

Distribution and population dynamics of box turtles in North and Central Arkansas

Two species of box turtles that have been designated as species of special concern will be studied. The distribution and abundance of the turtles will be examined. We will also utilize genetic markers to study population structure and hybridization between the two species within the genus *Terrapene*. Initial surveys suggest that populations are highly fragmented and have been strongly impacted by habitat loss. Genetic analyses suggest that hybridization is occurring between the two species and may further threaten the rarer ornate box turtle with extinction as three-toed box turtles become dominant in the ornates favored habitat –grasslands. Surveys will be conducted in the Arkansas River Valley, in the Ozark and Ouachita Mountains. Also all known 14 previous locations in Arkansas for the ornate box turtle will be resampled. Tissue will be collected for genetic analyses and turtles will be marked and released for population estimates and survival analyses. We will also examine the influence of restoration effects in grasslands and forests on population abundance.

A pre-proposal submitted to the Arkansas Game and Fish Commission, State Wildlife Grant Program

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Total Project Cost:
\$217,595

Requested SWG Funding:
\$98,671

In-kind/Match:
\$118,924

Introduction

Two species of box turtle are found in Arkansas: the ornate box turtle (OBT; *Terrapene ornata ornata*), and the three-toed box turtle (TBT; *Terrapene carolina triunguis*). Both species have been listed as species of special concern in Arkansas due mainly to anecdotal evidence that both are declining along with available habitat. Population estimates have not been made for any locations in Arkansas and the distribution data is incomplete. For example, according to Trauth et al. 2004 the TBT was not found in southern Franklin County in grasslands where the largest populations of OBT were located. However, recent surveys have shown that the TBT is currently the dominant species in these grasslands (Huxel, unpublished data).

The OBT is a grassland species and avoids heavily forested areas, but will utilize adjacent forest margins (Reagan 1974). The TBT can utilize both grasslands and heavily forested areas. The diets are similar however the OBT tends to be more of a carnivore while the TBT has a more omnivorous diet. The OBT is rarer known from only 14 sites in Arkansas, most likely due to the limited range of extant grasslands in the state (Trauth et al. 2004). As most of Arkansas is forested, more habitat is available to the TBT and it has a wider distribution relative to the OBT. However, forests in Arkansas have changed dramatically over the past 100-150 years with the loss of chinquapin due to chestnut blight and logging possibly resulting in TBT moving into marginal habitats such as grasslands. Potentially the encroachment of TBT into prairies could negatively impact the OBT. As noted above, the TBT appears to be the dominant species in three prairies surveyed in the Arkansas River Valley (Huxel, unpublished data). In addition to competitive interactions between the two species, earlier studies have hinted at hybridization between the two species. To address this we collected blood samples for genetic analyses to investigate whether hybridization was occurring in the natural setting of the three prairies in the Arkansas River Valley where the two are sympatric. Of seven individuals tested, one appears to be a hybrid (see genetic analyses below).

We have surveyed 6 prairies that are in various stages of restoration using fire and mowing in attempts to return the vegetation to “native” prairie. In addition, we have surveyed 4 forested sites. Currently our recapture rate is too low to provide for estimates of population size and survival. We have marked and released a total of 101 individuals. Determining the species identification is not straightforward given that there appears to be a significant number of hybrids with a range of morphological traits. Based upon best methods and some genetic information (see below) we believe that our total includes 8 OBT, 63 TBT, and 30 purported hybrids. Most of our effort has

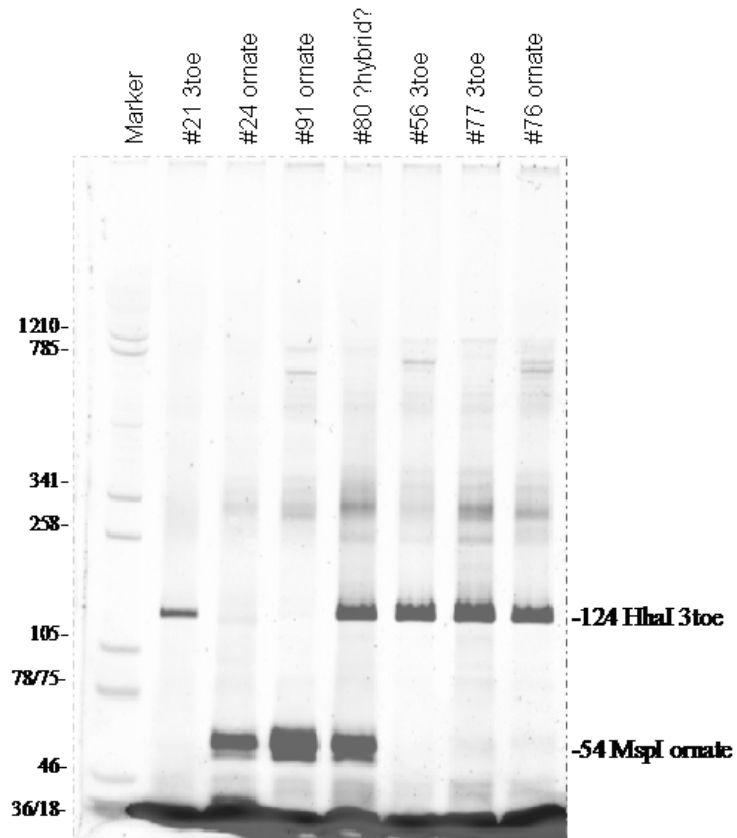


Figure 1. Electrophoresis gel of PCR products to distinguish between OBT and TBT.

been at the Cherokee Prairie in the Arkansas River Valley and we have marked 61 individuals there. No OBT or hybrids have been found in forested locations, but the majority of individuals in the prairies appear to be TBT. Further genetic analysis will aid in our ability to identify individuals to species or as hybrids.

Genetic analyses

We compared Genbank entries for *T. ornata ornata* RNA fingerprint protein 35 (R35) gene, intron (DQ649464) and for *T. carolina triunguis* intron (DQ649463). Multiple polymorphisms were identified but one SNP affected a MspI restriction site in *T. o. ornata*. Based on this SNP, we designed conserved PCR primers to amplify a 298 base region encompassing the MspI site and a upstream HhaI site for a PCR-RFLP test. The reverse primer was 5' Hex-labeled for easy detection. Products from both species should be digested by HhaI to yield a 124 base Hex labeled Hex labeled band. After PCR amplification, aliquots of the PCR products were first resolved on 1.5% agarose gels and detected on a Molecular Dynamics Typhoon 9500 Scanner to estimate the amount of product. Based on the relative quantification, aliquots were diluted in 20 μ l of 1x PCR buffer and digested with 3 units each of HhaI and MspI at 37°C for one hour. The digests were subjected to PAGE analysis (Figure 1). From the analysis we can deduce that there are homozygous turtles that conform with their overt phenotypes (samples #21, 24, 91, 56 and 77). However, sample #80 appears to be heterozygous and thus a hybrid. Surprisingly, turtle #76 had an ornate shell and 4 toes, but was homozygous for the 3-toe (*T. c. triunguis*) SNP. This could either be because this SNP is not diagnostic for these species (polymorphic in both species), or that the 3-toe SNP has been introgressed through hybridization and backcrosses into the *T. o. ornata* population sufficiently to become homozygous in an overtly *T. o. ornata* turtle. The simplest explanation is the former, but additional genotype data is required to determine which explanation is correct.

Based on these results we identified individuals that appeared to be pure *T. ornata ornata* and *T. carolina triunguis*. We used recommended PCR primers (Primmer et al. 2002) to PCR portions of the TGF β , and HNF α genes from these two samples. Each amplicon originated from exonic sequences to encompass a single intron. The PCR products were purified, quantified and sequenced using the PCR primers in the UA DNA Resource Center. For HNF α we obtained 862 bases of overlapping sequence in which we identified 5 potential SNPs. However, none of the SNPs distinguished *T. o. ornata* from *T. carolina triunguis*, all 5 SNPs were polymorphisms (double reads) in the *T. o. ornata* product and were either identical double-reads (1 SNP) or homozygous (4 SNPs) in the 3-toe sample. For TGF β we obtained 986 bases and identified 2 SNPs in the *T. ornata* product that were homozygous in the *T. c. triunguis* sequence, and one SNP that appears to be homozygous A in the *T. o. ornata* sample, and C in the *T. c. triunguis* sample. Sequence data for this same region from a PCR product from the perplexing turtle #76 showed it to be homozygous C. Therefore, based on 2 different genes we have compelling data that either *T. c. triunguis* and *T. o. ornata* share the same nuclear SNPs or hybridization has been going on for quite some time. We propose to sequence additional intron sequences for SNP identification and the mtDNA D-loop region. There are PCR primers for VNTR loci for these two species (Buchman et al. 2009) but it is unlikely that unique repeats would be identified. In addition, Dr. John Wiens (SUNY Stonybrook) has related that he has a paper in review on sequence analysis of some fast evolving nuclear loci in these species (Dr. Wiens was the originator for the original R35 sequences in Genbank).

Proposed Work

We propose to survey all 14 prairie sites in which there are historical records of OBT (Trauth et al. 2004) and to survey forest locations in the Ouachita National Forest, Ozark National, Madison County Wildlife Management Area, and the Fort Chaffee Wildlife Management Area. All turtles found will be measured for length and width, height of carapace, weighed, marked, and released.

Data will be collected on temperature, humidity, geographical location, elevation, activity of the turtle, and habitat found in. We will also use radio telemetry and geo-referencing of capture locations in three prairies and three forests to examine habitat use and to determine home range size. We will collect blood samples for genetic analysis to examine population structure and the extent of hybridization (for the sympatric populations). We expect that with increased sampling we will improve our recapture rate and thus be able to provide population size and survival estimates that could be used to better manage habitat for the two species. We will also assess restoration efforts on the population dynamics of the two species; hopefully to see which restoration methods best suit their long-term persistence. Our proposed study should allow for best management practices to be discerned based upon habitat type and availability. We expect that OBT will need further protection via listed as a federally endangered species in Arkansas.

Estimated Budget for 2 year project

Graduate Student	Salary	\$20,000/yr
	Benefits	\$1,526/yr
	Tuition	\$5,670/yr
PCR primers (labeled and unlabeled)		\$600/yr
PCR product purification, DNA sequencing charges		\$200/yr
PCR reagents, gels, disposables		\$3,200/yr
Travel Costs (estimated mileage 15,000 mi/yr @ \$.42/mi)		\$6,300/yr
Housing Costs (2 nights/month @ \$50/night)		\$2,400/yr
Materials including radio tags		\$2,500/yr
Trimble GPS unit with antennae		\$5,400
10% overhead (based upon AGFC requested funds)		\$8,479
	Total	\$98,671

Matching

Salaries and benefits for Drs. Huxel (25%) and Rhoads (10%)		\$27,912/yr
DNA Sequencer		\$30,000
Thermocycler for PCR		\$4,000
Centrifuge		\$16,500
Freezer for DNA samples		\$6,500
Computers and Software		\$5,000
Radio telemetry receiver		\$1,100
	Total	\$118,924

Matching is based upon limited understanding of what types of charges are allowed in matching. Equipment costs are estimates of replacement value.

Buchman A, Deaton R, Randle C, Brummel T, Wilson E et al. (2009) Isolation and characterization of polymorphic microsatellite loci for the three-toed box turtle (*Terrapene carolina triunguis*) and cross-amplification in other *Terrapene* species. *Molecular Ecology Resources* (in press).

Primmer C, Borge T, Lindell J, Saetre G-P (2002) Single-nucleotide polymorphism characterization in species with limited available sequence information: high nucleotide diversity revealed in the avian genome. *Mol Ecol* 11(603-612).

Reagan, DP (1974) Habitat selection in the three-toed box turtle, *Terrapene carolina triunguis*. *Copeia* 63:512-527.

Trauth, SE, Robison, HW, Plummer, MV. (2004) *The Amphibians and Reptiles of Arkansas*. University of Arkansas Press.

Qualifications

Dr. Huxel is an Assistant Professor at the University of Arkansas in the Department of Biological Sciences. His PhD is in Ecology and he has been studying box turtles for two years. Dr. Huxel has published over 20 journal articles and book chapters as well as co-editing a book entitled "Food webs at the landscape level."

Dr. Rhoads is a Professor at the University of Arkansas in the Department of Biological Sciences. Dr. Rhoads has been studying genetics for nearly 30 years and has written numerous journal articles and book chapters. Dr. Rhoads has worked on a number of organisms from microbes to rattlesnakes to birds and now turtles.