



Original Article

Low Bottleneck Detection in Long-Lived Species Despite Lost Genetic Diversity: A Case Study of Tuatara and Eastern Massasauga Rattlesnakes

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Abstract

Population bottlenecks can reduce genetic diversity and may lead to inbreeding depression. However, some studies have provided evidence that long lifespans buffer negative genetic effects of bottlenecks. Others have cautioned that longevity might merely mask the effects of genetic drift, which will still affect long-term population viability. We used microsatellite data from actual populations of tuatara (*Sphenodon punctatus*) and eastern massasaugas (*Sistrurus catenatus*) as a starting point for simulated population declines to evaluate the performance of bottleneck tests under a range of scenarios. We quantified losses in genetic diversity for each scenario and assessed the power of commonly used tests (i.e., M-ratio, heterozygosity excess, and mode-shift) to detect known bottlenecks in these moderate- to long-lived species. Declines in genetic diversity were greater in bottlenecks simulated for eastern massasaugas, the shorter-lived species, and mode-shift and heterozygosity excess tests were more sensitive to population declines in this species. Conversely, M-ratio tests were more sensitive to bottlenecks simulated in tuatara. Despite dramatic simulated population declines, heterozygosity excess and mode-shift tests often failed to detect bottlenecks in both species, even when large losses in genetic diversity had occurred (both allelic diversity and heterozygosity). While not eliminating type II error, M-ratio tests generally performed best and were most reliable when a critical value (M_c) of 0.68 was used. However, in tuatara simulations, M-ratio tests had high rates of type I error when M_c was calculated assuming $\theta = 10$. Our results suggest that reliance on these tests could lead to misguided species management decisions.

Subject Area: Conservation Genomics and Biodiversity

Key words: genetic diversity, heterozygosity excess, longevity, mode-shift, M-ratio, population bottleneck

Large reductions in population size, or population bottlenecks, are increasingly prevalent in wildlife populations due to mounting anthropogenic impacts such as habitat destruction, climate change, the introduction of invasive species, overexploitation, and interactions among these factors (Ceballos et al. 2017). Population bottlenecks often lead to reduced genetic diversity and increased inbreeding (Nei et al. 1975; Frankham 1995; Allendorf and Luikart 2007). Reduced genetic diversity leaves populations vulnerable because they have a lessened ability to adapt and respond to environmental change (Frankham 1996, 2005; Frankham et al. 1999), while inbreeding and the related buildup of deleterious alleles can decrease a population's average fitness (Mills and Smouse 1994; Crnokrak and Roff 1999). Interactions between genetic effects and demographic stochasticity can create a positive feedback loop that augments the probability of extinction (i.e., an extinction vortex; Gilpin and Soulé 1986).

Because of the threat bottlenecks pose to population viability, knowing whether a population has experienced a reduction in size is often of conservation interest. However, conservationists rarely know the historic size of a population or its historic levels of genetic diversity. Other options for inferring whether a significant bottleneck has occurred include comparing the genetic diversity of the population of interest to museum specimens (e.g., Hoelzel et al. 2002; Wisely 2002) or to conspecific populations with known stability (e.g., Ohnishi et al. 2007), but these data are often unattainable or unreliable. Therefore, due to their relative ease and accessibility, methods that infer past bottlenecks using current genetic data collected from a single point in time are widely employed in the scientific literature (Peery et al. 2012). Three of the most common methods applied to microsatellite data include the M-ratio (Garza and Williamson 2001), heterozygosity excess (Cornuet and Luikart 1996), and mode-shift (Luikart et al. 1998) tests.

These 3 bottleneck detection tests have different underlying assumptions and focus on different aspects of variation in the data. The M-ratio is calculated as the number of alleles divided by range in allele size, and it is expected that this ratio will decrease when a population experiences a bottleneck (Garza and Williamson 2001). This generally occurs in loci with ≥ 5 alleles because the loss of any allele reduces the numerator in the ratio while only the loss of the largest or smallest alleles reduces the denominator (Garza and Williamson 2001). Therefore, the calculated M-ratio for a population can be compared to the ratio expected for a population in mutation-drift equilibrium (i.e., the critical value or M_c) to infer whether a bottleneck has occurred (Garza and Williamson 2001). The heterozygosity excess test is based on the assumption that allelic richness decreases at a faster rate than heterozygosity (H_E) during population bottlenecks (Cornuet and Luikart 1996). Thus, heterozygosity, as defined by Nei (1973), will be greater in a recently bottlenecked population than expected for a population in mutation-drift equilibrium, when taking into account the actual number of observed alleles (Cornuet and Luikart 1996; Piry et al. 1999). The mode-shift test assumes that when population size is greatly reduced, low-frequency alleles are lost from a population more readily than intermediate frequency alleles (Luikart et al. 1998). Consequently, the mode of a population at mutation-drift equilibrium occurs at low allele frequency, while the mode of a recently bottlenecked population occurs at intermediate allele frequency (Luikart et al. 1998; Piry et al. 1999).

Previous studies have noted that negative impacts of bottlenecks on genetic diversity appear to be reduced in long-lived species (Kuo and Janzen 2004; Hailer et al. 2006; Lippé et al. 2006). Hailer et al. (2006) suggested that this could be a consequence of longevity

buffering declines in genetic diversity, where longer generation times result in a much slower loss of diversity on timescales measured in years. However, Kuo and Janzen (2004) cautioned that longevity may only mask the effects of genetic drift. Bottlenecks will still affect population viability via greater vulnerability to demographic stochasticity and an eventual reduction in fitness if populations are not restored.

Reptiles provide a useful model to test these hypotheses due to the long lifespans of many species. Additionally, the low vagility of many reptiles coupled with isolated patches of suitable habitat may result in limited to no migration between populations. Therefore, genetic variation lost during a bottleneck is less likely to be regained through gene flow, and bottlenecks experienced by these populations are likely to be of greater impact than in more vagile species. Here, we used observed data for 2 reptile species, the eastern massasauga (*Sistrurus catenatus*) and the tuatara (*Sphenodon punctatus*), to assess the effects of their longevity on bottleneck severity and detectability.

The eastern massasauga is a small rattlesnake with a distribution extending from the midwestern United States into Ontario, Canada (Harding 1997). Many populations throughout the species' range have been impacted by habitat loss and fragmentation, among other threats (Szymanski 1998). Consequently, this species is listed as threatened under the US Endangered Species Act (US Fish and Wildlife Service 2016) and Canada's Federal Species at Risk Act (Environment Canada 2012). The eastern massasauga is a wetland specialist, often occurring in isolated patches of habitat and exhibiting small home-range size and short daily movements (Reinert and Kodrich 1982; Weatherhead and Prior 1992; Johnson 2000; Moore and Gillingham 2006; Bailey et al. 2012). Significant genetic differentiation has been observed, even between geographically close populations (~5–7 km apart; Gibbs et al. 1997; Chiacchi and Gibbs 2010; Sovic et al. 2019). Depending on locality, individuals sexually mature between 2 and 6 years of age (Szymanski et al. 2015); longevity is generally unknown in the wild, but captive *Sistrurus* spp. have lived up to 20 years (Snider and Bowler 1992).

The tuatara is the only extant species of the ancient reptilian order Rhynchocephalia. Endemic to New Zealand, this species was extirpated from the 2 main islands, likely due to habitat destruction and predation by invasive rats (Miller et al. 2012). Natural populations are now limited to small offshore islands, with high genetic differentiation observed among island populations (MacAvoy et al. 2007; Hay et al. 2010; Miller et al. 2010). Estimates of sexual maturity range from 9 to 20 years of age (Castanet et al. 1988; Cree et al. 1992; Allendorf and Luikart 2007) and the estimated maximum longevity of wild tuatara (based on mark-recapture data) is at least 91 years (Moore et al. 2007).

In this study, we simulated instantaneous population bottlenecks that varied in level of severity (i.e., length of time and proportional decrease in population size), with initial parameters based on microsatellite data observed from tuatara and eastern massasaugas. This allowed us to assess whether the M-ratio, heterozygosity excess, or mode-shift tests can reliably detect known (simulated) population bottlenecks in moderate- to long-lived species. Additionally, we analyzed the effects of bottlenecks on genetic diversity by quantifying pre- and post-bottleneck measurements of allelic richness and heterozygosity. Based on previous studies (e.g., Kuo and Janzen 2004; Williamson-Natesan 2005; Hailer et al. 2006; Peery et al. 2012; Hoban et al. 2013), we hypothesized that detection of bottlenecks would be imperfect, with false negatives (type II error) more prevalent than false

positives (type I error), and that these standard bottleneck tests would have diminished power to detect population declines in long-lived species.

Materials and Methods

Eastern Massasaugas, Bruce Peninsula Data

We obtained genetic data from the Dryad Digital Repository (Dileo et al. 2013a, 2013b). Data used in our analyses include microsatellite genotypes at 12 loci collected for 162 eastern massasaugas sampled from the northern Bruce Peninsula in Ontario, Canada between May 2008 and October 2010 (Dileo et al. 2013b; Supplementary Material 1). Sampling was conducted with an effort to represent the continuous distribution of the species in this area and occurred mainly along roadways (see Figure 1 in Dileo et al. 2013b). The Bruce Peninsula is at the species' northern range limit (Weatherhead and Prior 1992) and comprises one of the largest extant populations of this species (Parks Canada Agency 2015). Additionally, Dileo et al. (2013b) identified massasaugas from the Bruce Peninsula as a genetically distinct panmictic population, suggesting that signals of possible population bottlenecks are unlikely to be confounded by unrecognized population structure. Furthermore, Sovic et al. (2019) did not detect a recent bottleneck in this population using maximum likelihood methods and genome-scale data.

Tuatara, Takapourewa Data

We obtained genotypes at 7 microsatellite loci collected from 272 individuals sampled from Takapourewa/Stephens Island, New Zealand between March 2003 and March 2007 (Moore et al. 2008; Supplementary Material 1). Sampling occurred at 8 sites on the island and was distributed throughout the available remnant forest and pasture landcover types (see Figure 1 in Moore et al. 2008). Despite extensive habitat loss from farming in the early 1900s (Brown 2000), Takapourewa contains the largest extant population of tuatara (Moore et al. 2007). Tuatara on the island are considered a single population, but evidence of low-level fine-scale genetic structuring was observed by Moore et al. (2008).

Genetic Diversity and Bottlenecks

For each species, we calculated mean number of alleles per locus (A), mean effective alleles per locus (A_E), observed heterozygosity (H_O), and expected heterozygosity (H_E). We tested all loci for deviations from Hardy-Weinberg equilibrium using χ^2 tests implemented in GenAlEx v.6.502 (Peakall and Smouse 2006, 2012). To test for linkage disequilibria, we performed pairwise exact tests between all loci in GenePop v.4.6 (Rousset 2008) using a Markov chain Monte Carlo method with 10 000 batches, 10 000 iterations, and 10 000 dememorizations, according to the algorithm of Guo and Thompson (1992). For Hardy-Weinberg and linkage disequilibria tests, we *a priori* set $\alpha = 0.05$ to evaluate significance and applied a sequential Bonferroni correction to account for multiple tests (Holm 1979; Rice 1989).

We ran 1,000 iterations in program BOTTLENECK v.1.2.02 (Piry et al. 1999) to test for evidence of a recent bottleneck in each species. We selected a 2-phase mutation (TPM) model with 90% single-step mutations, as recommended by Garza and Williamson (2001) based on available published data on microsatellite mutation, and we assumed a variance (σ_s^2) of 12 as recommended by Piry et al. (1999). We tested for heterozygosity excess (Cornuet and Luikart 1996) using a 1-tailed Wilcoxon test with $\alpha = 0.05$, and we evaluated allele frequency distributions using the mode-shift test (Luikart et al. 1998).

We calculated the M-ratio for each dataset using the formula $M = \frac{k}{(R+1)}$, where k represents the number of alleles for a locus and R represents the range of allele sizes divided by the repeat unit (e.g., di-, tri-, or tetra-nucleotide; Garza and Williamson 2001). To assess whether a recent bottleneck had occurred, we compared each estimated M-ratio to 2 different critical values (M_c). The first value ($M_c = 0.68$) was identified by Garza and Williamson (2001) as a reasonable cutoff for assuming a recent population bottleneck. For the second value, here termed calculated M_c , we used the Critical_M.exe software and the conservative parameterization recommended by Garza and Williamson (2001; i.e., proportion of 1-step mutations = 0.90 and mean size of multi-step mutations = 3.5). We set θ to

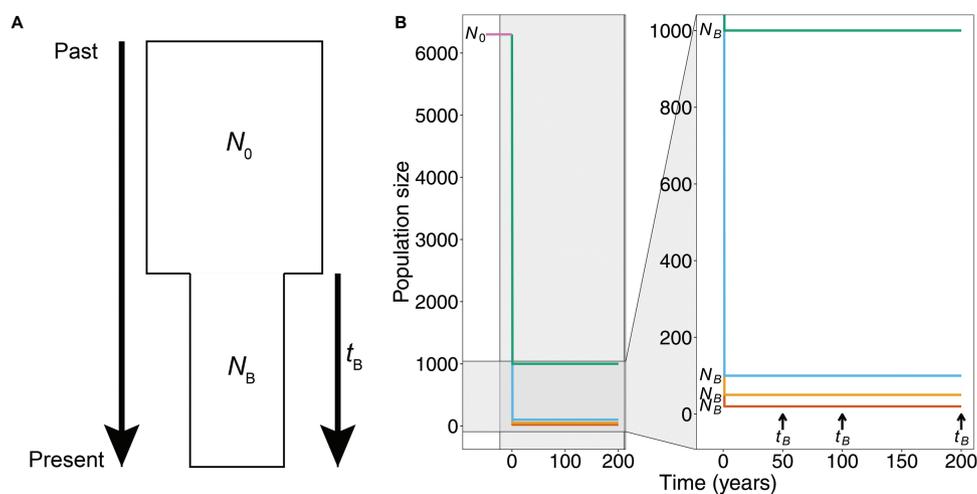


Figure 1. Model parameters and conditions used in simulations. (A) Diagram depicting a simulated population bottleneck, where a pre-bottleneck starting population size N_0 is reduced to a bottlenecked population size N_B and remains at its reduced size for length of time t_B . (B) Plot of population size through time for each value of N_B simulated (i.e., 20, 50, 100, 1000) with each value of t_B assessed (i.e., 50, 100, 200) indicated with an arrow. The value shown for N_0 (6300) represents the census size estimate for the population of eastern massasaugas used to obtain allele frequencies for initiating the simulation. The census estimate (and N_0) for the tuatara population was 25 000. Scenarios in which no bottleneck occurred (i.e., $N_B = N_0$) were also simulated for each species. See online version for full color.

a value of 10, which assumes a mutation-drift equilibrium effective population size of 5000 and a mutation rate of 5×10^{-4} (Garza and Williamson 2001).

Simulations

We used program BOTTLESIM v.2.6 (Kuo and Janzen 2003) to simulate multiple bottleneck scenarios for each species. We selected this software based on its ability to simulate allele frequency changes over time in species with overlapping generations (Kuo and Janzen 2003, 2004). Simulations varied by 1) length of time (t_b): 50, 100, and 200 years, 2) starting population size (N_0): estimated census population size and one order of magnitude above and below, and 3) bottlenecked population size (N_b): 20, 50, 100, 1000, and N_0 (the lattermost $N_b = N_0$ scenario representing a constant population size with no bottleneck). Simulated eastern massasauga populations with a starting population size of 630 (one order of magnitude below the estimated N_0) were not modeled with $N_b = 1000$ as this would constitute an increase in population size. For each scenario, we simulated an instantaneous bottleneck in the population, which remained constant at its reduced N_b size for t_b years (Figure 1). Each starting (i.e., pre-bottlenecked) population was assigned allele frequencies corresponding to the actual data observed from the respective species, so that all simulations reflected empirical populations. Thus, all starting populations for a given species effectively had the same genetic effective population size (N_e), so varying the N_0 parameter only tested whether BOTTLESIM was sensitive to the initial census size specified. For all simulations, we performed 1000 iterations and set the reproductive system as dioecy with random mating, a 1:1 sex ratio, and 100% generational overlap. Setting generational overlap at 100% means that all individuals in the population are randomly assigned an age value at the start of the simulation that ranges from age zero to the longevity of the given species (Kuo and Janzen 2003).

The remaining simulation parameters (i.e., starting population size, age at sexual maturity, and longevity) were based on the natural history and demography of each species. For eastern massasaugas, an estimated census population size of 6300 individuals was obtained from Miller's (2005) population estimate for the northern Bruce Peninsula mainland. We set age of sexual maturity at 5 years based on average age of first reproduction in females from a nearby population (Rouse 2005), and we set longevity at 12 years based on the estimated maximum breeding age for the Bruce Peninsula population (Miller 2005). For tuatara, we conservatively estimated a population size of 25 000 based on Newman's (1987) estimate of ~30 000–50 000 individuals. We set age of sexual maturity at 14 years and conservatively assumed a longevity of 80 years based on records reported by Cree et al. (1992) and Moore et al. (2007).

To discern any effects of using a greater number of microsatellite loci in the eastern massasauga dataset compared to the tuatara dataset, we randomly selected 7 loci from the eastern massasauga dataset. Using this reduced dataset, we conducted a set of simulations with the same values for t_b and N_b as described above and set N_0 equal to estimated census population size. All other simulation parameters remained the same.

We separately evaluated the sensitivity of our results to variation in simulated longevity and age of sexual maturity (hereafter jointly referred to as life history parameters) within each species. Accordingly, we again simulated bottlenecks using the values for t_b and N_b described above and set N_0 equal to estimated census population size for each species. Age of sexual maturity and longevity were set at 10 years and 50 years, respectively, for both species. Simulating

the same set of values for these parameters for each species also allowed us to make comparisons across the 2 species where these life history parameters were the same, but initial effective population size (represented as initial genetic diversity) differed.

For each simulated scenario, BOTTLESIM produced 1000 independent datasets, each constituting one panmictic population, which included genotypes of all individuals immediately following the final year of the bottleneck. We randomly subsampled 90% of individuals from each simulated post-bottleneck population, with an upper limit of 150 sampled individuals, using the PopGenKit package in R (Paquette 2012). We did this in order to produce a more realistic sampling situation because it is rare to sample every individual from a population. Each subsampled population was analyzed using program BOTTLENECK with the same parameters and tests implemented with the original datasets (see *Genetic Diversity and Bottlenecks* section). To determine the power of each test to detect a bottleneck for a given scenario, we calculated the proportion of simulated populations with heterozygosity excess ($P < 0.05$) and the proportion of populations with a shifted mode. We also calculated the proportion of simulated populations for each scenario that had M-ratios below the M_c values previously described. To batch process the large number of M-ratio calculations required (i.e., 1000 simulated populations per bottleneck scenario), we created a custom workflow in R (www.r-project.org) to calculate M-ratios from a GENEPOP file (Supplementary Material 2). We quantified the average decrease in genetic diversity for each bottleneck scenario using the output provided by BOTTLESIM, which consisted of mean A , A_E , H_O , and H_E (for all iterations combined).

Results

Eastern Massasaugas, Bruce Peninsula Data

Three loci (*scu5*, *scu211*, and *sca2a5*) had heterozygote deficiency and were not in Hardy-Weinberg equilibrium after applying a sequential Bonferroni correction. We did not detect linkage disequilibria between any pairs of loci ($P > \text{Bonferroni-corrected } \alpha$ in all cases). We retained all loci for all simulations and analyses. Mean number of alleles per locus (A) was 10.83 (SE = 1.23) and mean effective alleles per locus (A_E) was 5.59 (SE = 0.65). Observed heterozygosity (H_O) was 0.751 (SE = 0.04) and expected heterozygosity (H_E) was 0.789 (SE = 0.03). A bottleneck was not detected using the heterozygosity excess test ($P = 0.48$), mode-shift test (allele frequencies displayed a normal L-shaped distribution), or M-ratio test (M-ratio = 0.816 vs $M_c = 0.68$ or calculated $M_c = 0.750$).

Tuatara, Takapourewa Data

Two loci (*H5H* and *C12F*) had heterozygote deficiency and were not in Hardy-Weinberg equilibrium after applying a sequential Bonferroni correction. We did not detect linkage disequilibria between any pairs of loci ($P > \text{Bonferroni-corrected } \alpha$ in all cases). We retained all loci for all simulations and analyses. Mean A was 14.29 (SE = 3.71), mean A_E was 6.50 (SE = 1.72), H_O was 0.755 (SE = 0.06), and H_E was 0.780 (SE = 0.05). A bottleneck was not detected using the heterozygosity excess test ($P = 0.85$), mode-shift test (allele frequencies displayed a normal L-shaped distribution), or M-ratio test (M-ratio = 0.742 vs $M_c = 0.68$ or calculated $M_c = 0.733$).

Simulations

Varying N_0 among simulation scenarios confirmed that BOTTLESIM is insensitive to starting population census size, demonstrating that

all populations assigned the same initial allele frequencies are affected the same by a given t_b and N_b , regardless of N_0 (Supplementary Material 3). Accordingly, the remainder of our results and discussion focus on scenarios run with N_0 = estimated census size (i.e., 6300 eastern massasaugas; 25 000 tuatara). No-bottleneck scenarios ($N_0 = N_b$) exhibited little or no loss of genetic diversity for massasaugas ($\leq 0.9\%$; Figure 2) and no loss of genetic diversity for tuatara (Figure 2).

In simulations run using full datasets and realistic life history parameters, among scenarios with a decrease in population size simulated for massasaugas, bottlenecks were detected with 0–77% power using the mode-shift test, 3–74% power using the heterozygosity excess test, and 0–100% power using M-ratio tests (Figure 3). Among the same scenarios simulated for tuatara, power to detect a bottleneck ranged from 0 to 35% using the mode-shift test, from 0% to 37% using the heterozygosity excess test, and from 9% to 100% using M-ratio tests (Figure 3). For both species, mean A and A_E decreased in all scenarios where a reduction in population size was simulated, and generally decreased proportionally to bottleneck severity (i.e., according to duration and post-bottleneck population size; Table 1; Figure 2). Mean H_O and H_E followed the same trend as mean A and A_E , but percent losses in heterozygosity were smaller than percent losses in allelic diversity for a given scenario (Table 1; Figure 2). For all measures of genetic diversity, percent losses were smaller in tuatara than in eastern massasaugas for each scenario (Table 1; Figure 2).

With the exception of bottlenecks simulated for eastern massasaugas with $N_b = 1000$ individuals or with $N_b = 100$ individuals and $t_b = 50$ years (using $M_c = 0.68$), M-ratio tests demonstrated the highest rates of bottleneck detection in both species, followed by heterozygosity excess tests, and mode-shift tests (Figure 3). Very few bottlenecks simulated for massasaugas were detected using mode-shift tests, except in the most extreme scenarios (e.g., $N_b = 20$ individuals; Figure 3). Among tuatara simulations, very few bottlenecks were detected using mode-shift tests or heterozygosity excess tests, even in extreme bottlenecks, and fewer bottlenecks were detected in tuatara simulations than in massasauga simulations using both of these tests (Figure 3). In contrast, using M-ratio tests, more bottlenecks were detected in tuatara simulations compared to massasauga simulations (Figure 3). Bottleneck detection generally increased corresponding to bottleneck severity, except in massasauga simulations using the heterozygosity excess test (Figure 3). Using this test for simulations in this species, detection rate peaked in the scenario with $N_b = 100$ individuals and $t_b = 200$ years, at which point detection of bottlenecks decreased with decreasing N_b (Figure 3).

For the M-ratio test applied to massasauga simulations, we observed little difference in bottleneck detection between the 2 types of M_c values used, but using calculated M_c generally resulted in a slightly higher probability of bottleneck detection (Figure 3). Among tuatara simulations, we observed greater differences in bottleneck detection between the 2 types of M_c values. Using calculated M_c rather than assuming $M_c = 0.68$ resulted in much higher bottleneck

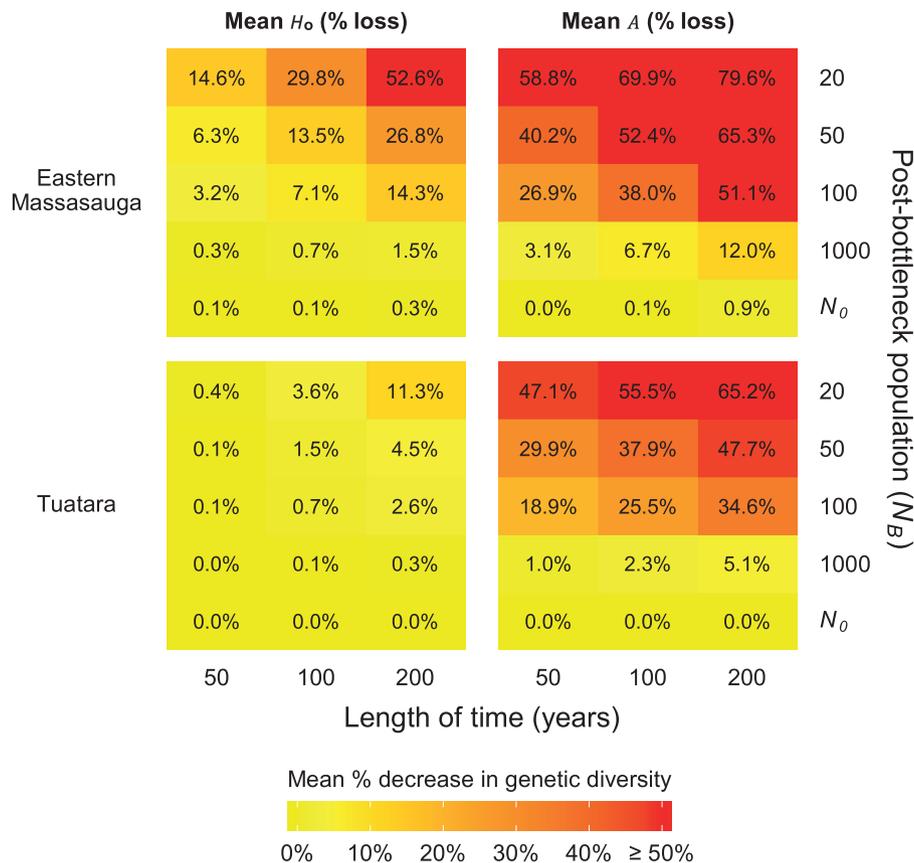


Figure 2. Mean percent decreases in observed heterozygosity (H_O) and mean number of alleles per locus (A) from simulated bottlenecks for tuatara and eastern massasaugas. Starting population census size (N_0) = 25 000 tuatara or 6300 massasaugas. Simulations varied by bottlenecked population size (N_b) and bottleneck duration. $N_b = N_0$ indicates simulations of constant population size (i.e., no bottleneck). See online version for full color.

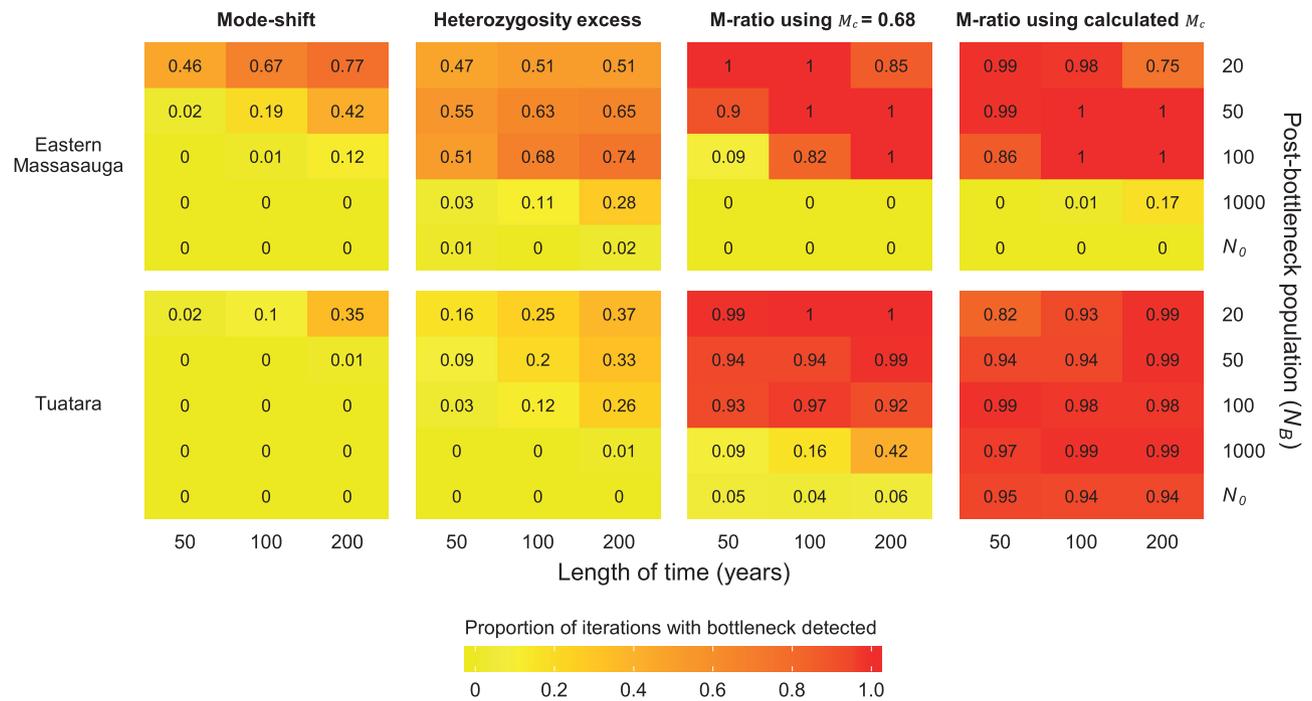


Figure 3. Proportion of simulated bottlenecks detected in tuatara and eastern massasaugas using common bottleneck tests. Starting population census size (N_0) = 25 000 tuatara or 6300 massasaugas. M-ratios were compared to either a critical value ($M_c = 0.68$ or M_c calculated using Critical_M.exe software and the parameters $\theta = 10$, proportion of 1-step mutations = 0.90, and mean size of multi-step mutations = 3.5. Simulations varied by bottlenecked population size (N_b) and bottleneck duration. $N_b = N_0$ indicates simulations of constant population size (i.e., no bottleneck). See online version for full color.

detection for bottlenecks with $N_b = 1000$ tuatara, but also yielded extremely high rates of false-positive results in constant population size ($N_0 = N_b$) scenarios (Figure 3). Assuming $M_c = 0.68$ resulted in a low percentage of false-positive results for tuatara simulations (4–6%), which was still higher than the rate of false positives resulting from heterozygosity excess or mode-shift tests.

Repeating the simulations using the reduced eastern massasauga dataset of 7 randomly selected loci resulted in similar losses in genetic diversity and bottleneck detection as conducting the analyses with all 12 loci. However, rates of bottleneck detection by the heterozygosity excess test were slightly higher using the full 12-locus dataset. Still, the trends described above between the eastern massasauga and tuatara results were the same regardless of whether the full or reduced dataset was used to make comparisons (Supplementary Materials 4 and 5).

Repeating the simulations for the full eastern massasauga dataset with increased life history parameter values resulted in reduced losses in genetic diversity compared to simulations conducted using the realistic life history values (Figure 4A). Similarly, the power of all tests to detect bottlenecks was generally diminished when eastern massasauga life history parameters were increased (Figure 4A). Likewise, repeating the simulations for the tuatara dataset using the decreased life history parameters relative to the realistic life history values resulted in greater losses in genetic diversity (Figure 4B). The power of bottleneck tests generally increased slightly or remained approximately the same when tuatara life history parameters were decreased, depending on the test and the bottleneck severity (Figure 4B).

When simulations using the same life history parameters for both species were compared, bottlenecks simulated for the tuatara dataset produced greater losses in allelic richness than those simulated for

the eastern massasauga dataset, but both datasets produced comparable losses in heterozygosity (Figure 5A). The power of mode-shift tests to detect bottlenecks was low and was similar for both species (range = 0–51%; Figure 5B). Heterozygosity excess tests performed better in eastern massasauga simulations, but the power difference between the 2 species was smaller compared to simulations using the realistic life history parameters for each species (Figure 5B). The power of M-ratio tests was greater for tuatara simulations and this difference in power was larger than when comparing simulations with realistic life history parameters (Figure 5B). M-ratio tests on the tuatara simulations resulted in false positives ranging from 5% to 6% when assuming $M_c = 0.68$ and from 93% to 96% when using calculated M_c (Supplementary Material 5).

Discussion

In this study, we used empirical microsatellite data from populations of tuatara and eastern massasaugas as a starting point for simulated population declines to evaluate the performance of bottleneck tests under a range of scenarios, for moderate- to long-lived species. Similar to Peery et al. (2012), we found that some of the most commonly employed methods for detecting recent bottlenecks are often unreliable. Among the methods we considered, the mode-shift test was generally least effective and the M-ratio test was typically most effective at detecting simulated bottlenecks. Unlike previous studies that assessed the reliability of these tests, we quantified pre- and post-bottleneck genetic diversity to compare known losses in allelic richness and heterozygosity to bottleneck detection. This allowed us to infer whether failures to detect bottlenecks in long-lived species are solely attributable to limited genetic diversity losses caused by the buffering effect of

Table 1. Tuatara and eastern massasauga population bottleneck simulation results

Tuatara					
N_B	t_B (years)	Calculated M_c	Mean M-ratio (SD)	% A_E lost	% H_E lost
20	50	0.62	0.57 (0.05)	26	6
20	100	0.62	0.54 (0.05)	36	10
20	200	0.62	0.51 (0.06)	48	17
50	50	0.68	0.62 (0.03)	13	2
50	100	0.68	0.60 (0.04)	20	4
50	200	0.68	0.57 (0.05)	30	7
100	50	0.71	0.66 (0.02)	7	1
100	100	0.71	0.63 (0.02)	12	2
100	200	0.71	0.61 (0.04)	20	4
1000	50	0.72	0.70 (0.01)	1	0
1000	100	0.72	0.69 (0.01)	1	0
1000	200	0.72	0.68 (0.01)	3	0
N_0	50	0.72	0.70 (0.01)	0	0
N_0	100	0.72	0.70 (0.01)	0	0
N_0	200	0.72	0.70 (0.01)	0	0
Eastern Massasauga					
N_B	t_B (years)	Calculated M_c	Mean M-ratio (SD)	% A_E lost	% H_E lost
20	50	0.65	0.56 (0.04)	45	20
20	100	0.65	0.53 (0.05)	58	34
20	200	0.65	0.60 (0.08)	69	56
50	50	0.71	0.64 (0.03)	27	9
50	100	0.71	0.58 (0.03)	40	16
50	200	0.71	0.54 (0.05)	54	29
100	50	0.73	0.71 (0.02)	16	4
100	100	0.73	0.66 (0.03)	26	8
100	200	0.73	0.59 (0.03)	39	15
1000	50	0.75	0.79 (0.01)	2	0
1000	100	0.75	0.78 (0.01)	4	1
1000	200	0.75	0.76 (0.02)	7	2
N_0	50	0.75	0.80 (0.01)	0	0
N_0	100	0.75	0.80 (0.01)	1	0
N_0	200	0.75	0.80 (0.01)	1	0

Starting population census size (N_0) = 25 000 tuatara or 6300 massasaugas. Simulations varied by bottlenecked population size (N_B) and bottleneck duration (t_B). Calculated critical values (calculated M_c) were compared to M-ratios as one method of testing for a population bottleneck. M_c values were calculated using Critical_M.exe software and the parameters $\theta = 10$, proportion of 1-step mutations = 0.90, and mean size of multi-step mutations = 3.5. Mean percent losses in effective number of alleles (% A_E lost) and expected heterozygosity (% H_E lost) were obtained from simulation outputs. $N_B = N_0$ indicates simulations of constant population size (i.e., no bottleneck).

long generation times. Our results demonstrate that even when large losses of genetic diversity have occurred (both allelic diversity and heterozygosity), mode-shift and heterozygosity excess tests are likely to produce a false negative result (i.e., fail to detect a bottleneck when one occurred). Thus, although longevity may buffer some losses in genetic diversity, as suggested by Hailer et al. (2006), these tests perform unreliably even when bottlenecks are severe enough to cause dramatic and biologically relevant losses of diversity.

We observed much smaller declines in genetic diversity for bottlenecks simulated in the longer-lived tuatara compared to the more moderate-lived eastern massasauga, supporting that longer generation time may lead to smaller losses in genetic diversity. Furthermore, manipulating longevity resulted in similar changes in genetic diversity. Specifically, when we artificially increased longevity in eastern massasauga simulations, we observed smaller losses in genetic diversity for this species and when we artificially decreased longevity in tuatara simulations, we observed larger losses

in genetic diversity. As Hailer et al. (2006) demonstrated, this makes sense given the expectation for the effect of time measured in (t) generations on current heterozygosity (H_t), given past heterozygosity (H_0) and effective population size (N_e): $H_t = H_0 \left(1 - \frac{1}{2N_e}\right)^t$ (Wright 1931). However, we caution that although long generation time decreases the rate of genetic drift in absolute time, this can also mask critical reductions in census size that make a population vulnerable to demographic stochasticity and result in a lack or delay of needed management action (Kuo and Janzen 2004).

Using mode-shift and heterozygosity excess tests, detection of bottlenecks was much greater for massasaugas, the species with larger losses in genetic diversity, than for tuatara. Surprisingly, however, detection of bottlenecks using M-ratio tests was typically greater for tuatara, despite smaller losses in genetic diversity. The initial tuatara dataset had higher allelic richness than the initial massasauga dataset and similar heterozygosity, but a lower M-ratio. The lower initial M-ratio of the tuatara population likely led to this higher rate of bottleneck detection using the M-ratio test. It is possible that the

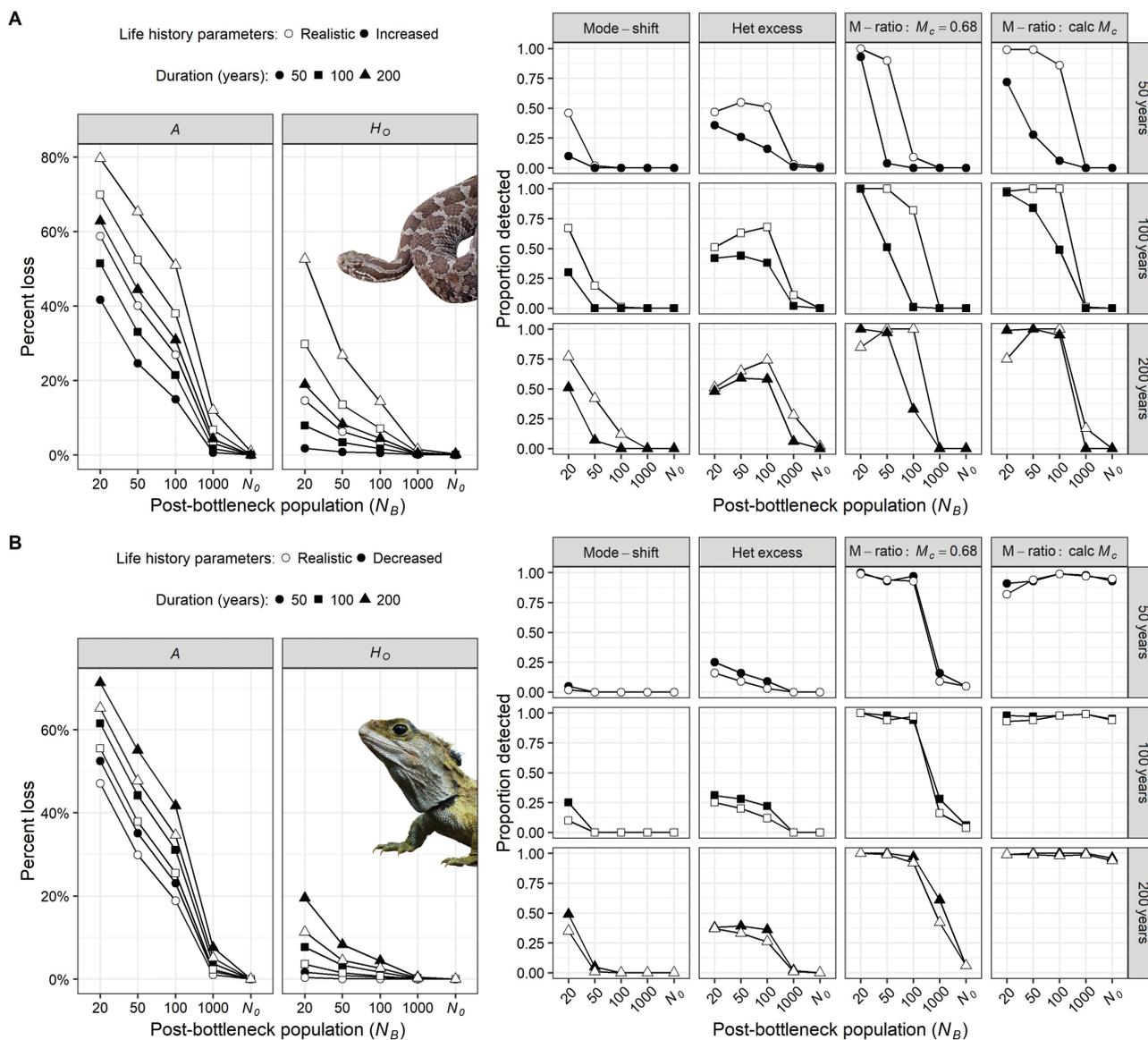


Figure 4. Comparison of simulation and test results within (A) eastern massasauga and (B) tuatara datasets simulated under 2 different sets of life history parameters: realistic values for the given species vs. 50-year longevity and 10-year age of maturity. Left panels show the mean percent loss in number of alleles (A) and observed heterozygosity (H_o) for 1000 iterations. Right panels show the proportion of iterations in which a bottleneck was detected using each test. To assess a positive M-ratio test, M-ratios were compared to either a critical value (M_c) = 0.68 or a M_c calculated using Critical_M.exe software and the parameters $\theta = 10$, proportion of 1-step mutations = 0.90, and mean size of multi-step mutations = 3.5 (calculated M_c). Starting population census size (N_0) = 25 000 tuatara or 6300 massasaugas. Simulations varied by bottlenecked population size (N_b) and bottleneck duration. $N_b = N_0$ indicates simulations of constant population size (i.e., no bottleneck). See online version for full color.

lower initial M-ratio is a consequence of true mutation parameters (i.e., mutation rate, proportion of single-step mutations, average size of non-single step mutations) differing between these species (Garza and Williamson 2001; Williamson-Natesan 2005; Peery et al. 2012). Alternatively, undetected population structure can result in smaller M-ratio values (Garza and Williamson 2001). The larger initial population size of tuatara may have also contributed to greater bottleneck detection using M-ratio tests. Because we simulated the same set of N_b values for both massasaugas and tuatara, tuatara experienced comparatively larger population declines for each N_b .

We also observed false-positive M-ratio results in tuatara simulations of constant population size with no genetic diversity loss. The rates of false positives were exceptionally high (94–95%) when

we used the calculated M_c value as a threshold for tuatara. Previous studies have demonstrated that incorrectly identifying microsatellite mutation model parameters can result in false positive or false negative bottleneck test results, and that the M-ratio test is particularly sensitive to departures from mutation model assumptions (Garza and Williamson 2001; Williamson-Natesan 2005; Peery et al. 2012). Specifically, underestimating the proportion of or mean size of multi-step mutations can yield a false positive M-ratio result, and the opposite can yield a false negative (Williamson-Natesan 2005). Underestimating θ (i.e., $4N_e\mu$, where μ represents the microsatellite mutation rate and N_e represents the effective population size at mutation-drift equilibrium) in determining M_c can also result in a false positive (Williamson-Natesan 2005). Conversely, for

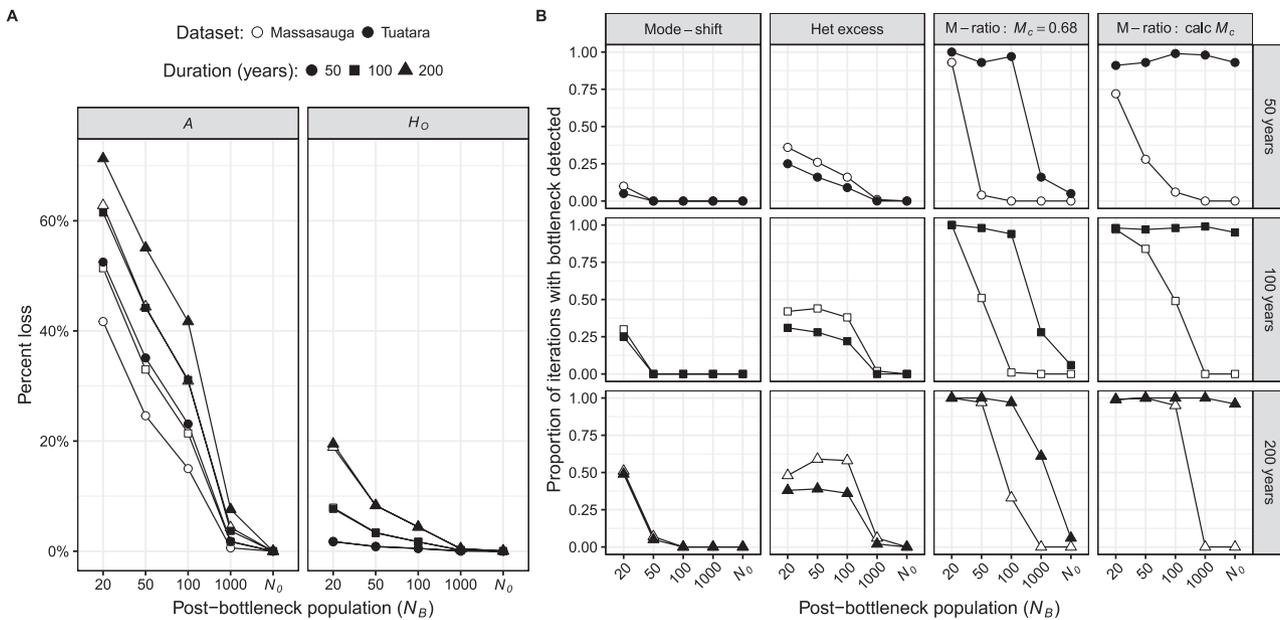


Figure 5. Comparison across species of simulation and test results using datasets simulated under identical, but unrealistic life history parameters: 50-year longevity and 10-year age of maturity. **(A)** Mean percent loss in number of alleles (*A*) and observed heterozygosity (H_o) for 1000 iterations and **(B)** proportion of iterations in which a bottleneck was detected using each test. To assess a positive M-ratio test, M-ratios were compared to either a critical value (M_c) = 0.68 or a M_c calculated using Critical_M.exe software and the parameters $\theta = 10$, proportion of 1-step mutations = 0.90, and mean size of multi-step mutations = 3.5 (calculated M_c). Starting population census size (N_0) = 25 000 tuatara or 6300 massasaugas. Simulations varied by bottlenecked population size (N_b) and bottleneck duration. $N_b = N_0$ indicates simulations of constant population size (i.e., no bottleneck).

the heterozygosity excess test, overestimating the proportion of multi-step mutations can yield a false-positive result, and the opposite can yield a false negative (Williamson-Natesan 2005; Peery et al. 2012). The mode-shift test is qualitatively based on graphing the proportion of alleles within each allele frequency class and does not assume a specific mutation model or rate (Luikart et al. 1998). While increasing the number of loci sampled can significantly increase the power of bottleneck tests to detect bottlenecks (Hoban et al. 2013; Davy and Murphy 2014), doing so can result in a much higher rate of false-positive results if mutation model parameters are incorrect (Williamson-Natesan 2005).

Unfortunately, the true values of mutation model parameters are not known for most species, so users of these tests often choose recommended values or default software settings. The values we selected to represent the mutation models included mean size of multi-step mutations of 3.5 (M-ratio test), variance in the size of multi-step mutations of 12 (heterozygosity excess test), and proportion of 1-step mutations of 0.90 (heterozygosity excess and M-ratio test). These were the most common values used for these bottleneck tests in a review of the literature conducted by Peery et al. (2012) and are values recommended by Garza and Williamson (2001) and Piry et al. (1999). Therefore, while these parameters may not represent the true mutation model for these species, they represent values likely to be used in testing for bottlenecks. Similarly, using a M_c value of 0.68 or calculating M_c using a θ value of 10 is common for comparing M-ratios to infer whether there has been a bottleneck. Using a θ value of 10 is based on assuming a mutation rate equal to an average microsatellite mutation rate (5×10^{-4} ; Selkoe and Toonen 2006) and a mutation-drift equilibrium effective population size of 5000 individuals. However, microsatellite mutation rates have been estimated to be as low as 10^{-6} or as high as 10^{-2} (Schlötterer 2000),

and it may be impossible to know the equilibrium effective population size of a population being studied.

To evaluate the sensitivity of our results to variation in life history parameters, we repeated our simulations with longevity set at 50 years and age of sexual maturity set at 10 years for both species (Figure 5). In doing so, we observed nearly identical heterozygosity losses between species, but larger allelic diversity losses in tuatara. Mode-shift tests performed similarly across species; however, heterozygosity excess tests continued to perform better for massasaugas, but by a smaller degree. As in the analyses using biologically realistic life history parameters, we found a greater statistical power of M-ratio tests in tuatara than in massasaugas and this difference increased in magnitude, likely because of the larger allelic diversity losses in this species under these contrived conditions. M-ratio false-positive rates remained comparable to when the more realistic life history values were used. Overall, lower values for life history parameters within a species typically resulted in greater losses in genetic diversity and correspondingly higher bottleneck detection. Thus, it appears that some, but not all, of the differences in bottleneck detection between these species are directly linked to longevity. The remaining differences are likely due to variation in starting allele frequencies, which is related to unequal effective population sizes. Using the empirical datasets, tuatara had higher allelic diversity at the start of simulations, but heterozygosity was similar between the species ($H_o = 0.751$ for massasaugas vs $H_o = 0.755$ for tuatara and $H_e = 0.789$ for massasaugas vs $H_e = 0.780$ for tuatara). These measures of genetic diversity differed between tuatara and massasaugas because we used actual allele frequency data to achieve more realistic results. Kuo and Janzen (2004) found that using actual allele frequencies and simulating overlapping generations resulted in greater (and more realistic) genetic diversity losses. It is likely that using a greater number of loci also contributed to higher heterozygosity

excess test power in massasaugas relative to tuatara, but to a lesser extent than the species' shorter longevity.

All bottleneck simulations resulted in greater losses in allelic diversity than heterozygosity. This was expected given the heterozygosity excess test hinges on the assumption that allelic richness decreases at a faster rate than heterozygosity (Cornuet and Luikart 1996). Heterozygosity is the most widespread estimate of genetic diversity used to compare populations because it is less sensitive to sample size and more comparable across populations than allelic richness (Allendorf and Luikart 2007). However, while heterozygosity is a useful indicator of short-term adaptability, allelic richness is more important to the long-term adaptive potential of populations (Allendorf 1986). Because lost alleles cannot be recovered without migration or mutation, this highlights the threat to population persistence for many reptiles and other species with low vagility occurring in isolated patches of suitable habitat. For an island inhabitant like the tuatara, gene flow may be impossible without human intervention (i.e., augmentation). Most remnant populations of eastern massasaugas also persist in small habitat "islands," and some on actual islands (e.g., Kudla et al. 2021), with no connectivity for gene flow.

Despite dramatic losses in population size and concurrent losses of genetic diversity (particularly allelic diversity), M-ratio, heterozygosity excess, and mode-shift tests were unreliable at identifying bottlenecks in both eastern massasauga and tuatara simulations, with high rates of both type I and type II error. These bottleneck tests will likely have even less power in real-world scenarios where population size reductions occur more gradually over time (Hoban et al. 2013). Therefore, reliance on these tests could result in misinformed species management and conservation decisions. False negatives could result in bottlenecked populations being overlooked and assumed stable, while false positives could result in unnecessary management interventions. Errors like these could mean misappropriation of already-limited conservation funds and misguided management efforts.

Supplementary Material

Supplementary material can be found at *Journal of Heredity* online.

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Data Availability

We have deposited the primary data underlying these analyses as follows:

- Raw tuatara microsatellite genotypes and simulated tuatara and eastern massasauga microsatellite genotypes: Dryad (doi:10.5061/dryad.wpzgmsbn3).
- R script for calculating M-ratios uploaded as [Supplementary Material 2](#).

References

- Allendorf FW. 1986. Genetic drift and the loss of alleles versus heterozygosity. *Zoo Biol.* 5:181–190.
- Allendorf FW, Luikart GH. 2007. *Conservation and the genetics of populations*. Malden (MA): Blackwell Publishing.
- Bailey RL, Campa H III, Bissell KM, Harrison TM. 2012. Resource selection by the eastern massasauga rattlesnake on managed land in southwestern Michigan. *J Wildl Manage.* 76:414–421.
- Brown D. 2000. *Stephens Island: ark of the light*. Blenheim (New Zealand): Cloudbay Publishing.
- Castanet J, Newman DG, Saint Girons H. 1988. Skeletochronological data on the growth, age, and population structure of the tuatara, *Sphenodon punctatus*, on Stephens and Lady Alice Islands, New Zealand. *Herpetologica.* 44:25–37.
- Ceballos G, Ehrlich PR, Dirzo R. 2017. Biological annihilation via the ongoing sixth mass extinction signaled by vertebrate population losses and declines. *Proc Natl Acad Sci U S A.* 114:E6089–E6096.
- Chiucchi JE, Gibbs HL. 2010. Similarity of contemporary and historical gene flow among highly fragmented populations of an endangered rattlesnake. *Mol Ecol.* 19:5345–5358.
- Cornuet JM, Luikart G. 1996. Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. *Genetics.* 144:2001–2014.
- Cree A, Cockrem JF, Guillelte LJ. 1992. Reproductive cycles of male and female tuatara (*Sphenodon punctatus*) on Stephens Island, New Zealand. *J Zool.* 226:199–217.
- Crnokrak P, Roff DA. 1999. Inbreeding depression in the wild. *Heredity (Edinb).* 83 (Pt 3):260–270.
- Davy CM, Murphy RW. 2014. Conservation genetics of the endangered spotted turtle (*Clemmys guttata*) illustrate the risks of "bottleneck tests." *Can J Zool.* 92:149–162.
- Dileo MF, Rouse JD, Dávila JA, Loughheed SC. 2013a. Data from: The influence of landscape on gene flow in the eastern massasauga rattlesnake (*Sistrurus c. catenatus*): insight from computer simulations. *Dryad Digital Repository*. Available from: <https://doi.org/10.5061/dryad.v22n5>.
- Dileo MF, Rouse JD, Dávila JA, Loughheed SC. 2013b. The influence of landscape on gene flow in the eastern massasauga rattlesnake (*Sistrurus c. catenatus*): insight from computer simulations. *Mol Ecol.* 22:4483–4498.
- Environment Canada. 2012. Species at risk public registry: eastern massasauga profile. Available from: https://wildlife-species.canada.ca/species-risk-registry/species/speciesDetails_e.cfm?sid=1220. Accessed 18 August 2019.
- Frankham R. 1995. Conservation genetics. *Annu Rev Genet.* 29:305–327.
- Frankham R. 1996. Relationship of genetic variation to population size in wildlife. *Conserv Biol.* 10:1500–1508.
- Frankham R. 2005. Genetics and extinction. *Biol Conserv.* 126:131–140.
- Frankham R, Lees K, Montgomery ME, England PR, Lowe EH, Briscoe DA. 1999. Do population size bottlenecks reduce evolutionary potential? *Anim Conserv.* 2:255–260.
- Garza JC, Williamson EG. 2001. Detection of reduction in population size using data from microsatellite loci. *Mol Ecol.* 10:305–318.
- Gibbs HL, Prior KA, Weatherhead PJ, Johnson G. 1997. Genetic structure of populations of the threatened eastern massasauga rattlesnake, *Sistrurus c. catenatus*: evidence from microsatellite DNA markers. *Mol Ecol.* 6:1123–1132.
- Gilpin ME, Soulé ME. 1986. Minimum viable populations: processes of species extinction. In: Soulé ME, editor. *Conservation biology: the science of scarcity and diversity*. Sunderland (MA): Sinauer Associates, pp. 19–34.
- Guo SW, Thompson EA. 1992. Performing the exact test of Hardy-Weinberg proportion for multiple alleles. *Biometrics.* 48:361–372.
- Hailer F, Helander B, Folkstad AO, Ganusevich SA, Garstad S, Hauff P, Koren C, Nygård T, Volke V, Vilà C, et al. 2006. Bottlenecked but long-lived: high genetic diversity retained in white-tailed eagles upon recovery from population decline. *Biol Lett.* 2:316–319.

- Harding JH. 1997. *Amphibians and reptiles of the Great Lakes Region*. Ann Arbor (MI): University of Michigan Press.
- Hay JM, Sarre SD, Lambert DM, Allendorf FW, Daugherty CH. 2010. Genetic diversity and taxonomy: a reassessment of species designation in tuatara (*Sphenodon*: Reptilia). *Conserv Genet*. 11:1063–1081.
- Hoban SM, Gaggiotti OE, Bertorelle G. 2013. The number of markers and samples needed for detecting bottlenecks under realistic scenarios, with and without recovery: a simulation-based study. *Mol Ecol*. 22:3444–3450.
- Hoelzel AR, Fleischer RC, Campagna C, Le Boeuf BJ, Alvord G. 2002. Impact of a population bottleneck on symmetry and genetic diversity in the northern elephant seal. *J Evol Biol*. 15:567–575.
- Holm S. 1979. A simple sequentially rejective multiple test procedure. *Scand J Stat*. 6:65–70.
- Johnson G. 2000. Spatial ecology of the eastern massasauga (*Sistrurus c. catenatus*) in a New York peatland. *J Herpetol*. 34:186–192.
- Kudla N, McCluskey EM, Lulla V, Grundel R, Moore JA. 2021. Intact landscape promotes gene flow and low genetic structuring in the threatened eastern massasauga rattlesnake. *Ecol Evol*. 00:1–13.
- Kuo CH, Janzen FJ. 2004. Genetic effects of a persistent bottleneck on a natural population of ornate box turtles (*Terrapene ornata*). *Conserv Genet*. 5:425–437.
- Kuo CH, Janzen FJ. 2003. BOTTLESIM: a bottleneck simulation program for long-lived species with overlapping generations. *Mol Ecol Notes*. 3:669–673.
- Lippé C, Dumont P, Bernatchez L. 2006. High genetic diversity and no inbreeding in the endangered copper redhorse, *Moxostoma hubbsi* (Catostomidae, Pisces): the positive sides of a long generation time. *Mol Ecol*. 15:1769–1780.
- Luikart G, Allendorf FW, Cornuet JM, Sherwin WB. 1998. Distortion of allele frequency distributions provides a test for recent population bottlenecks. *J Hered*. 89:238–247.
- MacAvoy ES, McGibbon LM, Sainsbury JP, Lawrence H, Wilson CA, Daugherty CH, Chambers GK. 2007. Genetic variation in island populations of tuatara (*Sphenodon* spp) inferred from microsatellite markers. *Conserv Genet*. 8:305–318.
- Miller HC, Allendorf F, Daugherty CH. 2010. Genetic diversity and differentiation at MHC genes in island populations of tuatara (*Sphenodon* spp.). *Mol Ecol*. 19:3894–3908.
- Miller KA, Miller HC, Moore JA, Mitchell NJ, Cree A, Allendorf FW, Sarre SD, Keall SN, Nelson NJ. 2012. Securing the demographic and genetic future of tuatara through assisted colonization. *Conserv Biol*. 26:790–798.
- Miller PS. 2005. Population viability assessment for the eastern massasauga rattlesnake (*Sistrurus catenatus catenatus*) on the Bruce Peninsula, Ontario, Canada. In: Third International Eastern Massasauga Symposium; 2005 Oct 12–14; Toronto, Ontario, Canada: Massasauga Recovery Team.
- Mills LS, Smouse PE. 1994. Demographic consequences of inbreeding in remnant populations. *Am Nat*. 144:412–431.
- Moore JA, Gillingham JC. 2006. Spatial ecology and multi-scale habitat selection by a threatened rattlesnake: the eastern massasauga (*Sistrurus catenatus catenatus*). *Copeia*. 2006:742–751.
- Moore JA, Hoare JM, Daugherty CH, Nelson NJ. 2007. Waiting reveals waning weight: monitoring over 54 years shows a decline in body condition of a long-lived reptile (tuatara, *Sphenodon punctatus*). *Biol Conserv*. 135:181–188.
- Moore JA, Miller HC, Daugherty CH, Nelson NJ. 2008. Fine-scale genetic structure of a long-lived reptile reflects recent habitat modification. *Mol Ecol*. 17:4630–4641.
- Nei M. 1973. Analysis of gene diversity in subdivided populations. *Proc Natl Acad Sci U S A*. 70:3321–3323.
- Nei M, Maruyama T, Chakraborty R. 1975. The bottleneck effect and genetic variability in populations. *Evolution*. 29:1–10.
- Newman DG. 1987. Burrow use and population densities of tuatara (*Sphenodon punctatus*) and how they are influenced by fairy prions (*Pachyptila turtur*) on Stephens Island, New Zealand. *Herpetologica*. 43:336–344.
- Ohnishi N, Saitoh T, Ishibashi Y, Oi T. 2007. Low genetic diversities in isolated populations of the Asian black bear (*Ursus thibetanus*) in Japan, in comparison with large stable populations. *Conserv Genet*. 8:1331–1337.
- Paquette SR. 2012. PopGenKit: useful functions for (batch) file conversion and data resampling in microsatellite datasets. In: R Packag. version 1. Available from: <https://cran.r-project.org/package=PopGenKit>.
- Parks Canada Agency. 2015. *Recovery strategy for the massasauga (Sistrurus catenatus) in Canada*. Ottawa (Canada): Parks Canada Agency.
- Peakall R, Smouse PE. 2006. GenAlEx 6: genetic analysis in Excel. Population genetic software for teaching and research. *Mol Ecol Notes*. 6:288–295.
- Peakall R, Smouse PE. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research—an update. *Bioinformatics*. 28:2537–2539.
- Peery MZ, Kirby R, Reid BN, Stoelting R, Doucet-Béer E, Robinson S, Vásquez-Carrillo C, Pauli JN, Palsbøll PJ. 2012. Reliability of genetic bottleneck tests for detecting recent population declines. *Mol Ecol*. 21:3403–3418.
- Piry S, Luikart G, Cornuet JM. 1999. BOTTLENECK: a computer program for detecting recent reductions in the effective population size using allele frequency data. *J Hered*. 90:502–503.
- Reinert HK, Kodrich WR. 1982. Movements and habitat utilization by the massasauga, *Sistrurus catenatus catenatus*. *J Herpetol*. 16:162–171.
- Rice WR. 1989. Analyzing tables of statistical tests. *Evolution*. 43:223–225.
- Rouse JD. 2005. *Killbear eastern massasauga management plan*. Ontario (Canada): Ontario Parks.
- Rousset F. 2008. genepop'007: a complete re-implementation of the genepop software for Windows and Linux. *Mol Ecol Resour*. 8:103–106.
- Schlötterer C. 2000. Evolutionary dynamics of microsatellite DNA. *Chromosoma*. 109:365–371.
- Selkoe KA, Toonen RJ. 2006. Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. *Ecol Lett*. 9:615–629.
- Snider AT, Bowler JK. 1992. *Longevity of reptiles and amphibians in North American collections*. Oxford (OH): Society for the Study of Amphibians and Reptiles.
- Sovic M, Fries A, Martin SA, Lisle Gibbs H. 2019. Genetic signatures of small effective population sizes and demographic declines in an endangered rattlesnake, *Sistrurus catenatus*. *Evol Appl*. 12:664–678.
- Szymanski J. 1998. *Status assessment for the eastern massasauga (Sistrurus c. catenatus)*. Fort Snelling (MN): US Fish and Wildlife Service.
- Szymanski J, Pollack C, Ragan L, Redmer M, Clemency L, Voorhies K, JaKa J. 2015. *Species status assessment for the eastern massasauga rattlesnake (Sistrurus catenatus)*. Fort Snelling (MN): US Fish and Wildlife Service.
- U.S. Fish and Wildlife Service. 2016. Endangered and threatened wildlife and plants; threatened species status for the eastern massasauga rattlesnake. *Fed Regist*. 81:67193–67214.
- Weatherhead PJ, Prior KA. 1992. Preliminary observations of habitat use and movements of the eastern massasauga rattlesnake (*Sistrurus c. catenatus*). *J Herpetol*. 26:447–452.
- Williamson-Natesan EG. 2005. Comparison of methods for detecting bottlenecks from microsatellite loci. *Conserv Genet*. 6:551–562.
- Wisely SM. 2002. Genetic diversity and fitness in black-footed ferrets before and during a bottleneck. *J Hered*. 93:231–237.
- Wright S. 1931. Evolution in mendelian populations. *Genetics*. 16:97–159.