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Population Genetics of the Copperhead at Its Most Northeastern Distribution

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Population genetic data are an important standard with which to gauge demography and gene flow within and among biodiversity units, and are often gathered on species of conservation concern. Yet an exclusive focus on 'conservation immediacy' can also have negative consequences. For example, it can shift monitoring efforts away from more abundant and/or widely distributed clades and, by so doing, promote a more myopic approach to conservation and management. A current example concerns North American pitvipers (Viperidae: Crotalinae), within which listed species of *Crotalus* and *Sistrurus* receive considerable population genetic attention whereas broadly distributed *Agkistrodon* is largely overlooked. To address this disparity, we used 22 polymorphic tetra-nucleotide microsatellite loci to explore genetic structure, diversity, and relatedness in a Connecticut population of Copperhead (*A. contortrix*). Three admixed genetic clusters were identified across five winter dens, with overall and sex-specific relatedness similar among dens and across the population. First- and second-order relationships were identified within the population, then juxtaposed against known den associations. Values for genetic structure, diversity, and effective population size are similar to those reported for populations of North American *Crotalus* and *Sistrurus*. However, the study population did not sustain a genetic bottleneck following recent anthropogenic habitat alterations, and this may reflect a potential resilience to environmental change, particularly when compared with North American *Crotalus* and *Sistrurus*. Our results underscore the importance of (1) quantifying population-level parameters in non-threatened crotalines so as to broaden and extend our understanding of anthropogenic impacts, and (2) evaluating population genetics in taxa that appear superficially resilient to anthropogenic modifications. The latter may also promote valuable comparative analyses with threatened and endangered taxa.

ANTHROPOGENIC climate change and habitat fragmentation are accelerating globally, much to the detriment of biodiversity and despite conservation efforts to the contrary (Opdam and Wascher, 2004; Thomas et al., 2004). Of particular concern is the recognition that extinction vortices (Gilpin and Soulé, 1986) are synergistically driven by environmental, demographic, and genetic aspects (Clark et al., 2011), and population data derived from an array of biodiversity elements are the necessary barometers to gauge their impacts. Mark–recapture and telemetry studies can track connectedness but not gene flow (Holycross and Douglas, 2007), yet can be broadened and extended by molecular genetic methods (Douglas and Douglas, 2010) such that additional data are obtained beyond the scope of traditional approaches (Levine et al., 2015).

While molecular studies are apparent in North American pitvipers (Viperidae: Crotalinae), their focus has largely been on threatened and endangered *Crotalus* and *Sistrurus* (e.g., Eastern Massasauga [Lougheed et al., 2000; Bushar et al., 2001; Chiucchi and Gibbs, 2010]; Desert Massasauga [Anderson et al., 2009]; New-Mexico Ridge-Nosed Rattlesnake [Holycross and Douglas, 2007]; Midget Faded Rattlesnake [Oyler-McCance and Parker, 2010]; Timber Rattlesnake [Bushar et al., 1998, 2014; Anderson, 2010; Clark et al., 2011]). While such a consistently narrow focus on species of conservation concern is often a recognized necessity, it also limits our ability to delineate on a broader scale the impacts of natural and anthropogenic stressors. Further, it reduces the impetus to monitor those forms of limited conservation concern while preventing an accumulation of background data for their eventual management. Finally, it ignores the resiliency potential of these more common, widespread forms in the face of a changing climate (Moritz and Agudo, 2013; Sutton et al., 2015; Young et al., 2015). Herein, we posit that such a knowledge gap exists in temperate North American crotalines, in that scant efforts have been directed towards understanding the population genetics of *Agkistrodon*, the third genus within this assemblage.

The paucity of population genetic studies for *Agkistrodon* is surprising, and for several reasons. First, few generalizations are found across taxa regarding the influence of landscape features and habitat change (Storfer et al., 2010), and the broad geographic distribution of this genus in North America (Gloyd and Conant, 1990) should promote such studies. The Copperhead is also the oldest lineage in the phylogeny of *Agkistrodon* (Douglas et al., 2009) and is therefore appropriate for evaluating important population-level parameters such as effective population size (N_e), genetic diversity, and genetic structure that may mainfest in the evolutionary trajectories of other North American crotalines. Additionally, the distribution of Copperhead is largely sympatric and often syntopic with threatened species of *Crotalus* and *Sistrurus* (Gloyd and Conant, 1990), such that comparative population-level analyses among these North American pitvipers may promote the concept of surrogate species in conservation and management (Caro and O'Doherty, 1999). Lastly, *A. contortrix* is a model for studies of reproductive ecology in that it is capable of facultative parthenogenesis (Booth and Schuett, 2011; Booth et al., 2012; Jordan et al., 2014), with male-biased fecundity and sexual selection (Levine et al., 2015), both of which can drive population genetic patterns.

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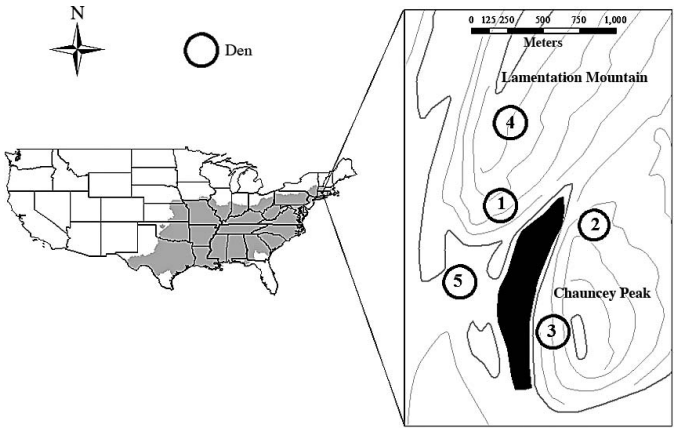

Fig. 1. DNA Samples were collected from 100 adult Copperheads (*Agkistrodon contortrix*) from 2001 to 2003 (Smith et al., 2009) for analyses of genetic structure, diversity, and relatedness. Individuals were collected from five dens (=1–5) located on Lamentation Mountain and Chauncey Peak, and separated by Bradley Hubbard Reservoir (=black), 4.74 km northwest of Meriden, Connecticut, USA. The study population is located in the northeastern portion of the range of *A. contortrix* (shown in gray).

Agkistrodon contortrix evolved from west to east in North America, with its northeastern 'subspecies' (Northern Copperhead, *A. contortrix mokasen*) being most recently derived (Douglas et al., 2009). This form extends from southern Illinois north and east to Connecticut and Massachusetts, a distribution in tandem with the Wisconsinian Glacier (Gloyd and Conant, 1990). Rapid range expansions to the north and east may have reduced heterozygosities post-Pleistocene, and population genetic studies are needed to evaluate these aspects against expectations implicit to rapid range expansions and their impacts on life history evolution. Additionally, there is disagreement over the importance of genetic diversity in peripheral vs. central populations, and an understanding of genetic structure in a population of *A. contortrix* at the distal edge of the species distribution will contribute to this debate (Garner et al., 2004). To accomplish these and other tasks, we employed 22 tetranucleotide microsatellite loci (Castoe et al., 2010) to quantify the demographic and genetic structure of a Copperhead population in Connecticut.

MATERIALS AND METHODS

Study site and sample collection.—A 486 ha site located within the basalt trap rock ridges of the Central Connecticut River valley, 4.75 km northwest of the city center of Meriden (Smith, 2007), provides habitat for *A. contortrix* and contains five winter dens (Smith et al., 2009; Fig. 1). These are located on Lamentation Mountain and Chauncey Peak, respectively, and are separated by a straight-line distance of approximately 200 m that is interrupted by Bradley Hubbard Reservoir (Smith et al., 2009; Fig. 1), which was constructed in the late 1800s and expanded in 1927 (www.ctparks.net/meriden/giuffrida/history). From 2001 to 2003, blood samples were collected from 100 adults (male = 50, female = 49, unknown sex = 1; Smith, 2007). Additionally, skin sheds were collected from 137 juveniles of known maternity for parentage analyses in a previous study (Levine et al., 2015), of which only the results are considered here to provide context for our estimates (i.e., only adults from the study population were analyzed in this study).

Microsatellite genotyping.—Genomic DNA was extracted using the PureGene® DNA Isolation Kit, with concentration standardized at 20.0 ng/uL. Genotype profiles were generated across 23 tetra-nucleotide microsatellite loci (Castoe et al., 2010). Polymerase chain reactions (PCR) consisted of 1X GoTaq® Flexi PCR buffer, 2.0–2.5 mM $MgCl_2$, 0.2 mM dNTP, 15.15 μM BSA, 0.2–0.65 mM forward and reverse primer, 0.5 units of GoTaq® Flexi *taq* DNA polymerase, and 20–40 ng DNA. The temperature profile was an initial denaturation of 95°C for 3 m followed by 15 cycles of 95°C for 45 s, 55°C for 45 s, and 72°for 30 s, followed by 16–19 cycles of 95°for 30 s, 55°C for 30 s, and 72°C for 15 s, followed by a final extension of 72°C for 5 m and conducted on GeneAmp® 9600 PCR System or Veriti® 96-Well Thermal cyclers. Microsatellite fragments were resolved on an ABI Prism 3730xl automated sequencer and sized after comparison to the LIZ500 size standard. Alleles were scored using PeakScanner™ (Applied Biosystems) software.

Population genetic analyses.—All loci in all individuals were evaluated for null alleles, large allele dropout, and genotyping errors in Micro-Checker Ver. 2.2.3 (Van Oosterhout et al., 2004). Departures from Hardy-Weinberg Equilibrium in the study population were examined in Genepop (Raymond and Rousset, 1995) using a Markov Chain method with 200 batches, 5000 iterations per batch, and with Bonferroni-adjusted $\alpha = 0.0002$. A Markov Chain test for linkage disequilibrium (LD) was also conducted in Genepop (batches/iterations as above).

GenAlEx 6.5 (Peakall and Smouse, 2006, 2012) was used to calculate observed (H_o) and expected (H_e) heterozygosity at each locus, and mean H_o, H_e, and number of alleles per locus (N_a) for all individuals and by sex and den affiliation. Friedman tests for nonparametric repeated measures analysis were used to compare H_e among groups (i.e., sex or dens), with each locus as a block (Marshall et al., 2009; Row et al., 2011).

Relatedness, effective population size, and bottlenecks.—Coancestry (Wang, 2011) with the triadic likelihood moment estimator (TrioML *r*; Wang, 2007) was used to gauge mean relatedness across the entire adult population, as well as by sex, den affiliation, and by sex within the largest den (=Den 1; Fig. 1). Mean relatedness was compared among groups by implementing the "Test Group Difference" option in Coancestry with 95% bootstapped confidence intervals ($N_{bootstraps} = 10{,}000$). Pairwise relatedness estimates derived by program Colony (Jones and Wang, 2009) identified those individuals with a probability of relatedness ≥ 0.95 and were used to determine first-order (=parent-offspring or full sibling) and second-order (=avuncular or half sibling) relationships. The ability of these microsatellite loci to delineate first- and second-order relationships with high accuracy was confirmed in a previous study (Levine et al., 2015) in which known maternity and sibships of 137 Copperheads exactly matched those inferred by Colony without the use of known parental data or sibships as priors.

Effective population size (N_e) was evaluated with the sibship assignment method (Wang, 2009) implemented in Colony (Jones and Wang, 2009) and the LD method (Hill, 1981) corrected for a finite sample size in program LDNe (Waples and Do, 2007, 2010) . In our LDNe analysis, alleles with frequencies <0.02 were excluded and jackknife-adjusted 95% confidence intervals were calculated (Waples and Do, 2007, 2010). Program Bottleneck (Piry et al., 1999; per

Table 1. Average observed (H_o) and expected (H_e) heterozygosities, and average numbers of alleles per locus (N_a) at 22 tetra-nucleotide microsatellite loci for a Connecticut population of adult Copperheads (*Agkistrodon contortrix*; $n = 100$). Estimates are also considered separately for male and female Copperheads in the population and with respect to den affiliations.

Group	*n*	H_o	H_e	N_a
Total	100	0.63±0.04	0.62±0.04	6.1±0.5
Females	49	0.65±0.04	0.63±0.04	5.7±0.4
Males	50	0.61±0.04	0.61±0.04	6.8±0.5
Den 1	62	0.61±0.04	0.62±0.04	5.8±0.4
Den 2	7	0.65±0.05	0.58±0.04	3.9±0.3
Den 3	10	0.63±0.04	0.60±0.04	4.6±0.3
Den 4	7	0.66±0.05	0.61±0.04	4.4±0.3
Den 5	14	0.69±0.05	0.62±0.04	5.0±0.4

Chiucchi and Gibbs, 2010) was used to test for the presence of a proximal bottleneck associated with the urban development and habitat fragmentation that has occurred in the vicinity of the study population in the past century. Stepwise Mutation (SMM) and Two-Phase (TPM) models were both employed as standards, with the TPM potentially more appropriate (Cornuet and Luikart, 1996). We ran 1000 iterations under TPM with 95% single-step mutations, 5% multistep mutations, and a multistep mutation variance of 12 (Piry et al., 1999). Statistical significance was assessed using the standardized differences test at $\alpha = 0.05$. We also applied the mode-shift test to assay for the presence of a more recent bottleneck (i.e., one that occurred in the last few dozen generations; Luikart et al., 1998), the hypothesis of which can be rejected when a frequency distribution of alleles is significantly L-shaped.

Genetic structure.—A Bayesian assignment method with admixture, correlated allele frequencies and the assumptions of a population without linkage disequilibrium and in Hardy-Weinberg Equilibrium (Structure 2.3.4; Pritchard et al., 2000; Falush et al., 2003, 2007; Hubisz et al., 2009) was used to test for genetic structure. Simulations (K = 1–7) exceeded the total number of dens in the study site ($n = 5$), with 12 independent runs at each K (Evanno et al., 2005) and no location priors. A burn-in of 100,000 iterations was followed by 800,000 Markov Chain Monte Carlo (MCMC) iterations, although multiple burn-in lengths (e.g., 50,000–100,000 iterations) and run lengths (e.g., 250,000–800,000 iterations) were tested and converged on the same value of K. Structure Harvester (Earl and vonHoldt, 2012) was employed to identify the greatest value of ΔK (per Evanno et al., 2005) and thus determine the most likely number of genetic clusters. Following identification of the most likely K, CLUMPP (Jakobsson and Rosenberg, 2007) was used to align cluster membership coefficients from each run of K, and Distruct 1.1 (Rosenberg, 2004) was employed to graphically display cluster membership coefficients for each individual. The Structure "Pop ID" option was implemented *a posteriori* to facilitate the grouping of individuals by den affiliation and to visualize the average cluster membership coeffients of each den in the study population by Distruct.

Structure analyses were also conducted with the removal of one individual from each first-order relationship pair ($n = 9$; Anderson and Dunham, 2008; Rodríguez-Ramilo and Wang, 2012), as it has been shown that such relationships have the capacity to bias Bayesian clustering algorithms (Anderson and Dunham, 2008; Rodríguez-Ramilo and Wang, 2012). However, second-order relationships identified in the previous analysis were not removed, as each second-order relationship family consisted of a small number of individuals (≤6), and such groups consisting of up to 17 individuals have been shown to have a negligible impact on Structure results (Anderson and Dunham, 2008). Each of the aforementioned processes were executed on the University of Arkansas High Performance Computing Cluster.

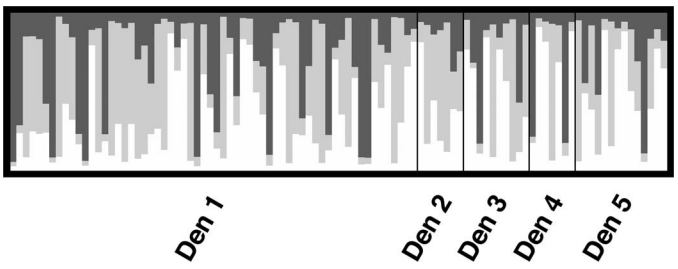

Fig. 2. Graphical depiction of three genetic clusters (K = 3; represented by varying shades of gray) in adult Copperheads (*Agkistrodon contortrix*; $n = 100$) from five dens in central Connecticut, as identified by Structure Harvester (with Evanno Method) after Bayesian clustering (Structure 2.3.4). Twelve runs of each value of K (=1–7) were tested, with 100,000 burn-in and 800,000 MCMC iterations, and without location priors. Each vertical bar represents a single individual and horizontal partitions represent the proportion of the individuals' genome that was assigned to each genetic cluster.

RESULTS

Population genetic diversity and structure.—One of the 23 loci was removed from analyses because it displayed departure from Hardy-Weinberg equilibrium and evidence of a null allele ($p = 0.12$). GenePop also detected LD between three pairs of loci, but these comparisons were less than expected by chance alone (Bonferroni-adjusted $\alpha = 0.0002$). There were no significant differences in H_e among the five dens (Friedman $\chi^2 = 4.22$, df = 4, $P = 0.38$; Table 1), nor among males and females (Friedman $\chi^2 = 1.64$, df = 1, $P = 0.20$; Table 1). A total of 144 alleles was recorded across 22 loci. Number of alleles per locus ranged from 2–11 and the average number per locus within each of the five dens (Table 1) ranged from 3.9 (±0.3) to 5.8 (±0.4). Three genetic clusters (Fig. 2) were identified by Structure Harvester ($\Delta K_{MAX} = 22.9$; Supplemental Appendix 1a), the same number identified when using the traditional method (Pritchard et al., 2000) in program Structure (mean Ln P(K) = –5576.6250). Clusters were distributed across all dens, with each individual in the study population displaying some proportion of each cluster (Fig. 2). Furthermore, the removal of full siblings from Structure analyses resulted in the same number of genetic clusters (Supplemental Appendix 1b).

Relatedness.—Mean relatedness (r) among adults was 0.039 (±0.005), with no significant difference among adult males or females (mean $r_{♂} = 0.042$ [±0.072]; mean $r_{♀} = 0.041$ [±0.080]; $r_{♂}$–$r_{♀} = 0.001$; 10,000 bootstrapped lower and upper 95% confidence intervals = –0.006–0.006). There were no significant differences in mean r among dens, nor among males (0.042±0.075) and females (0.042±0.085) in the largest den (Den 1; $r_{♀}$–$r_{♂}$ = –0.000026; 10,000 bootstrapped lower and upper 95% confidence intervals = –0.010–0.011).

Nine first-order relationships were detected by program Colony with a probability ≥0.984: five within Den 1 (one

male-male dyad; two male-female dyads; two female-female dyads), two between individuals from Dens 1 and 5 (male-female dyads), one between individuals from Dens 1 and 4 (female-female dyad), and one between individuals from Dens 2 and 5 (male-female dyad). Importantly, there were no first-order relationships identified that consisted of greater than two individuals. In contrast, 169 second-order relationships were detected with a probability ≥0.985. Of these, 70 second-order relationships were among individuals from Den 1 (the largest den), including 43 male-female dyads, 13 female-female dyads, and 14 male-male dyads. However, the maximum number of individuals in a second-order relationship family was six.

Effective population size and bottlenecks.—Effective population size (N_e) calculated with the sibship assignment method by program COLONY was equal to 93.0 (lower and upper 95% CI = 68 and 129, respectively), while that estimated with the LD method corrected for finite population size by program LDNe was 110.6 (jackknifed lower and upper 95% CI = 89.7 and 140.8, respectively). In program BOTTLENECK, we failed to reject the null hypothesis of no significant heterozygote excess under both SMM and TPM (P = 0.994 and 0.889, respectively). This result, in conjunction with a distinctly L-shaped allele frequency distribution derived from BOTTLENECK's mode-shift test, allowed us to reject the possibility of a recent bottleneck (i.e., occuring in the last few dozen generations).

However, an historical bottleneck was detected with the standardized differences test implemented in BOTTLENECK (P = 0.037) which identifies bottlenecks that occured within the past two to four N_e generations (Piry et al., 1999). When employing the more conservative estimate of N_e derived by the sibship assignment method in program COLONY (=93.0; 95% CI = 68–129) and multiplying by 2 and 4 (as above), we estimated that the bottleneck occurred 186 to 372 generations ago. When using the value of N_e estimated by the LD method corrected for finite sample size in program LDNe (=110.6; 95% CI = 89.7–140.8) and similarly multiplying by two and four, the bottleneck was estimated to occur from 221.2 to 442.4 generations ago. Assuming a three-year average generation interval for Copperhead (Fitch, 1960) and considering the lower and upper estimates derived from the two N_e estimators, the bottleneck would have occurred from approximately 558–1327 years before 2001 (i.e., 673–1443 A.D.).

DISCUSSION

Genetic variability in Copperhead and other North American pitvipers.—Genetic variability in our study population is similar to that reported for other North American pitvipers (e.g., Eastern Massasauga [Gibbs et al., 1997; Chiucchi and Gibbs, 2010], Desert Massasauga [Anderson et al., 2009], New Mexico Ridge-Nosed Rattlesnake [Holycross and Douglas, 2007], Midget Faded Rattlesnake [Oyler-McCance and Parker, 2010]; Appendix 1) including the threatened Timber Rattlesnake (Clark et al., 2008, 2010). Throughout their distribution, Copperhead and Timber Rattlesnake are largely sympatric, often syntopic, common to rocky woodlands (Trauth et al., 2004) and frequently share overwintering dens (Gloyd and Conant, 1990). Comparable habitat preferences and several life-history similarities between the two (Appendix 2) may yield similar selective agents and induce parallel levels of genetic diversity, as previously demonstrated for garter snakes (Manier and Arnold, 2005), salmonid fishes (Gomez-Uchida et al., 2009; Ackerman et al., 2013), amphibians (Phillipsen et al., 2011), and freshwater invertebrates (Marten et al., 2006). However, comparisons of microsatellite estimates of genetic variability across taxa should be considered with caution, as variation in microsatellite mutation rates among taxa (Ellegren, 2004) and disparity in sample sizes among studies may affect the reliability of comparisons.

Genetic structure in the study population.—No differences in genetic structure were found among Copperhead dens in this study, a pattern also reported for other temperate pitvipers (Timber Rattlesnake [Anderson, 2010; Bushar et al., 2014], Desert Massasauga [Anderson et al., 2009], Eastern Massasauga [Chiucchi and Gibbs, 2010]) and consistent with gene flow or dispersal among dens. The reservoir that bisects our study site seemingly has no effect on gene flow or dispersal, as inferred from the presence of each genetic cluster across all dens and from the indentification of first- and second-order relationships among individuals with den affiliations on opposite sides of the reservoir.

Interestingly, we detected the same number of genetic clusters (=3) in our study population as was recently reported for northern populations of Timber Rattlesnake, with each Timber Rattlesnake population similarly representing all genetic clusters but in different proportions (Bushar et al., 2014). The presence of genetic structure among northern populations of Timber Rattlesnake was attributed to a combination of geographic factors and genetic drift, as several distant populations were assayed (Bushar et al., 2014). Because only one population of Copperhead was evaluated in the present study, the extent to which geographic relationships to other populations may influence the genetic structure of our study population is unknown.

However, three lines of evidence from both the present study and previous work on the study population (Smith et al., 2009; Levine et al., 2015) lend support to the idea that the genetic structure of this population may be influenced by direct or indirect gene flow with other populations. First, previous work on the relationship between the study population with individuals collected from 3 km away indentified a single second-order relationship between individuals from the former and latter (Levine, 2013). Second, there were no first-order relationships identified in the study population that consisted of greater than two individuals and average population relatedness was only slightly greater than zero, yet previous work on the study population (Smith et al., 2009; Levine et al., 2015) found the average size of 11 single paternity litters to be equal to 6.82 (±2.89). The descrepancy between full-sibling family size (Levine et al., 2015) and the maximum first-order relationship size identified by the present study may be due to factors such as low juvenile survivorship and/or low capture probability, but may also be influenced by emigration. Third, although the study population has been extensively and repeatedly surveyed (Smith, 2007; Smith et al., 2009), a previous parentage analysis with juveniles and adults collected from the study population found that paternity of 71 of 137 (=51.8%) offspring was assigned to 15 unknown males that had never been captured (Levine et al., 2015). Athough this result could be due to factors such as long-term sperm storage by females (Schuett and Gillingham, 1986) or "sneaky" males that mate while eluding detection (Prosser et al., 2002), it is also possible that males from adjacent

populations mated with study population females. Such a scenario is plausible considering that males in the study population move extensive distances throughout the breeding season and that mating occurs in late summer away from winter hibernation sites (Smith et al., 2009). Future work will be required to determine the extent to which the aforementioned factors influence genetic structure in the study population.

Genetic similarity among Copperhead dens.—Co-habitation in winter dens may increase among related individuals, particularly when den availability is low or declining. Such resource sharing is seemingly commonplace among vertebrates (e.g., snakes [Bushar et al., 1998; Clark et al., 2008], birds [Dickinson and Hatchwell, 2004], mammals [Kitchen et al., 2005; Banks et al., 2011]). Suitable hibernation sites are scarce for reptile populations at northern extremes of their ranges, and resource sharing among kin is a persuasive argument for elevated within-den relatedness. For example, Timber Rattlesnake in a Pennsylvania population were more closely related to den members than to individuals from more distant dens (Bushar et al., 1998). However, Timber Rattlesnake hibernating together in a New York population were neither related (i.e., with *r* values not significantly different from zero) nor significantly different from the population average (Clark et al., 2012). In our study, genetic relatedness within dens did not differ from overall relatedness (as above), and two possible explanations are offered. First, dens in the study area are closer (<200 m; Smith, 2007) than those recorded by Bushar et al. (1998). Also, mean distances moved by males in our study were >2X that of females (3,994 vs. 1,714 m, respectively; Smith et al., 2009). Therefore, a lack of within-den relatedness may simply be due to easier movements among dens, particularly coupled with the potential for spatial and thermal similarities among dens. Second, a lack of genetic relatedness may simply reflect the capacity for panmixia. Individuals in the study population reproduce in late summer, a situation different from more southerly populations where breeding occurs both in spring and fall (Smith et al., 2009). Given that breeding in our study population does not occur immediately following hibernation, individuals from different dens have a greater opportunity to move about within the habitat prior to reproduction.

Effective population size and genetic bottlenecks.—Effective population size of the Copperhead population in this study falls within the range reported for Timber Rattlesnake and Eastern Massasauga (Clark et al., 2008; Chiucchi and Gibbs, 2010). An N_e of ~500–1000 is considered necessary to offset the effects of long-term environmental change (Franklin and Frankham, 1998) and is likely an underestimate given assumptions of an idealized population (e.g., equal sex ratios, all adults reproduce, panmixia). The N_e of our study population (93.0–110.6) is but 19–22% of the lower estimate provided by Franklin and Frankham (1998) and is likely influenced by the male-biased sex ratio and fecundity in the study population (Levine et al., 2015). This value of N_e suggests a potential vulnerability to stochastic environmental events, to include climate change (Gilpin and Soulé, 1986; Spielman et al., 2004; Palstra and Ruzzante, 2008). Despite the recommendation by Franklin and Frankam (1998), studies in other species of Viperidae (e.g., Eastern Massasauga [Chiucchi and Gibbs, 2010], Timber Rattlesnake [Clark et al., 2008], Adder [Madsen et al., 1996; Ursenbacher et al., 2009]) suggest that lower values of N_e may actually be commonplace. However, estimates of N_e in reptiles are relatively scarce (Lougheed et al., 1999) and largely restricted to species of conservation concern in Viperidae (i.e., Eastern Massasauga, Timber Rattlesnake, Adder), further underscoring the importance of evaluating population genetic parameters across genera to include those species of lesser concern. Additionally, estimates of N_e derived from single-sample estimators are sensitive to a variety of factors common to natural populations such as overlapping generations, incomplete sampling, and population sub-structure (Holleley et al., 2014; Waples et al., 2014). Therefore, until implications are better understood, N_e values should be used with caution to inform management decisions.

We detected an historical bottleneck for our study population within the span of 673 to 1443 A.D. Although we did not test for the direct cause of this pattern, one hypothesis is that the historical bottleneck that we detected is an artifact of the Medieval Climate Anomaly (MCA: 800–1300 A.D.). The MCA provoked temperature and precipitation changes on a global scale (Trouet et al., 2009), to include a persistent drought ('megadrought') in North America with impacts most prevalent in western and central regions of the continent (Oglesby et al., 2012). However, Laird et al. (2012) have documented the presence of the MCA megadrought in more northern and eastern regions of North America as well, a significant finding in that these regions have not historically experienced such impacts. These results juxtapose with earlier research (Hupy and Yansa, 2009) that demonstrated the sensitivity of forest communities in Michigan to late Holocene climate change, with species dominance changing in response to small variations in precipitation and temperature. Because the study population lies at the northeastern limit of the range of *A. contortrix* and may therefore be more vulnerable to stochastic environmental change than central forms (Eckert et al., 2008), it is possible that climate changes caused by the MCA contributed to the historical bottleneck that we detected, particularly considering the overlap in the timing of these two events. Future work will be necessary to evaluate the effect that the MCA and other factors may have had on genetic bottlenecks in temperate crotalines, as the MCA has relevance as a natural analog to current anthropogenic climate change. A final caveat is that our estimate of the timing of the historical bottleneck is derived from our two estimators of N_e, and therefore retains the sensitivity of N_e estimators to the previously mentioned factors which may affect its accuracy.

We unexpectedly failed to detect a bottleneck coincident with the construction of the Bradley Hubbard Reservoir (at the center of the study site), which occurred from the late 1800s to 1927 (www.ctparks.net/meriden/giuffrida/history). Additionally, development and expansion of the city of Meriden (ca. 1661 A.D.–present; www.cityofmeriden.org/About/History_of_Meriden/) did not elicit a decline in N_e. Both types of anthropogenic activities have had deleterious effects on other North American pitvipers, including Timber (Clark et al., 2011) and Midget Faded Rattlesnake (Oyler-McCance and Parker, 2010), and our inability to detect a similar effect in the study population is a further example of the variation in response to environmental change that may be experienced across a taxon. However, genetic bottlenecks may require a more extensive time span to be manifested, depending on factors such as the size of the population and the number of elapsed generations (Frankham et al., 2010). Additionally, microsatellite mutation rates may vary across

loci and species (Ellegren, 2004), which may also explain why a recent bottleneck in response to urbanization was not detected in the study population while similar processes have caused bottlenecks in other crotalines.

Conclusion.—Population genetic parameters often remain unstudied unless or until a species become the focus of conservation concern. This reality has an unfortunate aspect in that it provides scant baseline information regarding natural genetic variation in a variety of species (Storfer, 1996; Hendrickson et al., 2003), and in the case of North American pitvipers, biases population genetic research efforts against an entire genus. Although studies of non-threatened species lack conservation immediacy, they can provide valuable data regarding the taxon-wide impacts of environmental change, particularly when threatened and endangered species are restricted to specific genera.

Our study provides a valuable baseline for the Copperhead, a non-threatened North American pitviper in most areas of its extensive range, and is an important addition to our understanding of North American pitviper molecular ecology. Its genetic diversity and structure are comparable to recently fragmented (~100–200 years) Timber Rattlesnake and Massasauga populations. Such comparisons have the potential to inform conservation decisions regarding deleterious effects of genetic bottlenecks, as well as the importance of genetic structure and habitat connectivity in conservation management.

DATA ACCESSIBILITY

The supplementary appendices referenced in this manuscript are available from The Copperhead Institute (http://www.copperheadinstitute.org/#!publications/cee5).

Supplementary Appendix 1. STRUCTURE HARVESTER (Earl and vonHoldt, 2012) was used to generate delta K plots from STRUCTURE 2.3.4 (Pritchard et al., 2000; Falush et al., 2003, 2007; Hubisz et al., 2009) analyses of (a) 100 adult Copperheads from a Connecticut population and (b) the same population with individuals removed that were first-order relatives ($n = 9$). STRUCTURE 2.3.4 runs tested K value 1–7 with 100,000 burn-in and 800,000 MCMC iterations, with 12 runs per K. K = 3 was identified as the most likely number of genetic clusters in both analyses.

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Appendix 1. Estimates recovered from the literature for populations of North American pitvipers for expected heterozygosities (H_e) with standard error (SE) where available as derived from analysis of nuclear microsatellite loci. Ranges of H_e are presented when multiple populations were examined in a single study. Sample size (*n*) and number of loci employed differ across studies. Abbreviation: ON = Ontario, Canada.

Taxon	*n*	# of loci	$H_e \pm$SE	State/Province	Study
Agkistrodon contortrix	100	22	0.62±0.04	CT	This study
Crotalus horridus	58	8	0.51±0.03	MO	Anderson, 2010
Crotalus horridus	131	9	0.50–0.66±NA	NY	Clark et al., 2010
Crotalus horridus	373	9	0.55–0.66±NA	NY	Clark et al., 2008
Sistrurus catenatus	388	19	0.49–0.77±NA	IL, NY, OH, PA, ON	Chiucchi and Gibbs, 2010
Sistrurus catenatus	78	6	0.695–0.764±NA	AZ, NM	Anderson et al., 2009

Appendix 2. Comparison of natural- and life-history attributes of adult *Agkistrodon contortrix* and *Crotalus horridus* from the northeastern extreme of their ranges. Both taxa occasionally occur in syntopy. Six New England states considered in our table are Connecticut, Maine, Massachusetts, New Hampshire, Rhode Island, and Vermont. The mid-Atlantic state of New York is also included. SVL = snout–vent length. MCP = minimum convex polygon.

Attribute	*A. contortrix*	*C. horridus*	Authority
Range female length (SVL cm)	medium (700–100+)	large (100–120+)	1–5
Range female mass (g)	medium (150–350+)	large (900–1500+)	1–5
Conspicuousness	inconspicuous	conspicuous (rattle)	1–3
Venomous bite	fatalities rare	fatalities not rare	1–3
Toxicity and yield	low to moderate	moderate to high	1, 3
Diet			
Breadth	large	limited	1–3, 6, 8
Invertebrates	yes	no	1–3
Vertebrates	yes	yes	
Ectotherms	yes	no	1–4
Spatial			
Home range (MCP)	moderate to large	moderate to large	1–2
Habitat	forests, variable	forests, variable	1
Winter aggregation (dens)	yes	yes	
Longevity	20 years or greater	20 years or greater	1–2
Female reproduction			
Age at maturity	3–4 years	7–11 years	1–3
Frequency	biennial (annual)	triennial (or greater)	1, 4
Litter size (average range)	3–8	4–14	1–4
Human persecution	low to moderate	high	1–3
State bounties	none	present (formerly)	1–3
Commercial value	low	high (skins, rattles)	1–2
Habitat destruction/loss	moderate to high	moderate to high	1–3
Population status			
Adults in population	low to high	low (extirpated MA)	1–3
Threatened or endangered	Massachusetts	all states	2, 9
Protected	Massachusetts	all states	2, 9

1 = Gloyd and Conant, 1990; 2 = Brown, 1993; 3 = Campbell and Lamar, 2004; 4 = Smith, 2007; 5 = Smith et al., 2009; 6 = Reinert and Zappalorti, 1988; 7 = Martin, 2002; 8 = Clark et al., 2010, 2011; 9 = Internet sites*

* Internet sites referenced:

1) http://www.ct.gov/deep/cwp/view.asp?a=2702&q=323462
2) http://www.maine.gov/ifw/wildlife/species/endangered_species/state_list.htm
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