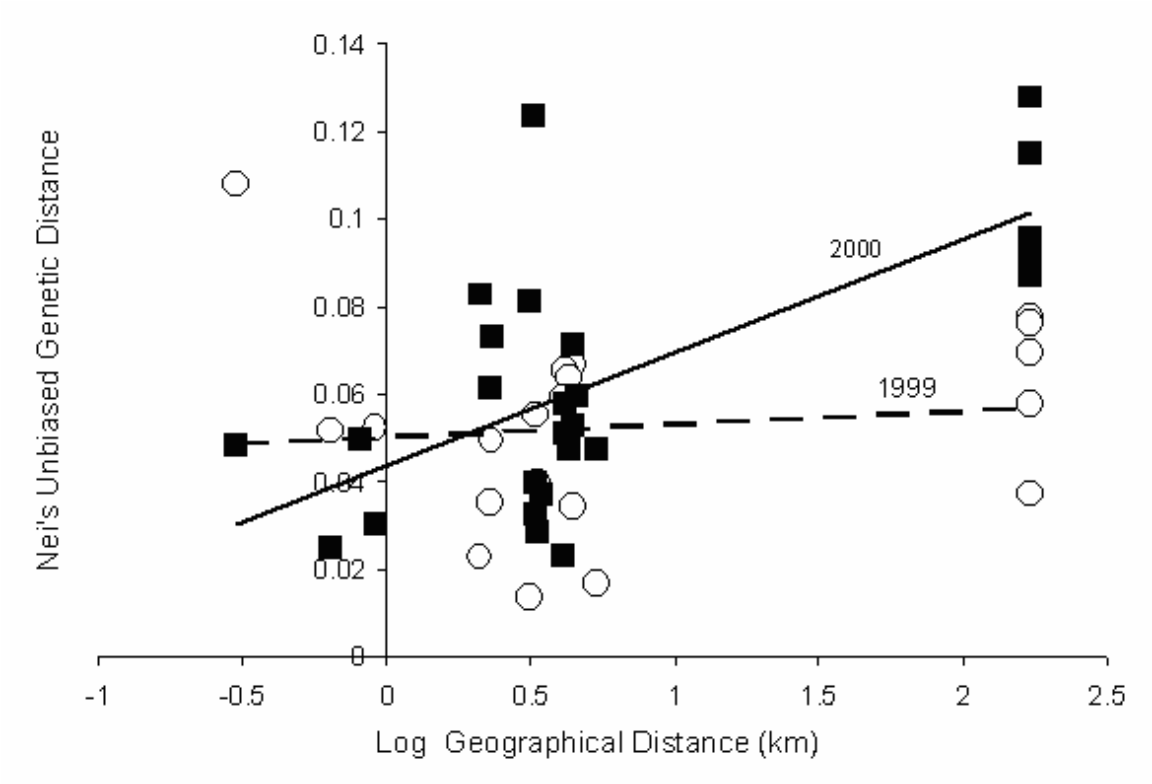

0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
-1
-0.5
0
0.5
1
1.5
2
2.5
2000
1999
Nei's Unbiased Genetic Distance
Log Geographical Distance (km)

Table 3.1. Wood frog populations ranked by the Gini-Simpson Index of genetic diversity (GSI) in 1999, 2000, and 2004. The number of embryos analyzed for each woodlot/year is given in parentheses.

1999		2000		2004	
Population	GSI	Population	GSI	Population	GSI
Huggins (24)	0.3428	Huggins (23)	0.2882	Huggins (22)	0.3383
Hill (21)	0.2580	Caesar Creek (11)	0.2579	Etter C. (23)	0.3012
Robinson N. (14)	0.2555	Ewing (20)	0.2506	Hill (19)	0.2987
Caesar Creek (7)	0.2547	Robinson N. (24)	0.2433	Heiser (20)	0.2982
Ewing (7)	0.2286	Hill (10)	0.2384	Dyer (20)	0.2922
Etter C. (22)	0.2195	Stump (16)	0.2380	Robinson N. (24)	0.2846
Stump (13)	0.1818	Etter C. (29)	0.2368	Ewing (22)	0.2840
		Carmean (18)	0.1969	Ludwick (21)	0.2746
				Frost (18)	0.2738
				Fritz (15)	0.2724

Continued

Table 3.1 continued.

1999		2000		2004	
Population	GSI	Population	GSI	Population	GSI
				Miller (21)	0.2703
				Stump (24)	0.2403
				Carmean (20)	0.2316
				Cassan (18)	0.2180
				Crall S. (24)	0.2176
				Cincibank (20)	0.1930

Table 3.2. AIC models predicting genetic diversity (GSI) of wood frog (*Rana sylvatica*) populations, ranked by AIC_C. The best model is shown in boldface. Model weights (ω_m) represent the probability that a given model is the best model in the set. Hydro = Hydroperiod (months); DistWood = Distance to nearest woodlot (m) containing a wetland; Woodlot = woodlot size (ha); LengthRR = total length of roads and railways (m) within 1 km of the woodlot

Model	K	AIC_C	ΔAIC_C	ω_m
Hydro	**3**	**-103.067**	**0.000**	**0.408**
Hydro; DistWood	4	-100.720	2.347	0.126
DistWood	3	-100.245	2.822	0.089
Hydro; LengthRR	4	-99.627	3.440	0.065
Hydro; Woodlot	4	-99.574	3.943	0.064
LengthRR	3	-99.572	3.495	0.064
Woodlot	3	-98.678	4.389	0.041
DistWood; LengthRR	4	-97.415	5.652	0.022
Woodlot; DistWood	4	-97.142	5.925	0.019
Hydro; Woodlot; DistWood	5	-96.751	6.316	0.016
Hydro; DistWood; LengthRR	5	-96.473	6.594	0.014
Woodlot; LengthRR	4	-96.281	6.786	0.012
Hydro; Woodlot; LengthRR	4	-95.463	7.604	0.008
Woodlot; DistWood; LengthRR	5	-93.748	9.319	0.004
Hydro; Woodlot; DistWood; LengthRR	6	-91.601	11.466	0.001

Table 3.3. Descriptive statistics and AIC parameter weights (ω_p) for variables included in our set of AIC models. AIC parameter weights represent the probability that a variable will be included in the best model. The sign of the relationship between genetic diversity and the variable is given in parentheses before the AIC parameter weight.

Variable	Mean ± SD	(ω_p)
Hydroperiod (months)	6.12 ± 1.54	(+) 0.701
Distance to Nearest Woodlot (m) containing wetlands	419.23 ± 323.30	(-) 0.289
Length Roads and Railways (m) within 1km of woodlot	3303.94 ± 1256.76	(+) 0.189
Woodlot Size (ha)	16.78 ± 20.26	(+) 0.148

Regression analysis indicated a positive relationship between hydroperiod and number of egg masses deposited in a breeding pond. This relationship was not significant in 1999 (p=.238) or 2000 (p=.166), but was significant in 2004 (p=.036).

DISCUSSION

Wood frogs typically live in wooded areas in the Midwestern United States and are highly philopatric. These characteristics lead to the expectation that wood frog populations within woodlots diverge genetically over time, with geographically closer populations diverging less due to occasional dispersal events. Our study supported this expectation. However, we failed to find support for the hypothesis that connectivity between woodlots, as measured by aggregate length of roads and railways surrounding woodlots, strongly influences genetic diversity. The lack of a strong relationship between genetic diversity and our measure of roads and railways may be due to the low volume of vehicular traffic in this rural area.

Huggins woodlot, which had the highest levels of genetic diversity in all three years, is part of a riparian corridor. Due to the presence of roadways separating it from the rest of the wooded riparian corridor, we did not consider Huggins a continuously forested habitat as we did Carmean. However, with little evidence that the roadways in this study area influence genetic diversity, the high diversity of Huggins woodlot may be at least partially related to its position within a

wooded corridor.

In several species of frogs and toads, individuals have been found to breed only every second or third year (Duellman and Trueb 1994). The genetic compositions of breeding populations may therefore vary among years. Temporally segregated populations have been documented in natterjack toads (*Bufo calamita*), with distinct populations breeding early and late within a breeding season (Sinsch 1992). Our data suggest that wood frogs may have genetically distinct temporal populations among years, because genetic distances were more different within the same population sampled in consecutive years than between some geographically separate populations (Figure 3.2).

Although the relationship was not significant in 1999, we found evidence for genetic structuring of populations by geographic isolation in our 2000 data ($p=0.040$; see Figure 3.3). This is consistent with a metapopulation model of wood frog population dynamics, featuring rare dispersal events between island populations. In the study landscape of southern Crawford County, dispersal would likely involve crossing large agricultural fields. Ditches along roadways, drainage ditches through fields, or fence rows may have provided dispersal corridors for varying distances during dispersal. However, no two sampling locations were directly connected by fence row or drainage ditch. Therefore, all dispersal events would require some crossing of agricultural fields without such aids.

Genetic diversity in wood frog populations within the agriculturally fragmented habitat of north central Ohio is primarily related to the hydroperiod of the

breeding pond, a critical habitat feature for many amphibians. Permanent ponds are often unsuitable for amphibians because they can support predatory fishes and larger populations of predatory insects. Conversely, vernal pools that dry too early can lead to high mortality rates of larval amphibians. A pool that holds water until all, or nearly all, larvae have metamorphosed would likely support the largest adult population. At large population sizes, however, density dependent factors can limit the survival rate of larvae and metamorphs. Berven (1990) found that, in wood frog populations, adult breeding population size was correlated primarily with larval survival, which varied greatly from year to year with pond hydroperiod. Because genetic diversity is lost more quickly in smaller populations, we expected genetic diversity to be lower at breeding ponds with shorter hydroperiods, and our analyses support this hypothesis. Our results indicate that recruitment of metamorphs may be limiting the population size and genetic diversity of some of these populations. Further, ponds with very short hydroperiods may not yield any metamorphs in dry years, possibly resulting in frequent genetic bottlenecks.

The results of this study indicate that relatively small woodlots within an agricultural landscape can support populations of wood frogs that may be as genetically diverse as populations within continuously forested habitat, as long as the hydroperiod of the pond is sufficiently long. Further, it emphasizes the importance of length of hydroperiod for maintaining genetically diverse amphibian populations.

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CHAPTER 4

RELATIONSHIPS OF GENETIC DIVERSITY WITH DEFORMITY AND MORTALITY RATES IN THE WOOD FROG (*RANA SYLVATICA*)

ABSTRACT

Genetic diversity is necessary for populations to evolve and is believed to be positively associated with individual fitness. We hypothesized that genetic diversity of a wood frog (*Rana* sylvatica) population would be inversely related to mortality and deformity rates of eggs and tadpoles from that population reared in the laboratory. We used RAPDs to assess genetic diversity of wood frog eggs collected from eight genetically distinct populations. We then compared the genetic diversity of each population with the mortality and deformity rates of lab-reared eggs and tadpoles. The Gini-Simpson Index of genetic diversity for our eight populations ranged from 0.1818 to 0.3428. We observed an overall mortality rate of 21.7%, and an overall deformity rate of 7.5%. Most mortality occurred at the egg stage, and deformities consisted of axial malformations and edema. Although there were weak negative correlations, our MANCOVA

analysis failed to find a significant relationship of genetic diversity with either deformity or mortality rate of wood frog eggs and larvae. This result may be due, at least in part, to the fact that the range of genetic diversity among populations was not broad enough to detect an effect.

INTRODUCTION

The diversity of genes within a species can be partitioned into three levels: within individuals, among individuals within populations, and among populations (Meffe and Carroll 1997). As genetic diversity is lost at the population level and more loci become monomorphic, the overall level of heterozygosity of individuals within the population declines. Conservation of genetic diversity is important because a reservoir of genes allows adaptation to changing environmental conditions and because heterozygosity is positively associated with several measures of fitness. The benefits of heterozygosity include greater developmental stability and higher rates of growth, survival, and fecundity (e.g., Mitton and Grant 1984; Allendorf and Leary 1986; Mitton 1993; Reed and Frankham 2003). Genetic diversity can be lost quickly in small, isolated populations, increasing the probability of extinction.

For several reasons, amphibians may be especially susceptible to loss of genetic diversity due to habitat fragmentation: (1) many species are highly philopatric, (2) amphibians are relatively small and natal dispersal distances are usually short, (3) they require moist microhabitats, making dispersal across open

areas difficult, and (4) their populations often undergo large fluctuations from year to year, which can lead to a faster erosion of genetic diversity.

Several studies have investigated relationships between genetic diversity and fitness in amphibians, with varying results. Rowe and colleagues (1999) found that growth and survival rates in natterjack toad (*Bufo calamita*) larvae were positively correlated with heterozygosity. Pierce and Mitton (1982) found a positive association between protein polymorphism and growth rate in tiger salamander (*Ambystoma tigrinum*) larvae early in their development, but the relationship was not evident later in development. Chazal and colleagues (1996) found no relationship between heterozygosity and body size of juvenile marbled salamanders (*Ambystoma opacum*) when larvae were reared under crowded conditions; when reared at low densities, however, more-homozygous individuals metamorphosed at a larger size. Wright and Guttman's (1995) study of wood frog (*Rana sylvatica*) populations in nature found no association between heterozygosity and weight of tadpoles.

Heterozygosity in tiger salamander larvae has been shown to be directly correlated with active oxygen consumption and inversely correlated with standard oxygen consumption (Mitton et al. 1986). Heterozygosity would therefore confer fitness because higher rates of active oxygen consumption indicate a greater aerobic capacity, whereas lower rates of standard oxygen consumption are associated with lower maintenance costs (Mitton et al. 1986). In the green treefrog (*Hyla cinerea*), reproductive success of females, but not body size, was found to be positively associated with heterozygosity (McAlpine 1993). Also in

green treefrogs, heterozygosity at the isocitrate dehydrogenase-2 locus was found to be associated with increased overwinter survival, but heterozygosity over the eight studied loci, in the aggregate, was not correlated with survival or mating success (McAlpine and Smith 1995).

Across large portions of the midwestern United States, agricultural landscapes predominate with small patches of woodland serving as habitat islands for many species. If dispersal between patches is lost or severely limited and if population sizes are small, amphibians in such habitat patches may lose genetic diversity over time. In a 15,450-ha study landscape in north-central Ohio, we found that populations of wood frogs inhabiting forest patches in an agricultural landscape had varying levels of genetic diversity, primarily correlated with the hydroperiod of the breeding pond (see Chapter 2). Genetic distance between populations was positively related to geographical distance, indicating that some dispersal may occur between nearby woodlots. In the present study, we determine whether fitness correlates in larval wood frogs from these different populations are related to the genetic diversities of the populations. In the laboratory, we reared larval wood frogs from eight different populations with varying levels of genetic diversity, and hypothesized that larvae from populations with higher genetic diversity would experience lower rates of mortality and deformity.

METHODS

Genetic Diversity -- Using RAPD markers, we obtained genetic diversity estimates for seven wood frog populations in southern Crawford County, Ohio, and one population in Warren County, Ohio. Within each breeding pond, approximately 30 eggs were removed from individual egg masses, with a maximum of 30 egg masses sampled per pond. Each sample of eggs from an egg mass was placed in a separate plastic vial of pond water and transported to the laboratory in a cooler. Eggs were maintained in the laboratory in aged, aerated tap water until the embryos were near hatching [Gosner stages 18-20 (Gosner 1960)]. Three randomly selected embryos from each egg mass were then removed from the egg capsules and each was placed in an individual autoclaved 1.5-ml Eppendorf tube. Five hundred µl of Longmire's solution (100 mM Tris pH 8.0, 100 mM EDTA, 10 mM NaCl, and 0.5% SDS) were added to each tube, and the embryos were carefully crushed using a pestle to avoid damaging the DNA. The samples were labeled and stored at 4ºC until DNA could be extracted.

Our methods for obtaining RAPD markers are described in detail in Chapter 3. Briefly, DNA was extracted from one randomly selected embryo using standard phenol-chloroform extraction. Amplification products were generated using RAPD primers in PCR (Williams et al. 1990), and were visualized using ethidium bromide fluorescence. We screened 18 RAPD primers from Operon Technology, Inc., and selected three that produced crisp banding patterns. Primers K-01 (5'

to 3' sequence CATTCGAGCC) and K-02 (5' to 3' sequence GTCTCCGCAA) were from Operon oligonucleotide kit K, while Primer 1-1 was a custom oligonucleotide (5' to 3' sequence GAAGCGCGAT). Strong, clear bands were scored as either present (1) or absent (0) using Kodak I.D. 2.0.2 software. A total of 32 loci were scored using these methods.

Because repeatability is sometimes a concern with the RAPD method, we assessed the repeatability of our banding patterns for each primer. We blindly scored a gel of 20 randomly selected individuals, and compared those banding profiles with the profiles on the original gels (adapted from Gibbs et al. 1994). For each locus, a repeatability index was calculated as the proportion of those fragments that were identical in the original and the repeatability gel. Of the 32 markers scored, 25 had a repeatability index of 80% or greater, and were therefore used in this analysis.

Collection and Rearing of Tadpoles -- We reared wood frog eggs/larvae in 36-cm X 24-cm plastic containers that were divided by nylon screening into 15 7.2-cm X 8-cm chambers. In each chamber we placed five eggs, one randomly selected from each of five randomly selected egg masses from a given population. These five egg masses were a subset of the total egg masses sampled for the RAPD analysis. Each of eight of the 15 chambers in each plastic container was thus filled with eggs from one of eight wood frog populations, and we created six plastic-container replicates. Eggs and the resulting larvae were maintained in aged, aerated tap water at room temperature (18.9°C to 20.6°C) with a 10:14-

hour light:dark cycle. Once the larvae had hatched, equal amounts of a pasty food mixture (¼ Tetramin flaked goldfish food, ¼ Wardley's Reptile Sticks, ¼ boiled lettuce, and ¼ boiled mashed broccoli) were fed to the tadpoles every other day, and the water in the containers was changed three times a week. Quantity of food was adjusted for stage of development, compensating for mortality. Survival was monitored daily, and any noticeable deformities in tadpoles were recorded. Tadpoles were maintained until emergence of front legs, at which time metamorphosis was considered essentially complete and the juvenile frogs were released at their natal ponds.

Data Analysis -- We used the average Gini-Simpson Index (GSI) (Gibbs 1998) to estimate genetic diversity within each wood frog population. GSI was calculated as $\sum [1- (q_1^2 + q_2^2)]$, where q_1 and q_2 are the proportions of individuals in a population possessing and lacking a RAPD marker, respectively. GSI, and thus genetic diversity, is maximized when half of the individuals in a population possess, and half lack, each marker. A one-factor, balanced MANOVA analysis was carried out using SPSS Release 11.5.1 software. GSI was the independent variable, and the three response variables were egg mortality rate, larval mortality rate, and deformity rate. Mortality and deformity rates were arcsine square root transformed for analysis. In addition, univariate ANOVAs associated with the MANOVA were examined for each response to determine the relative importance of the relationships between GSI and each fitness measure. We used a Bonferroni adjustment for our univariate analyses, with $\alpha = 0.017$, to

maintain an overall experiment-wise error of $\alpha = 0.05$. We also analyzed observed power to obtain β levels for our tests.

RESULTS

GSI ranged from 0.1818 to 0.3428 in our 8 wood frog populations. We observed two main types of deformities in the larval wood frogs: axial malformation (dorsal or lateral tail flexure or wavy tail), and edema (Figure 4.1). The mean number of individuals that died or were deformed per chamber of five eggs/tadpoles was 1.08 ± 0.71 and 0.375 ± 0.64, respectively (Table 4.1). Most mortality occurred at the egg stage. We recorded a total of 18 deformed tadpoles with 15 exhibiting axial abnormalities, two showing edema, and one having both axial abnormality and edema. Only six of the most severely deformed individuals died before metamorphosis.

The MANOVA did not find a statistically significant relationship between GSI and any of our response variables (Pillai's test, $p=0.090$). Individual ANOVAs also failed to find a significant relationship between GSI and our fitness measures, although the relationship with egg mortality rate was nearly significant (Table 4.1). Power for our overall MANOVA was high ($\beta=0.086$), but lower for our individual ANOVAs (Table 4.1).

Figure 4.1. Two main types of deformities observed in lab-reared wood frog larvae: (a) tail flexure, and (b) edema.

A.

B.

Table 4.1. Descriptive statistics for dependent and independent variables used in MANOVA analysis, and individual ANOVA p- and β-values for the relationship between each dependent variable and GSI (Gini-Simpson Index of genetic diversity). Mortality and deformity rates are given as the proportion, before arcsine square-root transformation, of individuals that died or were deformed per chamber of five individuals from a particular wood frog population.

Variable	Mean ± StDev	Maximum	Minimum	p	β
GSI	0.241 ± 0.048	0.343	0.182		
Egg Mortality Rate	0.179 ± 0.138	0.600	0.000	0.075	0.290
Larval Mortality Rate	0.037 ± 0.079	0.200	0.000	0.405	0.604
Deformity Rate	0.075 ± 0.128	0.400	0.000	0.308	0.541

DISCUSSION

We were unable to find any statistically significant relationship between genetic diversity of wood frog populations and our fitness measures (egg mortality rate, larval mortality rate, and deformity rate). However, even though not significant, there were negative trends between genetic diversity and mortality rates and deformity rates. In particular, the relationship between genetic diversity and egg mortality rate, while not statistically significant ($p=0.075$), may be biologically significant. Given these results, one may conclude either that genetic diversity is unrelated to deformity and mortality rates in wood frog eggs and larvae, or that our experiment was not robust enough to detect the relationship. If genetic diversity is related to mortality and deformity rates, but we were unable to detect the relationship, this could be due to one or more factors. There may have been insufficient replicates, making the statistical power of the test too low. Our study had six replicates per population, and responses (mortality and deformity rates) were highly variable (Table 4.1). The power of our multivariate test was high ($\beta=0.086$), but for our individual ANOVAs was moderate to low (Table 4.1). Additionally, we may have examined a range of genetic diversity insufficient to detect a difference in fitness. Genetic diversity of the eight studied populations ranged from 0.1818 to 0.3428, which is not a high level of variation. For example, GSI values for populations of redback salamanders (*Plethodon cinereus*) in a fragmented landscape in Connecticut, USA, ranged from 0.00 to 0.50 (Gibbs 1998). Drought conditions during the

spring of 1999 prevented us from collecting eggs at many known wood frog populations in our study area. Had a greater number of populations been available for sampling, it is likely that a greater diversity of population GSIs would have been found. Given the weakly positive association between GSI and our fitness measures, further study with a broader range of population GSIs would be informative.

We observed two main categories of deformities in our laboratory-reared wood frog larvae, axial malformation and edema. These types of deformities have been associated with pollutants or nutritional stress (Harris et al. 1998; Marco et al. 1999; Rosenshield et al. 1999; Wright and Whitaker 2001). 7.5% of the tadpoles in this experiment were deformed, but only a third of those died before metamorphosis. Upon metamorphosis, those frogs that displayed axial malformations as larvae appeared "normal." In the three cases in which a tadpole exhibited edema, the deformity was fatal, preventing the tadpole from swimming or feeding normally. As no significant relationship was found between genetic diversity and deformity rates, it is possible that variables other than genetic factors are responsible for the observed deformities, such as exposure to pollutants when eggs were deposited in the breeding ponds, or inadequate nutrition in the laboratory.

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CHAPTER 5

THE INTERACTION BETWEEN GENETIC DIVERSITY AND AN ENVIRONMENTAL STRESSOR AFFECTS WOOD FROG (*RANA SYLVATICA*) FITNESS

To be submitted to the journal, Science, by S.L. Weyrauch and T.C. Grubb, Jr.

ABSTRACT

Amphibian declines in recent decades have spurred much research into potential causes, included synergistic interactions. Genetic diversity may buffer populations against environmental vicissitudes. We hypothesized that wood frogs (*Rana sylvatica*) from populations with lower genetic diversity are more susceptible to UV-B radiation than those from populations with higher diversity. In a controlled test, we found a strong effect of UV-B treatment on larval mortality and deformity rates, but not on egg mortality. We found significant effects of

genetic diversity on egg mortality rate, and larval mortality and deformity rates. Further, we found that the interaction between UV-B treatment and genetic diversity significantly affected larval mortality rates. This is the first study to document that loss of genetic diversity causes increased vulnerability to an environmental stressor. Differences in genetic diversity between populations, coupled with environmental stressors, may help explain patterns of amphibian decline.

INTRODUCTION

Declines in many amphibian populations have been observed in recent decades, leading to much research into potential causes (e.g., Blaustein and Wake 1990; Wake 1991; Houlahan et al. 2000; Collins and Storfer 2003). Some studies have focused on single factors, such as UV-B radiation (e.g. Blaustein et al. 1994a), disease (e.g. Blaustein et al. 1994b), or pollutants (e.g. Dunson et al. 1992). However, studies of amphibian declines have increasingly involved investigations of potential synergistic interactions among multiple environmental factors (e.g. Carey 1993; Pounds and Crump 1994; Pahkala et al. 2002a).

Increased exposure to ultraviolet-B radiation (280-320 nm wavelength) has been widely implicated in amphibian declines, either as a single factor or in conjunction with other factors. Increasing levels of UV-B radiation are reaching the earth's surface due to ozone depletion (Stolarski et al. 1992; Kerr and McElroy 1993). The global nature of increasing levels of UV-B radiation has

made it an attractive hypothesis for world-wide amphibian declines, whether by itself or in combination with other factors. UV-B radiation damages DNA, creating mutations such as cyclobutane pyrimidine dimers (Licht and Grant 1997). Photolyase enzymes can repair the damage, but the level of such enzymatic activity varies from species to species, and between conspecific individuals (Blaustein 1994a; van de Mortel 1998; Belden and Blaustein 2002). Blaustein and colleagues' (1994a) study was the first to document that current levels of UV-B radiation could be directly increasing mortality in some amphibian species. Although not all studies have found a detrimental effect of natural levels of UV-B radiation on amphibians (e.g. Grant and Licht 1995; Blaustein et al. 1996), considerable evidence now exists that UV-B radiation may be an important factor in at least some declining amphibian populations (e.g. Blaustein et al. 1995; Hays et al. 1996; Anzalone et al. 1998; Lizana and Pedraza 1998). Exposure to UV-B radiation can also affect survival indirectly by weakening the immune system and making amphibians more susceptible to diseases (Kiesecker and Blaustein 1995). Further, UV-B radiation has been found to interact synergistically with low pH (Long et al. 1995) and various toxic chemicals (Blaustein et al. 2003).

In this study, we investigate another potential synergistic interaction that may be important in understanding amphibian declines, a low genetic diversity/high UV-B radiation synergism. Genetic diversity may buffer populations against environmental vicissitudes. When amphibian populations are fragmented (naturally or anthropogenically), they may lose genetic diversity over time. We

hypothesize that populations with lower levels of genetic diversity may be more susceptible to UV-B radiation. Such an interaction might occur if, for example, genetic drift results in populations losing alleles for higher photolyase activity. Particularly with regard to increasing levels of UV-B radiation, such loss of genetic diversity could be harmful to amphibian populations. Recently, some studies have begun to address differential effects of UV-B radiation on amphibians from different populations within the same species (Belden et al. 2000; Pahkala et al. 2002a; Pahkala et al. 2002b). In particular, studies have investigated whether populations that naturally experience greater levels of UV-B radiation are better adapted to those conditions and thus suffer lower mortality and deformity rates than conspecific populations that are naturally exposed to lower levels of UV-B radiation, as in latitudinal (Pahkala et al. 2002b) or elevational gradients (Belden et al. 2000). Similarly, we propose that genetically diverse amphibian populations may be better capable of repairing UV-B-induced DNA damage and thus have lower mortality and deformity rates than those with less genetic diversity.

In 2004, we collected wood frog (*Rana sylvatica*) eggs from 12 populations in Crawford County, Ohio, and analyzed their genetic diversity. We also reared eggs/tadpoles from those populations under three UV-B radiation treatments. We predicted that eggs and tadpoles from populations with lower genetic diversity exposed to the highest level of UV-B radiation would experience the greatest mortality.

METHODS

Genetic Analysis -- Using RAPD markers, we obtained genetic diversity estimates for 12 wood frog populations in 12 separate island woodlots within the agricultural landscape of Crawford County, Ohio. Within each breeding pond, approximately 30 eggs were removed from individual egg masses, with a maximum of 25 egg masses sampled per pond. Each sample of eggs from an egg mass was placed in a separate plastic vial of pond water and transported to the laboratory in a cooler. Three randomly selected embryos from each egg mass were maintained in the laboratory in aged, aerated tap water until the embryos were near hatching [Gosner stages 18-20 (Gosner 1960)]. These embryos were then removed from their egg capsules and each was placed in an individual autoclaved 1.5-ml Eppendorf tube. Five hundred µl of Longmire's solution (100 mM Tris pH 8.0, 100 mM EDTA, 10 mM NaCl, and 0.5% SDS) were added to each tube and the embryos were carefully crushed using a pestle to avoid damaging the DNA. The samples were stored at 4ºC until DNA could be extracted.

DNA was extracted from one randomly selected embryo from each egg mass using standard phenol-chloroform extraction. Amplification products were generated using RAPD primers in PCR (Williams et al. 1990), and were visualized using ethidium bromide fluorescence. We screened 18 RAPD primers from Operon Technology, Inc., and selected three that produced crisp banding patterns. Primers K-01 (5' to 3' sequence CATTCGAGCC) and K-02 (5' to 3'

sequence GTCTCCGCAA) were from Operon oligonucleotide kit K, while Primer 1-1 was a custom oligonucleotide (5' to 3' sequence GAAGCGCGAT). Strong, clear bands were scored as either present (1) or absent (0) using Kodak I.D. 2.0.2 software. A total of 32 loci were scored using these methods.

Because repeatability is sometimes a concern with the RAPD method, we assessed the repeatability of our banding patterns for each primer. We blindly scored a gel of 20 randomly selected individuals, and compared those banding profiles with the profiles on the original gels (adapted from Gibbs et al. 1994). For each locus, a repeatability index was calculated as the proportion of those fragments that were identical in the original and the repeatability gel. Of the 32 markers scored, 25 had a repeatability index of 80% or greater and were therefore used in this analysis.

Experimental Protocol -- Wood frog eggs/tadpoles were reared in 36-cm X 24-cm plastic containers that were divided by nylon screening into 12 12-cm X 6-cm chambers (Figure 5.1). We placed eight eggs in each chamber, one randomly selected from each of eight randomly selected egg masses from a given population. These eight egg masses were a subset of the total egg masses sampled for the RAPD analysis of genetic diversity. Assignment of populations of wood frog eggs to chambers within each box was random.

UV-B treatments were similar to those of Blaustein and colleagues (1994a). In the first treatment, eggs were exposed to unfiltered sunlight. In the second treatment, eggs were shielded by a UV-B blocking filter (Mylar sheets). In the

third treatment, eggs were covered with acetate sheets which allowed the passage of most UV-B radiation. This third treatment served as a control for using a cover in the UV-B-blocking treatment. We created six replicates for each of the three UV-B treatments, in a double Latin square design (Figure 5.2). The individual chamber was considered the primary sampling unit, making the sample sizes in various analyses $\leq$ 216 (i.e. 12 chambers per box X 18 boxes). The experiment was carried out on open ground in Newark, Ohio (40° 1'N, 82° 28' W). There were no trees or other obstructions near the experiment, permitting direct sunlight to reach the eggs.

Boxes were placed in an artificial pool. Twelve 1-cm-diameter holes (one for each chamber) were created below water level in the sides of each plastic box and covered with nylon screening to allow circulation of water between the artificial pool and the box. In addition, 12 1-cm-diameter holes were created above the water level in the sides of each plastic box to allow air circulation (Figure 5.1). The pool and boxes were allowed to fill naturally with rainwater. The pool and boxes were 5.5 cm deep when full. Once the larvae hatched, an equal amount of a pasty food mixture (¼ Tetramin flaked goldfish food, ¼ Wardley's Reptile Sticks, ¼ boiled lettuce, and ¼ boiled mashed broccoli) was fed to the tadpoles twice a week. Quantity of food was adjusted for stage of development, compensating for mortality. Survival was monitored twice a week, and any noticeable deformities in tadpoles were recorded. UV-B measurements were taken at the experimental site daily at solar noon, using a Solarmeter Model 6.2 UV-B meter. The water temperature of each container was then measured

Figure 5.1. Experimental box for rearing wood frog (*Rana* sylvatica) eggs from 12 different woodlots in Crawford County, Ohio. Each of the twelve chambers in a box contained eight eggs, one from each of eight egg masses, from one woodlot. Assignment of woodlot populations to the twelve chambers was random.

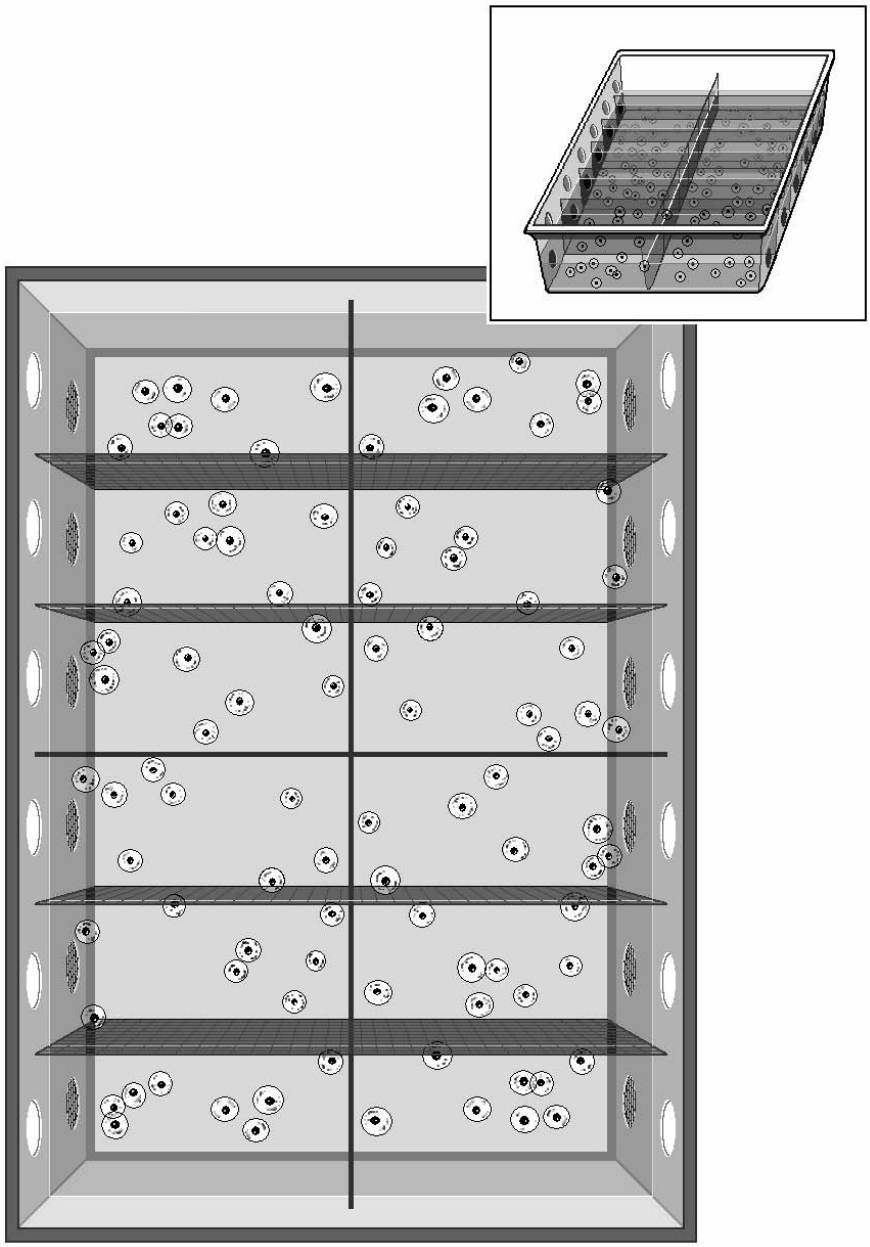
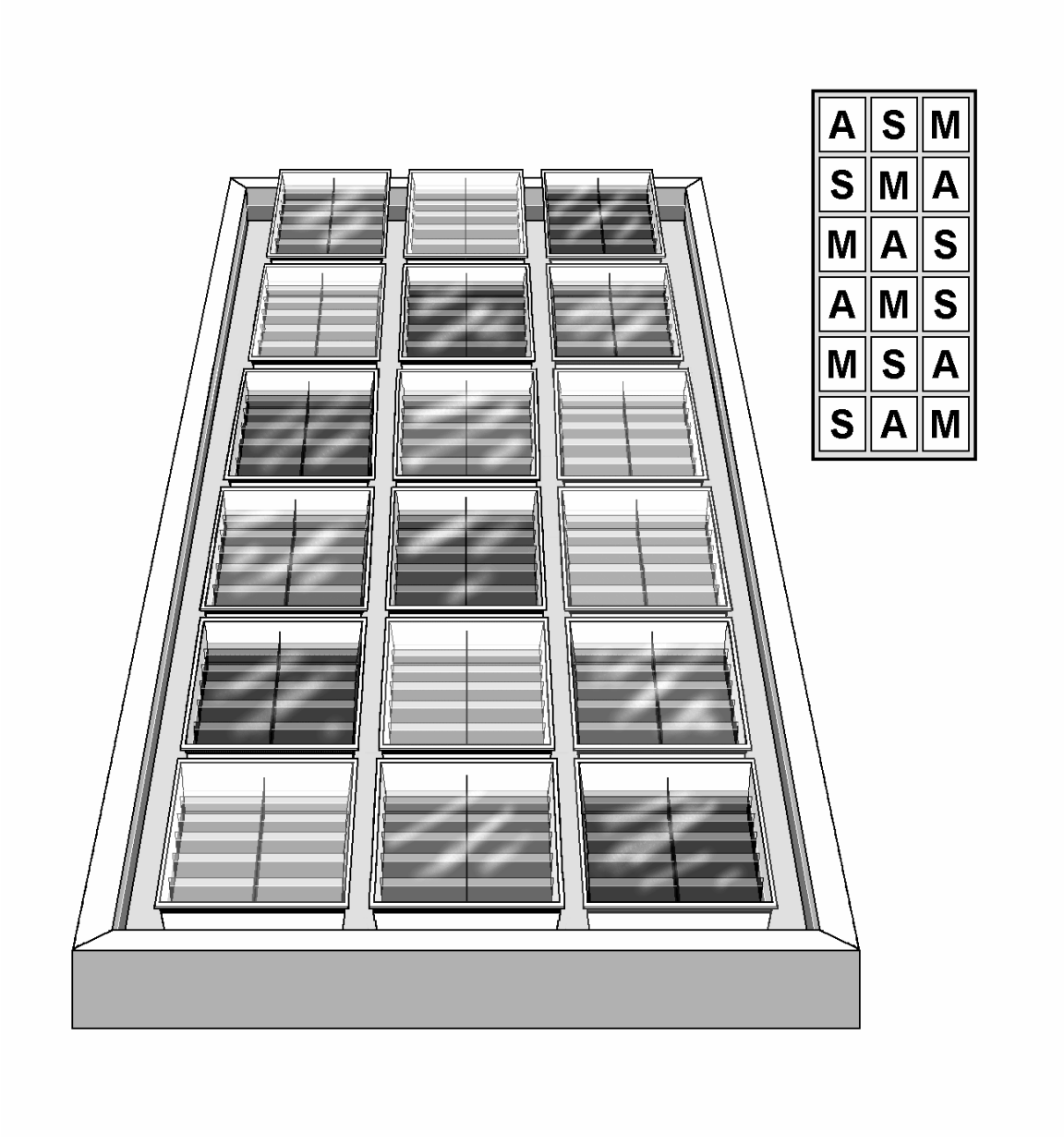


Figure 5.2. Experimental setup in which wood frog (*Rana sylvatica*) eggs and larvae from each of 12 different populations were reared in separate chambers within each plastic box. There were six replicates for each UV-B treatment: natural sunlight filtered by UV-B-blocking Mylar ("M"), natural sunlight filtered by UV-B-transmitting acetate ("A"), and unfiltered natural sunlight ("S"). Treatments were assigned to each row randomly, within the constraints of a double-Latin-square design.


A S M
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using a digital thermometer to determine if there were differences among treatments in temperature. The experiment was begun on 31 March 2004 and was terminated on 27 April 2004.

Data Analysis -- We used the average Gini-Simpson Index (GSI) (Gibbs 1998) to estimate genetic diversity within each wood frog population. GSI was calculated as $\sum [1- (q_1^2 + q_2^2)]$, where q_1 and q_2 are the proportions of individuals in a population possessing and lacking a RAPD marker, respectively. GSI, and thus genetic diversity, is maximized when half of the individuals in a population possess, and half lack, each marker.

We used MINITAB Release 13 software to perform one-way ANOVAs to investigate the relationship between UV treatment (Mylar, acetate, and direct sun treatments) and water temperature for each date temperatures were recorded. When ANOVA found significant differences between treatments on a particular day, we conducted paired t-tests to determine which treatments were significantly different. We performed a two-factor, balanced MANOVA analysis to determine whether GSI and UV-B treatment or their interaction were related to egg mortality, larval mortality, and deformity rates. Mortality and deformity rates were arcsine square root transformed for analysis. In addition, univariate ANOVAs associated with the MANOVA were performed for each response to examine the relative importance of GSI and UV-B treatment or their interaction on each fitness measure. For these univariate tests, the α level was Bonferroni-corrected (Sokal and Rohlf 1995) to control for experimentwise error.

RESULTS

UV-B measurements at the experimental site ranged from 25 to 285 μW/cm^2 (Figure 5.3). Mylar filters blocked 90% of UV-B radiation, while acetate filters blocked 36%. On 11 of the 29 days of the experiment, the average water temperature for the six replicates of the direct-sun treatment was significantly less than that for the Mylar-filtered or acetate-filtered treatment (on average, a 1.1°C difference). Temperatures were never significantly different between Mylar and acetate treatments. Mean temperature at solar noon for the direct-sun treatment was 19.8°C (range = 9.8°C -27.3°C), 20.9°C (range = 9.6°C -28.7°C) for the acetate-filtered treatment, and 20.9°C (range = 9.9°C-28.3°C) for the Mylar-filtered treatment. Therefore, we performed MANOVAs both with and without the data for the direct-sun treatment, as the temperature difference may have confounded the results.

Mean mortality and deformity rates for all three treatments are given in Table 5.1. Most mortality occurred at the larval stage. We found a total of 186 deformed tadpoles, with 4 exhibiting edema, 181 having axial malformations (i.e., dorsal and lateral tail flexure and wavy tail), and 1 showing both axial malformations and edema. Descriptive statistics for GSI and for dependent variables in our MANOVAs are shown in Tables 5.2 and 5.3.

When all three treatments were included in our analysis, MANOVA found significant relationships between our response variables and GSI (Pillai's criterion, p=0.000), UV treatment (Pillai's criterion, p=0.000), and the interaction

Figure 5.3. UV-B measurements taken at solar noon at the site of the experiment from 30 March to 27 Arpil 2004.

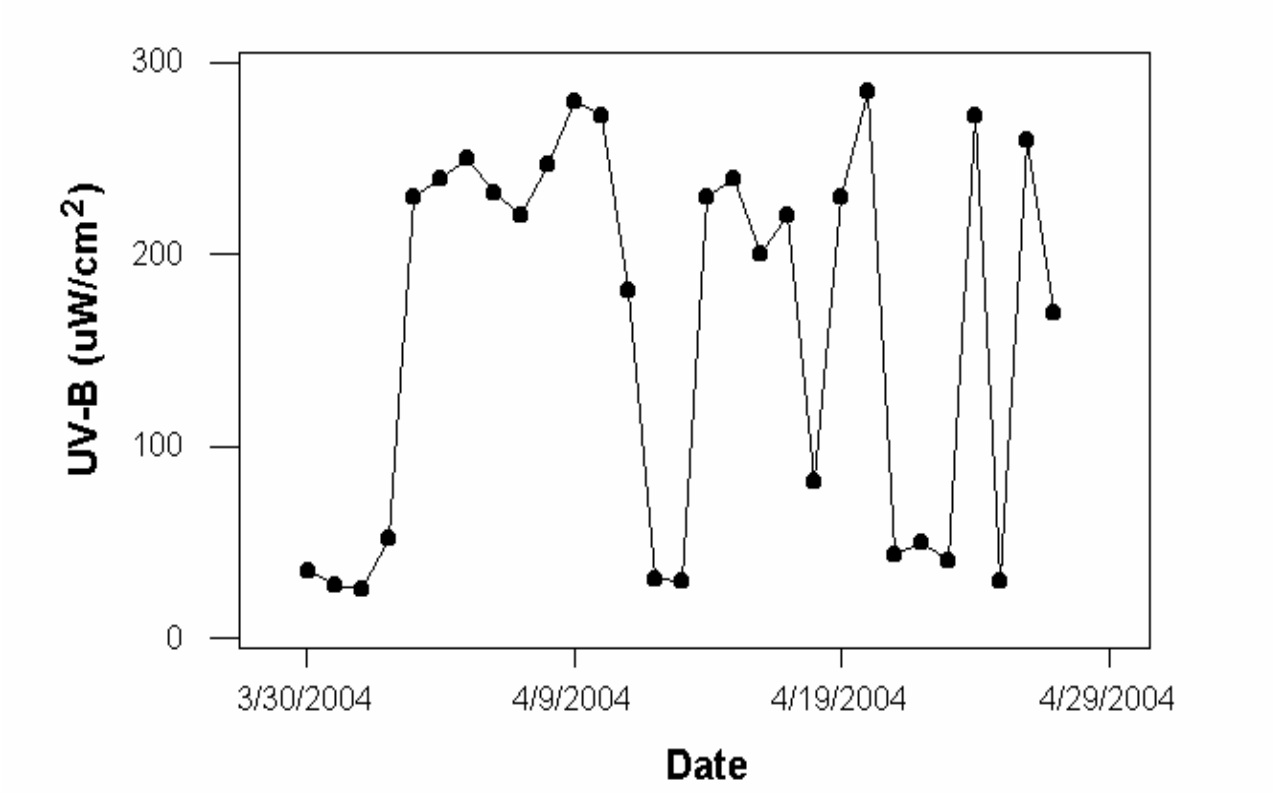

300
200
100
0
UV-B (uW/cm2)
3/30/2004
4/9/2004
4/19/2004
4/29/2004
Date

Table 5.1. Mean mortality and deformity rates of wood frog (*Rana sylvatica*) eggs or larvae for each UV-B treatment, independent of GSI.

	UV-B Treatment		
Variable	Mylar (N=72)	Acetate (N=72)	Direct Sun (N=72)
Mean Egg Mortality ± SD	0.045 ± 0.070	0.053 ± 0.088	0.033 ± 0.076
Mean Larval Mortality ± SD	0.083 ± 0.086	0.637 ± 0.200	0.645 ± 0.205
Mean Deformity ± SD	0.014 ± 0.040	0.139 ± 0.132	0.156 ± 0.140

Table 5.2. Descriptive statistics for GSI and for dependent variables used in the MANOVA with direct-sun treatment included. Mortality and deformity rates are given as the proportion (before arcsine square root transformation) of wood frog (*Rana sylvatica*) eggs or larvae that died or were deformed per group of eight.

Variable	N	Mean	StDev
GSI	216	0.2687	0.0400
Egg Mortality Rate	216	0.0440	0.0787
Larval Mortality Rate	216	0.4632	0.3160
Deformity Rate	216	0.1030	0.1297

Table 5.3. Descriptive statistics for dependent and independent variables used in the MANOVA with direct-sun treatment omitted. Mortality and deformity rates are given as the proportion (before arcsine square root transformation) of wood frog (*Rana sylvatica*) eggs or larvae that died or were deformed per group of eight.

Variable	N	Mean	StDev
GSI	144	0.2687	0.0400
Egg Mortality Rate	144	0.0495	0.0799
Larval Mortality Rate	144	0.3698	0.3232
Deformity Rate	144	0.0764	0.1156

between GSI and UV treatment (Pillai's criterion, p=0.031). Using a Bonferroni correction, our α level for univariate tests was 0.017 to maintain an overall experimentwise error of 0.05. Examination of the univariate tests associated with MANOVA revealed that egg mortality rate was significantly associated with genetic diversity, but not UV treatment or the interaction term (Table 5.4). Larval mortality rate was significantly related to UV treatment, GSI, and the interaction term. Deformity rate was significantly related to GSI and UV treatment, but not the interaction term. The boxplot in Figure 5.4 contrasts deformity and mortality rates between the two populations from our study with the highest and lowest genetic diversities.

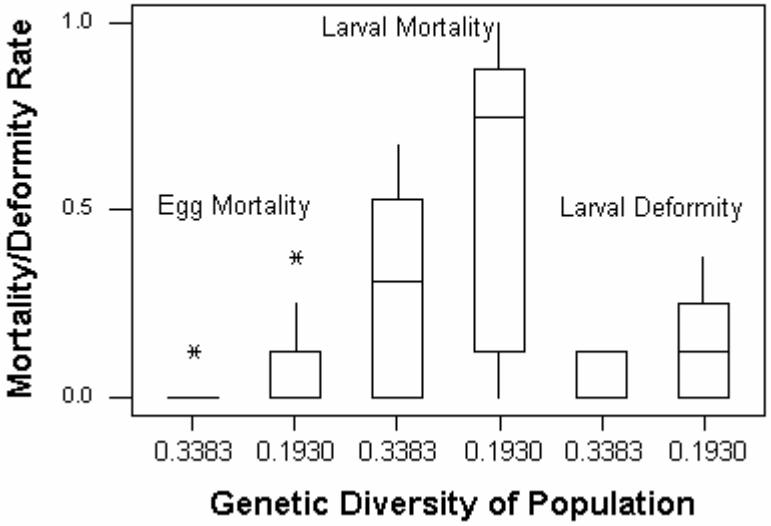
When we omitted the direct-sun treatment from our analyses to account for the possible confounding effect of water temperature, MANOVA still showed a significant relationship between our response variables and UV treatment (Pillai's criterion, p=0.000), GSI (Pillai's criterion, p=0.000), and the interaction term (Pillai's criterion, p=0.047). The univariate tests showed that egg mortality rate was significantly related to GSI (p=0.000), but not UV treatment or the interaction term. Larval mortality rate was strongly related to GSI, UV treatment, and the interaction term. Deformity rate was significantly related to UV treatment (p=0.000) but not GSI or the interaction term (Table 5.5).

Table 5.4. Results of the univariate tests (ANOVAs) associated with MANOVA (direct-sun treatment included) for the effects of genetic diversity (GSI) and UV-B treatment and their interaction on mortality and deformity rates of wood frog (*Rana sylvatica*) eggs and larvae. Alpha levels were Bonferroni-corrected to 0.017. Significant effects are shown in boldface.

Response Variable	Independent Variable	DF	SS	MS	F	P
Egg Mortality Rate	**GSI**	**11**	**1.2213**	**0.1110**	**4.26**	**0.000**
	UV Treatment	2	0.0932	0.0466	1.79	0.170
	GSI*UV Treatment	22	0.8077	0.0367	1.41	0.116
	Error	180	4.6960	0.0261		
Larval Mortality Rate	**GSI**	**11**	**1.7770**	**0.1616**	**5.97**	**0.000**
	UV Treatment	**2**	**19.0102**	**9.5051**	**351.43**	**0.000**
	GSI*UV Treatment	**22**	**1.1088**	**0.0504**	**1.86**	**0.014**
	Error	180	4.8684	0.0270		
Deformity Rate	**GSI**	**11**	**1.0860**	**0.0987**	**2.36**	**0.009**
	UV Treatment	**2**	**3.3718**	**1.6859**	**40.36**	**0.000**
	GSI*UV Treatment	22	0.5882	0.0267	0.64	0.891
	Error	180	7.5183	0.0418		

Figure 5.4. Egg mortality, larval mortality, and deformity rates of wood frogs (*Rana sylvatica*) from populations with the highest and lowest genetic diversities, as calculated by the Gini Simpson Index. Panels A and B show these data with the direct sun treatment included (A) and excluded (B). The bottom of a box is the first quartile (Q1), the top of a box is the third quartile (Q3), and the horizontal line across a box is the median. Whiskers extend to the highest and lowest values within the range defined by: lower limit = Q1 - 1.5(Q3-Q1); upper limit = Q3 + 1.5(Q3-Q1). Asterisks represent outliers.

A.


Mortality/Deformity Rate
1.0
0.5
0.0
Larval Mortality
Egg Mortality
Larval Deformity
0.3383
0.1930
0.3383
0.1930
0.3383
0.1930
Genetic Diversity of Population

B.

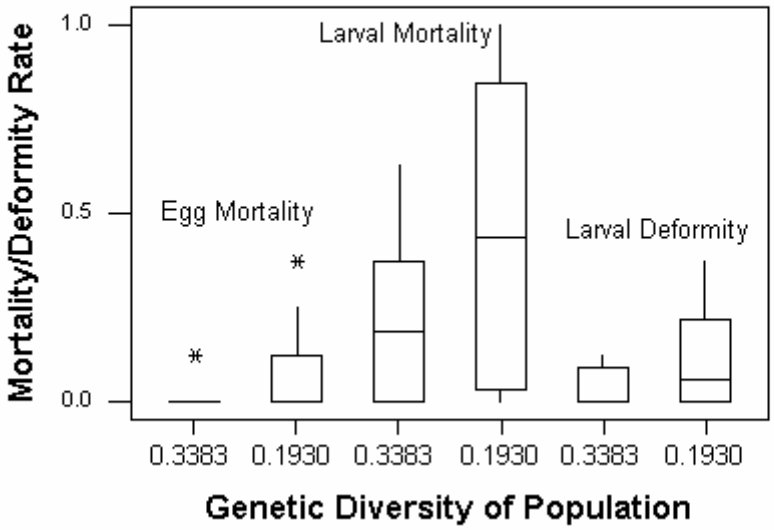

Mortality/Deformity Rate
1.0
0.5
0.0
Larval Mortality
Egg Mortality
Larval Deformity
0.3383
0.1930
0.3383
0.1930
0.3383
0.1930
Genetic Diversity of Population

Table 5.5. Results of the univariate tests (ANOVAs) associated with MANOVA (direct-sun treatment omitted) for the effects of genetic diversity (GSI) and UV-B treatment and their interaction on mortality and deformity rates of wood frog (*Rana sylvatica*) eggs and larvae. Alpha levels were Bonferroni-corrected to 0.017. Significant effects are shown in boldface.

Response Variable	Independent Variable	DF	SS	MS	F	P
Egg Mortality Rate	**GSI**	**11**	**1.1653**	**0.1059**	**3.78**	**0.000**
	UV-Treatment	1	0.0136	0.0136	0.49	0.487
	GSI*UV-Treatment	11	0.2497	0.0227	0.81	0.629
	Error	120	3.3598	0.0280		
Larval Mortality Rate	**GSI**	**11**	**0.8509**	**0.0774**	**2.62**	**0.005**
	UV-Treatment	**1**	**14.0982**	**14.0982**	**477.56**	**0.000**
	GSI*UV-Treatment	**11**	**0.8083**	**0.0735**	**2.49**	**0.008**
	Error	120	3.5426	0.0295		
Deformity Rate	GSI	11	0.5493	0.0499	1.47	0.151
	UV-Treatment	**1**	**2.2562**	**2.2562**	**66.50**	**0.000**
	GSI*UV-Treatment	11	0.3702	0.0337	0.99	0.458
	Error	120	4.0715	0.0339		

DISCUSSION

Our data indicate that a synergistic interaction between UV-B radiation and genetic diversity can influence amphibian fitness. This is the first time such an interaction between genetic diversity and a potential environmental stressor has been demonstrated. Both analyses with and without the direct-sun treatment found significant interaction effects. Univariate analyses demonstrated that the interaction between genetic diversity and UV-B radiation exposure significantly increased mortality rates in wood frog (*Rana sylvatica*) larvae, but not egg mortality or larval deformity rates.

In addition to demonstrating, for the first time, such an interaction effect, this study adds to the body of work reporting negative effects of natural levels of UV-B radiation, alone, on amphibians. Our analyses found significant effects of UV-B radiation on survival of larval wood frogs. The mean larval mortality rate was nearly eight times higher, and mean deformity rate was nearly ten times higher, under a UV-B transmitting filter (acetate) than under a UV-B blocking filter (Mylar) (Table 5.1). Egg mortality rate was not significantly affected by UV treatment. Other studies have similarly documented that amphibian embryos are less susceptible to UV-B-related mortality than are larval amphibians (Crump et al. 1999; Tietge et al. 2001).

In our study, cloudy conditions limited UV-B exposure during the first four days of the experiment (Figure 5.3). Because most eggs had hatched by the seventh day, embryos may not have experienced sufficient levels of UV-B to

result in significant mortality differences among treatments. Further, the gelatinous membrane surrounding the embryo may offer some protection against UV-B (Licht and Grant 1997; Ovaska et al. 1997).

We also found evidence supporting our hypothesis that genetic diversity, alone, may affect egg mortality, larval mortality, and deformity rates in wood frogs (Tables 5.4 and 5.5; Figure 5.4). Although the relationship between egg mortality and genetic diversity was significant, the difference in mean egg mortality rates between the populations with the highest and lowest genetic diversities was small, as overall mortality rate at the egg stage was low. The population with the highest genetic diversity had a mean egg mortality rate of 0.021, and the population with the lowest genetic diversity had a mean egg mortality rate of .056. Differences in larval mortality rates and deformity rates may be more biologically significant (Figure 5.4).

With shallow water exposed to direct sunlight, our experiment was designed to maximize exposure of eggs and larvae to natural UV-B radiation. In natural ponds, larvae can behaviorally regulate their UV-B exposure, finding deeper or more shaded areas of a pond. Further, natural pond water typically has high levels of dissolved organic compounds, reducing penetration of ultraviolet radiation into the water column. Therefore, it is likely that any UV-B-related mortality and deformity rates of wood frog tadpoles in nature would be lower than those observed in our experiment.

Most studies that have found a significant effect of natural levels of UV-B radiation have found the effect at high elevations or low latitudes, where UV-B

radiation is more intense. This experiment has shown that natural levels of UV-B radiation at a relatively low altitude (269 m) in central Ohio, United States, can significantly affect survival of wood frog tadpoles. Further, we have demonstrated that populations with lower levels of genetic diversity may be more susceptible to the effects of UV-B radiation than those with higher genetic diversities. It is possible that genetic diversity is interacting with a variety of other factors to influence amphibian survivorship. Future research should consider the genetic diversity of amphibian populations when attempting to understand declines.

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