THESIS

INFERRING MATING BEHAVIOR AND REPRODUCTIVE SUCCESS OF (*AGKISTRODON CONTORTRIX*) USING MOLECULAR PARENTAGE ASSIGNMENT TESTS

Submitted by

Jennifer Kendall

Graduate Degree Program of Ecology

In partial fulfillment of the requirements

For the Degree of Masters of Science

Colorado State University

Fort Collins, Colorado

Spring 2012

Master's Committee:

Advisor: Julie A. Savidge
Co-Advisor Michael E. Douglas

Marlis R. Douglas
Christopher Richards
Geoffrey Givens

ABSTRACT

INFERRING MATING BEHAVIOR AND REPRODUCTIVE SUCCESS OF (*AGKISTRODON CONTORTRIX*) USING MOLECULAR PARENTAGE ASSIGNMENT TESTS

Historically, behavioral and ecological research on snakes has been limited due to the secretive and cryptic nature of these species. Readily available molecular techniques have enhanced the study of reproductive behavior and advanced our understanding of mating patterns (such as multiple paternity) that were previously deemed ambiguous. Instead they have been revealed as often prevailing and strongly influential on genetic population structure. Underlying biological questions about the social and behavioral movements associated with mating can be addressed by correlating the results of population genetic analyses with known social structure and can be used to make inferences regarding landscape genetics and gene flow.

During a three-year (2001—2003) ecological study of a geographically isolated population of Northern Copperhead (*Agkistrodon contortrix*) in Meriden, Connecticut, U.S.A., blood samples from 254 individuals (117 adults and 137 juveniles) were collected for extraction and amplification of genomic DNA. Five microsatellite DNA markers, derived from species closely related to the study organism, were used to identify individuals in the population and to infer paternity. These analyses revealed aspects of mate selection, reproductive ecology, and sociality in Northern Copperhead.

No spatial genetic structure among dens could be determined with the available data and thus influence of den structure on mating patterns could not be inferred. In contrast, genetic structure in the form of three distinct genetic clusters was identified within the population. Paternity tests identified a nonrandom mating pattern by which individuals showed a clear pattern of intra-cluster mating. Causation underlying this phenomenon remains unclear, as further ecological and genetic data would be needed.

ACKNOWLEDGMENTS

I would like to thank my advisors, Julie A. Savidge, Marlis R. Douglas and Michael E. Douglas who gave me the opportunity to develop a higher education and for their continued guidance and support throughout my Master's research. Their words of encouragement and belief in my success gave me the confidence to persevere through some of the most challenging circumstances.

I would like to gratefully acknowledge Chuck Smith at the University of Connecticut whose dissertation research yielded the tissues samples for my study; his field data were extremely useful in the interpretation of the genetic results of my research. I would also like to thank Geoffrey Givens who served as external member of my committee and who provided insights into statistical intricacies of my data analyses.

I would also like to thank my invaluable committee member Chris Richards, a truly amazing and patient mentor. His input was crucial in the development of this project and his commitment to seeing me succeed kept me dedicated to my goals.

My sincerest thanks to Marie Turner, my dearest friend, a fellow grad student, a past coworker and a master editor; without her friendship and belief in my ability I may have lacked the resolve to survive the "snake fight."

Many thanks to the people who invested their time to lend a helping hand during the progression of my degree by offering an ear to explore an idea, a crash course in an computer software, a lesson in statistics or hours of free child care, including: Adam Henk, Pat Reeves, Dave Noroski, Natalie Score, Amanda Moore and all my friends at NCGRP.

Thanks to my Mother and Father who set the standards for their children by teaching the importance of pursuing what you believe no matter what challenges come your way and also for their commitment to helping their children achieve their goals.

To my dear husband Matt who has shown amazing love, and dedication to the success and accomplishments of his family. To my sons Camron and Quinn who I hope through my actions I will instill the same lessons my parents gave me: Never give up, you can achieve anything you put your mind to.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Prior to the application of molecular techniques in behavioral studies (Avise 2004) the complexities of mating systems were defined in a variety of organisms solely by observational data (Emlen & Oring 1977), with a caveat that behaviors of cryptic and elusive species were difficult to directly observe and hence largely unstudied. In addition, the limitations inherent to an observational approach either provoked inaccurate assessments of behaviors and mating strategies in the wild (Gibbs et al. 1990; Hughes 1998), or forced the use of laboratory situations that constrained the natural behaviors of study species (Krebs & Davies 1997). This was particularly true for snakes (Duvall et al. 1993; Prior et al. 1997; Shine 2003), with many species generally understudied due to their (perceived) intractability as research subjects (Bonnet et al. 2002). These difficulties were compounded historically by the perception that snakes were largely asocial, solitary, and lacked extensive parental care (Bushar et al. 1998; Clark 2004; Clark et al. 2008), thus diminishing them as subjects for comparative behavioral studies.

Researchers now routinely identify individuals based on microsatellite (msat) DNA profiles (Bushar et al. 1998), and apply these data to determine fine-scale genetic structure (Prior et al. 1997; Elmer et al. 2007) and gender-biased dispersal (Lane & Shine 2011). These results, combined with *in situ* observations (Shine & Bonnet 2000), suggest that reptiles in general, and snakes in particular, are actually quite complex behaviorally, with a much more nuanced social system than previously theorized (Graves & Duvall 1990; Prior et al. 1997; McCracken et al. 1999; Prosser et al. 1999, 2002).

This study evaluates the social and mating behaviors in the Northern Copperhead (*Akistrodon contortrix mokasen*) through an analysis of spatial genetic structure. Copperheads overwinter in communal hibernacula (dens), most often in rocky encroachments (Fitch 1960). Such shelters are numerically limited and hibernacula may be utilized that also support a variety of other snake species (i.e., colubrids, viperids, and natricines). However once hibernacula are identified, individual snakes can be easily captured, tagged, and sampled for DNA analysis prior to spring egress. This event initiates a five-month dispersal period during which individuals establish home ranges and mate (reviewed in Ernst & Ernst 2003). Males migrate from a hibernaculum to surrounding areas and often return to the same site in the fall. Females follow a similar pattern, but differ from males in that they often congregate in communal basking areas while gravid, largely due to the paucity of such microhabitats within dense forest (Ernst & Ernst 2003). Thus, the natural history of this snake establishes it as a good candidate species for a molecular evaluation of its behaviors.

The Northern Copperhead is not actively managed, yet another pitviper (Timber Rattlesnake, *Crotalus horridus*) is listed as "vulnerable–to-potentially-extirpated" in one third of its range (to include Connecticut) (Nature Serve 2007). Timber Rattlesnakes embody many of the same biological, behavioral and social characteristics as Copperheads, suggesting potential for generality in reproductive ecology across taxa. Given this, a comprehensive examination of the social and genetic structure (e.g., fine scale, spatial genetic structure and parentage estimation) of the more common Northern Copperhead may illuminate patterns of mating behavior in Timber Rattlesnake.

This study combines genetic with previously collected ecological data (Smith 2007; Smith et al. 2009) to address overarching patterns of paternity and overwintering behavior in the Northern Copperhead. Correlation between estimates of genetic structure and mating success may identify processes that structure the genetic variability of the study species. To accomplish this, the following questions were examined: (a) Is there genetic diversity in the study species? (b) If so, is it spatially structured with respect to individual dens? (c) Can spatial structure be used to corroborate relatedness among dens and/or genetic clusters of individuals? and (d) Does genetic structure reflect observed mating behavior in the species?

METHODS

Study Area

The study site (Figure 1), in the Connecticut River Valley 4.75 km northwest of Meriden, consists of 485.6 ha of basalt trap rock (Smith 2007). A significant portion of its eastern aspect was once an active limestone quarry defined by two distinguishable basalt ridges. The first (Chauncey Ridge, 914 m in length, 200 m above mean sea level) is in the central region of the study area and consists of exposed cliffs to the west that slope abruptly toward the Bradley Hubbard Reservoir and wetlands to the east. The second (Lamentation Ridge, 3.2 km in length, 219 m above mean sea level) is longer than Chauncey Ridge but similarly configured in that it slopes westward toward the reservoir with basalt cliffs, talus slopes and a wooded area. Northern Copperhead is continuously distributed throughout the study site, which is situated at the extreme northeastern range of the species.

Tissue Sampling

From 2001-2003 Smith (2007) conducted dissertation research on spatial and reproductive ecology of Northern Copperhead at the site. He collected 254 individuals (Table 1) using an intensive search method and transported captured snakes to the University of Connecticut to measure body mass, snout to vent length (SVL) and tail length and to ascertain gender. He also tagged each snake with a Passive Integrative Transponder (PIT) tag and extracted blood (0.1 ml) from the caudal vein prior to returning each snake to the site of capture. Blood samples were preserved in 100% ethyl alcohol and stored at -20 C for subsequent molecular analyses.

Spatial Sampling

Of the 254 individuals collected during 2001-2003, Smith (2007) implanted 35 adults (20 males and 15 females) with radio-transmitters to obtain spatial data for estimation of home ranges and to identify den affiliation. Snakes were collected at the time of egress from hibernacula and Smith et al. (2009) identified five main dens (Table 2): Point of Lamentation, 141, Middle Lamentation, c29 Chauncey Side, and Middle Chauncey. Smith (2007) confirmed fidelity of adults to their ascribed dens over all three years of the study. He found a total of 101 adults associated with these five main dens.

Number of individuals associated with each main den varied considerably (Table 2). The largest den, (141) was located at the center of the study site, and contained 63 adults. Two other main dens, (Point of Lamentation and Middle Chauncey, were used by considerably fewer adults (N=14 and 10, respectively), whereas the remaining two main dens, Middle Lamentation and c29 Chauncey Side, contained even fewer adults (both N=7).

In my study, I used affiliation with the main dens as reference for over-wintering and permanent residency within the study area. I considered snakes associated with these five main dens as "*residents*" (Table 2). For analytical purposes, neonates were considered affiliated to the maternal den.

Over the three-year period, Smith (2007) found an additional 16 adults within the area, but these were not collected at the time of egress from one of the five main dens. They may have emigrated from surrounding populations or failed to make the seasonal ingress to one of the five

main den locations. For nine of these individuals, he recorded den location as: Black Pond, Old Lyme, Rock Wall at Dam, and Wassel Reservoir (Table 2). For the remaining seven individuals Smith (2007) could not confirm affiliation with any known den. In my study, I considered these individuals as "*non-residents*" (Table 2).

Paternity Sampling

For aspects of his reproductive ecology study, Smith (2007) collected gravid females in the field and held them at the laboratory until parturition. Mothers and their offspring were subsequently released back to their collection site. These known mothers produced a total of 20 clutches: six gave birth to 34 offspring in 2001, eight to 64 offspring in 2002, and six to 33 offspring in 2003. One female was collected in 2002 and 2003 and gave birth to six offspring in each year. Thus, 19 females produced a total of 20 clutches ("*assigned neonates*"; Table 3).

Smith (2007) found another six individual neonates in 2001 without detecting a female nearby as potential mother. His field notes document that these six neonates had little or no sheds and thus could not be identified conclusively as siblings. I included them in my molecular analyses to test if a maternal genotype could be determined ("*unassigned neonates*"; Table 3).

DNA Extraction and Genotyping

Genomic DNA was extracted from all 254 tissues samples, representing 117 adults and 137 juveniles, following the procedures described in Douglas et al. (2006). Five microsatellite DNA primer sets designed for Ridgenose Rattlesnake *Crotalus willardi* (Holycross et al. 2002*)* successfully amplified DNA fragments in *A. contortrix* (CW06, CW14, CW15, CW23, and

CW24; Table 4). For initial tests, amplified products were separated electrophoretically on a SEA 2000 (Elchrom Scientific, Cham, Switzerland) using 3% agarose gels (14 minute runtime at 200 V and 25°C), and visualized after 45 minutes staining in ethidium bromide (EtBr) to verify fragment size.

For genotyping, forward primers were labeled with a fluorescent dye and all five primer sets were pooled into a multiplex panel (Table 4). This allowed me to amplify all five loci in a single polymerase chain reaction (PCR) under the following conditions: 10-µl reaction volume, containing 20 ng of genomic DNA, 1.7 µl water, 1.9 µl 5X GoTaq® Flexi buffer, 2.0µl $MgCl_2$ (25mM), 0.8µl dNTP (1.25 mM), 0.2µl BSA (1 mg/ml), 1.0 µM each of forward and reverse primer, and 0.5 u of GoTaq® Flexi DNA polymerase (Promega Corp., Madison, WI). PCR reactions were conducted on an ABI GeneAmp 2400 (ABI: Applied Biosystems, Inc., Foster City, CA), programmed for MP1 at 95° denaturing for 3 minutes, 15 cycles were run at 95° C for 45 seconds, annealing at 58° C for 45 seconds, and extension at 72° C for 30 seconds, followed by 25 cycles at 95° C for 30 seconds, 60° C for 30 seconds, and 72° C for 15 seconds, with a final extension at 72° C for 3 minutes and a final hold at 20° C.

Microsatellite fragments were visualized on an ABI Prism 3100 using standard electrophoresis parameters. An internal size standard (Liz500) was run with each sample. GENESCAN 3.7 (ABI) was used to examine peak allele profiles, and sizes were determined in GENOTYPER 3.7 (ABI). Genotypes were imported into an EXCEL spreadsheet and scores were rounded to the nearest whole number. Allele calls were manually verified to assure that alleles were correctly binned into discrete size classes.

Database Compilation and Data Partitioning

Genotypic data were first aligned and individuals with no or too many missing allele scores removed, reducing the number of genotypes to 238 (Table 1). Each individual was then aggregated with previously recorded observational and ecological data [i.e., PIT, sex, age, mother-offspring-relationship, clutch assignment, den assignment, GPS coordinates and field notes: Smith (2007)], then cross-checked manually to ensure a correct match for each individual. Genotypes were partitioned into four data sets (Table 1) appropriate for particular analyses (Figure 2) and formatted for input into various genetic data analyses software.

The first data set, referred to as "*all samples,*" used the greatest number of available genotypes (N = 238) and was comprised of 115 adults plus 123 offspring (Table 1). The second data set contained 99 genotypes and consisted of snakes considered "*resident adults*"; this data set represented 49 males, 48 females and two individuals of unknown sex (Table 1). For each individual, affiliation with one of the five main dens had been confirmed (Smith 2007). A third data set was compiled by adding neonates from resident females to the second data set and is referred to as "*all residents"* (Table 1). This increased the number to 197 genotypes (99 adults and 98 offspring). A fourth data set, referred to as "*parentage*" (Table 1) was generated for paternity analysis. It consisted of genotypes of all offspring (123 neonates), known mothers (19 females), and possible candidate fathers (55 males plus two individuals of unknown sex).

Overall genetic diversity and structure was assessed with the data set "*all samples"* using *F*-statistics and Bayesian assignment tests. To evaluate if genetic structure was associated with den affiliation, data sets "*resident adults"* and "*all residents*" were employed in analyses again

using *F*-statistics and Bayesian assignment tests. Data set "*parentage*" was used to identify fathers of known mother-offspring couplets. Results from paternity analyses were used to evaluate associations between genetic clusters and mating patterns (see Figure 2).

Descriptive Statistics

Genetic diversity was assessed with *F*-statistics (F_{ST}) in a two-level hierarchy that described differentiation within and among dens (FSTAT; Goudet 1995). In addition, allelic richness Goudet (1995) was calculated within dens using a rare fraction method to correct for sample size variation across dens. Genetic Data Analysis (GDA) was also used to compute private alleles and pair-wise genetic distances among dens (Lewis & Zaykin 2001).

Model-based Clustering

Whereas *F*-statistics require the determination of an *a priori* hierarchy, Bayesian clustering does not. The latter instead allows genetic clusters to be based solely on linkage disequilibria among loci, and thus reveals novel biological information not associated with collection data (i.e., geographic information, gender, etc.). The program STRUCTURE (Prichard et al. 2000) groups genotypes in a way that optimizes linkage disequilibria among clusters and minimizes it within clusters. One unique feature of STRUCTURE is its ability to fractionally assign individuals to different clusters (i.e., admixture), and thus identify immigrants (Pritchard et al. 2000; Excoffier & Heckel 2006).

STRUCTURE was initially run with K (the number of clusters) = 1-10, burn-in = 100,000, and Markov chain Monte Carlo (MCMC) replications after burn-in = 100,000. An

ancestry model of admixture and correlated allele frequencies between populations enabled fractional assignment of individual genotypes to multiple populations by probability of membership. Genetic subdivision of K was then rigorously tested using two complementary approaches.

The first method evaluates change in ΔK which is an ad hoc quantity related to the second order rate of change of the log posterior probability between successive K values and is equal to the mean of [(|L”(K)|)/sd(L(K))] (Evanno et al. 2005). Via simulation, these authors determined that this method accurately establishes the highest level of partitioning for the data.

The second method estimates stability of cluster composition across multiple MCMC runs for each K value. The similarity coefficient quantifies the similarity of results for an ordered pair of structure runs within each value of K. This method quantifies whether individual genotypes are assigned to the same clusters repeatedly and whether clusters contain the same individual genotypes across multiple runs. Missing data can cause certain individuals to be assigned to different clusters across MCMC runs, and this method offers additional confidence in that it identifies the most likely K as well as the partition resulting in the most stable assignments (Rosenberg et al. 2002).

An additional Bayesian assignment method similar to STRUCTURE was also used to independently confirm the value of K. The software STRUCTURAMA does not estimate admixture but rather jointly estimates K clusters and assigns individual genotypes to K clusters (Huelsenbeck & Andolfatto 2007). I ran 50 MCMC chains in STRUCTURAMA and used the

mode for final K and cluster composition. By using each of the described methods K is rigorously validated in complementary ways.

Parentage Assignment

Since maternal parentage was *a priori* known for clutches, paternal parentage was calculated using CERVUS (Marshall et al. 1998). Parentage simulations were set with the following parameters: 10,000 cycles, 57 potential fathers (two individuals of unknown gender were included as potential fathers); 95% of the candidate parents estimated as being sampled; 97.3% of loci typed; and 0.5% of loci mistyped.

Genotyping errors were expected because of the relatively small size of the population, relatedness among individuals, and the limited set of microsatellite loci from which to gauge likelihood estimates of paternity. However, genotyping error rates are still expected to be low (0.5%) because each individual was typed at least twice for each locus, with discrepancies resolved by scoring individuals multiple times.

RESULTS

Genotype Data

A total of 254 blood samples were available (Smith 2007), representing 56 males, 59 females, and 137 neonates (Table 1). The gender of two individuals could not be determined, but in my analyses I included them as adults (i.e., potential paternal genotypes). Across all 254 samples, I was able to successfully generate microsatellite genotypes for 238 individuals, including 115 adults and 123 offspring (Table 1).

Genetic Structure based on Bayesian Clustering

Clustering algorithms in STRUCTURE using the data set "*all samples*" identified three genetic clusters within the population; each is referred to in Table 5 by its assigned color: Blue (B), Green (G) and Red (R). Genetic partitioning into clusters was supported by all three methods used to determine K (i.e., Rosenberg et al. 2002; Evanno et al. 2005; Huelsenbeck & Andolfatto 2007). Especially strong evidence came from the stability of the correlation coefficient across multiple runs (i.e., the same individuals were assigned to the same genetic cluster virtually every time).

To examine if spatial genetic structure existed according to known den affiliation, the data set "*resident adults*" was used for an identical analysis; however no partitioning of the data was found (K=1). This outcome was likely due to limitations of the software STRUCTURE to detect such fine scale structuring with small N or few loci. This analysis was repeated with the data set "*all residents*" (including resident adults and offspring) and genetic clustering was detected (K = 2-3). The minimal difference found among genetic partitions generated by the first

and third tests seems to validate the assumption that STRUCTURE's ability to detect signals of genetic partitioning becomes restricted with few samples and/or loci.

Genetic Diversity by Den Affiliation

Genetic diversity within and among dens was evaluated using the data set "*resident adults*" (Table 6). F_{ST}-values showed no significant level of differentiation among dens (F_{ST} = 0.010; 95 % CI = -0.010/+0.016). Similarly, low values of the inbreeding coefficient (***f***) also showed that individuals affiliated with a particular den do not appear to be more closely related. Moderately high values of allelic richness were likely the result of the large number of individuals deriving from den 141. The non-significant F_{ST} values, low inbreeding coefficients and a lack of private alleles strongly indicate gene flow among dens. In other words, individuals are not mating assortatively by den.

Genetic Diversity by Cluster Assignment

Clusters were significantly differentiated (F_{ST} =0.120; 95 % CI =0.069/0.173) with a comparative test in GDA producing similar results (Table 5). Variation among the three genetic clusters was 12%. Inbreeding coefficients and values of allelic richness based upon cluster (rather than den) also support assortative mating and are more reliable than the previous analysis by den due to a more balanced sample sizes among clusters (Table 5).

Spatial Distribution of Genetic Clusters

To examine distribution of the three genetic clusters among dens, cluster assignment of resident adults was displayed in pie charts for each den (Figure 3). A noticeable trend showed

that the three dens on Lamentation Ridge contained all three clusters, while the two dens on Chauncey Ridge contained only two of the three clusters each. This suggests that gene flow among dens maintains a certain degree of admixture.

Of the three dens on Lamentation Ridge (Figure 3) the largest den (141) had nearly equal ratios of genotypes assigned to cluster B and G, but only 13% to cluster R. Middle Lamentation also showed a similar distribution of genotypes in cluster B and G, with only 14% assigned to cluster R. Point of Lamentation again had the largest proportion of individuals assigned to clusters B (31%) and G (46%) but had a higher percentage of assignment to cluster R (23%) compared to the other two dens on Lamentation Ridge.

Neither den on Chauncey Ridge contained all three clusters. The majority of adults affiliated with dens on Chauncey Ridge were assigned to cluster G, with Middle Chauncey at 70% and c29 Chauncey Side at 71%. The two dens differed in the assignment of the remainder of individuals, with the former having assigned 30% to cluster R, and the latter had 29% assigned to cluster B.

A Fisher's exact test was performed to determine whether den and cluster assignment were independent of each other (Table 7). This would reveal if the distribution of individuals in the three clusters is spatially structured. Fisher's exact test rejects the null hypothesis of independence if the *p*-value is small. The returned *p*-value was 0.097 (Table 7), and thus the null hypothesis that den and cluster affiliation are independent cannot be rejected. Weak inference is likely due to small sample sizes for some dens and a limited number of loci examined.

Paternity Analysis

Comparison of genotypes revealed that all neonates from clutches with known mothers ("*assigned neonates"*) represented full- or half-sibs. Relatedness of the six "*unassigned neonates"* could not be resolved, due to incomplete genotypes. There is some evidence that these individuals might represent siblings, as four of the six shared at least one, sometimes two, common alleles, but the missing maternal genotypes prevented further resolution.

Of the 20 clutches available for analysis with known mothers ("*assigned neonates*"), 14 fathers were identified with a likelihood confidence of 85% or greater using CERVUS. Of the clutches that could be matched with parental genotypes, eight represented a single male/female pairing (Table 8) and six represented multiple male/female pairings (Table 9).

Six of 20 clutches (30%) could not be positively matched to a father, given the genetic data available. CERVUS could not produce a greater than 85% likelihood value for three of these six families and closer inspection of clutches with known mother ("*assigned neonates"*) revealed no potential father from the 57 potential males collected. Given this information, it is likely the true father was not sampled. Two clutches had possible paternal matches between two sampled fathers, but again, without an 85% likelihood value. It is also possible these clutches were a product of multiple paternity, but our genetic data did not have the power to unequivocally establish this.

A noticeable pattern across all documented successful matings (i.e., those represented with neonates in this study) is that females appear to mate preferentially with males from the

same genetic cluster. Seven of the eight single male/female pairings (87.5%) mated with a male from the same genetic cluster (Table 8), whereas three of the six females from multiple male/female pairings (50%) also successfully mated with at least one or more males from her same genetic cluster (Table 9). In the other three multiple pairings, the identified father was from outside the female's genetic cluster. However, as the other father remained unknown, it is still possible that even these females mated with at least one male of the same genetic cluster.

Once paternal identity was determined, paternity assignments were used to assess the probability of successful mating (i.e., were the observed data different from what would be expected if mating were random, such as promoting intra-vs inter-cluster parings). To illustrate: assume a female would randomly mate with any of the 58 possible males; the expected probability of her mating with a male from her own genetic cluster should be proportional to the percentage of the males that are assigned to that cluster. In our data set, the frequency of males assigned to cluster B is 34.5% (Table 10), so the expectation is that a cluster B female should mate with a cluster B male 34.5% of the time. In contrast, observed data for single male/female pairings revealed that cluster B females mated with cluster B males 100% of the time (Table 8). Furthermore, the trend among cluster B individuals was particularly pronounced. When the binomial probability of intra-cluster pairings vs. inter-cluster pairings was calculated, all six females assigned to cluster B showed intra-cluster mating. The probability of this occurring by chance six times in six trials is 0.0015% and supports a consistent trend of within-cluster mating. Overall, this pattern occurred in seven of eight single male parings and three of six multiple male pairings and strongly suggests that mating may not be random with respect to cluster membership.

DISCUSSION

Snakes, once perceived as intractable research subjects, are actually quite amenable to population genetic analyses. This has simultaneously reinforced the need for combined ecological and genetic data so as to more efficiently decipher true patterns of social mating systems in these and other organisms. In this study, three years of observational data on spatial and reproductive ecology (Smith 2007; Smith et al. 2009) were augmented by microsatellite DNA analysis to examine genetic structure and relatedness among a geographically isolated population of Northern Copperhead (*A. contortrix*) in Connecticut. Molecular analyses document a complex behavioral and social mating system in this pitviper.

How is the Population Structured?

Diversity and differentiation were examined using a number of complementary analyses and the findings were somewhat surprising. Limited overall genetic variation was expected for the population, given its isolated location on the fringe of the range. Such populations are likely to be more monomorphic, have lower levels of heterozygosity and allelic diversity and be at increased risk of inbreeding and genetic drift (Blows & Hoffmann 1993). This is in sharp contrast to populations at centers of distributions, where more preferable habitat enhances polymorphism, effective population sizes, gene flow and colonization. However, despite these predictions, the study population of Northern Copperhead is relatively diverse genetically, with three distinct and stable genetic clusters that point to the existence of a non-random mating pattern.

The Bayesian assignment test identified sufficient differentiation to partition genotypes into three distinct gene pools (i.e., genetic clusters), but genetic affiliation did not reflect den affiliation. All three gene pools were represented in nearly every den, and each could also be detected throughout the population. The results of the Fisher's exact test did not allow rejection of the null hypothesis of independence, so we cannot assume that there is any non-random association among dens and genetic structure. Additionally, F_{ST} analysis found no evidence of genetic structure associated with den. If there was an influence of den structure on patterns of mating it could not be inferred with the data available. Although spatial structure was lacking, the genetic structure maintained within the population implies the existence of a non-random pattern generated by some aspect of natural history other than den affiliation.

Distribution of genetic clusters hints at potential gene flow among dens. Genotypes assigned to cluster R appear to migrate to other dens along the southwest side of Bradley Reservoir, crossing over the reservoir into Middle Chauncey. Genotypes from cluster B show a similar effect with individuals migrating from the northwest to the northeast point of the reservoir, again crossing over the reservoir into c29 Chauncey Side. Interestingly, all five dens contained genotypes from cluster G with the largest assignment (71%) to the southeast side of the reservoir and the smallest (14%) in the north.

Do Paternity Tests Reflect a Mating Pattern?

For paternity analysis this study included several advantages: (a) high probability that the population had been fully sampled due to presence of geographic barriers enclosing the study area (Smith et al. 2009); (b) continuous observation of individuals from departure to arrival at

hibernacula over a three-year period (Smith 2007); and (c) a discrete breeding season. These supported the assumption (above) that a large percentage of the candidate paternal population (95%) was *a priori* genotyped, and thus part of the existing data set.

Results from paternity assignment were evaluated so as to uncover mechanisms responsible for the maintenance of the observed genetic clustering patterns. Paternity analysis distinctly identified all individuals in the population and assigned paternity to 14 of the 20 (70%) clutches with known mothers ("*assigned neonates"*). In some cases, multiple fathers were assigned to clutches, confirming the presence of multiple paternity in this species.

There was also strong evidence for nonrandom mating associated with these 14 clutches. However, it is unclear which (of several) social or genetic processes led to a lack of spatial structure. One factor may be some level of cryptic developmental incompatibility between cluster-specific genotypes, or even some type of assortative mating strategy [a pattern or preference of mating as defined by Burley (1983)] through which individuals display preferential mate choices.

Identifying the specific processes behind these preferences can provide clues to a more comprehensive understanding of mating behavior. There is considerable evidence that behavioral mechanisms do exist by which individuals discriminate among potential mates. Interest in such behaviors can be traced back to Darwin's (1871) theory of sexual selection, and has since had strong influence on the fields of evolutionary biology and population genetics. Assortative mating has been previously demonstrated in snakes, for example, when male mate choice was

based on female body size (Shine & Bonnet 2000; Shine 2003). Similar social and mating behaviors have been observed in other reptiles such as Sand Lizards (*Lacerta agilis*) where assortative patterns were again based on female body size (Olsson & Madsen 2001). Birds (Freeburg 1996; MacDugall & Montgomerie 2003), arthropods (Crespi 1989) and fishes (Foote & Larkin 1988) also exhibit various forms of assortative mating.

The above studies clearly show a pattern of mate choice. However, assortative mating is difficult to confirm because, in many cases, the controlling mechanism is often inferred or even unknown (Burley 1983). This is also true for this study. Although a very clear pattern of intra-cluster mating is deduced from the genetic data, the causation underlying this phenomenon remains obscure. Further, such inferences must be made cautiously, particularly when examining a smaller, outlying population from a broader distribution, and one within which atypical mating behavior is hypothesized.

Caveats

The genetic structure found among clusters of Northern Copperhead was statistically supported, but with the caveat that a small number of microsatellite loci was used, thus limiting cluster assignment and an evaluation of fine-scale genetic diversity. The genetic perspective was instead coarse-grained and the results can be more appropriately couched as hypotheses awaiting additional genetic and/or population data. Five microsatellite loci provided each individual with a distinct genotype and confirmed identities and relationships among mother/offspring and siblings. The molecular data also identified the genotypes of the true father(s). However, as one would expect, paternal determination was ambiguous in cases where DNA quality was poor or

sampling incomplete. Certainly, molecular results would be more robust if additional loci were used and complete genotypes could have been obtained for all of the 254 blood samples.

Sampling of the population could be performed in a number of ways; genotypes were partitioned into three alternative data sets and analyses were carried out to ascertain that no bias was present. For example, one must consider that there is a kinship structure to any natural sampling scheme and the addition of offspring could impart analytical biases driven by kin groups; the observed differentiation among genetic clusters would be inflated. However, if this were the case, one would expect partitioning of genotypes into numbers of genetic clusters close to number of clutches (e.g., K=20 with 20 clutches). Existing limitations of this molecular data set include low numbers of loci and small sample sizes. By using the data set "*all samples*" (a combination of all adults and neonates) the number of genotypes available for analyses was nearly doubled. Therefore it seems prudent to include all genotypes, simply because it increases the power of the Bayesian assignment test to detect subtle genetic structure.

The abundant ecological data (Smith 2007; Smith et al. 2009) also offered an opportunity to associate spatial and genetic information. However, radio telemetric data was not gathered for successfully mated pairs (since this cannot be *a priori* known when individuals are selected for transmitter implants), tracked individuals were a small fraction of the population (a problem inherent to radio-tracking studies), and the sampled area was limited. This molecular study also represents but a single population located at the most northern extent of the species' geographic range. This makes it difficult to associate molecular data generated in this study with habitats and social environments typical of other Northern Copperhead, much less the remaining subspecies

of *A. contortrix*. Optimally, results of this study could be contrasted with data derived from other populations of Northern Copperhead at the periphery of the range. This underscores the importance of expanding future studies in both scope (i.e., multiple populations) and geography (multiple sites) across the species range.

Conclusions

This study examined several questions about diversity and differentiation in a small population of Northern Copperhead by combining ecological and genetic data to augment insights into the natural history of this species. Findings illustrate how genetic analyses can provide data previously unavailable to behavioral ecologists. The molecular markers used herein provide a measure of individual reproductive success which is an important step toward resolving key features of mating behavior, to include estimates of multiple paternity. The patterns of genetic diversity discovered in this study demonstrate that genetic differentiation may not always be distributed according to den affiliation. Genetic structure may in fact, be influenced more strongly by developmental and/or behavioral processes, such as non-random mating.

Distribution of genetic diversity can be used to inform conservation efforts and provoke further research on threatened and endangered species such as Timber Rattlesnake, which occurs in the same geographic location and ecological niche as the Copperhead. This study suggests previously unknown and unexpected behavioral complexities in *A. contortrix*, and should inspire further research into the processes underlying these patterns, as well as their adaptive significance.

Table 1. Overview of 254 blood samples (Tissues) collected for the genetic survey of Northern Copperhead (*Agkistrodon contortrix mokasen*) and partitioned for various analytical steps (rows). Gender could be identified for all adult individuals (Males and Females, respectively) save two individuals (Unknown). Genetic profiles (Genotypes) could be derived for 238 samples, and 197 of these represented individuals associated with one of the five main dens (Residents). For paternity assignment tests (Parentage) all males, neonates, plus two adults of unknown gender were evaluated, but only known mothers were included.

	Males	**Females**	**Unknown**	**Adults**	**Neonates**	**Total**
Tissues	56	59	2	117	137	**254**
Genotypes	55	58	2	115	123	**238**
Residents	49	48	2	99	98	**197**
Parentage	55	19	2	76	123	**199**

Table 2. Number of Northern Copperhead (*Agkistrodon contortrix mokasen*) affiliated with dens at the study site over a three-year period from 2001-2003 (Smith 2007). (A) Individuals associated with one of the five main dens were considered "*residents*"; (B) individuals associated with other sites or for which no den affiliation could be determined were considered "*non-residents*"; the latter were excluded in calculations of global statistics and spatial structure. See Figure 3 for geographic location and spatial relationship among the five main dens.

(A)

Main Dens	Adults	Neonates
Point of Lamentation	14	18
141	63	86
Middle Lamentation	7	
c29 Chauncey Side	7	
Middle Chauncey	10	7
Residents	**101**	**111**

(B)

Other Sites	Adults	Neonates
Black Pond	4	11
Old Lyme	3	
Rock Wall at Dam	1	9
Wassel Reservoir	1	
Unknown Den	7	6
Non-Residents	**16**	**26**

Table 3. Northern Copperhead (*Agkistrodon contortrix mokasen*) neonates detected at the study site over a three-year period (from Smith 2007). Provided for each year and across all years are: number of clutches (Clutches) with number of neonates within each of these clutches (Assigned Neonates) and total number of neonates (Total Neonates). In 2001, six apparently newly born individuals were found, but could not be assigned to a specific clutch (Unassigned Neonates).

	Clutches	Assigned Neonates	Unassigned Neonates	Total Neonates
2001	6	2,5,6,4,10,7	6	40
2002	8	6,8,11,6,9,13,6,5	0	64
2003	6	6,8,5,4,4,6	0	33
Totals	20	131	6	137

Table 4. Characteristics of five microsatellite DNA loci (Locus) developed for *Crotalus willardi* by Holycross et al. (2002) and used in *Agkistrodon contortrix* (this study). Listed for each locus are: fluorophore (fluorescent dye) used to label the forward primer (Dye); DNA sequence comprising the microsatellite (Motif); number of base pairs (bp) sequenced for the clone from which the locus was derived (Clone); range of allele sizes (in bp) detected across eight individuals from the study population [Allele (bp)]; number of alleles detected across all 238 genotyped individuals [Allele (N)]; and frequency of most common allele [Allele (*f*)].

Locus	Dye	Motif	Clone (bp)	Allele (bp)	Allele (N)	Allele (*f*)
*CW*06	6FAM	$(GA)_{19}$	120	100-120	4	0.56
*CW*14	6FAM	$(AC)_{24}$	160	130 -170	6	0.54
*CW*15	VIC	$(CAT)(TAT)(CAT)_{14}$	150	130-150	7	0.31
*CW*23	NED	$(TG)_{18}(AG)_{22}$	300	200-270	5	0.41
*CW*24	PET	$(CTT)_{49}$	200	240-400	16	0.19

Table 5. Genetic diversity across three genetic clusters identified from 238 Northern Copperhead (*Agkistrodon contortrix mokasen*) collected in the study site. Data were derived from genotyping five microsatellite loci. Listed for each genetic cluster is number of individuals genotyped (N), plus descriptive statistics, including inbreeding coefficient (f), average allelic richness (AAR), expected heterozygosity (H_E), observed heterozygosity (H_O), number of alleles (A), and private alleles (PA). Cluster acronyms refer to color displayed in pie charts in Figure 3.

Genetic Cluster	N	f	AAR	H_E	H_O	A	PA
Cluster B	91	-0.056	5.3	0.54	0.57	28	3
Cluster G	90	0.015	5.5	0.65	0.64	29	2
Cluster R	57	0.09	7.3	0.72	0.65	37	11

Table 6. Genetic diversity of 99 "*resident adults*" Northern Copperhead (*Agkistrodon contortrix mokasen*) collected from five main dens in the study site. Data were derived from genotyping five microsatellite loci. Listed for each den is number of individuals genotyped (N) plus descriptive statistics, including inbreeding coefficient (f), average allelic richness (AAR), expected heterozygosity (H_E), observed heterozygosity (H_O), number of alleles (A), and private alleles (PA).

Main Dens	N	f	AAR	H_E	H_O	A	PA
Point of Lamentation	13	-0.164	4	0.68	0.78	26	0
141	62	0.024	3.8	0.65	0.63	30	1
Middle Lamentation	7	0.102	4.1	0.75	0.68	24	1
c29's Chauncey Side	7	0.035	3.8	0.61	0.59	20	0
Middle Chauncey	10	0.103	4.4	0.72	0.65	26	0

Table 7. Results of Fishers exact test for allocation of 99 "*resident adults*" Northern Copperhead (*Agkistrodon contortrix mokasen*) to three identified genetic clusters (B, G, R). Samples were collected from five main dens (Table 2), and genotypes were derived from five microsatellite loci. Genetic clusters were identified based on a larger data set ("*all samples*"; N=238) using a Bayesian assignment test as implemented in program STRUCTURE, with K=3 the most stable distribution; $p = 0.0974$.

Main Dens	B	G	R
Point Lamentation	4	6	3
141	29	25	8
Middle Lamentation	3	3	1
c29 Chauncey Side	2	5	0
Middle Chauncey	0	7	3

Table 8. Representation of genetic clusters in single male/female pairings in Northern Copperhead (*Agkistrodon contortrix mokasen*). Paternal genotypes were derived from genotypes of known mother/offspring couplets and matched with males in the population using CERVUS. Letters were used to represent family groups after paternity analysis (Male/Female Pairing). Genetic clusters were identified using Bayesian assignment tests and letters reflect assignment of maternal (Mother Cluster) and paternal (Father Cluster) genotypes to one of three gene pools: B, G or R (see Figure 3).

N Offspring	Male/Female Pairing	Mother Cluster	Father Cluster
10	♀♂*A*	*G*	*G*
6	♀♂*D*	*B*	*B*
5	♀♂*F*	*B*	*B*
6	♀♂*J*	*B*	*B*
6	♀♂*M*	*B*	*B*
4	♀♂*S*	*B*	*B*
4	♀♂*R*	*B*	*B*
5	♀♂*N*	*G*	*R*

Table 9. Representation of genetic clusters in multiple male/single female pairings in Northern Copperhead (*Agkistrodon contortrix mokasen*). Paternal genotypes were derived from genotypes of known mothers/offspring couplets and matched with males in the population using CERVUS. Letters were used to represent family groups after paternity analysis (Male/Female Pairing). Genetic clusters were identified using Bayesian assignment tests and letters reflect assignment of maternal (Mother Cluster) and each paternal (Father A Cluster and Father B Cluster) genotypes to one of three gene pools: B, G or R (see Figure 3). For some paternal genotypes, no known male could be assigned (?).

N Offspring Father A	N Offspring Father B	Male/Female Pairing	Mother Cluster	Father A Cluster	Father B Cluster
4	*2*	♀♂♂*T*	*G*	*G*	*G*
2	*3*	♀♂♂*Q*	*G*	*B*	*?*
4	*2*	♀♂♂*O*	*G*	*B*	*?*
5	*1*	♀♂♂*H*	*G*	*G*	*?*
9	*4*	♀♂♂*L*	*R*	*G*	*?*
6	*2*	♀♂♂*P*	*G*	*G*	*?*

Table 10. Frequency of male/female pairings by genetic cluster in Northern Copperhead (*Agkistrodon contortrix mokasen*). Three genetic clusters (Cluster) were identified using Bayesian assignment tests (see Figure 3 and Table 5). Provided is assignment of 58 potential males to a particular genetic cluster (Males). If mating was independent of genetic cluster, paternal genotypes would proportionally represented genetic clusters (Expected Probability). However, observed representation of paternal genotypes of assigned to a particular genetic cluster in single male/female pairings (SMFP) and multiple male/female pairings (MMFP) deviate from random expectations. For one of the 58 males collected at the study site, no genotype could be derived and assignment to a genetic cluster is unknown (No cluster).

Cluster	Males	Expected Probability	Observed SMFP	Observed MMP
Blue	20	34.5%	100.0%	0.0%
Green	23	39.7%	50.0%	40.0%
Red	14	24.1%	0.0%	0.0%
No cluster	1	1.7%	0.0%	0.0%

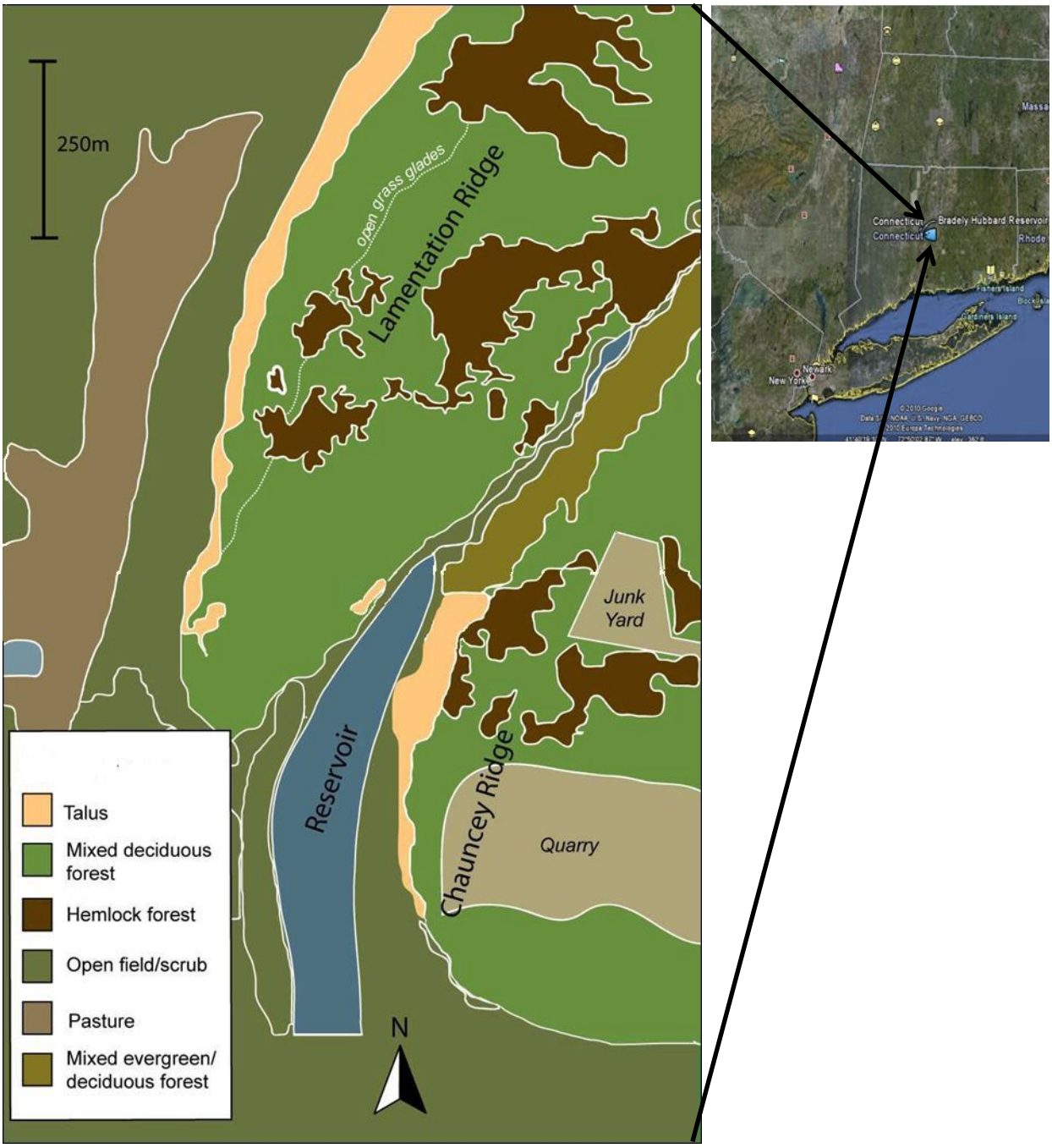

Figure 1. Location of study site in Connecticut. Insert depicts physiographic map of the study area (modified after Smith et al. 2009), showing land coverage/vegetation type (explained in legend), and quarry site in reference to reservoir.

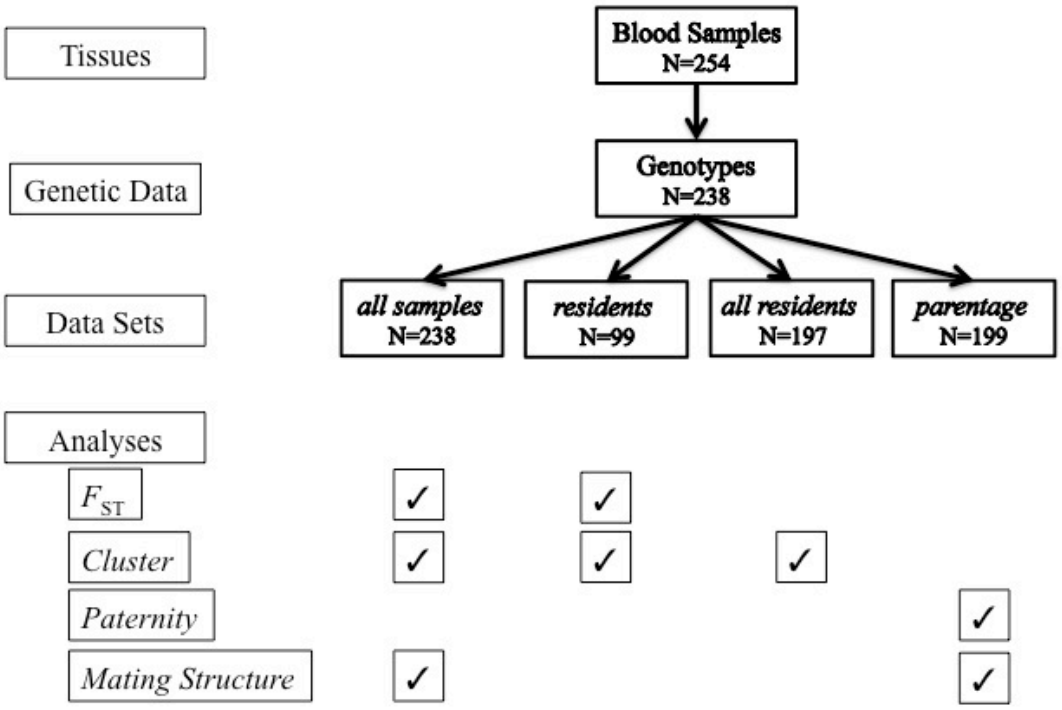

Figure 2. Flow chart of samples and genetic data used in this study. Tissues consisted of 254 blood samples collected from Northern Copperhead (*Agkistrodon contortrix mokasen*) in Connecticut (Smith 2007). Genetic data were generated by extracting genomic DNA from blood and screening across five microsatellite loci, resulting in 238 genotypes. Individual genotypes were associated with ecological data from Smith (2007) and partitioned into four data sets for statistical evaluation. Analyses examined genetic variation (F_{ST}), genetic structure (*Cluster*), parentage (*Paternity*) and assortative mating (*Mating Structure*) by corroborating paternity with genetic assignment. See Table 1 for composition of data sets.

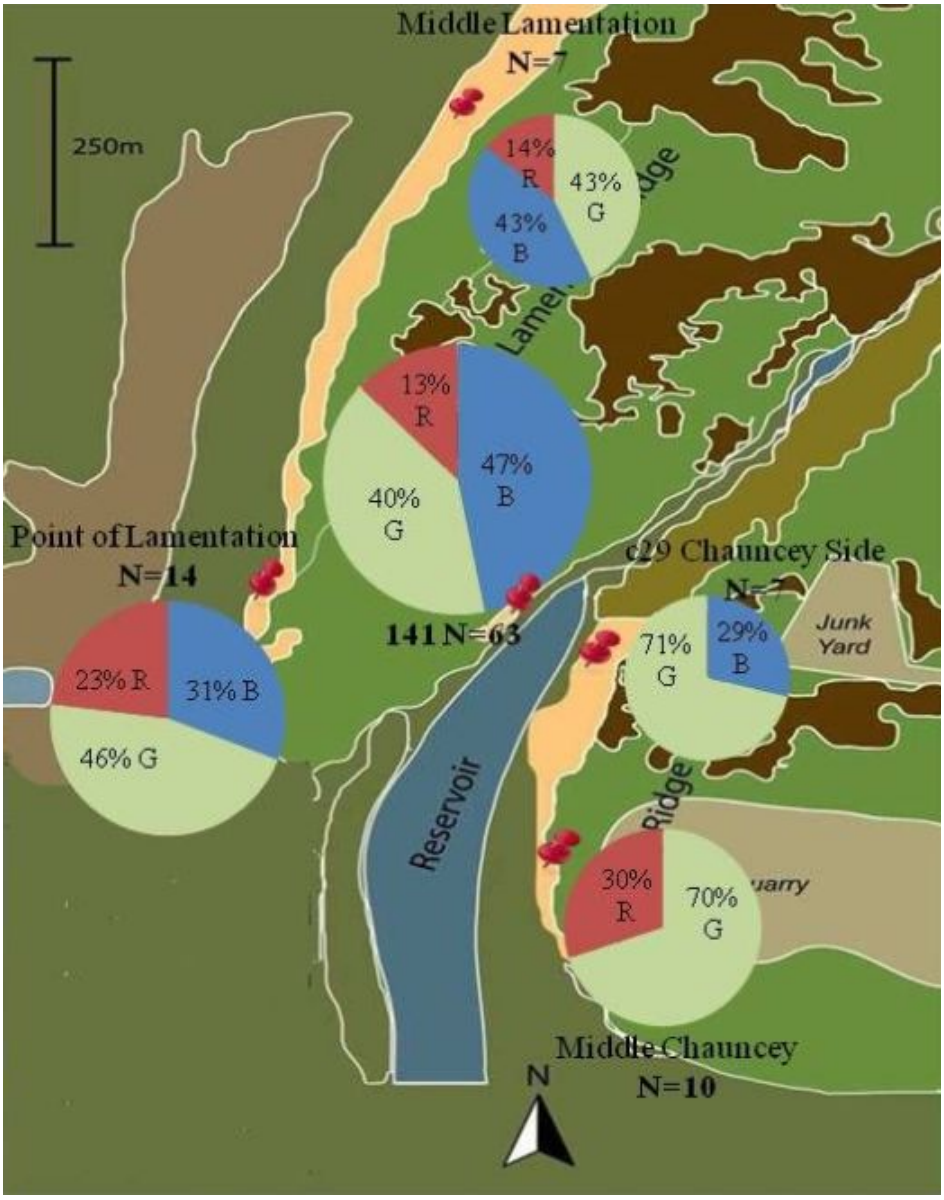

Figure 3. Locations of five main dens at the study site. Shown for each den are name and number of resident adults associated with that particular den. A pie chart reflects proportional assignment of adults at each den to three genetic clusters identified via a Bayesian assignment test. Genetic clusters are referred to by the color applied in the pie chart: B=Blue, G=Green, and R=Red.

LITERATURE CITED

Avise JC (2004) *Molecular markers, natural history and evolution*. Sinauer Associates, Sunderland, Massachusetts.

Blows MW, Hoffmann AA (1993) The genetics of central and marginal populations of *Drosophila serrata. I*. Genetic variation for stress resistance and species borders. *Evolution*, **47**, 255-1270.

Bonnet X, Lourdais O, Shine R, Naulleau G (2002) Reproduction in a typical capital breeder: costs, currencies, and complications in the aspic viper. *Ecology*, **83**, 2124-2135.

Burley N (1983) The meaning of assortative mating. *Ethology and Sociobiology*, **4**,191-203.

Bushar LM, Reinert HK, Gelbert L (1998) Genetic variation and gene flow within and between local populations of the Timber Rattlesnake, *Crotalus horridus*. *Copeia*, **1998**, 411-422.

Clark RW (2004) Kin recognition in rattlesnakes. *The Royal Society Biology Letters,* **271**, 243-245.

Clark RW, Brown WS, Stechert R, Zamudio KW (2008) Integrating individual behavior and landscape genetics: the population structure of timber rattlesnake hibernacula. *Molecular Ecology*, **17,** 719-730.

Crespi B (1989) Causes of assortative mating in arthropods. *Animal Behavior*, **38**, 980-1000.

Darwin CR (1871) *The descent of man and selection in relation to sex.* Vol 2. John Murray, London, England.

Douglas ME, Douglas MR, Schuett GW, Porras LW (2006) Evolutions of rattlesnakes (Viperidae; *Crotalus*) in the warm deserts of western North America shaped by Neogene vicariance and Quaternary climate change. *Molecular Ecology*, **15**, 3353-3374.

Duvall D, Schuett GW, Arnold SJ (1993) *Snakes: Ecology and Behavior*. McGraw-Hill, New York, New York.

Elmer KR, Davila JA, Lougheed SC (2007) Applying new inter-individual approaches to assess fine-scale population genetic diversity in a neotropical frog, *Eleutherodactylus ockendeni*. *Heredity*, **99**, 506-515.

Emlen ST, Oring LW (1977) Ecology, sexual selection and the evolution of mating systems. *Science*, **197**, 215-223.

Ernst CH, Ernst EM (2003) *Snakes of the United States and Canada*. Smithsonian Books, Washington, D.C.

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software structure: a simulation study. *Molecular Ecology*, **14**, 2611-2620.

Excoffier L, Heckel G (2006) Computer programs for population genetics data analysis: a survival guide. *Nature Review Genetics*, **7**, 745-758.

Fitch HS (1960) *Autecology of the Copperhead*. Museum of Natural History, University of Kansas, Lawrence, Kansas.

Foote CJ, Larkin PA (1988) The role of male choice in assortative mating of anadromous and non-anadromous sockeye salmon (*Oncorhynchus nerka*). *Behavior*, **106,** 43-62.

Freeburg TM (1996) Assortative mating in captive cowbirds is predicted by social experience. *Animal Behavior*, **52**, 1129-1142.

Gibbs LH, Weatherhead PJ, Boag PT, White BN, Tabak LM, Hoysak DJ (1990) Realized reproductive success of polygynous Red-Winged blackbirds revealed by DNA markers. *Science*, **250,** 1394-1396.

Goudet J, (1995) FSTAT (Version 1.2): A computer program to calculate *F*-statistics. *Journal of Heredity*, **86**, 485-486.

Graves BM, Duvall D. (1990) Spring emergence patterns of wandering garter snakes and prairie rattlesnakes in Wyoming. *Journal of Herpetology*, **24**, 351-356

Holycross AT, Douglas ME, Higbee JR, Bogden RH (2002) Isolation and characterization of the microsatellite loci from a threatened rattlesnake (New Mexico Ridge-nosed Rattlesnake, *Crotalus willardi obscures*). *Molecular Ecology Notes*, **2**, 537-539.

Huelsenbeck JP, Andolfatto P (2007) Inference of population structure under a dirichlet process model. Genetics, **175**, 1787-1802.

Hughes C (1998) Integrating molecular techniques with field methods in studies of social behavior: a revolution results. *Ecology*, **79**, 383-399.

Lane A, Shine R (2011) Intraspecific variation in the direction and degree of sex-biased dispersal among sea-snake populations. *Molecular Ecology*, **20**, 1870-1976.

Lewis PO, Zaykin D (2001) Genetic Data Analysis: Computer program for the analysis of allelic data. Version 1.0 (d16c). Free program distributed by the authors over the internet from http://lewis.eeb.uconn.edu/lewishome/software.html.

Krebs JR, Davies NB (1997) *Behavioral Ecology: An Evolutionary Approach*. Blackwell Scientific, Oxford, England.

MacDugall AK, Montgomerie R (2003) Assortative mating by carotenoid-based plumage colour: a quality indicator in American goldfinches, *Carduelis tristis*. *Naturwissenschaften,* **90**, 464-467.

Marshall TC, Slate J, Kruuk L, Pemberton JM (1998) Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular Ecology*, **7**, 639-655.

McCracken GF, Burghardt GM, Houts SE (1999) Microsatellite markers and multiple paternity in the garter snake *Thamnophis sirtalis*. *Molecular Ecology*, **8**, 1475-1479.

Nature Serve. 2007. Nature Serve Explorer: An online encyclopedia of life [web application]. Version 6.2. NatureServe, Arlington, Virginia. http://www.natureserve.org/explorer

Olsson M, Madsen T (2001) Promiscuity in sand lizards (L*acerta agilis*) and adder snakes (*Vipera berus*): causes and consequences. *American Genetic Association,* **92**, 190-197.

Pritchard JK, Stephens P, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics,* **155**, 945-959.

Prior KA, Gibbs LH, Weatherhead PJ (1997) Population genetic structure in the black rat snake: Implications for management. *Conservation Biology,* **11**, 1147-1158.

Prosser MR, Gibbs LH, Weatherhead PJ (1999) Microgeographic population genetic structure in the northern water snake, *Nerodia sipedon sipedon* detected using microsatellite DNA loci. *Molecular Ecology,* **8**, 329-333.

Prosser MR, Weatherhead PJ, Gibbs LH, Brown GP (2002) Genetic analysis of the mating system and opportunity for sexual selection in northern water snakes (*Nerodia sipedon*). *Behavioral Ecology,* **13**, 800-807.

Rosenberg NA, Pritchard JK, Weber JL, Cann HM, Kidd KK, Zhivotovsky LA, Feldman MW (2002) Genetic structure of human populations. *Science,* **298**, 2381-2385.

Shine R (2003) Reproductive strategies in snakes. *Proceedings of the Royal Society of London B,* **270**, 995-1004.

Shine R, Bonnet X (2000) Snakes: a new 'model organism' in ecological research? *Trends in Ecology and Evolution,* **15**, 221-222.

Smith CF (2007) *Sexual Dimorphism, and the Spatial and Reproductive Ecology of the Copperhead Snake, Agkistrodon contortrix*. University of Connecticut. Connecticut: United States. ProQuest Dissertations & Theses (PQDT). Web. 12 Sep. 2011.

Smith CF, Schuett GW, Earley RL, Schwenk K (2009) The spatial and reproductive ecology of the copperhead (*Agkistrodon contortrix*) at the northeastern extreme of its range. *Herpetological Monographs,* **23**, 45-73.