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CRYPTIC VARIATION AND EXAMINATION OF HYBRID CHARACTERISTICS IN LEOPARD FROGS OF SOUTHWESTERN SOUTH DAKOTA, USA

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Abstract.—**Hybridization and introgression between distinct populations or species may promote or suppress local adaptation. Leopard frogs in the *Lithobates* (= *Rana*) *pipiens* complex have had widespread hybridization throughout their North American range. In South Dakota (SD), USA, Plains Leopard Frogs (*L. blairi*) are known to hybridize with Northern Leopard Frogs (*L. pipiens*); however, the distribution of *L. blairi* or hybrids therein in the southwestern part of the state is unresolved. We used morphometric and phenotypic characters to assess potential hybridization of leopard frogs at multiple sites in the southern Black Hills (BH) and Buffalo Gap National Grasslands (BGNG) of southwestern SD. We found subtle morphometric variation among population sites, which may represent local adaptation. Moreover, we identified a BGNG site with multiple individuals that expressed subtle or more pronounced phenotypic characters intermediate of *L. blairi* and *L. pipiens*, suggesting occurrence of introgression. This locality was most proximal to known hybrid sites to the east. Our data add resolution to the possible extent of *L. blairi* × *L. pipiens* hybridization in southwestern SD. Further studies, including genomic analyses, could elucidate the extent of introgression as well as infer any effects therein on local adaptation. Such data would be valuable for conservation efforts, especially in sympatric regions where they exist as sensitive species of concern.**

Key Words.—amphibians; Black Hills; Buffalo Gap; conservation; introgression; *Lithobates blairi*; *Lithobates pipiens*; phenotype

INTRODUCTION

Contact between closely related taxa could yield hybridization (i.e., interbreeding between distinct population segments or species) and introgression (i.e., allele transfer from one gene pool entity into another), phenomenon driven in part by the environment, distribution and dispersal, and selection pressures (Chunco 2014; Harrison and Larson 2014). Introgression may be disadvantageous by increasing extinction rates via genetic swamping, wherein genotypes underlying ecologically important phenotypes do not match the environment in which the organism lives (Frankham et al. 2002). Alternatively, adaptive introgression (Hedrick 2013) can have positive effects by facilitating novel and advantageous phenotypes (Chunco 2014; Moran et al. 2021).

Among amphibians, introgression through hybridization may produce relatively fit genotypes suitable for a particular environment but can also offset larval fitness of parental amphibian species in certain rearing environments (Parris 2001a,b). For instance, Ryan et al. (2009) discovered reduced larval size and survival of native California Tiger Salamanders (*Ambystoma californiense*) resulting from predation effects by hybrids of native (*A. californiense*) × introduced (Barred Tiger Salamander, *A. tigrinum mavortium*). Hybrids can negatively affect other syntopic taxa and alter community structure and species assemblages within an area (Ryan et al. 2009). Amphibians could face further stresses if warming climate trends cause migration towards cooler environments (Bickford et al. 2010), which could lead to clustering and subsequently increased rates of hybridization and introgression (Chunco 2014; Pabijan et al. 2020).

Leopard frogs in the *Lithobates* (= *Rana*) *pipiens* complex are widespread in North America and have had a storied history in evolutionary biology, with several taxonomic elevations and delimitations (Pace 1974; Hoffman and Blouin 2004; O'Donnell and Mock 2012). Hybridization within the *L. pipiens* complex has occurred in many places throughout its range, including the southeastern Appalachian states, Great Lakes region, central Plains states, and the southwestern U.S. (Sage and Selander 1979; Parris 2001b; O'Donnell and Mock 2012; Gustin and Richter 2013). In South Dakota (SD), USA, two leopard frog species occur: Plains Leopard Frogs (*L. blairi*) in the southeastern quadrant relative to the Missouri River drainage and Northern Leopard Frogs (*L. pipiens*) distributed ubiquitously throughout the state (Fischer et al. 1999; Ballinger et al. 2000; Smith and Quinn 2012; Dodd 2013). Both *L. blairi* and *L. pipiens* are listed as sensitive species under the U.S. Department of Agriculture, Forest Service Region 2 (Rocky Mountain Region; https://www.fs.usda.gov; Smith and Keinath 2007), and *L. blairi* is designated a species of special concern in South Dakota (https://sdherps.org).

Hybridization between *L. blairi* and *L. pipiens* has occurred in southeastern SD, resulting in phenotypic intermediates (Dunlap and Kruse 1976). Putative *L. blairi* × *L. pipiens* hybrids predominantly resemble *L. pipiens*, showing only sparse *L. blairi* morphological characteristics (e.g., tympanum spot; Dunlap and Kruse 1976; Smith and Quinn 2012). Phenotypic intermediates have also been observed about 250 km west of the nearest known pure *L. blairi* population in Tripp County, SD (Ernst 2001; Smith and Quinn 2012). This indicates that *L. blairi* may be more widely distributed in SD than realized, or that prior contact and introgression between species has cryptically advanced *L. blairi* alleles westward in the region. There is some genetic evidence to support the latter supposition in southwestern SD (Ernst 2001; Di Candia and Routman 2007); however, gaps in the geographic distribution of potential hybrids in southwestern SD remain unresolved (Smith 2003).

We set out to obtain morphological data to provide insight into potential *L. blairi* × *L. pipiens* hybridization in southwestern SD. Our specific objectives were to assess differences in morphological body size and coloration phenotypes between two populations in southwestern SD and identify any phenotypic characteristics of hybridization. Based on the morphological depictions of *L. blairi* × *L. pipiens* hybrids by previous authors (Dunlap and Kruse 1976; Smith and Quinn 2012), we expected leopard frogs of southwestern SD (e.g., Custer and Fall River counties) to predominantly resemble *L. pipiens* and those with subtle intermediate characters to indicate possible hybrids. Our results provide further insight into the morphometric variation among leopard frogs in southwestern SD and highlight opportunities for additional sampling and genomic surveillance for hybridization. This may be of importance for wildlife managers given the sensitivity of *Lithobates* populations in the region (Smith 2003; Smith and Keinath 2007), which could be further jeopardized by any deleterious effects of introgression (Smith and Quinn 2012).

MATERIALS AND METHODS

Study site.—We surveyed leopard frogs in lentic wetlands in the southern Black Hills (BH; Custer County) and Buffalo Gap National Grasslands - Fall River Ranger District (BGNG; Fall River County) in southwestern SD (Fig. 1). Climate in the montane BH is highly variable in temperature and precipitation but tends to follow patterns of xeric to mesic transition with elevational gain (Hoffman and Alexander 1987; Froiland 1990). The BGNG region is semi-arid with mixed prairie grasslands and is characterized by high winds and annual precipitation between 30–60 cm, albeit with high evaporation rates (Ernst 2001; Platt et al. 2006). Wetlands in the BH include several lotic hydrological networks, small ponds, and artificial reservoirs (Fontaine et al. 2001), whereas the BGNG has many small lentic ponds and artificial water catchments (Ernst 2001).

Sampling and scoring.—In October of 2014, we surveyed lentic body edges at BH and BGNG sites and captured frogs by hand or with dip nets (Graeter et al. 2013). We used calipers to measure snout-urostyle length (SUL), head-width, and lengths of the femur

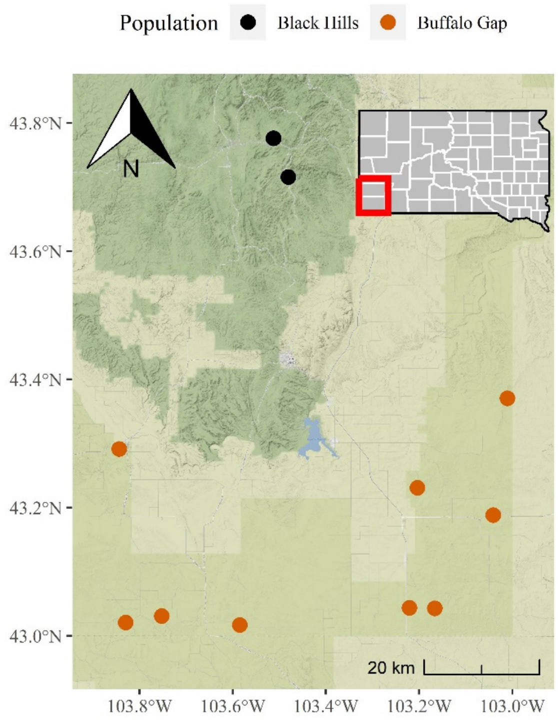

FIGURE 1. Sampling sites of leopard frogs at lentic wetlands in southwestern South Dakota, USA, October 2014. Sites in the Black Hills (dark green shading) are in black and Buffalo Gap National Grassland (light green shading) are in orange. (Map data from Stamen Maps©).

and tibiofibula in millimeters. We considered frogs to be adults if SUL ≥ 51 mm (Dodd 2013). We used the presence of enlarged thumb size to determine the sex adults (males larger than females; Conant and Collins 1998). Several BGNG frogs vocalized breeding calls, including while in hand during processing, which aided in deducing sex of individuals (Bubac et al. 2017). Using pigmentation descriptions of leopard frogs as provided by regional and South Dakota authorities (Conant and Collins 1998; Fischer et al. 1999; Ballinger et al. 2000; Smith and Quinn 2012; https://amphibiaweb.org; https://sdherps.org), we categorically scored individuals according to coloration and other morphological phenotypic traits (e.g., thigh wash patterns and absence/ presence of tympanum spots; Table 1). We also counted the total number of snout spots and spots located between the dorsolateral folds (DLFs). We noted if the DLFs were continuous, broken, or indistinct. We processed and released all frogs at their site of capture. Logistics and resource limitations prevented additional sampling.

***Statistical analyses*.**—We performed Factor Analysis of Mixed Data (FAMD) in the R package FactoMineR (Lê et al. 2008) to explore qualitative and quantitative (i.e., morphometric/ coloration) traits of individuals (Jaeger et al. 2001). We omitted trait factors with little variance (e.g., thigh pattern and thigh wash coloration), examined quantitative traits with Pearson correlation matrix, and used Shapiro-Wilk tests to confirm normality of the remaining continuous variables. Because leopard frogs from BH (i.e., higher elevations) would most likely be *L. pipiens* (see Smith 2003; Smith and Quinn 2012), cluster separation or individual differentiation in multivariate space for localities outside of BH could identify variability within the *L. pipiens* complex in SD.

We used Logistic Regression to assess morphological differences in frogs between BH and BGNG populations, the response variable. We performed backwards stepwise selection (Venables and Ripley 2013) from a full model fitted with the following predictors: SUL, femur length, head-width, and tibiofibular length. We used corrected Akaike's Information Criterion (AICc) scores and model weights in the AICcmodavg package (Mazerolle 2020), where the optimal model had the lowest ΔAICc > 2 (Jenkins and Quintana-Ascencio 2020). We repeated this process with sex as the response variable but did not separate populations due to sample size. Unless specified, we set α to 0.05 and report means ±1 standard deviation where applicable. We performed all statistical analyses in R version 4.1.1 (R Core Team 2021). We used R packages ggmap (Kahle and Wickham 2013) and sp (Bivand et al. 2013) to generate the sampling map.

RESULTS

We collected data on 27 post-metamorphic leopard frogs: four individuals from two sites in the BH and 23 individuals from nine sites in the BGNG (Fig. 1). We classified 20 frogs as adults based on SUL, one as a subadult, and six as undetermined (not measured). Five individuals were female, 13 were male, and nine were of undetermined sex. We did not detect any aberrant color morphs known from the region (e.g., lacking spots or exhibiting heavy mottling instead of spotting; see Smith and Quinn 2012); all individuals were either brown/tan, olive, or green in base coloration and mostly exhibited typical *L. pipiens* morphological phenotypic variation. Most leopard frogs (n = 23) had a single snout spot, three had no spots, and one had two spots. The mean number of spots between DLFs was 13.0 ± 2.9 spots. The number of snout spots was correlated with the number of spots between DLFs ($r = 0.66$), but these traits were not strongly correlated with any measured morphometric attribute ($r \leq 0.32$). There was correlation

Table 1. Qualitative trait variables (character) used for leopard frogs species in southwestern South Dakota, USA, October 2014. Superscripts depict local and regional descriptions for Northern Leopard Frogs (*Lithobates pipiens*) and Plains Leopard Frogs (*L. blairi*): References are A = Conant and Collins (1998); B = Ballinger et al. (2000); C = Fischer et al. (1999); D = Smith and Quinn (2012); and E = web authorities (amphibiaweb.org or sdherps.org).

Character	*Lithobates pipiens*	*Lithobates blairi*	Sublevels
Base color	Greenish to brown ^{A,B,C,D,E}	Greenish to brown ^{A,C,D,E}; tan ^{B,E}	Brown/tan; olive; green
Venter color	Cream to solid white ^{C,E}	Cream to solid white ^{E}	Cream; white
Throat color	White ^{C,E}	White but sometimes mottled/ stippled ^{E}	Mottled; white
Thigh wash color	Greenish, may have light yellow tint ^{C}	Greenish but sometimes yellow ^{A,C,E}	Greenish; yellow
Thigh wash pattern	non-reticulated ^{E}	Mottled reticulations ^{E}	Reticulated; non-reticulated
Dorsolateral folds (DLFs)	Continuous ^{A,B,C,D,E}	Broken posteriorly ^{A,B,C,D,E}	Broken; continuous; indistinct
Spot halos	Light-edged ^{A,C,E}	Light outline ^{E}	White; non-white; neither
Tympanum spot	Absent ^{A,B,C,D,E}	Distinct ^{A,B,C,D,E}	Absent; distinct; indistinct
Snout spot	May have spot ^{C}	Usually dark spot ^{A,C,E}	None, one, two

Table 2. Descriptive statistics of body morphology (in millimeters) characteristics for leopard frogs in southwestern South Dakota, USA, October 2014. Data are for males, females, and sex undetermined and are shown as mean ±1 standard deviation for measurements in millimeters.

Body morphology	Female (n = 5)	Male (n = 11)	Undetermined (n = 5)
Femur length	36.1 ±4.3	32.5 ±3.9	27.7 ±3.8
Head width	16.7 ±1.5	14.8 ±2.6	13.5 ±1.7
Snout-urostyle length (SUL)	78.2 ±4.3	71.3 ±9.0	61.5 ±11.0
Tibiofibula length	41.9 ±1.6	38.6 ±4.4	32.7 ±3.2

among all four morphometric body attributes (SUL, femur length, head-width, and tibiofibular length; $r \geq 0.66$), with tibiofibular length exhibiting the strongest correlation with the other morphometrics ($r \geq 0.79$; Table 2).

Candidate hybrids.—One female (BGNG, 43.189°N, −103.041°W) had an indistinct and partially broken DLF on one side, but not inset medially as commonly observed in *L. blairi*, a potential indication of a hybrid individual (Ernst 2001; Smith and Quinn 2012). Also, an adult male (43.371°N, -103.010°W; northeastern-most BGNG site in Fig. 1) exhibited multiple characteristics intermediate of *L. blairi* and *L. pipiens* including a reticulated thigh pattern and distinct tympanum spots, yet the DLFs were unbroken (see Fig. 2). Two additional individuals at that site had indistinct tympanum spots, but otherwise resembled typical *L. pipiens*. The BGNG sites with intermediate characteristics were the easternmost that we sampled and plausibly closest to any population known to have *L. blairi* × *L. pipiens* alleles (Ernst 2001; Di Candia and Routman 2007).

Multivariate examination of coloration and morphology.—The first three dimensions of multivariate space captured 62.1% of the total variance. Morphometric attributes, population, and coloration drove the first three dimensions, respectively. The most contributing and representative quantitative variable was tibiofibula length. Samples from BH clustered somewhat separately from BGNG (Fig. 2), representing variability among the morphometric and coloration characteristics sampled. All other qualitative factors, including tympanum spot (Fig. 2), did not separate in

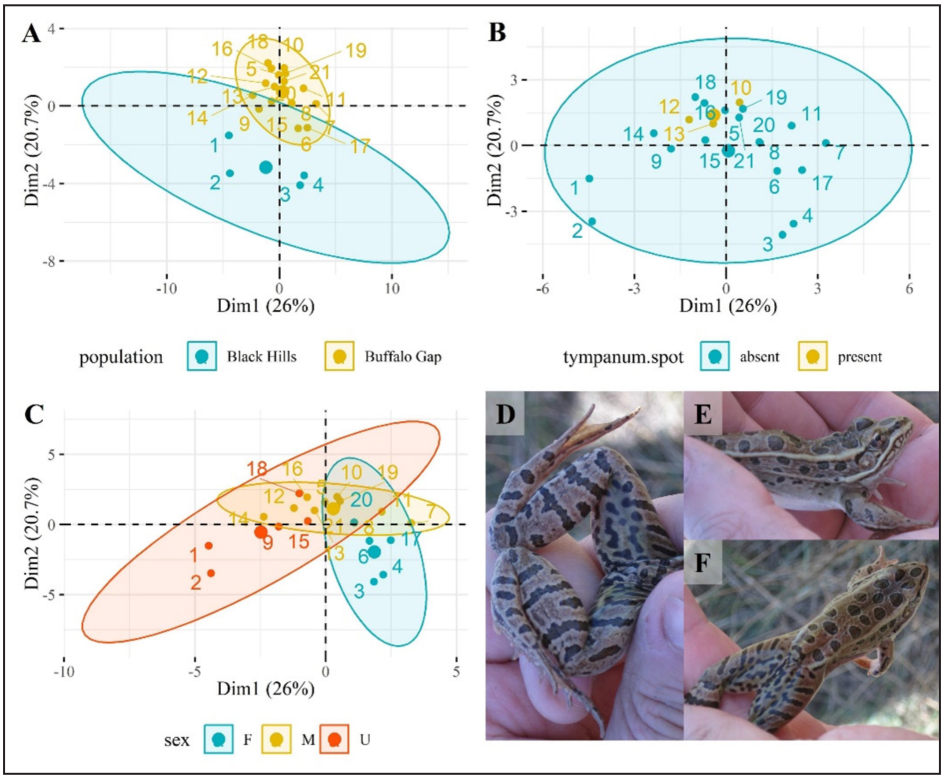

Figure 2. Multivariate biplots of variation in individual leopard frogs in southwestern South Dakota, USA, October 2014. Data are partitioned by (A) population; (B) tympanum (ear) spot; and (C) sex (F = female; M = male; U = unknown). Inset photographs of an individual expressing intermediate characteristics of a putative *Lithobates blairi* × *L. pipiens* hybrid, including (D) reticulated thigh patterns, (E) prominent lip stripe and distinct tympanum spot, but with (F) continuous dorsolateral folds, broken in pure L. blairi. (Photographed by Christine Bubac).

Table 3. Logistic Regression model results of body size characteristics of leopard frogs by population (upper) and sex (lower) in southwestern South Dakota, USA, October 2014. Model comparisons use corrected Akaike Information Criterion (AICc); no fitted models outperformed the null intercept at explaining variation in the response variable (i.e., ΔAICc < 2). Body size metrics are summarized in Table 2.

Response	Model coefficients	k	AICc	ΔAICc	AICc Weight	Cumulative Weight
Population	~ SUL	2	23.27	---	0.43	0.43
	~ SUL + headwidth	3	24.04	0.78	0.29	0.71
	~ 1 (null)	1	24.81	1.55	0.20	0.91
	~ SUL + headwidth + tibiofibula	4	26.71	3.45	0.08	0.99
	~ SUL + headwidth + tibiofibula + femur	5	30.21	6.94	0.01	1.00
Sex	~ SUL	2	22.03	---	0.54	0.54
	~ 1 (null)	1	23.52	1.49	0.26	0.79
	~ SUL + femur	3	24.31	2.28	0.17	0.97
	~ SUL + headwidth + femur	4	27.79	5.76	0.03	1.00
	~ SUL + headwidth + tibiofibula + femur	5	32.15	10.13	0.00	1.00

multivariate space. We observed some morphometric differences between sexes, but complete separation was not apparent (Fig. 2).

The morphometric traits examined did not significantly differ between populations (Table 3). That is, no fitted model outperformed the null intercept (i.e., ΔAICc < 2) and no fitted model coefficients were significant ($P > 0.05$). Similar results occurred for sex; no fitted model was 2 AICc units lower than the null intercept model (Table 3). Although there was morphometric variation among individuals in southwestern SD (as shown in FAMD analyses), we did not detect phenotypic distinction between populations or sexes.

Discussion

With changing environmental conditions that could influence the spatial distributions of species (Bickford et al. 2010), it is important to understand where introgression might occur and how it will affect adaptation (Hedrick 2013; Chunco 2014). Based on the morphological characters observed in the field, we surmise that nearly all leopard frogs sampled for the current study were *L. pipiens*. At least four of the 23 (17.4%) individuals from BGNG displayed morphologically diagnosable characteristics, however, indicative of *L. blairi* × *L. pipiens* hybrids. Although multivariate FAMD analyses revealed some separation between the BH and BGNG populations, these differences were subtle. The extent of phenotypic variation observed between populations may be indicative of local adaptation; habitats differ between the BH and BGNG areas, largely due to elevation and hydrological parameters. Our data shed some light on the extent of cryptic variation existing in southwestern SD. Of body morphometric characters, tibiofibular length may be important in deciphering finite population-level differences, albeit likely not as a standalone diagnostic.

Our phenotypic findings corroborate support for *L. blairi* × *L. pipiens* hybridization in southwestern SD (Dunlap and Kruse 1976; Smith and Quinn 2012). The sites with obvious or subtle *L. blairi* characteristics (e.g., extent of tympanum spotting, broken DLFs, thigh pattern) are proximal to where introgression could be expected given prior sampling of the region (e.g., Ernst 2001; Di Candia and Routman 2007; O'Donnell and Mock 2012). We did not detect any hybrid characteristics from BH samples; *L. blairi* is likely absent from forested (and elevated) regions in the Black Hills (Smith 2003; Smith and Quinn 2012).

Introgression between *L. blairi* and *L. pipiens* may have occurred for longer periods and across a broader geographic extent than previously thought. In southwestern South Dakota and northwestern Nebraska, for example, nuclear alleles from *L. blairi* have been detected in *L. pipiens* populations (Ernst 2001; Di Candia and Routman 2007; O'Donnell and Mock 2012). Mitochondrial and nuclear DNA analyses suggests that introgression may be unidirectional: female *L. pipiens* mating with male *L. blairi* or *L. pipiens* possessing *L. blairi* alleles that result in individuals with *L. blairi* morphology in allopatric *L. pipiens* regions where *L. blairi* alleles are present (Di Candia and Routman 2007). This could in part explain the BGNG sites that exhibited hybrid characteristics, especially due to spatial proximity to known pure *L. blairi* or introgressed populations.

Phenotypic variation in other adaptive traits, including life history and behavior, are also expected to vary with hybridization and introgression (Chunco 2014; Harrison and Larson 2014). While surveying for the current study at BGNG sites, Bubac et al. (2017) recorded the latest known seasonal breeding phenology by *L. pipiens*, where adult males possessed enlarged thumbs and were vocally calling (diurnally). In SD, *L. pipiens* are typically springtime breeders (e.g., April-June), whereas *L. blairi* normally exhibit breeding behavior in June with extension into September (Smith 2003; https://sdherps.org). Environmental cues likely influence anuran calling phenology and could result in documenting late-season breeding characteristics. For example, Boreal Chorus Frogs (*Pseudacris maculata*) also exhibited record-late calling in the BGNG (Blais et al. 2015). Investigating the genotypes underlying phenotypic variation, while modeling environment × single nucleotide polymorphism (SNP) interactions to account for phenotypic plasticity (Gienapp et al. 2017), would provide insight into the genetic basis of ecologically important traits as well as relationships between genotypes and phenotypes in hybrid individuals. For instance, could late-season breeding characteristics of *L. pipiens* (earlier breeder) be influenced from allele introgression by *L. blairi* (later breeder)? Because *L. blairi* typically inhabit drier habitats and may be more tolerable of anthropogenic disturbances (e.g., agriculture land-use changes) than *L. pipiens* (Di Candia and Routman 2007), would *L. blairi* × *L. pipiens* introgression represent adaptive benefits in a changing climate? Or could introgression be swamping genetic diversity in the long term?

Herein, we have provided evidence of morphological variation that suggests *L. blairi* × *L. pipiens* hybrids may be more prevalent in southwestern SD than realized. Additional sampling of leopard frogs in southwestern SD, including obtaining tissue samples *in situ* or from museum specimens, is warranted to confirm the spatiotemporal extent of introgression, gene flow, and population genetic risks (e.g., swamping; Smith and Quinn 2012; Pabijan et al. 2020). High throughput DNA sequencing techniques should be used to incorporate large volumes of SNPs or other genetic markers in analyses to accurately resolve the extent of introgression and gene flow between these taxa in SD (see O'Donnell and Mock 2012), especially at the sites identified with putative hybrids. High throughput studies could also help explain the extent of local adaptation in heterogeneous environments (Harrison and Larson 2014). Such studies can inform effective conservation management strategies for sensitive fauna in the Great Plains and Black Hills ecosystems, including *L. blairi* and *L. pipiens,* as they experience warming, drying, and other disturbances attributed to anthropogenic climate change (Fontaine et al. 2001; Brown 2006; Smith and Quinn 2012).

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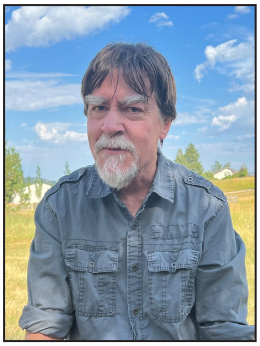

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