Contributed Paper

Roads, Interrupted Dispersal, and Genetic Diversity in Timber Rattlesnakes

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Abstract: *Anthropogenic habitat modification often creates barriers to animal movement, transforming formerly contiguous habitat into a patchwork of habitat islands with low connectivity. Roadways are a feature of most landscapes that can act as barriers or filters to migration among local populations. Even small and recently constructed roads can have a significant impact on population genetic structure of some species, but not others. We developed a research approach that combines fine-scale molecular genetics with behavioral and ecological data to understand the impacts of roads on population structure and connectivity. We used microsatellite markers to characterize genetic variation within and among populations of timber rattlesnakes (*Crotalus horridus*) occupying communal hibernacula (dens) in regions bisected by roadways. We examined the impact of roads on seasonal migration, genetic diversity, and gene flow among populations. Snakes in hibernacula isolated by roads had significantly lower genetic diversity and higher genetic differentiation than snakes in hibernacula in contiguous habitat. Genetic-assignment analyses revealed that interruption to seasonal migration was the mechanism underlying these patterns. Our results underscore the sizeable impact of roads on this species, despite their relatively recent construction at our study sites (7 to 10 generations of rattlesnakes), the utility of population genetics for studies of road ecology, and the need for mitigating effects of roads.*

Keywords: conservation genetics, *Crotalus horridus*, genetic connectivity, habitat fragmentation, population structure, roads, snakes, timber rattlesnake

Carreteras, Dispersión Interrumpida, y Diversidad Genética en Serpientes de Cascabel

Resumen: *La modificación antropogénica del hábitat a menudo crea barreras al movimiento de animales, transformando hábitat previamente continuo en un conjunto de islas de hábitat con baja conectividad. En la mayoría de los paisajes las carreteras son un elemento que puede funcionar como barrera o filtro para la migración entre poblaciones locales. Aun las carreteras pequeñas y recién construidas pueden tener un impacto significativo sobre la estructura genética de las poblaciones de algunas especies, pero no de otras. Desarrollamos un método que combina la genética molecular de escala fina con datos conductuales y ecológicos para entender los impactos de las carreteras sobre la estructura y conectividad de la población. Utilizamos marcadores de microsatélite para caracterizar la variación genética dentro y entre las poblaciones de serpientes de cascabel (*Crotalus horridus*) que ocupan hibernáculos (madrigueras) comunales en regiones divididas por carreteras. Examinamos el impacto de las carreteras sobre la migración estacional, la diversidad genética y el flujo entre poblaciones. Las serpientes en hibernáculos aislados por carreteras tuvieron una diversidad genética significativamente menor y una mayor diferenciación genética que las serpientes en hibernáculos en hábitat continuo. Los análisis de asignación genética revelaron que la interrupción de la migración estacional era el mecanismo subyacente en estos patrones. Nuestros resultados enfatizan el impacto de las carreteras sobre esta especie, construcción en nuestros sitios de estudio (siete a 10 generaciones*

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de serpientes de cascabel), la utilidad de la genética de poblaciones para estudios de ecología de carreteras y la necesidad de mitigar los efectos de las carreteras.

Palabras Clave: carreteras, conectividad genética, *Crotalus horridus*, estructura de la población, fragmentación de hábitat, genética de la conservación, serpientes, serpiente de cascabel

Introduction

Fragmentation of natural habitats is an important cause of biodiversity loss (Meffe & Carroll 1997). As remaining habitat patches become isolated by the inhospitable matrix of anthropogenic development, fragmented populations become susceptible to inbreeding and possible extinction due to genetic erosion and stochastic demographic processes (Frankham et al. 2002). Roadways are one of the most ubiquitous forms of habitat fragmentation in terrestrial environments (Forman & Alexander 1998). A network of roads now carves through much of the global terrestrial habitat and has fragmented the landscape, leading to lowered population connectivity. Many conservation biologists argue that the effects of roads on natural populations are one of the most pressing conservation issues (reviewed by Forman & Alexander 1998), and recent studies continue to document the deleterious effects of roads on individuals and populations in a wide variety of taxa (Gibbs & Shriver 2002).

The most immediate and direct effect of roads is mortality associated with vehicular collisions (Mazerolle 2004). In addition, because the vulnerability of individuals to roads can depend on both age and sex, road mortality can also result in demographic and structural changes to populations because it alters individual survival and dispersal (Row et al. 2007). Behavioral studies show that many taxa exhibit some degree of road avoidance (Shepard et al. 2008). Combined, direct mortality and road avoidance can create barriers to gene flow and thus cause populations to become genetically isolated. Isolated populations exhibit increased genetic drift and inbreeding, which can lead to losses of rare alleles, diminished heterozygosity, and, ultimately, decreased population fitness due to erosion of genetic variation required for the evolutionary response to environmental change (Frankham et al. 2002).

Recently, high-resolution molecular markers have advanced studies of population structure, dispersal, and conservation (Selkoe & Toonen 2006) in the field of landscape genetics (Manel et al. 2003). This discipline quantifies the effects of natural and anthropogenic landscape features on population genetic structure. In the context of conservation biology, landscape genetics can provide tools to mitigate deleterious effects of anthropogenic habitat modification (Manel et al. 2003). This approach is particularly powerful in quantifying the effects of roads on population genetic structure of taxa for which direct measures of dispersal are otherwise difficult (Epps et al. 2005; Riley et al. 2006).

The degree to which roads act as barriers depends on characteristics of the road (Clevenger et al. 2003) and life-history attributes of the focal species (Wiens 1997). Several researchers have found that the effects of roads on population genetics depend on road size and traffic volume (Keller & Largiader 2003; Riley et al. 2006; Marsh et al. 2008). Yet, most roads are relatively recent barriers and thus may not have been in place long enough to affect connectivity in some long-lived species (Edwards et al. 2004). Nonetheless, in some taxa, the genetic effects of anthropogenic fragmentation have been measured after only a few generations (Moore et al. 2008). This high variance underscores the importance of understanding what characters mediate species' responses to different types of habitat fragmentation. For example, many philopatric species exhibit genetic structure at a very fine spatial scale because of the complex ecological and social constraints leading to natal philopatry and delayed dispersal (McDonald et al. 1999). By combining fine-scale genetic analyses with studies of social organization, mating systems, and life-history characteristics, we can develop research approaches that will allow for more effective management of populations that persist in fragmented habitats.

We used microsatellite markers to examine the effects of roads on genetic diversity and connectivity among populations of timber rattlesnakes (*Crotalus horridus*). We had detailed information about timber rattlesnake population genetic structure, habitat use, dispersal movements, mating system, and foraging ecology (reviewed in Brown 1993; Brown 2008). Thus, we used this species as a model to investigate general processes underlying population-level responses to habitat fragmentation in philopatric, terrestrial vertebrates. We sampled timber rattlesnake populations in contiguous and road-fragmented habitats to test the following predictions: populations in areas with more roads have reduced genetic diversity; separation of hibernacula by roads increases genetic differentiation; and these two patterns result specifically from fewer matings between individuals from hibernacula separated by roads. In general, we sought to reveal the degree to which roads may affect the population genetic structure of philopatric species with limited direct dispersal, and the mechanisms by which genetic connectivity in such systems can be interrupted.

Methods

Study Species

Timber rattlesnakes are cryptic, long-lived vertebrate predators at or near the top of the food chain in temperate deciduous forests. The distributional range of timber rattlesnakes has contracted significantly over the last century, and remaining populations are often small and isolated (Brown 1993). Although this species has suffered historically from direct persecution by humans, habitat loss and fragmentation are the main causes of population declines (Martin et al. 2008). In the northernmost parts of its range, *C. horridus* populations are centered around communal wintering dens (hibernacula) and both sexes exhibit high levels of philopatry to their natal hibernaculum (Clark et al. 2008). During the spring, individuals migrate to surrounding forested habitats to forage and mate. Both adult females and males show this seasonal dispersal, but adult males typically move farther from the hibernacula (3–7 km, compared with 1–3 km in females [reviewed in Brown 1993]). The fine-scale genetic structure of timber rattlesnake populations shows that snakes with hibernacula in contiguous forests, within 3–4 km of each other, exhibit high levels of genetic connectivity (Bushar et al. 1998; Clark et al. 2008).

Population Sampling and Genotyping

We collected tissue samples, including some shed skins, from 131 individuals from five hibernacula and used the 373 samples from the 14 hibernacula included in Clark et al. (2008). The five new sites were within 3 km of the original hibernacula, but in each case they were isolated from other sites by a roadway (Fig. 1). We genotyped all samples at nine microsatellite loci (Villarreal et al. 1996; Holycross et al. 2002). Sample sizes from our five new sites were comparable to those sampled previously (Table 1). Procedures for sample collection, DNA extraction, polymerase chain reactions, and genotyping are described in Clark et al. (2008). The 19 hibernacula included in this study were in four different regions of New York (U.S.A.): six in the Adirondack region (AD), five in Sterling Forest (SF), six in Bear Mountain (BM), and two in Chemung County (CH) (Fig. 1).

We identified the average annual daily traffic (AADT) volume of all roads in our study areas with data provided by the New York State Department of Transportation.
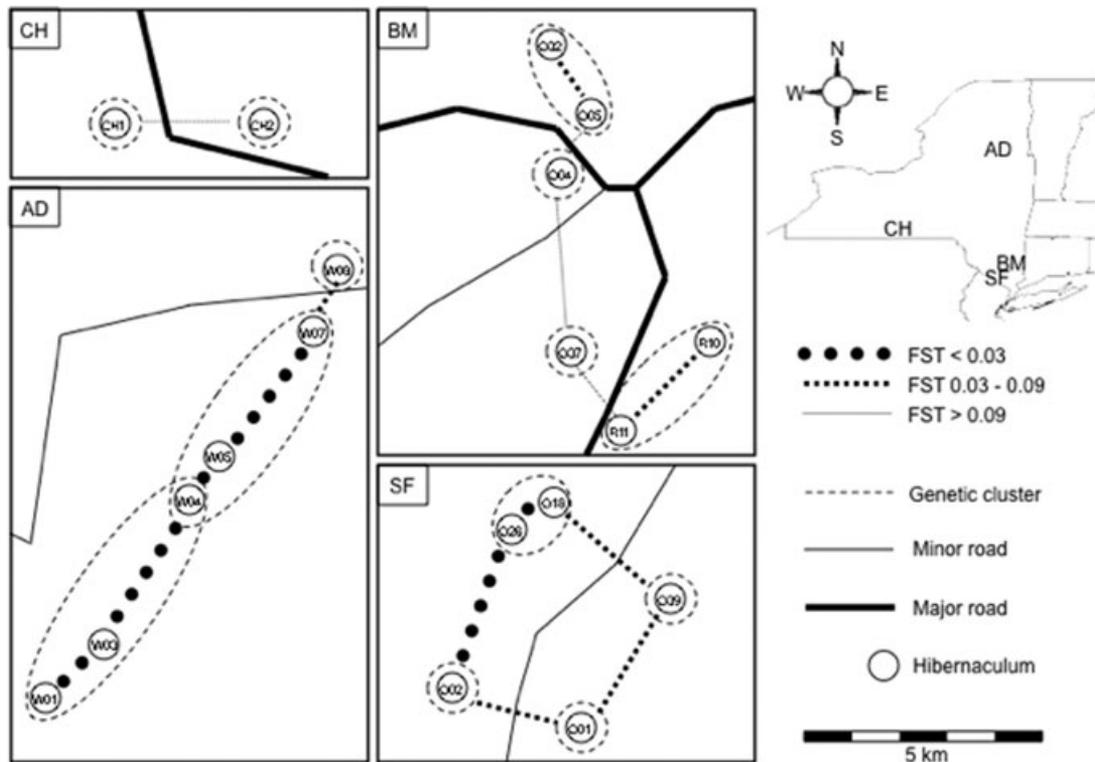

Figure 1. Location of Crotalus horridus *hibernacula and roads in four regions of New York State (CH, Chemung County; BM, Bear Mountain; AD, Adirondacks; SF, Sterling Forest). Hibernacula assigned to the same genetic deme in our Bayesian assignment analyses are encircled by dashed lines. The thickness of dotted lines corresponds to higher levels of population connectivity as measured by pairwise* F_{ST}. *Thus, line thickness is inverse to* F_{ST} *value: populations with higher* F_{ST} *values are connected by thinner lines.*

Table 1. Sample sizes, levels of heterozygosity, allelic richness, and genetic diversity of all 19 timber rattlesnake hibernacula sampled in four regions of New York State.*

Region	Hib	n	H_e	H_o	A	HW
Adirondack	W-01	57	0.55	0.50	4.83	*Cw*C24f, 7–144
	W-03	18	0.57	0.58	5.07	
	W-04	15	0.60	0.59	5.55	
	W-05	32	0.56	0.53	5.06	*Cw*A29f
	W-07	18	0.59	0.51	5.12	
	W-08	39	0.55	0.49	4.63	
Sterling Forest	O-18	22	0.63	0.59	5.98	*Cw*A29f
	O-26	27	0.66	0.63	6.21	
	O-02	54	0.61	0.57	5.87	*Cw*A29f
	O-01	31	0.63	0.60	5.58	
	O-39	18	0.55	0.49	4.60	
Bear Mountain	O-32	17	0.57	0.57	4.17	
	O-05	24	0.59	0.61	3.82	
	O-04	31	0.59	0.62	4.67	
	O-37	23	0.58	0.57	4.12	
	R-10	19	0.61	0.60	5.08	
	R-11	21	0.60	0.59	4.44	*Cw*A29f
Chemung County	C-01	20	0.62	0.55	4.62	
	C-02	18	0.50	0.48	4.43	

*Key: Hib, hibernaculum; n, number of individuals genotyped; H_e, expected heterozygosity; H_o, observed heterozygosity; A, allelic richness; HW, loci with significant Hardy–Weinberg disequilibrium after Bonferroni correction.

We classified roads as either minor (AADT < 3,000) or major (AADT > 10,000) barriers (Fig. 1). All focal roads were paved for motorized traffic in the late 1920s or early 1930s; thus, they may have been barriers to rattlesnake populations for approximately 80–90 years. Roads were not associated with any major natural barriers (e.g., mountains, rivers, ridges) that could act as barriers to snake movement (Clark et al. 2008). A single exception is the road in CH that parallels a river (approximately 10 m wide) and a 200-m-wide strip of farmland.

Genetic Diversity and Differentiation

We used GIMLET (Valiere 2002) to identify shed skins that were duplicate genotypes and removed them from the analysis. We analyzed genotypes at all nine loci and 19 populations in MICROCHECKER 2.0 (Van Oosterhout et al. 2004) to estimate null allele frequency and identify other genotyping problems. We used GENEPOP 3.1 (Raymond & Rousset 1995) to calculate observed and expected heterozygosities. To test for deviations from Hardy–Weinberg (HW) expectations at each locus and hibernaculum, we used a Monte Carlo chain method (1000 dememorizations, 100 batches, 1000 iterations), following the algorithm of Guo and Thompson (1992). We applied a Bonferroni correction to correct for multiple tests, which resulted in a table-wide significance level of $\alpha = 0.05$ (adjusted *p* value = 0.001). We used FSTAT

2.1 (Goudet 1995) to test for linkage-disequilibrium at each locus over all populations (adjusted $p = 0.00139$), to estimate allelic richness of individuals at each hibernaculum and to compute pairwise F_{ST} values and their significance among sites.

Landscape Genetic Analysis

We previously showed that a habitat-path distance that takes into account availability of habitat suitable for thermoregulation and gestation explains more variation in genetic distance among populations than does Euclidian distance (Clark et al. 2008). Our goal here was to infer the distribution of genetic variation across natural landscapes; therefore, we included only populations in areas not bisected by roads or other anthropogenic disturbances. We used the same habitat-path distance developed previously to generate expected values for genetic distances between pairs of hibernacula separated by roads. We define the expected habitat-path distance as that predicted by the slope of a regression between habitat-path distance and genetic distance for all pairs of hibernacula not separated by roads. The observed values of divergence were then compared with the values predicted in the absence of roads to determine the additional genetic cost of major and minor roads between pairs of populations. After confirming normality and equal variance in the data, we compared expected values to observed values with a paired *t* test. We used an unpaired

t test to compare the observed genetic distance for hibernacula separated by minor roads and major roads.

Genetic Demes

To examine the impact of roads on genetic clustering, we used STRUCTURE 2.1 (Pritchard et al. 2000) to identify the most likely number of genetic clusters (*K*) present and the proportion of membership in those clusters for each hibernaculum. STRUCTURE uses a Bayesian algorithm to cluster genotypes in the absence of any geographic information. We used a model allowing admixture and correlated gene frequencies and ran 500,000 steps after a burn-in of 100,000. We determined these values were sufficient for accurate estimation of *K* by convergence of key summary statistics (Pritchard et al. 2000). We conducted 10 independent runs for each value of *K* and retained the simulation with the highest likelihood. Hibernacula were assigned to the cluster with the highest coefficient of membership (*Q*) for all individuals combined.

We first ran STRUCTURE with hibernacula pooled from all four regions to determine whether all our regional samples were in fact genetically independent. Upon confirming in this overall analysis that our four sample regions were independent genetic demes, we ran STRUCTURE separately for each region to identify the impact of roads on these specific groups of populations. We used average *Q* values summed across all individuals to represent the geographic distribution of genetic variation across hibernacula in each region. To determine the effect of roads on admixture, we computed the average *Q* each individual showed to the next nearest genetic cluster (as opposed to the cluster to which it was assigned). We then used a one-way analysis of variance (ANOVA) with post hoc comparisons that assumed unequal variances to compare clusters separated by roads with those not separated by roads.

Gene Flow across Roads

In a previous study we used parentage analysis to identify parent–offspring pairs within and among hibernacula (Clark et al. 2008). Here, we used these same methods to identify parent–offspring pairs and quantify the frequency of interhibernaculum pairings that occurred across roads. We used NEWPAT (Wilmer et al. 1999) to identify parentage in our sample and to estimate the significance of parent–offspring pairs. We estimated the age of presumptive parents from a combination of rattle characteristics (width of basal segment relative to terminal segment of the rattle) and body size (total length and weight) (Brown 1991). Because demographic studies show that minimum age at maturity for timber rattlesnakes is approximately 5 years (Brown 1991; Martin et al. 2008), parent-offspring pairs were only considered

valid if they were significant at the $\alpha = 0.05$ level and if the estimated ages of the pair were at least 5 years apart.

In our previous study of hibernacula in contiguous habitat, we found 18 parentage assignments that occurred between hibernacula separated by habitat-path distances from 0.1 to 3.7 km (Clark et al. 2008). We used the data from Clark et al. (2008) to generate expected numbers of interhibernaculum parent–offspring pairs under a null assumption of no effect due to roads. To generate these expected values, we conducted a nonparametric test on these data to determine whether there was any correlation between the habitat-path distance separating the hibernacula and the number of parent–offspring pairs found between them. Upon confirming a lack of such a correlation, we assumed all hibernaculum pairs occurring within a habitat-path distance of at least 3.7 km had an equal probability of containing a parent–offspring pair. We then generated expected values under the assumption that roads would have no effect on the distribution of parent–offspring pairs occurring between hibernacula within a 3.7-km habitat-path distance. We compared expected values with our observed values with a chi-square test.

Results

Genetic Diversity and Differentiation

For all 19 hibernacula combined, we observed significant departures from HW equilibrium for *Cw*a29f at 4 of the 19 populations and for *Cw*C24f and 7–144 at one population each (Table 1). MICROCHECKER showed that locus *Cw*a29f had a significant probability of null alleles at four populations, and loci 5a, 7–144, and 7–87 each showed a significant probability of null alleles at one population. FSTAT indicated no linkage disequilibrium between any of the loci over all populations. Because we found no consistent patterns of HW disequilibrium or null alleles across all populations, all loci were retained for analysis. The average allelic richness for the five road-isolated hibernacula was 4.5 (SD 0.2) (Table 1), which is significantly lower than the average allelic richness of 5.1 (0.7) for hibernacula not isolated by roads ($t = 2.1$, df = 17, $p = 0.01$). Population divergences as measured by F_{ST} revealed significant levels of differentiation among all five road-isolated hibernacula (Table 2).

Landscape Genetics

The landscape-genetics model developed from populations in continuous habitat predicted an average F_{ST} value of 0.03 (SD 0.01) for our hibernacula separated by minor roads and 0.05 (0.02) for hibernacula separated by major roads. The actual observed values were 0.06 (0.03) (minor roads) and 0.12 (0.04) (major roads). For hibernacula separated by both road types, the observed values

Table 2. Pairwise population F_{ST} for sampled timber rattlesnake hibernacula in four study regions in New York State indicating which hibernaculum pairs are separated by roads and which are not.

Region and hibernaculum	W-01	W-03	W-04	W-05	W-07
Adirondacks					
W-03	0.01				
W-04	0.00	0.00			
W-05	0.01	0.01	0.00		
W-07	0.03[a]	0.03[a]	0.02	0.03	
W-08	0.05[a,b]	0.05[a,b]	0.04[a,b]	0.04[a,b]	0.07[a,b]
Bear Mountain	O-05	O-32	O-04	O-37	R-10
O-32	0.05[a]				
O-04	0.11[a,c]	0.14[a,c]			
O-37	0.0[a,c]	0.08[a,c]	0.13[a,c]		
R-10	0.08[a,c]	0.13[a,c]	0.12[a,c]	0.08[a,c]	
R-11	0.14[a,c]	0.19[a,c]	0.20[a,c]	0.13[a,c]	0.05[a]
Sterling Forest	O-01	O-39	O-18	O-26	
O-39	0.05[a]				
O-18	0.02[a,b]	0.04[a,b]			
O-26	0.04[a,b]	0.06[a,b]	0.00		
O-02	0.06[a,b]	0.08[b]	0.04	0.02	
Chemung County	C-01				
C-02	0.13[a,c]				

[a] *Significant* F_{ST} *values.*
[b] *Pairs of dens separated by minor roads.*
[c] *Pairs of dens separated by major roads.*

were significantly higher than the predicted values (minor roads: $t = 2.63$, $p < 0.03$; major roads: $t = 6.94$, $p < 0.001$). Additionally, the average F_{ST} value for hibernacula separated by major roads was significantly greater than for minor roads ($t = 4.80$, $p < 0.01$)

Genetic Demes

We first performed STRUCTURE analyses including all hibernacula in all four sampled regions. This analysis revealed that 16 genetic demes were present among our 19 sampled hibernacula ($K = 16$; posterior probability $\sim$ 1.0). None of these genetic clusters included hibernacula from different regions (Fig. 2). These results indicated that despite the geographic proximity of the SF and BM samples, hibernacula in different regions belonged to different genetic demes and that genetic structuring also occurred among hibernacula within each region.

Given these results, we performed STRUCTURE analyses for each geographic region independently to determine the most likely number of independent genetic demes (K) within each region. For region AD, $K = 3$ (posterior probability $\sim$ 1.0). Individuals from hibernacula south of the road showed high levels of admixture between clusters 1 and 2 (Table 3, mean percent membership Q values), whereas the hibernaculum to the north of the road assigned to an independent genetic cluster (Fig. 3). The hibernaculum north of the road showed the highest proportion of cluster membership within AD, despite its close geographic proximity to W-07 south of the
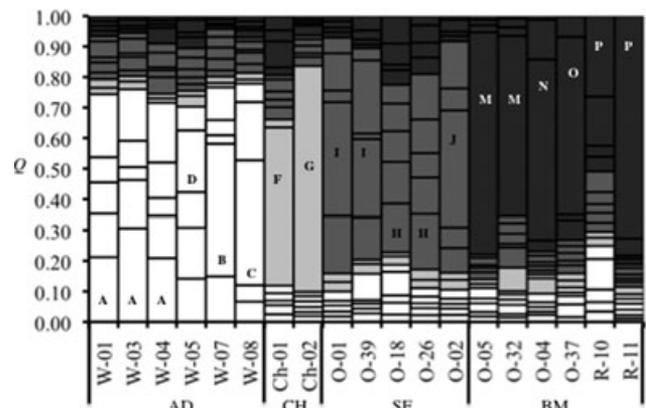

Figure 2. Average coefficient of membership, Q, for individuals sampled at each hibernaculum in each of 16 demes defined by STRUCTURE when all regions (CH, Chemung County; BM, Bear Mountain; AD, Adirondacks; SF, Sterling Forest) are pooled. Each hibernaculum is divided into 16 sections representing average Q values of that population for each of the 16 demes (demes A–P). Demes are shaded according to the geographic region from which the major proportion of their membership derives. Adirondack (AD) individuals belong primarily to genetic demes A–E, Chemung County (CH) individuals to demes F–G, Sterling Forest (SF) individuals to demes H–L, and Bear Mountain (BM) individuals to demes M–P. Each hibernaculum is labeled with the letter of the deme to which it shows the highest Q value.

Table 3. Proportion of genetic cluster or deme membership, *Q*, of individuals from each hibernaculum (Hib) in each cluster (1–4) defined by STRUCTURE with all regions analyzed separately.

	Adirondacks				*Bear Mountain*			
Hib	*1*	*2*	*3*	*Hib*	*1*	*2*	*3*	*4*
W-01	0.54*	0.27	0.19	O-05	0.02	0.88*	0.04	0.05
W-03	0.51*	0.32	0.17	O-32	0.02	0.90*	0.06	0.02
W-04	0.40*	0.40*	0.20	O-04	0.02	0.06	0.17	0.76*
W-05	0.3	0.45*	0.25	O-37	0.07	0.09	0.82*	0.02
W-07	0.27	0.60*	0.13	R-10	0.58*	0.07	0.23	0.12
W-08	0.12	0.20	0.68*	R-11	0.92*	0.02	0.05	0.01

	Sterling Forest				*Chemung County*		
Hib	*1*	*2*	*3*	*4*	*Hib*	*1*	*2*
O-01	0.29	0.16	0.06	0.49*	Ch-01	0.95*	0.05
O-39	0.57*	0.07	0.04	0.32	Ch-02	0.07	0.93*
O-18	0.24	0.36*	0.15	0.25			
O-26	0.27	0.38*	0.13	0.23			
O-02	0.14	0.23	0.51*	0.13			

*The highest average membership for each hibernaculum.

road (Fig. 3). In region SF, STRUCTURE identified four genetic demes (posterior probability ∼ 1.0). As in AD, hibernacula on the same side of a minor road showed more admixture with each other (Table 3; Fig. 3). In region BM, STRUCTURE identified four genetic demes (posterior probability ∼ 1.0). Hibernacula isolated by roads in this region both formed separate genetic demes, whereas hibernacula not separated by roads occurred in the same genetic deme (Table 3; Fig. 3). In region CH, STRUCTURE identified two genetic demes (posterior
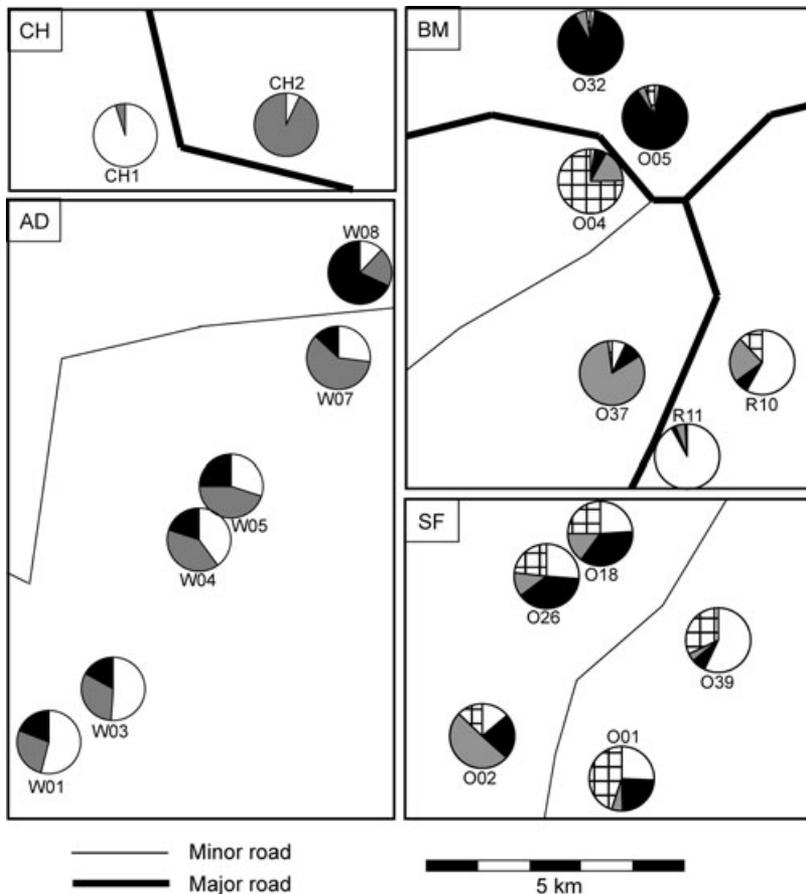

Figure 3. Genetic assignment results from the program STRUCTURE for timber rattlesnakes from four different regions of New York State with each region analyzed independently. The position of pie charts corresponds to the relative geographic location of hibernacula within each region. Pie chart sections represent the average proportion of membership (Q) individuals exhibited to each genetic deme identified by STRUCTURE. Three genetic demes were identified in the Adirondacks (AD) (black, gray, and white), two in Chemung County (CH) (gray and white), four in Bear Mountain (BM) (black, gray, white, and hatched), and four in Sterling Forest (SF) (black, gray, white, and hatched). Solid lines indicate the presence of either a minor road (thin lines) or major road (thick lines).

probability $\sim$ 1.0). The two hibernacula in this region, which are separated by a major road, formed separate demes with very low levels of admixture (Table 3; Fig. 3).

Over all four regions and 19 hibernacula, none of the genetic clusters identified by Bayesian assignment tests spanned either major or minor roads; hibernacula belonging to the same genetic deme were always on the same side of a road (Fig. 1). Genetic clusters not separated by roads showed more admixture than those separated by minor roads, which in turn showed more admixture than those separated by major roads (Fig. 3). Individuals from genetic clusters not separated by roads showed an average membership with neighboring clusters of $Q = 0.25$ (SD 0.25), whereas this shared membership was $Q = 0.17$ (0.19) for demes separated by minor roads, and $Q = 0.07$ (0.16) for demes separated by major roads ($F = 35.3$, $p < 0.01$ for overall significance and for all post hoc comparisons between groups).

Gene Flow across Roads

Parentage analyses identified 20 parent–offspring pairs that occurred between hibernacula separated by a habitat-path distance of 3.7 km or less. Across all four regions in our study, 52% of hibernaculum pairs within this distance were not separated by roads, 32% were separated by minor roads, and 16% were separated by major roads. Under the null assumption that roads have no effect on the probability of parent–offspring pairings, we would expect approximately 6 of 20 pairings to span minor roads and 3 of 20 to span major roads. We observed only two pairings that spanned minor roads (one between W-05 and W-07, one between O-01 and O-26) and none that spanned major roads ($\chi^2 = 12.1$, df = 2, $p = 0.002$). These data indicate that matings occurred much more frequently between hibernacula when there was no road separating them.

Discussion

Our results demonstrate a significant effect of roads on genetic structure, connectivity, and gene flow among populations of *C. horridus*. Isolation by roads was associated with lower genetic diversity of populations and significant increases in genetic differentiation, as measured by allelic richness, F_{ST} values, and Bayesian assignment to genetic demes. Furthermore, our parentage analysis showed that the probable mechanism for reduced connectivity is fewer matings between individuals from hibernacula separated by roads. In each case we tested our empirical observations against a null hypothesis of expected values derived from continuous populations in the same area; therefore, our data allow us to link patterns of population genetics with specific mechanisms inferred from the paternity analyses. This fine-scaled analysis, repeated over four geographic regions, underscores the significance of roads as barriers to dispersal and natural population processes for timber rattlesnakes and perhaps for other species with similar ecological traits.

Our results indicate roads are extremely effective barriers to gene flow, given how recently they were constructed and the long generation time of northern populations of timber rattlesnakes. In the northeastern United States, the generation time for timber rattlesnakes is approximately 10 years (Brown 1991); therefore, paved roads in these regions have been a barrier for only seven to eight rattlesnake generations. We infer that the barrier effect of roads must be strong, given that genetic structure measured by most molecular markers carries the signature of historical connectivity, which can mask the impact of more recent anthropogenic barriers (Richmond et al. 2009). For example, populations of desert tortoises bisected by roads are not yet genetically differentiated (Edwards et al. 2004). In contrast, paved roads affect the genetic structure of several other terrestrial animals (Kuehn et al. 2007; Marsh et al. 2008). Studies of grizzly bears (*Ursus arctos*), bighorn sheep (*Ovis canadensis*), and agile frogs (*Rana dalmatina*) corroborate our finding that roads cause significant levels of population differentiation in very few generations (Epps et al. 2005; Proctor et al. 2005; Lesbarreres et al. 2006).

Interpreting the effects of roads on population genetics requires consideration of the history of human transportation corridors used before road construction. Although the paved roads in our study areas have been used by motor vehicles for about seven generations of timber rattlesnakes, these areas contain corridors that likely have been used by humans for much longer (Burr 1829). Additionally, from the late nineteenth century to the late twentieth century, the percentage of forested land in New York increased from approximately 25% to 61% (Considine 1984). Thus, given their habitat requirements (Reinert 1984), rattlesnake populations likely experienced a period of significant habitat fragmentation when forested habitat was at its lowest (Stechert 1982). These various impacts have probably resulted in a complex demographic history of bottlenecks and expansions that may have contributed to the rapid differentiation of isolated populations.

Our genetic results complement behavioral studies that show significant impacts of paved roadways on snake movement and habitat use. Snakes appear to avoid both paved and unpaved roads (Shine et al. 2004; Andrews & Gibbons 2005) and to suffer high mortality when they do attempt to cross roads (Rosen & Lowe 1994). In an experimental study, Andrews and Gibbons (2005) demonstrated that timber rattlesnakes cross roads slowly (approximately 10 cm/s) and individuals stop moving and remain immobile for up to a minute or more in response to any traffic noise. From their behavioral data, they estimated that timber rattlesnakes crossing roads would suffer an 80% mortality rate on roads with a traffic

volume of 3,000 cars/day, a traffic level corresponding to the minor roads in our study and a mortality rate close to 100% on major roads with traffic volumes of more than 9,000 cars/day (Andrews & Gibbons 2005). Our finding that even minor roads significantly decrease genetic connectivity corroborates the behavioral observations of Andrews and Gibbons (2005) and represents the first estimate of the genetic consequences of this limitation in gene flow for viperid snakes.

Because northeastern populations of timber rattlesnakes are restricted to mountainous terrain, it could be argued that roads traversing these areas are preferentially built along lowland passes or parallel to ridges, geographic features that themselves could be barriers to gene flow. Nevertheless, our habitat-path distance measure accounts for the influence of topographic features separating hibernacula; therefore, we do not believe the patterns we observed are due only to the nonrandom placement of roads. A singular feature among our four regions that may represent a natural barrier to movement is a river that runs parallel to a major road in region CH. This river, however, is slow moving and is not likely to deter seasonally migrating timber rattlesnakes, which swim readily across rivers and lakes much greater than 10 m wide (W.S.B. & R.S., unpublished data). In addition, the degree of differentiation shown by the two populations in CH was similar to that shown between populations separated only by roads.

Our sampling design allowed us to infer a possible mechanism responsible for the severing of genetic connectivity. In timber rattlesnakes, summer mating movements, primarily by males, are responsible for gene flow among neighboring hibernacula (Clark et al. 2008). Our data indicate that intervening roads strongly interrupt breeding-season dispersal. Parentage analysis provided a "snapshot" of recent gene flow between hibernacula and showed that matings occurred mostly between hibernacula not separated by roads. This result is most likely due to the combined effects of road avoidance and road mortality; a disproportionate number of *C. horridus* found dead on roads are males killed during the mating season (Aldridge & Brown 1995).

Our results, combined with previous behavioral and ecological studies, indicate that management and conservation plans for snakes need to account for adverse effects of roads. One form of mitigation could be installation of underpasses or culverts placed strategically at migration concentration points. Several studies indicated that such mitigation efforts could be effective in reducing road mortality for amphibians and reptiles (Yanes et al. 1995; Dodd et al. 2004; Aresco 2005). Because breeding rattlesnakes move on a direct course (Brown et al. 1982) and may not be inclined to deflect to a lateral culvert, under-road passages would be most effective if equipped with drift fences to direct snakes to a tunnel (Aresco 2005). Further research to evaluate the effectiveness of road-crossing structures for large, terrestrial snakes is necessary. Nevertheless, the restoration of genetic connectivity needs to be tested directly because movement of individuals across roads does not necessarily lead to effective gene flow (Riley et al. 2006), and a recent review of wildlife overpasses highlights the lack of evidence that such structures have a positive effect on genetic connectivity (Corlatti et al. 2009). Although the effectiveness of road passages for maintaining genetic connectivity still needs to be tested, they remain one of the few viable options for mitigating the effects of roads, given continued anthropogenic development and potential increases in vehicular density.

Our study adds to a growing body of literature indicating that even anthropogenic habitat modification that does not destroy a large amount of habitat can create significant barriers to gene flow (Keyghobadi 2007; Belkenhol & Waits 2009). The effects of roads on connectivity will result in the eventual isolation of subpopulations, with negative genetic consequences that may only become evident after a number of generations. Given the relatively short time during which paved roads have been in place, it is alarming that even the smaller roads in our study already have had a detectable effect on the genetic connectivity of a long-lived, late-maturing vertebrate species. Our findings underscore an urgent need for road mitigation methods as an active component of wildlife conservation efforts.

Acknowledgments

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