Conserv Genet (2012) 13:1133–1143
DOI 10.1007/s10592-012-0360-z

RESEARCH ARTICLE

Inbreeding, body condition, and heterozygosity-fitness correlations in isolated populations of the endangered eastern massasauga rattlesnake (*Sistrurus c. catenatus*)

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Received: 3 March 2012 / Accepted: 19 April 2012 / Published online: 4 May 2012

Abstract Endangered species often occur in small populations that may have a greater risk of short-term extinction due to the negative effects of inbreeding depression. The eastern massasauga (*Sistrurus c. catenatus*) is an endangered rattlesnake that is found in isolated populations of varying size throughout its range. Here, we investigate whether variation in an indirect measure of individual fitness (relative body condition) can be explained by genome-wide levels of genetic variation (based on 19 microsatellite loci) and other factors. To do this, we use genetic and phenotypic data from individual snakes sampled from 14 populations throughout the species' range. We tested for levels of inbreeding by comparing observed mean multilocus heterozygosity (MLH) for each population (an estimate of average inbreeding) with the expected distribution under random mating. We then looked for evidence of heterozygosity-fitness correlations (HFCs) using a measures of individual MLH and relative body condition. In all but one population, observed MLH values are indistinguishable from those generated under a model of random mating implying low levels of inbreeding in most populations. There was significant variation in both mean MLH and mean body condition within and among populations but evidence for inbreeding depression was equivocal: in support, there were some high (but largely non-significant) HFCs effect sizes within a number populations, including one that showed significant evidence for both inbreeding and a HFC. Overall, however, there was no significant correlation between MLH and body condition across all populations after controlling for non-genetic factors such as sex, season of capture and year of capture. Our results suggest that among-population and individual differences in fitness (measured as body condition) in these snakes are better explained by short-term ecological factors rather than genetic mechanisms, but leave open the possibility that limited undetected effects of inbreeding depression are present.

Keywords Inbreeding · Multilocus heterozygosity · Relative body condition · Heterozygosity-fitness correlations · Microsatellite DNA loci · Eastern massasauga rattlesnake · *Sistrurus c. catenatus*

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Introduction

Endangered species that persist in small isolated populations may suffer increased risk of extinction due to the negative effects of inbreeding (Frankham 2005; Oostermeijer et al. 2003; Reed and Frankham 2003; Lopez-Pujol et al. 2005). Such inbreeding depression has been observed in many taxa with small populations in the wild and in captivity (see Crnokrak and Roff 1999; Keller and Waller 2002) and as a result the minimization of inbreeding is a goal of many conservation programs (e.g. Amos et al.

2001; Boakes et al. 2007). However, not all animals found in small isolated populations show evidence for inbreeding depression (e.g. Gyllenstrand and Seppa 2003; Lippe et al. 2006), possibly because the observed effects of inbreeding are highly dependent on the specific demographic and evolutionary history of the organisms in question and hence can vary from species to species (Frankham et al. 2002). Thus, there is a need to examine the relationship between inbreeding and fitness on a case-by-case basis in endangered taxa that exist in small isolated populations to determine if inbreeding depression is of concern.

Where pedigrees are unavailable, data from molecular markers offer an alternative method for assessing levels of inbreeding in wild populations (Frankham et al. 2002). Specifically, because inbreeding increases identity disequilibrium of loci across the genome, inbred individuals will be more homozygous across multiple loci than their outbred counterparts in proportion to the severity of inbreeding (Frankham et al. 2002). In this situation, average multilocus heterozygosity (MLH) among individuals within a population will provide an estimate of average level of individual inbreeding within a population. Numerous metrics have been proposed for quantifying MLH; one that has been widely-used is the proportion of loci within an individual that are heterozygous (Chapman et al. 2009). If inbreeding occurs and has a negative effect on fitness, then correlations should arise between MLH and measures of individual fitness (Szulkin et al. 2010) and the strength of these heterozygosity-fitness correlations (HFCs) can be measured using the magnitude of the correlation between individual MLH and fitness (Chapman et al. 2009; Szulkin et al. 2010). A number of studies have shown a significant positive relationship between fitness and MLH from microsatellite loci (Amos et al. 2001; Bean et al. 2004; Coltman et al. 1998; David 1998; Gage et al. 2006; Hansson et al. 2001; Slate et al. 2000) but the use of HFC correlations to detect the effects of inbreeding depression remains controversial largely due to issues related to detectability of both inbreeding and fitness effects (see discussion in Chapman et al. 2009; Grueber et al. 2011).

The eastern massasauga rattlesnake (*Sistrurus c. catenatus*) is an endangered reptile where inbreeding depression may occur as it exists in a series of small isolated populations that have had little to no gene flow between them over historical timescales (Chiucchi and Gibbs 2010). Population declines throughout the range attributed to habitat fragmentation and destruction have lead the United States Fish and Wildlife Service to list this snake as a candidate sub-species for endangered or threatened status within the United States (Szymanski 1998) and it is classified as a federal Species at Risk in Canada (Government of Canada 2010). Given the long-term isolation of these populations, it is possible that these snakes could suffer from fitness costs associated with prolonged inbreeding as has been observed in other snakes (Ujvari et al. 2002; Madsen et al. 1996). However, it could also be the case that these snakes do not suffer from the costs of inbreeding because repeated exposure to non-random mating has led to the exposure of deleterious alleles to selection, hence their removal from the population (Keller and Waller 2002). Assessing the role of genetic mechanisms compared to ecological factors as determinants of fitness variation in these snakes would be useful in developing broad, range-wide conservation strategies for these organisms (e.g. Szymanski 1998; Upton et al. 2003).

Here, we explore whether there are genetic fitness costs to massasauga rattlesnakes living in small, isolated populations over long periods of time. We do this in two ways. First, we test for evidence of inbreeding within 14 populations of the eastern massasauga rattlesnake using population-wide estimates of average inbreeding compared to expectations based on random mating. Next, to test for inbreeding depression we investigate the relationship between fitness (estimated as relative body condition of individuals) and MLH within populations and across a pooled sample. Overall, this study is the first to explore the possible effects of inbreeding depression in a range-wide sample of populations in this endangered snake.

Methods

Samples

Tissue samples, mass, and snout-to-vent length (SVL) data were collected from 253 snakes from 14 populations throughout the range of the eastern massasauga (Fig. 1, Appendix). Samples were collected in seven different years and across different months within the active season of these snakes (April to September). Sample sizes per population varied from 10 to 46 individuals. These samples represent a subset of those analyzed by Chiucchi and Gibbs (2010).

Estimating genetic diversity

For genetic analyses, we used genotypic data previously generated for a portion of the individuals analyzed in Chiucchi and Gibbs (2010). Briefly, individuals were genotyped at 19 microsatellite loci as detailed in Anderson et al. (2010) and loci were checked for deviations from Hardy–Weinberg equilibrium (HWE) as described in Chiucchi and Gibbs (2010). The number of loci out of HW and the extent of deviations were low: only 6 % of locus-by-population tests were significant and estimates of null allele frequencies at individual loci were <8 % (Chiucchi and

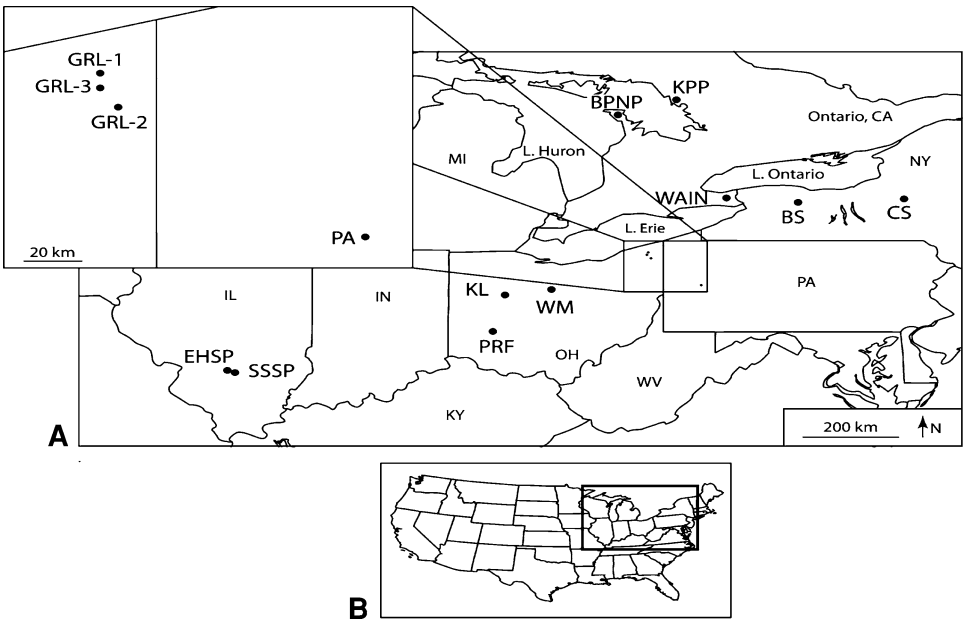

Fig. 1 **a** Sampling locations for *S. c. catenatus* populations showing the Great Lakes and relevant state abbreviations. Population codes for the sampling locations are as follows: *EHSP* Eldon Hazlet State Park, *SSSP* South Shore State Park, *PRF* Prairie Road Fen, *KL* Killdeer Plains Wildlife Area, *WM* Willard Marsh Wildlife Area, *GRL*-1 Grand River Lowlands 1, *GRL*-2 Grand River Lowlands 2, *GRL*-3 Grand River Lowlands 3, *PA* Western Pennsylvania, *BS* Bergen Swamp, *CS* Cicero Swamp, *WAIN* Wainfleet Bog, *BPNP* Bruce Peninsula National Park, *KPP* Killbear Provincial Park. **b** *Inset* shown in the relative position of the sampling locations within the United States and Canada

Gibbs 2010). Individual MLH was measured as the total number of heterozygous loci divided by the total number of genotyped loci for a given individual (Slate et al. 2004). Most individuals were genotyped at 19 loci however, a minor number of individuals [$n = 17$ (7 %)] were genotyped at 18 of the 19 loci. For population comparisons, mean MLH was calculated as the average of the individual heterozygosity estimates for a given population.

Estimates of inbreeding

As inbreeding increases within a population, individuals are expected to have increased levels of genome-wide homozygosity and therefore, on average, decreased MLH estimates compared to a randomly mating population (Slate et al. 2004). To estimate levels of average levels of inbreeding on a population basis, we calculated the MLH for each individual as described above and then calculated the mean MLH value for each population. We then compared the observed mean MLH to the expected distribution of MLH assuming a random mating population using a permutation test as described by Castric et al. (2002). To do so, we used MATLAB (2010R) to simulate 1,000 randomly mating populations resampling existing data for each population by permuting alleles within a locus and then calculating the mean MLH for each simulated population. If a population is experiencing significant inbreeding, the observed mean MLH of our populations is expected to be <95 % of the permuted mean MLH values. A P value was then calculated as the number of simulated values less than the observed mean MLH values and significance was determined at the 95 % level after correcting the level of significance for multiple tests using the B–Y method (Narum 2006).

Estimates of body condition

To estimate body condition, we used length and mass data collected both by us and a number of colleagues (see Acknowledgements) for the 253 snakes for which genotypic data were also available. For each snake, mass (in grams) and snout-vent length (SVL, in mm) was recorded along with sex, female gravid status, and date of capture. Body condition was used as an estimate of fitness with more "fit" individuals considered to be those in better condition. Previous work has documented that $\sim$50 % of the variance in body condition estimates is due to variation in stored body fat in snakes (Weatherhead and Brown 1996) while the other 50 % (unexplained variance) is likely stored in places other than fat bodies, for example in liver and/or muscle tissue (Madsen and Shine 2002). Therefore, we assume that variation in condition in massasaugas reflects the amount of fat an individual snake contains and should be nearly as reliable an estimate of body condition as actual measurements of an individual's percent fat (Weatherhead and Brown 1996).

To measure body condition, we calculated the scaled mass index (Peig and Green 2009), an index that standardizes mass to a specific fixed body length [in grams per cm (gpc)] and is designed to incorporate allometric changes in scaling that are observed in many species. This index has recently been shown to outperform other methods of determining body condition from mass and length estimates because its use of model II linear regression, specifically standardized major axis regression, incorporates the likelihood that both variables have some underlying error rate associated with their measurement (Peig and Green 2010). To estimate condition using this measure mass and SVL were log-transformed and Model II Regression in R version 2.11.1 (lmodel2 function; Legendre 2008) was used to calculate the slope (b_{SMA}) of the best-fit line from a standardized major axis regression. L_o was calculated as the mean SVL from the entire dataset (mean SVL = 497 mm). We combined all treatment groups into one analysis under the assumption that snakes in different treatment groups store fat and energy reserves in similar ways and so allometric relationships between length and weight are similar. A small number of gravid females were removed from all analyses because they are expected to be heavy relative to their length and can potentially bias the relationship of mass to SVL for the species.

Because length and weight data were collected by different researchers using two different techniques to estimate SVL (the tube and squeeze box methods; Bertram and Larsen 2004), we were concerned about the impact of measurement error on estimates of body condition. Therefore, we estimated both among-researcher and among-technique error in measurement of body condition in the following way. Two researchers, each of whom had contributed data to this study, recorded measurements from a captive population of 15 individuals from a congeneric species [pygmy rattlesnakes (*Sistrurus miliarius*)] using both the tube method and the squeezebox method. The mass and SVL of each person's measurements were converted into a scaled mass index as described above and grouped by researcher and technique. We then compared the magnitude of difference between researchers and technique to the observed differences in scaled mass index among our populations.

Heterozygosity-fitness correlations

We looked for evidence of HFCs based on microsatellite and body condition variation both within and across populations. The strength of HFCs can vary among populations due to differences in genetically-effective population size (N_e) and demographic history (Chapman et al. 2009) and N_e has been documented to vary among the populations analyzed here (Chiucchi and Gibbs 2010). Therefore, we estimated the potential effect size of HFCs in each of the 14 populations under study by calculating the Pearson product moment correlation (r) between MLH and relative body condition for all individuals sampled from each population as suggested by Chapman et al. (2009). We also tested whether there was a significant negative correlation between r and N_e using population-specific values for N_e from Chiucchi and Gibbs (2010) as expected if inbreeding effects were more pronounced in small populations (Chapman et al. 2009).

To conduct a more comprehensive analysis of the factors affecting body condition across populations we used general linear mixed models (GLMM's) to test which of a number of variables in addition to MLH could explain the most variation in body condition (Zuur et al. 2009). In all models, body condition was the response variable with heterozygosity (as individual MLH), season of capture, year, and sex treated as independent variables. Season of capture [spring (April and May captures), summer (June and July) and autumn (August and September)], year and sex were all included as independent variables because they all could potentially be responsible for any differences in body condition. Initially, all independent variables were included in the model (full model) along with their interaction terms. However, all interaction terms were non-significant so we removed them from further analysis. Independent variables were then removed sequentially as determined by Akaike's information criterion (AIC) until removal of a given term failed to improve model fit (minimal model) (Zuur et al. 2009). In all models, population was treated as a random effect. Models were estimated using restricted maximum likelihood (REML) methods and F and P values from the full and minimal models are reported. Likelihood ratio tests were used to determine the significance of random effects by comparing models with and without random effects included. All tests were performed using the nlme package in R version 2.11.1 following guidelines in Zuur et al. (2009).

Environmental differences between populations

We used the methods of Wooten and Gibbs (2012) to explore whether environmental differences between populations covaried with differences in mean body condition. To identify specific climate and vegetation variables that best explained the geographic range occupied by *S. c. catenatus*, they conducted a principle components analysis (PCA) using point-specific environmental data for 19 temperature and precipitation and four land cover variables (see Appendix S1 in Wooten and Gibbs 2012) extracted from DIVA-GIS v. 4.0 (Hijmans et al. 2001) and ArcGIS v. 9.3 based on *S. c. catenatus* occurrence data reported in HerpNet (www.herpnet.org). They found that the first five PCs explained 89.23 % of the variance, with 46.64, 23.16,

8.60, 6.34, and 4.50 % for factors 1–5, respectively (see Table 2 in Wooten and Gibbs (2012) for biological interpretations of each factor). We used the statistical models developed in Wooten and Gibbs (2012) to estimate scores for each of PC factors 1–5 for each population based on their geographic location. We assume that these scores summarize environmental differences between populations that result from long-term differences in abiotic factors and vegetation. We then calculated the correlation between mean relative body condition of snakes in each population and PC scores for factors 1–5 to assess whether these long-term environmental differences could account for variation in relative body condition.

Results

Levels of inbreeding

Permutation analyses indicate that inbreeding relative to expectations under random mating is rare in these snake populations despite their isolation. Mean observed MLH across all individuals in each population ranged from 0.497 in PRF to 0.761 at KL (Table 1). After adjusting the critical *P* value for multiple tests, evidence for inbreeding was observed only in the prairie road fen (PRF) population in Ohio (mean observed MLH: 0.529; predicted 95 % CI for MLH under random mating: 0.532–0.619; $P = 0.01$). All other populations had mean MLH estimates within the range of values expected for a randomly mating population (Table 1).

Heterozygosity-body condition correlations

There was a strong positive linear relationship between log-transformed mass and log-transformed SVL ($r^2 = 0.932$, $P < 0.0001$) for the pooled sample of 262 snakes from 14 wild populations. This supports the use of residuals from this regression to estimate the relative body condition of individuals.

Within some populations (e.g. SSSP, PRF, KL, BS, and BPNP—see Fig. 1 legend for population names) the correlations between MLH and individual body condition were positive and high (>0.1; see Chapman et al. 2009) as expected if HFCs are present. However, only the correlation for PRF was significantly different from zero ($P < 0.01$) after adjusting the critical *P* value for multiple comparisons (Table 1; Fig. 2). We note that PRF is also the only population to also show significant evidence for inbreeding. Furthermore, eight of the 14 correlations were in fact negative, albeit non-significantly so. Finally, we found a predicted negative but non-significant correlation between

Table 1 Summaries for each population of (a) tests for inbreeding and (b) effect sizes for heterozygosity-fitness correlations (HFC)

Population (*N*)	Estimated Ne[a]	MLH[b] Observed mean MLH (95 % CI)	*P* value	HFC Effect Size[c] *r* value	*P* value
EHSP	440	0.687 (0.651–0.746)	0.32	−0.381	0.221
SSSP	440	0.654 (0.620–0.710)	0.30	0.507	0.093
PRF	180	0.529 (0.532–0.619)	**0.01**	0.550	**0.015**
KL	635	0.767 (0.763–0.804)	0.06	0.155	0.286
WM	590	0.689 (0.648–0.737)	0.42	−0.084	0.784
GRL-1	420	0.596 (0.514–0.611)	0.93	−0.045	0.892
GRL-2	190	0.543 (0.454–0.543)	0.97	−0.055	0.843
GRL-3	75	0.647 (0.583–0.667)	0.81	−0.032	0.969
JEEC	350	0.562 (0.549–0.679)	0.07	−0.148	0.613
BS	355	0.560 (0.461–0.547)	0.99	0.152	0.547
CS	400	0.542 (0.521–0.571)	0.34	−0.239	0.197
WAIN	80	0.634 (0.611–0.722)	0.11	−0.045	0.886
BPNP	1100	0.700 (0.661–0.744)	0.50	0.311	0.382
KPP	495	0.628 (0.564–0.650)	0.83	0.032	0.928

[a] N_e values for each population are derived from a range-wide joint estimation of θ ($4N_e\mu$) and gene flow as described in Fig. 4a in Chiucchi and Gibbs (2010)

[b] MLH is calculated as the proportion of heterozygous loci per individual and averaged for individuals within a given population. 95 % CI's were constructed by randomizing alleles for each locus to create 1,000 permuted populations from which mean MLH was calculated for each. *P* values were calculated as the number of permutations less than the observed mean value for each population. Significant values after correction for multiple tests are in bold

[c] *r* is estimated as the Pearson moment correlation between relative body size and MLH for each individual within a given population. Sample sizes for each population are given in Appendix

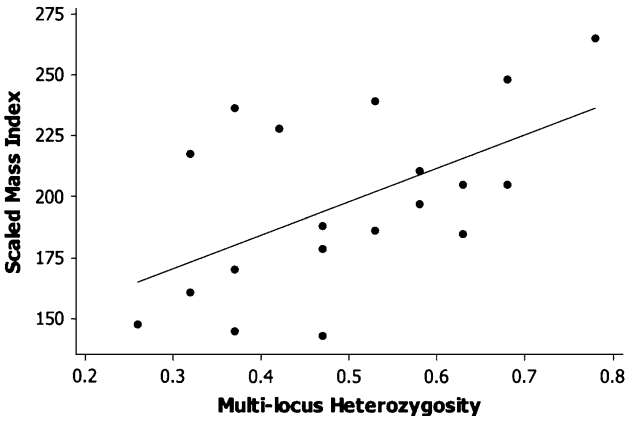

Fig. 2 Multilocus heterozygosity-fitness correlation for individual snakes ($n = 19$) from the Prairie Road Fen, Ohio population (see Fig. 1 for population location)

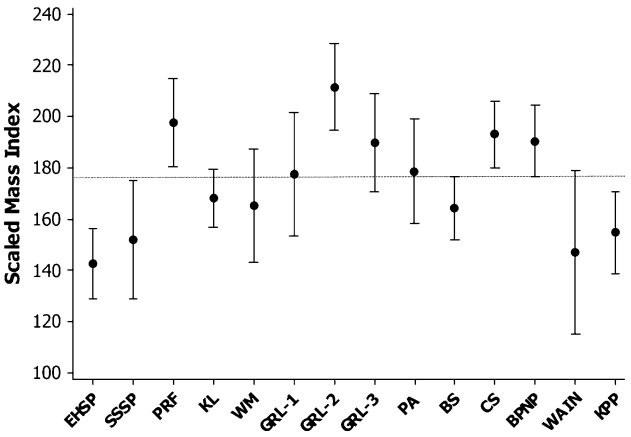

Fig. 3 Mean body condition (*filled circle*) with 95 % CI (indicated by *black bars*) among wild populations of *S. c. catenatus.* The *dashed line* shows the mean scaled mass index of 175.17 gpc. Populations are shown as they roughly occur from west to east in the range of the snake. Sample sizes for each population are given in Appendix

population N_e and effect size values ($r = -0.27$; $P = 0.37$). These results argue that while HFCs may be present in a small number of these rattlesnake populations, they are not common, suggesting that the effect of inbreeding depression on individual fitness is limited.

There is substantial variation among populations in heterozygosity and condition. ANOVA's revealed significant differences among populations for average body condition ($F = 4.89$, $P < 0.001$, Fig. 3) as well as individual heterozygosity ($F = 12.75$, $P < 0.0001$; data shown in Table 1). However, the GLMM analysis showed no evidence for a significant association between individual heterozygosity and body condition. The full GLMM resulted in a lower AIC value than any reduced models so we only report the results from this model. Overall, GLMM's failed to detect any evidence of a correlation between genetic variation and body condition: MLH and all other independent variables (sex, age and time) were not significant predictors of body condition in all models tested (Table 2). However, there was a highly significant effect of population (Table 2, $P = 0.0001$).The average population scaled mass index ranged from 142.3 to 211.4 g and the 95 % confidence intervals for mean population body condition revealed seven populations to be in average condition, four populations above average and three populations below average (Fig. 3).

Table 2 Results from the general linear mixed effects model with body condition as the response variable and heterozygosity, sex, season of capture and year of capture as the independent variables

	Full Model (AIC = 2508.54)	*P* value
Heterozygosity (MLH)	$F_{1,224} = 0.215$	0.643
Sex	$F_{1,224} = 0.069$	0.794
Season of capture	$F_{2,224} = 2.061$	0.129
Year of capture	$F_{6,224} = 1.981$	0.083
Population (random effect)	$L = 15.97$	0.0001

Population of capture was included as a random effect and its significance was determined using a likelihood ratio test by comparing models with and without the random effect included

Measurement error cannot account for observed population differences in body condition. Both researcher and method effects were estimated to be <2.0 gpc (analyses not shown). These values are substantially lower than the observed mean scaled mass index differences among populations (range: 142.3–211.4 gpc; SD: 38.7 g).

Finally, PC factor scores summarizing environmental variation vary between populations (see Fig. 4 for a bivariate plot of factor 1 and 2 scores for each population) suggesting significant long-term differences between populations in abiotic- and vegetation-based environmental variation. However, none of the correlations between mean relative body condition and PC factor scores 1–5 was significant although the correlation between condition and PC three scores was almost significant (Table 3). This argues that long-term habitat-based environmental differences between populations cannot account for differences in mean relative body condition between populations of these snakes but rather that ecological factors that show variation over limited time scales may be more important.

Discussion

Our major results are that (i) there was evidence for inbreeding in only one population suggesting that in general inbreeding is rare; (ii) there is only weak evidence for inbreeding depression as shown by lack of a correlation between MLH and body condition in all but a single population although effect sizes for potential HFCs are high albeit non-significant in other populations; (iii) body

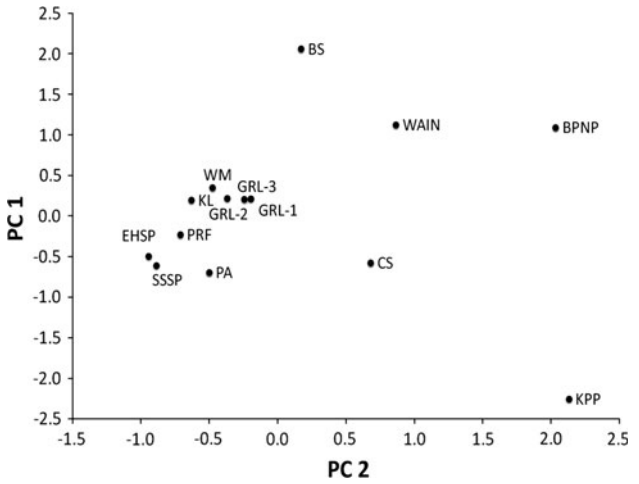

Fig. 4 PC 1 and 2 factor scores summarizing variation in temperature, precipitation, and vegetation variables for *S. c. catenatus* populations shown in Fig. 1 (see Sect. "Methods" for details). Factor 1 describes variation in temperature and explains 47.1 % of the overall variation while factor 2 describes variation in temperature and seasonal precipitation and explains 23.1 % of overall variation (Wooten and Gibbs 2012)

Table 3 Peason correlation coefficients (r) between mean relative body condition and scores for PC factors 1–5 and associated P values

PC factor	r value	P value
Factor 1	0.00	1.00
Factor 2	0.10	0.73
Factor 3	0.60	0.023
Factor 4	−0.19	0.52
Factor 5	−0.06	0.84

False discovery rate corrected P value for this set of analyses was 0.022 (Narum 2006)

condition shows significant variation among populations that is independent of effects due to MLH, sex, season of capture or year of capture. Below, we discuss the evolutionary, ecological and conservation implications of these results.

Lack of observed inbreeding

Observed MLH estimates fall in the range expected under random mating in 13 of 14 populations examined suggesting that inbreeding is rare despite the long term isolation of these populations. This result makes sense for the three larger populations (BPNP, KL, and WM) where the genetically effective population size is estimated to be >500 and hence inbreeding is expected to be infrequent. It is not expected for the remaining populations which all have N_e values of <500 individuals where inbreeding effects are expected to be much higher (Chapman et al. 2009). A possible reason for this inconsistency is that heterozygosity estimated from the number of microsatellite loci used here may not be a reliable estimator of genome-wide heterozygosity and therefore may be a poor predictor of inbreeding (Balloux 2004; DeWoody and DeWoody 2005; Slate et al. 2004). In support of this possibility, Grueber et al. (2011) found a correlation of only 0.33 between individual values for MLH and presumably more accurate pedigree-derived estimates of inbreeding in a threatened bird that lived in small populations. They argue that, in fact, upwards of 200 microsatellite loci are needed to detect any evidence of inbreeding depression using these methods given expected effect sizes for HFCs in natural populations of vertebrates (Grueber et al. 2011). Genetic markers based on "Next Generation" sequencing technology such as RADSeq loci (Davey and Blaxter 2011) offer and alternative approach to generating large numbers of loci for HFC analyses. Another way to increase the accuracy of molecular data in detecting inbreeding in future studies of these populations would be to use molecular data to infer pedigree relationships between individual which can then be used to directly estimate inbreeding coefficients (Santure et al. 2010) although this data is difficult to collect for wild snakes.

Heterozygosity-fitness correlations

Our results provide little evidence for HFCs in these snakes and suggest little evidence for inbreeding depression. The strongest evidence for this conclusion comes from the facts that there is little evidence for significant inbreeding these populations which is necessary for HFCs to be present in natural populations (Szulkin et al. 2010). We emphasize that our result does not mean that inbred individuals are not present in these populations or that these individuals do not suffer reduced fitness. Rather, MLH is an estimate of the average population-wide level of inbreeding and the fact that observed MLH estimates are close to that predicted under random mating implies that observed levels of inbreeding are rare hence the overall impact of inbreeding on individual fitness is low.

The best-known examples of inbreeding in snakes come from small populations of adders in northern Europe (Madsen et al. 1996) and Antiguan racers from the small island of Antigua (Daltry et al. 2001). Both species have been shown to suffer some extreme effects of inbreeding depression but each has a population size that is smaller than almost all massasauga populations in this study. The European adders have an estimated population size (census size) of ~40 animals (Madsen et al. 1996) whereas the Antiguan racer population was last estimated to harbor only 80 animals (Daltry et al. 2001). In both of these populations, the loss of genetic diversity due to inbreeding, in combination with drift, has led to severe morphological deformities. Further, when inbreeding depression is observed it often results in a

reduction in growth rate, fertility, fecundity and resistance to disease (Charlesworth and Charlesworth 1987). Currently, there is no evidence for these issues in massasauga populations. Our smallest population (WAIN, estimated census size = 160 animals) is approximately four times the size of the European adder population studied by Madsen et al. (1996) and shows no evidence of inbreeding depression. Thus, the lack of detectable effects of inbreeding in most massasauga populations may be due to the fact that they are still sufficiently large to avoid these effects.

Nonetheless, despite these results we think it is premature to dismiss the possibility that inbreeding depression has no impact on the fitness of these snakes. Our reasons are as follows. First, we did observe significant evidence for inbreeding and HFCs in one population (PRF) and the high although non-significant correlations between MLH and condition observed in some of the other smaller populations are strongly suggestive of HFCs being present but not detectable due to insufficient statistical power in our data sets. Indeed, power analyses indicate that >2–3 × numbers of individuals are required for the observed correlation coefficients to achieve significance at the 0.05 level of significance (analyses not shown). Szulkin et al. (2010) emphasize that it is important to consider the effect size of HFCs when assessing whether they occur rather than focusing only on whether they achieve statistical significance or not because effect sizes are expected to be low even though they may be biologically relevant.

Second, there are biological reasons why inbreeding depression may be difficult to detect in natural populations that may apply to these snakes. For example, there may have been repeated exposure of deleterious alleles from mating between closely related individuals and subsequent purging of genetic load via genetic drift (Glemin 2003). Purging could lead to detectability issues since individuals with higher inbreeding coefficients might be selected against at a very early age and simply not sampled in this study because selection has already acted. This would create a situation where no HFC is detected even though strong inbreeding effects are present due to the fact that both heterozygosity and fitness were measured after selection had already operated. In addition, the effects of inbreeding depression may only be observed in stressful environments (Keller et al. 1994; Harrison et al. 2011) and such conditions may not have been present when the current data was collected from the snake populations. Finally, for logistic reasons, we only examined fitness effects of inbreeding using a single indirect measure of fitness. More comprehensive analyses using life history traits directly related to survival and/or reproduction as has done in other studies that have detected significant HFCs (Chapman et al. 2009) or direct assessment of adaptive variation at specific loci (e.g. Ujvari et al. 2002) may yield evidence for the effect of genome-wide levels of variation on fitness.

Geographic variation in body condition

Of the variables considered, population identity had by far the strongest association with variation in body condition. Geographic variation in fitness-related traits such as condition could be due to local adaptation resulting from genetic differences (Bronikowski and Arnold 1999) or plasticity in response to local environmental conditions (Madsen and Shine 2008). Although these populations show the high degree of genetic structure required for local adaptation to be maintained (Chiucchi and Gibbs 2010), we feel that environmental influences are likely more significant because of the repeated demonstration that ecological factors such as prey availability have a significant influence body condition in snakes (Gregory 2006; Reading 2004; Taylor et al. 2005; Madsen and Shine 2008; Sperry and Weatherhead 2008). This conclusion is also supported by the observation that populations that are geographically very close to one another (e.g. GRL- 1, 2, and 3—see Chiucchi and Gibbs 2010) can have snakes with substantially different body conditions (Fig. 3) suggesting local ecological effects. The populations analyzed here have a range-wide distribution and are found a variety of ecologically distinct habitats (e.g. prairie and bog habitats—see Szymanski 1998) that could lead to variation in body condition through number of mechanisms (see below). Analyses using the methods outlined in Wooten and Gibbs (2012) confirm that there are significant long-term ecological differences between sites in terms of variation related to temperature and precipitation and vegetation (Fig. 4) although this variation does not covary with body condition suggesting that short-term ecological factors that show significant variation through time are likely more important).

In this respect, two factors that seem potentially important and could show short-term differences between populations are variation in prey abundance and composition and disease incidence. In terms of variation in prey, there is evidence for variation in abundance and diversity of small mammals at each population that could influence snake diet (Reading 2004; Sperry and Weatherhead 2008). Specifically, analysis of diet using stable isotope techniques show evidence for among-population level variation in diet in these snakes (Chiucchi JE, unpublished data). For example, shrews represent an important group of prey for eastern massasaugas (Weatherhead et al. 2009; Chiucchi JE, unpublished data) and data from one species, the masked shrew (*Sorex cinereus*), suggests their abundance varies across habitats within the state of Ohio (Pietkiewicz and Harder, in prep). This could have a significant impact on the condition of snakes in each population by providing the opportunity to feed more often in locations with higher shrew (or small mammal) densities. Fitzgerald et al. (2004) provide another example of a threatened snake in which female condition and hence reproductive rates could be influenced by prey availability.

Differences in the prevalence of disease could also be responsible for differences in body condition among populations. Massasaugas in southern Illinois have been shown to contain high levels of infection with ophidian paramyxovirus (Allender et al. 2008; Allender et al. 2006). Two of the populations in this study with below average body condition are located in southern Illinois, EHSP and SSSP. Whether the lower observed body condition in these populations is due to the presence of ophidian paramyxovirus is unknown although this virus is known to have severe lethal issues in captive populations (Jacobson 1993). Though we do not have data on infection rates for other population this raises the possibility that rates of infection could vary among populations and contribute to the observed differences in body condition. Surveys of disease prevalence across populations studied here using the techniques described in Allender et al. (2008) could be used to explore this possibility.

Reproductive implications of variation in body condition

Body condition has been shown to vary in other snake species and evidence suggests it may play an important role in determining if female snakes will reproduce successfully. There is empirical evidence suggesting a minimum threshold for body condition exists whereby females below this threshold fail to become reproductively active and females above the threshold tend to successfully reproduce (Naulleau and Bonnet 1996). This can have even greater effects in populations that are extremely isolated like the eastern massasauga because many population sizes are relatively small with little to no chance of recruitment via migration from other populations (Chiucchi and Gibbs 2010). If poor body condition results in females losing opportunities to breed this can have severe impacts to the overall health of the population (Gregory 2006). Female massasaugas do not breed every year but rather every second or third year, which results in a mating system where few females breeding each year (Reinert 1981). Therefore, a population with many females below the body condition threshold may decrease reproductive effort leading to a reduction in population birth rate and ultimately lowering the long-term viability of a population.

Conclusions

A long-standing issue in conservation biology has been assessing the impact of genetic versus ecological factors on the viability of populations of endangered species (Lande 1988). Our study provides strong circumstantial evidence that ecological factors related to population location have a significant impact on relative body condition in eastern massasauga rattlesnakes. Should additional research determine that these are related to habitat this opens the way for direct management of habitat characteristics as a way of enhancing the long-term viability of populations of these snakes. Future work should focus on identifying what the ecological factor(s) are that drive variation in body condition. The impact of genetic factors operating through inbreeding depression is less clear. Our findings suggest that inbreeding is rare in most populations hence the impact of inbreeding depression is likely to be limited. Nonetheless, there is evidence that inbreeding depression can occur in particular populations and so this possibility needs to be considered in developing conservation plans for individual populations of this species. Clarification of the significance of inbreeding depression will come from long-term studies of individuals in multiple populations of different sizes in which associations between pedigree-based estimates of inbreeding are compared to direct estimates of fitness related to survival and reproduction.

Acknowledgments This study would not have been possible without the very generous assistance with the collection of samples and data by a variety of people. Many individuals have helped but in particular, we thank Jeff Davis, Michael Dreslik, Brian Fedorko, Tony Frazier, Dan Harvey, Matt Kowalski, Greg Lipps, Chris Parent, Chris Phillips, Kent Prior, Kevin Shoemaker, Michelle Villenueve, Pat Weatherhead, and Doug Wynn for their enthusiastic help with finding and collecting samples from rattlesnakes, Doug Wynn for help with measurements of captive snakes, Jose Diaz, Christine Anderson, and Mike Murphy for assistance with the lab work, Jessica Wooten for help with analyses, Tony Fries, Matt Holding, Mike Sovic and Pat Weatherhead for comments on the paper and Carolyn Caldwell (Ohio Division of Wildlife) for her long-term, sustained support of this work. This research was supported by funds from the Ohio Division of Wildlife and Ohio State University.

Appendix

See Table 4.

Table 4 Summary of data collected for estimating body condition across years and season for each population

Population	Year							Season			Sex		Total
	1993	1994	2003	2004	2005	2006	2007	Spring	Summer	Autumn	Male	Female	
EHSP	0	0	0	0	10	2	0	12	0	0	6	6	12
SSSP	0	0	0	0	10	2	0	12	0	0	4	8	12
PRF	0	0	0	0	7	1	11	8	9	2	4	15	19

Table 4 continued

Population	Year							Season			Sex		Total
	1993	1994	2003	2004	2005	2006	2007	Spring	Summer	Autumn	Male	Female	
KL	0	0	8	16	9	0	13	30	9	7	13	33	46
WM	0	0	0	0	5	4	5	0	11	3	8	6	14
GRL-1	0	0	0	14	0	0	0	1	3	10	3	9	14
GRL-2	0	0	0	0	2	4	9	6	6	3	7	8	15
GRL-3	0	0	0	0	2	10	7	8	10	1	6	11	19
PA	0	0	0	0	0	14	0	0	14	0	6	8	14
BS	0	0	0	0	0	8	10	0	10	8	6	12	18
CS	0	0	0	0	0	23	8	1	21	9	10	21	31
BPNP	0	0	0	14	0	0	0	1	6	7	14	0	14
KPP	15	0	0	0	0	0	0	1	10	4	5	10	15
WAIN	0	10	0	0	0	0	0	0	1	9	6	3	10
Total	15	10	8	44	45	68	63	80	110	63	98	150	253

The number of males and females captured from each population is also shown. The total for each column is shown at the bottom

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