BioOne COMPLETE

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Danielle R. Bradke,[1,2] Eric T. Hileman,[3] Jeffrey F. Bartman,[4] Lisa J. Faust,[5] Richard B. King,[3] Nathan Kudla,[1] and
Jennifer A. Moore[1]

[1]*Biology Department, Grand Valley State University, Allendale, Michigan, USA*
[3]*Department of Biological Sciences, Northern Illinois University, DeKalb, Illinois, USA*
[4]*Biology Department, Eastern Michigan University, Ypsilanti, Michigan, USA*
[5]*Alexander Center for Applied Population Biology, Lincoln Park Zoo, Chicago, Illinois, USA*

Abstract.—Destruction and fragmentation of wildlife habitat often results in small, isolated populations that are highly susceptible to extirpation. In many cases, however, estimates of population size are lacking, precluding accurate assessments of population viability and sound conservation management recommendations. The Eastern Massasauga (*Sistrurus catenatus*) is a federally threatened pitviper species that has been extirpated throughout much of its historic range attributable to agricultural conversion of wetland habitat and other synergistic threats. Population size is generally unknown among extant massasauga populations, making site-specific management difficult. In this study, we estimated genetic effective population size (N_e) and census population size (N_c) for Eastern Massasaugas at two sites in southwest Michigan. For each population, we used mark–recapture models to estimate N_c and the linkage disequilibrium method to estimate N_e. Our results revealed small N_c, with approximately 108 (95% CI = 87–165) and 148 (95% CI = 102–295) adults estimated at our study sites in Cass County and Barry County, respectively. Estimates of N_e were even smaller: approximately 29.5 (95% CI = 21.2–43.1) for Cass County and 44.2 (95% CI = 30.8–69.3) for Barry County. Additionally, N_e/N_c ratios were similar across study sites, suggesting some stability in this ratio for Eastern Massasaugas, at least for populations in close proximity. Although we did not detect high levels of inbreeding or relatedness in either population, we caution that these small populations could become increasingly vulnerable to extirpation from unpredictable threats such as disease and climate change.

As human populations rapidly grow and exploit natural areas, wildlife populations across the globe are increasingly impacted by habitat loss and fragmentation (Lande, 1999). One consequence of this exploitation is a rising number of small and isolated populations (Allendorf and Luikart, 2009), which are vulnerable to extirpation from a variety of factors. Specifically, small, isolated populations tend to have higher rates of inbreeding, lower genetic diversity, and increased fixation of deleterious alleles compared to large populations (Frankham, 1995a; Lande, 1995; Lynch et al., 1995). Small populations also have greater sensitivity to demographic stochasticity (i.e., natural random variation in vital rates and sex ratios; Lande, 1993; Mills, 2012). Moreover, when a population is too small, genetic and demographic influences may generate positive feedback between one another and interact with environmental stressors to produce an "extinction vortex" (Gilpin and Soulé, 1986; Fagan and Holmes, 2006).

Reptiles represent some of the most imperiled species on the planet, with habitat destruction, fragmentation, and degradation among their greatest threats (Gibbons et al., 2000; Ananjeva et al., 2015; Tingley et al., 2016). Other forces driving reptile extinctions include overexploitation, climate change, disease, invasive species, and pollution (Gibbons et al., 2000; Tingley et al., 2016). Narrow geographic distributions, ties to specialized habitat, slow life histories, and temperature-dependent sex determination are among the factors that make many reptile species particularly susceptible to these threats (Tingley et al., 2013; Böhm et al., 2016a,b). Additionally, reptiles are the least understood group of terrestrial vertebrates in terms of viability, with less than half of documented species evaluated by the International Union for Conservation of Nature (Tingley et al., 2016). Of the 45% of reptile species evaluated, an estimated 20% are at risk of extinction while another 19% lack enough data to determine their status (Tingley et al., 2016). Population-level data, in particular, are generally lacking among reptiles and especially for snakes (Böhm et al., 2013).

Collecting population-level data can be difficult, especially when species are cryptic in nature, which is true of many snakes (Fitch, 1987); however, sufficient data are critical for making informed and effective conservation decisions. For instance, population viability analysis is a common tool for evaluating population extinction risk in vulnerable species, but incorporating inaccurate or nonsite-specific data can yield unrealistic and misleading results (Lande et al., 2003; Hileman et al., 2018b).

The Eastern Massasauga (*Sistrurus catenatus*) is a small pitviper species with an affinity for shallow wetland habitat and is distributed across the Great Lakes region of North America (Harding, 1997). Wetlands in this region have been a major target for agricultural conversion (Dahl and Johnson, 1991), making habitat loss and fragmentation the principal cause of population decline in this species (Szymanski, 1998; United States Fish and Wildlife Service, 2016). Eastern Massasauga populations are also threatened by human persecution, road mortality, and an emerging snake fungal disease caused by *Ophidiomyces ophiodiicola* (Shepard et al., 2008; Allender et al., 2016; Baker et al., 2016; Lorch et al., 2016). Because of population declines, the Eastern Massasauga is currently listed as threatened under the U.S. Endangered Species Act (U.S. Fish and Wildlife Service, 2016) and Canada's Federal Species at Risk Act (Environment Canada, 2012).

Massasaugas are secretive and have cryptic coloration, so monitoring and collecting data on their populations generally is difficult. Across the species' known historical distribution, only 46% of populations have been confirmed extant, whereas the rest are extirpated (26%) or have unknown status (28%; Szymanski et al., 2015). Most confirmed extant populations lack estimates of population size important for assessing long-term viability (White et al., 2002; Szymanski et al., 2015).

The population parameter generally of greatest interest to managers is census size (N_c), which represents the number of reproductively mature adults in a population (Luikart et al., 2010). N_c is important to monitor because it indicates how vulnerable a population is to demographic stochasticity (Lande, 1993; Mills, 2012). Additionally, biological interactions such as competition, mating, and cooperative social behaviors may be influenced by N_c.

A second, and arguably equally important, parameter to monitor is the genetic effective size of a population (N_e). This parameter represents how many individuals would be in an "idealized" population (e.g., one with constant population size, equal family sizes, and a 1:1 sex ratio) that is experiencing genetic drift or inbreeding at the same rate as the population of interest (Wright, 1931). Therefore, any life-history attributes leading to deviations from an idealized population can cause N_e to be lower than N_c (Hare et al., 2011). N_e is important because it indicates how vulnerable a population is to genetic drift. Populations with smaller N_e experience stronger effects of genetic drift, which decreases genetic diversity via random changes in allele frequencies that result in fixation of alleles (Wright, 1931). With less genetic diversity to act on, the process of selection is constrained and populations are less adaptable to environmental change. Furthermore, populations with small N_e generally experience high levels of inbreeding, which can lead to reduced fitness (Keller and Waller, 2002; Reed, 2005).

Monitoring both N_e and N_c is ideal because each parameter is important to understanding population viability and the impacts of management decisions. If there is a consistent relationship between these parameters within a particular species or taxa, managers may be able to collect data on only one and infer the other, making monitoring efforts more efficient (Luikart et al., 2010). Comparing $N_e : N_c$ ratios across populations will help determine whether any stability in this ratio exists. Assessing variation in this ratio and its relationship to life history, demography, and the environment can also help elucidate the relative influence of these factors on N_e (Cooper et al., 2009; Luikart et al., 2010; Waples et al., 2013) and be used to guide management.

Here, we collected data on two Eastern Massasauga Rattlesnake populations in southwest Michigan. We used mark–recapture models and microsatellite genotypes to obtain estimates of N_c and N_e and calculate basic measures of genetic diversity (e.g., H_o, H_e) and inbreeding (F_{IS} and relatedness). Our objectives were to 1) provide baseline estimates of population size important to monitoring this federally threatened species, 2) assess consistency in the N_e/N_c ratio across sites, and 3) evaluate N_e estimates for perceptible effects on genetic diversity or rates of inbreeding.

MATERIALS AND METHODS

Study Sites and Field Methods.—Our two study sites are located in southwest Michigan and are ∼86 km apart (Fig. 1). The first site, in Cass County, is on 1,052 ha of private property composed of prairie, meadow, wetland, forest, open water, cropland, private roads, and buildings. The second site, in Barry County, is located on ∼277 ha of privately owned land that includes forest, wetland, open water, old-field, prairie, and buildings and is bisected by a public dirt road. Both sites are actively managed for Eastern Massasaugas (e.g., controlled burns, invasive species removal) and a large proportion of the land cover surrounding each site is agricultural or otherwise developed. Within a 5-km radius

FIG. 1. Locations of the two Eastern Massasauga study sites in southwest Michigan: Cass County (black) and Barry County (black cross-hatching).

around the center of each study area, ∼72% of land in Cass County and 48% of land in Barry County is classified as either "developed" or "planted/cultivated" for livestock/crop production (based on the 2011 National Land Cover Database; Homer et al., 2015).

We used a combination of visual encounter surveys, drift fences with funnel traps, and artificial cover objects to capture snakes within an ∼64 ha survey area at the Cass County site and an ∼20 ha survey area at the Barry County site. Surveys at the Cass County site were conducted from 28 March to 8 October 2012 and surveys at the Barry County site were conducted from 28 April to 30 August 2015. Surveyors recorded search effort (i.e., time looking for snakes), captured snakes using tongs, and secured individuals in cloth bags within buckets until processing. We recorded all capture locations using handheld GPS units.

We measured total length to the nearest 0.1 cm using a squeezebox (Quinn and Jones, 1974) and flexible measuring tape. We restrained snakes in clear plastic tubes, measured tail length (starting at the posterior end of the anal plate) with a ruler, and subtracted tail length from total length to obtain snout–vent length (SVL; Fitch, 1987). We determined sex via cloacal probing (Schaefer, 1934) and palpated females for the presence of embryos. We classified females as adults if SVL was ≥45.1 cm, the length of the smallest gravid female observed from 2010–2016 (Cass County) and 2013–2016 (Barry County). We based adult male size on the smallest male with motile sperm detected via cloacal smear at the Cass County site (i.e., 43.3 cm SVL; R. B. King, pers. com.). We marked each individual with a subcutaneous Avid passive integrated transponder (PIT) tag (Avid Identification Systems, Inc., Norco, CA; Gibbons and Andrews, 2004) and stored blood collected from the caudal vein in either 95% or 100% ethanol. After processing, we released snakes at their respective capture locations.

Laboratory Methods.—We extracted DNA from ~10 μL of blood using Qiagen DNEasy kits following standard manufacturer protocols. We used primers developed by Anderson et al. (2010) to amplify 17 microsatellite loci from each DNA sample. Each 10 μL PCR reaction consisted of 20–100 ng DNA, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl$_2$, 1 μL of 0.5 mg/mL bovine serum albumin, 0.2 μL deoxynucleotide solution mix (0.2 mM of each), 1 unit Taq DNA Polymerase, 0.6 μL primers (2 μM of each, with fluorescently labeled forward primer), and 5 μL double-distilled H$_2$O. We amplified markers using an Eppendorf Mastercycler nexus gradient thermal cycler and followed the protocol in Anderson et al. (2010), except for modified annealing temperatures. Loci and respective annealing temperatures were as follows: *Scu200* (62°C), *Scu201* (62°C), *Scu202* (60°C), *Scu203* (60°C), *Scu204* (62°C), *Scu205* (60°C), *Scu206* (62°C), *Scu208* (62°C), *Scu209* (62°C), *Scu210* (56°C), *Scu211* (56°C), *Scu212* (56°C), *Scu213* (56°C), *Scu214* (56°C), *Scu215* (50°C), *Scu216* (56°C), and *Scu217* (50°C). For all PCR runs, we included a negative control for each locus to detect any contamination. Following amplification, fragment analysis was performed with an ABI3730 DNA Analyzer (Applied Biosystems, Corp., Foster City, CA) at the University of Arizona Genetics Core. We scored fragments using PeakScanner vers. 2.0 (Applied Biosystems).

Genetic Analyses.—To assess genotype accuracy, we reamplified and genotyped a random sample of 12% of individuals (15 of 124) from our full data set (that included juveniles and adults captured in years not included for this study). Based on this, we calculated an allele scoring error rate (i.e., incorrect alleles/total alleles). We used Micro-Checker vers. 2.2.3 (Van Oosterhout et al., 2004) to check the full data set for null alleles, large allele dropout, and stuttering. We used FreeNA (Chapuis and Estoup, 2007) to estimate null allele frequencies according to Dempster et al. (1977). To verify that our study sites represented two distinct populations, we used program STRUCTURE vers. 2.3.4 (Pritchard et al., 2000; details in Appendix 1). For all other descriptive statistics and analyses, we included only genotypes from adults captured during the years of this study.

We calculated the number of alleles (N_a), effective number of alleles (effective N_a), observed heterozygosity (H_o), and expected heterozygosity (H_e) for each locus using GenAlEx v6.503 (Peakall and Smouse, 2006, 2012). We used GenAlEx to estimate mean pairwise relatedness according to Queller and Goodnight (1989). We used FSTAT vers. 2.9.3.2 (Goudet, 2001) to calculate F_{IS} according to Weir and Cockerham (1984) and test whether F_{IS} per population was significantly positive or negative. Using GENEPOP vers. 4.6 (Rousset, 2008), we tested for deviations from Hardy-Weinberg equilibrium using exact tests and tested for linkage disequilibria using pairwise exact tests. We performed all analyses separately for each population. For Hardy-Weinberg and linkage disequilibria tests, we used the default settings, a priori set α = 0.05 to evaluate significance, and applied a sequential Bonferroni correction to account for multiple tests (Holm, 1979; Rice, 1989).

To estimate contemporary effective population size (N_e), we used the linkage disequilibrium (LD) method. This single-sample method estimates N_e based on the frequency of correlations between alleles located at separate loci (i.e., linkage disequilibrium; Hill, 1981). Linkage disequilibrium is inversely related to N_e, because in physically unlinked neutral loci it results mainly from genetic drift, which becomes a stronger evolutionary force as population size decreases (Waples and Do, 2010). We implemented the LD method using NeEstimator vers. 2.1 (Do et al., 2014), which uses the Jones et al. (2016) improved

jackknife method for estimating 95% confidence intervals achieved by jackknifing over individuals (rather than pairs of loci as in vers. 2.01). We used a random mating model and excluded alleles with frequencies <0.02 as recommended by Waples and Do (2010) to best balance the bias associated with keeping rare alleles and the precision lost by removing them.

Census Size Estimation.—Our estimates of census size (N_c) represent abundance estimates of reproductively mature adults within each study area during the respective survey period. We used Huggins closed-capture models (Huggins, 1989, 1991; Alho, 1990) in Program MARK vers. 8.1 (White and Burnham, 1999), which allowed us to censor individuals held for captive parturition at the Cass County site. We truncated the data sets to include ≤4 mo of data to approximate the assumptions of closure (i.e., no births, deaths, recruitment, immigration, or emigration). To maximize the data retained for this analysis, we selected time periods that included the greatest number of captures. For the Cass County site, we used captures from 17 April to 14 August and, for the Barry County site, we used captures from 28 April to 14 August. For each site, we pooled our data into seven capture occasions. To assess whether truncated data sets adequately met the assumptions of geographic closure, we tested for violations of these assumptions using Pradel (1996) models in Program MARK vers. 8.1 following the approach of Boulanger et al. (2002). For these models, we allowed all estimated parameters to vary with sex and recapture rates to vary with time. We used likelihood ratio tests to compare models allowing only immigration or only emigration to a null model. We also compared a model allowing both immigration and emigration to models allowing only immigration or only emigration. We estimated overdispersion for global models using the Fletcher $\hat{c}$, where a value of ~1 indicates the data are adequately independent and identically distributed (Fletcher, 2012; White and Cooch, 2017).

For the Huggins closed-capture analyses, we constructed a set of 16 candidate models that included combinations of variables expected to affect capture (p) and recapture (c) probabilities (Table 1). We constrained these parameters to be constant or allowed them to vary by sex, search effort, or time, including additive or interactive effects (Table 1). We also considered embellishments of the models described above, incorporating an additive effect of behavior. In the behavior models, p and c differed from one another by a constant, presumably due to a handling effect (i.e., "trap happy" or "trap shy"; Otis et al., 1978). We ranked models using Akaike's information criterion adjusted for small sample size (AICc; Akaike, 1973; Burnham and Anderson, 2002) and model averaged according to AICc weight to account for model selection uncertainty. We calculated lognormal 95% confidence intervals for each estimate. Summary statistics are reported as means ± SE.

RESULTS

We genotyped 47 (24 female, 23 male) and 53 (35 female, 18 male) adult Eastern Massasaugas from Cass County and Barry County, respectively. One locus (*Scu200*) was removed from the data set before running any analyses. This locus was described as a tetranucleotide repeat by Anderson et al. (2010); however, scoring conflicted with the published repeat motif (e.g., some heterozygotes had alleles two base pairs apart). For the remaining 16 loci, alleles per locus ranged from 2–12 (mean = 6.5 ± 0.68) for Cass County, and from 2–13 (mean = 7.2 ± 0.77) for Barry County (Appendix 2). Observed heterozygosity for a

TABLE 1. Candidate models used to estimate abundance of Eastern Massasaugas at two sites in southwest Michigan. K = number of parameters. Capture probability (p) and recapture probability (c) are modeled as equal (=) or as differing by an additive constant (+b). Variables considered for an effect on p and c include: time, sex, and effort. Effort refers to total search time (h) per sampling occasion. Additional notation: (*) interaction term; (+) additive term; (.) invariant parameter.

Model	K
$p(.)=c(.)$	1
$p(sex)=c(sex)$	2
$p(effort)=c(effort)$	2
$p(time)=c(time)$	7
$p(sex+effort)=c(sex+effort)$	3
$p(sex+time)=c(sex+time)$	8
$p(sex*effort)=c(sex*effort)$	4
$p(sex*time)=c(sex*time)$	14
$p(.)=c(.)+b$	2
$p(sex)=c(sex)+b$	3
$p(effort)=c(effort)+b$	3
$p(time)=c(time)+b$	8
$p(sex+effort)=c(sex+effort)+b$	4
$p(sex+time)=c(sex+time)+b$	9
$p(sex*effort)=c(sex*effort)+b$	5
$p(sex*time)=c(sex*time)+b$	15

given locus varied widely, ranging from 0.15–0.91 (mean 0.70 ± 0.05) in Cass County, and from 0.08–0.94 (mean 0.69 ± 0.06) in Barry County (Appendix 2). The F_{IS} value calculated across all loci was −0.035 ($P = 0.04$) for Cass County and 0.010 ($P = 0.29$) for Barry County. Mean pairwise relatedness was the same for each population ($R = -0.02 ± 0.01$).

We calculated an allele scoring error rate of 0.2% and corrected the error before proceeding with further analyses. We did not detect evidence of allelic dropout or stuttering using Micro-checker. Based on Micro-checker and FreeNA, one locus (Scu206) demonstrated evidence of null alleles but only in the Barry County population, with an estimated frequency of 8.4% (Appendix 2). Because the estimated null allele frequency was 0% for this locus at the Cass County site, we retained it for further analyses. No loci significantly deviated from Hardy-Weinberg equilibrium. We detected evidence of linkage disequilibrium at the Cass County site in 10 of 120 pairs of loci and at the Barry County site in 7 of 120 pairs of loci (Appendix 3). Five pairs of loci with linkage disequilibria were consistent across sites (Appendix 3). Effective population size estimated using the linkage disequilibrium method was 29.5 (jackknife 95% CI = 21.2–43.1; Fig. 2) for Cass County and 44.2 (jackknife 95% CI = 30.8–69.3; Fig. 2) for Barry County.

We surveyed 568.8 h from 17 April to 14 August 2012 at the Cass County site. Pooling data to account for low encounter probabilities resulted in 132 captures of 74 unique adults (30 gravid females, 13 nongravid females, 31 males). We did not detect evidence of overdispersion for the global model in our Huggins or Pradel analyses (Fletcher $\hat{c}$ = 0.99 and 1.00, respectively). Likelihood ratio tests between Pradel models revealed no evidence of geographic closure violations (null vs. emigration, $\chi_2^2 = 3.89$, $P = 0.14$; null vs. immigration, $\chi_2^2 = 3.87$, $P = 0.14$; emigration vs. migration, $\chi_2^2 = 3.95$, $P = 0.14$, immigration vs. migration, $\chi_2^2 = 3.97$, $P = 0.14$). Five of the 16 candidate models included in the Cass County N_c analysis received AICc support (Table 2). The top-ranked model, which included additive effects of sex and search effort on encounter probability, received 42% of AICc weight. The second-, third-,
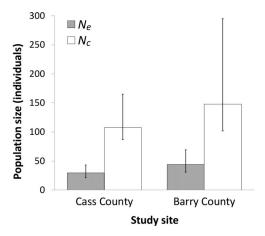

FIG. 2. Eastern Massasauga effective population size (N_e) and census population size (N_c) estimates for the two study sites located in southwest Michigan. Data were collected during 2012 for the Cass County site and 2015 for the Barry County site. Error bars represent 95% confidence intervals.

and fifth-ranked models were all variants of the top-ranked model, but each one included an additional parameter for an additive behavioral effect, an interaction between sex and effort, or both. These additional parameters were considered uninformative, however, because the model deviance did not decrease by at least two units with the addition of either parameter (Arnold, 2010; Table 2). The fourth-ranked model included an additive effect of sex and time on encounter probabilities, indicating weak support for time as an informative parameter because this model received 14% of AICc weight. Our model-averaged abundance estimate was 108 (95% CI = 87–165; Fig. 2), including 46 females (95% CI = 44–56; Fig. 3) and 62 males (95% CI = 42–119; Fig. 3). Using the point estimates for N_e and N_c, we estimated an N_e/N_c ratio of 0.27 for this site.

We surveyed 462.3 h from 28 April to 14 August 2015 at the Barry County site. Pooling data to account for low encounter probabilities resulted in 166 captures of 80 unique adults (45 gravid females, 6 nongravid females, 29 males). We did not detect evidence of overdispersion for the global model in our Huggins analysis (Fletcher $\hat{c}$ = 1.00) or Pradel analysis (Fletcher $\hat{c}$ = 1.03). Likelihood ratio tests between Pradel models revealed no evidence of geographic closure violations (null vs. emigration, $\chi_2^2 = 3.36$, $P = 0.19$; null vs. immigration, $\chi_2^2 = 1.06$, $P = 0.59$; emigration vs. migration, $\chi_2^2 = 2.83$, $P = 0.24$, immigration vs. migration, $\chi_2^2 = 5.14$, $P = 0.08$). Nine of the 16 candidate models included in the Barry County N_c analysis received AICc support (Table 2); however, the top two models received 82% of the total weight and, therefore, contributed the most to the model-averaged estimate. The top-ranked model included additive effects of sex, search effort, and behavior on encounter probability, and received 55% of AICc weight. The second-ranked model was identical to the top model, except it included an interaction between sex and effort; however, the addition of this interaction term did not reduce the model deviance by at least two units, indicating that it was an uninformative parameter (Arnold, 2010). The remaining models suggest weak support for time as an informative model parameter. Our model-averaged abundance estimate was 148 adults (95% CI = 102–295; Fig. 2), including 63 females (95% CI = 54–97; Fig. 3) and 85 males (95% CI = 46–212; Fig. 3). Our estimated N_e/N_c ratio for this site was 0.30.

TABLE 2. Model selection for adult Eastern Massasauga abundance estimates in A) Cass County, Michigan, 2012, and B) Barry County, Michigan, 2015. Only models garnering $\geq 1\%$ of AICc weight (w_i) are shown. Models are in ascending ΔAICc order. K is the number of parameters. Deviance is the difference in $-2\log(\mathcal{L})$ of the current model and $-2\log(\mathcal{L})$ of the saturated model, where $\mathcal{L}$ is the maximized likelihood for each model. Capture probability (p) and recapture probability (c) are modeled as equal (=) or as differing by an additive constant (+b). Explanatory variables considered for p and c include time, sex, and effort. Effort refers to total search time (h) per sampling occasion. Additional notation: (*) interaction term; (+) additive term.

Model	AICc	Δ AICc	w_i	$\mathcal{L}$	K	Deviance
A) Cass County, Michigan						
p(sex+effort)=c(sex+effort)	497.15	0.00	0.42	1.00	3	501.42
p(sex+effort)=c(sex+effort)+b	498.58	1.44	0.21	0.49	4	500.83
p(sex*effort)=c(sex*effort)	499.18	2.03	0.15	0.36	4	501.42
p(sex+time)=c(sex+time)	499.34	2.19	0.14	0.33	8	493.37
p(sex*effort)=c(sex*effort)+b	500.61	3.46	0.07	0.18	5	500.81
B) Barry County, Michigan						
p(sex+effort)=c(sex+effort)+b	628.85	0.00	0.55	1.00	4	584.78
p(sex*effort)=c(sex*effort)+b	630.28	1.44	0.27	0.49	5	584.18
p(sex+time)=c(sex+time)	633.75	4.91	0.05	0.09	8	581.49
p(sex)=c(sex)+b	634.21	5.36	0.04	0.07	3	592.17
p(sex+time)=c(sex+time)+b	634.37	5.52	0.03	0.06	9	580.04
p(sex+effort)=c(sex+effort)	635.16	6.31	0.02	0.04	3	593.12
p(sex*time)=c(sex*time)	635.71	6.86	0.02	0.03	14	570.94
p(sex*effort)=c(sex*effort)	636.09	7.24	0.01	0.03	4	592.02
p(sex*time)=c(sex*time)+b	636.34	7.50	0.01	0.02	15	569.46

DISCUSSION

Our results demonstrate small effective and census population sizes for Eastern Massasaugas at both study sites. We estimated an effective population size of ~30 individuals at the Cass County site and ~44 individuals at the Barry County site. Both of these N_e estimates are below the minimum thresholds commonly advocated to avoid extinction, where effective populations <50 are considered vulnerable to inbreeding depression in the short term (Franklin, 1980; Soulé, 1980) and populations <500 risk diminished adaptive potential from genetic drift in the long term (Franklin, 1980). Moreover, recent follow-up work on Franklin's and Soulé's rules for critical N_e provided evidence that minimum effective sizes should be even larger: ≥100 to avoid inbreeding depression within five generations and >1,000 for long-term viability (Frankham et al., 2014).

The effective population size estimator we used in this study has become popular in recent years because of its ability to
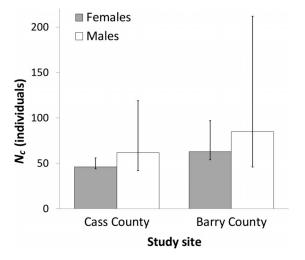

FIG. 3. Eastern Massasauga census population size (N_c) estimates for females and males at the two study sites located in southwest Michigan. Data were collected during 2012 for the Cass County site and 2015 for the Barry County site. Error bars represent 95% confidence intervals.

produce precise estimates for populations with relatively small N_e (i.e., < 200 individuals), using samples taken at a single point in time (Waples and Do, 2010). This estimator assumes discrete generations, however, which is rarely true in nature and is not true of Eastern Massasauga populations. To limit potential bias associated with violating this assumption, we included only adults in our data set, which were randomly sampled from our study site. Robinson and Moyer (2013) found this to be the best sampling approach for approximating true N_e in simulated populations of iteroparous species exhibiting various life histories, with all N_e estimates generated using this sampling strategy coming within 15% of true N_e. In simulating populations of species with a broader range of life-histories, however, Waples et al. (2014) found that even estimates based on randomly sampled adults resulted in N_e estimates that were biased low. Therefore, although N_e could be underestimated in our study, even a large percent bias in N_e (e.g. ~30%; Waples et al., 2014) would be relatively small in terms of actual number of individuals.

Contemporary effective population size estimates produced using genetic methods are scarce for other species of snakes, but our estimates fall within the range of those published (Table 3). Of 15 populations of federally threatened Giant Gartersnakes (*Thamnophis gigas*), the smallest reported N_e was 14.6 (95% CI = 10.6–32.0) and the largest was 82.0 (95% CI = 54.0–260.6; Wood et al., 2015). Likewise, estimates of N_e for 11 populations of locally threatened adders (*Vipera berus*) ranged from 4.1 (95% CI = 2.8–6.3) to 63.4 (95% CI = 29.9–597.5) when the linkage disequilibrium method was used (Ursenbacher et al., 2009; but note that this range omits five estimates with 95% CIs that spanned infinity). In a population of federally threatened New Mexico Ridge-Nosed Rattlesnakes (*Crotalus willardi obscurus*), N_e was estimated to be 220 (90% credible interval = 103–293; Holycross and Douglas, 2007), and more recent estimates for three populations of this species ranged from 25 to 70, but no estimates of error were reported (Davis et al., 2015). For Timber Rattlesnakes (*Crotalus horridus*) in New Jersey, where they are state listed as endangered, effective size was estimated for eight populations, but only one ($N_e = 4.0$; 95% CI = 2.4–10.7) had confidence intervals that did not span infinity (Bushar et al.,

TABLE 3.   Summary of contemporary effective population size (N_e) estimates published for snakes. Only estimates generated using genetic methods and reported with 95% confidence (*or 90% credible) intervals (CI) that do not span infinity are shown. The conservation status for each species applies to its specified study location(s). Methods used to estimate N_e include linkage disequilibrium (LDNe), approximate Bayesian computation (ABC), sibship assignment (Sibship), and a temporal likelihood-based estimator (LB).

Species	Conservation status	Location (population)	N_e	CI	Method	Source
Crotalus atrox	No special status	Sonoran Desert (I10W), AZ	78	58–112	LDNe	Herrmann et al., 2017
		Sonoran Desert (I10E), AZ	104	70–192	LDNe	
		Sonoran Desert (CT), AZ	108	78–170	LDNe	
Agkistrodon contortrix	No special status	Central CT	110.6	89.7–140.8	LDNe	Levine et al., 2016
			93.0	68–129	Sibship	
Crotalus horridus	State endangered	Highlands, NJ	4	2.4–10.7	LDNe	Bushar et al., 2015
Crotalus willardi obscurus	Federally threatened	Animas Mountains, NM	220	103–293*	LB	Holycross and Douglas, 2007
Sistrurus catenatus	Federally threatened	Barry County, MI	44.2	30.8–69.3	LDNe	This study
		Cass County, MI	29.5	21.2–43.1	LDNe	
		Bruce Peninsula, ON, Canada	53[a]	32–100[a]	Sibship	DiLeo et al., 2013
		Killbear, ON, Canada	34[a]	19–61[a]	Sibship	
		South of Parry Sound, ON, Canada	52[a]	31–105[a]	Sibship	
		Carlyle Lake, IL: 2002	19–26[b]	15–35[b]	LDNe	Baker, 2016
		2007	24–25[b]	19–32[b]	LDNe	
		2012	20–30[b]	15–40[b]	LDNe	
Thamnophis gigas	Federally threatened	American West, CA	54	42.7–125.5	ABC	Wood et al., 2015
		Badger Creek, CA	82	54.0–260.6	ABC	
		Colusa NWR, CA	44.6	33.1–115.1	ABC	
		Conaway Ranch, CA	55.1	40.9–120.3	ABC	
		Gilsizer Slough, CA	32.8	22.7–73.2	ABC	
		Gray Lodge, CA	13.3	11.1–20.0	ABC	
		Los Banos Creek, CA	14.6	10.6–32.0	ABC	
		Natomas East, CA	39.7	29.4–88.8	ABC	
		Natomas West, CA	63.7	39.8–174.6	ABC	
		North Yolo, CA	21.1	17.0–44.2	ABC	
		Sutter East of Bypass, CA	23.4	18.0–36.6	ABC	
		Sutter West of Bypass, CA	33.6	26.9–59.5	ABC	
		Volta Wildlife Area, CA	18.9	15.1–33.3	ABC	
		White Slough, CA	41.1	30.7–107.5	ABC	
		Yolo Wildlife Area, CA	44.6	30.8–109.6	ABC	
Vipera berus	Endangered in Switzerland	Alps (UR), Switzerland	11.7	6.7–24.8	LDNe	Ursenbacher et al., 2009
		Jura Mountains (BR), Switzerland	63.4	29.9–597.5	LDNe	
		Jura Mountains (CH1), Switzerland	39.3	28.3–58.1	LDNe	
		Jura Mountains (CH2), Switzerland	37.1	25.2–59.5	LDNe	
		Jura Mountains (PM), Switzerland	17.6	9.9–39.5	LDNe	
		Jura Mountains (FR2), France	4.1	2.8–6.3	LDNe	
		Jura Mountains (RO), France	7.1	4.0–15.2	LDNe	
		Jura Mountains (VC), France	14.4	6.7–89.9	LDNe	
		Massif Central (MA1), France	27.8	16.4–67.2	LDNe	
		Massif Central (MA2), France	5.3	3.0–12.8	LDNe	
		Rennes, France	35.0	16.6–424.8	LDNe	

[a] We approximated the values for *S. catenatus* in ON, Canada using fig. S4 in the supporting information of DiLeo et al. (2013).

[b] N_e estimates and 95% CIs for *S. catenatus* in Carlyle Lake, IL were presented as ranges in Baker (2016), based on applying three different minimum allele frequency ("P_{crit}") values per estimate.

2015). Even among nonthreatened snakes, N_e can be relatively small. In Ohio and Ontario, the mean N_e estimated among 10 populations of Common Gartersnakes (*Thamnophis sirtalis*) was 97 (range = 35–275) and N_e estimated for a population of Plains Gartersnakes (*Thamnophis radix*) in Illinois was 25, but no estimates of error were reported (King, 2009). For a Connecticut population of Eastern Copperheads (*Agkistrodon contortrix*), an N_e of 110.6 individuals (95% CI = 89.7–140.8) was estimated using the linkage disequilibrium method (Levine et al., 2016). Similarly, estimates of N_e for three subpopulations of Western Diamond-Backed Rattlesnakes (*Crotalus atrox*) ranged from 78 (95% CI = 58–112) to 108 (95% CI = 78–170; Herrmann et al., 2017). Conversely, median values of N_e were estimated to range from 32,419 to 41,722 individuals for Yellow Sea Kraits

(*Laticauda saintgironsi*) in the South Lagoon of New Caledonia (Bech et al., 2016). The scarcity of larger contemporary N_e reported for snakes may be attributable to the lack of estimators capable of reliably estimating N_e when the value of this parameter is large (i.e., large populations are typically estimated as infinite; Waples and Do, 2010; Luikart et al., 2010).

We are aware of only two other Eastern Massasauga studies where contemporary N_e was estimated. Contemporary N_e estimates for three populations in Ontario, Canada, ranged from about 34 to 53 individuals (with 95% CIs spanning about 19 to 105 individuals; see supporting information fig. S4 from DiLeo et al., 2013). In the other study, N_e estimates for a single population in Illinois varied from 19 to 30 individuals across 2002 to 2012 (with confidence intervals spanning 15 to 40

individuals; Baker, 2016). These estimates are surprisingly similar to ours. Chiucchi and Gibbs (2010) found evidence suggesting that Eastern Massasauga populations within 25 km of each other, located in Ohio, Illinois, and Pennsylvania, have been small and isolated for thousands of years. Therefore, this species may have been long adapted to persisting in small isolated patches of habitat. The relatively high levels of genetic diversity we observed, coupled with low inbreeding coefficients and low relatedness within each of our study populations supports potential adaptations for tolerating small N_e. For instance, massasaugas may be able to recognize and avoid mating with kin, similar to kin discrimination demonstrated in Timber Rattlesnakes (Clark, 2004), which could prevent inbreeding. The relatively high proportion of negative F_{IS}-values per locus per population we observed (50%; Appendix 2) may also indicate outbreeding and support kin recognition. Multiple paternity, which has been documented in Eastern Massasaugas (Stedman et al., 2016), may also reduce the effects of inbreeding and bolster genetic diversity (Stockley et al., 1993; Jennions and Petrie, 2000).

Although this species may have adaptations to cope with isolation and small N_e, compounding contemporary threats likely increase the vulnerability of populations to extirpation. Snake fungal disease was recently detected at both sites in this study (Allender et al., 2016), and the population-level implications of this disease are currently unknown (Lorch et al., 2016; Hileman et al., 2018a). Furthermore, environmental stochasticity is expected to escalate with climate change, likely impacting these populations in unpredictable ways. Hence, our small N_e estimates may be of greater concern presently than they would have been under past conditions.

Broad recommendations similar to those for effective population size are not available for census population size, because extinction risk from nongenetic factors is expected to be more contingent on life history and environmental characteristics specific to each population (Lynch et al., 1995). We are aware of only three other Eastern Massasauga populations with published estimates of abundance. In Cicero Swamp, New York gravid female abundance estimates made between 2006 and 2014 ranged from 9–41 individuals (Johnson et al., 2016). Based on these estimates, the authors extrapolated to infer a maximum population size of 164 adult rattlesnakes (Johnson et al., 2016). On Beausoleil Island, Ontario annual adult abundance estimates ranged from 35–77 individuals during 1993–2007 (Jones et al., 2017). In Carlyle Lake, Illinois, annual adult abundance estimates ranged from 18–69 individuals during 1999–2010 (Dreslik et al., 2017). Our estimates of 108 adults for Cass County and 148 adults for Barry County fall within the range of these other populations.

Results from the multimodel inference approach we used to estimate N_c indicated that Eastern Massasauga encounter probabilities were influenced by sex and search effort at both study sites. This is not surprising, as we expected increased search effort to result in higher rates of capture. Additionally, gravid females generally bask in open areas to stimulate embryological development, resulting in greater female detection (Bonnet and Naulleau, 1996) and, therefore, more precise estimates of population sizes for females than males (Fig. 3). For the Barry County population, model selection also supported a behavioral effect on encounter probabilities, where recapture probabilities were higher than capture probabilities in both sexes, but the magnitude of this effect was greatest for females. This "trap happy" response is likely a consequence of surveyor

bias, reflecting the tendency of surveyors to increase search effort in areas where snakes were previously found.

Luikart et al. (2010) suggested that N_e and N_c could be used interchangeably as surrogates for one another, assuming there is a consistent relationship between these parameters. Our $N_e : N_c$ ratios were consistent across study sites (0.27 for Cass County and 0.30 for Barry County), which may indicate some stability in this ratio for Eastern Massasaugas, at least for populations in close proximity (~86 km in this case). Numerous factors affect the $N_e : N_c$ ratio, however, including life history, environmental characteristics, and demography (Lee et al., 2011; Waples et al., 2013; Schrey et al., 2016; Waples, 2016), some of which demonstrably vary across the Eastern Massasauga's range (Jones et al., 2012; Hileman et al., 2017). Indeed, $N_e : N_c$ ratios have been reported to vary across different species (Frankham, 1995b; Palstra and Ruzzante, 2008; Palstra and Fraser, 2012), among populations of the same species (Belmar-Lucero et al., 2012), and even temporally within the same population (Ardren and Kapuscinski, 2003). Population census size itself may affect the ratio if variation in reproductive success decreases as N_c becomes smaller (i.e., genetic compensation; Palstra and Ruzzante, 2008), causing a disproportionately small reduction in N_e compared to N_c.

Implicit in our ratios of $N_e : N_c$ is the assumption that N_c sizes have been stable for at least one generation (i.e., ~5 yr; Sovic et al., 2016), because our estimates of N_e apply to the parent generation of the samples used to estimate them (Waples and Do, 2008). This assumption may be reasonable given that both sites are actively managed to maintain Eastern Massasauga populations and we have observed no detectable changes in estimated abundance from 2011–2016 at the Cass County site (Hileman et al., 2018b) and from 2013–2017 at the Barry County site (DRB, unpubl. data). Additional data are needed to evaluate if the $N_e : N_c$ relationship we observed for Michigan Eastern Massasauga populations holds geographically and temporally. If this ratio is not stable, future research should investigate what factors have the greatest influence on $N_e : N_c$ variation for this species.

Effective management of small wildlife populations is of increasing importance in conservation biology as humans continue to modify the landscape. Our results demonstrate that remnant populations of federally threatened Eastern Massasaugas can be very small, especially in terms of genetic effective size. Despite small N_e, we did not detect high levels of inbreeding or relatedness in either study population. Still, we caution that these populations could become increasingly vulnerable to extirpation from unpredictable threats such as disease and climate change.

Northern Illinois University's Institutional Animal Care and Use Committee (permit LA10-001).

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APPENDIX 1

Population structure analysis

To confirm that our study sites were two distinct populations, we performed a STRUCTURE analysis using a burn-in period of 50,000 Markov chain Monte Carlo (MCMC) iterations, 500,000 MCMC iterations after burn-in, the admixture model, and correlated allele frequencies. We used values for *K* that ranged from 1 to 4 and ran each *K*-value 10 times to verify consistent results across runs. We did not include prior location information. We then used STUCTURE HARVESTER Web v 0.6.9 (Earl and vonHoldt, 2012) to implement the Evanno (2005) method to calculate Δ*K*, which demonstrated strong support for *K* = 2 (Table A1). The STRUC-TURE results for all runs of *K* = 2 consistently assigned all individuals from the Cass County population to one cluster and all individuals from the Barry County population to the other cluster (Fig. 1A).

TABLE A1. Results from the Evanno (2005) method implemented in STUCTURE HARVESTER to identify the most likely value of *K*.

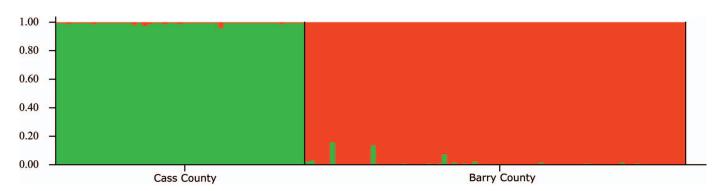| K	Mean LnP(K)	SD	Ln′(K)	|Ln″(K)|	ΔK
1	−6829.7	0.47	–	–	–
2	−6107.3	0.25	722.4	613.8	2469.5
3	−5998.8	0.98	108.5	30.6	31.3
4	−5920.9	23.55	77.9	–	–

FIG. A1. STRUCTURE bar plot for *K* = 2 population clusters, which was the most likely value of *K* identified using STUCTURE HARVESTER. The plot is grouped by study site location along the x-axis and displays each individual as a vertical bar. The y-axis displays the probability of assignment into either cluster, with each cluster represented by a different color.

APPENDIX 2. Genetic diversity at 16 microsatellite loci in two southwest Michigan populations of Eastern Massasaugas. Values reported are for number of alleles (N_a), effective number of alleles (effective N_a), allele size range, observed heterozygosity (H_o), expected heterozygosity (H_e), inbreeding coefficient (F_{IS}), and null allele frequency estimated according to Dempster et al. (1977) as implemented in FreeNA (Chapuis and Estoup, 2007).

	Locus															
Population (N)	scu201	scu202	scu203	scu204	scu205	scu206	scu208	scu209	scu210	scu211	scu212	scu213	scu214	scu215	scu216	scu217
Cass Co. (47)																
N_a	7	5	4	4	9	2	5	3	8	9	8	9	7	8	12	4
Effective N_a	4.8	2.6	2.7	2.2	4.6	1.6	1.5	1.2	5.5	6.5	5.7	6.4	4.9	5.7	8.0	2.5
Size range (bp)	213–245	171–185	231–255	147–163	189–233	188–194	176–192	181–185	176–227	293–329	341–369	189–225	167–195	111–157	200–268	167–181
H_o	0.77	0.68	0.72	0.64	0.72	0.47	0.38	0.15	0.85	0.85	0.89	0.85	0.77	0.85	0.91	0.64
H_e	0.79	0.62	0.63	0.55	0.78	0.38	0.33	0.14	0.82	0.85	0.82	0.84	0.80	0.83	0.87	0.60
F_{IS}	0.04	−0.08	−0.14	−0.15	0.09	−0.22	−0.14	−0.05	−0.03	0.00	−0.07	0.00	0.05	−0.02	−0.04	−0.06
Null alleles	0.007	0.000	0.000	0.000	0.034	0.000	0.000	0.000	0.000	0.000	0.000	0.006	0.012	0.000	0.000	0.002
Barry Co. (53)																
N_a	7	6	4	5	8	2	5	5	5	10	9	12	7	11	13	6
Effective N_a	3.3	3.5	2.1	3.6	6.2	1.1	2.6	1.6	3.1	7.1	6.6	6.6	4.1	6.9	9.1	3.2
Size range (bp)	213–253	177–189	243–255	147–163	189–221	188–194	160–192	179–187	170–218	289–333	329–373	193–241	155–187	111–159	196–332	173–185
H_o	0.77	0.64	0.49	0.70	0.79	0.08	0.66	0.32	0.77	0.87	0.85	0.94	0.79	0.85	0.79	0.70
H_e	0.70	0.71	0.52	0.72	0.84	0.11	0.62	0.38	0.68	0.86	0.85	0.85	0.75	0.85	0.89	0.69
F_{IS}	−0.10	0.11	0.07	0.04	0.07	0.30	−0.05	0.16	−0.13	0.00	0.01	−0.10	−0.04	0.02	0.12	0.00
Null alleles	0.000	0.016	0.000	0.026	0.023	0.084	0.000	0.025	0.000	0.000	0.012	0.000	0.000	0.000	0.028	0.000

APPENDIX 3. Pairs of loci exhibiting significant linkage disequilibria (LD) in Eastern Massasauga populations at two sites in southwest Michigan. * indicates pairs of loci with significant LD at both sites.

Population	Locus 1	Locus 2	P-value
Cass Co.	Scu204*	Scu205*	< 0.00001
	Scu213*	Scu201*	< 0.00001
	Scu202*	Scu210*	< 0.00001
	Scu202*	Scu217*	< 0.00001
	Scu212*	Scu216*	< 0.00001
	Scu214	Scu216	< 0.00001
	Scu201	Scu216	< 0.00001
	Scu215	Scu216	< 0.00001
	Scu210	Scu217	< 0.00001
	Scu213	Scu216	< 0.00001
Barry Co.	Scu204*	Scu205*	< 0.00001
	Scu213	Scu212	< 0.00001
	Scu213*	Scu201*	< 0.00001
	Scu202*	Scu217*	< 0.00001
	Scu212*	Scu216*	< 0.00001
	Scu202*	Scu210*	0.00028
	Scu212	Scu201	0.00038