5-2023

Analysis of the Population Structure and Migration Habits of the Northern Leopard Frog (Lithobates Pipiens) at Midewin National Tallgrass Prairie

Gretchen A. Brinkman
Olivet Nazarene University, gretie.bird@gmail.com

Analysis of the Population Structure and Migration Habits of the Northern Leopard Frog (*Lithobates Pipiens*) at Midewin National Tallgrass Prairie

Gretchen A. Brinkman

ACKNOWLEDGEMENTS

Special thanks are due to the Pence-Boyce Research Grant bestowed on this project in the summer of 2022, which financially supported essential research expenses necessary to complete this project. I would also like to express appreciation for the Hippenhammer Grant providers for granting this project consistent financial support throughout the years of its development and completion. Thanks also to the University of Utah's Core Genomics Facility for working with me to supply genotyping/sequencing data for such a large data set. Without this help, the level of analysis required for this research would have been nearly unattainable. I also greatly appreciate the constant support the faculty and members of the Honors Program at Olivet Nazarene University have given me throughout my entire undergraduate experience. All your patience, guidance, and pushes for improvement are what have allowed this thesis to come to fruition—something I will always be grateful for.

Finally, my deepest thanks to my mentor, Dr. Troendle, who has guided through highs and lows, supported me during failures, and helped me tremendously with the sample collections required to complete this project. Not only has working with my mentor allowed me to complete a project of this size, but Dr. Troendle has also cultivated a passion for this area of study within me. I cannot thank you enough for being the guiding light towards my creation of a research project that I am immensely proud of.

ABSTRACT

Massive amphibian declines of recent years have pushed researchers to pursue population genetics surveys and assess the status of these essential components of many ecosystems. The Northern Leopard Frog (*Lithobates pipiens*) has continuously experienced population declines across the continental United States due to a combination of habitat losses and environmental changes. Midewin National Tallgrass Prairie houses a considerable portion of *L. pipiens* in Illinois, and the two creek watersheds studied within this location provide ideal conditions to support these animals. Because this prairie restoration project is a relatively recent development, further assessments regarding the population structure and degree of migration within these *L. pipiens* populations are needed. Analysis of seven microsatellite loci specific to *L. pipiens* revealed significant deviation in Hardy-Weinberg Equilibrium for most populations, supporting the large estimated migration rate of 7.197 migrants per generation. Genotype clustering analysis also implied large instances of gene flow between populations—a likely effect of many migration events. Additionally, high levels of genetic diversity (H_o = 0.792-0.873) and total private alleles (46) were surprisingly maintained in all observed populations, suggesting large population sizes. These findings indicate the presence of an incredibly large, dynamic, and biodiverse *L. pipiens* population that has not previously been defined in this area. With this information, further protection of the Midewin populations can be achieved to avoid the genetic diversity and population size loss associated with *L. pipiens* groups located in the western United States.

Keywords: Population genetics, *L. pipiens*, Midewin National Tallgrass Prairie, Illinois, migration, microsatellite loci, gene flow, genetic diversity

INTRODUCTION

Scientists have recorded global amphibian population declines since the 1980s and early 1990s, when concerns about amphibian disappearances influenced an increase in observational studies (Hayes & Jennings 1986). At around this time, reports of over 500 frog and salamander populations considered their declines to be of special conservation concern (Blaustein & Kiesecker 2002). In a more recent study, upwards of 1,000 data-deficient amphibian species were determined to be threatened with extinction (González-del-Pliego et al. 2019). Amphibians play many important roles in ecosystems, and the continual population losses will have critical future implications on ecological function (Rogers and Peacock 2012). These consistent downward trends have greatly effected biodiversity and species health, leading to urgent calls for population research and conservation efforts (Seaborn & Goldberg 2020; González-del-Pliego et al. 2019; Beebee & Griffiths 2005; Blaustein & Kiesecker 2002). Additionally, because they are frequently exposed to various contaminants when using ponds to reproduce, amphibian population trends are often used as biological indicators. In these cases, researchers can preemptively identify potential dangers in an environment by observing how amphibian populations respond to them (Smith 2003). Ultimately, the scientific and environmental purpose of amphibians—in addition to their intrinsic value, should encourage further goals of protecting their biodiversity worldwide.

Specifically within the class Amphibia, species in the family Ranidae (or true frogs) that are native to North America have experienced repeated instances of local population declines and range reductions since the late 1800s (Hayes & Jennings 1986). As a member of the Ranidae family, the

Northern Leopard Frog (*Lithobates pipiens*) has not escaped this fate. Many researchers have reported declines of these populations in Nevada (Rogers & Peacock 2012), Washington state (Seaborn & Goldberg 2020), and the national parks of South Dakota and Wyoming (Smith 2003). In some areas, previously identified populations have disappeared completely (Werner 2003). In 2012, a petition to list the *L. pipiens* as threatened in the western United States under the Endangered Species Act of 1973 was submitted to the United States Fish and Wildlife Service (U.S. Fish and Wildlife Service 2012). After reviewing a previous petition from 2009, the U.S. Fish and Wildlife Service concluded that the proposed threatened status *L. pipiens* was not warranted in this twelve-month finding, regardless of the concerning population losses in western states (U.S. Fish and Wildlife Service 2012). Because *L. pipiens* is experiencing declines, continuous research on population health must be conducted to potentially aid their protection from further losses.

Likely causes of ranid frog declines

Many hypotheses have been brought forward to explain the dramatic and continuous amphibian declines worldwide. Some of these hypotheses include competition, habitat alteration, predation, exploitation, contaminants, and pathogens as major contributors to amphibian population loss (Hayes & Jennings 1986). For example, the resource competition and predation of North American ranid frogs by the American Bullfrog (*Lithobates catesbeianus*) has been noted to have the potential to eliminate *L. pipiens* populations, along with many other factors (Hayes & Jennings 1986). Habitat changes are the most probable cause of the loss of western ranid populations. This is frequently observed as a loss of suitable nursery environments and refuges for hibernating adult frogs, resulting in a major negative impact on the species' overall survival (Rogers & Peacock 2012; Hayes & Jennings 1986). Similarly, excessive road traffic can also have an impact on amphibian migrations critical to locating suitable breeding environments (Beebee & Griffiths 2005). Dynamic climate changes are another instance of habitat change, and these changes can heavily influence amphibian populations by causing a varying yet detectable effect on amphibian breeding phenology (Beebee & Griffiths 2005). Additionally, droughts and unusually dry seasons have been speculated to even cause the extinction of some non-ranid amphibians and could be yet another origin of stress on ranid populations (Smith 2003). Finally, viruses and fungal infections are another probable influence on *L. pipiens* populations, with these types of diseases being frequently identified in mass mortality events (Smith 2003). Chytridiomycosis, one of these lethal fungal infections, has been blamed for worldwide amphibian declines (Smith 2003; Rogers & Peacock 2012) and has been known to negatively impact some *L. pipiens* populations as well (Voordouw et al. 2010). Because of the damage these external factors can have on any species of ranid frog, it is incredibly important to understand what could be influencing populations while conducting a genetic assessment on *L. pipiens* populations.

The Northern Leopard Frog (*Lithobates pipiens*)

L. pipiens is a species of frog taxonomically grouped into the family Ranidae. These frogs are characterized by round, white-margined dark spots on the dorsal side and dorsolateral folds with some occasional pigment variations between isolated populations (Rogers & Peacock 2012). *L. pipiens* is a slim, medium-sized frog with average weights ranging from 16 to 80 grams and average lengths ranging from 5 to 11 centimeters in adults (U.S. Fish and Wildlife Service, n.d.). This species of frog has previously been known to range from the northern half of the United States far into the north of Canada with some of the southernmost populations located in the western

United States (Smith 2003). Populations have been steadily declining nationwide, however, and the presumed distribution of these frogs has been dramatically altered after extensive population research (Seaborn & Goldberg 2020; Rogers & Peacock 2012; Smith 2003; Werner 2003). With sightings of living populations varying so wildly between researchers, a recent, more accurate map of *L. pipiens* distribution has yet to be accepted.

Previous research has identified that the migration habits of *L. pipiens* generally consist of an initial movement from overwintering sites to breeding sites in the spring, then a dispersal to grassy meadows during the warm summer, and finally a fall migration where adults return to overwintering sites once again (Smith 2003). Migrations between upland sites occur seasonally within their home range, and the frogs often prefer habitats with adequate moisture during summer temperatures to aid in reproductive success (Smith 2003). Since *L. pipiens* generally have breeding periods from the middle of March through the entirety of May, shallow water systems such as streams, ponds, and creeks help retain necessary moisture (Illinois Department of Natural Resources 2020).

Survey locations

As mentioned previously, *L. pipiens* prefers to inhabit areas with adequate moisture and shallow water systems to aid in survival in summers and breeding periods. Within Midewin National Tallgrass Prairie, where these frogs are abundant, both aquatic and terrestrial environments fulfill the requirements necessary for *L. pipiens* survival. Midewin National Tallgrass Prairie was established in 1996, making it one of the first national tallgrass prairies developed within the United States (The Nature Conservancy, n.d.). The location is a large-scale prairie restoration project aiming to restore approximately 20,000 acres of farmland into functional tallgrass prairie that can support a variety of native or previously threatened species (The Nature Conservancy, n.d.). Because of the land's protection of environmentally sensitive and endangered midwestern plant and animal species, Midewin also has the continued protection of the Nature Conservancy (The Nature Conservancy, n.d.). The area of Midewin National Tallgrass Prairie is mostly separated into two distinct watershed environments, Grant Creek and Prairie Creek, the predominant *L. pipiens* collecting locations. Prairie Creek runs through a majority of Midewin National Tallgrass Prairie before meeting with the Kankakee River. Alternatively, only a portion of Grant Creek runs through Midewin before also merging into the Kankakee River. Before the dry summers, both creeks branch out, forming small streams and ponds throughout Midewin National Tallgrass Prairie. This event creates optimal breeding sites for *L. pipiens* populations throughout the tallgrass prairie.

Research objective

Identifying the environmental influences on populations is essential to further understanding how differing conditions can shape genetic variation within a species. In restoration areas like Midewin National Tallgrass Prairie, *L. pipiens* has populations that are geographically isolated by watershed environments. These individual watersheds act as experimental replicates with varying resources, competing populations, and levels of contamination. Aquatic-bound organisms, like *L. pipiens*, often experience a strong influence in their population structure due to their heavy reproductive reliance on water sources and the terrestrial areas draining into these water sources (Loxterman & Keeley 2012). Although previous research has identified significant genetic diversity in other aquatic organisms separated by watersheds (Seaborn & Goldberg 2020; Kremer et al. 2017;

Loxterman & Keeley 2012; Rogers & Peacock 2012), there is a lack of knowledge on how *L. pipiens* at Midewin National Tallgrass Prairie is affected by these barriers, specifically because the two surveyed watersheds are geographically close—with a small overall survey radius of about 1.5 km (Figure 1, Table 1). The goal of this research is to determine the population structure of the Prairie Creek and Grant Creek watersheds at Midewin National Tallgrass Prairie and to assess the degree of migration between four *L. pipiens* subpopulations within these two watersheds environments.

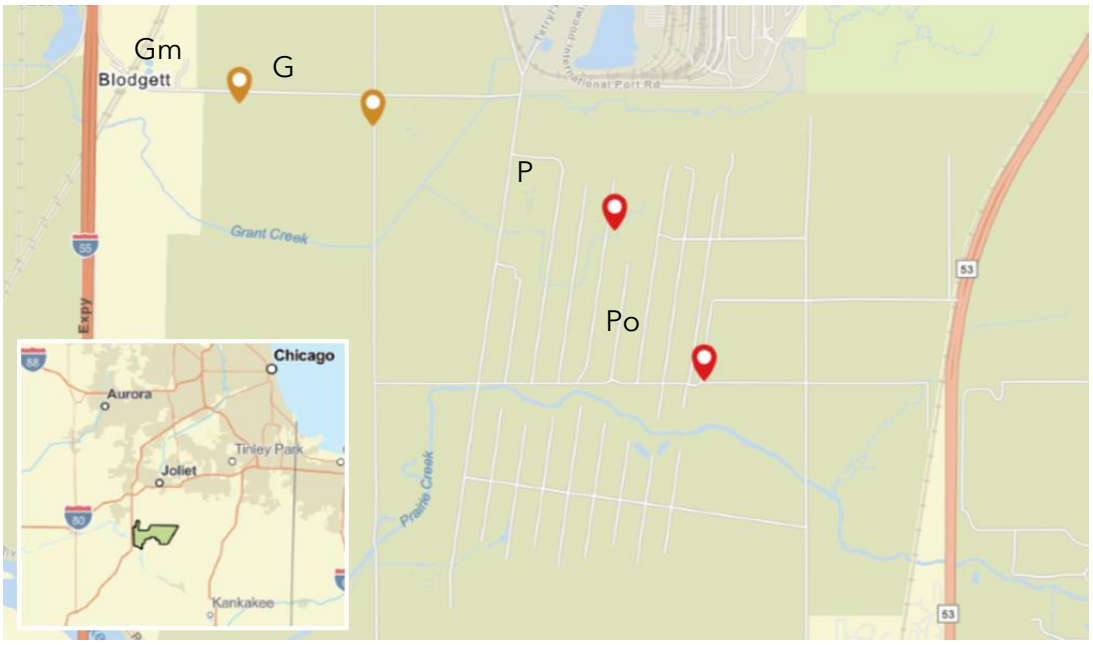

Figure 1: Map of Midewin National Tallgrass Prairie
L. pipiens survey locations at Midewin National Tallgrass Prairie (Wilmington, IL, USA). Included are the Prairie Creek (P and Po) and Grant Creek (Gm and G) watershed populations.

METHODOLOGY

Sample collection

At Midewin National Tallgrass Prairie (Wilmington, Illinois), toe clip and dorsal dermal swab collections of *L. pipiens* were obtained within two locations at each of the two observed watershed regions—Grant Creek (labelled *G* and *Gm*) and Prairie Creek (labelled *P* and *Po*). *L. pipiens* specimens were collected using dip nets from a variety of aquatic and terrestrial environments. After the frogs were captured with dip nets and then restrained with gloved hands, sterile omniswabs were used to collect the frog's dermal cells and surgical grade scissors were used to collect 2-3 mm of toe tissue from each organism. To collect an adequate quantity of dermal cells, the sterile omniswabs were run back and forth along the frog's dorsal surface five times (Ringler 2018) and then ejected into a 1.5 mL microcentrifuge tube similarly to the toe clip samples to be stored at 0°C until further analysis (Prunier et al., 2012). A total of 71 frogs were sampled at Midewin using these methods; of these 71 frogs, 36 were sampled in the Prairie Creek watershed and 35 were sampled in the Grant Creek watershed. Additionally, for easy identification of each frog's exact location, the latitude and longitude coordinates of the collection site were recorded for each specimen (Appendix A).

Table 1: Survey site distances

Pairwise representation of approximate distances in meters between each of the four *L. pipiens* survey locations at Midewin National Tallgrass Prairie.

	G	Gm	P
Gm	739.75 m	—	—
P	1502.26 m	2191.75 m	—
Po	2407.46 m	3019.6 m	1008.72 m

DNA extraction and PCR amplification

The DNA extraction of toe clip samples utilized QIAGEN's DNeasy® Blood and Tissue Kit and the corresponding tissue extraction protocol provided in the handbook. Dermal swab samples were extracted using the same kit; however, the kit's alternative protocol specifically for omniswabs was used instead. After extraction, samples were only used in the following downstream reactions if they surpassed the limit of 5 ng/µL DNA in 50 µL aliquots. DNA concentrations of each sample were quantified using the ThermoFisher NanoDrop™ UV-vis spectrophotometer. A total of seven fluorescently labelled microsatellite primer pairs for *L. pipiens* were used to amplify the extracted DNA samples as shown in Table 2 (Hoffman et al. 2003). These seven microsatellite primers were chosen for both their previous successful uses in many studies involving the genotyping of *L. pipiens* and for their relatively similar annealing temperatures. Each PCR reaction mixture contained 0.5 U Promega™ Taq Polymerase, 1 Promega™ PCR buffer mix, 1.5 mM $MgCl_2$, 0.1 mM of each dNTP, and 0.8 mM of each primer (Hoffman et al. 2003). The reactions occurred in 25 µL volumes that contained 1-6 µL of previously extracted template DNA to ensure approximately 30 ng of DNA were in each reaction. The specific parameters for the subsequent PCR amplification are as follows: an initial cycle at 94°C (2 min); 36 cycles of 94°C (30s), then the specific annealing temperature (30s), then 72°C (1 min); and a final extension of 72°C (2 min). After completion of PCR, 5 µL of each reaction was run through 2% agarose gel to confirm that DNA amplification had occurred correctly.

Table 2: Microsatellite primer pairs

Microsatellite sequence information for *L. pipiens* as described by Hoffman et al. in 2003. The annealing temperature (T_a), number of base pairs, and number of alleles are also provided. An asterisk indicates a 5' fluorescent-labelled primer.

Locus	Sequences (5'-3')	T_a (°C)	No. BP	No. alleles
Rpi100	*GGACTGGGGAGTTTCATCC	62	174-222	9
	AAGTCCTATCCCTAGTATGATACAC			
Rpi101	*AACGCACAGCAAAGGAGTAA	62	161-201	9
	CAAGGGATGACTTAGAAAGGG			
Rpi102	*GTGTGTGTGTTTATTTACTG	54	118-152	5
	CTTCCATTTTAATTGTGT			
Rpi103	*TTGAACAGGTATATCTAATAAAGT	56	135-211	10
	TGCTTCCATTTTAATTGTGTC			
Rpi104	*CAGGGCAATGTGGAATGTGGA	62	226-230	2
	AGGACCACTCAGGTACAAAATGTTCT			
Rpi107	*GTGGTCTTATTACATTTCTTAC	57	161-223	8
	GCCAGTGAGTGTAGATAGAT			
Rpi108	AAATAACTCCTGGGAAATGT	57	272-298	7
	*CATCCCAAAGAGTCATATC			

DNA genotyping and statistical analysis

After amplification was confirmed, each sample was prepared as per the request of the University of Utah's Genomics Core Facility for genotyping. Once the genotypes were obtained, population analysis of the *L. pipiens* at Midewin National Tallgrass Prairie was conducted using a combination of the software packages GENEPOP (Rousset 2008, Raymond & Rousset 1995; version 4.8.2), GenAlEx (Peakall & Smouse 2006, 2012; version 6.51), and STRUCTURE (Hubisz et al. 2009; Falush et al. 2007, 2003; Pritchard et al. 2000; version 2.3.4). Each program calculated various measurements needed to assess genetic diversity, population structure, and degrees of migration.

GENEPOP was utilized to test each *L. pipiens* population for Hardy-Weinberg Equilibrium (HWE), determine the presence of linkage disequilibrium (LD) among all loci, and calculate the average number of *L. pipiens* migrants per generation. HWE is a denotation given to populations that have exhibited genotype frequencies that remain constant between subsequent generations due to a lack of external, genotype disturbing events such as mutation, migration, selection pressure, or genetic drift (Edwards 2008). This is one of the most commonly used methods of confirming genetic stability, and any deviations of HWE usually indicates events that have decreased heterozygosity, usually via genetic drifting. Increased observed heterozygosities can also occur in response to genotype disturbing events—typically in instances of high mutation or migration.

Measurements of LD are used in combination with HWE assessments and provide information on the independence of alleles. When there is no evidence for LD, populations are often simultaneously in HWE, and a proportionally large heterozygote frequency is maintained (Slatkin 2008). HWE tests were conducted using the Markov chain method using 1000 batches and 10,000 iterations per batch. The presence of LD was determined using the same batch and iteration per batch number. Finally, the number of migrants per generation, when within the constraints of one to ten migrants per generation as described by Mills and Allendorf, determines whether the risk for deleterious allele fixation or genetic drift events is decreased (1996).

The GenAlEx program was used to calculate F-statistics (F_{is}, F_{it}, and F_{st}), Nei's G-statistics (Nei 1973), the number of different alleles (N_a), the number of effective alleles (N_e), the number of private alleles of each locus and population, the number of migrants (Nm) per locus, and the expected (H_e) and observed (H_o) heterozygosity. Most of these measurements were taken to observe the levels of genetic diversity between populations and microsatellite loci, and in the case of the F-statistics and Nei's G-statistics, the genetic differences between the sampled locations.

Finally, the STRUCTURE program was utilized to develop an estimated genotype cluster (k) bar plot of the subpopulations' allele distributions using Bayesian analysis. The orientation and number of k genotype clusters aids in determining the levels of gene flow between populations and to what degree any instances of genetic isolation occur at. The results from this program are primarily used to support and connect the data received from the previous two programs, GENEPOP and GenAlEx.

RESULTS

Population genetic diversity

Genotypes of all *L. pipiens* samples from each sampling location at Midewin National Tallgrass Prairie (populations abbreviated *G, Gm, P,* and *Po*) were obtained for each microsatellite locus. The average expected heterozygosity (H_e) of all seven loci across each population was at expected levels of approximately 84.3-88.2%, but they were slightly lower than those noted for other northeastern *L. pipiens* populations at 89.3-91.5% (Hoffman et al. 2004). Average observed heterozygosity (H_o) ranged from 79.2-87.3%. These two values (H_e and H_o) were then compared to assess whether each population was in Hardy-Weinberg Equilibrium (HWE), and by using GENEPOP and GenAlEx software, it was revealed that most of the sampled *L. pipiens* populations appeared to be significantly out of HWE. Significant deviations from HWE existed in populations *Gm* (p = 6.981E-03), *P* (p = 3.80E-08), and *Po* (p = 6.10E-04), and only one population (*G*) was in HWE (p = 0.148) as shown in Table 3. Additionally, the GENEPOP and GenAlEx programs revealed little evidence for the presence of linkage disequilibrium (*LD*) between any alleles.

Table 3: GenAlEx heterozygosity results

The mean (SE) over all microsatellite loci in each of the four *L. pipiens* locations at Midewin National Tallgrass Prairie (Wilmington, IL, USA). *N* = population size, N_a = number of different alleles, N_e = number of effective alleles, H_o = observed heterozygosity, and H_e = expected heterozygosity. *Note deviations in HWE:* *** $p \leq 5E\text{-}08$, ** $p \leq 0.005$, * $p \leq 0.05$

Pop	N	N_a	N_e	H_o	H_e
G	19.000 (0.000)	15.286 (1.409)	9.560 (1.073)	0.872 (0.030)	0.882 (0.021)
Gm	15.714 (0.184)	12.286 (1.229)	7.965 (0.882)	0.873 (0.023) *	0.862 (0.019)
P	15.857 (0.404)	11.857 (1.421)	8.360 (1.275)	0.792 (0.057) ***	0.847 (0.040)
Po	13.857 (0.261)	12.000 (1.134)	7.748 (1.037)	0.852 (0.046) **	0.843 (0.038)
Total	16.107 (0.376)	12.857 (0.672)	8.408 (0.525)	0.847 (0.021) ***	0.859 (0.015)

Inbreeding coefficients (F_{is}) were also obtained for all populations across each of the seven microsatellite loci and ranged from -0.066 to 0.171 (mean [SE] = 0.011 [0.030]) with Rpi107 representing the maximum. Measurements of F_{it} were also taken across each locus and ranged from -0.017 to 0.204 (mean [SE] = 0.045 [0.029]) with Rpi107 once again representing the maximum (Table 4). The fixation index was taken for each microsatellite locus, appearing slightly larger for each locus rather than each population (mean [SE] = 0.035 [0.003]; Table 4). Private alleles were present in every surveyed *L. pipiens* population (Table 5).

Table 4: F-statistic results

The F-statistics of all *L. pipiens* populations at Midewin National Tallgrass Prairie (Wilmington, IL) for each microsatellite locus. F_{is} = inbreeding coefficient of individual to subpopulation, F_{it} = inbreeding coefficient of individual to total population, F_{st} = fixation index, *Nm* = number of migrants.

Locus	F_{is}	F_{it}	F_{st}	Nm
Rpi100	-0.027	0.004	0.029	8.292
Rpi101	-0.025	0.008	0.032	7.491
Rpi102	0.051	0.083	0.034	7.083
Rpi103	-0.039	0.000	0.038	6.269
Rpi104	-0.066	-0.017	0.046	5.193
Rpi107	0.171	0.204	0.040	5.931
Rpi108	0.009	0.033	0.024	10.121
Mean	0.011	0.045	0.035	7.197
SE	0.030	0.029	0.003	0.623

Table 5: Private alleles

Total number of private alleles found within each sampled *L. pipiens* population.

	Population			
	G	**Gm**	**P**	**Po**
Total	18	9	5	14

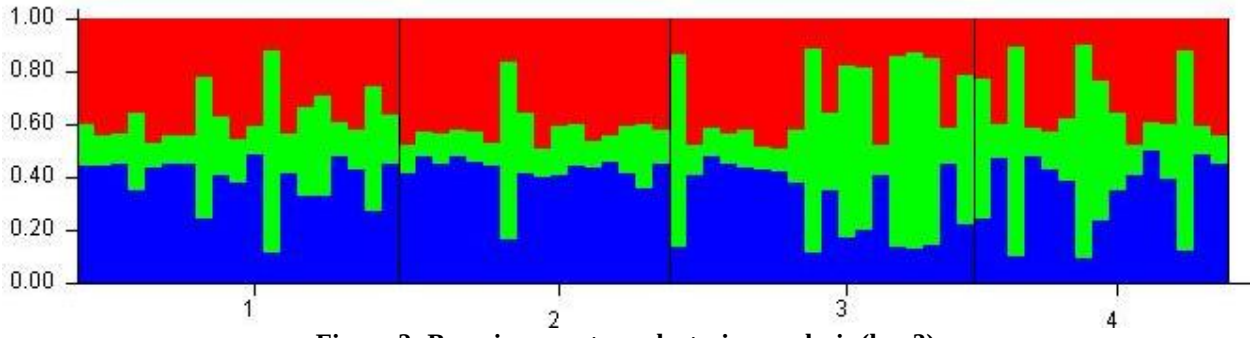

Figure 2: Bayesian genotype clustering analysis (k = 3)

Bayesian genotype clustering for the *L. pipiens* populations at Midewin National Tallgrass Prairie. Population 1 is *G* of Grant Creek, population 2 is *Gm* of Grant Creek, population 3 is *P* of Prairie Creek, and population 4 is *Po* of Prairie Creek.

Population structure and degree of migration

A pairwise F_{st} test reveals consistently low values throughout all compared populations (0.018-0.029), indicating high levels of interbreeding between the sampled locations (Table 6). This is further supported through STRUCTURE Bayesian genotype clustering (k = 3) of the populations, showing evidence of population differentiation (Figure 2). A pairwise test based on Nei's measurements of genetic identity and distance revealed relatively high levels of similarity between most populations (Table 7). An average of approximately seven migrants were estimated to have occurred between all four *L. pipiens* populations per generation (Table 4).

Table 6: Genetic variance results

Pairwise population matrix of genetic variance (F_{st}) values between the four sampled locations of *L. pipiens* to measure population structure.

	G	**Gm**	**P**	**Po**
G	0.000			
Gm	0.018	0.000		
P	0.021	0.028	0.000	
Po	0.025	0.029	0.020	0.000

Table 7: Nei's measurements

Pairwise population matrix of Nei's genetic distance below diagonal and Nei's genetic identity above diagonal.

	G	Gm	P	Po
G	-	0.752	0.744	0.686
Gm	0.285	-	0.670	0.661
P	0.296	0.400	-	0.765
Po	0.377	0.414	0.268	-

DISCUSSION

Population genetic diversity

Although average H_e for all microsatellite loci across each population was lower than previously recorded measurements of *L. pipiens* populations in the northeastern United States (0.893-0.915; Hoffman 2004), Midewin populations exhibited a relatively high H_e range of 0.843 in population *Po* to 0.882 in population *G* (Table 3). Additionally, the H_e of Midewin populations was quite similar to those measured from North Dakota (0.81-0.88; Mushet et al. 2013) and were noticeably higher than the H_e of Nevada's drastically declining remnant populations (0.411-0.482; Rogers and Peacock 2012). These generally high H_e values for *L. pipiens* at Midewin indicate similarly high levels of genetic diversity within all populations. From this, population *G* was found to have the highest H_e and subsequently the highest genetic diversity of the four populations. The H_o for populations *Gm*, *P*, and *Po* significantly diverted from the corresponding H_e, indicating Hardy-Weinberg Disequilibrium (HWD)—that of which can be induced by mutations, selection pressures, migrations, or genetic drifting events within these populations (Table 3). Population *G*, on the other hand, was the only tested subpopulation that remained in HWE. Of the populations out of HWE, two (*Po* and *Gm*) showed significantly higher H_o while one (*P*) showed significantly lower H_o which could both be influenced by the events mentioned previously (Table 3).

Opposing evidence for genetic drifting events being the cause of the observed HWD was indicated by the low F-statistics (F_{it} and F_{st}) average for all populations. These low, and sometimes even negative, values imply high levels of genetic diversity that are consistent with low genetic differentiation of the individual or subpopulation to the total sampled group (Table 4). Essentially, because each population has high genetic diversity in these circumstances, it is unlikely that they are recovering from or currently experiencing a genetic drifting event. This is because in those scenarios, very few genotypes survive and drastically lower overall genetic diversity. Additionally, the inbreeding coefficients (F_{is}) for each population also opposed the possibility for genetic drift since they were quite low for each microsatellite locus, consistent with previous conclusions of the relatively high genetic diversity within Midewin populations. Low F_{is} values, like those shown for all loci, indicate a general avoidance of inbreeding and overall high levels of heterozygosity for each locus (Table 4). Private allele analyses indicated a larger number of rare genotypes—thus

larger population sizes and higher preservation of diversity—present in all measured populations at Midewin National Tallgrass Prairie (Table 5).

Population structure and degree of migration

Each sampled population of the Prairie and Grant Creeks largely appear to be interbreeding (rather than inbreeding) between each other when observing pairwise F_{st} values (0.018-0.029, Table 6). Compared to contemporary measurements of genetic divergence in other northeastern *L. pipiens* populations (0.043 average), the surveyed Midewin populations were less genetically differentiated (Hoffman et al. 2004). This difference can likely be attributed towards the small survey radius of about 1.5 km compared to the larger distances of the study in 2004 by Hoffman et al., and this data largely supports the likelihood that migration could be causing HWD in these populations. Additionally, *L. pipiens* populations in the northwestern United States, particularly the remnant populations of Nevada, experienced some instances of low pairwise F_{st} values between sampling locations of a similar distance as the Midewin populations (F_{st} = 0.0124), and these were considered indicative of minimal differentiation (Rogers & Peacock 2012). Next, pairwise representations of Nei's original measurements on genetic distance and identity were taken from the GenAlEx program (Table 7). These values also revealed generally high levels of relative genetic similarity between all sampled locations at an average of 0.713. This average opposes the likelihood of a barrier (whether that be sympatric or allopatric) between populations that is preventing interaction and encouraging subsequent speciation. Of the Nei's genetic identity measurements, the two Prairie Creek locations were the most genetically similar, whereas populations *Gm* and *Po* were the least similar (Table 7). The least genetically similar populations, *Gm* and *Po*, were simultaneously the farthest in distance from one another (≈ 3 km), likely attributing to the relatively lower—albeit considerably high—genetic similarity. All results of these measurements (the F-statistics and Nei's measurements) support the idea that the *L. pipiens* survey locations at Midewin National Tallgrass Prairie are likely a singular, interbreeding population rather than multiple, isolated subpopulations.

STRUCTURE Bayesian genotype clustering analysis (where k = 3) additionally provides further evidence of minimal genetic differentiation between the surveyed populations at Midewin. The horizontal genotype clusters that are fairly consistent sizes across all populations, which indicates high levels of gene flow and expression between all populations (Figure 2). In the case that these survey locations were genotypically isolated, *k* clusters would appear vertically, and in some cases colored clusters would appear solid throughout a designated population. The STRUCTURE genotype clustering histograms of *L. pipiens* throughout North Dakota are an example of these highly genetically isolated populations. This is primarily due to landscape and geographical isolation of subgroups via large rivers and long distances—variables that are not present throughout the surveyed locations of Midewin (Waraniak et al. 2019). Furthermore, the cluster number *k* of the Midewin populations was chosen based off logarithmic possibilities as shown further in Appendix B. Migration rates between Midewin populations were also estimated to be about 7.197 migrants per generation when accounting for the size of the populations studied. This is well within recommended migrations between populations (one to ten migrants per generation) to maintain adequate levels of genetic diversity and avoid the effects of deleterious allele fixation (Mills and Allendorf 1996). When considering the number of private alleles for each population in addition to the number of migrants, the overall population sizes of each sampled location at Midewin must be incredibly large to maintain such a high degree of genetic variability.

CONCLUSION

The maintenance and preservation of genetic diversity of *L. pipiens* is considered to be essential for the western regions of the United States where researchers have noted drastic losses of populations and diversity (Seaborn & Goldberg 2020, Rogers & Peacock 2012, Smith 2003 & Werner 2003). To avoid any similar degradation events, areas in the eastern United States must encourage a similar mindset. The *L. pipiens* populations of Midewin National Tallgrass Prairie show signs of large, genetically diverse, and ultimately healthy populations of these animals. Even though there are significant deviations in Hardy-Weinberg Equilibrium, none appear to be the result of bottlenecking, colonization, or other genetic drifting events. Instead, large instances of migration per generation appear to be the influence of these deviations while surprisingly avoiding any loss of population-specific, private alleles. These two characteristics of the Midewin *L. pipiens* populations heavily implies the possibility for enormous population sizes, which is an essential component for the maintenance of their genetic diversity. The tested locations of both the Grant Creek and Prairie Creek watersheds also showed a high instance of gene flow—likely an effect of the high migration rate—that suggests the watersheds are quite interconnected and provide no geographic barriers to prevent interbreeding. Additionally, an interesting relationship between Nei's original measurements on genetic distance and identity hints at the possibility of a potential genetic distance/geographic distance relationship within these populations. This potential relationship, as well as further analyses into genetic development of the populations over time could both be investigated to further understand the dynamics of the *L. pipiens* populations at Midewin National Tallgrass Prairie.

REFERENCES

Beebee, Trevor J.C. & Griffiths, Richard A. (2005). The Amphibian Decline Crisis: A Watershed for Conservation Biology? *Biological Conservation*, 125: 271–85.

Blaustein, A. R. & Kiesecker, J.M. (2002). Complexity in Conservation: Lessons from the Global Decline of Amphibian Populations. *Ecology Letters*, 5: 597–608.

Edwards, A. W. F. (2008). Anecdotal, Historical and Critical Commentaries on Genetics: G. H. Hardy (1908) and Hardy–Weinberg Equilibrium. *Genetics*, 179(3): 1143–50.

Falush, D., Stephens, M., & Pritchard, J. K. (2007). Inference of Population Structure Using Multilocus Genotype Data: Dominant Markers and Null Alleles. *Molecular Ecology Notes*, 7(4): 574–78.

Falush, D., Stephens, M., & Pritchard, J. K. (2003). Inference of Population Structure Using Multilocus Genotype Data: Linked Loci and Correlated Allele Frequencies. *Genetics*, 164: 1567–87.

González-del-Pliego, P., Freckleton, R. P., Edwards, D. P., Koo, M. S., Scheffers, B. R., Pyron, R. A., & Jetz, W. (2019). Phylogenetic and Trait-Based Prediction of Extinction Risk for Data-Deficient Amphibians. *Current Biology*, 29(9): 1557–63.

Hayes, M. P. & Jennings, M. R. (1986). Decline of Ranid Frog Species in Western North America: Are Bullfrogs (*Rana catesbeiana*) Responsible? *Journal of Herpetology*, 20(4): 490–509.

Hoffman, E. A., F.W. Schueler, & M.S. Blouin. (2004). Effective Population Sizes and Temporal Stability of Genetic Structure in *Rana pipiens*, the Northern Leopard Frog. *Evolution* 58: 2436–45.

Hoffman, E., Ardren, W., & Blouin, M. (2003). Nine Polymorphic Microsatellite Loci in the Northern Leopard Frog (*Rana pipiens*). *Molecular Ecology Notes*, 3: 115–16.

Hubisz, M. J., Falush, D., Stephens, M., & Pritchard, J. K. (2009). Inferring Weak Population Structure with The Assistance of Sample Group Information. *Molecular Ecology Resources*, 9: 1322–32.

Illinois Department of Natural Resources. (2020, October 21). *Northern leopard frog*. Illinois Department of Natural Resources. https://www2.illinois.gov:443/dnr/education/Pages/WAFTNorthernLeopardFrog.aspx

Kremer, C. S., Vamosi, S. M., & Rogers, S. M. (2017). Watershed Characteristics Shape the Landscape Genetics of Brook Stickleback (*Culaea inconstans*) in Shallow Prairie Lakes. *Ecology and Evolution*, 7(9): 3067–79.

Loxterman, J., & Keeley, E. (2012). Watershed Boundaries and Geographic Isolation: Patterns of Diversification in Cutthroat Trout from Western North America. *BMC Evolutionary Biology*, 12(38): 1–16.

Mills, L. S. & Allendorf, F. W. (1996). The One-Migrant-per-Generation Rule in Conservation and Management. *Conservation Biology*, 10(6): 1509–18.

Mushet, D. M., Euliss, N. H., Chen, Y., & Stockwell, C. A. (2013). Complex Spatial Dynamics Maintain Northern Leopard Frog (*Lithobates pipiens*) Genetic Diversity in a Temporally Varying Landscape. *Herpetological Conservation and Biology*, 8(1): 163–75.

The Nature Conservancy. (n.d.). *Visit Midewin National Tallgrass Prairie (overview)*. The Nature Conservancy. Retrieved May 12, 2022, from https://www.nature.org/en-us/get-involved/how-to-help/places-we-protect/midewin-national-tallgrass-prairie/

Nei, M. (1973). Analysis of Gene Diversity in Subdivided Populations. *Proceedings of the National Academy of Sciences*, 70: 3321–23.

Peakall, R. and Smouse P. E. (2012). GenAlEx 6.5: Genetic Analysis in Excel. Population Genetic Software for Teaching and Research-An Update. *Bioinformatics*, 28: 2537–39.

Peakall, R. and Smouse P. E. (2006). GENALEX 6: Genetic Analysis in Excel. Population Genetic Software for Teaching and Research. *Molecular Ecology Notes*, 6: 288–95.

Pritchard, J. K., Stephens, M., & Donnelly, P. (2000). Inference of Population Structure Using Multilocus Genotype Data. *Genetics*, 155: 949–59.

Prunier, J., Kaufmann, B., Grolet, O., Picard, D., Pompanon, F., & Joly, P. (2012). Skin Swabbing as a New Efficient DNA Sampling Technique in Amphibians, and 14 New Microsatellite Markers in the Alpine Newt (*Ichthyosaura alpestris*). *Molecular Ecology Resources*, 12(3): 524–31.

Raymond M. & Rousset, F. (1995). GENEPOP (version 1.2): Population Genetics Software for Exact Tests and Ecumenicism. *Journal of Heredity*, 86: 248–49

Ringler, E. (2018). Testing Skin Swabbing for DNA Sampling in Dendrobatid Frogs. *Amphibia-Reptilia*, 39(2): 245–51.

Rogers, S. D., & Peacock, M. M. (2012). The Disappearing Northern Leopard Frog (*Lithobates pipiens*): Conservation Genetics and Implications for Remnant Populations in Western Nevada. *Ecology and Evolution*, 2(8): 2040–56.

Rousset, F. (2008). Genepop'007: A Complete Reimplementation of the Genepop Software for Windows And Linux. *Molecular Ecology Resources*, 8: 103–6.

Seaborn, T., & Goldberg, C. S. (2020). Integrating Genetics and Metapopulation Viability Analysis to Inform Translocation Efforts for the Last Northern Leopard Frog Population in Washington State, USA. *Journal of Herpetology*, 54(4): 465–75.

Slatkin, M. (2008). Linkage Disequilibrium — Understanding the Evolutionary Past and Mapping the Medical Future. *Nature Reviews Genetics*, 9: 477–85.

Smith, B. E. (2003). Conservation Assessment for the Northern Leopard Frog in the Black Hills National Forest South Dakota and Wyoming. *United States Department of Agriculture Forest Service.*

U.S. Fish and Wildlife Service (USFWS). (2012). Endangered and Threatened Wildlife and Plants; 12-Month Finding on a Petition to List the Northern Leopard Frog in the Western United States as Threatened; Proposed Rule. *Federal Register* 76(193): 61896–931.

U.S. Fish and Wildlife Service. (n.d.). Northern Leopard Frog (*Rana pipiens*) | U.S. Fish & Wildlife Service. *FWS.Gov*. Retrieved September 23, 2022, from https://www.fws.gov/species/northern-leopard-frog-rana-pipiens

Voordouw, M. J., Adama, D., Houston, B., Govindarajulu, P., & Robinson, J. (2010). Prevalence of the Pathogenic Chytrid Fungus, Batrachochytrium Dendrobatidis, in an Endangered Population of Northern Leopard Frogs, *Rana pipiens*. *BMC Ecology*, 10(6): 1–10.

Waraniak, J. M., Fisher, J. D. L., Purcell, K., Mushet, D. M., & Stockwell, C. A. (2019). Landscape Genetics Reveal Broad and Fine—Scale Population Structure Due to Landscape Features and Climate History in the Northern Leopard Frog (*Rana pipiens*) in North Dakota. *Ecology and Evolution*, 9: 1041–60.

Werner, J. K. (2003). Status of the Northern Leopard Frog (*Rana pipiens*) in Western Montana. *Northwestern Naturalist*, 84: 24–30.

SUPPLEMENTAL MATERIALS

Appendix A Latitude and longitude coordinates of each *L. pipiens* survey location at Midewin National Tallgrass Prairie.

Population	**Latitude**	**Longitude**
G	41.37659	-88.17347
Gm	41.3745087	-88.1807584
P	41.369242	-88.1608812
Po	41.3639141	-88.1525273

Appendix B Logarithmic likelihoods for *k* and Δ*k* for the Bayesian genotype clustering analysis.

K	**Reps**	**Mean LnP (K)**	**Stdev LnP (K)**	**Ln' (K)**	**|Ln''(K)|**	**ΔK**
1	4	-2490.950	1.008	—	—	—
2	4	-2927.875	500.662	-436.925	358.875	0.716
3	4	-3005.925	428.184	-78.050	329.725	0.770
4	4	-2754.250	93.330	251.675	—	—