FINAL PERFORMANCE REPORT

As Required by

THE ENDANGERED SPECIES PROGRAM

TEXAS

Grant No. TX E-145-R

F12AP00860

Endangered and Threatened Species Conservation

Post de-listing demographic and genetic monitoring of the

Concho water snake (*Nerodia paucimaculata*)

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FINAL REPORT

STATE: _____Texas_____ GRANT NUMBER: _____TX E-145-R-1___

GRANT TITLE: Post de-listing demographic and genetic monitoring of the Concho water snake (*Nerodia paucimaculata*)

REPORTING PERIOD: _____1 Septeber 2012 to 31 August 2016_

OBJECTIVE(S). To use both demographic and genetic methods of assessing population viability to conduct 3 years of post-delisting monitoring of the Concho water snake and establish a recommended protocol for the long-term monitoring of this species.

Segment Objectives:

Task 1: Sept 2012 - Mar 2013. Identify 18 sites for monitoring and contact landowners to obtain permission to work on their property. (These sites will be based on previous surveys and those listed in the Post Delisting Monitoring Plan (Dixon et al. 1998, USFWS 2011).

Task 2: Apr 2013 – Jul 2013. Begin monitoring and genetic sampling at the 18 sites by trapping and active searching at all sites for the Concho water snake using a mark-recapture survey design. Also, during this time period, we will begin systematically paddling the Colorado River drainage to search riffle sections in order to assess the complete distribution of the Concho water snake.

Task 3: Aug 2013 – mid Sep 2013. Continue monitoring and genetic sampling at the 18 sites by trapping and actively searching for neonate snakes.

Task 4: Oct 2013 – Apr 2014. Analyze data from the first year, including census population estimation and viability analysis. Microsatellite genotyping of tissue samples and genetic analyses to estimate Ne and gene flow.

Task 5: Apr 2014 – Jul 2014. Monitor and collect genetic samples of snakes at the 18 sites with trapping and active searching. Also we will continue the total distribution search for the snake.

Task 6: Aug 2014 –Sep 2014. Monitoring and genetic sampling at the 18 sites by active searching for neonate snakes

Task 7: Oct 2014 – Apr 2015. Analyze data from the first and second year, including population estimations and viability analysis. Genotyping of tissue samples from the second year and population genetics analyses to estimate Ne and gene flow.

Task 8: Apr 2015 – Jul 2015. Monitor and collect genetic samples of snakes at the 18 sites with trapping and active searching. Also we will continue the total distribution search for the snake.

Task 9: Aug 2015 – Sep 2015. Monitoring and genetic sampling at the 18 sites by active searching for neonate snakes.

Task 10: Apr 2015 – Sep 2015. In this last year, data analysis and genotyping will be done concurrently with field work. Final data analyses over the entire project will be completed.

Significant Deviations:

None.

Summary Of Progress:

Please see Attachment A; Excel file also submitted (sent electronically) containing details of collections, including date of capture, sex, morphometrics, GPS data (dd), PIT tag IDs, and miscellaneous observations.

Location: Mitchell, Coke, Tom Green, Runnels, Concho, Coleman, McCulloch, Brown, Mills, San Saba, Lampasas Counties, Texas.

Cost: ___Costs were not available at time of this report, they will be available upon completion of the Final Report and conclusion of the project.___

Prepared by: _Craig Farquhar_____   Date: _30 October 2016_

Approved by: _____ Date:_____30 October 2016_____
                        C. Craig Farquhar

ATTACHMENT A

POST DE-LISTING DEMOGRAPHIC AND GENETIC MONITORING OF THE CONCHO WATERSNAKE (*NERODIA PAUCIMACULATA*): FINAL REPORT

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INTRODUCTION

The Concho watersnake (*Nerodia paucimaculata*) is a relatively small natricine snake endemic to the Concho and Colorado rivers of central Texas (Whiting et al. 1997). It was initially federally listed as a threatened species due to its small geographic range, specific habitat requirements, and potential effects of modification to its habitat. Extensive field work was conducted in the late 1980's and early 1990's to understand its natural history and to determine the effects of dams on the snake, specifically the construction of O. H. Ivie reservoir (Dixon et al., 1988; 1989; 1990; 1991; 1992; Greene et al., 1994; 1999; Whiting et al., 1997; 2008). Evidence from this work suggested that the Concho watersnake was not restricted to the riffle sections but that they would also use habitats within lakes, especially areas with rocks along the shoreline. However, these large impoundments may potentially restrict connectivity of populations and thus, restrict gene flow. The US Fish and Wildlife service removed the Concho watersnake from the federal endangered species list (Federal Register 76:66780-66804; July 8, 2011). The Concho watersnake Recovery Plan (U.S. Fish and Wildlife Service, 1993) listed 3 criteria for post-delisting monitoring of the species: (1) assurance of adequate instream flows; (2) stable, viable populations in the 3 main reaches of the species' range; and (3) assurance of movement of adequate number of Concho watersnakes among the 3 reaches to counteract adverse effects of fragmentation caused by the O.H. Ivie Reservoir. FWS has determined that the threats to this species have been reduced to the point where the species no longer meets the definition of threatened or endangered (USFWS 2011). However, detailed quantitative data are needed to determine whether these criteria continue to be met.

The Endangered Species Act requires "delisted" species to be monitored for at least 5 years to ensure that the status of the species does not change, and thereby become threatened following the removal of the legal protections under the Act. Some biologists, including at least 1 reviewer solicited by FWS, expressed concerns during the comment period for the proposal to delist the species. Specifically, these biologists emphasized the need for rigorous post-delisting monitoring to ensure that populations of this species continue to be viable. The ESA Section 6 grant program requested a proposal to conduct 3 years of post-delisting monitoring of the Concho watersnake.

Herein, we describe the findings of our 3 year post-delisting monitoring study. We used demographic (mark-recapture) and genetic (effective population size and connectivity estimates) data for post-delisting monitoring with the goal of providing critical information needed to assess the status of the Concho watersnake populations after de-listing. Both genetic and demographic data are needed to fulfill criteria 2 and 3 (stated above) of the recovery plan outlined by the USFWS (1993). Specifically, demographic monitoring is needed to obtain estimates of distribution, census population sizes, and fluctuations in population size over time (criterion 2) and to assess habitat use. However, it is labor intensive to continue demographic monitoring over the long-term. Furthermore, demographic methods (e.g., radio telemetry) provide limited information on snake dispersal between populations, are expensive, and often yield small sample sizes, thus, making it difficult to infer connectivity. Fortunately, genetic data offer an efficient means to monitor changes in population sizes over

time and to estimate movement (i.e., gene flow) among locations (Luikart et al. 2010). Robust estimates of the effective population size (N_e) from molecular markers are necessary to assess population viability and adaptive potential under changing environmental conditions (e.g., habitat alterations, drought) because N_e reflects the levels of genome-wide genetic diversity in the population (Franklin and Frankham 1998; Willi et al. 2006; Luikart et al. 2010).   Molecular data are frequently used to estimate connectivity among populations as genetics data can generate information on both historical and contemporary levels of gene flow, identify first generation migrants, and individuals with mixed ancestry (i.e., maternal and paternal lineage from different populations; Luikart et al. 2010). Our set goals, contingent upon appropriate sample sizes and random sampling, were to use genetic and demographic data form the Concho watersnake to monitor changes in N_e and census size, respectively, over time (criterion 2) especially in relation to river flow conditions, and to assess connectivity among the 3 reaches of the river, especially in relation to any potential obstruction caused by the O.H. Ivie and E.V. Spence Reservoirs (criterion 3). However, as we outline in our findings, sample sizes of Concho watersnakes were too small to carry out these ideal analyses. Prolonged drought conditions persisting into 2013 and 2014 likely precluded snake activity. With more rainfall in 2015, sampling was better. Nonetheless, locations on the upper Colorado River where the Concho watersnake was previously known to occur remained dry. Hence no Concho watersnakes were found in the upper most reaches downstream of Spence Reservoir and upstream of Hwy 277 of the Colorado River. In addition, even with intensified efforts, no Concho watersnakes were found on the Concho River, which was noted has having habitat characteristics that are not archetypical of Concho watersnake ecology and that differed from that of Colorado River locations where the snake was found. Below we provide details of our demographic surveys and a preliminary genetic assessment based on the sample of snakes we were able to catch.

Task-8- Spring/Summer Surveys

April-June 2015

   The USFWS requested a clarification of the 2015 interim report regarding post-delisting monitoring site substitutions and additions from task 8. The purpose of task 8 was to complete the population monitoring surveys outlined in task three of the initial proposal. As outlined in the post-delisting monitoring plan (PDMP), Concho watersnake surveys were to be conducted at 18 sampling locations along the Colorado and Concho River, two tributaries of the Colorado River and three reservoirs (Table 1, see also S1 Figures 1-4). During the course of the 2013 to 2015 surveys it became necessary to alter the location of some of the sites recommended by the PDMP.   The Concho watersnake had previously been captured at all of the sites that were added during our surveys. All site additions and substitutions were made after consulting with J. Dixon and based on his recommendation for sites where stable Concho watersnake populations had been collected during his research. Rusk and Smith_AR were both dry during the 2013-2015 surveys and were replaced with Hwy 277 and CR 293. A small stretch of the Colorado River Hwy 208 south of Robert Lee, TX near Rusk had water for a short period of time in 2014 and was surveyed at that time. Rusk and Smith continued to be dry. Both Cervenka Dam and Blair are private properties whose owners declined to allow their properties to be surveyed. Potter Falls was substituted for Blair. Twelve Mile bridge was added to replace Cervenka Dam but is not considered a substitution because they are located too far apart. Smith on the Lower Colorado River and Glassscock are also privately owned and the owners declined to allow surveys on their property. Heatchen Ranch, Bell Street Bridge and the South Fork sites were added to increase sampling along the Concho River but are not considered substitutions.

Distance of river or reservoir sampled varied among sites depending on the type of habitat that was in a reach of the stream. To account for this, river distance was standardized to one mile of river per survey site that encompassed the majority of the riffle habitat available. When this standardization was not possible due to small numbers of riffles or long distances between riffle habitats, larger segments of the river were surveyed. The purpose of this standardization was to ensure reproducibility in monitoring efforts of the Concho watersnake in the years following the initial three years of the post-delisting monitoring plan. For sites that were surveyed in both 2013 and 2014, survey distance, location and trap number were kept consistent between years.

Table 1. Post-delisting monitoring site locations for the Concho watersnake 2013-2015. The original 18 sites recommended in the post-de-listing monitoring plan are numbered 1-18. The sites that were substituted or added during these surveys are numbered 19-26. Site Numbers correspond S1 Figures 1-4). Four sites from the original PDMP were not surveyed between 2013 and 2015 are marked NS in the GPS location. Eight new sites were added. All eight sites have previously been documented to have Concho watersnakes.

	Site Name	Site Number in PDMP	Added Site #	Upstream		Downstream	
				Latitude	Longitude	Latitude	Longitude
Reservoir	E.V. Spence 1	1a		31.918269°	-100.551140°		
	E.V. Spence 2	1b		31.916186°	-100.531181°		
	E.V. Spence 3	1c		31.906604°	-100.536601°		
Upper Colorado River	Rusk	2		31.883025°	-100.471309°		
	Smith_AR	3		31.849105°	-100.388879°		
	Robert Lee 208		19	31.885631°	-100.480420°	31.885852°	-100.472568°
	Hwy 277 S-Bronte-		20	31.847704°	-100.291970°	31.839605°	-100.263778°
	CR 293		21	31.812718°	-100.226190°	31.809190°	-100.217972°
	Cervenka Dam	4		NS			
	Blair	6		NS			
	Lake Ballinger-Colorado	7		31.729364°	-100.043121°		
	Hwy 83-Colorado	5		31.730221°	-99.941243°	31.731215°	-99.917710°
	Elm Creek-Colorado	8		31.760825°	-99.955570°	31.785338°	-99.841420°
	Potter Falls		22	31.682696°	-99.877002°	31.679240°	-99.832191°
	12 Mile Bridge		23	31.657718°	-99.845776°	31.636338°	-99.711060°
Reservoir	O.H.Ivie Reservoir-1-Concho Recreation Area	13a		31.555571°	-99.711988°	31.557208°	-99.711060°
	O.H.Ivie Reservoir-2-Colorado	13d		31.488207°	-99.682464°		
	O.H.Ivie Reservoir-3-Colorado	13c		31.562679°	-99.679879°	31.556878°	-99.674645°
	O.H.Ivie Reservoir-4-Colorado	13b		31.526948°	-99.650987°	31.532853°	-99.639831°
Lowe	Frees Dam-	14		31.498513°	-99.661599°	31.497889°	-99.657992°

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	Smith	15		NS			
	Cooper (Hwy 283)	16		31.454991°	-99.399751°	31.439263°	-99.375066°
	Theriot (River Bend Ranch)	17		31.415028°	-99.330595°	31.415397°	-99.308853°
	HWY 377	18		31.462438°	-99.185997°	31.467554°	-99.161125°
Concho River	**FM_380 (Veribest)**	9		31.470594°	-100.341020°	31.477100°	-100.332018°
	Vinson Dam	10		31.513502°	-99.960596°	31.515898°	-99.956678°
	Haechten Ranch		24	31.542153°	-100.177977°	31.549301°	-100.161608°
	South Fork		25	31.192697°	-100.504962°	31.203171°	-100.507572°
	Bell Street Bridge		26	31.45275°	-100.413578°	31.452957°	-100.405907°
	Paint Rock	11		31.513170°	-99.915770°	31.529875°	-99.894900°
	Glasscock	12		NS			

Traps were partially submerged and placed along natural structures within the river in order to direct the path of snakes into the trap. Traps were also baited with "Little Stinker" Catfish bait to attract fish into the trap and subsequently attract watersnakes. Traps were placed in riffles and adjacent pools up and downstream from the riffle. Traps were checked twice each day. The number of traps set and the duration of each trapping effort were recorded. Traps were left in place for approximately 72 hours, depending on the weather, flow conditions, and capture success rates.

Each segment of river and reservoir habitat was also actively searched for two to four hours depending on the length of river and flow conditions. Concho watersnakes frequently take refuge under rocks in and along the edge of the river, as well as along other sorts of debris found along river banks. Also, snakes can frequently be seen basking on vegetation overhanging the river. We conducted surveys overturning (and replacing) potential refuge sites and scanning potential basking sites in search of snakes. All snakes located by sight were captured when possible. If capture attempts were unsuccessful, the species of snake and GPS coordinates of the location where the individual was sighted were recorded. At each survey site, the total length of the shoreline searched, and the names and number of individuals involved were also recorded. GPS locations of all snakes captured were recorded.

The 2013 and 2014 surveys were conducted during extreme drought conditions (USGS Annual water Reports). The 2015 spring and summer sampling season experienced the largest influx of rain that West-Central Texas had experienced since the Concho watersnake post-delisting monitoring was initiated in 2013 (Table 2). Despite the heavy rains during this time, the upper portion of the Colorado River continued to experienced low water conditions. However the middle and lower portions of the Colorado River and the entire stretch of the Concho that was surveyed had noticeably more water for the duration of the spring and summer surveys compared to the 2013 and 2014 survey period. Between May 11 and May 31, 2015, access to the sections of the Colorado and Concho Rivers was restricted and intermittent due to high water. For this reason, several sets of four-day sampling periods were interrupted and then reinitiated when the rivers were once again safe to access. Despite the limited access during this time, the large amounts of rain and flooding experienced in May 2015 resulted in significantly more snake activity during the late summer and fall surveys than were seen in 2013 or 2014. The number of days spent sampling on the Concho River was increased from 4 days per site to 16 days per site. This was done in an attempt to increase the detection of Concho watersnakes as none were detected along this river in the 2013 and 2014 surveys.

Task 9- Late Summer/Early Fall Surveys

Late summer and early fall surveys were conducted in order to detect the presence of neonate snakes. Each of the sites where Concho watersnakes were detected during the summer of 2015 surveys was revisited in the fall of 2015. These surveys were conducted following the protocols outlined in Task 5. Chris Chapa and Diego Araujo from the USFWS Texas Fish and Wildlife Conservation office assisted in all aspects of the late summer surveys of 2015. Due to the small size of the neonate snakes and their propensity to be found under rocks rather than basking or active feeding, additional time was devoted to each site to actively search the survey area by flipping rocks. Neonate snakes were collected at the Freese Dam, Hwy 83, 12 Mile Bridge, and Hwy 283 sampling sites, as well as within O.H. Ivie reservoir at the Concho Recreation Area. Neonate snakes were detected at all of the sampling locations where adult Concho watersnakes were encountered during the summer surveys of 2015 except Potter Falls. During the 2013 and 2014 field seasons, the monitoring sites along the Concho River yielded few watersnakes (i.e., other *Nerodia spp*.) in general relative to the Colorado River sites. Additionally, no Concho watersnakes were detected on the Concho River during the course of these surveys. For these reasons, all Concho sites were surveyed again during the September surveys to increase the likelihood that Concho watersnakes would be detected. **Despite these additional efforts, Concho watersnakes were never detected at Concho River sampling locations.**

Task 10:   Analysis of Genetic Monitoring and Demographic Monitoring Data

Molecular Methods*--Library Preparation and Microsatellite Development*--In 2013, a tissue sample collected from Hwy 277 survey site in 2013 was submitted to the Sequencing and Genotyping Facility at the Cornell Life Sciences Core Laboratory Center (Ithaca, NY) in order to develop a microsatellite library. DNA was extracted with Qiagen DNeasy Tissue kits (Hilden, Germany). Genomic DNA was digested using three restriction enzymes (*AluI*, *RsaI* and *Hpy 166 II*) in three different reactions. Restriction enzymes were the heat de-activated. Digested and ligated DNA fragments were enriched for microsatellites via hybridization to 3'-biotinylated oligonucleotide repeat probes. The enriched fragments were captured using streptavidin-coated magnetic beads and amplified using Platinum *Taq* polymerase (Invitrogen) and both a universal and an indexed Illumina primer. PCR products were examined on an agarose gel and quantified. Equal amounts of each restriction enzyme library were pooled and fragments between 300 and 600 bp were recovered and sequenced on an Illumina MiSeq with a 2 x 250 paired end at 10X coverage. The raw data was imported and assembled in SEQMAN NGEN v. 11. The two assemblies resulted in both single contigs (i.e., those contigs that did not assemble with another conting) and metacontigs (i.e., contigs that did assemble with other contigs). Both single and metacontigs were scanned for microsatellite loci and primer pairs were designed using MSATCOMMANDER v. 1.0.3.

A subset of the resulting library of potential primers and loci were selected to encompass variation in product size, repeat count, and dimeric, trimeric and tetrameric repeats. The M13 method for genotyping was used by attaching a 20-bp tag to the 5' end of the forward primer (Schulke 2000). A short tail sequence (GTTTCTT) was added to the 5'end of the reverse primer to reduce polyadenylation (Brownstein et al. 1996). Primers were screened using DNA extracted from the anterior portions of 6 *Nerodia paucimaculata* individuals by a 5% chelex and 0.2 mg/mL protienase K in 200 µL volume reactions. The samples were incubated at 56º C for 2 hours and then boiled at 100º C for 8 minutes. PCR amplification was performed in 10 µL reactions containing 3.1 µL ultrapure water, 5 µl 2X Qiagen Type-IT kit Master Mix, 0.16 µL fluorescent-labeled M13

primer (Applied Biosystems: FAM), 0.08 µL M13-labeled forward primer, 0.16 µL of 10 µM reverse primer and 1.5 µL of genomic DNA. The thermocycler profile was 94 ºC for 5 minutes, 31 cycles of 94ºC for 30 seconds, 56º C for 45 seconds, 65º C for 45 seconds, followed by nine cycles of 94ºC for 30 seconds, 53ºC for 45 seconds 65 ºC for 45 seconds and extension at 65 ºC for 10 minutes. PCR-product was visualized on a 2% agarose gel run in 0.5X TBE buffer at 95 V for 45 minutes. Primers that yielded discrete bands in the expected product size range were sent to the DNA Analysis Facility on Science Hill at Yale University (Princeton, NJ, USA) and visualized on a 3730xl 96-capilary Genetic Analyzer with 500-LIZ size standard. Samples were genotyped on GeneMarker V2.6.4. Initially, we tested primer pairs for 48 loci in a subset of 6 Concho watersnakes. Of these 48, 18 were found to amplify consistently and produced bands that could be scored reliably. We then genotyped 110 Concho watersnake individuals collected between the 2013 to 2015 sampling years at these 18 loci. Of these 18 loci, 5 loci were not included in final analyses; 2 were effectively monomorphic so contained no information and 3 showed very strong deviations from Hardy-Weinberg Equilibrium (details on analyses below) suggesting technical problems with these loci. Thus, the final data set contained 13 loci genotyped across 110 Concho watersnakes.

Genetic Diversity and Equilibrium Tests

As an initial assessment of potential population structuring and genetic diversity, samples were grouped into 3 regions: the Upper Colorado River (UCR), Lower Colorado River (LCR), and O.H. Ivie Reservoir. Samples from all three years were grouped into these three *a priori* delimited groups due to small sample sizes within each given sampling location and/or year (S2, data tables). Gene diversity (H_s), the number of alleles per locus (An), and allelic richness (A_R) (rarefied number to the lowest region sample size of $n = 21$) were calculated in FSTAT (version 2.39; Goudet 2001). The number of private alleles was calculated using GenAlEx (version 6.502; Peakall and Smouse 2006). Estimates of F_{IS} (which quantifies the proportional change in heterozygosity due to deviations in Hardy Weinberg Equilibrium, HWE; $F_{IS} = 0$ if there is HWE) and randomizations to test if F_{IS} deviated from 0 were conducted in in FSTAT (version 2.39; Goudet 2001). Tests of pairwise linkage disequilibrium (LD) between loci was performed in Genepop (Version 4.2; Raymond and Rousset 1995).

Population Structure and Connectivity

N_e of each of the three regions was estimated using the linkage disequilibrium estimator that was revised by Waples (2006) and is implemented in the software LDNe (Waples and Do 2008). An overall test of genetic differentiation (and estimation of F_{ST}) along with pairwise tests of genetic differentiation (and pairwise estimation of F_{ST}) were conducted among the three regions using FSTAT. These tests rely on *a priori* delimitations of populations, thus we also conducted 2 individual based clustering analyses: 1) Principal Coordinate Analysis (PCoA) (as implemented in GenAlEx), which is a multivariate method that is not based HWE or linkage equilibrium, and 2) the model based clustering implemented in STRUCTURE 2.3.4 (Pritchard et al. 2000), which partitions individuals based on the assumptions of HWE and linkage equilibrium. The input parameters for STRUCTURE were correlated allele frequencies, and the admixture model. STRUCTURE was run with 300,000 iterations with a burn-in of 100,000 iterations for K (i.e., the number of possible clusters) values 1 to 10 with 10 replications of each possible K value.

Demographic Methods

Mark-recapture data for the Concho watersnake from 2013 to 2015 surveys were originally planned to estimate population viability and population size estimates. However, Concho watersnakes were only recaptured during the 2015 sampling season and even then, there was an extremely low recapture rate. Thus, only data from the 2015 season were used to estimate population size. No estimate of changes in population

size across the three years was feasible with the current data set. Population size model implementation was conducted using the program MARK (White and Burnam 1999). Models based on Huggins (1989) were selected for use as only the probability of the first recapture and the probability of recapture are required to obtain derived estimates of population size.   The models assessed were constant (M_0), constant probability of capture (M_t,), time varying probability of recapture, and behavioral response to recapture (M_b.). The suitability of these three models, which incorporate different sources of variance for capture probabilities and variation by time, was assessed using AIC_c (AIC-corrected). The model best fitting the data (the lowest AIC value) was used to estimate population size (Burnham and Anderson 2002).

Summary of Results 2013-2015

  Field Surveys--A total of 371 watersnakes were captured between 2013 and 2015 surveys (S2, data tables). The yellow-bellied watersnake (*N. erythrogaster*) was the most commonly captured snake and accounted for 51.2% of the total snake captures. The diamond-backed watersnake, (*N. rhombifer*) accounted for 16.4% of the total captures. Concho watersnakes accounted for 32.5 % of the total number of snakes captured. Of the 120 total number of Concho watersnakes captured, 113 were unique individuals and 7 were recaptures (S2, data tables). Recaptures of Concho watersnakes occurred only during 2015 surveys. Four of the seven recaptures occurred within one month of the individuals' initial capture date. Two were captured one year after their initial capture date. One of these latter two individuals that was originally marked in 2014 was captured twice in 2015. Four additional Concho watersnakes were observed but were not captured. Thus, a total of 117 individual Concho watersnakes were observed during the course of these surveys. Tissue samples were collected from 110 of the 113 caught Concho watersnakes. Of the captured Concho watersnakes, 54.8 % were neonates, 24.7 % were juveniles and 20.4% were adults (classification of age group was based on snout-vent-length (SVL): adult males >380 mm SVL, adult females >420 mm SVL, juvenile females <420 mm SVL, neonates <250 mm SVL; Greene et al 1999). **Of the 113 unique individuals captured, 5 were captured in 2013, 11 were captured in 2014 and 97 were captured in 2015.** There were 34 Concho watersnakes captured during the spring/summer sampling of 2015. This is in contrast to the 5 and 7 individuals captured during the spring/summer seasons of 2013 and 2014, respectively. The major increase in captures in 2015 was due largely to the presence of Concho watersnakes in O.H. Ivie reservoir, which accounted for 58 of the 97 Conchos captured in 2015. Moreover, 44 of the 97 Conchos captured in 2015 were neonates from the fall sample.

  Catch-per-unit-effort (CPUE) for the Concho watersnakes was calculated for both foot-search and minnow trap by reach and by year (Table 2). The amount of survey effort required to find Concho watersnakes was considerable across all years. The 2015 sampling period yielded the largest CPUE for both the upper and lower Colorado Rivers and O.H. Ivie Reservoir. In 2013 for instance, the CPUE on the upper Colorado River was 1 Concho watersnake for every 156 search hours. This increased in 2015 to one Concho watersnake caught per every 17.92 active search hours. No Concho watersnakes were captured or observed on the Concho River during 2013-2015 surveys despite extensive trapping and search effort (Tables 2 and 3).

Table 2. Catch-per-unit effort (CPUE) for each reach of the Colorado River system for each year. CPUE is calculated as the number of Concho watersnakes caught by hand or by trap per reach divided by the total number of search hours/trap hours per reach for each year. Asterisks indicate that no snakes were caught for a given reach or year for that particular capture method.

Reach	2013	2014	2015

	Trap Hours	Search Hours	Trap Hours	Search Hours	Trap Hours	Search Hours
Spence Reservoir	**	**	**	0/24	**	0/24
Upper Colorado River	1/9035	1/156	0/17872	1/54.25	1/6341.25	1/17.92
O.H. Ivie Reservoir	0/6450	0/36	**	0/24	**	1/1.67
Lower Colorado River	1/1087	0/8	1/5586	1/31.8	1/864	1/9.86
Concho River	0/11235	0/116	0/9930	0/134	0/1862.5	0/262.5

The Concho watersnake was detected at 7 of the 25 total surveyed sites (including dry sites and across all years) between the 2013-2015 surveys (Table 3). Gravid females were captured, tagged and released at 5 of the 7 sites where Concho watersnakes were present (Cooper, Freese Dam, Potter Falls, O.H. Ivie, and Hwy 83) during the 2015 surveys. Neonate snakes were detected at all sites where Conchos where detected in 2015 surveys except Hwy 277 and Potter Falls. Recaptures occurred at Freese Dam, Cooper and O.H. Ivie locations.

Populations were detected within the core of the Concho watersnake's range between Hwy 277 S of Robert Lee and northwest of Hwy 283 south of Santa Ana (Site Name: Cooper) (Table 3). The presence of gravid females and neonates at these locations also indicates that some recruitment was taking place. The seven sites where Concho watersnakes have been detected are all areas where the river continues to flow even during periods of drought and where riffles are consistently present during the spring and summer months. Concho watersnakes were detected at O.H. Ivie reservoir at the Concho Recreation Area for the first time during the 2015 sampling season. No Concho watersnakes were found along sampling sites of the Concho River.

Table 3. Presence and absence at the post-delisting monitoring plan sites (PDMPS) for the post-delisting monitoring of the Concho watersnake from 2013 to 2015. X indicates sites where Concho watersnakes were detected in a particular year. 0 indicates sites that were surveyed but no Concho watersnakes were found. Unmarked sites indicate that site was not surveyed that year. Dry indicates sites that did not have water during the site the summer site visits.

	Site Name	2013	2014	2015
	Percent of Sites Occupied by Concho watersnakes (excludes dry sites)	**20%**	**15%**	**31%**
Reservoir	**Spence Reservoir**	0	0	0

	Rusk	Dry		Dry
	Smith	Dry		Dry
	Robert Lee 208	Dry	0	Dry
	Hwy 277 S-Bronte	X	0	0
Upper Colorado River	**CR 293**	0	0	0
	Lake Ballinger		0	0
	Hwy 83	X	X	X
	Elm Creek	0	0	0
	Potter Falls			X
	12 Mile Bridge		0	X
	O.H.Ivie Reservoir-1-Concho Recreation Area	0	0	X
Reservoir	**O.H.Ivie Reservoir-2-Colorado**	0	0	0
	O.H.Ivie Reservoir-3-Colorado	0	0	0
	O.H.Ivie Reservoir-4-Colorado	0	0	0
	Frees Dam-Colorado	X	X	X
Lower Colorado River	**Cooper-Colorado (Hwy 283)**		X	X
	Theriot-Colorado (River Bend Ranch)		0	
	HWY 377-Colorado		0	0
	FM_380-Concho (Veribest)	0	0	0
	Vinson Dam	0		0
Concho River	**Haechten Ranch**	0	0	0
	South Fork	0		
	Bell Street Bridge		0	
	Paint Rock	0	0	0

Hydrological Monitoring

Hydrological data was collected from 7 of the 8 USGS stream flow gauges from 2010 to 2015 (Tables 4-6). Steam flow data (ft^3/sec) was collected from site 08138000 the Colorado River at Winchell, TX for only 2010 and 2011 as monitoring by the USGS at this site was discontinued after 2011. The annual mean discharge for the upper Colorado River was lowest during the 2013 sampling season, which also corresponded to the lowest number of Conchos collected and the smallest CPUE values. In contrast, 2015 had the highest annual mean discharge for the upper Colorado River sites, the least number of days with an average discharge of 0 ft^3/sec. Mean annual discharge from Stacy Dam decreased during from the 2013 to 2015 sampling period. Both monitoring sites in the Concho River had higher mean annual discharge values and fewer days with a mean discharge of 0. Despite the higher stream flows in the Concho for the last five years, no Concho watersnakes were detected on the Concho River. With the exception of the Concho River monitoring sites, greater stream flow levels and fewer days per year with 0 stream flow were associated with larger CPUE values.

Unfortunately, correlational analyses between hydrological data and current Concho watersnake demographic data were not possible due to the following reasons. First there was an extremely small number of Concho watersnakes captured during the 2013 and 2014 sampling periods. In addition, as noted above under Task 8, there was variation in sampling at sites on the upper and lower Colorado between years (for example Hwy 283, 12 Mile Bridge and Potter Falls were not surveyed in 2013). Lastly, to increase our chances of finding Concho watersnakes, we increased surveys during the 2015. Thus, sampling effort was not consistent across years.

Table 4. Hydrological data from the USGS monitoring station in the Upper Colorado River. Annual harmonic mean not calculated for years where one or more months mean discharge is 0 ($1/0 = \infty$).

Annual Statistics for Stream Flow (ft^3/sec) 08123850 Colorado River above Silver, TX						
	2010	**2011**	**2012**	**2013**	**2014**	**2015**
Annual Mean	19.67	41.67	43.43	35.19	26.74	76.68
Annual Median	3.35	2.41	15.37	3.62	44.67	44.67
Annual Peak	74.5	429	260	148	192	271
Annual Harmonic Mean	1.49	**	**	0.292	**	3.53
Number of Days with mean of 0	17	164	188	17	97	0

Annual Statistics for Stream Flow (ft^3/sec) 08124000 Colorado River at Robert Lee, TX						
	2010	**2011**	**2012**	**2013**	**2014**	**2015**
Annual Mean	0.815	0.04	0.58	0.015	0.031	0.73
Annual Median	0.145	0.04	0.015	0.01	0.016	0.03
Annual Peak	0.5	0.11	0.04	148	0.205	7.11
Annual Harmonic Mean	**	**	**	**	**	**
Number of Days with mean of 0	69	160	229	196	188	82

Annual Statistics for Stream Flow (ft^3/sec) 08127000 Elm Creek at Ballinger, TX						
	2010	**2011**	**2012**	**2013**	**2014**	**2015**
Annual Mean	13.92	1.09	16.49	2.37	10.17	24.45
Annual Median	7.82	0.06	0.115	0.14	1.38	2.43
Annual Peak	61.1	9.46	190	26	70	149.8
Annual Harmonic Mean	**	**	**	**	**	0.049
Number of Days with mean of 0	140	208	276	163	226	27

Annual Statistics for Stream Flow (ft^3/sec) 0812638 Colorado River near Ballinger, TX

	2010	2011	2012	2013	2014	2015
Annual Mean	4.39	0.37	22.33	10.79	8.98	13.39
Annual Median	2.105	0.06	0.44	0.58	1.25	1.04
Annual Peak	15.5	1.51	258	61.4	52	75.3
Annual Harmonic Mean	**	**	**	0.70	0.75	1.16
Number of Days with mean of 0	153	161	157	0	0	0

Table. 5. Hydrological data from the USGS monitoring station in the Lower Colorado River. Annual harmonic mean not calculated for years where one or more months mean discharge is 0 ($1/0 = \infty$). No data was collected from site number 08138000 Colorado at Winchell, TX after 2011 as this station is no longer in operation.

Annual Statistics for Stream Flow (ft^3/sec) 08136700 Colorado River neat Stacy, TX						
	2010	2011	2012	2013	2014	2015
Annual Mean	3.90	6.06	8.63	11.71	6.177	5.94
Annual Median	2.93	6.13	5.59	4.07	3.85	4.29
Annual Peak	8.87	8.1	33.5	61.3	20.7	15
Annual Harmonic Mean	2.79	5.56	0.11	1.36	3.18	3.83
Number of Days with mean of 0	9	0	61	22	41	0

Annual Statistics for Stream Flow (ft^3/sec) 08138000 Colorado River at Winchell, TX						
	2010	2011	2012	2013	2014	2015
Annual Mean	32.64333	1.3225	--	--	--	--
Annual Median	16	0.055	--	--	--	--
Annual Peak	117	4.32	--	--	--	--
Annual Harmonic Mean	5.9361	**	--	--	--	--
Number of Days with mean of 0	40	203	--	--	--	--

Table. 6. Hydrological data from the USGS monitoring station in the Concho River. Annual harmonic mean not calculated for years where one or more months mean discharge is 0 ($1/0 = \infty$). No data was collected from site number 08138000 Colorado at Winchell, TX after 2011 as this station is no longer in operation.

Annual Statistics for Stream Flow (ft^3/sec) 08136000 Concho River at San Angelo, TX						
	2010	2011	2012	2013	2014	2015
Annual Mean	17.81	13.48	19.98	8.99	23.31	20.52
Annual Median	16.65	8.57	10.15	9.43	8.43	10.7

Annual Peak	35.3	53.1	82.9	16.1	186.2	82.2
Annual Harmonic Mean	15.86	9.02	10.31	7.50	7.46	10.97
Number of Days with mean of 0	0	0	0	0	0	0

Annual Statistics for Stream Flow (ft^3/sec) 08136500 Concho River at Paint Rock, TX						
	2010	**2011**	**2012**	**2013**	**2014**	**2015**
Annual Mean	24.25333	18.48167	65.38833	17.90917	91.96408	31.701
Annual Median	22.7	18.35	12.43	13.75	11.85	13.65
Annual Peak	48.5	65.9	569	49.8	963	102.9
Annual Harmonic Mean	18.5123	**	0.2222	5.1971	2.1555	10.082
Number of Days with mean of 0	0	81	80	45	25	0

Genetic Diversity and Equilibrium Test Results

Microsatellite Diversity --Thirteen microsatellite loci were amplified from 110 Concho watersnakes that were sampled between 2013 and 2015 (Tables 7-9). The mean number of alleles per locus within each region was LCR = 4.615, O.H. Ivie = 3.385, and UCR = 3.23. There was no significant difference in allelic richness between the three populations ($F_{2,36}$ = 2.8, p = 0.07). Gene diversity (H_s) did not differ significantly among the three regions ($F_{2,36}$ = 0.134, p= 0.87) (means among loci within regions, LCR = 0.516, O.H. Ivie = 0.479, and UCR = 0.504). The number of private alleles was highest in the LCR (AP= 1.15), and low in both O.H. Ivie (AP=0.077) and the UCR (AP=0).

In the LCR and O.H. Ivie, only 1 locus (a different one in each region) showed significant departure from HWE, i.e., F_{IS} significantly > 0 (Tables 7 and 8). In the UCR, 4 loci showed significant departure from HWE, i.e., F_{IS} significantly > 0 (Table 9). The multilocus estimate of F_{IS} was significantly greater than 0 in LCR and UCR, but not O.H. Ivie. There was significant linkage disequilibrium between pairs of loci within each of the three regions. There are 78 pairwise comparisons with 13 loci. At a nominal alpha level of 0.05, one would expect 4 comparisons to be significant by chance alone. In the LCR, 14 of 78 pairwise comparisons were significant (exact binomial p < 0.001). In the O.H. Ivie sample, 24 of 78 pairwise comparisons were significant, and in the UCR, 12 of 78 comparisons were significant.

Exploration of the data set for similarities in multilocus genotypes (MLG) revealed that a number of individuals had genotypes that differed at only one or two loci. Because of the similar MLGs and because a large number of neonates were collected in the same location on the same day (especially in the O.H. Ivie reservoir), there was the possibility that sibling groups were represented in the data set. As the presence of family groups is known to inflate estimates of population subdivision among defined population units (Allendorf and Phelps, 1981), we analyzed the data two ways. First, we used the full data set (results in Tables 7-9 are based on the full data set, N = 110). Second, we created a reduced data set that attempted to reduce

potential sibling structure ($N = 58$). To create the reduced data set, potential family groups were roughly identified by grouping neonate individuals collected within a two day period at the same or very close GPS location. We then randomly selected a single individual from each of these groups to retain in the data set.

Table 7. Measures of microsatellite diversity and tests of HWE for the Concho watersnake in the Lower Colorado River.

Locus	N[a]	An[b]	A_R[c]	H_O[d]	H_S[e]	F_{IS}[f]
NePa_1277	32	4	3.313	0.469	0.539	0.131
NePa_2048	32	3	2.848	0.094	0.150	0.376
NePa_9972	32	2	2	0.219	0.246	0.111
NePa_21288	32	3	2.885	0.406	0.490	0.171
NePa_22153	32	4	3.619	0.438	0.569	0.230
NePa_5932	32	5	4.312	0.500	0.509	0.018
NePa_1470	32	7	6.299	0.719	0.713	-0.008
NePa_12681	32	5	4.847	0.469	0.567	0.173
NePa_9687	32	4	3.885	0.625	0.653	0.042
NePa_11248	32	4	3.862	0.188	0.285	**0.343**
NePa_2287	32	5	4.963	0.594	0.666	0.109
NePa_12577	32	9	7.509	0.688	0.762	0.098
NePa_2349	32	5	4.618	0.594	0.558	-0.063
Mean/Multilocus F_{IS}		4.62	4.23	0.461	0.516	**0.106**

[a] Number of individuals genotyped

[b] The number of alleles per locus

[c] Allelic richness (rarefied number based on sample size of 21)

[d] Observed heterozygosity

[e] Gene diversity

[f] Bolded F_{IS} values indicate significant deviations from HWE ($p < 0.05$)

Table 8. Measures of microsatellite diversity and tests of HWE of for the Concho watersnake in the O.H. Ivie Reservoir.

Locus	N[a]	An[a]	A_R[b]	H_O[d]	H_S[g]	F_{IS}[f]
NePa_1277	57	2	2	0.509	0.414	-0.228
NePa_2048	57	2	1.368	0.018	0.018	0.000
NePa_9972	57	2	2	0.421	0.422	0.003
NePa_21288	57	2	2	0.596	0.502	-0.188
NePa_22153	57	3	2.752	0.632	0.524	-0.205
NePa_5932	56	5	4.374	0.714	0.733	0.026
NePa_1470	57	6	5.774	0.596	0.679	0.121
NePa_12681	57	4	3.997	0.754	0.716	-0.053
NePa_9687	57	3	2.964	0.544	0.518	-0.050
NePa_11248	57	4	3.332	0.333	0.368	0.094
NePa_2287	57	3	2.751	0.123	0.150	0.182
NePa_12577	57	5	4.214	0.632	0.638	0.010
NePa_2349	55	3	2.998	0.436	0.544	**0.197**

| Mean/Multilocus F_{IS} | | 3.39 | 3.121 | 0.485 | 0.479 | -0.013 |

[a] Number of individuals genotyped

[b] The number of alleles per locus

[c] Allelic Richness (rarefied number based on sample size of 21)

[d] Observed heterozygosity

[e] Gene diversity

[f] Bolded F_{IS} values indicate significant deviations from HWE ($p < 0.05$)

Table 9. Measures of microsatellite diversity and tests of HWE for the Concho watersnake in the Upper Colorado River.

Locus	$N^{[a]}$	$An^{[a]}$	$A_R^{[b]}$	$H_O^{[d]}$	$H_S^{[g]}$	$F_{IS}^{[f]}$
NePa_1277	21	2	2	0.333	0.398	0.162
NePa_2048	21	2	2	0.571	0.500	-0.143
NePa_9972	21	2	2	0.286	0.250	-0.143
NePa_21288	21	3	3	0.429	0.502	0.147
NePa_22153	21	3	3	0.238	0.469	**0.492**
NePa_5932	21	5	5	0.429	0.719	**0.404**
NePa_1470	21	4	4	0.619	0.719	0.139
NePa_12681	21	4	4	0.619	0.676	0.085
NePa_9687	21	3	3	0.381	0.557	0.316
NePa_11248	21	3	3	0.238	0.436	**0.454**
NePa_2287	21	3	3	0.381	0.514	0.259
NePa_12577	21	6	6	0.429	0.599	**0.284**
NePa_2349	21	2	2	0.143	0.217	0.341
Mean/Multilocus F_{IS}		3.23	3.23	0.392	0.504	**0.223**

[a] Number of individuals genotyped

[b] The number of alleles per locus

[c] Allelic Richness (rarefied number based on sample size of 21)

[d] Observed heterozygosity

[e] Gene diversity

[f] Bolded F_{IS} values indicate significant deviations from HWE ($p < 0.05$)

Population Structure and Connectivity Results

In the full data set, pairwise F_{ST} values were 0.144 between LWC and O.H. Ivie, 0.058 between LWC and UPC, and 0.144 between O.H. Ivie and UPC and all were significant with $p < 0.001$. The overall F_{ST} of 0.129 for the three populations was significant, $p = 0.001$. The PCoA is in agreement with the pairwise differentiation tests; i.e., three groups largely corresponding to the three regions fall out in multivariate space of axes 1 and 3 of the PCoA (Figure 2). Likewise the results of the STRUCTURE analysis were concordant with both the pairwise F_{ST} tests and the PCoA. The most likely *K* value based on log likelihood scores and *ΔK* suggest that there were three clusters that largely corresponded to our *a priori* delimitations of the UCR, LCR and O.H. Ivie Reservoir. However, notice that in both the PCoA and the STRUCTURE results, some individuals from the UCR cluster with the LCR.
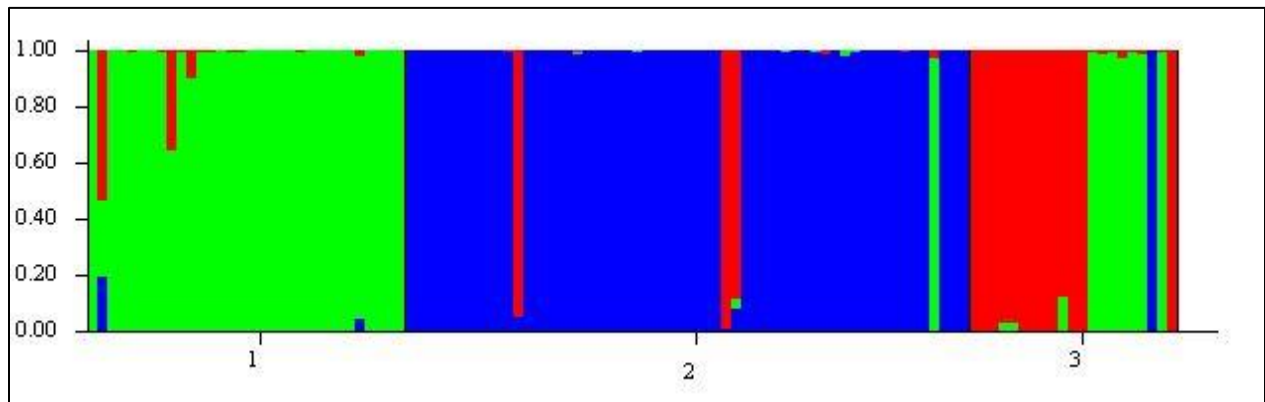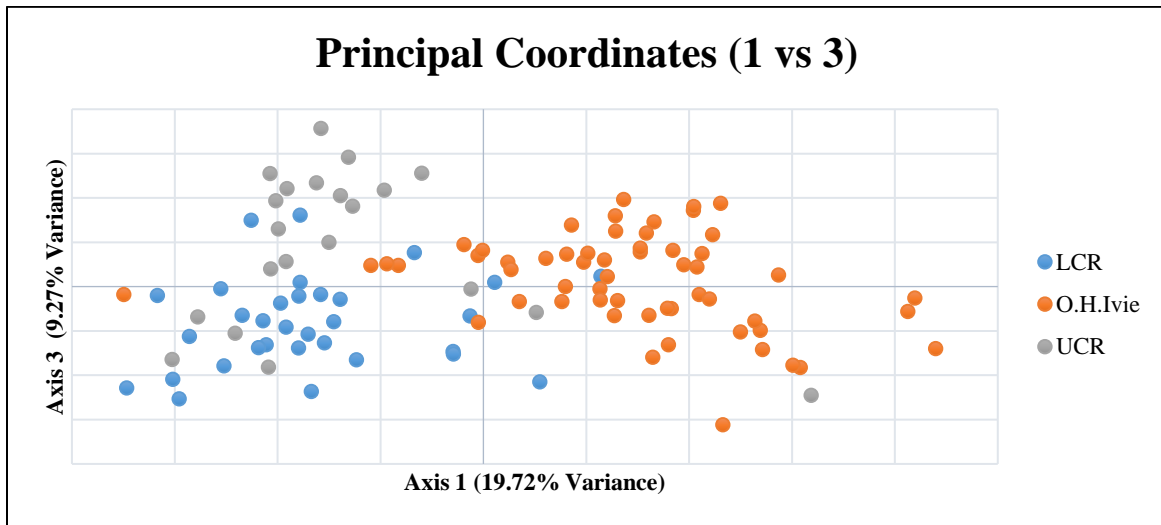
Figure 2. Results of the PCoA (top) and STRUCTURE (bottom) analysis for the complete data set ($N = 110$). PCoA shown for axis 1 vs. 3 (29% of the total variance). Three largely delimited clusters are noticeable in the PCoA: the UCR (grey individuals), LCR (blue) and the O.H. Ivie reservoir (orange). STRUCTURE plot at $K = 3$ (bottom): LCR samples (pop 1), O.H. Ivie (pop 2), and UCR (pop 3). Individuals are the bars aligned along the x-axis. The y-axis indicates the posterior probability of assignment of the individual to one of three clusters (indicated by the three colors).

When the potential sibling groups were removed from the dataset, pairwise F_{ST} values were 0.183 between LWC and O.H. Ivie, 0.024 between LWC and UPC, and 0.172 between O.H. Ivie and UPC and all were significant at $p < 0.001$. The overall F_{ST} value was 0.137 and was significant ($p < 0.001$). We note that the comparisons between UCR and O.H. Ivie and LCR and O.H. Ivie both increased. Thus, even after removing the potential effect of siblings, there remained a signature of population genetic differentiation from the O.H. Ivie population to the UPC and LCR samples. The pairwise F_{ST} value between the UCR and LCR decreased, but remained significant ($p = 0.03$).

The PCoA is in agreement with the pairwise differentiation tests; i.e., two groups fall out in multivariate space of axes 1 and 2 of the PCoA, one consisting primarily of O.H. Ivie. The UCR and LCR are less distinct (as was seen with the pairwise F_{ST}), but there are 3 individuals from the UCR that clearly fall outside the LCR samples (top left 3 grey dots in Figure 3). STRUCTURE identified a most likely K value of 2 based on both log

likelihood scores and *ΔK*. The two populations corresponding to UCR and LCR form a single population in this analysis that is distinct to the O.H. Ivie population.
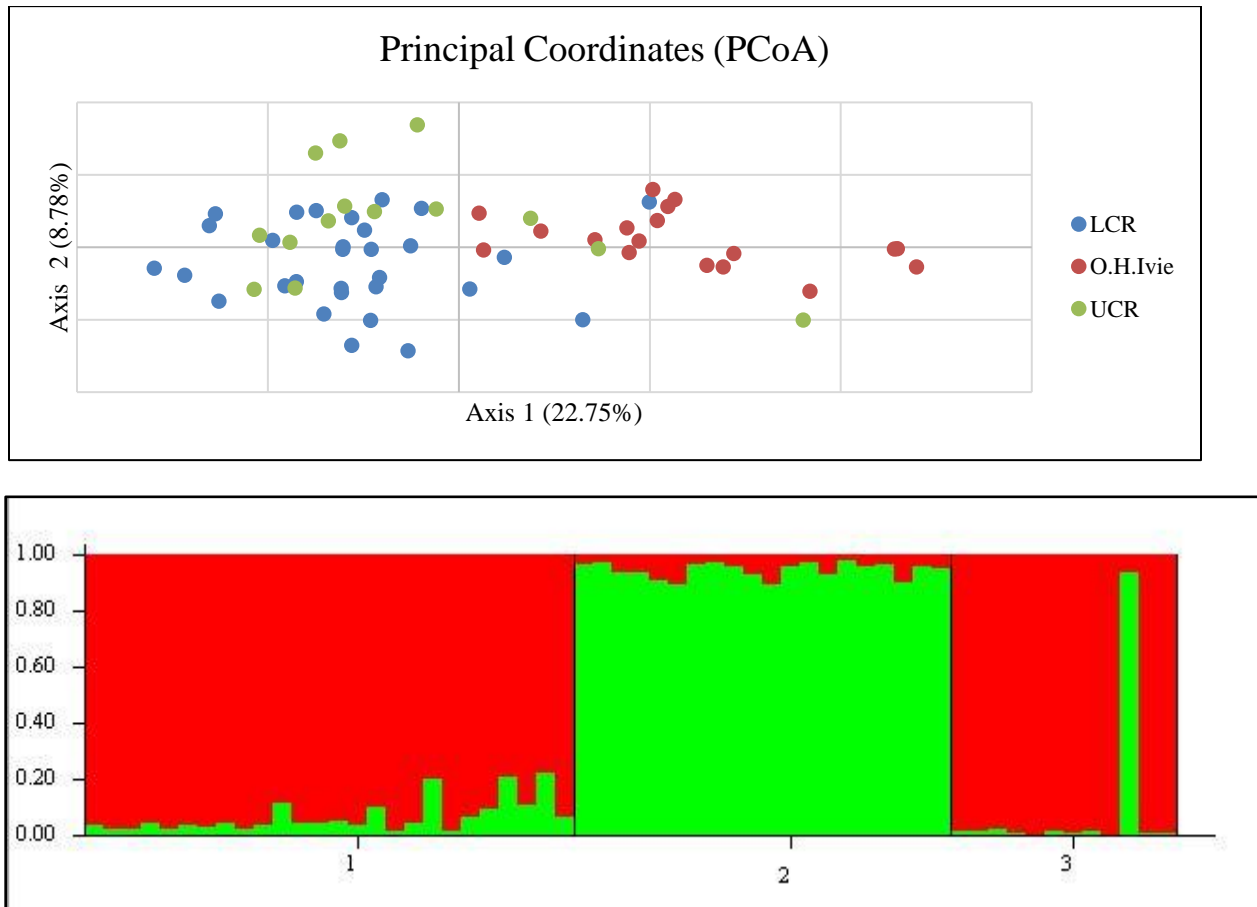

Figure 3. Results of the PCoA (top) and STRUCTURE (bottom) analysis for the partial data set with potential sibling groups removed ($N = 58$). PCoA shown for axis 1 vs. 2 (32.05 % of the total variance). Two delimited clusters are noticeable in the PCoA: LCR (blue) and the O.H. Ivie reservoir (orange). But 3 individuals from UCR (grey) fall outside these clusters. STRUCTURE plot (bottom) indicates 2 populations, LCR samples (pop 1), O.H. Ivie (pop 2), and UCR (pop 3). Individuals are on the bars aligned along the x-axis. The y-axis indicates the posterior probability of assignment of the individual to one of two clusters (indicated by the two colors).

Effective Population Size –We note these N_e estimates should be interpreted with caution. The primary assumption behind using the linkage disequilibrium method (Waples 2006) is that genetic drift is the only cause of the linkage disequilibrium in the sample. Thus, the method assumes a closed population (i.e., no migration; Waples and England 2011). If in the population sample there are a few migrants that originated from highly diverged populations, LDNe estimates of N_e will be decreased due to the linkage disequilibrium generated by admixed individuals. Also, if there is not a random sample of individuals such that only a few sib groups are sampled, then N_e estimates will be lower than if a random sample was obtained. In other words, N_e estimates will be a reflection of the number of breeders that generated that sample of individuals, thus the effective number of breeders, N_b, provides a better description of the estimate generated. Removing potential siblings

from the data (as we did in the reduced data set) does not address this latter problem as only a few families would have been sampled from the start. Thus, removing siblings from the data set should not greatly impact the results. Indeed, this is what we observed (compare Tables 10 and 11). In general, we feel these N_e estimates should be interpreted as the number of effective breeders, N_b that generated the sampled individuals. They can only be regarded as the effective sizes of the regions if we assume random samples of the regions were obtained.

N_b (Table 10) was estimated to be the highest in the LCR with 19.1 individuals, followed by the UCR and O.H. Ivie with 10.7 and 7.5 individuals each. When LDNe was estimated using the reduced data set where potential sibling groups removed, the estimates for LCR and UCR remained similar and within the confidence intervals of the estimates generated from the full data set (Table 11). O.H. Ivie had a lower N_b in the reduced data set suggesting we may have artificially removed excess families using the demographic information alone. Future analyses will center on a more formal analysis of sibship reconstruction as a means to remove sibship bias in tests of population differentiation. Nonetheless, the O.H. Ivie remained differentiated from the LCR and UCR, so we believe this signature to be real.

Table 10. Effective population size estimates using LDNe method for the full data set ($N = 110$). Lowest allele frequency cutoff was 0.03 for LCR and O.H. Ivie, and 0.02 for UCR based on the guidelines of Waples and Do (2010).

	N	r^2	N_b	Confidence Intervals
LCR	32	0.052183	19.1	13.5 – 28.3
O.H. Ivie	57	0.048756	10.7	7.7- 14.6
UCR	21	0.084792	7.5	3.5 – 13.0

Table 11. Effective population size estimates using LDNe method with potential sibling groups removed ($N = 58$). Lowest allele frequency cutoff was 0.02 for LCR, 0.03 for O.H. Ivie, and 0.05 for UCR) based on the guidelines of Waples and Do (2010).

	N	r^2	N_b	Confidence Intervals
LCR	26	0.05024	17.4	11.8-27.6
O.H. Ivie	20	0.10470	4.8	2.6-8.9
UCR	12	0.11111	10.9	3.4-46.9

Demographic Results

In 2015, a total of 103 Concho watersnakes were captured, 7 of which were recaptures. There were no Concho watersnakes recaptured in the Upper Colorado River. Recaptures occurred only at Freese Dam (1 individual), O.H. Ivie (3 individuals), and Hwy 283 (2 individuals, one of which was recaptured twice). Recaptured Concho watersnakes occurred only in 2015. Recaptured individuals from Freese Dam and O.H. Ivie were captured within a month of their initial capture, while the individuals captured at Hwy 283 were originally marked in 2014. The results of the genetic data suggest that O.H. Ivie and the Lower Colorado sites (Freese Dam and Hwy 283) are different populations, thus these sites are considered separately. The recapture at Freese Dam was within a 1 month period whereas the recaptures at Hwy 283 were a year apart, thus separate estimates are generated for each of these. In each sampling location model M_0 (assumes constant probability of capture) yielded the lowest AICc (AIC corrected) value (Tables 12-17). Population size estimates based on the model with lowest AICc values were 59.66 ± 6521.12 individuals (CI = 58-4889.92) for the O.H. Ivie

Reservoir. This estimate was based on 61 total captures in 2015 of which 3 were recaptures within a month of sampling. The Freese Dam population is estimated to be 12.64, but a standard error and, thus CI cannot be generated from this estimate because of only 1 recapture. This estimate was based on 15 total captures in 2015 of which there was a single recapture. Hwy 283 had an estimated population size of 22.022 ± 2778.31 (CI=22.00-320.77). This estimate was based on a total of 16 captures (4 in 2014 and 12 in 2015) where there were 3 recaptures (2 encounters for one snake) in 2015. We note that our estimates violate key assumptions of these models. The estimates from O. H. Ivie and Freese Dam violate the no-births assumption as captures from May and the fall (when birth is reported to happen) were combined. In addition to the no-birth assumption, the constant time interval assumption was violated in the Hwy 283 sample. **Due to the extremely low sample sizes, mark recapture rates, and violation of assumptions, population size estimates herein are included for heuristic purposes only and should not be considered accurate, as indicated by the large standard errors and confidence intervals associated with the estimates.**

Table 12. Candidate model for live-capture mark-recapture population size estimates based on mark-recapture data for the O.H. Ivie populations from 2015.

Model	AICc	AICc weight	Model Likelihood	Number of Parameters	N	SE	95% CI Lower	95% CI Upper
M_0	205.26	0.99	1.0	11	59.66	6521.128	58.00	4883.92
M_T	221.35	0.000033	0.0003	9	63.729	11106.133	58.002	11810.610
M_S	367.9019	0.000	0.000	2	3.6×10^9	0.114×10^{10}	17901762	0.75×10^{10}

Table 13. Model selection criteria and goodness-of-fit tests for the tests in Table 12.

Model	χ^2	df	α
M_0	20.15	2	<0.0001
M_T	180.912	9	<0.0001
M_S	160.76	7	<0.001

Table 14. Candidate model for live-capture mark-recapture population size estimates based on mark-recapture data for Freese Dam Location in 2015.

Model	AICc	AICc weight	Model Likelihood	Number of Parameters	N	SE	95% CI Lower	95% CI Upper
M_0	74.02	1.000	1.0	11	12.64	0.00	14.00	14.00
M_T	81.6585	0.00727	0.022	12	12.32	0.000	14.00	14.00
M_S	92.94	0.00008	0.001	2	44.53	90.979	15.57	606.90

Table 15. Model selection criteria and goodness-of-fit tests for the tests in Table 14.

Model	χ^2	df	α
M_0	-5.404	2	**
M_T	38.119	2	<0.0001

M$_S$	32.716	2	0.0003

Table 16. Candidate model for live-capture mark-recapture population size estimates based on mark-recapture data for Hwy 283 in 2015

Model	AICc	AICc weight	Model Likelihood	Number of Parameters	N	SE	95% CI Lower	95% CI Upper
M$_0$	149.90	0.8727	1.0	13	22.002	2778.31	22.00	320.77
M$_T$	153.947	0.1156	0.022	11	22.009	468.02	22.00	105.63
M$_S$	158.53	0.0168	0.000	2	1366467.7	37104612	8894.47	0.210x10^9

Table 17. Model selection criteria and goodness-of-fit tests for the tests in Table 16.

Model	χ^2	df	α
M$_0$	8.36	2	0.015
M$_T$	31.74	11	0.008
M$_S$	23.378	9	0.005

Discussion and Recommendations

Population size estimates

A total of 117 Concho watersnakes were captured or observed during the course of 2013-2015 surveys. Demographic population size estimates (heuristic value only) based on mark-recaptures from 2015 were about 59 individuals for O.H. Ivie, 12 individuals for Freese Dam, and 22 individuals for Hwy 283. Combined in the LCR region, there would be about 34 individuals. We caution that these demographic population size estimates are likely unsuitable for any use in population management decisions due to the very small sample sizes and low mark-recapture rates.

A considerable amount of effort was required to detect the presence of Concho watersnakes and the search success paralleled weather conditions. In the UCR, where snakes were captured each year during the 2013 to 2015 surveys, the CPUE ranged from 1 Concho watersnake per 156 active search hours in 2013 to 1 Concho watersnake per 17.92 active search hours in 2015 when there was more rainfall (Table 2). In general, a significant amount of time was required to find a single individual even during periods of normal rain fall. Previous studies have demonstrated a significant decrease in watersnake catchability and detection during periods of drought (Green et al. 1994, Whiting et al. 1997, Scott et al. 1989). However, because there are no population size estimates prior to the delisting, it is not possible to determine if population sizes have decreased or if sampling was negatively impacted by extreme weather conditions. Additional future sampling seasons will be required to determine if the small sample sizes reflect snake activity in relation to weather conditions or if the snake populations do indeed have small census population sizes. Although not directly comparable, we note that historical studies (between years 1987-1996) examining the natural history of the Concho snake were able to obtain samples sizes upwards of 300 individuals in a given year (Whiting et al. 2008).

Ideally, both mark-recapture and genetic monitoring would continue in the 2017 and 2018 summer and fall seasons now that stream discharge has increased at all monitoring locations and more than 100 individuals

have been tagged (USGS Daily Water Conditions). Our highest overall numbers of captured individuals (of all snake species) and greater recaptures came during periods of increased rainfall and hence, stream flow. Additional mark-recapture surveys would allow for more robust model building and survivorship estimates that would increase the utility of using these methods for future monitoring purposes.

Genetic diversity and structure

Measures of genetic diversity (gene diversity or allelic richness) were modest and not significantly different among the three regions. Gene diversity was slightly higher (0.47 to 0.51) in our samples compared to that reported from 5 loci by Rodriguez et al. (2012) (0.43 in the UPC and 0.42 in the Concho River). However, caution is advised in making such a comparison as the same markers were not used in both studies.

The results of the population structure analyses indicated significant pairwise structure among the 3 *a priori* grouped regions. The clustering results (PCoA and STRUCTURE analyses) based on individuals were largely consistent with the *a priori* groupings. This clustering by region supports the contention of significant structure among these generalized regions. Taken at face value, the O.H. Ivie sample is clearly differentiated from the UCR and LCR samples. In both, the PCoA and STRUCTURE results, the UCR appears to contain individuals from 2 groups: one that clusters with the LCR samples and one that is unique. This admixture (i.e., the Wahlund effect) may explain the excess deviations from HWE (i.e., positive F_{IS}) we observed in the UPC region (Table 9).

Because our sampling records indicated several occurrences where neonate snakes were collected at the same location within 1 or a few days of one another (especially in the O.H. Ivie reservoir) , there was the distinct possibility that sibling snakes were sampled. After reducing the data set, there was still significant pairwise F_{ST} among the three regions. Again the PCoA of the reduced data set was in agreement with these results. However, notice the 3 individuals in the UCR that fall outside of the bulk of the LCR samples (top left 3 grey points in Figure 3 PCoA plot). The UCR sample at 12 Mile Bridge consisted of 10 neonates and 2 older snakes. These individuals formed the unique cluster in the full data set (Figure 2, red cluster STRUCTURE plot). Using only 1 of these neonates and the 2 older snakes, STRUCTURE was not able to identify this unique cluster in the reduced data set (Figure 3, STRUCTURE plot). With so few individuals representing a third cluster in the data set, the algorithm in STRUCTURE would not have enough information (i.e., allele frequency information) to form a distinct cluster. Nonetheless, these 3 individuals still fell outside the cluster of points in the PCoA (top left 3 grey points in Figure 3 PCoA plot), indicating there is likely some unique local genetic structuring within the Colorado River itself. More samples would be needed to formally test this hypothesis, but in general, we interpret that there remains significant genetic structure even after the potential for sibling groups is removed. Overall, the 3 *a priori* delimited regions are supported, but where the UPC is actually an admixed region containing individuals similar to the LCR and individuals that form a unique cluster. The O.H. Ivie sample forms its own unique cluster.

The Rodriguez et al. (2012) study had 34 snakes spanning a large region of the upper Colorado River and 30 snakes from the Concho River. They found an F_{ST} of 0.27 (based on 5 microsatellite loci) between these 2 regions and they found 2 mtDNA haplotypes that were largely associated with the 2 rivers. Given that the O.H. Ivie reservoir population is located slightly less than 1 mile downstream of the confluence between the Colorado and the Concho River, it is possible that the population subdivision between the UPC-LCR to the O.H. Ivie reservoir population is driven by a descendent Concho River population that has colonized this area. However, we were unable to directly test this hypothesis as we were not able to collect any snakes from the Concho River itself. We plan to sequence the mtDNA region used in Rodriguez et al. (2012) study to see if we observe the predominant Concho River mtDNA haplotype to be more common in O.H. Ivie population sample. However, the mtDNA in itself will still largely be circumstantial because it is not a fixed genetic marker.

Again, a conclusive test of the hypothesis of a Concho River origin of the O.H. Ivie population requires samples from the Concho River itself.

What is more perplexing is why the UCR and LCR, which lie on either side of the O.H. Ivie, contain individuals that are more similar to one another than to individuals from a location that is in the middle of two (S1, Figures 1-2). In fact, the UCR samples that cluster with the LCR are from a more northern location (HWY 83). The more southern samples from 12 Mile Bridge are the ones that form a more unique cluster. We are unaware of any translocation efforts with the Concho watersnake either above or below the O.H. Ivie reservoir; however, records should be reexamined to determine if any translocations have occurred.

Overall, we observed significant amounts of LD in the 3 regions. This LD can be generated by genetic drift (i.e., small N_e) and/or admixed populations assuming our set of microsatellites are not under a common selective pressure (which is a safe assumption given our library was randomly generated). Admixture in the UCR is likely given the results of the clustering analyses (Figures 2 and 3). The effect of admixed samples on the LDNe method of estimating N_e was analyzed by Waples and England (2011). The LDNe method may overestimate local N_e if there is a migration rate greater than 0.1 or underestimate N_e if a there are a few migrants from a highly diverged population. We cannot tease these factors apart because sample sizes would be too small if we were to separate the UCR into its different clusters and then estimate N_e separately for each. Taken at face value, the LDNe estimates suggest that the local effective breeding populations are small and on the order of 10-20 individuals within each of *a priori* delimited sampling areas. If we compare the genetic estimates to the demographic estimates, the genetic estimates are lower (e.g., ~20 in the LCR for the genetic and 33 in the combined demographic for the LCR). Genetic estimates are typically smaller than census estimates, so this is not surprising. In general, though both the demographic and genetic estimates suggest population sizes are relatively small across the span of the Colorado River that we collected snakes. Clearly, more data are needed, but given our inability to collect decent sample sizes compared to historical studies, we hypothesize there are no more than a few hundred Concho snakes.

Monitoring Thresholds

The USFWS post- delisting monitoring plan (PDMP, USFWS 2011) cites 3 monitoring thresholds that would suggest healthy populations of the of the Concho watersnake during the post-delisting monitoring period: 1) Snakes must be present at 75% of the monitoring sites each year, 2) found in at least one site on each of the 3 reaches, and 3) found to be reproducing.

1). Snakes must be present at 75% of the monitoring sites each year

During the 2013 to 2015 Concho watersnake surveys, 117 snakes were captured or observed over the three year period. **The percent of monitored sites in a given year (excludes dry sites) at which Concho watersnakes were detected was 20% in 2013, 15% in 2014 and 31% in 2015** (Table 3)**.** Concho watersnakes were confirmed in only 7 of the 22 monitored sites (excludes dry sites) for the 3 year period. Even during the 2015 sampling season, when stream flow was highest and the number of biologists engaged in surveys was also highest, the percentage of sites were Concho watersnakes were present was less than the 75% recommended by the PDMP. Concho watersnakes were not found in Spence Reservoir, the uppermost reaches of the Colorado between Spence and Bronte, TX or in Ballinger Municipal Lake. There were no snakes captured in artificial riffle habitat between Spence and Bronte TX during the 2012 to 2015 surveys because there is no longer water in this section of the river. Concho watersnakes were also not found in Spence Reservoir or Ballinger Municipal Lake during the surveys conducted from 1991 to 1996 (Whiting et al. 2008). The 7 sites where Concho watersnakes were present on the Colorado River were located in the remaining quality habitat along the Colorado River from Hwy 277 to Hwy 283. The largest sample sizes were concentrated within O.H. Ivie reservoir, directly below Stacy Dam and at Hwy 283. Reproductive females with

embryos and neonates were found in 5 of the 7 sites, all located between Hwy 83 to Hwy 283, indicating that the Concho watersnake is actively reproducing in this stretch of the Colorado River.

2). Found in at least one site on each of the 3 reaches.

No snakes were captured or observed on the Concho River despite significant trapping and search effort from 2013-2015. Given that the stream flow rates were higher along the Concho River than the Colorado River, even during periods of drought, and the number of days with no stream flow was very low, there may be other factors contributing to the lack of detectable Concho watersnakes (or small population sizes in general) along the Concho River. For example, water quality, aquatic vegetation, algal blooms and mineralization of river bed stone would suggest that the Concho River is highly eutrophic and disturbed (Janecka, personal observation). There are few riffles along the Concho River and the habitat is better characterized by long stretches of a deep river channel. When there are riffles, fish communities in the riffles consist of only a single species of fish, compared to the 6 or more species found in Colorado River riffles (Janecka personal observation). Due to the significant search effort required to locate a single Concho watersnake across all 3 survey years, quantification of habitat quality, quantity, and prey abundance were not feasible during the course of these surveys. Future monitoring may wish to give more attention to fine habitat characteristics, though more resources for additional personnel will be required for such a venture.

It is important to note that the Colorado and Concho Rivers have been under extreme drought conditions for a little over 10 years (USGS Year end Reports). The upper reaches of the Colorado River from above Spence Reservoir to south of Bronte, TX have had no water for the last 3 years. Artificial habitat created for the snake in the upper Colorado River is no longer under water and so is clearly not being used by the snakes. Both O.H. Ivie reservoir and Spence Reservoir are less than half of their normal fill levels. CRMWD is not required to maintain flow regimes for the snake under extreme drought and so releases from the reservoirs have been minimal.

3). Found to be reproducing.

Reproductive females with embryos and neonates were found in 5 of the 7 sites, all located between Hwy 83 to Hwy 283, indicating that the Concho watersnake is actively reproducing in this stretch of the Colorado River and in O.H. Ivie reservoir.

Concluding Remarks

Both mark-recapture and genetic estimates of population size suggest that the present population may be quite small, possibly due to persistent drought conditions along the Colorado or other factors resulting in a population bottleneck. However, it is also possible that these numbers were deflated due to reduced detection probability of a cryptic snake with short activity periods. For this reason, we recommend that sampling in years 2017-2019 (years 6-8 of the PDMP) be intensified from the original sampling scheme outlined in the PDMP (USFWS 2011). We recommend that sampling occur in both spring/summer and late summer fall of both years and that both mark-recapture and genetic monitoring continue along the Colorado River. In addition to the mark-recapture and genetic monitoring surveys, studies that focus on estimating detection probabilities and site-occupancy modeling estimates that account for environmental covariates would likely prove a valuable tool for the conservation of this species. Such studies would help to quantify the amount of survey effort required to make quantitative management decisions (Durso et al. 2011). Alterations of the methods described by Durso et al. (2011) would be required however, as the majority (80%) of the Concho watersnakes captured in on the Colorado were caught by hand rather than in traps. We also recommend that the Concho River be reassessed for potential monitoring sites, as Concho watersnakes were not detected at either the sites recommended by the USFWS for monitoring or the sites added during the 2013-2015 surveys.

In all, both demographic and genetic descriptors of the Concho watersnake populations were hampered by inadequate sample sizes. However, we highlight this inadequate sampling is not the result of poor sampling protocols. All the standard methods of snake capture were employed and numerous person and trap hours were put into this project. It is our professional opinion that the inadequate sampling is likely the result of two factors. First, there has been a culmination of drought years, which persisted in the years of 2013 and 2014, and this drought period may have led to decreased snake activity in general. Not until 2015 did rainfall levels, and consequently Concho snake captures, increase substantially. Nonetheless, even within this last year where both our sampling effort intensified and "mother-nature" provided more ideal sampling conditions, we still did not detect the Concho watersnake on the Concho River. Moreover only 6 of 15 (40%) non-Concho River PDMP sites were positive for Concho watersnakes (Table 3). This falls short of the 75% required to be still called a "healthy population". Second, it is likely the drought itself has had negative impacts on the populations. For example, there are several dry regions in the upper Colorado River. It is unlikely that a stream specialist such as the Concho watersnake would persist in the absence of stream habitat. Thus, the inability to collect decent sample sizes is likely a reflection of decreased population sizes (or even absent populations). On the Concho River itself, lack of water is not the problem. However, as outlined above, there are several major habitat differences between the Concho River and Colorado River such that the Concho River habitat is not "ideal" for the Concho watersnake. Given our sampling effort was greater on the Concho River than the Colorado River in 2015 and that we still did not find snakes on the Concho River, it is our opinion that Concho watersnake populations on the Concho River are unlikely to be doing well.

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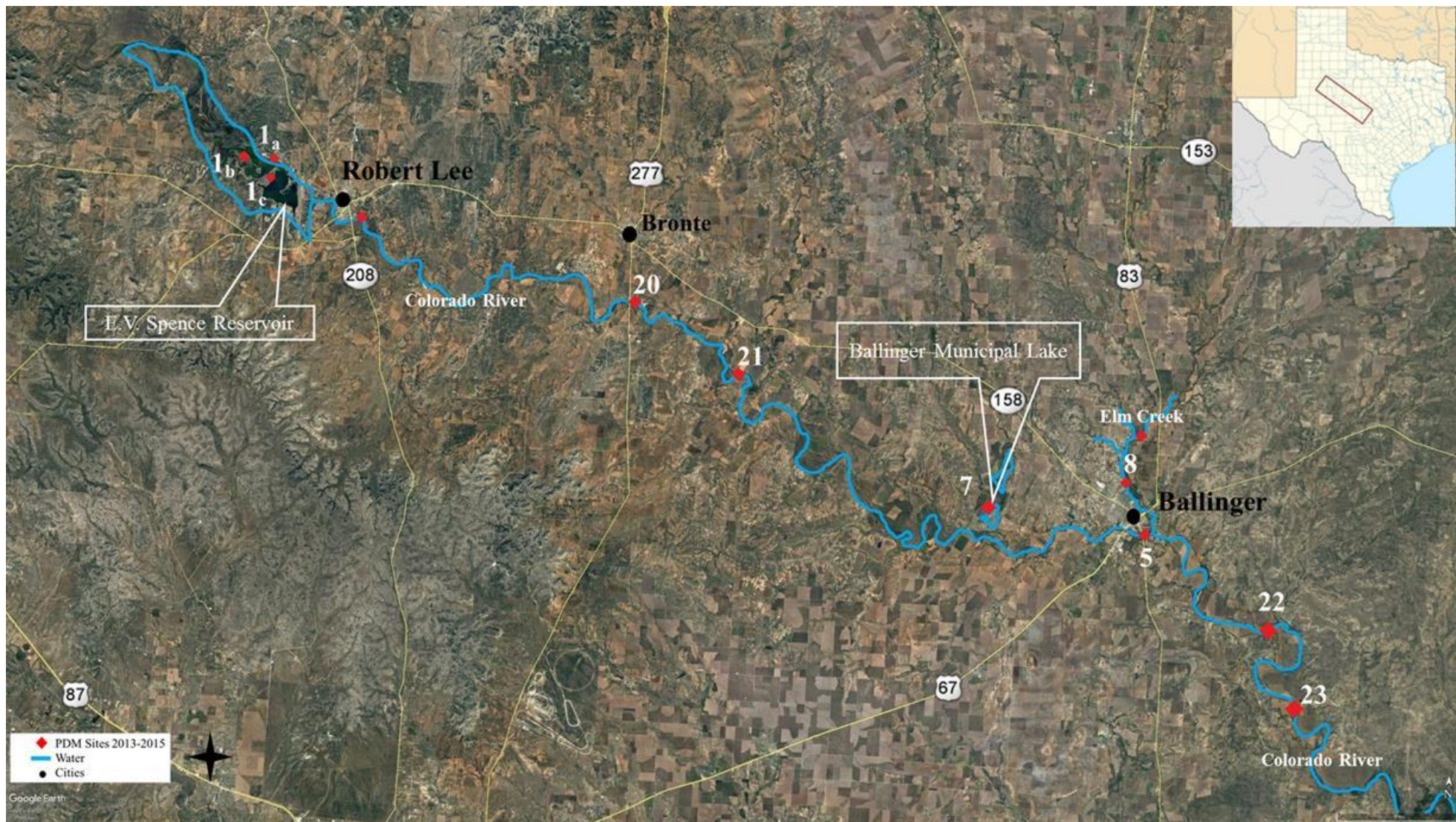
S1. Figure 1. Location of post delisting monitoring sample sites for Spence Reservoir and the upper Colorado River used during the 2013-2015 surveys. Site numbers correspond to Table 1 of the Post-Delisting monitoring plan and Table 1 of the final report.

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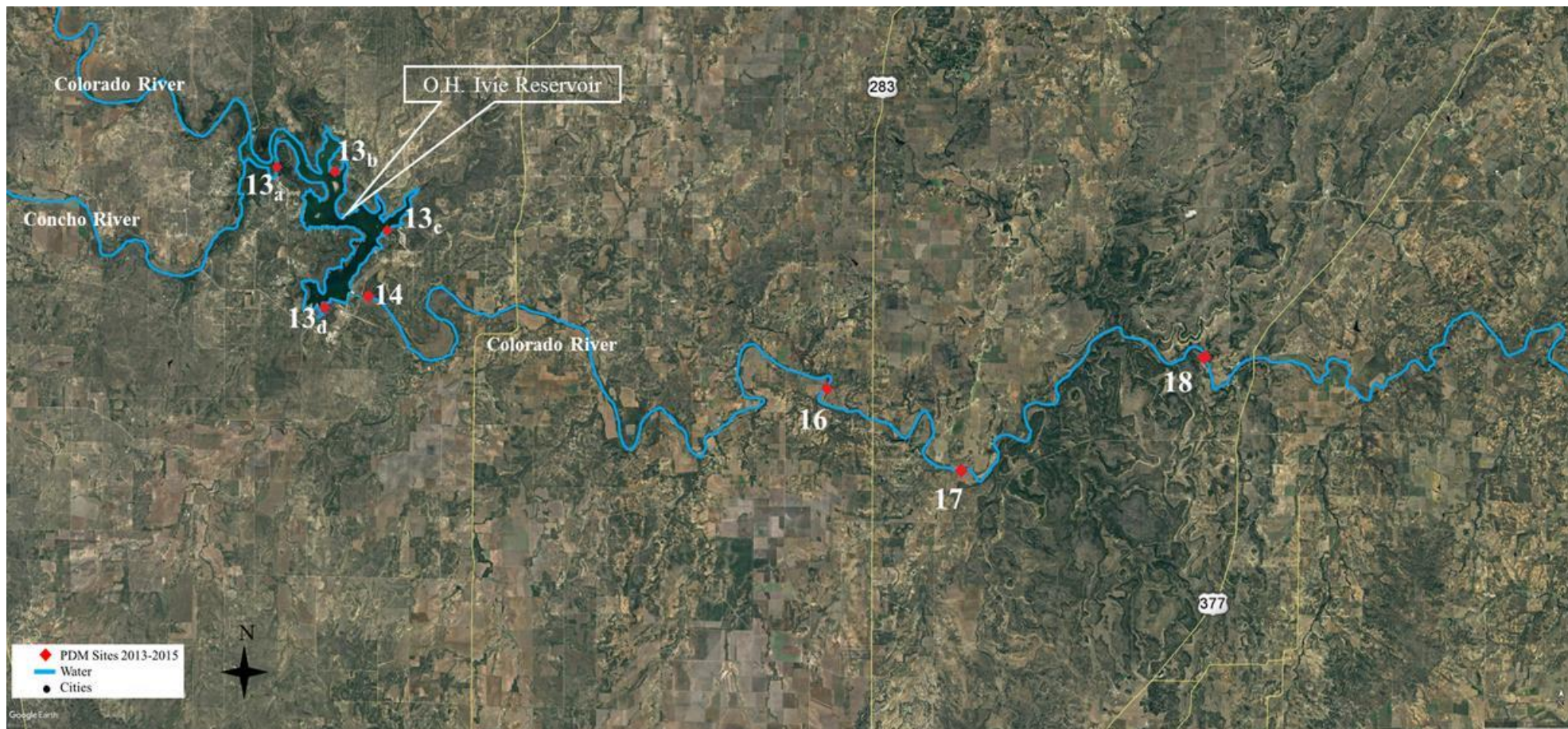
S1. Figure 2. Location of post delisting monitoring sample sites on O.H. Ivie reservoir and the lower Colorado River used during the 2013-2015 surveys. Site numbers correspond to Table 1 of the Post-Delisting monitoring plan and Table 1 of the final report.
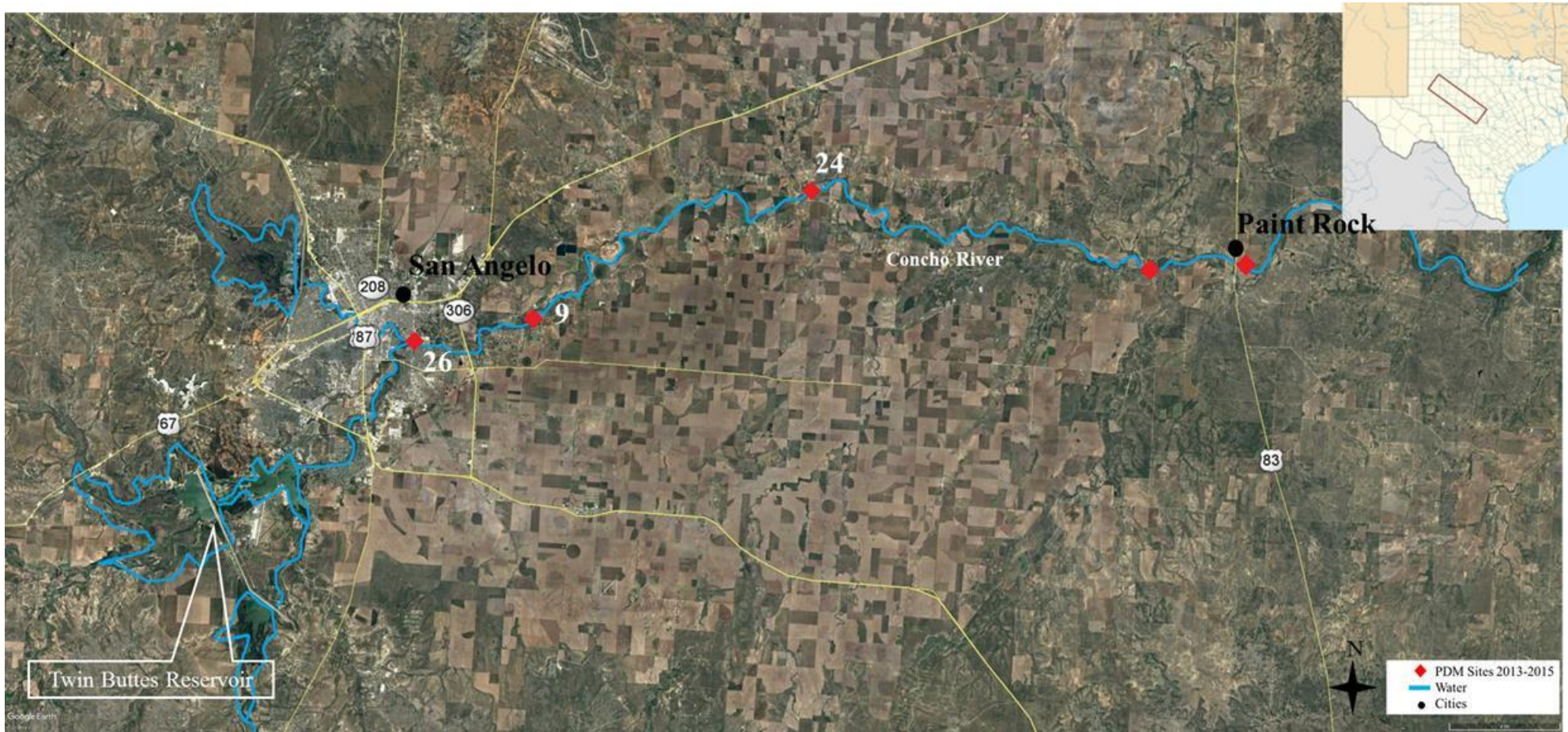
S1. Figure 3. Location of post delisting monitoring sample sites on the Concho River used during the 2013-2015 surveys. Site numbers correspond to Table 1 of the Post-Delisting monitoring plan and Table 1 of the final report.

S1. Figure 4. Location of post delisting monitoring sample sites on the Concho River used during the 2013-2015 surveys. Site numbers correspond to Table 1 of the Post-Delisting monitoring plan and Table 1 of the final report.