Color and Pigment Polymorphisms of Northern Leopard Frogs on a Prairie Landscape

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Abstract.—Variation allows populations to adapt to changing conditions. As human activities continue to alter environments and evolutionary processes, it becomes increasingly important to conserve standing genetic variation. Despite technical advances in population genetics, it is still useful to have inexpensive methods of detecting and monitoring genetic variation, particularly in traits that potentially influence fitness. In the Northern Leopard Frog, *Lithobates pipiens* (= *Rana pipiens*), genetically determined color (green [dominant: G] or brown [recessive: g]) and two pigment pattern polymorphisms (Burnsi/spotless [B] or spotted [b]; Kandiyohi/mottled [K] or non-mottled[k]) are hypothesized to have adaptive benefits. We assessed spatiotemporal patterns of these polymorphisms during two time periods in one of the largest remaining grasslands in North America. The frequency of the dominant green phenotype remained consistent from the early-to-late 2000s; however, we observed Kandiyohi phenotypes more frequently during 2001–2002 compared to 2009–2010. By contrast, we observed dominant Burnsi phenotypes more frequently in the latter time period. Although not statistically significant, we observed green phenotypes more frequently in areas with less water on the landscape and in locations closer to tree cover. Burnsi phenotypes were more common in wetlands that did not dry out and Kandiyohi phenotypes were more common in wetlands with aquatic vegetation, although not significantly. No pigment polymorphism was associated with body size. We found no indication of spatial structure, suggesting ample gene flow. The correlations were generally weak, but some were consistent with hypotheses of adaptive benefits. This genetically determined phenotypic variation could be important under changing climactic conditions or if land uses change.

Key Words.—Burnsi; Kandiyohi; *Lithobates pipiens*; population genetics; *Rana pipiens*; Sheyenne National Grasslands

Introduction

As human activities continue to alter local and global environments, it has become increasingly important to understand anthropogenic effects on ecological and evolutionary processes. Determining how wildlife populations respond to disturbances requires historical baseline and current population data (Vellend et al. 2013), including data on genetic variation. Although the goal of many conservation programs is to manage population abundances or distributions (Redford et al. 2011), genetic variability will ultimately determine the ability of populations to adapt to disturbances (Willi et al. 2006; Caballero and García-Dorado 2013). Despite calls for genetic monitoring in conservation and wildlife management programs (Schwartz et al. 2007; Caballero et al. 2010), genetic monitoring is often not included (Laikre 2010), perhaps because of the expense or lack of technical, laboratory, and statistical expertise required to conduct molecular genetic assessment (DeYoung and Honeycutt 2005). For species that exhibit visible morphological polymorphisms, however, relatively inexpensive observational studies may provide useful information on genetic variation and can be implemented in conjunction with other conservation efforts with little to no additional technology, training, or cost (Elledge et al. 2009).

Long before population geneticists had the available array of molecular techniques that allowed them to assess genetic variability at the nucleotide or protein level, many classic studies of evolution used visible morphological traits to detect, quantify, and analyze patterns of population genetic variation (Ludwin 1951; Jones et al. 1977; Bishop and Cook 1980). The key feature of these systems is that phenotypic variation occurred in a small number of discrete states for each trait, and the mode of inheritance could be worked out by cross-breeding individuals of known phenotype according to specific designs (Myles et al. 2009). Observation of phenotype ratios in the field would then yield some information on population genetic variability, at least for that locus; however, alleles with known phenotypic effects cannot be assumed to be selectively neutral, rather, they could be current or future targets of selection (Holderegger et al. 2006). Thus, documenting spatial and temporal data on visible polymorphisms can be useful for understanding population variability and potential adaptive responses to disturbances or changing conditions.

In amphibians, color and pattern polymorphisms are well-documented and the mode of inheritance is

known in some species (Hoffman and Blouin 2000). The Northern Leopard Frog (*Lithobates pipiens* = *Rana pipiens*) exhibits at least three genetically based dorsal color and pattern polymorphisms, including variation in color, spots, and mottling (Merrell 1970). Green color is dominant to brown (Fogleman et al. 1979), spotless (termed Burnsi) is dominant to spotted (Moore 1942), and mottled (termed Kandiyohi) is dominant to non-mottled (Volpe 1955). Although these expressed phenotypes were originally incorrectly identified as three distinct species (*R. pipiens*, *R. burnsi*, and *R. kandiyohi*; Weed 1922), additional research established that the traits are inherited as simple Mendelian characters with two alternate alleles at three independent loci in a single species, *L. pipiens* (Anderson and Volpe 1958; Merrell 1972). Green and brown forms are both relatively common and widespread, whereas the Burnsi and Kandiyohi forms are rare and geographically restricted (Volpe 1955; Merrell 1965). Because rare alleles are more prone to stochastic loss during periods of reduced population sizes (Allendorf 1986; Fuerst and Maruyama 1986), their presence may be indicative of the recent magnitude of effective population size. Although specific gene-fitness relationships are not well understood, several reports indicate certain color and pattern polymorphisms provide benefits to Northern Leopard Frog populations under certain environmental conditions (Merrell and Rodell 1968; Dapkus 1976; Corn 1981; Schueler 1982; Hoffman et al. 2006). Thus, documenting the frequency and spatial distribution of these phenotypes in relationship to environmental characteristics may be important for future conservation programs.

Our primary objective was to assess and document individual, temporal, and spatial patterns of pigment polymorphisms in Northern Leopard Frogs in the Sheyenne National Grasslands (SNG), one of the largest remaining contiguous prairies in North America (Cunningham and Johnson 2006). Two field studies have determined that all three genetic variants occur in the SNG. The first sample period (Bly 2004) was during the summers of 2001 and 2002 and the second spanned the summers of 2009 and 2010. Beyond determination of presence and frequency of phenotypes, we tested a series of hypotheses about patterns at the individual, population, and landscape levels. At the individual level, we tested size-phenotype relationships which could be indicative of any potential effects of phenotype on growth or survival. At the population level, we tested for temporal differences in phenotype frequencies between the two studies, which would reflect effects of selection or genetic drift. We also tested for spatial structure in the trivariate pattern of phenotypic variation. Lastly, we tested for spatial association of phenotypes with wetland and landscape characteristics, which could be indicative of local adaptation.

MATERIALS AND METHODS

Study site: Sheyenne National Grasslands.—The SNG is situated within Richland and Ransom counties on the western edge of the Tallgrass Prairie ecoregion in southeastern North Dakota, USA. The SNG consists of roughly 280 km² of contiguous (although not pristine) Tallgrass Prairie (Cunningham and Johnson 2006) used predominantly for grazing. The surrounding landscape is dominated by crop production. Northern portions of the SNG contain large sand hills that descend into the Sheyenne River Valley, which provides the only contiguous stand of forest across the SNG. The central portions of the SNG are characterized by small hummocks and Bur Oak (*Quercus macrocarpa*) savannas, which gradually turn into the flat prairie observed in southern portions of the SNG. The SNG contains a large number of wetlands embedded in a grassland matrix and the Sheyenne River is a relatively permanent water feature. For a more complete description on the SNG see Manske and Barker (1988) and Gustafson and Newman (2016).

Sampling periods.—During amphibian wetland occupancy surveys in 2001 and 2002, we recorded phenotypic polymorphisms from 161 (2001: 76; 2002: 85) Northern Leopard Frogs (Bly 2004). Because of low precipitation and drying of some wetlands in 2002, we could not sample several of the same locations that we surveyed the previous year. We recorded general locations for all frogs, and subsequently grouped them into four regions based on geographic proximity (Fig. 1; regions 1–4).
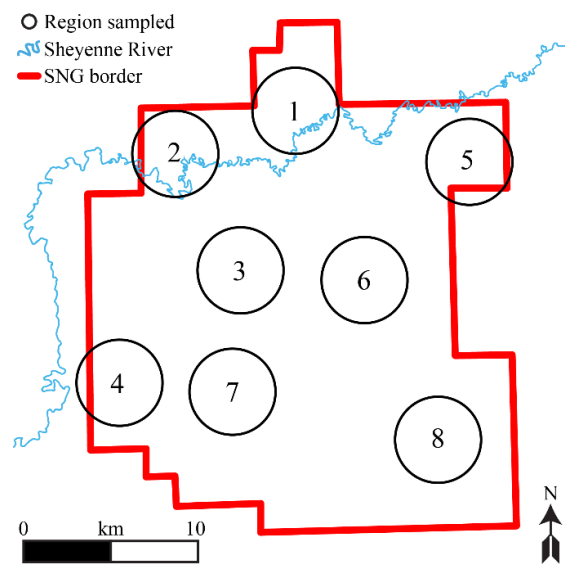

FIGURE 1. Map of the Sheyenne National Grasslands (SNG) showing sampling regions. We sampled Regions 1–4 in 2001–2002, and Regions 1–8 in 2009–2010.

Figure 2. Genetically determined pigment color and pigment pattern polymorphisms in Northern Leopard Frogs (*Lithobates pipiens*). Green (G) is dominant to brown (g), Burnsi (B: spotless) is dominant to spots (b), and Kandiyohi (K: mottling) is dominant to non-mottling (k). Because phenotypes are expressed by dominant markers, a plus sign (+) indicates a homozygote or heterozygote. The green frog exhibiting Burnsi and Kandiyohi phenotypes (G+B+K+) is incompletely expressing the Burnsi phenotype (Volpe 1961). (Photographed by Kyle Gustafson).

During amphibian wetland occupancy surveys in 2009 and 2010, we recorded phenotypic polymorphisms from 703 (2009: 495; 2010: 208) Northern Leopard Frogs. We recorded precise geographic coordinates using a geographic positioning system for each frog and subsequently grouped collection locations into eight regions (including regions 1–4 surveyed in 2001 and 2002) based on geographic proximity (Fig. 1; regions 1–8).

Pigment pattern characterization.—Upon capture, we measured frog snout-vent length (SVL) to the nearest mm. We recorded the dorsal color and pigmentation patterns of each frog (Fig. 2), including color (brown/ green), presence of mottled pigmentation (Kandiyohi), and absence of spots (Burnsi). We released frogs at the site of capture unless sampled for parasitic helminths (Gustafson et al. 2013). Because the inheritance of all three traits involves dominance, genotype and allele

frequencies can only be inferred. Thus, we based most comparisons on phenotype frequencies. We conducted all analyses in program R (R Development Core Team 2010), unless otherwise noted.

Genetic analyses.—We regressed the binary phenotypic state on size (SVL) using logistic regressions. We restricted these analyses to the 703 frogs collected in the second time period because we did not record SVL during the first time period. We restricted landscape- and wetland-scale analyses to frogs from the second time period because we captured all frogs at specific wetlands and recorded individual geographic coordinates. Wetland characteristics included presence-absence of aquatic vegetation, whether the site was a man-made cattle pond, and whether the wetland completely dried during either summer. Based on the location where each frog was captured, we calculated several measures of proximity to landscape features, including distance to croplands, tree cover, the Sheyenne River, and roads. We also calculated several measures of landscape composition, including the surrounding proportion of croplands, trees, water, and the number of wetlands. We calculated landscape composition measures using superimposed 100 m buffers centered on each collection site. Buffers > 100 m contained significant areas of overlap and were not independent (Gustafson and Newman 2016). We did not detect multicollinearity among predictor variables and obtained all landscape data from publicly available geospatial databases described by Gustafson and Newman (2016).

We used binomial generalized linear mixed models to test wetland and landscape associations with the presence-absence of pigment polymorphisms in Northern Leopard Frogs (Bolker et al. 2009). To account for lack of independence of animals captured at the same wetland (i.e., pseudoreplication) in tests for wetland and landscape effects, we treated wetland as a random effect in all models. Treating wetland as a random effect mitigates the concern about correlated phenotypic states that might be present among individuals at the same site within the sampling period as a result of kinship, rather than because of direct effects of landscape or wetland characteristics.

Based on analyses of photographs, we did not recapture any of the same individuals. We implemented the global model, including all wetland and landscape measures, in package lme4 (Bates et al. 2015). Subsequently, we used best-subsets model-selection methods based on sample size-corrected Akaike information criterion (AICc) scores to determine the relative importance of each variable (Burnham and Anderson 2002). We averaged β estimates, standard errors, 95% confidence intervals, and Wald Z test statistics across models for those with similar statistical support (ΔAICc < 2). We implemented model selection with package MuMIn (Bartoń 2016). For relative comparison and easier interpretation, we standardized all predictor variables to mean of zero with equal variances. We plotted well-represented variables using logi.hist.plot and plot.error (de la Cruz Rot 2005).

Population genetic structure.—To test for genetic structure, we used the spatially-explicit Bayesian population assignment program GENELAND 4.0 (Guillot et al. 2005). We restricted population genetic structure analyses to frogs from the second time period because we captured all frogs at specific wetlands and have individual geographic coordinates for that time period. Because each phenotype is determined by dominant genes and heterozygotes cannot be differentiated from dominant homozygotes, we used phenotypes and geographic location data to estimate population structure (Guillot et al. 2012). In GENELAND, the number of genetic clusters (K) is optimized by the model. We implemented 15 spatial models allowing K to vary from 1–10. Each run included 1,000,000 iterations, a thinning interval of 10,000, and a 25% burn-in period prior to extracting model output.

Temporal analyses.—For temporal analyses, we tested differences in phenotype frequencies and phenotype combinations between time periods (i.e., 2001–2002 vs 2009–2010). We used Chi-squared tests ($\alpha = 0.05$) for all temporal analyses.

RESULTS

Phenotype frequencies.—All phenotypes determined by dominant markers were less frequent than homozygous recessive phenotypes (Appenix 1). Brown wild-type (i.e., spotted and not mottled) frogs were most common (55.3%), followed by green wild-type frogs (36.5%). Although all other phenotypic combinations were rare (< 5%) overall, the Burnsi phenotype was more frequent (5.3%) than the Kandiyohi phenotype (3.3%), ranging from 1.6–13.6% regionally, compared to 1.6–27.3% for Kandiyohi (Appendix 1). Regional locations with the smallest sample sizes, however, tended to have higher frequencies of rare phenotypes (Fig. 3).

Individual size-phenotype relationships.—Color was not correlated with snout-vent length (Wald Z = 0.98, df = 702, P = 0.321). Similarly, the Burnsi (Wald Z = 0.89, df = 702, P = 0.345) and Kandiyohi phenotypes (Wald Z = 0.89, df = 702, P = 0.444) were not correlated with snout-vent length.

Landscape- and wetland-scale analyses.—Overall, frog phenotypes exhibited weak relationships with
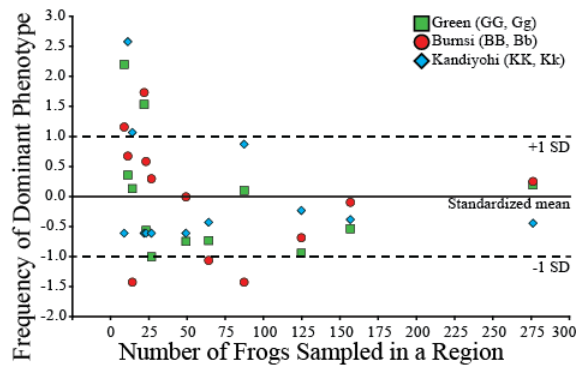
Figure 3. Standardized frequencies of dominant phenotypes of Northern Leopard Frogs (*Lithobates pipiens*) in each of eight sampling areas show upward bias in geographic sampling regions with low samples sizes. Because phenotype frequencies are on difference scales, each phenotype was standardized to a mean of zero for visualization purposes.

wetland and landscape characteristics. The best supported models for each polymorphism included only 1–2 environmental explanatory variables with importance > 0.5 and accounted for only a small proportion of the spatial variation in phenotype occurrence based on comparison of null deviance to model residual deviance (Tables 1 and 2). Although not significant, frogs with green phenotypes were more frequently observed in locations where only a small proportion of the surrounding landscape was composed of water (Fig. 4A). This means frogs with brown phenotypes were found more commonly in areas of higher water density. Although not significant, frogs with green phenotypes tended to be more common in areas closer to tree cover (Fig. 4B); conversely, brown phenotypes were found further from tree cover. The proportion of water surrounding a location and distance from tree cover were not strongly correlated ($r^2 = 0.007$). Although not significant, frogs with Burnsi

phenotypes were more frequently observed in wetlands that did not dry out throughout the sampling season (Fig. 5). All other relationships between color or pattern polymorphisms and wetland or landscape characteristics were clearly not significant. Aquatic vegetation was commonly included in Kandiyohi phenotype models, with Kandiyohi phenotypes more frequently observed in wetlands with aquatic vegetation; however, aquatic vegetation was not significant in single predictor variable models (Wald $Z = 1.37$, df = 699, $P = 0.170$).

Population genetic structure.—In all 15 models, GENELAND converged on K = 1. There was no support for additional K clustering. Thus, there was no evidence for spatial structure based on trivariate combinations of genetically-determined phenotypes.

Temporal analyses.—Because regional sample sizes containing fewer than 25 individuals exhibited signs of sampling size-related bias (Fig. 3), we restricted our temporal analyses to samples sizes > 25. Further, we excluded region as a factor in this analysis because the population genetics analysis found no spatial structure. When analyzed together, frequencies of dominant phenotypes (green, Burnsi, and Kandiyohi) between time periods (2001–2002 vs. 2009–2010) were significantly different ($\chi^2 = 10.2$, df = 2, $P = 0.006$). This was primarily driven by the significantly higher proportion of Kandiyohi phenotypes in the first time period ($\chi^2 = 40.2$, df = 1, $P < 0.001$) and to a lesser extent by the higher proportion of Burnsi phenotypes in the second time period ($\chi^2 = 2.20$, df = 1, $P = 0.144$). Frequencies of the green phenotype among time periods were nearly equivalent ($\chi^2 = 0.09$, df = 1, $P = 0.756$). When put into their eight possible combination, however, frequencies of phenotype combinations were significantly different between time periods ($\chi^2 = 62.8$, df = 7. $P < 0.001$),
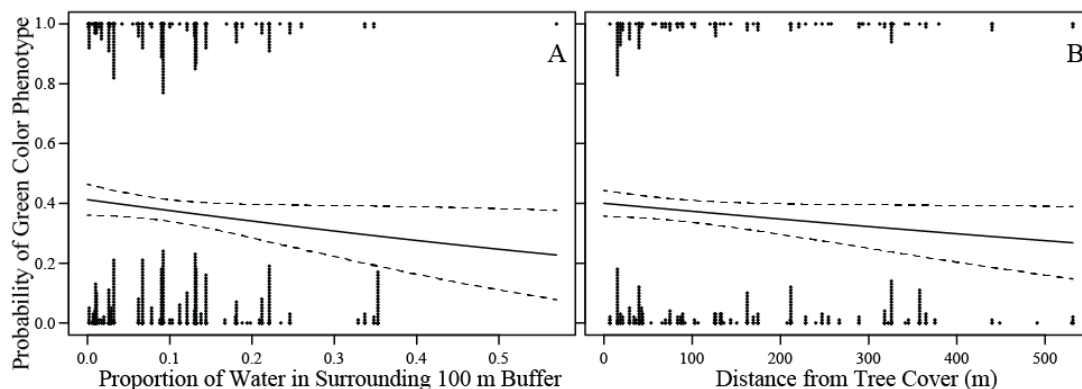

Figure 4. Probability of the green color polymorphism of Northern Leopard Frogs (*Lithobates pipiens*; solid line) relative to (A) the surrounding proportion of landscape composed of water and (B) distance from tree cover. These two variables were presented because they had the strongest relationships with phenotype, despite being non-significant ($P > 0.050$). Stacked dots on the upper/lower x-axes indicate frogs with/without green phenotypes, respectively. Zero-values are not shown because of overlapping dots; however, the intercept represents the frequency at X = 0. Dashed lines represent 95% confidence intervals.

TABLE 1. Model averaging results for landscape- and wetland-scale analyses. Variables are sorted by importance value which accounts for the presence of the variable in the models and model weight. For direct variable comparisons, all variables were standardized to a mean of 0. Higher absolute values of beta estimates indicate a greater effect. AqVeg: wetland had aquatic vegetation or did not; CattlePond: wetland was either a manmade cattle pond or not; Crops100m: amount of surrounding landscape within a 100 m buffer that is croplands; DistAg: wetland distance to nearest cropland; DistRiver: wetland distance to the Sheyenne River; DistRoad: wetland distance to nearest road; DistTrees: wetland distance to nearest forest; Dried: wetland dried out during the sampling season; Trees100m: amount of surrounding landscape within a 100 m buffer that is forest; Water100m: amount of surrounding landscape within a 100 m buffer that is water; Wetlands100m: the number of wetlands within a 100 m buffer around the wetland.

Factor	Importance	Beta	± 95% CI	Z	P
Green phenotype					
Water100m	0.84	-0.31	0.34	1.74	0.082
DistTrees	0.79	-0.32	0.35	1.77	0.077
DistRiver	0.17	-0.20	0.36	1.11	0.267
AqVeg	0.13	-0.20	0.35	1.12	0.264
Trees100m	0.1	-0.17	0.40	0.85	0.397
DistRoad	0.1	-0.15	0.36	0.81	0.418
Crops100m	0.06	-0.12	0.34	0.71	0.477
CattlePond	0.05	0.09	0.31	0.53	0.594
DistAg	0.05	-0.07	0.34	0.40	0.686
Dried	0.05	-0.06	0.34	0.37	0.713
Wetlands100m	0.05	0.05	0.42	0.22	0.825
Burnsi phenotype					
Dried	0.73	-1.95	2.34	1.63	0.104
Crops100m	0.64	-2.96	3.84	1.51	0.131
Trees100m	0.27	-1.67	2.67	1.23	0.220
Wetlands100m	0.08	0.82	2.05	0.78	0.436
DistTrees	0.08	0.66	1.83	0.71	0.480
Water100m	0.07	0.47	1.81	0.51	0.609
AqVeg	0.06	-0.25	1.93	0.25	0.802
Kandiyohi phenotype					
AqVeg	0.76	5.00	7.10	1.38	0.168
Dried	0.17	2.21	4.04	1.07	0.284
Water100m	0.13	1.49	4.04	0.72	0.471
Crops100m	0.09	-2.51	5.72	0.86	0.391
DistRoad	0.08	1.54	3.98	0.76	0.448
DistRiver	0.07	-1.82	5.72	0.62	0.533
CattlePond	0.07	-1.51	5.29	0.56	0.576
Wetlands100m	0.06	0.68	4.23	0.31	0.755
Trees100m	0.06	-0.57	4.50	0.25	0.805

which was primarily driven by higher frequency of brown, spotted, Kandiyohi frogs in the first time period.
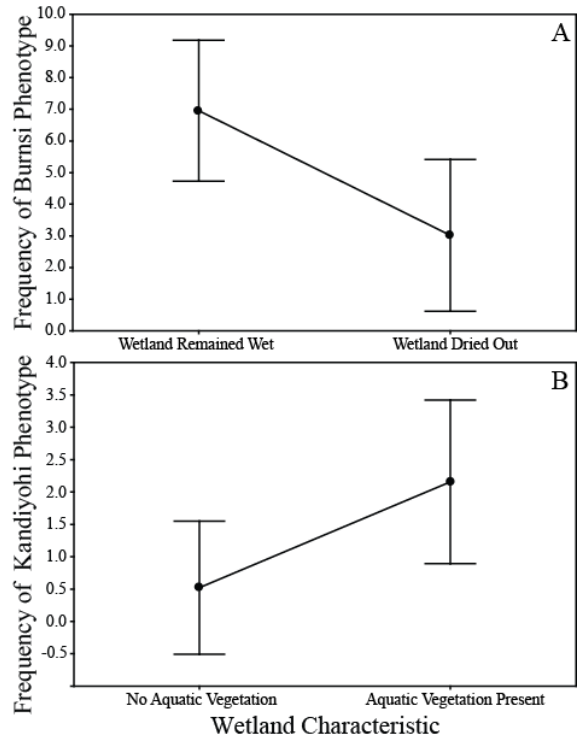

FIGURE 5. Frequency of pattern polymorphisms of Northern Leopard Frogs (*Lithobates pipiens*) relative to wetland characteristics. Burnsi phenotypes (A) were more frequently observed in wetlands that did not dry out whereas Kandiyohi phenotypes (B) were more commonly observed in wetlands with aquatic vegetation. Means ± 95% confidence intervals are presented.

DISCUSSION

We found distinct differences in patterns of polymorphism in three genetically-determined components of dorsal pattern. This is the first study to detect all known trivariate phenotype combinations in the wild in a single area. Given the relatively low frequency of the dominant phenotypes (green: 38.6%; Burnsi: 5.3%; Kandiyohi: 3.3%), we could have expected to observe less than one frog (0.58 frogs out of 864) exhibiting all three dominant traits based on the low probability of 0.00067. Thus, our observation of a green, mottled frog exhibiting incomplete expression of the Burnsi phenotype is fortuitous. Color variation was more evenly distributed in our sample (40% green and 60% brown) than Burnsi or Kandiyohi morphs, which appears to be typical for this polymorphism across the range of Northern Leopard Frogs (Fogleman et al. 1979; Hoffman et al. 2006). The frequency of the spotless (Burnsi) form on the SNG was less than the values found in four other locations in southeastern North Dakota, but similar to values found in many other locations in Minnesota and South Dakota (McKinnell

TABLE 2. Best-subsets model-selection results for landscape- and wetland-scale analyses showing models with a ΔAICc < 2. Model structure, degrees of freedom, log likelihoods (LL), model deviance (Dev.), ΔAICc, and model weight (AICω) are shown. Model variables are defined in the caption of Table 1.

Phenotype	Model	df	LL	Dev.	ΔAICC	AICω
Green	DistTrees+Water100m	4	-462.935	925.9	0	0.124
	DistRiver+DistTrees+Water100m	5	-462.455	924.9	1.07	0.073
	DistRiver+AqVeg+Water100m	5	-462.517	925	1.19	0.069
	Water100m	3	-464.627	919.4	1.36	0.063
	Ag100m+DistTrees+Water100m	5	-462.679	925.4	1.52	0.058
	DistTrees+AqVeg	4	-463.699	921.3	1.53	0.058
	DistTrees+Trees100m+Water100m	5	-462.700	925.4	1.56	0.057
	DistTrees	3	-464.765	923.8	1.64	0.055
	DistRiver+Water100m	4	-463.787	924.6	1.7	0.053
	Cattlepond+DistTrees+Water100m	5	-462.793	925.6	1.74	0.052
	DistRoad+DistTrees+Water100m	5	-462.794	925.6	1.75	0.052
	DistAg+DistTrees+Water100m	5	-462.852	925.7	1.86	0.049
	DistTrees+Dried+Water100m	5	-462.867	925.7	1.89	0.048
	Null	2	-465.904	915.2	1.9	0.048
Burnsi	Ag100m+Dried	4	-143.592	232.8	0	0.163
	Dried+Trees100m	4	-143.988	233	0.79	0.109
	Ag100m	3	-145.013	230	0.82	0.108
	Dried	3	-145.219	229	1.23	0.088
	Ag100m+Dried+Trees100m	5	-143.232	234.2	1.31	0.085
	Null	2	-146.272	227.4	1.32	0.084
Kandiyohi	Ag100m+Dried	4	-143.592	232.8	0	0.163
	Dried+Trees100m	4	-143.988	233	0.79	0.109
	Ag100m	3	-145.013	230	0.82	0.108
	Dried	3	-145.219	229	1.23	0.088
	Ag100m+Dried+Trees100m	5	-143.232	234.2	1.31	0.085
	Null	2	-146.272	227.4	1.32	0.084

et al. 2005) and Kandiyohi frequencies were similar to those reported by Merrell (1965) for other populations in the prairie pothole region of the United States.

Despite several field studies (Merrell 1965, 1970; Hoffman and Blouin 2000) and laboratory experiments (Volpe 1955; Anderson and Volpe 1958; Volpe 1961; Merrell 1972) examining phenotype frequencies and patterns of inheritance, the mechanisms responsible for persistent genetic variation at these loci are not well understood (Merrell and Rodell 1968; McKinnell et al. 2005; Hoffman et al. 2006). Thus, we tested a series of hypotheses about phenotype patterns at the individual, population, and landscape levels that may be indicative of selection or adaptation. Despite reports of certain morphs having faster development (Merrell 1972; Corn 1981) or different seasonal survival rates (Merrell and Rodell 1968; Dapkus 1976), we did not observe any relationships between individual frog size and phenotype, indicating color and pattern polymorphisms do not have a strong, or at least detectable, association with this fitness-related trait.

At the population level, we found a lack of significant differences between sampling periods at the most variable locus, responsible for the green-brown polymorphism, suggesting alleles at this locus are temporally stable. Color may simply not be under strong selection over the entire study area, at least during the time encompassed by our two sampling periods. In contrast, we found the mottled (Kandiyohi) morph at a significantly greater frequency in the first sampling period (2001–2002) than the second sampling period (2009–2010), whereas the spotless (Burnsi) morph was significantly more common in the latter sampling period. Kandiyohi morphs are hypothesized to develop

faster than wild-type frogs and Burnsi phenotypes have been observed to have higher survival rates than wild-type frogs, which is hypothesized to be a function of increased overwintering survival (Merrell and Rodell 1968; Merrell 1972; Dapkus 1976). Although these changes in allele frequencies could be indicative of selection or drift, these statistically significant differences must be interpreted with caution.

Spatial Bayesian structure analyses revealed no evidence of population structure. Aside from the question of the role of selection in determining allele frequencies in color and pattern polymorphisms (Merrell 1972; Hoffman et al. 2006), even detecting differences in color frequencies (binomial proportions) is statistically problematic with small sample sizes (e.g., fewer than 50 per population), unless differences are strong. For example, with sample sizes of 50 for each of two populations, the power to detect a significant difference in color frequency (with $\alpha = 0.05$ in a 2-sided test) is 98% when the frequencies are 0.3 versus 0.7 for one of the colors (e.g., brown) in the two populations, but drops to under 50% when the frequencies are 0.4 versus 0.6, and to less than 18% when the frequencies are 0.6 versus 0.7, as they were for our two areas with larger sample sizes (power calculated in Cytel StatXact version 6; Cytel, Cambridge, Massachusetts, USA). Expected values of FST for differentiation in allele frequencies based on a single locus depends on the heterozygosity of the locus as well as the sample size and rate of gene flow (Beaumont and Nichols 1996). Subtle differentiation associated with high levels of gene flow (e.g., FST = 0.01) for a biallelic locus would be difficult to detect even with uniform overall allele frequencies (maximum expected heterozygosity), but there is a reasonable chance of detecting differentiation with somewhat stronger differentiation (e.g., FST = 0.05) and greatly improved sensitivity with more loci (Ryman et al. 2006). Detecting temporal changes in genetic composition for a particular population or location suffers from the same problems as measuring spatial population structure. In other words, our results are consistent with the view that there is substantial gene flow, but certainly do not provide compelling evidence for this to the exclusion of alternative hypotheses. The slight variation in allele frequencies among areas does not contradict this argument, because with low overall frequencies of dominant phenotypes, differentiation due to drift at these loci is expected to be greater, despite any homogenizing effect of gene flow. McKinnell et al. (2005) arrived at the same hypothesis, suggesting genetic drift was almost certainly a factor in explaining geographic differences in the frequency of dorsal pigment variants in Minnesota and the Dakotas.

Landscape- and wetland-scale analyses, which could reveal patterns indicative of local adaptation, did suggest some associations with phenotypes. Previous reports indicate brown morphs develop faster than green morphs, which presumably would give them an advantage in prairie wetlands where there is less green vegetation than forested wetlands (Corn 1981; Hoffman et al. 2006). By contrast, previous researchers have hypothesized green morphs are favored in forested habitats (Schueler 1982; Hoffman et al. 2006).

Although the trends were weak and not significant, we observed phenotype-landscape relationships consistent with both hypotheses. We observed more green morphs in wetlands closer to tree cover and brown morphs further away. Given that the net effect of selection is likely to be weak across this heterogeneous landscape, and that Northern Leopard Frogs can produce hundreds of offspring that can move relatively large distances, future studies assessing phenotype-landscape relationships should do so over a larger distance on a landscape that has a strong linear tree-cover gradient. We also observed relatively more brown morphs in areas with a high density of water on the surrounding landscape. The mechanism for this apparent relationship is currently unclear.

Previous reports indicate Kandiyohi morphs metamorphose sooner than other morphs (Merrell 1972); however, we did not observe a significant relationship between hydroperiod and Kandiyohi phenotype. Instead, we observed Burnsi morphs to be more frequent in more permanent wetlands and Kandiyohi morphs to be present in wetlands with aquatic vegetation. Previous researchers have not reported these relationships and future experiments will be needed to identify any potential mechanisms for these relationships.

Obviously, none of these phenotypic values are allele frequencies, but we can at least conclude that despite sometimes small sample sizes and potentially small effective population sizes associated with large fluctuations in abundance, skewed sex ratios at breeding sites, and highly uneven reproductive success among individuals that are often ascribed to pond-breeding anurans (Hoffman et al. 2004; Merrell 1968; Rowe and Beebee 2004; Scribner et al.1997), Northern Leopard Frog populations on the SNG and elsewhere often maintain genetic variation in dorsal color and pattern loci. Although selection would confound the interpretation of spatial and temporal variation in allele frequencies if ecological conditions varied among locations or through time, Hoffman et al. (2006) found no evidence that selection has influenced the distribution of color morphs in a broad geographic analysis in which population structure based on color allele frequency was compared with structure based on neutral molecular markers. The prospect that color or pattern might be associated with fitness, though, raises the intriguing possibility that the genetic composition of populations

might affect population dynamics, particularly in response to climate variation (Alho et al. 2010; Rudh and Qvarnström 2013). That would make genetic monitoring of any loci involved more compelling, albeit for different reasons than simply as markers for genetic variability and population structure.

In summary, although we detected genetic variability based on three visible polymorphisms, the value of our results is limited by the low resolution associated with low overall variation at two of the loci, and the small number of loci which can be monitored in this way. The principal advantages of monitoring these loci are the modest marginal cost (it is practically free if done in conjunction with other field monitoring) and limited training required. Color and pattern polymorphisms are also common in other anurans (Hoffman and Blouin 2000), potentially allowing monitoring of multiple species by this means. Even so, it is critical to obtain frequency estimates based on adequate sample sizes per location, and for any more refined purpose (e.g., evaluation of spatial pattern) a suitable sampling design is required, which may or may not be concordant with designs intended for other purposes. If future studies establish firmer connections with fitness for any of the loci, quantifying the allelic composition of populations might be considered for improving understanding of population dynamics and responses to changing environmental conditions. If genetic studies, however, are deemed sufficiently important for monitoring levels of genetic variation, for elucidation of population structure, or for identification of management units, we strongly advocate the use of modern molecular approaches for the much greater resolution and power that are attainable (Schwartz et al. 2007). On this point we anticipate that ongoing rapid advances in molecular techniques will finally enable detection and quantification of genetic variation in ecologically important traits, such as life-history characteristics, physiological tolerances, and morphological features including color polymorphisms.

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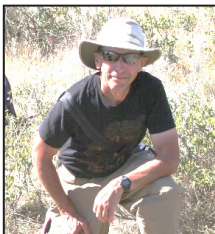
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APPENDIX 1. Phenotype frequencies of color (green or brown), mottling (Kandiyohi or non-mottled), and spotting (Burnsi or spotted) of Northern Leopard Frogs (*Lithobates pipiens*) based on sampling location and sampling period.

Location region	Year of study	Sample size	Green (G+)				Brown (gg)				Sum Green	Sum Burnsi	Sum Kandiyohi
			Kandiyohi (K+)		Not mottled (kk)		Kandiyohi (K+)		Not mottled (kk)				
			Bursi (B+) G+K+B+	Spotted (bb) G+K+bb	Bursi (B+) G+kkB+	Spotted (bb) G+kkbb	Bursi (B+) ggK+B+	Spotted (bb) ggK+bb	Bursi (B+) ggkkB+	Spotted (bb) ggkkbb			
All data pooled													
All	Both	864	0.1	0.4	1.6	36.5	0.2	2.6	3.4	55.3	38.6	5.3	3.3
Data pooled by study													
All	01-02	161	0.0	0.6	1.2	37.9	0.0	9.3	1.2	49.7	39.8	2.5	9.9
All	09-10	703	0.1	0.3	1.7	36.1	0.3	1.0	3.8	56.6	38.3	6.0	1.7
Data pooled by region within study													
1	2001-02	87	0.0	1.1	0.0	41.4	0.0	11.5	0.0	46.0	42.5	0.0	12.6
1	2009-10	276	0.4	0.4	4.0	38.8	0.0	0.7	2.9	52.9	43.5	7.2	1.4
2	2001-02	11	0.0	0.0	0.0	45.5	0.0	27.3	9.1	18.2	45.5	9.1	27.3
2	2009-10	157	0.0	0.0	0.0	35.0	1.3	0.6	4.5	58.6	35.0	5.7	1.9
3	2001-02	14	0.0	0.0	0.0	42.9	0.0	14.3	0.0	42.9	42.9	0.0	14.3
3	2009-10	64	0.0	0.0	0.0	32.8	0.0	1.6	1.6	64.1	32.8	1.6	1.6
4	2001-02	49	0.0	0.0	4.1	28.6	0.0	0.0	2.0	65.3	32.7	6.1	0.0
4	2009-10	125	0.0	0.8	0.0	29.6	0.0	2.4	3.2	64.0	30.4	3.2	3.2
5	2009-10	22	0.0	0.0	4.5	54.5	0.0	0.0	9.1	31.8	59.1	13.6	0.0
6	2009-10	9	0.0	0.0	0.0	66.7	0.0	0.0	11.1	22.2	66.7	11.1	0.0
7	2009-10	23	0.0	0.0	0.0	34.8	0.0	0.0	8.7	56.5	34.8	8.7	0.0
8	2009-10	27	0.0	0.0	0.0	29.6	0.0	0.0	7.4	63.0	29.6	7.4	0.0