Biogeography and Population Genetic Structure of the Cottonmouth , *Agkistrodon piscivorus,* in the Alapahoochee Watershed

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ABSTRACT

This study examines the biology of the Florida Cottonmouth, *Agkistrodon piscivorus conanti*, in the Alapahoochee watershed, Lowndes and Echols Counties, Georgia. Included is a biogeographical investigation incorporating population genetics, a diet survey, analysis of length/weight relationships, analysis of sex ratios, and observations on reproductive ecology. Collections occurred over a two year period in 2008 and 2009. Data recorded in the field and laboratory included location, sex, snout-vent length, weight, gut contents and condition of the snake at time of collection. DNA was extracted and analyzed based on published microsatellite primer pairs for *Crotalus contortix* and cross amplified in *Agkistrodon piscivorus* that were tested for use in our study. Overall sex ratios did not deviate from those expected in this study but, the ratios were definitely skewed relative to the year collected and location from which they were collected. The majority of guts examined had no contents, and the remainder had small meals suggesting snakes were foraging at the time of capture. Length-weight relationships were comparable for both males and females. Presence of eggs and embryos showed that ovulation occurs in May followed by a 3 to 4 month gestation period with birth occurring by end of August. The genetic analyses in this study involve only three microsatellite loci and limited numbers of individuals per population. One locus, CH4B, was highly divergent between populations indicating that the populations sampled may be exhibiting restricted gene flow.

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DEDICATION

I dedicate this thesis to my parents, Dicky and Vickie Kirkley, and to my sons, Aiden and Josh Kirkley, who have all suffered and sacrificed so that I could achieve this goal.

Chapter I

INTRODUCTION

The present study is an ecological survey of the Florida Cottonmouth, *Agkistrodon piscivorus conanti* in the Alapahoochee watershed in Lowndes and Echols Counties, Georgia. Included are a biogeographical investigation incorporating population genetics, a diet survey, a length/weight relationships analysis, sex ratio analysis and observations on reproductive ecology. The major hypothesis is that the two major creeks of the Alaphoochee watershed, Grand Bay Creek and Mud Creek, are separated by geological, anthropological and hydrological barriers and maintain separate breeding populations of *Agkistron piscivorus conanti*.

The southeastern United States (U.S.) maintains the highest diversity of reptiles and amphibians in North America (Conant & Collins, 1991; Whiles & Grubaugh, 1996). With hot summers and mild winters and average rainfall of more than 127 cm per year, herptofauna have historically flourished in this environment. Southern Georgia and northern Florida are within the Coastal Plain region which is rich in swamps, wetlands and slow-flowing rivers. Water snakes, *Nerodia spp*. and *Agkistrodon piscivorus*, are abundant in Georgia and northern Florida.

The Alapahoochee water shed is made up of Grand Bay Creek, Knight's Creek and Mud Creek, which merge to form the Alapahoochee River. The Alapahoochee flows south across the Florida state line before emptying into the Alapaha River, which flows into the Suwannee River. The river system is a mosaic of habitats, mostly swamp and flatwoods in the head waters and then further south in Echols County the river banks are relatively steep with sandy hills and rocky cliffs (Barnett et al., 2007).

Taxonomy and Systematics

Agkistrodon piscivorus belongs to the family Viperidae, subfamily Crotalinae, or the pit vipers. This subfamily includes all native U.S. venomous snakes except for coral snakes. The genus *Agkistrodon* consists of the "moccasins," the copperheads (called the Highland Moccasins), and the Cottonmouths (sometimes called the Water Moccasins). Members of *Agkistrodon* have all characteristics of the pit vipers including facial pits with which they sense the body temperature of prey when aiming to strike (Conant and Collins, 1998).

Distribution, Behavior and Ecology

Pit vipers range from Canada to southern Argentina and from extreme southeastern Europe through southern central Asia and Malaysia. The pit viper clade *Agkistrodon* (copperheads, cottonmouths, and cantils) includes three widely distributed, polytypic species, collectively spanning a broad range of body sizes, diets, life history traits, and habitats. Copperheads and cottonmouths occur throughout much of the eastern U.S. at sites ranging from canyons and upland temperate deciduous forests to coastal swamps and offshore keys, whereas cantils inhabit tropical deciduous forest and thorn scrub in México and Central America (Campbell & Lamar 1989; Gloyd & Conant 1990). The range of *A. piscivorus* is from southeastern Virginia to central Texas to extreme southern Florida. Cottonmouths are a snake of lowlands found in swamps, lakes, rivers, rice fields, and ditches. They can be found sunning on branches, logs, or stones at water's edge and sometimes wander~~s~~ away from its normal habitat in search of food (Conant & Collins, 1998). The three subspecies, western *A. p. leucostoma*, eastern *A. p. piscivorus*, and Florida *A. p. conanti* intergrade extensively in Florida, Georgia, Alabama, and Mississippi (Conant & Collins, 1998). Cottonmouths eat frogs, salamanders, snakes, lizards, small turtles, baby alligators, birds, and small mammals, but their mainstay are fishes hence the scientific name piscivorus or "fish eating" (Conant & Collins, 1998). Kofron (1979) speculated that *A. piscivorus* is a habitat specialist and probably a food generalist in the Atchafalaya River Basin in Louisiana only finding them in abundance in bottomland forest.

Site Location and Description

The Alapahoochee basin (Figure 1) is composed of two major creeks, Grand Bay Creek and Mud Creek, which drain Lowndes County, Georgia, as well as parts of Lanier County and Echols County. Mud Swamp (42.5 hectares) lies at the headwaters of Mud Creek in central western Lowndes County and flows east and southeast until it's convergence with Grand Bay Creek, which forms the Alapahoochee River. Grand Bay Swamp (5,263 hectares) is drained by Grand Bay Creek and originates in northeastern Lowndes County. Grand Bay Creek flows north and east into the southwestern corner of Lanier County before turning south where it becomes the border between Lowndes and Echols counties. Between these two creeks are the City of Valdosta, Moody Air Force Base, multiple major highways including Interstate 75 and extensive acreage devoted to agriculture and the timber industry (Barnett et al., 2007).

Mud Creek flows through an industrial park and is also influenced by a water treatment plant and the City of Valdosta. Grand Bay Creek is mostly influenced by agriculture, Moody Air Force Base and timber tracts (Barnett et al., 2007). As Grand Bay Creek and Mud Creek converge to form the Alapahoochee River, the habitat changes from slow moving black water, typically associated with flat woods, to faster flowing waters bound by ravines and limestone bluffs (Barnett et al., 2007).

Collecting sites on Mud and Grand Bay Creeks (Figure 1) were typified by broad flat flood plains dominated by black gum and cypress trees. Substrate in the creeks is typified by silt and mud bottoms with areas of higher currents often having a hardpan nature. The courses of the creeks above and below the sites are often braided, especially during high water. Near the convergence of the creeks there is a considerable reduction in the width of the adjoining flood plains. There is also considerable change in the floristic nature of the area as pinelands grade into mixed deciduous forests. River substrate is typified by sand bottoms with occasional pools with mud and silt bottoms.

The Alapahoochee River shows much deeper dissection into the sands and gravels overlying Pleistocene limestone. While showing some characteristics similar to Mud creek and Grand Bay creek, the northern portion of the Alapahoochee has a course more tightly constrained by moderately high hills and more nearly vertical banks. Elevation above the bed of the river ranges from 3 to 4 meters. Farther south the river cuts well into the Pleistocene limestone with stretches of the river being bordered by very steep banks and limestone cliffs. This area is typified by springs and seeps as well as small ephemeral and permanent creeks. Elevation above the river bed ranges from four to 10 meters and during normal and low water conditions extensive riffles and small waterfalls can be found. The river bed is typified by numerous rocks, rocky outcrops, stretches of flat rock, and sand bottom pools (Barnett et al., 2007).

Chapter II

MATERIALS AND METHODS

Field Protocol

Snakes were collected in spring, summer and early fall 2008 and 2009. In general, collecting was done with one researcher on either side of a creek or the river's main channel, walking in the same direction. Collecting took place by following the main channels of Grand Bay Creek and Mud Creek. Global Position System (GPS) coordinates (Table 1) were recorded from each starting point and sampling occurred upstream and downstream from each starting point when possible. Snakes were captured by use of snake tongs so as to avoid handling live specimens. When collected, snakes were euthanized in the field according to Georgia Department of Natural Resources Collecting permit 1734 or found as road kills adjacent to collecting sites (Figure 1). Euthanizing was carried out based on the American Society of Ichthyologists and Herpetologists guidelines (ASIH Guidelines, 2004) by blunt force blow to the head upon capture in the field, which also permitted safer handling of specimens in the field (see Animal Use Proposal, Appendix C). Tail tips were removed for DNA tissue samples and the remainder of each specimen stored on ice and subsequently placed in a -20C freezer. Half of each tissue sample was stored in 4M guanidinium thiocyanate (GIT) at room temperature and the other half was stored on ice and subsequently placed in a -80C freezer.

Table 1. Descriptions of Collecting Sites.

Site	Description
1	Mud Swamp (Langdale Lake)
2	Mud Creek at St. Augustine Rd.
3	Mud Creek at Inner Perimeter Rd.
4	Mud Creek at Water Treatment Plant
5	Knights Creek
6	Mud Creek at Vann Rd.
7	Alapahoochee River at Culpepper Rd.
8	Grand Bay at Knight's Academy Rd.
9	Grand Bay Hunting Club at Howell Rd.
10	Grand Bay at Old State Rd.
11	Grand Bay Hunt Club (Cemetery)

Site descriptions are graphically displayed in Figure 1.

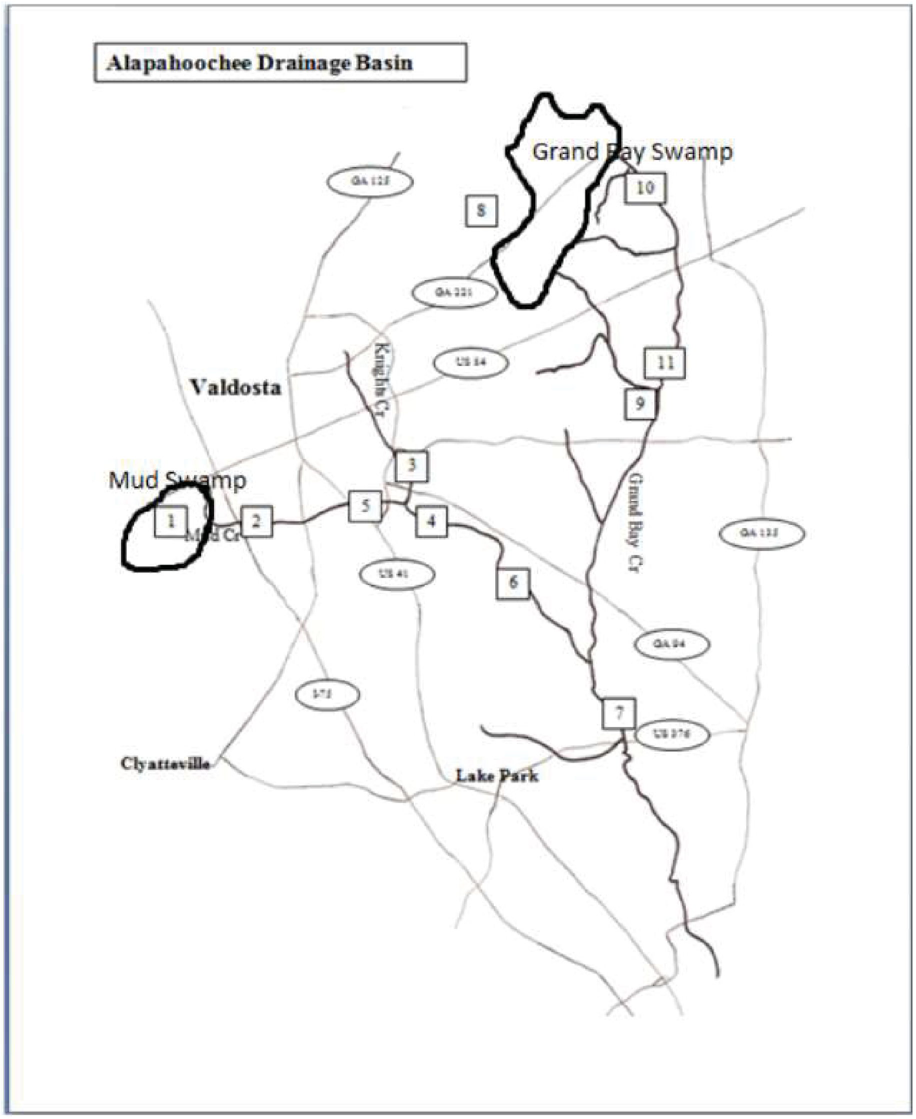

Figure 1: Collection sites in the Alapahoochee River Basin. Numbers indicate points of origin for collection sites. Sites are labeled 1-11. For site descriptions see Table 1.

Laboratory Protocol

Sex, best estimates (given the trauma to the head) of snout-vent length (SVL), weight (wt), and gut contents were recorded for each snake when possible; however, the gut content of some road kills could not be determined. Embryos in gravid females were labeled as to the female collected from and stored in a -20^{O}C freezer.

Tissue samples, (40 tail tips and one shed skin), stored in 4 M GIT were minced, and subsequently placed in 1 ml of fresh 4M GIT homogenization buffer in Eppendorf microcentrifuge tubes. After incubating for 5 min at room temperature, samples were homogenized using a loose fitting pestle and incubated for an additional 5 min at room temperature to ensure cell lysis. To purify the DNA and remove protein, samples were then extracted with 1 ml of 1:1 phenol/chloroform and centrifuged for 20 min using a Hermle Z180M (Wehingen, Germany) at 5000 RPM. Large bore disposable Pasteur pipettes were used to collect the sample supernatant reducing the potential for shearing of nucleic acids. One additional extraction with 1:1 phenol/chloroform was performed to further purify DNA and remove any remaining proteins. Phenol/chloroform extractions were followed by several extractions using 24:1 chloroform/isoamyl alcohol until a clean interface was present between the organic and aqueous phases of the sample. The final supernatants were collected in a clean mirocentrifuge tube and 10 M ammonium acetate (0.2 volumes) was added to the supernantants followed by cold 95% ethanol (2 volumes) to precipitate nucleic acids. Samples were incubated over night at -20^{o}C. Pellets were formed by centrifugation and the ethanol was poured off ensuring the pellets remained in the tube. Pellets were washed twice with cold 70% ethanol to remove salts and then allowed to dry in air. The pellets were then dissolved in 1X TE buffer (1 mL) at 37^{o}C for 24 hours and held in refrigeration until needed.

Microsatellite loci PCR primers previously developed for *Crotalus horridus* (Clark, 2006) have been shown to "cross amplify" loci in *A. piscivorus conanti* (McCleary, 2009).

These published primer pairs were tested for use in our study. Three of these primer pairs amplified informative loci (CH1A, CH4B, and CH2E), two other primers (CH4C and CH4D) were also tested but did not cross amplify, consistent with McCleary (personnel communication, 2007). Primers were purchased, laboratory ready with standard desalting from Integrated DNA Technologies® (IDT®; Coralville, IA). Primers were labeled with IRDye® 700 (LiCor®; Lincoln NE) fluorescent dye compatible with the LiCOR® (Lincoln, NE) 4300 infrared genotyping system. Stock solutions for all primers were 100uM in IDTE (1X TE buffer made by IDT®; Coralville, IA) buffer, pH 8.0 and stored at -20^{o} C.

Polymerase Chain Reaction

PCR protocols were all performed in 25 uL volume reactions using New England BioLabs® (Ipswich, MA) reagents. Reactions contained 2.5 uL of NEB's 10X Standard *Taq* Reaction Buffer (100mM KCl, 100mM $(NH_4)_2SO_4$, 200mM Tris-HCl, 2mM $MgS0_4$, 0.1% Triton X-100, pH of 8.8) . Reactions contained 1 uL of standard dNTP mix solution (100mM each), 6.0 uL of sample DNA, 1.0 uL of forward primer, 1.0 uL of reverse primer and 0.5 uL of *Taq* Polymerase. Thirteen uL of PCR-grade H20 was added to adjust final reaction volumes to 25uL. PCR was conducted using a Bio-Rad MyCycler™ Thermal Cycler (Bio-Rad; Hercules, CA). PCR parameters were a single soak at 95^{o}C for 1 min. followed by 45 cycles of 95oC for 1 min., 54^{o}C for 1 min., , 72^{o}C for 2 min., and finished at 4^{o}C until the samples were removed for gel electrophoresis.

Gel Electrophoresis

Gel electrophoresis and microsatellite allele scoring was conducted using the LiCOR® (Lincoln, NE) 4300 Sequence Analyzer/Genotyping System. Polyacrylamide gels 23 cm long and 0.25 mm thick were used to differentiate microsatellite fragment lengths. The polyacrylamide gels were made from 30% acrylamide stock solution (28.5 g acrylamide and 1.5 g of bis-acrylamide (28:2) in 100 mL of ddH2O filtered). Gels (4.5%) were made using 75 mL of 30% stock, 50 mL of 10X TBE buffer, and 42 g of urea (7 M) in 500 mL of ddH2O (Theophilus, 1996). A 10% ammonium persulfate (APS) (Bio-Rad Laboratories; Hercules, CA) (0.1 g ammonium persulfate to 1.0mL ddH2O) and UltraPure™ Tetramethylethylenediamine (Temed) (Invitrogen' Carlsbad, CA) were used as gel polymerization catalysts. For every 20 mL of 4.5% acrylamide, 150 uL of APS and 15 uL of Temed were used. After polymerizing for one hour shark tooth combs were inserted approximately 1 mm into the top of the gel trough to form loading wells. The gel assembly was then placed into the LiCOR® (Lincoln, NE) 4300 Sequence Analyzer/Genotyping apparatus with 1X TBE buffer. Gels were pre-run at 1500 volts for 20 min to preheat the gel. The gel was then loaded with 50-350 or 50-700 size standards (LiCOR® Biosciences, 2011b) and PCR products were run at 1500 volts until completed, which was approximately one and a half hours. Digital images were taken of each gel and the sample window using the Saga$^{GT™}$ software developed for microsatellite research that accompanies the LiCOR® (Lincoln, NE) 4300 Sequence Analyzer/Genotyping System. Saga$^{GT™}$ digitizes electrophoresis, finds lanes, locates standards, calibrated band sizes, and scores microsatellite alleles (LiCOR® Biosciences, 2011a).

Statistical Analyses

Microsatellites were amplified from genomic DNA using the primers listed above. The fragment length differences, due to differences in the number of repeats of the microsatellite sequences, were scored as allelic differences.

Excel Microsatellite Toolkit version 3.1.1 (2008), an add-on for Microsoft Excel®, was used to convert the allele size data into a standard format which could be analyzed by GENEPOP. Statistical analyses were calculated using GENEPOP, an online population genetics software (Raymond and Rousset, 1995b; Rousset, 2008). F-statistics, Rho-statistics, exact G tests (genotypic variation) and Hardy-Weinberg (HW) heterozygote deficiency/excess tests were performed using GENEPOP. Contingency tables used for F-statistics, Rho-statistics and exact G tests are located in Appendix A. F_{is} compares genetic variability between the individual and the subpopulation. F_{st} compares genetic variability between the subpopulation and the total population. F_{it} compares genetic variability between the individual and the total population (Wright, 1978). Rho-statistics compare individuals and subpopulations in the same manner except they are taking into account the higher mutation rates of microsatellites by assuming they mutate according to the stepwise mutation model (SSM) (Valdes et al., 1993). Exact G-test is the comparison of observed genotypic distributions to expected genotypic distributions. Mud Creek population and Grand Bay population were compared over all loci using Fisher's (1939) method. Hardy-Wienberg (HW) deficiency/excess tests determines if any excess or deficiency exits in heterozygosity of a locus in a population. A deficiency in heterozygosity may indicate lower genetic diversity and an excess in heterozygosity may indicate the contrary. A global U-test determines a summary descriptor for heterozygote deficiency/excess at all loci for each population and over both populations.

Chapter III

RESULTS

A total of 41 *Agkistrodon piscivorus conanti* were collected (20 males and 20 females). Four males and 13 females were collected from Mud Creek sites. Seven females and 16 males were collected from Grand Bay sites. Although two *A. piscivorus piscivorus* were observed at study sites 6 and 9 both eluded capture. All snakes collected were *A. piscivorus conanti* and hereafter will be referred to as *A. piscivorus*

Length and Weight

Length/weight grouped linear regression analysis (Figure 2, Table 2) showed that slopes for both males and females are significant. However, the difference between slopes is not significant (Figure 2, Table 2) indicating that both males and females have similar slopes based on their regression analyses.

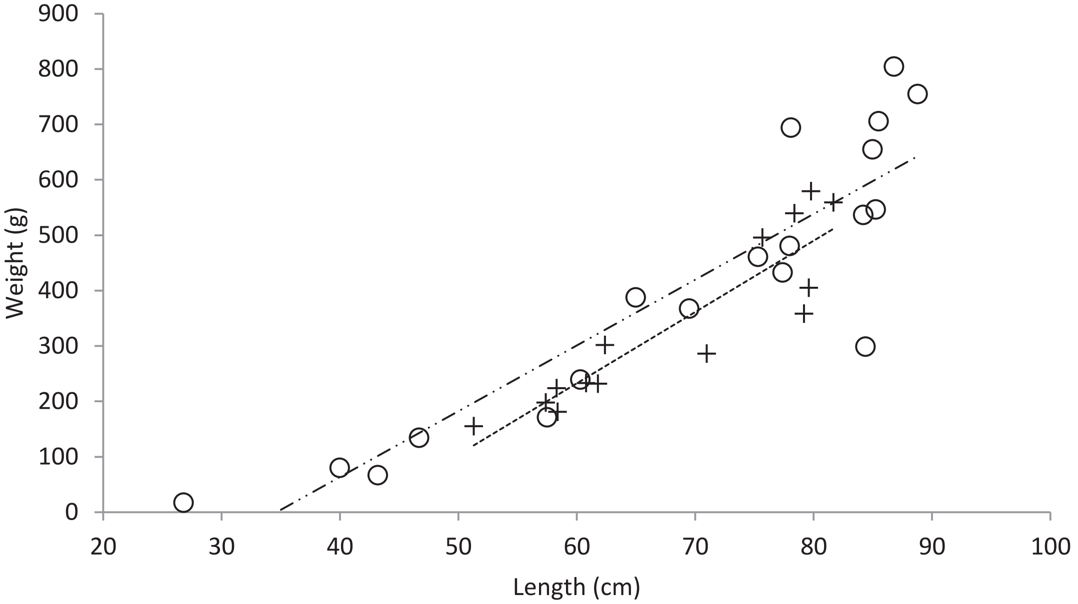

Figure 2: Linear regression of SVL against weight for males and females. Data from both the Mud Creek and Grand Bay Creek samples are combined. Female data points = O. Male length/weight = +. Linear regression female = dashed/dotted line. Male linear regression = dashed line

Table 2. Length/Weight Grouped linear regression.

Source of variation	SSq	DF	MSq	VR	P
Common slope	339969.6291	1	933996.629	99.792	< 0.0001
Between slopes	2456.1935	1	2456.193	0.262	0.613
Separate residuals	233985.2259	25	9359.409		
Within groups	1170438.0486	27			

SSq = sum of squares, DF = degrees of freedom, MSq = mean sum of squares, VR = value R, P = P-value

Sex Ratios

A single proportion test was used to examine sex ratios involving the number of males and females. Out of 39 snakes sexed there were 20 males and 19 females. Assuming a normal 1:1 sex ratio, there was no significant difference between the sexes

(Proportion = 0.512821, Exact (Clopper-Pearson) 95% confidence interval = 0.347802 to 0.675819, Binomial two sided P > 0.9999)

Diet

Of 37 snakes sampled, 11 (29.73 %) had contents in the gut. The gut contents were varied with one round tailed muskrat, *Neofibir alleni,* three fish, one kinosternid turtle, three frogs, one *Coluber constrictor*, one unidentifiable red mass, and one snake had a piece of wood and leafy debris in its gut.

Reproduction

Five females were collected in 2008, 13 females were collected in 2009 and one was collected in 2010 during other field work. Of the 19 females collected, five were gravid and three of those five were collected on 14 June, 2009, in the same area. Gravid females had five to seven eggs each. Of the three collected on 14 June, none of the eggs showed any visible signs of embryonic development. However they were not dissected. Another female collected on 30 July, 2009, had five eggs, all of which contained well developed embryos. A second gravid female collected 11 August, 2009, had five eggs all of which contained nearly completely developed embryos with little remaining yolk.

Biogeography

No *A. piscivorus* were found past the convergence of Mud Creek and Grand Bay Creek, supporting previous observations from sampling in this area which involved numerous collections over a 1.5 year period by Hightower (2008) working on the life history of the crayfish *Procambarus spiculifer*, and a study of the fish assemblages (Bechler, personal communication) and invertebrate assemblages as part of a Nonpoint

Source Management Program study (Barnett et al., 2007). *Nerodia taxispilota* and *N. fasciata* were observed during this study and the others mentioned above in the Alapahoochee River below the confluence of the two creeks, but during this study none were collected.

Genetic Analysis

Gel images were captured by the software; however, the scoring of alleles is not shown in the image (Appendix A). The detailed data file images produced (Appendix A) show the scoring of each allele in Grand Bay and Mud Creek (LiCOR® Biosciences, 2011a). The approximate F and R values are displayed in Table 3 (Raymond & Rousset, 1995b; Rousset, 2008; Weir & Cockerham, 1984). The guidelines for interpreting F_{st} were also used for interpreting Rho_{st} and are shown in Table 4 (Wright, 1978). CH4B shows significant genetic differentiation. CH1A and CH2E shows little genetic differentiation (Table 4).

Table 3. Wright's (1978) Estimates for F_{st}

Little genetic differentiation	Moderate genetic differentiation	Great genetic differentiation	Very great genetic differentiation
0.0-0.05	0.05-0.15	0.15-0.25	> 0.25

Table 4. F-statistics and Rho-Statistics.

	F- statistics			Rho- statistics		
Locus	$F_{is^\wedge}$	$F_{st^\wedge}$	$F_{it^\wedge}$	$Rho_{is^\wedge}$	$Rho_{st^\wedge}$	$Rho_{it^\wedge}$
CH1A	0.1129	-0.0050	0.1084	-0.3560	0.0209	-0.3276
CH4B	0.1611	0.5267	0.6030	0.9407	0.9400	0.9964
CH2E	0.0079	0.0125	0.0203	-0.6159	0.0017	-0.6132
MultiLocus	0.0792	0.1948	0.2585	0.6465	0.9268	0.9741

F_{is} = variation between the individual and the subpopulation. F_{st} = variation between the subpopulation and the total. F_{it} = variation between the individual and the total. The Rho statistics are denoted with the same convention

Exact G-test (Table 5) was used to test if the two populations have the same distribution of genotypes. The sum of all loci probabilities were used to generate a rejection zone (see contingency table Appendix B). An impartially estimated *P*-value of the exact G-test is produced for the population pair based on the best fit of the observed genotype to the contingency table (Raymond & Rousset, 1995a; Raymond & Rousset, 1995b; Rousset, 2008). The null hypothesis for this test is that all genotypes are drawn from the same distribution. The large *P*-values for CH1A and CH2E support H_o while CH4B has a *P*-value of < 0.0001 rejecting $H_{o.}$ The standard error (S.E.) in Table 5 is the standard deviation between the observed and expected distributions (Larson & Farber, 2009).

Fisher's method for finding a *P*-value from a Chi squared value was used for the population pair across all loci (Table 6) (Fisher, 1935; Raymond & Rousset, 1995a; Raymond & Rousset, 1995b; Rousset, 2008).

Table 5. Genotypic Distributions of Population Pair.

Exact G-test	CH1A		CH4B		CH2E	
Population pair	P-Value	S.E.	P-Value	S.E.	P-Value	S.E.
MC & GB	0.18377	0.0061	0.0000	0.0000	0.4013	0.0087

Table 6. Genotypic Distribution (Fisher's Method).

Population Pair	Chi-squared	Df	P-Value
MC & GB	Infinity (∞)	6	Highly Sign.

P-value for the population pair across all loci.

HW exact tests were performed (Table 7 & Table 8) to determine deviations from the HW equilibrium. HW excess and deficiency tests determine if excess or deficiency in heterozygosity is present. H_o is "random union of gametes is occurring." A global U test determined deficiency and excess for both populations at all loci (Table 9).

Table 7. Mud Creek HW Exact Test.

Locus	Probability P-Value	Probability S.E.	Defic./Excs	P-Value	S.E.
CH1A	0.0804	0.0070	Deficiency	0.2225	0.0097
			Excess	0.8100	0.0095
CH4B	0.0312	0.0140	Deficiency	0.0150	0.0062
			Excess	0.9866	0.0060
CH2E	0.0024	0.0015	Deficiency	0.2819	0.0274
			Excess	0.7476	0.0256
Chi2 : 24.0647 Df : 6.0000 Prob : 0.0005					

The probability P-value is given for the $H_{o.}$ The P-value for excess-deficiency is given for the $H_{1.}$

Table 8. Grand Bay HW Exact Test.

Locus	Probability *P*-Value	Probability S.E.	Defic./Excs	*P*-Value	S.E.
CH1A	0.3810	0.0288	Deficiency	0.4811	0.0316
			Excess	0.5621	0.0315
CH4B	1.0000	0.0000	Deficiency	1.0000	0.0000
			Excess	0.9339	0.0013
CH2E	0.0022	0.0011	Deficiency	0.1326	0.0274
			Excess	0.9024	0.0177
Chi2 : 14.1504 Df : 6.0000 Prob : 0.0280					

The probability *P*-value is given for the $H_{o.}$ The *P*-values for excess and deficiency are given for the $H_{1.}$

Table 9. Global U Test.

Population	Deficiency/Excess	*P*-Value	S.E.
MC	Deficiency	0.0197	0.0073
	Excess	0.9803	0.0073
GB	Deficiency	0.2523	0.0244
	Excess	0.7477	0.0244
All Populations with all Loci	Deficiency	0.0416	0.0104
	Excess	0.9584	0.0104
	Chi2 : 38.2151	Df : 12.0000	Prob: 0.0001

P-values are given for heterozygote excess and deficiency over all loci for each population. Excess and deficiency *P*-values are also given for both populations and all loci along with Chi-squared value. MC = Mud Creek, GBC = Grand Bay Creek.

Chapter IV

DISCUSSION AND CONCLUSIONS

Diet

It is well documented that *A. piscivorus* are generalists when it comes to choice of prey (Allen & Swindell, 1948; Birkenholz, 1963, 1972; Bothner, 1974; Eskew et al., 2009; Gloyd & Conant, 1990; Goodman, 1958; Himes, 2003; Kardong, 1975; Lee, 1968; Lillywhite & McCleary, 2008; Lillywhite et al., 2008; Savitzky, 1983; and Wharton, 1966). Allen & Swidell (1948) reported Cottonmouths taking various species of snakes, including rattlesnakes, many frog species, hatchling alligators, mice, rats, young rabbits, birds, bats, squirrels, lizards, bird eggs, fish and turtles as prey. Fish and frogs are the most common prey source, but cottonmouths have even been known to be cannibalistic (Allen and Swindell, 1948). *Agkistrodon piscivorus* will congregate at drying ponds or pools and gorge on dying fish as long as the supply remains (Allen & Swindell, 1948; Bothner, 1974). There are even previous reports of *A. piscivorus* preying on *N. alleni* (Birkenholz, 1963, 1972; Lee, 1968) and chips of wood (Goodman, 1958). Although a small percentage (29.73%) of the sample population had meals present, the highly variable contents supports the previous literature that *A. piscivorus* is an opportunistic generalist predator. Most of the snakes sampled were void of any stomach content. The meals present were small compared to the size of meals known to be taken by this species. This leads to the conclusion that most, if not all, snakes collected were foraging at the time of capture. It should be noted that the habitat complexity and known cryptic nature of this species prevented the researchers from finding snakes on many outings (5.93 man hours per snake). When snakes were found, they were usually in sparse vegetation and easily seen. It could be speculated that the snakes only risked making themselves obvious to potential predators when they were in search of a beneficial meal. Finally, while it is argued that snakes were out primarily foraging, it is possible that some were also thermoregulating and lying in sunny areas.

Reproduction

The principle that natural selection favors parental investment into each sex equally was proposed by Fisher (1930). Although Fisher's principle has been largely accepted, supporting data for sex ratio is minimal beyond the observation that most organisms have a 1:1 sex ratio (Shine & Bull, 1977). Data from previous studies on sex ratios in snakes show that most snakes have equal sex ratios with a few exceptions (Shine & Bull, 1977). Snake sex ratios may be seasonally skewed (Iverson, 1990). Only 5 of the 20 females collected in this study were taken in 2008. Drought was prevalent in 2008 with much of the watershed reduced to still pools until tropical storm Fay flooded areas in Florida as well as southern Georgia (http://pubs.usgs.gov/of/2010/1142/pdf/ofr2010-1142.pdf). Subsequently most of the snakes collected that year were found dead on the stretch of road near Grand Bay (Site 8) and the majority of those were males. Closer to normal rain conditions returned in 2009 and 14 females were collected. Although overall sex ratios did not deviate from the expected in this study, they were definitely skewed relative to the year collected and relative to the creek drainage from which they were collected. Grand Bay Creek produced more males while Mud Creek produced more females. Drought followed by subsequent flooding led to an obvious increase in road kills of males on Knight's Academy Road near the entrance to the Grand Bay Wildlife Management Area. One could suggest that the skewed sex ratio of road kills was due to males attempting dispersal.

Some studies have reported annual reproductive cycles in *A. piscivorus* (Kofron, 1979; Arny, 1948) in Louisiana while others (Burkett, 1966; Wharton, 1966) report biennial reproduction in other parts of its range. However, there is evidence that frequency of reproduction is largely dependent on size and fat reserves (Blem, 1981; 1982; 1997). Ovulation is thought to start by May, followed by a 3 to 4 month gestation with birth occurring by August (Kofron, 1979; Burkett, 1966; Wharton, 1966 and Arny, 1948). Sample size in this study is not sufficient to make inferences about an annual verses biennial reproductive cycle. No females collected in 2008 were gravid, while five were in 2009. However, very few females were collected in 2008. Our results do support the findings that ovulation occurs in May (three females collected in June had well yolked eggs) followed by a 3 to 4 month gestation period (one female collected in July had eggs with developing embryos and yolk) with birth occurring by end of August as one female collected on August 11 had eggs with nearly completely developed embryos and nearly no visible yolk.

Biogeography

The Alapahoochee River watershed has been the site of several recent studies involved in fish, crayfish, and a management survey (Bechler, 2006; Chaney & Bechler 2006; Barnett et al., 2007; Hightower & Bechler, 2013). *Agkistrodon piscivorus* had not been observed in 13 years of field collections on the Alapahoochee River south of the confluence of Mud Creek and Grand Bay Creek, and none were observed during this study. The geology shifts and stream morphology changes from wide, slow-moving streams in flatwood habitats to swifter moving water bound by sand and limestone bluffs. Because snakes have not been observed does not mean that snakes are not present. The failure to observe Cottonmouths may indicate lower abundances. These snakes are extremely cryptic in nature and the habitat use in this area of the basin is unknown.

Agkistrodon piscivorus have been known to disperse considerable distances over land (Roth, 2005) and have even traversed sea barriers on flotsam (Lillywhite et al., 2008; Wharton, 1966; Parkinson et al., 2000). Although many factors exist that would impede dispersal from one creek to another (i.e., urbanization, heavy agriculture, distance, highways, behavior), the question arises as to whether there are enough barriers to prevent individuals from either creek from interbreeding? It seems unlikely that these barriers form a totally impenetrable barrier. *Agkistrodon piscivorus* have been known to use linear home ranges when inhabiting stream/river habitats (Roth, 2005). Roth (2005) used radio telemetry and found *A. piscivorus* most often within 10 m of the stream. Kofron (1979) suggested *A. piscivorus* has an affinity for habitats with still or slow moving waters in bottomland forests. If the movement patterns and home ranges of *A. piscivorus* in this study are consistent with Kofron (1979) and Roth (2005) then they would be expected to stay north (Grand Bay Creek) and west (Mud Creek) of the faster flowing Alapahoochee River and maintain linear home ranges along their respective creeks and proximate wetlands. If true, it is likely that dispersal from one creek to another would rarely be attempted. However, it must be noted that Cottonmouths have been found more than a 1.6 km from water (Gloyd & Conant, 1990) If the few animals that would disperse do not make it to the other creek it could be due to roads and other anthropological or geographical barriers. Therefore, effective barriers to gene flow and genetic divergence of populations is possible.

Population Genetics

The genetic analyses in this study involve only three microsatellite loci and limited numbers of individuals per population (Grand Bay n = 24; Mud Creek n = 17). Conclusions drawn from these microsatellite data are therefore tentative. Population samples were low because of the difficulty in finding snakes in abundance in the habitat. Loci CH1A and CH2E showed little genetic differentiation (F_{st} = -0.005003 and 0.012504 respectively Rho_{st} = 0.020930 and 0.001687 respectively). However, CH4B showed a significant indicating great to very great population differentiation when all loci are considered (Multi Locus F_{st} = 0.1948 & Multi Locus Rho_{st} = 0.9268). This suggests that gene flow might be restricted in the populations that were sampled. The null hypothesis for the Exact G test is that “genotypes are drawn from the same distribution in all populations.” Once again, when looking at individual loci, rejection of H_o only occurs for the CH4B locus. When Fisher's method is used to determine the P-value, taking all populations and all loci into account H_o is rejected.

The deficiency and excess of heterozygosity was determined for each population for each locus and overall for both populations using HW probability and exact tests. The H_o is that "random union of gametes is occurring." The probability over all loci for each population was low suggesting that H_o cannot be rejected (Global U test). No locus in either population showed strong evidence of heterozygote deficiency or excess.

Due to small sample sizes and restricted numbers of loci surveyed, these data tentatively indicate that the CH4B locus is highly divergent between Grand Bay creek and Mud creek snake populations. Additionally, it should be noted that the CH4B locus was highly polymorphic in the Mud Creek population. Allele size range for the CH4B locus exceeded the 144-166 range reported by McCleary (2009). Alleles for the CH4B locus in our samples ranged from 250 bp to 490 bp. More research is needed to determine whether the apparent divergence between the Grand Bay and Mud Creek populations is real. In order to better understand potential genetic structuring between Grand Bay and Mud Creek, *A. piscivorus* populations in the Alapahoochee watershed sample sizes need to be significantly increased and additional microsatellite loci need to be analyzed. Since this study began, additional microsatellite markers have been successfully developed and cross amplified for use in *A. p. conati* (McCleary, 2009). Future research should also include the analysis of populations from the Alapahoochee watershed samples and Florida samples.

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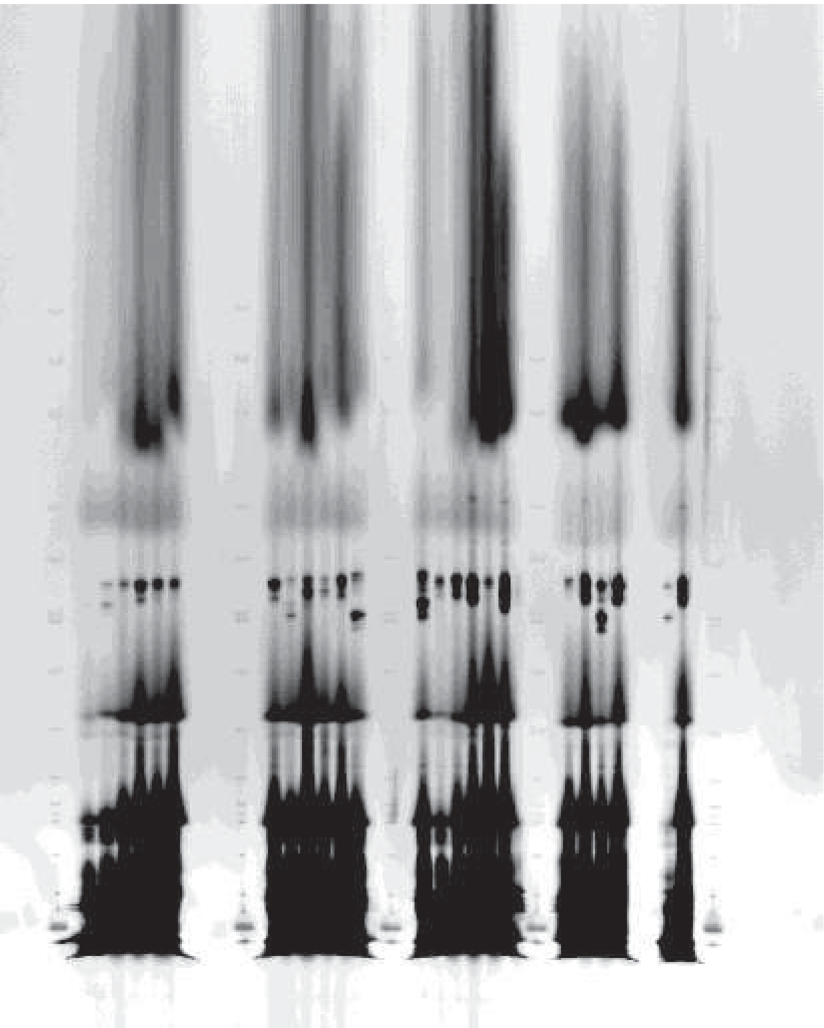
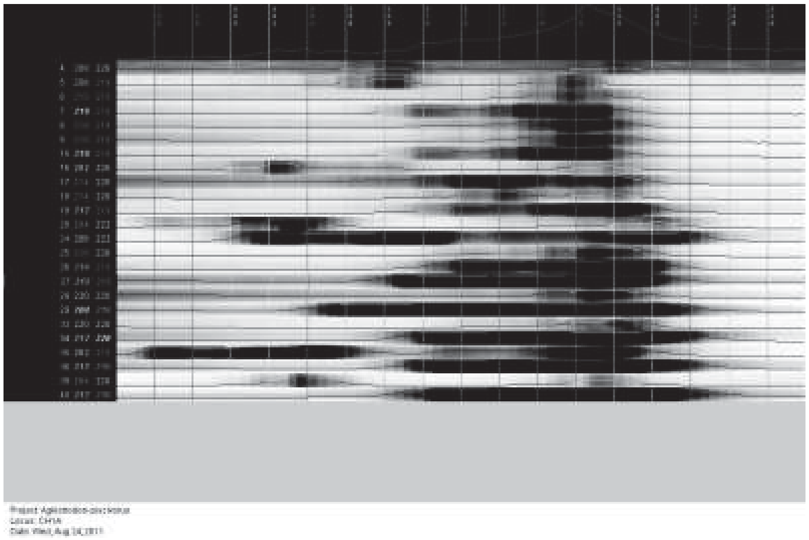
APPENDIX A:

Example Gel Image and Detailed Data Images Captured by Saga$^{GT™}$

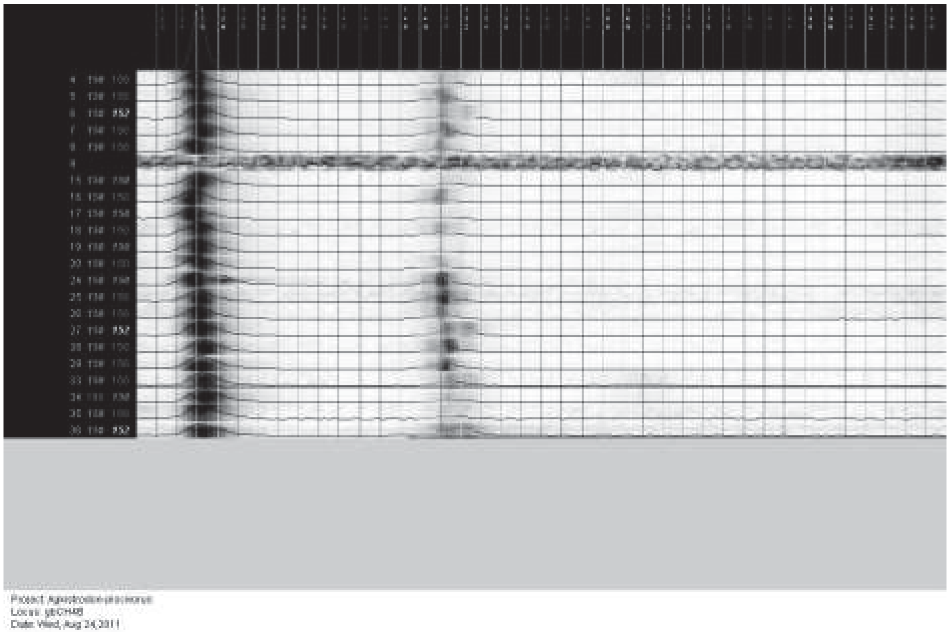
Example gel image (Saga$^{GT™}$; LiCOR® Biosciences, 2011a).



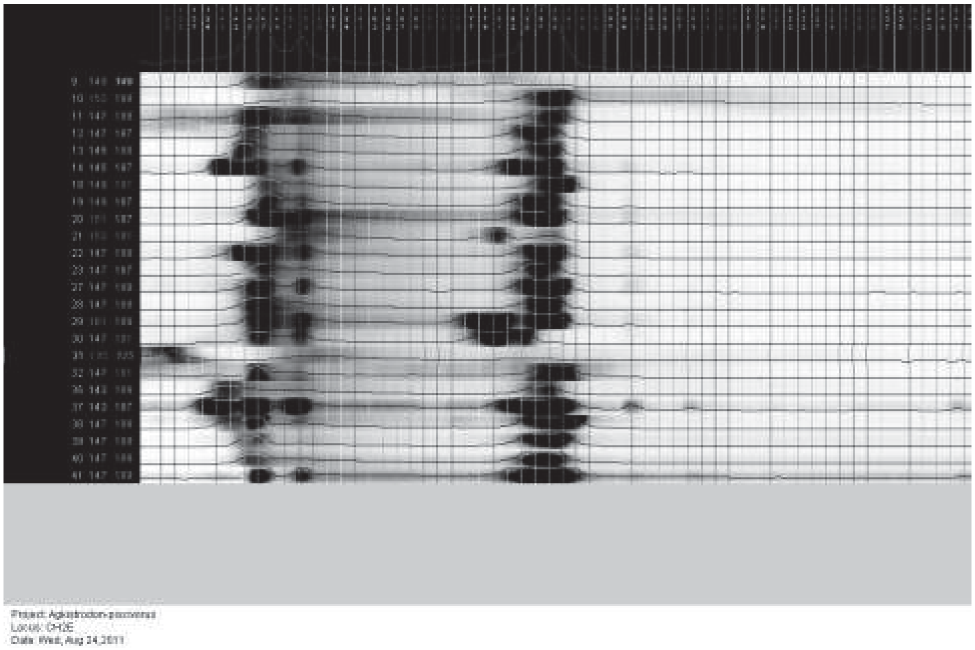
Grand Bay CH1A detailed data image (Saga$^{GT™}$; LiCOR® Biosciences, 2011a).



Grand Bay CH4B detailed data image (Saga$^{GT™}$; LiCOR® Biosciences, 2011a).

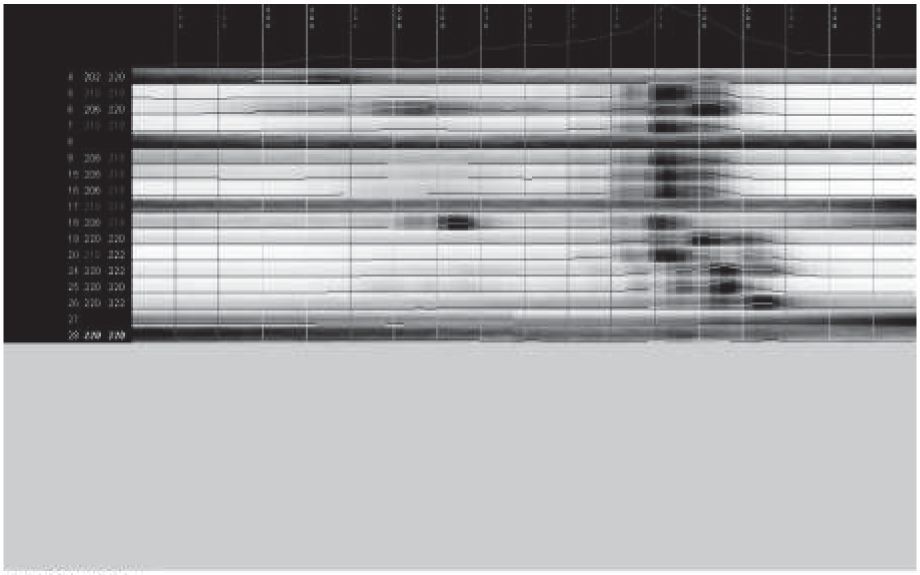


Grand Bay CH2E detailed data image (Saga$^{GT™}$; LiCOR® Biosciences, 2011a).



Project: Agkistrodon-piscivorus
Locus: CH2E
Date: Wed, Aug 24,2011

Mud Creek CH1A detailed data image (Saga$^{GT™}$; LiCOR® Biosciences, 2011a).



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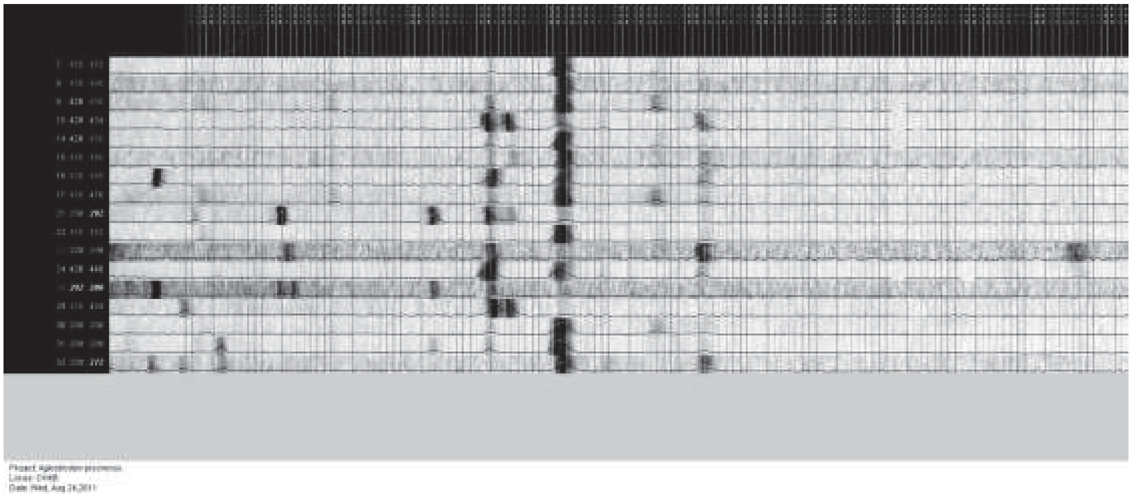
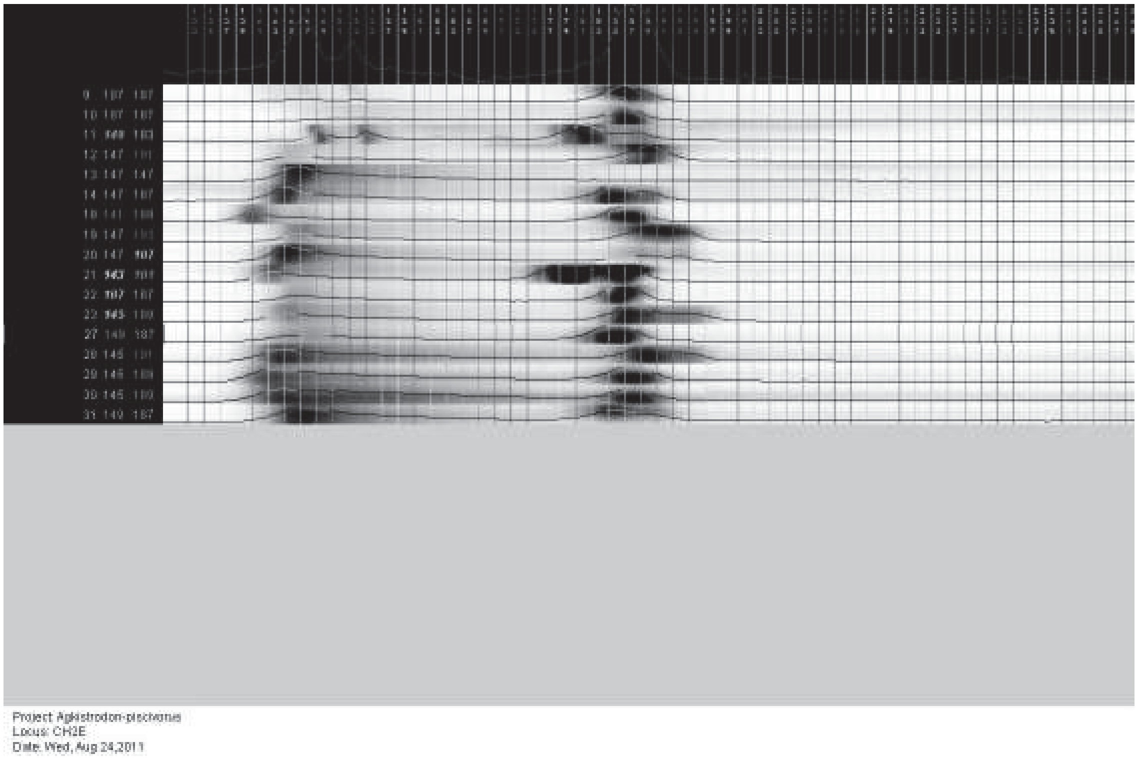
Locus: CH1A
Date: Wed, Aug 24,2011

Mud Creek CH4B detailed data image (Saga$^{GT™}$; LiCOR® Biosciences, 2011a).



Mud Creek CH2E detailed data image (Saga$^{GT™}$; LiCOR® Biosciences, 2011a).



Project Agkistrodon-piscivorus
Locus: CH2E
Date: Wed, Aug 24,2011

APPENDIX B:

Contingency Tables for F-statistics and Genotypic Distributions.

Contingency table for the CH1A locus.

Genotype		Population		Total
		Mud Creek	Grand Bay	
212	216	0	1	1
202	218	0	1	1
206	218	1	0	1
208	218	3	2	5
210	218	0	2	2
212	218	0	3	3
214	218	0	1	1
218	218	3	3	6
202	220	1	1	2
204	220	0	1	1
206	220	1	0	1
208	220	0	1	1
212	220	0	1	1
214	220	0	2	2
216	220	0	1	1
220	220	3	2	5
204	222	0	1	1
206	222	0	1	1
218	222	1	0	1
220	222	2	0	2
	Total	15	24	39

Contingency table for the CH4B locus.

Genotype		Population		Total
		Mud Creek	Grand Bay	
150	150	0	20	20
150	152	0	3	3
250	292	1	0	1
292	308	1	0	1
310	310	1	0	1
330	330	2	0	2
330	372	1	0	1
428	434	1	0	1
430	434	1	0	1
428	448	1	0	1
428	450	2	0	2
430	450	1	0	1
450	450	2	0	2
450	476	1	0	1
450	490	2	0	2
	Total	17	23	40

Contingency table for the CH2E locus.

Genotype		Population		Total
		Mud Creek	Grand Bay	
135	135	0	1	1
147	147	1	0	1
149	149	0	1	1
143	181	1	0	1
147	181	0	1	1
153	181	0	1	1
149	183	1	0	1
143	187	0	1	1
145	187	0	1	1
147	187	2	2	4
149	187	2	1	3
151	187	0	1	1
187	187	3	0	3
141	189	1	0	2
143	189	0	1	2
145	189	3	1	4
147	189	0	8	8
153	189	0	1	1
181	189	0	1	1
145	191	1	0	1
147	191	1	0	2
149	191	0	1	1
147	193	1	0	1
	Total	16	24	40

APPENDIX C:

Animal Use Proposal

Animal Use Proposal

In 2007, I developed an Animal Use Proposal (AUP) for the study of three snakes (Agkistrodon piscivorus, Nerodia taxispilota and Nerodia faciata) in the Alapahoochee Water Shed. The proposal addressed the collection and euthanizing of these species of snakes as part of a board scale analysis on their biology and population genetics. However, in the process of developing the thesis proposal and preparing for the initiation of field work in 2008, delivery of the AUP form to the Valdosta State University Institutional Animal Care and Use Committee failed to the be properly completed. As such, field work and the associated euthanizing of snakes in the field was carried out without proper approval from the Institutional Animal Care and Use Committee.

When developing the protocol that would be followed in the field for euthanizing the snakes, the protocol developed based on the guidelines established by American Society of Ichthyologists and Herpetologists (ASIH Guidelines, 2004, http://www.asih.org/sites/default/files/documents/) where employed. This protocol involved crushing the head upon capture in the field. This protocol was specifically selected so as it permitted safer handling of specimens in the field such that once the head was crushed, the venomous water moccasin, Agkistrodon piscivorus, was not able to bite and envenomate the collectors who had to process the snakes and take tissue samples for the genetic analyses in the field.

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