CONSERVATION AND MANAGEMENT OF MIDGET FADED RATTLESNAKES



STATE WILDLIFE GRANT FINAL REPORT

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Executive Summary

Midget faded rattlesnakes (*Crotalus oreganus concolor*) have a Wyoming distribution that is restricted to the vicinity of the Flaming Gorge Reservoir and is potentially threatened from impending energy development in the area. Previous research has identified important aspects of midget faded rattlesnake life history and ecology but additional information is needed regarding current population status, specific locations of important habitat for rattlesnakes, and factors affecting connectivity among populations. To this end, we had four objectives:

- 1) Use previous observations to develop a fine-scale model of denning habitat across the entire Wyoming range of the subspecies.
- 2) Use previous radio-telemetry data to develop a fine-scale model of foraging habitat.
- 3) Use tissue samples collected in 2000-2002 and 2009-2010 to conduct a landscape genetic analysis to assess levels of genetic diversity, population subdivision, and identify environmental factors influencing population connectivity.
- 4) Continue demographic monitoring of focal dens to assess current status of midget faded rattlesnakes with respect to number of individuals, size distribution at den sites, and percentage of gravid females.

We used several candidate models and independent validation surveys to build a model of den habitat; this model had high accuracy (85%) and contained only two variables (distance to rock outcrop and annual temperature range). We also produced a model of foraging areas based on past radio-telemetry points that identified distance to rock outcrop and mean growing season temperature as an important variable influencing foraging habitat. Genetic data indicated that midget faded rattlesnake populations have lower genetic diversity than most other rattlesnake populations, effective population sizes less than 50, and evidence of an historic bottleneck. We detected four genetic population clusters across the area, and three sites across the area appeared to be important source sites. We tested the correlation of several landscape variables with rattlesnake gene flow at several spatial scales. We found that distance and growing season precipitation were significant in models at all scales; these are the same variables found in the foraging model and provide an independent validation of the model. Roads, growing season precipitation, and terrain ruggedness were important variables at fine scales, whereas percent bare ground and transformed aspect were selected at broader spatial scales. We used our resistance surface to develop a current map of gene flow. Highest connectivity exists around the main reservoir, with lowest connectivity north of I-80. More survey data are needed to determine the status of populations in the Little Mountain area, an area of importance for both energy development and wildlife conservation. Our demographic monitoring identified a strongly female-biased sex ratio, which may be partially explained by a reduction in adult male rattlesnakes. Fecundity was relatively high, with approximately 50% of adult females gravid. Overall, our results suggest that midget faded rattlesnakes are susceptible to disturbance, and that roads are likely to have the most widespread impact on rattlesnakes. We recommend that the den model be used to protect dens from direct disturbances, and to minimize roads and road

traffic in important rattlesnake habitat, as well as continued surveys to monitor population demography and identify dens on the eastern side of the reservoir.

1. Introduction and Objectives

Midget faded rattlesnakes (*Crotalus oreganus concolor*) are a subspecies of the western rattlesnake limited to the Colorado Plateau region of Wyoming, Colorado and Utah, and in Wyoming are only found in Sweetwater County surrounding the Flaming Gorge Area. It is listed as a NSS1 species of greatest conservation need in the 2010 Wyoming state wildlife action plan (Wyoming Game and Fish Department 2010). It has several characteristics that distinguish it from other western rattlesnakes. These include small size, year-round presence at rocky denning habitat by some individuals, and a neurotoxic venom component that does not change from juvenile to adult (Mackessy et al. 2003; Parker 2003). The snakes appear to occur in lower densities than other rattlesnakes, and are probably more spatially restricted in movements due to their small size.

Energy development (primarily oil, gas and wind) is expanding across southern Wyoming and has the potential to influence wildlife populations and their habitats (Copeland et al. 2009). This is particularly true around the area of the Flaming Gorge Reservoir in Sweetwater County. This area represents lands primarily managed by the Forest Service (as the Flaming Gorge National Recreation Area), Bureau of Land Management, and some private ownership (generally managed for grazing interests). Aside from land managed as the recreation area, the Flaming Gorge region is being considered and/or leased for energy development, although a large portion of the area is yet to be developed. In addition to the direct impact of the drilling/installation sites, all forms of energy development have a larger footprint mainly characterized by a network of roads used for access. Therefore, if not properly planned, energy infrastructure could strongly impact wildlife populations. In the case of midget faded rattlesnakes, there is concern related to how energy exploration will directly disrupt den habitat as well as indirectly reduce connectivity, but models of den habitat or connectivity corridors are currently lacking.

Although we are not aware of any studies examining the direct impact of energy development on snake populations, there are certainly known life history characteristics of midget faded rattlesnakes that suggest they are highly susceptible to energy development. Midget faded rattlesnakes, like many northern temperate snakes, rely on communal hibernacula for overwintering habitat. In the case of midget faded rattlesnakes, these dens are rock outcrops that are relatively small in size and limiting across the landscape. In addition to overwintering habitat, denning areas are critical habitat for shedding, gestation, parturition, and year-round residence for many gravid females and juveniles (Parker and Anderson 2007). Individuals will typically use the same denning areas every year. Therefore, disruption to the denning habitat has a high probability of eliminating most if not all of the individuals that use that den. Second, roads can lead to high mortality and fragmentation across snake populations (Andrews et al. 2006). For instance, Jochimsen (2006) demonstrated high mortality (93% of all snakes observed on roads) in southeast Idaho, an assemblage that included the closely related Great Basin rattlesnake (*Crotalus oreganus lutosus*). Roads are most likely to adversely affect rattlesnake populations when they occur across the migratory corridors adult midget faded rattlesnakes use

to reach foraging and mating grounds, or if they occur directly through foraging and mating habitat. Disturbance to migratory or foraging habitat would not have the same instant effect of den destruction, but would likely lead to fragmentation and population declines over time.

The majority of our knowledge on midget faded rattlesnakes in Wyoming came from dissertation research conducted by Josh Parker on these populations in 2000-2002 (Parker 2003; Parker and Anderson 2007; Oyler-McCance and Parker 2010a), although there has been additional research on the ecology of this subspecies (Ashton and Patton 2001; Ashton 2003a-b; Mackessy et al. 2003). Such research demonstrates that each den typically has smaller numbers of individuals compared to other rattlesnake species (< 100 individuals); individuals have a 1-2 month long staging period around the den area that includes shedding aggregations. After this staging period, adult males and non-gravid females typically move away from the den to active foraging and mating areas, typically around 1 kilometer from the den. Gravid females and juveniles typically remain within 100 meters of the den. Individuals almost always return to the same den each year. Finally, an initial genetic study (Oyler-McCance and Parker 2010a) demonstrated genetic subdivision across the areas, with dens near disturbed areas more genetically different than others. However, there was no evidence that the Flaming Gorge reservoir genetically divided rattlesnake populations. As part of this research, Parker collected 426 tissue samples.

Our research expanded and took advantage of this previous research to address objectives that would provide managers with the necessary information to successfully protect populations of midget faded rattlesnakes under the threat of landscape development. We had four main objectives:

- 1) Use previous observations to develop a fine-scale model of denning habitat across the entire Wyoming range of the subspecies.
- 2) Use previous radio-telemetry data to develop a fine-scale model of foraging habitat.
- Use tissue samples collected in 2000-2002 and 2009-2010 to conduct a landscape genetic analysis to assess levels of genetic diversity, population subdivision, and identify environmental factors influencing population connectivity.
- Continue demographic monitoring of focal dens to assess current status of midget faded rattlesnakes with respect to number of individuals, size distribution at den sites, and percentage of gravid females.

2. Materials and Methods

2.1. Field and Demographic Methods

We sampled snakes at known den locations (based on Parker 2003) across the Flaming Gorge Area in 2010 and 2011. We focused our efforts on ten den sites, although we also sampled snakes opportunistically at other sites. For each snake captured, we recorded sex, number of rattles, pattern of blotches (for individual ID), any apparent prey items, number of enlarged follicles, snout-vent length, total length and mass. We inserted a PIT tag into each individual and collected at least $10 \ \mu$ l of blood from the caudal vein for genetic analysis. We also recorded UTM location coordinates for each individual. We visited each den site multiple times, although number of sites visits was not standardized.

2.2. Den Modeling Methods

We used known den site locations collected by Parker (2003), the Wyoming Game and Fish species database, and individual observations at dens to develop models of den habitat. In collaboration with the Wyoming Natural Diversity Database (WYNDD), we used the maximum entropy modeling method, MAXENT 3.3.2 (Phillips et al. 2006, Elith et al. 2010), to build models of den habitat. MAXENT requires only presence data and is a statistical method that outputs a continuous probability layer based on the distribution of environmental layers that best describe the characteristics of species locations. MAXENT has been demonstrated to be one of the best modeling methods for describing species distributions (Elith et al. 2006). This is also the modeling method preferred by WYNDD for their modeling efforts. We initially used a large number of environmental variables developed by WYNDD. These variables can be broken down into a number of categories including terrain, landscape structure, land cover, soil/bedrock, hydrology, and climate. These are all types of variables that could be relevant to midget faded rattlesnake habitat use.

While MAXENT is a powerful modeling tool, it (like other modeling approaches) is very dependent on the observational data being used for the model. For instance, if many observations are collected from a small area, but the large number of observations is simply due to more intense sampling effort in that area, then the model will be biased toward the environmental values in this small area. On the other hand, a greater number of observations in an area may truly reflect the actual abundance, and the environmental characteristics at that site should be reflected in the final model. Therefore, we developed four models based on different sets of observations. The observations per se) (N = 46), 2) all den points and all snake observations at dens (N = 170), 3) den observations spatially filtered such that only 1 observation per 1 kilometer radius is allowed (N = 21), and 4) all snake observations (whether known to be a den site or not), filtered by 1 km (N = 67). We chose to include this fourth model because some of the database observations likely occurred at unconfirmed dens and we wanted to simulate the typical modeling effort of using all observations; this would allow us to understand whether our focused den modeling effort provides greater insight than a typical modeling effort.

Because MAXENT requires only presence data, it must generate "pseudoabsences" to take the place of true absence data. The pseudoabsences typically are random points across the study area that describes the available environmental conditions and are therefore necessary to model the range of environmental variables that correlate best with observations. The choice of pseudoabsences can also bias the final model, especially if available points are sampled from areas that have not been sampled. Therefore, to ensure that the pseudoabsences represented areas that were actually sampled, we created a polygon that enclosed all snake observations, and randomly selected 10,000 points within this polygon. MAXENT then extrapolates the model to predict across the entire study area. Finally, we were interested in understanding what environmental variables best described denning habitat. Therefore, we narrowed our models to reflect the ten most explanatory variables for each model. We did this by first running a model with all potential environmental variables, and selected the 20 variables that had the highest variable contribution to the model. We then reran this model with the 20 variables and iteratively reduced the number of variables to 10 by stepwise elimination of the variable that was most redundant, and thus the models would lose the least amount of gain by elimination of that variable.

Once a 10 variable model was developed for each of the four observation sets, we conducted a field validation study in July and August 2010 to test whether our den models accurately represented denning habitat, and which of the four models performed best. To allow us to test all four models simultaneously, we created an ensemble model of all four models. To do this, we first converted the continuous den models to a binary habitat model. MAXENT estimates a number of statistical thresholds that can be used to convert a continuous model to a categorical or binary model. We used the maximum training plus specificity threshold (suggested by WYNDD) to create the binary models. We then added together four binary models to create a categorical model with five categories that indicated the number of candidate models that predicted den habitat. We conducted our validation surveys using a random stratified design based on the ensemble model. We randomly selected twelve rock outcrops (based on a topographic model) in each category, for a total of 60 validation sites. At each site, we searched for two man-hours looking for rattlesnakes or shed skin. We took advantage of the fact that gravid female and juvenile midget faded rattlesnakes remain at the den site year-round, and individuals remain very near the den to shed. Therefore, in July and August we can confirm den sites by the presence of gravid females, juveniles, or shed skins. To increase the chances of successful detection of snakes at den sites, we only searched during peak hours for snake activity, which in mid-summer is morning.

We then created an error matrix for each of the four candidate models based on the validation surveys. An error matrix is a four cell table that classifies the number of correct predictions (both correct presences and absences) as well as the number of incorrect predictions (Jenkins et al. 2009). We restricted the data for our error matrix to be from sites that actually contained suitable habitat, as a small number of random points did not actually contain outcrops suitable for snake hibernacula. From this error matrix, we calculated four summary statistics that have been used to assess model accuracy. These metrics included overall model accuracy (# correct predictions / total sites visited), sensitivity (proportion of presences that were correctly predicted), specificity (proportion of absences that were correctly predicted), and an overall summary metric, the true skill statistic (TSS; calculated 1 – sensitivity + specificity). We calculated each of these metrics for different threshold cutoffs for each model to determine the best categorical threshold. We ranked each model for each of the four metrics and determined which set of observation points best described den habitat. In the process of our validation study,

we became aware of the successful adaptation of AIC model selection to MAXENT models (Warren and Seifert 2011). Therefore, for our final model based on the validation study, we used AIC to choose the model with the best set of environmental variables.

2.3. Foraging Modeling Methods

We were also interested in developing a model of foraging habitat for rattlesnakes based on the radio-telemetry data of Parker and Anderson (2007). We used similar methods as with the den model to create the foraging model. However, we were unable to field validate the foraging model as it would have required intensive radio-telemetry to verify. Furthermore, although we had 45 potential radio telemetry points in core foraging areas, we had to reduce the number of observations to nine for the final modeling. This was to reduce autocorrelation of points and spatial bias. For instance, we only used one point per individual (selected randomly) because multiple points for each individual snake are not independent. Second, some individual snakes may have used similar areas, and therefore we filtered out points that were very close to another point. Although this limits our sample size, MAXENT has been successfully used with small samples size (Pearson et al. 2007). We selected pseudoabsence data using the same method as for the den model. We used AIC to select the environmental variables in the final model. To create a binary foraging model, we used the statistical threshold that was best supported based on the den model validation, as we did not have validation data to identify a threshold empirically.

2.4. Genetic Laboratory Methods

We used blood samples collected both by Parker (2003) and by us in 2009-2010. Therefore, some of the samples are the same as used in Oyler-McCance and Parker (2010a), but we have also added a large number of additional samples. For samples that had not already had DNA extracted by Oyler-McCance and Parker (2010a), we used the Qiagen DNEasy Blood and Tissue Extraction Kit (Qiagen, Inc.). We used 19 different microsatellite loci as genetic markers for the study. Microsatellite genetic markers are excellent for fine scale genetic studies because they are highly variable due to increased mutation rates and therefore tend to develop differences much sooner than other genetic markers. Therefore, if populations have been recently fragmented, microsatellites have the greatest chance of detecting those differences. The 19 loci we used were taken from four different sources based on three different species. Thirteen of the loci were developed specifically for midget faded rattlesnakes (Oyler-McCance et al. 2005, Oyler-McCance and Parker 2009b), four were originally developed for timber rattlesnakes (Crotalus horridus; Villarreal et al. 1996), and the remaining two were originally developed for ridge-nosed rattlesnakes (Crotalus willardi; Holycross et al. 2002). We used the Qiagen Multiplex PCR kit to run PCR reactions on all nineteen loci into four reactions. We present the protocols for each multiplex in Appendix 1. PCR products were then run on an ABI 3130x1 automated sequencer (Applied Biosystems, Inc.) and genotyped using ABI GENEMAPPER software. All laboratory analyses were conducted in the Laboratory of Ecological, Evolutionary, and Conservation Genetics (LEECG) at the College of Natural Resources at University of Idaho.

To ensure consistency of genotypes and data quality, we randomly selected 10% of sampled individuals to rerun.

2.5. Preliminary Genetic Analyses

We first tested for the presence of null alleles using the software FREENA (Chapuis and Estoup 2007). Null alleles occur occasionally in microsatellite loci and are defined by allele(s) that do not amplify during PCR, leading to apparent homozygotes or individuals that do not produce PCR product at that locus. We eliminated loci from further consideration if the estimated frequency of null alleles was greater than 0.1. Our next step was to identify any related individuals (siblings or parent-offspring). We expected the presence of highly related individuals because of our focal den sampling. It is important to identify closely related individuals before continuing with genetic analysis because their presence can lead to violations of population genetic assumptions and can bias results so that less gene flow is inferred than actually occurs. We used the software COANCESTRY (Wang 2010) to identify individual pairs with a relatedness of 0.5 or greater. This level of relatedness is characteristic of siblings and parent/offspring pairs. For each pair of related individuals identified, we randomly selected one individual to remove from the final dataset. Finally, we ran tests for Hardy-Weinberg equilibrium and linkage disequilibrium using GENEPOP (Raymond and Rousset 1995) to detect any violations of population genetic assumptions.

2.6. Genetic Diversity Analyses

We estimated indices of genetic diversity using the software GDA (Lewis and Zaykin 1996) and FSTAT (Goudet 2001). These included allelic richness, observed heterozygosity, expected heterozygosity, and inbreeding coefficient. Allelic richness is the number of alleles per locus, corrected for uneven sample size. Heterozygosity is the proportion of individuals that have two different alleles at a locus; observed heterozygosity is the actual average heterozygosity in the sample and expected heterozygosity is what would occur under random mating and equilibrium conditions. The inbreeding coefficient is estimated by the difference between observed and expected heterozygosity, and a positive inbreeding coefficient indicates individuals are more homozygous than expected, which could be an indication of inbreeding and loss of genetic diversity in the population (although there could be other explanations as well). A negative inbreeding coefficient suggests that individuals are less related to each other than would be expected normally. We estimated effective population size using the program ONESAMP (Tallmon et al. 2008), which uses multiple effective population size estimates to provide a single estimate of effective population size for each population. Effective population size can most easily be described as the breeding population size, and varies significantly from census population size. Effective population size can be a better indicator of long-term population persistence as it is the better determinant of future trends in genetic diversity and evolutionary potential of the population. Finally, given the multiple anthropogenic pressures on midget faded rattlesnake populations, we performed three tests for recent population size reductions. First, we tested for heterozygosity excess (relative to equilibrium expectations) which is a short-term

signature of a very recent population size decline (Cornuet and Luikart 1996). This was implemented in the software BOTTLENECK (Cornuet and Luikart 1996) using a two-phase mutation model and a Wilcoxon sign rank test. Second, we conducted a mode-shift analysis. In a non-bottlenecked population, the majority of microsatellite alleles will be of low frequency in the populations. In reduced populations, low frequency alleles will be randomly lost, and therefore a shift of most alleles from low to intermediate frequency signifies a decline (Luikart et al. 1998). This test is also implemented in BOTTLENECK. Finally, we estimated the M-ratio of each den site (Garza and Williamson 2001). The M-ratio is the ratio of the number of alleles to the allelic size range. The loss of alleles during a decline should reduce the M-ratio. Garza and Williamson (2001) used simulations to suggest a critical M-ratio value of 0.68. A value below this is indicative of population decline. In comparison to the heterozygosity excess and mode-shift tests, M-ratios may carry the signature of population declines over a longer time.

2.7. Genetic Clustering Methods

We used several methods to determine the degree of population structuring and scale of gene flow across the study area. First, we used a Bayesian population clustering algorithm to group dens together by genetic relatedness. There are several Bayesian clustering programs and we decided to use BAPS 5 (Corander et al. 2003) for two reasons. First, we were interested in examining the genetic connectivity at den sites and not for individuals alone and BAPS is suited for clustering at the population level. Second, BAPS has the fastest processing time of any of the clustering programs with comparable performance to more time-intensive methods (Latch et al. 2006). We used the "clustering of groups of individuals" option. Second, we ran a principal component analysis (an ordination procedure) that decomposes the information contained in all the genetic loci to a limited number of axes. This method only provides a visualization of genetic structure, but it allows us to infer the relative genetic distances among sites. Finally, to estimate migration levels among dens and determine if any dens serve as important sources, we used the Bayesian method implemented in BayesAss (Rannalla and Mountain 2003). We used the default parameters and ran 10 different trials to ensure consistency.

2.8. Landscape Genetic Methods

Our final objective was to determine which environmental layers were most important for genetic connectivity. Our general approach was to calculate values of important environmental variables along paths between dens, and then statistically correlate the values of those variables with a measure of genetic distance using multiple regression on distance matrices (MRDM). MRDM has been demonstrated to be one of the best statistical methods for landscape genetic analysis (Balkenhol et al. 2009). There are several options for modeling paths among populations to measure landscape variables, ranging from straight lines to least cost paths that attempt to describe the path of least resistance given a cost surface (Spear et al. 2010). We used the movement ecology of rattlesnakes to guide our choice of model path. Most rattlesnakes, including midget faded rattlesnakes, initially follow a straight-line path until they reach their main activity ranges (Parker and Anderson 2007). In midget faded rattlesnakes, the core activity

ranges identified by Parker and Anderson (2007) averaged 27 hectares for females and 56 hectares for males. Therefore, a least cost path may not describe rattlesnake movement very well because at least a portion of migratory movements for rattlesnakes are straight-line. However, the use of focal activity ranges suggests that a linear line connecting dens is probably also insufficient. Therefore, we decided to use a corridor approach (Emaresi et al. 2011) in which we buffered a straight line between dens and calculated average environmental layer values within that buffer. This accounts for both the general straight line movement of snakes and the short distance movements snakes undergo in the activity range. We tested three potential buffers to identify which best correlated with rattlesnake gene flow; the radius of each buffer was 30 meters, 100 meters or 250 meters. We identified nineteen different variables that we predicted could correlate with genetic connectivity (Table 1).

All variable layers were provided by WYNDD and were the same as those used for the habitat modeling. In most cases, we simply took the average value of the raw layer without any modification. However, in some cases we tested whether the variable responses in the habitat models better predicted gene flow than the raw data values. For instance, in the den model, when the distance to outcrop exceeds 750 m, there is a zero probability of den occurrence. Therefore, to represent the den model, we reclassed any value greater than 750 as 750, as any value greater than 750 provides no additional information to the den model. We conducted similar adjustments for the other variables that were included in the den and foraging models (see results for details). Additionally, some of the variables (roads, water, and basking habitat) were categorical and not continuous variables. Therefore, instead of an average, we calculated the proportion of the total buffer area occupied by these classes. The measure of genetic distance that is more appropriate for situations in which recent habitat modification may have affected population connectivity (Bowcock et al. 1994, Murphy et al. 2010).

To ensure the independent variables that we used in the regression models were independent, we first regressed each independent variable against each other to determine any high correlations among variables. We used an r² of 0.7 as our cutoff (Murphy et al. 2010); any variables with a correlation above this could not be included in the same model. We used AIC to determine the best supported model among all possible models. Finally, after determining the most supported set of variables, we wished to visualize genetic connectivity across the landscape. To do this, we used electrical circuit theory (McRae 2006) to model gene flow across the landscape. This method can model multiple paths of current (gene flow) across the landscape. To build these maps of genetic connectivity, we created a resistance surface using all the variables present in the best supported model. We standardized all variables so that they ranged from 1-10, and added all layers together. As we wished to represent gene flow across the entire area, we estimated current among all known dens (whether we had genetic samples or not) based on the resistance surface that best described gene flow among our focal dens. The maps were built using CIRCUITSCAPE V 3.5 (Shah and McRae 2008). Table 1. List of environmental layers used in landscape genetic models, with description of how the variable was used in genetic models.

Environmental Layer	Description
Terrain	
Terrain ruggedness	average value of ruggedness measure
Compound topographic index	average value/ represents wetness
Slope	average value of slope
Transformed aspect	average value/represents heat load
Distance to outcrop (dens)	distance to rock outcrops adjusted to represent response curve of den model
Distance to outcrop (foraging)	distance to rock outcrops adjusted to represent response curve of foraging model
Basking	proportion of buffer that contained basking habitat as defined by Clark et al. 2008
Land cover/structure	
Percent shrub	average shrub cover
Percent bare ground	average bare ground cover
Shrub height	average shrub height
Major roads	proportion of interstates and highways in buffer
All roads	proportion of all roads (paved and unpaved) in buffer
Water	proportion of water in buffer
Climate	
Frost-free period	average frost-free period
Precipitation of the warmest quarter	average precipitation of warmest quarter
Warmest quarter mean temperature	average warmest quarter temperature
Temperature range (dens)	temperature range adjusted to represent response curve of den model
Wettest quarter mean temperature (foraging)	wettest quarter mean temperature adjusted to represent response curve of foraging model
Precipitation of the wettest quarter	average precipitation of the wettest quarter

3. Results

3.1 Den Model Results

The four different candidate models all produced different results (Fig. 1). All predicted distributions centered around the reservoir, but they varied primarily in the spatial extent of prediction. The most constrained model used all snake observations at dens and primarily reflected known den sites. The models built using only physical den features or den observations spatially filtered had intermediate extent, and the model using all observations predicted a wide distribution (Fig. 1). Additionally, the variable importance was different among models. The den physical features model had warmest quarter temperature as the most important variable, the model with all den observations was best described using distance to permanent standing water, the model of spatially filtered den observations had distance to rock outcrop as most important, and finally the model with all observations had precipitation of the driest quarter. Therefore, the specific observations used altered model results in important ways.

The different den models also had different accuracy based on our validation surveys, and different models performed better depending on the specific metric (Fig. 2, Table 2). The only model that performed poorly at all four metrics was the model based on all den observations. The model based on only physical den locations had the greatest value of overall model accuracy (74%) and the TSS (0.49), which are overall metrics of model accuracy. The model using all observations after filtering was highly successful with regard to sensitivity (the metric that measures how well model identifies actual known dens) as all identified dens were identified by this model. The den model based on filtering all den observations had high specificity (77%), which means this model was good at identifying locations that would not have den habitat. While we valued sensitivity more highly than specificity as we wanted to identify actual dens (and not just negative locations), we felt the all observations model predicted too broad an extent to be useful for applied management. Therefore, we chose the physical den location model as our final den model because it had the best scores for the overall metric.

We took one additional step to improve the final den model. Examination of the results of the den validation surveys suggested that our training dataset was missing higher elevation dens on the eastern side of the reservoir. As a result, we decided to include one of the higher elevation dens identified in the validation efforts into our training dataset and rerun the physical locations den model. This, in combination with the use of AIC model selection, allowed us to improve the final den model. This method worked successfully, as each of the metrics improved in the final model. The overall model accuracy was 85%, the sensitivity was 95%, specificity was 75% and the TSS value was 0.70. This level of model accuracy was achieved using the maximum test sensitivity plus specificity threshold. Thus, we selected this as our final den model, and the spatial extent appears to be restricted enough to be useful for on the ground site selection and management (Fig. 3). There is one den site (from the WGF database) that is outside our final model. We searched near this site and were unable to find snakes, so it is currently unclear the importance of this area for midget faded rattlesnakes. The selected model

was simple in that only two variables were included: distance to rock outcrop and annual temperature range (Fig. 5). Den probability is reduced quickly as distance from rock outcrop increases, in accordance with previous knowledge of the species (Fig. 4a). The other variable was less expected, and had a narrow distribution of values of temperature range that were suitable for denning habitat (Fig. 4b).



Figure 1. Model results of four candidate models. Blue color indicate areas not predicted to have any rattlesnake den habitat, and warmer colors indicate greater den probability.



Figure 2. Ensemble model based on candidate models. Red represents areas predicted by 3-4 models, yellow represents areas predicted by 2 models, light blue represents areas predicted by one model and dark blue areas were predicted by no models.

Table 2. Model accuracy results for each candidate model.	Values in bold and italics represent the highest value for
that category.	

8,				
	Den	All Den	Den	All Points
	Features	Points	Filtered	Filtered
Accuracy	74%	67%	64%	67%
Sensitivity	85%	60%	50%	100%
Specificity	64%	72%	77%	36%
TSS	0.49	0.33	0.27	0.36



Figure 3. Final den model, converted to a binary model based on the maximum test sensitivity plus specificity threshold. Red indicates areas predicted to contain denning habitat.



Figure 4. Response curves of variables included in final den model with den probability on y-axis. A) distance to rock outcrop B) annual temperature range.

3.2. Foraging Model Results

The foraging model selected by AIC was similar to the den model in that only two variables were included. The best model threshold in the den model (maximum test sensitivity plus specificity) was not applicable to the foraging model as we did not have test validation data. Therefore, we used the threshold cutoff that had the second highest TSS in the den validation data, which was the minimum training presence. The map of foraging habitat is largely complementary to den habitat, with some discrepancies in areas north of I-80 (Fig. 5). The northwest portion of the study area has extensive predicted foraging habitat. Part of the White Mountain plateau is predicted as foraging habitat, whereas only the slopes are predicted as denning habitat. Finally the area between the towns of Green River and Rock Springs is

predicted to have denning habitat, but has very little predicted foraging areas (Fig. 5). The two variables included in the foraging model are distance to rock outcrop and growing season temperature (Fig. 6). However, instead of the constricted distributions present in the den models, the response curves in the foraging model are more (although not completely) linear. Foraging likelihood increases as distance to outcrop decreases and growing season temperature increases.



Figure 5. Map of foraging model prediction, using a binary threshold of minimum training presence. Red indicates model prediction.



Figure 6. Response curves of variables included in final foraging model with foraging probability on y-axis. A) distance to rock outcrop B) growing season temperature.

3.3. Preliminary Genetic Analyses and Genetic Diversity

Across 13 populations, we genotyped an average of 48 individuals per den (collected from both 2000-2002 and 2010), with a range of 13-123 (Table 3). Of the 19 loci we tested, we estimated high probability of null alleles in three loci (MFRD5, MFR12, MFRD6; see Appendix 1), and these loci were eliminated from further analyses. As expected based on known rattlesnake life history and a high number of juveniles captured, there were a large number of related individuals at each den. The percentage of unique family groups at each den was only 60%, so we removed on average 40% of individuals for further analysis (Table 3). Therefore, our average sample size after accounting for related individuals was 29. There were no violations of linkage equilibrium and limited violations of Hardy-Weinberg equilibrium at some dens and loci. There were no consistent Hardy-Weinberg violations among loci, but three dens did have violations at more than one locus. These were EN, SBF and SR. All three of these den areas are den complexes that are made up of multiple dens and the mix of individuals from

different dens within the same complex likely explains the violations. We decided to retain these den complexes because there were a number of loci at each complex that did not have violations and due to the importance of these dens for the overall dataset.

Overall, genetic diversity is low to intermediate across midget faded rattlesnakes. Allelic richness averaged less than four per locus and an average expected heterozygosity of 0.56 (Table 3). There was relatively little variation in these values among dens, suggesting that reduced diversity relative to other subspecies is characteristic of midget faded rattlesnakes in Wyoming. There was a pattern of the lowest allelic richness values occurring at several of the sites that are immediately adjacent to the reservoir. Observed heterozygosity was somewhat lower than expected heterozygosity (0.53), and thus there was an average inbreeding coefficient of 0.06. Therefore, it is possible that some degree of inbreeding is occurring throughout the area. Two dens have especially high inbreeding coefficients: DS and EB. Both of these dens are on the edge of the midget faded rattlesnake range in Wyoming (and DS is isolated by I-80) (Fig. 1). Effective population size averages 25 across dens, which is similar to the sample size of unrelated individuals at each den (Table 3). Effective population size appears to plateau around 45 at the larger dens. Therefore, it is unlikely that any den complex has an effective size greater than 50. There was no evidence of recent population size declines based on heterozygosity excess and only one site (CC) had a shifted allele frequency. In contrast, all sites had an M-ratio lower than 0.68, indicative of population declines (Table 3). The combination of low M-ratio with lack of heterozygosity excess suggests that a broad scale decline occurred within hundreds to thousands of years, but may not be attributable to events within the past few decades.

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Site	Ν	Nfam	Ne	Ar	He	Ho	f	M-ratio
BT	27	15	22 (18-28)	4.14	0.59	0.59	-0.01	0.58
BR	32	20	20 (17-27)	3.41	0.52	0.53	-0.01	0.56
CC	13	9	12 (10-14)	3.63	0.54	0.54	0.001	0.54
DS	22	20	24 (20-32)	4.05	0.57	0.48	0.16	0.54
EB	19	15	17 (15-24)	3.71	0.54	0.44	0.18	0.61
EN	119	56	44 (36-64)	4.08	0.57	0.53	0.07	0.62
ESBF	38	23	23 (19-31)	4.21	0.57	0.55	0.04	0.55
FH	13	13	14 (12-18)	3.71	0.59	0.55	0.07	0.61
LD	31	24	25 (21-33)	4.23	0.56	0.52	0.06	0.55
SBF	123	69	45 (37-60)	4.23	0.6	0.58	0.04	0.63
SM	55	26	26 (21-34)	4.07	0.56	0.55	0.03	0.53
SQ	21	18	19 (16-25)	3.53	0.54	0.5	0.08	0.59
SR	110	68	38 (30-52)	4.03	0.59	0.54	0.07	0.64
Average	47.92	28.92	25.3 (20.9-34)	3.93	0.56	0.53	0.06	0.58

Table 3. Estimates of genetic diversity across den sites. Site locations are as in Fig. 1. N = total size, Nfam = number of unique unrelated families, Ne = effective population size, Ar = allelic richness, He = expected heterozygosity, Ho = observed heterozygosity, and f = inbreeding coefficient.

3.4. Population structure results

The thirteen dens we sampled were divided into four different population clusters using the Bayesian method (Fig. 7). Three of these clusters were relatively restricted, with the fourth cluster covering a large portion of the study area. Only one site clustered completely by itself, DS. This is the site that is separated from all other sites by I-80 and had a high inbreeding coefficient. The other two restricted clusters were a Blacks Fork River cluster (ESBF, SBF, SM) and a southern reservoir cluster (EN, SQ, BR, EB). Note that the southern reservoir cluster spans both sides of the Flaming Gorge reservoir. The final broad cluster was also made up of sites on both sides of the Green River or Flaming Gorge Reservoir.



Figure 7. Map of clustered population based on the Bayesian program BAPS. Each circle represents the approximate locations of genetic clusters.

The PCA ordination clustering provides insight into how these four clusters are related to each other (Fig. 8). In general, the PCA agrees with the Bayesian clustering, although the placement of LD and CC is less consistent between the two methods. The PCA axes can best be interpreted as the primary differentiation along the x-axis and secondary differentiation along the y-axis. Following this logic, the Blacks Fork cluster is the most divergent group, with minimal separation of the other three clusters along the x-axis. Along the y-axis, DS and the southern

reservoir cluster diverge in opposite directions. Accounting for both PCA axes, the broadest cluster is the central hub of genetic connectivity across the area, and there is likely little direct connectivity among the Blacks Fork sites, southern reservoir sites or DS.

We identified three sites that consistently had very little migration into them from other sites, but provided a significant proportion of migrants into other populations: BR (which is a source for EB, EN, and SQ), SBF (which is a source for ESBF and SM) and SR (which is a source for BT). Therefore, these three areas represent key sites for midget faded rattlesnakes in their respective population clusters. The same analysis also confirmed the isolation of DS – it received little migration and did not provide migrants to other sites.



Figure 8. PCA ordination of genetic differentiation. X-axis represents most significant axis of genetic differences and the y-axis represents the next greatest axis of genetic differentiation.

3.5. Landscape Genetic Results

Landscape variables were significantly correlated with genetic distance and the landscape models explained up to 47% of genetic distance among sampled dens (Table 4). Using AIC as the model selection metric, 11 models across the three buffer sizes had some support. The best supported model was based on the 100 meter buffer and included transformed aspect, percent bare ground, topographic distance, temperature during the growing season, and roads (Table 4). There was increased gene flow (and thus reduced genetic distance) along northern/eastern slopes and higher growing season temperatures, and reduced gene flow across areas with bare ground and areas with roads. However, this best model only had AIC weight of 0.16, and there were several models that had similar support. Therefore, to describe the important variables for gene flow, we used a model averaging approach in which variable importance was assessed as the

cumulative value of AIC weights of the models in which the variable appears. Overall, models at the 100 meter buffer had a cumulative AIC weight of 0.52, followed by 250 meters at 0.27 and 30 meters at 0.21. A total of seven variables were included in at least one model (Table 4-5). These seven variables formed three tiers of overall variable importance. Two variables, topographic distance and temperature during growing season, were present in every model (variable importance = 1), although growing season temperature was best represented by the foraging model response curve in the 30 meter buffer models. The second tier was percent bare ground and roads, with a variable importance of 0.62-0.64. Finally, transformed aspect, precipitation during growing season and ruggedness had the lowest importance, ranging from 0.3-0.4 (Table 5).

Table 4. Landscape genetic results based on a multiple regression on distance matrices (MRDM) models. Models were selected based on AIC weights. Buffer represents the buffer around the straight line connecting dens within which variable values were estimated. Direction is the correlation direction of the variable with genetic distance D_{ps} .

Buffer	Variables	Direction	r²	AICc	AIC wt
100	t aspect	-	0.47	-260.8	0.16
	bare	+			
	distance	+			
	temp_growing	-			
	roads	+			
100	t aspect	-	0.46	-260.7	0.16
	bare	+			
	distance	+			
	temp_growing	-			
100	bare	+	0.46	-260.1	0.12
	distance	+			
	roads	+			
	temp_growing	-			
30	distance	+	0.46	-259.7	0.1
	precip_growing	-			
	road	+			
	temp_growing_for	-			
	ruggedness	-			
100	distance	+	0.46	-259.4	0.08
	precip_growing	-			
	road	+			
	temp_growing	-			
	ruggedness	-			
250	t aspect	-	0.45	-259.3	0.08
	bare	+			
	distance	+			

	temp_growing	-			
250	bare	+	0.45	-259.2	0.07
	distance	+			
	road	+			
	temp_growing	<u>-</u>	_	_	_
30	distance	+	0.43	-258.9	0.06
	precip_growing	-			
	road	+			
	temp_growing_for	-			
	ruggedness	-			
250	distance	+	0.44	-258.7	0.06
	precip_growing	-			
	temp_growing	-			
	ruggedness	-			
250	bare	+	0.43	-258.7	0.06
	distance	+			
	temp_growing	-			
30	bare	+	0.44	-258.5	0.05
	distance	+			
	road	+			
	temp_growing_for	-			

Table 5. Variable importance results for each variable based on AIC weights. Overall is the variable importance across all buffer sizes, and the columns for each buffer size represents the variable importance just using supported models at that buffer size.

Variable	Overall	30 m	100 m	250 m
aspect	0.4	0	0.62	0.3
bare	0.62	0.24	0.85	0.78
distance	1	1	1	1
precip_growing	0.3	0.76	0.15	0.22
roads	0.64	1	0.69	0.26
ruggedness	0.3	0.76	0.15	0.22
temp_growing	1	1	1	1

However, the variable importance varies at different scales. In fact, with the exception of distance and growing season temperature (present in all models), no variable had similar importance across each buffer. Growing season precipitation and ruggedness had high importance only in the 30 m buffer. Roads were present in every 30 m model, had high importance at 100 m, and low importance at 250 m. Transformed aspect had highest importance in only the 100 m models, and percent bare ground was important in both the 100 and 250 m buffers.

Finally, the map of current across the study area based on the top resistance surface provided further insight into areas of high gene flow, and areas that are more isolated (Fig. 9). The greatest area of current is around the confluence of the Blacks Fork and the Green River; this is also a well sampled area for dens. The lowest degree of connectivity is north of I-80; despite a relatively high density of known dens, there are consistently low levels of current among these dens. This map also illustrates areas in need of increased sampling, namely the east side of the reservoir, along the Green River south of SR, and near the Utah border. If in fact these areas do not contain many additional dens, then they each are areas of low connectivity, but if additional dens are found, then connectivity may be much higher than depicted.

3.6. Population Sampling

Across 2010 and 2011, we captured a total of 308 unique rattlesnakes at our focal dens, for an average of 31 per den (Table 6). The sex ratio was skewed toward females, with 1.47 female snakes for every 1 male. On average, 54.5% of adult female snakes at each den were gravid, suggesting a reproduction rate of every other year (we used a 40 cm SVL as our cutoff for adult females, as the smallest gravid snake was this size). Across all snakes, there is a bimodal pattern in size distribution (Fig. 10); there is a peak of first year snakes, a decline of juveniles and small adults, and a second peak of larger adult snakes. When we separated the size frequency histograms by sex, we see divergent patterns (Fig. 11-12). Both sexes share the peak of first-year snakes, but females retain the overall bimodal patterns (Fig. 11), whereas males have a decline in individuals greater than 45 cm (Fig. 12). Thus, adult male snakes appear to be missing from the sample relative to large adult females.



Figure 9. Current map produced by circuit theory illustrating connectivity across the landscape based on the best resistance surface. Warmer colors represent higher current. The degree of current is highly affected by den locations as well as resistance surface.

Site	Ν	Μ	F	% gravid
BR	16	3	13	50
CC	15	5	10	86
DS	10	3	7	71
EB	8	2	6	100
EN	44	15	29	57
ESBF	23	9	14	38
FH	16	6	10	33
SBF	89	41	48	43
SM	31	13	18	36
SR	67	32	35	31

Table 6. Numbers of individuals, number of males and females and percent gravid females across ten focal dens used for demographic monitoring in 2010-2011. Site abbreviations are as in Fig. 1. N represents total number captured, M number of males, F number of females.



Figure 10. Snout-vent length size distribution across all snakes captured in 2010-2011.



Figure 11. Snout-vent length size distribution across all females captured in 2010-2011.



Figure 12. Snout-vent length size distribution across all males captured in 2010-2011.

4. Discussion and Conclusions

The combination of habitat modeling and landscape genetics provided us with key insights into midget faded rattlesnake management and conservation in Wyoming that would not be available with either method alone. The habitat modeling provided us with a rigorously tested model of den habitat that can be immediately used by managers to identify important habitat for midget faded rattlesnakes. While not able to be independently validated, our foraging model also provides a first step to identifying areas important during the active season. However, the modeling efforts did not provide information on population status or connectivity among those foraging areas. The genetic methods provide important information on the genetic status of populations and the level of connectivity among dens. Furthermore, our landscape genetic analyses identified specific variables that are most important for rattlesnakes moving across the greater landscape. Finally, our focal den sampling provided important demographic information about the overall population as well.

In general, our research suggests that midget faded rattlesnakes are currently stable in Wyoming, but are vulnerable to further disturbance. We did not detect evidence of declines at known dens, and in fact, levels of reproduction appeared to be much higher than observed by Parker (2003). However, adult males appear to be reduced in our sample, and this reduction was not seen by Parker (2003). Genetic diversity is relatively low across all populatons, and gene flow among dens is strongly restricted by distance. Finally, roads (both paved and unpaved) are a significant restrictor of gene flow across the area (and may explain the reduction of adult males). The greatest threat to midget faded rattlesnakes is energy development, which consists of both direct habitat alteration and indirect alteration through the access road network. The reliance of rattlesnakes on stable den locations is an obvious constraint, and the den model should allow managers to protect specific den locations from development. However, our results imply that this may not be sufficient, and the

accompanying road network may be a more persistent threat. Roads reduce gene flow among dens and isolated dens will lead to erosion of genetic diversity. The current low levels of genetic diversity mean there is less of a "safety net" at this parameter, and any isolation will more quickly lead to deleterious genetic effects (such as inbreeding depression). We also detected a decrease in adult male snakes, which would be consistent with road mortality effects based on the higher susceptibility of male snakes (Jochimsen et al. 2006). The loss of male snakes would decrease the effective population size, which would increase loss of genetic diversity independent of population isolation. Thus, management of both exact development locations and road effects will be needed to ensure continued persistence of midget faded rattlesnakes across its Wyoming range. Below, we discuss in more detail and provide context for each of our study objectives.

4.1. Den Model

This study represents a rare instance in which a distribution model has been field validated, and our validation data demonstrate that the modeling approach was quite successful at identifying den habitat. Therefore, managers should largely feel comfortable using this model to manage midget faded rattlesnakes in the face of development, although we do have two caveats or areas of uncertainty. First, the model is highly reliant on correctly predicting rock outcrops, which are the most critical components of midget faded rattlesnake dens. We predicted rock outcrops using a topographic position index, and in general, the model did an excellent job of correctly identifying outcrop habitat. However, there were a handful of areas in which slopes predicted to be outcrops had limited exposed rock and thus were not suitable for rattlesnake dens. As a result, we recommend that at the very least, managers conduct initial site visits to predicted denning areas that are of interest, and confirm that suitable outcrop habitat exists. If there are suitable outcrops, then we have high confidence that there will be midget faded rattlesnakes in that area. The second consideration with the den model is some uncertainty regarding rattlesnake habitat in the Little Mountain area. Our den model does not predict habitat in a majority of the Little Mountain area, with most of the predicted dens within a few kilometers of the reservoir. Despite this, there is one den observation in the Little Mountain area that is outside of our extent of predicted dens. This observation was present in the Wyoming Game and Fish database. As part of our validation surveys, we did survey some sites in this area and did not find snakes near the database observation. We do not know whether this observation represents a peripheral den that is unlikely to persist through time, or whether the Little Mountain area is more important for rattlesnakes than indicated by our den model. The Little Mountain area is currently an area of much concern for several parties and is of interest for wildlife conservation, oil and gas development, grazing and wind interests. Thus, this should be an area of high importance to better understand midget faded rattlesnake population status. If surveys do detect midget faded rattlesnakes more broadly in this area, then the den model should be re-run to account for this previously missing data.

In addition to identification of den areas, the models provided insight into what factors determine successful denning habitat. Surprisingly, only two variables were included

in the final den model. Therefore, it is fairly simple to describe good den habitat, but it is highly restrictive as well. Distance to rock outcrops was already known to be an important variable, and we expected that temperature would be important for overwintering. However, the temperature variable included (annual range) had a very narrow distribution of values suitable for dens. This leads to the question of how strongly climate change might influence midget faded rattlesnake distribution. If future change shifts the distribution of annual temperature range, then some of the dens currently suitable might be unsuitable in the future. An avenue for future research would be to model predicted changes to estimate whether future temperature conditions will shift midget faded rattlesnake distribution, and if snakes are likely to be able to successfully shift and persist if conditions change.

4.2. Foraging Model

The foraging model we developed provided additional insights into important variables for rattlesnakes away from the denning area. Although the model was based on a much smaller number of points than the den model, we had a set of radio telemetry points collected by Parker and Anderson (2007) that allowed us to have high confidence that our occurrence points reflected actual foraging habitat. In addition, MAXENT has been demonstrated to work relatively well with small numbers of points (Pearson et al. 2007), although more data points are always preferred. We also received independent validation through the landscape genetic data as to the strength of our foraging model. The foraging model included two variables: distance to rock outcrops (essentially dens) and mean temperature during the growing season. In the landscape genetic models, distance and growing season temperature were the only variables included in every model. We consider the genetic models to be an indirect validator of the foraging model because mating (and thus gene flow) occurs away from the den on the foraging grounds for midget faded rattlesnakes (Parker and Anderson 2007). Thus, the variables that affect gene flow should be highly correlated with the variables important for foraging. As a result, we have confidence in our foraging model, despite the lower number of occurrences.

The predicted area of foraging habitat largely overlaps with concentrations of den habitat, although there are some key differences. The area surrounding the Flaming Gorge reservoir is an area of key importance for midget faded rattlesnakes with respect to both dens and foraging. The Little Mountain area is not predicted to have much foraging habitat, but we also lacked radio telemetry data from Little Mountain, so similar caveats are in order for this area as we discussed with the den model. Interestingly, the I-80 corridor between Green River and Rock Springs is predicted to have essentially no foraging habitat. This is probably the case currently, but the foraging model did not contain any anthropogenic variables, so it may be coincidental with the development along that corridor. However, it does suggest that the interstate occurs in areas that midget faded rattlesnakes may have rarely used for foraging (although there was clearly important den habitat that used to exist around Rock Springs). Two areas outside the Flaming Gorge Reservoir area appear to be especially good foraging habitat. The Blacks Fork river corridor has an extensive prediction of foraging habitat, although more limited extent of den habitat. Outside of sites near Highway 530, the Blacks Fork corridor has not been surveyed for snakes (largely due to access issues). In addition to the foraging model, the genetic data provide some further evidence that the Blacks Fork may be an important rattlesnake area. The sites we sampled along the Blacks Fork had slightly higher levels of genetic diversity than other sites, and were divergent from another sites, despite the lack of any obvious barriers to other reservoir sites. If the projection of extensive foraging habitat along the Blacks Fork is accurate, then this area likely provides sufficient habitat for rattlesnakes and is an important population center that does not require migration from other sites. The other area suggested as suitable foraging habitat for rattlesnakes is the White Mountain plateau. This was not an area we previously considered important rattlesnake habitat, but two rattlesnakes were observed on the White Mountain road this summer (D. Jochimsen, personal communication), and thus White Mountain could be important rattlesnake habitat as well.

4.3. Population and Landscape Genetics

Our population genetic data were an extension of a dataset published by Oyler-McCance and Parker (2010). We used the same samples as that paper, but included additional samples (all samples collected in 2010 as well as unused samples from 2000-2002). We also included seven more loci than in the previous paper. In addition, we had some methodological differences such as accounting for related individuals that was not conducted by Oyler-McCance and Parker (2010). As a result, even though our findings are largely concordant, we observed a slightly higher level of genetic diversity (average allelic richness was 3.93 compared to 3.59 in the previous study). This is probably attributable to increased sample size (both individuals and loci) that would add more alleles. Both studies find restricted gene flow across the area, although our study was able to detect more clear genetic clusters than Oyler-McCance and Parker (2010). The biggest discrepancy is the bottleneck results. Oyler-McCance and Parker (2009) identified several sites that had heterozygosity excess, whereas we did not identify any sites that fit this pattern. It is somewhat unclear what is causing this difference, but as we identified increased diversity with our additional sample size, the previous differences could be due to the combination of smaller sample size and inclusion of related individuals that may have given a false bottleneck signature.

We did detect evidence of population size reductions using the M-ratio, which has a much longer time period before it recovers to normal ratios (Garza and Williamson 2001). The fact that all sites had low M-ratios points to a broad historical event that caused the population decline. Midget faded rattlesnakes are a recent evolved subspecies of the western rattlesnake, and the formation of the (sub)species likely occurred during the Pleistocene (Douglas et al. 2002). If this is the case, the low M-ratios may be due to a founder effect associated with the isolation of the subspecies during the Pleistocene. This would also account for the consistently low diversity across all sites. If this is true, then the Wyoming population of midget faded rattlesnakes is inherently sucsceptible to genetic effects since it has already gone through one large bottleneck. Therefore, they are going to exhibit negative

consequences of fragmentation and isolation more quickly than other species that have greater baseline diversity.

Our landscape genetic results provided evidence that environmental factors strongly influence gene flow, but these factors can be scale dependent. In general, midget faded rattlesnakes are largely constrained by disperal limitations (leading to an isolation by distance pattern) and temperature. Therefore, the greatest factors influencing gene flow are not purely anthropogenic (although climate change certainly might change gene flow patterns). However, the next level of important variables, roads and bare ground, are where anthropogenic disturbance has contributed to reduction of gene flow. There are several aspects of the correlations with these two variables that give insight into midget faded rattlesnake management. To begin, density of all roads (paved and unpaved) had a greater correlation than interstates and major highways alone. In fact, there were no models in which major roads alone had a greater correlation than all roads. Thus, even unpaved roads lead to detrimental effects, which includes access roads related to energy development. A second interesting aspect of the road correlation was that it varied depending on the buffer used. At 30 meters, all supported models included roads, whereas at 250 meters, roads had a low variable importance. An implication of such a pattern is that roads exert fine-scale impacts on midget faded rattlesnakes, but are less influential at a broad scale. In other words, roads hinder movement and gene flow when the snakes encounter them, but there is not likely to be a strong "road-effect zone" (Forman et al. 2003) in this situation. The lack of broad scale effects is likely tied to the prevalence of unpaved roads compared to the greater footprint that a paved road would have. The scale difference also implies that roads are most disruptive to straight line movements to foraging grounds (although a road built through a foraging ground would lead to high disturbance). In contrast to roads, bare ground is less important at fine scales, and has its highest importance at the 100 m and 250 m buffer. Therefore, snakes may move relatively easily over bare ground on straight-line movements, but bare ground affects the suitability of foraging and activity areas. Thus, areas important for foraging should be protected from activities such as site development or overgrazing that would reduce vegetation, but relatively small patches of bare ground not in core areas would be less important.

Across the landscape, our current map demonstrates that the area around the reservoir has high connectivity, which means that there are multiple pathways among which successful gene flow takes place. In the northern area however, there is much higher resistance to gene flow, and maintaining intact suitable habitat is much more important for connectivity. The map does demonstrate that there are unlikely to be narrow corridors that can be the main focus of management efforts. From a conservation standpoint, this has both postivie and negative implications. The positive outlook is that there are wide areas of suitable habitat, and fine-scale disturbances to any one small area is less likely to harm snakes (with the exception of direct den destruction). The negative aspect is that it is difficult to make focal attempts to protect midget faded migration routes, and broad scale disturbance (such as extensive roads) are likely to affect many snakes. The efficacy of the current map is also highly dependent on our knowledge of den locations. An ideal current map would be based on every existing den on the landscape. Unfortunately, we will never have that level of data, but the increased sampling effort north of I-80 and west of the reservoir north of Holmes Crossing allows us to have high confidence in the current map in these areas. However, more den information is greatly needed around Little Moutain and south of Holmes Crossing to understand migratory corridors in these locations. If den locations are as sparse in this region as our models predict (and suitable rocky habitat is less abundant on the west side of the reservoir), then the existing dens face threats from isolation, but if there are unidentified dens, then connectivity is likely higher than our model suggests.

There have been a few genetic studies with rattlesnakes that allow us to compare the genetic diversity and population structure of midget faded rattlesnakes with other species. To our knowledge, the only other genetic study on a western rattlesnake subspecies was conducted by Parsons (2009) on Great Basin rattlesnakes (Crotalus oreganus lutosus) in southeastern Idaho. This study found a higher level of genetic diversity in these snakes, with an average expected heterozygosity of 0.69 in Great Basin rattlesnakes compared to our average of 0.56. There was little evidence of isolation by distance in Great Basin rattlesnakes at finer scales, as compared to strong isolation by distance in midget faded rattlesnakes. Thus, midget faded rattlesnakes are much more susceptible to isolation and drift effects than a related subspecies. This study also conducted a landscape genetic analysis and tested the correlation of distance, den suitability, number of dens and road crossings with genetic distance. The only variable that correlated with gene flow was probability of den suitability across a 1-3 km buffer. This is different than our study, in which we did not find support for variables associated with denning habitat (although it is possible that including more variables would lead to a model with greater support than den suitability). Surprisingly, there was little correlation of roads with genetic distance in Great Basin rattlesnakes, which may be due to the fact that a large proportion of the study area is restricted and has low traffic.

The other rattlesnake species that have been studied genetically are the timber rattlesnake (*Crotalus horridus*) and the massasauga (*Sistrurus catenatus*). Timber rattlesnakes have had a number of genetic investigations in the northeast (Clark et al. 2008; Clark et al. 2010; Clark et al. 2011), and in general display higher genetic diversity and higher gene flow than midget faded rattlesnakes. Overall allelic richness for timber rattlesnakes in New York was over 5 and there was no significant isolation by distance (Clark et al. 2008). Clark et al. (2011) did describe an extremely isolated population of timber rattlesnakes in New Hampshire that is displaying signs of inbreeding depression and had an allelic richness of only 2. Therefore, midget faded rattlesnakes have greater diversity in midget faded rattlesnakes could lead them to levels observed in this inbred population. The two landscape factors that have been tested in timber rattlesnakes are roads and basking habitat (defined by slope and aspect). Both were found to significantly correlate with timber rattlesnake gene flow (Clark et al. 2008; Clark et al. 2010). Our research is consistent with

respect to roads, but not to basking habitat. In fact, we used the exact basking model as Clark et al. (2008) and found no correlation. Thus, basking habitat is unlikely to be a limiting factor for midget faded rattlesnakes. Massasauga rattlesnakes are much more comparable to midget faded rattlesnakes with respect to size (although not other ecological aspects), and Chiucchi and Gibbs (2010) found almost identical average levels of genetic diversity in massasaugas across their range as we observe in midget faded rattlesnakes. This study also observed strong population structure in massasaugas and found that there was a strong historical component to this isolation. Therefore, small-sized rattlesnakes may share common features that lead to restricted gene flow and reduced diversity, and thus these snakes may be more susceptible to isolation and fragmentation effects than most rattlesnakes.

4.4. Demographic monitoring

Several trends were readily apparent from snakes captured in 2010-2011 as compared to snakes captured in 2000-2002 by Parker (2003). The number of first year snakes was much higher in 2010-2011, as approximately 30% of all captures were first year snakes, compared to less than 20% captured by Parker (2003). We suspect this pattern is probably best explained by weather differences between the two time periods. Examining precipitation from 1999-2001 and 2009-2010, 2009 was consistently the highest year for precipitation and 2000-2001 were consistently the driest years (Prism 2011). Parker (2003) did have a higher proporiton of 2-3 year old snakes than we saw, which may be explained by relatively high precipitation in 1999 before the drier years of 2000-2001. However, longterm continuous monitoring is needed to understand whether precipitation consistently explains reproductive output and to predict the potential effects of future climate change on rattlesnake recruitment. The other striking difference is the reduction in large adult male snakes. Of all the male snakes that Parker (2003) captured, 38% were greater than 50 cm SVL. In contrast, we only found 29% of male snakes that were in this category. We also observed a greater female-biased sex ratio than did Parker (2003), although both samples were female biased. Interestingly, observed sex ratio in populations of other western rattlesnake subspecies tends to be male-biased (Diller and Wallace 1996; Diller and Wallace 2002; Jenkins et al. 2009), although Jenkins et al. (2009) did observe one den that had a female biased sex ratio. This den also had significantly lower body condition and fecundity than other dens in their study area, so the reduction in males could have long-term consequences.

We do have to consider the possibility that our sampling methods are biased toward females and that is leading to our observed pattern. This is a valid concern because adult males generally leave the dens during the active season, whereas gravid females and juveniles tend to stay at the den year-round. This may have slightly biased our sampling, but the vast majority of sampling effort was at the dens in the spring and early summer, when both sexes of all age classes should be available for capture. Therefore, we do not think that the lack of males is simply due to our sampling protocol. Furthermore, we used the same general sampling strategy as Parker (2003), and thus the relative differences between the two studies should be reliable. We propose the most likely explanation for the reduction in males

to be related to road mortality, as the greater distances traveled by males as well as the increased time away from the den make them more likely to cross roads than females.

4.5. Management Recommendations and Future Research

- 1) Use the den model as a guide to protect areas from direct development. The successful performance of the den model means that it should be a useful and accurate tool for planning for energy development. Areas identified in the den model should be avoided as much as possible when developing the Flaming Gorge area. If an area of high priority for development falls within an area of predicted midget faded rattlesnake habitat but there is flexibility in placement, then we would suggest site visits to ensure that suitable rock outcrop habitat exists and determine which areas have the highest densities of snakes. Because of the extreme disturbance (and likely extirpation) that developing a den site would cause, we stress this should be an action of last resort for snake management.
- 2) <u>Reduce the road footprint associated with development and recreational activities.</u> Roads are likely the largest detrimental impact to midget faded rattlesnakes in this area, probably through direct mortality rather than indirect effects. Roads are a necessity for any energy development, but planning that reduces the spatial extent of the road network as well as avoids key midget faded rattlesnake habitat areas will be for rattlesnake populations. Furthermore, we suspect that most road effects occur when snakes are traveling from dens to core activity areas. Therefore, road mortality would be highest July-September when snakes are actively moving, with limited impact the rest of the year. Therefore, efforts to restrict the amount of traffic July-September and/or limit peak road traffic to times of rattlesnake inactivity (i.e., hot afternoons) should significantly reduce rattlesnake roadkill.
- 3) Initiate additional surveys in the Little Mountain area. Our modeling and genetic efforts spanned the entire range of the midget faded rattlesnake in Wyoming, but it became clear from our research and development pressures that the Little Mountain area is still somewhat uncertain with regard to rattlesnake status. Our limited surveys in the area suggest that the Little Mountain area is important for rattlesnakes close to the reservoir, but less so further east. However, the past observation of snakes on Little Mountain could mean that we are underestimating the importance of this area for rattlesnakes. There are important discussions currently ongoing as to how to manage Little Mountain, and the area could see significant energy development. Therefore, we propose a priority research objective to be intensive surveys (designed like our model validation surveys) to focus primarily on the Little Mountain area. Results of such surveys would have great implications for midget faded management and persistence east of the reservoir. It would also be beneficial to have increased surveys south of Holmes Crossings to the Utah border, but as much of this area is contained within the National Recreation Area, it is less of a priority than Little Mountain._
- 4) <u>Continue population monitoring at focal dens.</u> Our demographic mark-recapture monitoring has been useful in understanding the age and sex structure of midget faded

rattlesnakes, and combined with Parker's (2003) data identified a decrease in adult male rattlesnakes. This is especially concerning as most of our focal den sites have less distrubance than most surrounding areas and are in core habitat. However, our inferences related to our monitoring efforts are limited due to a small temporal sample size and discontinuous sampling. We recommend that a few focal dens be continuously sampled at least in spring to continue to track demographic trends. This will help explain what factors ultimately influence recruitment and detect any further declines.

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Appendix 1

Multiplex PCR conditions for midget faded rattlesnakes (locus names are as in Villareal et al. 1996, Holycross et al. 2002, Oyler-McCance et al. 2005, Oyler-McCance and Parker 2010) All runs have 7 µl total volumes/sample

All multiplexes have: 3.5ul Qiagen Master Mix/sample

0.7 µl Q solution/sample

1 µl template DNA/sample

- $0.7 \ \mu l \ primer \ mix$
- 1.1 µl water

All primer volumes are equal for both forward and reverse primers, and are at 5 μ M stock concentrations.

<u>Multiplex 1-</u> [95 deg 15 min, (94 deg 30 s, 60 deg 90 s, 72 deg 60 s)32X, 60 deg 30 min] MFR15 – 2 μ m MFRD5 – 2 μ m MFRD3 – 2 μ m MFRT1 – 3 μ m

<u>Multiplex 2</u> – [95 deg 15 min, (94 deg 30 s, 60 deg 90 s, 72 deg 60 s)29X, 60 deg 30 min] CH5 – 2 μ m CWB23 – 2 μ m MFR22 – 2 μ m MFR9 – 2 μ m MFRD7 - 2 μ m

<u>Multiplex 3</u> – [95 deg 15 min, (94 deg 30 s, 60 deg 90 s, 72 deg 60 s)32X, 60 deg 30 min] CH7-144 – 2 μ m CH7-150 – 2 μ m CH7-187 – 2 μ m CWD15 – 2 μ m MFR12 – 2 μ m MFR17 - 2 μ m

<u>Multiplex 4</u> – [95 deg 15 min, (94 deg 30 s, 60 deg 90 s, 72 deg 60 s)35X, 60 deg 30 min] MFR29 – 1 μ m MFR3 – 1.5 μ m MFRD2 – 3.5 μ m MFRD6 – 1.5 μ m

Dilute PCR product 1:15 (1 µl template PCR product, 0.15 µl LIZ500 size standard, 13.85 µl water)

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