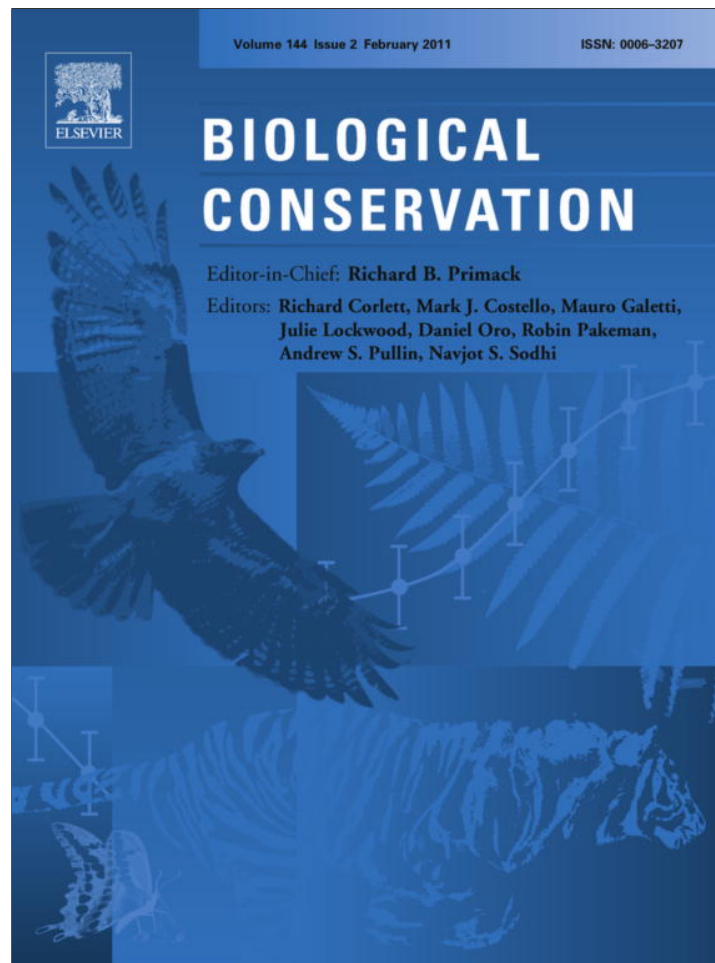


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## Decline of an isolated timber rattlesnake (*Crotalus horridus*) population: Interactions between climate change, disease, and loss of genetic diversity

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## ABSTRACT

Extinction of populations from anthropogenic forces rarely has a single cause. Instead, population declines result from a variety of factors, including habitat loss, inbreeding depression, disease, and climate change. These impacts often have synergistic effects that can lead to rapid decline in isolated populations, but case studies documenting such processes are rare. Here, we describe the recent decline of the last known population of timber rattlesnakes (*Crotalus horridus*) in the state of New Hampshire. We used polymorphic nuclear DNA markers to compare genetic diversity of this population to other populations in the region that are not isolated. We also compare results from ongoing field monitoring of these populations. Genetic analyses reveal that the New Hampshire population lacks genetic diversity and exhibits signs of a recent bottleneck. New Hampshire snakes also exhibited high levels of morphological abnormalities (unique piebald coloration, amelanistic tongues) indicative of inbreeding depression. Furthermore, after a year with exceptionally high summer rainfall, a skin infection of unknown etiology caused significant mortality in the New Hampshire population, whereas other surveyed non-inbred populations were unaffected. This case study demonstrates how different anthropogenic impacts on natural environments can interact in unexpected ways to drive threatened populations toward extinction.

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### 1. Introduction

Humans are currently causing one of the most rapid and widespread extinction events of Earth's history (Pimm et al., 1995). This loss of biodiversity threatens the long-term stability of modern society (Chapin et al., 2000). Although global extinctions have a root cause (growing human population), the mechanisms by which threatened populations are driven extinct are varied, complex, and synergistic (Forester and Machlis, 1996). In general, anthropogenic developments such as agriculture, housing, and transportation infrastructure result in loss and fragmentation of habitat used by other species; this loss of connectivity in turn leads to lower genetic diversity and inbreeding depression in impacted populations, making them more susceptible to diseases and stochastic environmental events (Frankham et al., 2002). Concurrently, human impacts are also causing an increase in frequency of stochastic environmental events through mechanisms such as altered wild-

fire regimes, invasive species introduction, and climate change (Coblentz, 1990; Meehl et al., 2000; Syphard et al., 2007).

The impacts of habitat fragmentation, disease, and climate change are all major areas of research in conservation ecology (Soule and Orians, 2001). Many natural populations are affected by several of these impacts concurrently. Multiple impacts can have interactive or synergistic feedbacks that drive populations toward extinction, a phenomenon termed an "extinction vortex" (Gilpin and Soule, 1986). However, examples from natural populations that demonstrate synergistic impacts are rare, and can serve as valuable case studies. For example, Glanville fritillary butterfly (*Melitaea cinxia*) populations are more susceptible to extinction if they are small, geographically isolated, and inbred (Saccheri et al., 1998), and extreme weather events adversely affected inbred song sparrows more strongly than outbred individuals (Keller et al., 1994).

Here, we describe the decline of an isolated population of timber rattlesnakes (*Crotalus horridus*). This population represents the last known occurrence of timber rattlesnakes in the state of New Hampshire, although historically this species was abundant throughout New England states, including New Hampshire (Palmer, 1992; Taylor and Marchand, 2006; Tynning, 1991). The New Hampshire population appears to be heading toward extinction

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due to a combination of genetic isolation, inbreeding, and stochastic weather events that render the population susceptible to opportunistic pathogens.

## 2. Method

### 2.1. Study species

The timber rattlesnake (*C. horridus*) is a medium-sized pitviper inhabiting deciduous forests and woodlands of eastern North America. In many regions, this is the only native rattlesnake species. Northeastern populations exhibit a dichotomous color polymorphism, with individuals being either predominantly yellow or black (Schaefer, 1969), referred to here as yellow-morph or black-morph individuals. In northern portions of their range, *C. horridus* populations are centered on communal wintering dens (hibernacula), and both sexes exhibit high levels of philopatry to their natal hibernaculum (Brown et al., 2007; Clark et al., 2008). During summer, individuals migrate to surrounding areas to forage and mate, with adult females and males typically moving 1–3 km and 3–6 km, respectively (Brown, 1993; Reinert and Zappalorti, 1988). Research on fine-scale genetic structure of 14 hibernacula in New York State shows that hibernacula within 3–4 km of each other with no intervening anthropogenic barriers exhibited high levels of genetic connectivity (Clark et al., 2008); however, hibernacula separated by roads and highways are genetically differentiated (Clark et al., 2010).

Timber rattlesnakes were formerly abundant and widespread throughout the eastern United States, with a range that extended northward into Vermont, New Hampshire, and Maine (Martin et al., 2008). Within the last century, timber rattlesnake populations have undergone dramatic declines and range contraction in these states. Recent surveys in the northeast US found that over 60% of populations are in decline, and, although this species has suffered historically from direct persecution by humans, habitat loss and fragmentation are the main causes of population declines (Brown, 1993). *C. horridus* has been extirpated from Canada, the US states of Maine, Michigan, Delaware and Rhode Island, and severely reduced in all of the New England states (Martin et al., 2008). Only one known hibernaculum persists in the state of New Hampshire (Taylor and Marchand, 2006; Tynning, 1991). Thus, like many species in modern landscapes, populations are disjunct, occur in highly fragmented habitats, and are much smaller than historical levels.

### 2.2. Population monitoring

Ecological monitoring of the only known timber rattlesnake population in New Hampshire was undertaken from 1992–2005 by an informal affiliation of environmental consultants and state officials. Initial surveys during 1992–1994 focused on visiting the hibernaculum and associated basking sites during emergence from and ingress to the den. Individual rattlesnakes were marked during this period by painting rattle segments (Brown et al., 1984). Sex, reproductive condition, relative size, coloration, and general appearance of individuals were also noted. Between 1995 and 2005, informal surveys were conducted sporadically. From 2006 until the current year (2010), formal surveys, including paint-marking individuals, have been conducted by biologists from the New Hampshire Fish and Game Department Nongame & Endangered Species Program. After initial paint marking, disturbance to individual snakes was minimized. Marked individuals were subsequently identified using binoculars, thereby minimizing disturbance to snakes, and photographs were taken of each individual encountered.

We compared results from surveys of the New Hampshire population (hereafter NH) to findings from a long-term monitoring program focused on extant *C. horridus* populations throughout New York state. One of us (RS) has been conducting annual monitoring of timber rattlesnake hibernacula throughout New York since 1968. We also have included data from the monitoring program of W.S. Brown, which includes annual mark-recapture surveys of hibernacula in the Adirondack region of northern New York since 1978 (hereafter AD). The AD populations, along with several in western Vermont, are the closest extant timber rattlesnake populations to the NH populations. A detailed genetic analysis of several AD populations is presented in Clark et al. (2008, 2010). Additionally, one of us (RS) has collected basic morphological data relevant to this study from 204 other timber rattlesnake populations throughout the northeastern states (New York, New Jersey, Vermont, Connecticut, Massachusetts, Pennsylvania). Precipitation data for northeastern states were obtained from the United States Department of Commerce National Climatic Data Center.

### 2.3. Genetic sampling

We obtained all available timber rattlesnake tissue samples collected from NH between 2005 and 2008, 34 samples in total. These samples were mainly in the form of shed skins found at basking and denning areas associated with the hibernaculum, but three samples were also obtained from individuals found dead in the field.

Genetic diversity of the NH population was compared to six hibernacula from AD that had recently been genotyped with these same microsatellite markers (Clark et al., 2008). Because the AD populations are some of the closest to NH, they serve as a meaningful comparison of levels of genetic variation in non-geographically isolated hibernacula.

DNA was extracted from all tissue types using Qiagen DNEasy Tissue Kits (QIAGEN), and the eluted product was used directly as template in polymerase chain reactions (PCR). Nine microsatellite loci were amplified: CwA29f, CwB6, CwB23, CwC24, and CwD15 (Holycross et al., 2002), and 5a, 5-183, 7-144, and 7-87 (Villarreal et al., 1996). PCR reactions were performed in 10  $\mu$ L reaction volumes containing 1  $\times$  PCR buffer (Invitrogen), 1.0–2.0 mM MgCl<sub>2</sub>, 0.02 mM each forward and reverse primer, 0.1 mM dNTP mix, 0.25 U Invitrogen Platinum Taq DNA polymerase, and 1  $\mu$ L template DNA ( $\sim$ 10 ng/ $\mu$ L). The PCR temperature profile was 94 °C for 5 min; 35 cycles of 90 °C for 1 min, either 60 (CwA29f and 7-144), 56 (CwD15 and 7-87) or 50 (all other loci) °C for 1 min, and 72 °C for 1 min; followed by a final 10 min extension at 72 °C. Fragment sizes were calculated by comparison with an internal size standard (LIZ500, Applied Biosystems) after electrophoreses on an ABI 3100 automated capillary sequencer. Allele sizes were scored using GENEMAPPER 3.0 (Applied Biosystems). Because of the degraded nature of many of these tissue samples, it was not possible to extract DNA from all of them. We attempted DNA extractions using a range of incubation conditions for any samples that could not be processed using the standard extraction protocols above.

We first used GIMLET (Valiere, 2002) to identify samples that had duplicate genotypes. Because of an observed lack of genetic diversity, samples with identical genotypes were not assumed to be from the same individual. We re-extracted and re-genotyped samples with identical genotypes to be sure that there was no cross contamination of samples. If collection information from samples indicated that they were from individuals distinct in age or sex, we assumed that samples represented distinct individuals who shared a genotype due to low levels of genetic diversity within the population.

We used several measures of genetic diversity to compare NH to AD. We measured mean number of alleles per locus and total proportion of polymorphic loci in each population. To control for differences in sample size between AD and NH, we randomly sub-sampled 20 individuals from any AD hibernaculum with a sample size greater than 20. Thus, comparisons of mean number of alleles per locus were all between populations with a sample size equal to or less than that of NH. We also used the rarefaction procedure in FSTAT 2.1 to calculate allelic richness (Goudet, 1995). Both allelic richness and alleles per locus were compared among hibernacula using repeated-measures ANOVA after log transforming data for normality.

Additionally, we tested for occurrence of a recent genetic bottleneck. One of the principal patterns resulting from a population bottleneck is loss of rare alleles (Luikart et al., 1998). We conducted a binomial test to determine if frequency of rare alleles (defined as those that occur at a frequency <0.1) in NH was less than expected based on the AD data. We also tested for a recent genetic bottleneck using BOTTLENECK (Cornuet and Luikart, 1996). We used the Wilcoxon test of deviation from mutation-drift equilibrium using the two-phase model of microsatellite evolution (Cornuet and Luikart, 1996). This approach reflects the finding that populations that have undergone a recent bottleneck often show heterozygosity excess at neutral loci, since allelic diversity is reduced faster than heterozygosity.

### 3. Results

#### 3.1. Population genetics

We were able to obtain tissue samples from 34 NH rattlesnakes, including 27 shed skins. We were able to extract viable DNA from 21 samples. Unusable samples were shed skins from 13 individuals that appeared too degraded to contain high quality DNA. GIMLET identified identical genotypes for two pairs of individuals. Two shed skins had identical genotypes and were similar in size, and so we assumed that these sheds were from the same individual, reducing our sample to 20. Reanalysis of the other pair of identical genotypes did not reveal any genotyping or extraction errors. Collection information associated with these samples indicated they could not have come from the same individual, so these samples were assumed to represent distinct individuals that shared identical genotypes due to low genetic diversity in the population. Our total sample size of 20 individuals was comparable to that obtained from the six AD hibernacula (average  $n = 29$ , range 15–57).

The NH population was found to be monomorphic for three of nine loci (Table 1). In contrast, none of the AD populations exhibited monomorphism at any loci. Furthermore, NH exhibited a lower average number of alleles per locus than all AD populations

(Table 1). Comparisons between hibernacula with repeated-measures ANOVA and a Tukey post hoc test with Bonferroni corrections showed that allelic richness and alleles per locus are both significantly lower for NH than for five of the six AD populations ( $P < 0.05$ ) (Fig. 1).

Tests in BOTTLENECK also indicate a significant probability of a recent bottleneck in NH ( $P = 0.04$ ), but not in any of the AD populations. Furthermore, only 28% of the alleles in NH were rare alleles, whereas an average of 55% of alleles in all AD populations were rare alleles. The proportion of rare alleles in NH is significantly lower than expected from the frequency of rare alleles in AD (binomial test,  $P = 0.01$ ).

#### 3.2. New Hampshire population surveys

Surveys of the NH population from 1995–2005 indicated that population size remained relatively constant at around 40 individuals (Taylor and Marchand, 2006). A total of forty snakes, including two neonates, were observed from 2006–2009. All individuals observed throughout this time period were black-morph (i.e., no yellow-morph snakes have been observed at this hibernaculum). Surveys documented several other morphological aberrations in individuals. At least four litters during this period included piebald colored (white on head, neck, and/or sides) neonates. Of 19 individual snakes observed during 1992–1993, two had piebald coloration, one neonate had a scoliotic spinal deformity, and at least two individuals had pink tongues rather than normal dark-colored tongues. During 2006, two adult and one immature animal were observed with various patches of unusual white skin coloration, and at least two individuals had pink tongues.

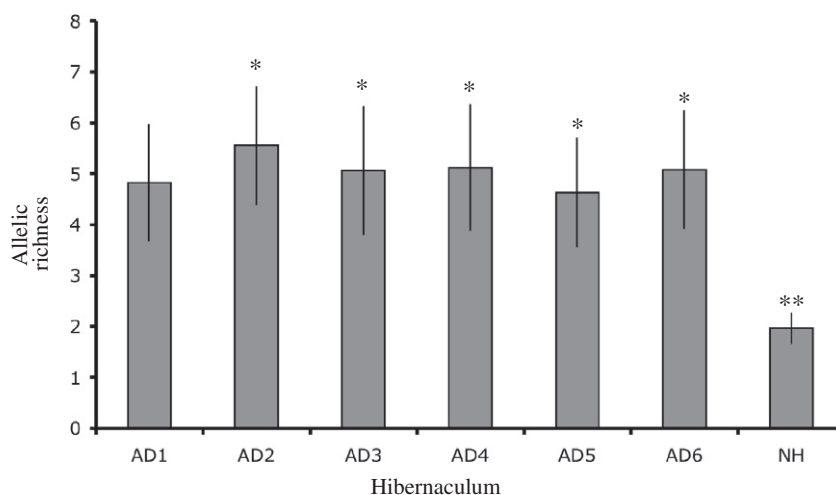
In 2006, many snakes displayed skin lesions around the head, chin, and body. One individual was found dead in early June, presumably from a severe fungal infection in the mouth. During fall, eight individuals remained visible outside of the hibernaculum area in October, and at least three of these lingered out of the den into the first week of November (typically timber rattlesnakes in the Northeast enter their hibernacula during the first week of October and do not emerge until early May). Five of these individuals have not been observed since the fall of 2006. During early spring 2007, two of these individuals (plus one other adult) were found dead. The remaining individual was observed alive during spring 2007, but had a severely swollen eye, and has not been observed subsequently. Over the past five annual surveys (2006–2010), only 19 individuals (plus 2 neonates) have been observed alive, even after intensive searches conducted throughout the active season. Our data thus indicate that the population declined by approximately 50% between 2006 and 2007. No individuals with piebald coloration or pink tongues have been observed since 2007.

The source of skin infection remains unknown, but appears fungal in origin. Scale and mouth samples were taken from three rattlesnakes for bacterial and fungal presence testing, which was completed at Tufts Cummings School of Veterinary Medicine Wildlife Clinic and Cornell University. All bacteria and a single fungus identified appear to be present in the natural environment. Even though the cause of the skin infections remains unknown, we noted that the years 2005 and 2006 were abnormally wet. Average monthly precipitation totaled for the state of New Hampshire for the May–October active season for 2006 was 101.3 cm, the highest on record from the United States National Climatic Data Center (Fig. 2); this value for 2005 was 91.4 cm, the second-highest on record following 2006. For comparison, the average amount of total precipitation for New Hampshire during May–October over the past 114 years is 57.1 cm. Increased cloudiness and humidity associated with high precipitation can be detrimental to timber rattlesnakes, as these conditions are correlated with reproductive failure in females (Martin, 1993, 2002).

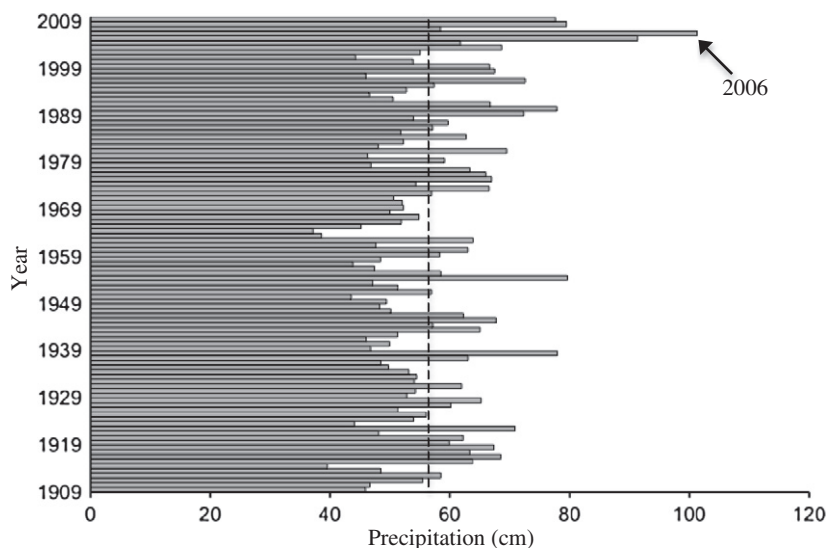
**Table 1**

The number of alleles identified at each of nine microsatellite loci (L1 through L9) for seven populations of timber rattlesnakes (*Crotalus horridus*). Populations AD1 through AD6 represent six populations from the Adirondack region of New York, whereas population NH represents one isolated New Hampshire population.

	AD1	AD2	AD3	AD4	AD5	AD6	NH
L1	3	4	2	2	4	3	2
L2	3	4	4	4	3	4	2
L3	7	7	5	5	4	6	1
L4	17	14	17	15	13	15	3
L5	2	5	4	4	4	3	2
L6	6	6	7	8	4	6	4
L7	3	2	3	2	2	3	1
L8	6	5	5	5	5	5	1
L9	3	3	3	3	3	3	2
Average	5.6	5.6	5.6	5.3	4.7	5.3	2.0



**Fig. 1.** Average allelic richness for six AD populations and one NH population of *Crotalus horridus*. Error bars indicate standard errors. Hibernacula denoted with \* were significantly different from that denoted with \*\* at  $P < 0.05$  (Tukey post hoc test with Bonferroni correction after repeated-measures ANOVA).



**Fig. 2.** Summer precipitation (total accumulated average precipitation for months of May through October) for the state of New Hampshire shown for the past 100 years (1909–2009). Dotted line shows average summer precipitation for these years. Data compiled from the United States Department of Commerce National Climatic Data Center.

### 3.3. Non-New Hampshire population surveys

The *C. horridus* population in New Hampshire is unusual in several respects when compared to conspecific populations elsewhere in the northeastern states: (1) Only one other population among the 204 we have surveyed is known to contain completely black-morph individuals, i.e., lacks individuals of the yellow color morph. (2) Both piebald and pink tongue coloration observed in NH have not been observed in any of the other surveyed populations. (3) Skin lesions that appear identical to those observed in New Hampshire have been observed in other populations after emergence from dens (informally referred to as “hibernation blisters”), but these lesions typically disappear after the affected snakes undergo ecdysis and no instance of mortality has resulted from the lesions (pers obs). (4) For the May–October months, 2006 was also the wettest year on record for the states of New York, Vermont, Massachusetts, and the fourth wettest year on record for Connecticut (average monthly precipitation totaled for May–October 2006 for these states was, respectively, 77.9, 89.1, 88.9, and 90.9 cm). Thus, even though 2006 was evidently an extreme year climatically throughout the northeast, no late-season basking, skin infections,

or other abnormalities were observed in any of the approximately 29 New York populations we surveyed in the fall of 2006, including the AD populations (W.S. Brown, personal communication).

### 4. Discussion

Our results show that the single and only remaining population of timber rattlesnakes in New Hampshire is depauperate with respect to genetic diversity. We found this population to be monomorphic for three of nine loci used in this analysis, whereas the same nine loci were polymorphic in all 19 *C. horridus* populations analyzed by Clark et al. (2010). The NH population also had significantly lower allelic richness, and, for remaining alleles, an excess of heterozygosity at neutral loci that is indicative of a recent population bottleneck (Luikart et al., 1998).

The NH population also exhibited gross morphological indicators of low genetic diversity and inbreeding depression. All NH snakes were dark morph individuals, whereas among the 204 other timber rattlesnake populations surveyed by us, only one was monomorphic with respect to color (a population surveyed by

R.S. and W.S. Brown in northern New York that is also geographically isolated). Approximately 10% of NH snakes exhibited a piebald coloration pattern that has not been observed in any of the other *C. horridus* individuals (approximately 7000) we have examined over the course of 42 years of field work. The tongue color aberration observed in NH also has not been observed by us in any other surveyed population.

The geographic isolation and lack of genetic diversity of the NH population have apparently rendered it more susceptible to stochastic environmental events, as predicted by the “extinction vortex” principle (Gilpin and Soule, 1986; Keller et al., 1994; Tanaka, 2000). It is well established that isolation of populations can lead to inbreeding depression, which in turn can lead to increased population susceptibility to disease (Frankham et al., 2002; Ilmonen et al., 2008; Townsend et al., 2009). Several fungal pathogens of snakes have been identified (Jacobson et al., 2000; Paré et al., 2003; Rajeev et al., 2009), including some that cause widespread mortality in free-ranging populations (Cheatwood et al., 2003). In the system described here, increased susceptibility to disease appears to have manifested as a result of other stochastic environmental events. We have often observed timber rattlesnakes emerging from hibernation with skin lesions (personal observations), but these are usually lost after the affected snakes undergo spring ecdysis. In captivity, these lesions (informally known as “hibernation blisters”) are exacerbated by humid conditions and can be colonized by bacteria and fungi, leading to severe local and systemic infections (Harvey-Clark, 1995). In the NH population, such infections appeared to cause high mortality during 2006. That year was the wettest year on record for New Hampshire for the May through October months, and immediately followed the previous wettest summer on record (Fig. 1). The behavioral response of rattlesnakes seen basking outside the hibernaculum in late fall 2006 is consistent with a response to infection or disease; ectotherms generally respond to pathogens by increasing their exposure to sunlight or heat, thereby raising their body temperature (Burns et al., 1996). However, 29 other populations we surveyed in the Northeast in the fall of 2006 showed no signs of infection or increased mortality. Thus, it appears that disease susceptibility in this system may result from interacting effects of habitat fragmentation, inbreeding depression, and climate change.

This combination of factors will most likely lead to the extinction of the NH population, unless it is actively managed. Past research on communally hibernating Viperids has indicated that isolated and inbred hibernacula can be “genetically rescued” by the introduction of novel alleles (Madsen et al. 1999, 1996, 2004). We recommend adopting the management strategy of Madsen et al., with a focus on restoring genetic variation from nearby non-isolated populations. If this is not done, it is likely that timber rattlesnakes will be extirpated from New Hampshire. This would be the first loss of this species in a US state in almost half a century.

Although forecasts of anthropogenic climate change for the northeastern US do not predict that summertime precipitation patterns will increase on average (Hayhoe et al., 2008), climatic variability in general is predicted to increase in magnitude and frequency (IPCC, 2001). This study serves as an example of how extreme weather events can interact with other anthropogenic impacts in unexpected ways to cause population declines. Unfortunately, as global change accelerates, this combination of deleterious local and global impacts will become more common. To preserve biodiversity, we will need to take action at both scales.

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