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IDENTIFICATION OF SPRING AND FALL SPAWNED EGGS AND THE GENETIC DISTINCTION OF GULF STURGEON IN THE CHOCTAWHATCHEE RIVER, ALABAMA-FLORIDA

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by

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ABSTRACT

The Gulf Sturgeon (GS; *Acipenser oxyrinchus desotoi*) is a threatened subspecies of Atlantic Sturgeon (*Acipenser oxyrinchus oxyrinchus*) which inhabits the Gulf Coast of the United States. Prior studies of Eurasian sturgeon species and Atlantic Sturgeon have revealed the existence of a dual spawning strategy (i.e. spring and fall). The presence of a fall spawn has also been proposed for GS, but only with the support of circumstantial evidence. This study used molecular techniques to investigate seasonality of GS spawning and the possibility that this has produced unique genetic groups in the Choctawhatchee River. Mitochondrial DNA from suspected GS eggs was used to verify the presence of a dual spawn. Microsatellite genotypes were used to characterize and quantify the extent of differentiation of genetic groups in the population. Egg samples were collected in spring and fall seasons of 2019 and 2020. Adult fin clips were collected in late summer and fall of 2018 to 2021. A total of 381 eggs were collected and sequences for 74 of these were confirmed as GS when compared to known haplotypes. STRUCTURE analysis of microsatellite genotypes identified two distinct genetic groups, presumably representing spring and fall spawning groups. Most individuals strongly assigned to a particular group with 44 fish identified as spring spawners and 67 as fall spawners. Only three individuals showed admixture between the two groups. The verification of a second spawn can have significant effect on how the species' recovery is managed.

ACKNOWLEDGMENTS

I must acknowledge and thank my committee chair and advisor Dr. Brian Kreiser for the opportunity to perform this research and pursue my Master's degree. Additionally, thanks is due to my other committee members, Dr. Jake Schaeffer and Steve Rider, as well as Dr. Dewayne Fox. Each played a critical role in collecting and analyzing samples, as well as production of this document. Funding for this project was provided in part by a Section 6 grant from the Alabama Department of Conservation and Wildlife Resources, Wildlife and Freshwater Fisheries Division. Finally, I would like to acknowledge my fellow lab mates, Biz, Chris, and Jake, for the daily support throughout this process.

DEDICATION

I owe this work and my dedication to the field of fisheries and aquatic sciences to my family and friends. While some have come and gone from my life, their influence and lasting memories have helped to guide me to where I am today and will persist as I continue forward.

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CHAPTER I – BACKGROUND

1.1 Natural Resources Management & Conservation Genetics

Acipenseridae (sturgeon family) is the most critically endangered family in the world with 17 species considered critically endangered and all 27 species being listed (IUCN Red List., 2022). Outliving the dinosaurs and remaining relatively unchanged over the past 200 million years, this family of fishes have developed a formula to survive, that is until now. The World Wildlife Fund (2020) notes world sturgeon populations decline is estimated to be 70% in the past century. The leading factors in the decline of sturgeon populations are overfishing, loss of migration routes, and habitat alterations (Flowers et al., 2009). Additionally, their capacity to overcome these threats is limited by their late age of maturity (Boreman, 1997).

Due to these threats, groups around the world are implementing conservation and management strategies. The National Wildlife Federation has defined conservation management as the preservation and protection of animals, plants, and their habitats. This approach can reach from specific geographic populations to entire species population or an entire ecosystem. Widescale protection of sturgeon may be beneficial for their status, but their economic value opens the door to the approach of resource management. Resource management is a more focused approach, allowing managers to define resource groups individually and apply unique management strategies for each. Such strategies can include captive breeding programs, creation and management of habitat patches and migration pathways, or removal of competitors and predators. Such strategies are utilized for many taxa and species. Lake Sturgeon (*Acipenser fulvescens*) are currently showing growth in total abundance and the number of populations after

decades of management programs controlling exploitation, improving habitat and water quality, and recruitment and stocking efforts (Bruch et al., 2017). Conversely, dependence on hatchery programs to maintain populations without proper management plans to address the underlying causes of population stresses, such as overfishing, habitat degradation or low genetic diversity can be counterproductive, evidenced by Beluga Sturgeon (*Huso huso*; Flowers et al., 2009) Doukakis et al. (2010) found that Beluga Sturgeon, a source of black caviar and sustained via hatchery supplementation, are being overfished, with many being harvested before second spawn, and concluded that reducing fishing pressure would be the most impactful recovery strategy.

Overfishing and bycatch have been major contributors to sturgeon mortality (Boreman, 1997). Management programs and the identification of threatened and endangered species have greatly reduced these threats. Current and future threats to sturgeon appear less direct than the harvest and bycatch of the species. Environment alterations have reduced the amount of suitable habitat for more than a century, with dams fragmenting rivers and making historical breeding grounds inaccessible or inadequate, as well as altering estuarine salinity levels which can impact algal activity and community composition (Flowers et al., 2009). Channelization and dredging alter the depth and substrate of natal rivers, further altering the breeding grounds as well as the habitats utilized by young-of-year anadromous and potamodromous sturgeon (Boreman, 1997). Heightened amounts of silt and chemicals from runoff and terrestrial land-use alteration can increase mortality of eggs, embryos, and larvae; low-level contaminants can have an unknown effect on the health and morphology of early life history stages of sturgeon (Fox et al., 2000). Increased nutrients are showing a particularly large effect on

estuarine and marine life in the Gulf of Mexico. A large hypoxic zone, commonly referred to as "the Dead Zone," develops each summer due to algal blooms, caused by the Mississippi River's increased nutrient load from spring rain and melting snow runoff. Other rivers along the northern gulf can exhibit similar effects on a smaller scale (Larkin and Adams, 2007; Lowery, 1998; Sulak et al., 2016). Additionally, as harvest restrictions have been created, the value of sturgeon eggs for caviar markets has increased while simultaneously creating illegal trade of the delicacy from the illegal harvest of the resource (Zabyelina, 2014).

The field of conservation is currently experiencing a boom in the study and usage of genetics to understand and reduce species and population extinctions, to the point where two journals, *Conservation Genetics* and *Conservation Genetics Resources*, have been created since 2000 solely to support the growing field of study. A principal objective of conservation biology is to maintain diversity. This rings true at both the community and population level. Human activities are causing or contributing to species extinctions that are lowering the diversity in communities, thereby upsetting the natural balance, causing other species' populations to increase or decrease, opening a niche to other organisms, some of which may be invasive, altering the ecosystem's resiliency after disturbances, and often decreasing the usefulness of the community for humans. At the species level, lack of genetic diversity reduces the species' potential to evolve over time as there are less unique heritable traits. Without unique heritable traits, populations can be less resilient and more susceptible to disturbance as individuals may lack heritable traits that would otherwise help them to persist (DeYoung and Honeycutt, 2005).

To combat loss of diversity, managers will first look to understand the source of the problem: if the community is lacking resources, experiencing frequent disturbance, or is suffering from habitat degradation. Species which are experiencing loss of diversity would be studied to determine the factors influencing the issue, with three major possible explanations: low reproduction, low population size, or low migration. Each of these issues can be observed in sturgeon due to a variety of reasons. Sturgeon are longer-lived fish with a relatively high age of first reproduction, leaving a large gap of time in which individuals can perish before they reproduce (Boreman, 1997). Species with low population numbers are more likely to experience genetic drift or inbreeding, each increasing homozygosity of the species and decreasing overall diversity (DeYoung and Honeycutt, 2005). Low migration represents a lower potential for a population to acquire new individuals, and therefore new or more unique or rare alleles.

After identifying potential causes of loss of genetic diversity, managers can choose to implement a variety of programs to introduce new genes into the populations or the community. Habitat improvement and general environmental cleanup efforts would help populations and communities to ensure they have the optimal conditions to grow and reproduce. Removal of invasive species would decrease the amount of competition or predation in the system. A species' genetic diversity can be improved in a variety of ways. Optimizing growth and survival by providing optimal habitat and protections helps populations with older ages of maturity. Propagation programs can be used to increase the population sizes, as well as introduce new populations, thereby decreasing the chance of genetic drift and inbreeding. Propagation also allows managers to control what genes are being produced and which populations are receiving the new stock.

Similarly, translocation programs can introduce new individuals from disconnected populations to contribute to natural breeding (Sarre and Georges, 2009). Creation and management of migration corridors can be used to link populations, allowing more regular and safer migration between populations and their genes.

In order to manage species effectively and efficiently, managers will often divide a species to be managed into management units (MU's) or evolutionary significant units (ESU's; Moritz, 1994). Each are meant to define independently reproducing populations and describe their population structure and genetics while allowing managers to make more targeted or specialized management decisions (Sarre and Georges, 2009). ESU's differ from MU's in that they have a longer-term focus due to observed phylogenetic or ecological distinctions which may represent an evolutionary diversion from other units.

1.2 *Acipenser oxyrinchus desotoi* of The Choctawhatchee River

This study will utilize molecular markers to confirm species and quantify population dynamics of Gulf Sturgeon (*Acipenser oxyrinchus desotoi*) eggs and fin clips. Gulf Sturgeon are a subspecies of the Atlantic Sturgeon, *Acipenser oxyrinchus oxyrinchus*, with each name referring to the species' location. Atlantic Sturgeon inhabit the Atlantic Coast and Gulf Sturgeon inhabit the Gulf Coast of the United States. Gulf Sturgeon are anadromous fishes, using natal honing to spawn in seven freshwater rivers ranging from the Florida Gulf Coast to Louisiana: the Suwannee, Apalachicola, Choctawhatchee, Yellow, Escambia/Conecuh, Pascagoula, and Pearl/Bogue Chitto rivers. The then grow to maturity in the brackish estuaries and nearshore waters of the Gulf of Mexico. Gulf Sturgeon of all life stages will emigrate from marine habitats of the gulf to riverine habitats, regardless of sexual maturity or spawning readiness, before the spring

spawning event (Andres et al., 2019). After spawning, these sturgeon will relocate down-river in freshwater holding areas until they emigrate back to their marine over-winter habitat in October and November (Sulak et al., 2016, Vick et al., 2018). Juveniles will make their way down river from where they were spawned, feeding and growing, until they emigrate to the estuarine/marine environment near the end of their first year (Allen et al., 2018, Peterson et al., 2013). Limited feeding occurs while the adults and subadults inhabit freshwater (Ross et al., 2009). Telemetry studies have revealed that in these overwintering habitats, estuaries, and offshore islands, east and west population will mix, contrary to historical assumptions (Vick et al., 2018). Unrestricted commercial fishing and habitat degradation, primarily the construction of dams which cut them off from their spawning locations or altered accessible sites, have decimated the population and limited its recovery potential, resulting in their listing as a threatened species under the Endangered Species Act (U.S. Fish and Wildlife Service, 1991).

Identifying and characterizing spawning habitat is a critical component of successfully managing threatened and endangered species. When all populations of a species exhibit low population numbers, reproduction, either natural or artificial, is the only means to increase population sizes. Many species, including Gulf Sturgeon, require certain parameters to be met of their spawning habitat, and to efficiently manage Gulf Sturgeon and maximize reproductive potential, managers and researchers must investigate what those parameters are and attempt to provide them. Inherent difficulties exist with identifying such information from fish and other aquatic organisms, as the limited visibility and inefficiency of sampling specimens limits the amount of knowledge that can be observed. Tagging of adults with acoustic transmitters allows for the

movements of individuals to be tracked; the timing of spawning movements can be timed, and anadromous fishes do not commonly continue up-river post-spawn, so researchers can investigate these furthest locations for evidence of spawning. Additional sampling efforts can be made to acquire ripe or spawning adults, as well as spawned eggs. Eggs are commonly collected by way of egg mats which provide an artificial surface conducive to capturing eggs. Fox et al. (2000) documented Gulf Sturgeon spawning and related movements in the Choctawhatchee River via radio telemetry; eggs were captured in egg samplers in the suspected spawning reaches, allowing the habitat to be characterized. These sites were flanked by steep bluffs and consisted of limestone or gravel substrates. Sturgeon eggs are adhesive: soft-bottom substrates can encapsulate the eggs and suffocate them, but the threat is diminished with the larger particle sizes of the upper river limestone and gravel substrates. Kreiser et al. (2008) sampled Gulf Sturgeon eggs from the Yellow River in a similar manner and sequenced their DNA to compare adult Gulf Sturgeon that had been sampled as well as to all available haplotypes on GenBank (http://www.ncbi.nlm.nih.gov/).

Molecular evidence in Gulf Sturgeon suggests the existence of a genetic structure of subpopulations based upon drainage, with some admixed individuals possessing alleles from more than one subpopulation (Dugo et al., 2004). Additionally, that same molecular data coincides with Mickle et al.'s (2014) morphometric study to suggest an east-west divide in Gulf Sturgeon, with the Pearl and Pascagoula River drainages representing the western region and the Escambia, Yellow, Blackwater, Choctawhatchee, Apalachicola, and Suwannee Rivers encompassing the east (Vick et al., 2018). Gulf Sturgeon generally exhibit breeding site philopatry, returning to spawn in the same rivers

from which they were spawned, though field surveys and molecular techniques have identified exceptions. Dugo et al. (2004) genotyped sturgeon samples from throughout the range, finding Choctawhatchee native sturgeon in the Yellow, Pascagoula, and Apalachicola systems, and Pearl natives admixed into the Pascagoula.

Traditionally, Atlantic Sturgeon and Gulf Sturgeon were assumed to only spawn in the spring season. Historical records detail the large numbers of sturgeon that could be visibly identified during their spawning migration upriver, and prior to their exploitation, were generally thought of as a nuisance for fishermen. Observations of possible fall runs had been made in Atlantic Sturgeon in the past, and dual-spawning is a common trait of European and Asian sturgeon (Berg, 1959), but little evidence was found to support the theory until recently. Randal and Sulak (2012) note the capture of ripe or recently spawned individuals, telemetry evidence of migration to the spawning sites, and age-length analysis of an age-0 fish as evidence for a fall spawn in the Suwanee River. Multiple studies in Atlantic Sturgeon have come to the same conclusion via adult and egg sampling, telemetry, and molecular data which suggests two distinct genetic groups in the James River, VA (Balazik et al., 2012, Hager et al., 2014, Balazik and Musick, 2015, Smith et al., 2015, Balazik et al., 2017). While an overarching protection plan for the subspecies is beneficial, due to the drainage fidelity and evidence of an east-west divide, additional division first into regions, then to individual MU's by drainage allows for more specialized management and protection.

This project will focus on the Choctawhatchee River system, whose head waters can be found in south-east Alabama and flows south, discharging in the Choctawhatchee Bay. Of the rivers which currently maintain a spawning population of Gulf Sturgeon, the

Choctawhatchee is distinctive as it is one of two which do not have a dam or impoundment on their mainstem, though one did exist on the Pea River tributary. Such structures have been found to negatively impact the life cycle and reproductive capabilities of many anadromous fishes by limiting their access to spawning sites and altering the flow and characteristics of water below the structures (Boreman, 1997, Flowers et al., 2009). Studies of the population abundance of the seven spawning populations from 1999 to 2008 estimate the Choctawhatchee to be the second largest population (2000-3000, >610mm TL), behind the Suwanee River (8877-9743, >1000mm TL; Sulak et al., 2016). In the Choctawhatchee River, ripe adults have been tracked to the upper river, where fertilized eggs have been located and sampled. The substrate consists of a notably greater amount of limestone beyond rkm 100 compared to the sandier lower river (Fox et al., 2000). Hard bottom or larger substrate particle sizes are thought to be preferred due to the adhesiveness of the eggs, where softer substrate may collect on the surface of the egg, limiting its access to dissolved oxygen, as well as the ability to use the interstitial spaces as protection. Upper reaches of the river would also be characterized by higher water quality metrics, such as lower temperature, higher DO, lower contaminants, and lower salinity, which would impact the development of growth of eggs, larvae and juvenile Gulf Sturgeon (Fox et al., 2000).

1.3 Research Goals

This project is a part of the ongoing work by the Alabama Division of Wildlife and Freshwater Fisheries (ALDWFF) to document dual spawning groups of Gulf Sturgeon at various sites within the Alabama reaches of the Choctawhatchee River system. Recent observations and research with Gulf Sturgeon have suggested that the

species practices a dual spawn, which would be consistent with the life-history displayed by their sister species, Atlantic Sturgeon, as well as other sturgeon species throughout the world (Randall and Sulak, 2012, Balzik and Musick, 2015). Historically, Gulf Sturgeon have been managed with a single spring spawning event in mind, but if a dual-spawn strategy can be confirmed, alterations to management plans could be proposed and implications of instream projects should be consider with respect to a second spawning group. Currently, seven MU's, delineated by natal river, are defined, but if a dual-spawn is confirmed with an accompanying degree of delineation based by spawning season, the number of MU's to be considered could be increased. This project first intends to investigate the presence of a second spawning event in the late summer and early fall period by confirming the species of eggs collected by ALDWFF using sequencing of mitochondrial DNA (mtDNA). The second goal is to genotype adults, collected either on the spawning grounds or detected via telemetry within the Choctawhatchee, using microsatellite data from fin clips. It is expected that the microsatellites will show distinct spawning groups based on the season in which they spawn, and the egg sequences will match known Gulf Sturgeon haplotypes.

CHAPTER II – SPRING AND FALL SPAWNING

2.1 Introduction

Gulf Sturgeon, *Acipenser oxyrinchus desotoi*, is a threatened species under the Endangered Species Act (U.S. Fish and Wildlife Service, 1991) and listed internationally under CITES Appendix II (IUCN Red List., 2022). Gulf Sturgeon is an anadromous species which inhabits the US Gulf Coast, with natal rivers ranging from the Suwannee River to the Pearl River. This species, like many other sturgeons, have suffered population declines and limited recovery potential due to overharvest, life history characteristics, and habitat and water quality alterations (World Wildlife Fund, 2020, Flowers et al., 2009, Boreman, 1997). While the Gulf Sturgeon have been protected and managed as a threatened species since 1991, their recovery has been long and slow, due in part to their life history; females can take at least 12 years to reach sexual maturity and may only spawn every two to 3 years (Sulak et al., 2016). Thus, only a few generations have been produced in the 32 years of protection.

The Choctawhatchee River originates as east and west forks and join together in southeast Alabama before flowing through the Florida panhandle and emptying into the Choctawhatchee Bay and the Gulf of Mexico near Destin, FL. The river drains 3,484 square miles, and is fed by various tributaries, most notably the Pea River. While the mainstem of the Choctawhatchee River is dam-free, one did exist on the Pea River at rkm 191 until being breached by flooding in December of 2015. This level of connectivity in a natal Gulf Sturgeon river only be surpassed by the Pascagoula River which remains unimpounded. Sulak et al. (2016) reported the Choctawhatchee hosts what is estimated, from sampling between 1999 and 2007, to be the second largest population of Gulf

Sturgeon behind the Suwannee River, with abundance estimated to range from 2000 to 3000 individuals more than 610 mm in total length.

Due to the documentation of dual-spawning strategies in Eurasian sturgeon species, researchers investigated and found evidence of the same in Atlantic Sturgeon, *Acipenser oxyrinchus oxyrinchus*, the sister-species of Gulf Sturgeon (Balazik et al., 2012, Hager et al., 2014, Balazik and Musick, 2015, Smith et al., 2015, Balazik et al., 2017). This has "spawned" the same question in Gulf Sturgeon. Do Gulf Sturgeon spawn in both the spring and fall, or only the spring? Some efforts and observations have already been made to suggest that Gulf Sturgeon do exhibit a second spawning event in the fall (Randall and Sulak, 2012). However, work by ADWFF has provided the first collections of Gulf Sturgeon eggs in the fall. Generally, previous studies of Gulf Sturgeon have verified spawning by microscopic examination of eggs (e.g., Fox et al. 2002; Heise et al. 2004). This study utilized genetic techniques (e.g., Kreiser et al. 2008) to identify collected eggs from fall and spring spawning events to verify and document their species, thus confirming whether a dual-spawning strategy is utilized by Gulf Sturgeon.

2.2 Methods

The ADWFF, under direction of Steve Rider, collected eggs in previously documented spawning areas in the Choctawhatchee River during the spring and fall of 2019 and 2020. Upon collection, eggs were preserved in 95% ethanol and sent to the Kreiser Laboratory at the University of Southern Mississippi. In the lab, before DNA extraction, eggs were photographed and their diameter measured with a Leica S8APO and Leica Application Suite V4.13. Diameter data was analyzed using a nonparametric

Kruskal-Wallis test in R (R Core Team, 2022). Eggs were removed from ethanol and soaked in distilled water for ten minutes before being blotted dry. DNA was then extracted using a DNeasy Tissue Kit (QIAGEN Inc., Valencia, CA). The polymerase chain reaction (PCR) was used to amplify a portion of the mitochondrial control region using the L15926 primer of Kocher et al. (1989) and another primer designed for Gulf Sturgeon (Kreiser et al. 2008; Gulf Sturgeon-CRH1: 5'-GTGCCATTCACTGTTTGTCC). Reaction conditions followed the methods of Kreiser et al. (2008) with a negative control employed for each set of reactions. Amplifications were gel checked on a 1% agarose gel to verify the success of the PCR. Successful reactions were sent to Eurofins Genomics (Louisville, KY) for Sanger sequencing using both the forward and reverse primers. Sequence data were edited and aligned with Sequencher v. 5.1 (GeneCodes Co., Madison, WI).

To confirm that these eggs were from Gulf Sturgeon, sequences were compared to Gulf Sturgeon control region sequences available on GenBank (http://www.ncbi.nlm.nih.gov/; accession numbers DQ088959-DQ088963 & KP997218). MEGA v. 7.0 (Kumar et al., 2016) was used to calculate the uncorrected *p* distances among these sequences and generate a neighbor-joining tree (Saitou and Nei, 1987) from this distance matrix. PopART (Leigh and Bryant, 2015) was used to generate a minimum spanning network of haplotypes.

2.3 Results

A total of 381 eggs were collected and sent to the Kreiser lab, 358 of which were measured for their diameter; some eggs had hatched into larval fish or had been crushed or desiccated and were unable to be measured (see **Table 2.1**). Fall 2020 eggs accounted

for 69% of eggs measured. Egg diameter was found to have significant difference in median among groups ($p<0.001$; see **Table 2.2, Figure 2.1**).

Table 2.1 *Gulf Sturgeon eggs*

Collection	# Eggs	# Eggs Measured	# Eggs Extracted	# Eggs Sequenced
Spring 2019	56	49	44	21
Fall 2019	32	22	27	16
Spring 2020	41	40	32	20
Fall 2020	252	251	59	17
Total	381	362	162	74

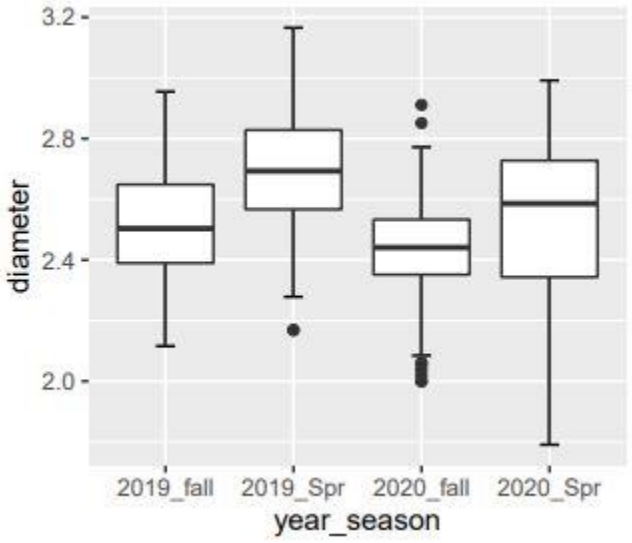
Number of Gulf Sturgeon eggs collected, measured, subjected to DNA extraction, and successfully sequenced for a portion of the mitochondrial control region

Table 2.2 *Summary statistics for Gulf Sturgeon egg diameter measurements*

Group	N	mean	SE	min	median	max
Spring 2019	48	2.67	0.03	2.17	2.69	3.17
Fall 2019	21	2.55	0.05	2.12	2.50	2.95
Spring 2020	38	2.53	0.05	1.79	2.59	2.99
Fall 2020	251	2.43	0.01	2.00	2.44	2.91

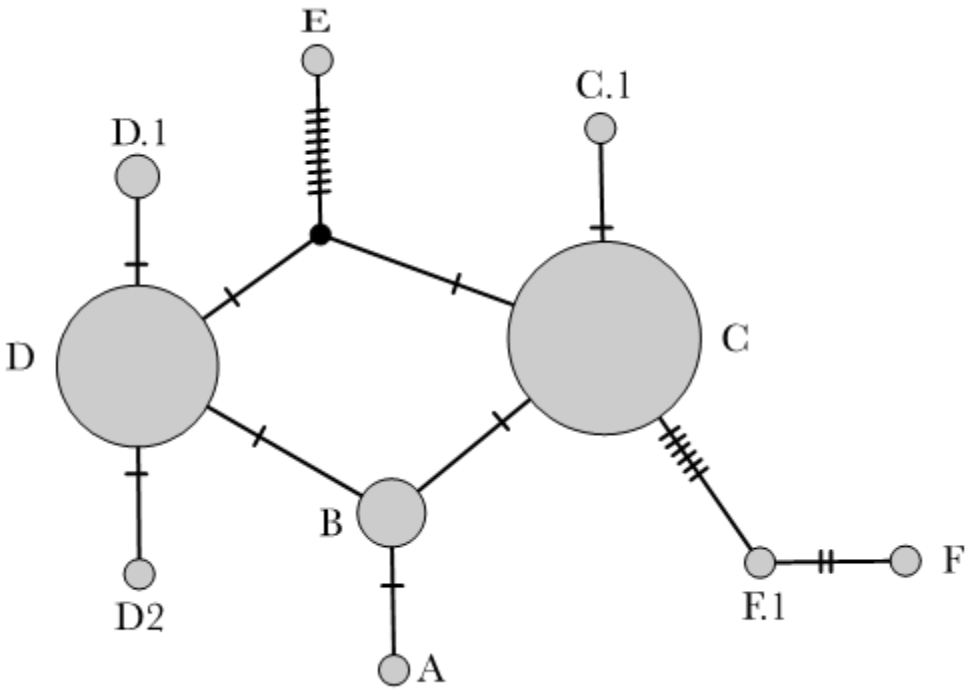
Sample size (N), mean, standard error (SE), minimum, median, and maximum

Figure 2.1 *Gulf Sturgeon egg size distribution*



Subsamples of each sample group had DNA extracted; some eggs visually appeared to be in a better state and thus were chosen for DNA extraction. DNA was extracted from 162 eggs, 105 of which were amplified, and 74 of which had enough amplified material to produce quality sequences. Each sampling group was fairly evenly represented in the sequenced eggs with 16-21 eggs sequenced per group. All 74 eggs sequenced were identified as Gulf Sturgeon from their mtDNA (**Appendix A**). Seven haplotypes were detected. Most eggs represented one of the three known Gulf Sturgeon sequences (haployptes B-D), while the remaining four haplotypes were new (C.1, D.1, D.2 and F.1) and differed by one or two base substitutions (*p* distance of 0.002-0.003) from known haplotypes (**Figure 2.2** and **Table 2.3**).

Figure 2.2 *Median joining network of the control region sequences from Gulf Sturgeon*



The size of the circle representing each haplotype reflects its frequency in this study. The hash marks indicate the number of base substitutions between haplotypes

Table 2.3 *Distances between Gulf Sturgeon mitochondrial control regions haplotypes*

	A	B	C	D	E	F	D.1	C.1	D.2	F.1
A	-	0.002	0.003	0.003	0.020	0.017	0.005	0.005	0.005	0.013
B	1	-	0.002	0.002	0.018	0.015	0.003	0.003	0.003	0.012
C	2	1	-	0.003	0.017	0.013	0.005	0.002	0.005	0.010
D	2	1	2	-	0.017	0.017	0.002	0.005	0.002	0.013
E	12	11	10	10	-	0.020	0.018	0.018	0.018	0.017
F	10	9	8	10	12	-	0.018	0.015	0.018	0.003
D.1	3	2	3	1	11	11	-	0.007	0.003	0.015
C.1	3	2	1	3	11	9	4	-	0.003	0.012
D.2	3	2	3	1	11	11	2	2	-	0.015
F.1	8	7	6	8	10	2	9	7	9	-

Uncorrected *p* distances are listed above the diagonal, number of base substitutions are listed below the diagonal

2.4 Discussion

The purpose of this study was to verify the identity of eggs collected in Gulf Sturgeon spawning grounds of the Choctawhatchee River both during and outside of the traditional spring spawning season. All 74 eggs sequenced were confirmed as Gulf Sturgeon eggs, and thus the historical assumption of a single spring spawn is unfounded. This study shows that Gulf Sturgeon do spawn outside of the spring season in the fall. Randall and Sulak (2012) previously published indirect evidence of dual-spawning such as observing ripe or just-spawned individuals and telemetry data documenting movement upriver to the spawning grounds outside of the normal spawning window. However, this study provides the first documentation of spawned eggs and verification of species via mitochondrial DNA.

Evidence exists which suggests that egg haplotypes can be correlated with region or natal drainage. In verifying a new spawning site in the Yellow River, Kreiser et al. (2008) sequenced 14 adult Gulf Sturgeon collected throughout the range and assigned arbitrary identification to the haplotypes, A-E. Four of the five individuals with C and D

haplotypes came from either the Choctawhatchee or Apalachicola Rivers. The Pearl and Pascagoula Rivers were represented by four E's and one B and C and the Yellow and Escambia Rivers were represented by two A's and three B's. The results of this study support this pattern of the geographic distribution of haplotypes. Most eggs in this study possessed haplotypes most common in the Choctawhatchee and Apalachicola (C = 39, D = 26), or were very similar to these haplotypes (C.1 = 1; D.1 = 2, D.2 = 1). However, some haplotypes are more widely distributed. Four B haplotypes, which were documented in the Yellow, Escambia, and Pearl previously, were identified, and one haplotype (F), which has been defined since Kreiser et al. (2008) and is associated with the Apalachicola, was closely represented by one egg with two base pair substitutions (F.1; Kreiser, Unpublished). This work supported the findings of Stabile et al. (1996), which identified five common haplotypes and three rarer ones that defined population structure across the Gulf Sturgeon range with some haplotypes correlating with specific drainages or the west, central, and east regions which were defined in the study.

Randall and Sulak (2012) hypothesized multiple spawning seasons may be a strategy to circumvent some of the difficulties associated with anadromy, such as insufficient or unfavorable water conditions at spawning sites. This adaptation, which likely came about due to natural environmental variability, may have played an important role in the survival of the species during and after their exploitation. Much of our knowledge of Gulf Sturgeon ecology and behavior is dependent upon the spring spawn paradigm. In order to better understand and manage the species, we need to be able to identify individuals as belonging to a particular spawning group. Unfortunately, mtDNA haplotypes alone do not possess sufficient resolution to classify individuals as belonging

to either the spring or fall spawning groups. However, other molecular markers (microsatellite loci) have proven useful in characterizing population genetic structure in Gulf Sturgeon (e.g., Dugo et al. 2004), and Kreiser et al. (unpublished data) has found evidence of some river systems possessing two distinct genetic groups, presumably indicative of a fall and spring spawning population. Further investigation of the utility of these markers to detect and differentiate between the two spawning groups is warranted and is the focus on my next chapter.

CHAPTER III – DISTINCT SEASONAL SPAWNING GROUPS

3.1 Introduction

Sturgeon, in general, are mysterious creatures. The benthic nature and seasonal comings and goings of anadromous sturgeons allows for many questions to be asked and answers sought. Anadromous fish migrations are nothing new; many cultures throughout history have in some way documented, celebrated, or otherwise acknowledged the movements of salmonids and sturgeon into their freshwater spawning grounds, but it wasn't until the 20th century that the idea of a dual spawn was postulated or documented in sturgeon. Berg (1959) evaluated records and documents from the mid to late 19th century for suspected dual spawning Eurasian sturgeon. Reports from late 19th and early 20th century shad and striped bass fisheries, respectively, provided the earliest evidence of dual spawning in Atlantic Sturgeon (Worth, 1904; Yarrow, 1874). Since then, sturgeon species after sturgeon species have drawn the same question from researchers: is a dual spawn exhibited? With the advent of molecular techniques, new methods can be utilized to not only confirm the species identification, but also to describe the population of origin.

Mitochondrial DNA analysis of Gulf Sturgeon eggs from the Choctawhatchee (Chapter 2) confirmed that Gulf Sturgeon do spawn in the fall outside of the traditional spring spawning period, as first proposed for the Suwannee River by Randall and Sulak (2012). As a listed species, this is potentially very impactful for the management and recovery potential of the species, as efforts to quantify reproduction to this point can be assumed to be an underestimate as they did not encapsulate all spawning individuals that year. With this knowledge comes new questions, including whether genetic

differentiation exists between individuals that spawn in the spring versus fall, that is to say are there two genetically distinct spawning populations in the Choctawhatchee River? There is already growing evidence that the closely related Atlantic Sturgeon exhibit a dual spawn in several river systems across there range (Balazik et al., 2012, Hager et al., 2014, Balazik and Musick, 2015, Smith et al., 2015), and that these spawning groups are genetically distinct (Balazik et al., 2017, Farrae et al., 2017).

Each listed species is evaluated, and management plans are formulated in order to recover the population. A common practice in these plans is to define management units, which are usually distinct groups or populations across the species range. With many anadromous fish, including Gulf Sturgeon, the defined MU's are the natal drainages as they are genetically distinct and limited admixture occurs between rivers. With the evidence of a fall spawn by Gulf Sturgeon in the Choctawhatchee River, it can be hypothesized that the dual spawning events are characterized by genetically distinct groups. If this hypothesis is supported by microsatellite data of adult Choctawhatchee Gulf Sturgeon, the establishment of additional MU's based on season of spawning may be justified.

3.2 Methods

The ADWFF, under direction of Steve Rider, collected fin clips from adult sturgeon caught in spawning waters during summer and fall seasons of 2019, 2020, and 2021. Upon collection, fin clips were preserved in 95% ethanol and sent to the Kreiser laboratory at the University of Southern Mississippi. Additional Gulf Sturgeon samples from the Choctawhatchee from lower in the drainage were obtained between 2018, 2019

and 2021 by Dr. Dewayne Fox (Delaware State University). DNA was then extracted using a DNeasy Tissue Kit (QIAGEN Inc., Valencia, CA).

Fourteen microsatellite loci were amplified (Atlantic Sturgeon- *Aox*B34, *Aox*D32, *Aox*D44, *Aox*D54, *Aox*D64, *Aox*D96, *Aox*D165, *Aox*D170, *Aox*D188, *Aox*D234, *Aox*D241, *Aox*D242, and *Aox*D297; Henderson-Arzapalo and King, 2002, & Lake sturgeon – LS68; May et al., 1997) using the conditions described in Dugo et al. (2004). PCR conditions utilized were: 12.5 µL reactions consisting of 1x *Taq* reaction buffer (New England Biolabs), 2.5-3mM $MgCl_2$, 200 mM dNTPs, 0.25 units of *Taq* polymerase, 0.16 mM of the M13 tailed forward primer (Schuelke, 2000), 0.16 mM of the reverse primer, 0.08 mM of the M13 labeled primer (LI-COR Inc.), 20-100 ng of template DNA, and water to the final volume. PCR cycling conditions consisted of an initial denaturing step of 94°C for 2 min followed by 35 cycles of 30 sec at 94°C, 1 min at 53-56°C and 1 min at 72°C. A final elongation step of 10 min at 72°C ended the cycle. See Dugo et al. (2004) for locus-specific annealing temperatures. Microsatellite alleles were visualized using a LI-COR 4300 DNA sequencer and scored with the Gene Image IR v. 3.55 software (LI-COR).

The sex of each individual was determined with molecular methods (sexotyping: *sensu*, S. Rider). I followed the methods for Gulf Sturgeon outlined in Sard et al. (in review) to amplify and visualize the *AllWSex2* locus, which are outlined hereafter. I multiplexed the *AllWSex2* locus with a microsatellite locus (AoxD165; Henderson-Arzapalo and King, 2002) to provide an internal positive control. These loci were amplified in 12.5 µL reactions. The PCR cycling conditions followed Dugo et al. (2004) with an annealing temperature of 56°C. These amplifications were then visualized on

acrylamide gels using a LI-COR 4300 DNA sequencer (LI-COR Inc., Lincoln, NE) and scored with the Gene Image IR v. 3.55 software (LI-COR).

I used STRUCTURE v. 2.3.4 (Pritchard et al., 2000) to determine the number of genetically distinct groups in the Choctawhatchee. This program uses a Bayesian approach to assign individuals to groups that minimizes linkage disequilibrium and deviation from Hardy-Weinberg equilibrium. I tested values of *K* (number of clusters) from 1-4 assuming correlated allele frequencies between groups. For each value of *K*, I ran 20 replicates with a burn-in of 250,000 generations followed by a subsequent 200,000 generations. I determined the best value of *K* by comparing the average likelihood scores and by examining the ΔK values (Evanno et al., 2005) as calculated by StructureSelector (Li and Lu, 2018).

Additional genetic diversity metrics were calculated to compare and quantify differentiation between any possible groupings. Calculated metrics consisted of the number of alleles (N_A), observed and expected heterozygosity (H_o and H_e, as calculated by GenAlEx 6.51; Peakall and Smouse, 2012), and allelic richness (A_R, as calculated by the package 'hierfstat'; Goudet, 2005). A non-parametric Kruskal-Wallis test was used to compare the measures of genetic diversity among sites in R (R Core Team, 2023), as Shapiro-Wilk tests of normality were all significant. The extent of differentiation among groups was measured using F_{ST} (Weir and Cockerham, 1984), calculated via the package 'hierfstat'.

3.3 Results

Adult Gulf Sturgeon fin clips collected during this study totaled 204: 63 collected by Steve Rider of the ADWFF in the summer and fall months of 2019-2021, 141

collected by Dewayne Fox of Delaware State University in 2018, 2019, and 2021. ADWFF collected fish specifically from spawning grounds, and these fish were presumed to represent spawning individuals. Seven fish were collected on 6/30/2020 and 7/20/2020 by ADWFF and were assumed to be spring spawners while the remaining 56 ADWFF fish were collected between August and October and were assumed to be fall spawners. Of the fall spawn fish, 17 individuals were found to be expressing gametes upon capture. Genotyping was attempted with all individuals. Some PCR amplifications failed and were unable to be scored, thus only individuals with five or fewer missing loci were used in the STRUCTURE analysis. The STRUCTURE analysis was performed using 126 individuals; ADWFF accounted for 60 samples (95% of ADWFF fish), DSU accounted for 66 (47% of DSU fish). The majority of samples were missing no more than one locus, and 87.3% of samples lacked no more than three loci (**Table 3.1**). A total of 102 fish (60 ADWFF, 42 DSU 2018) were genetically analyzed to determine sex. Of these, 85 of the scores of sex were definitive and 17 of the scores were ambiguous (51 males, 34 females, 2 suspected males, and 15 suspected females).

Table 3.1 *Frequency and missing loci data of Gulf Sturgeon analyzed via STRUCTURE*

Missing loci	#	%
0	49	38.9
1	30	23.8
2	16	12.7
3	15	11.9
4	12	9.5
5	4	3.2
Total	126	100

Figure 3.1 *LnP(K)*

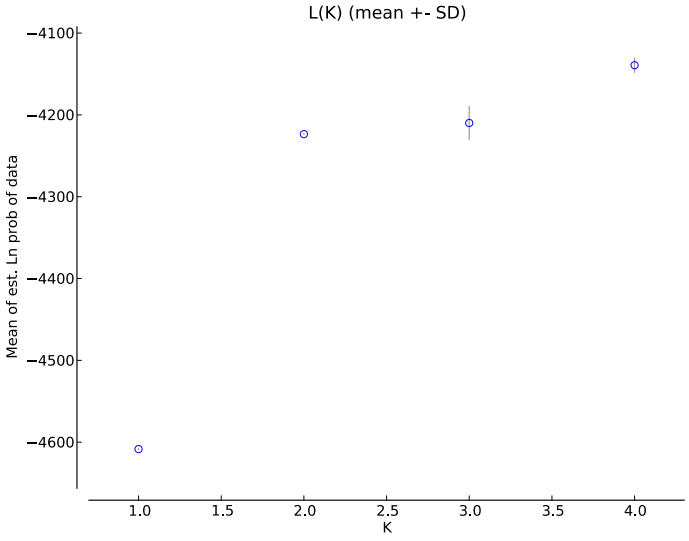
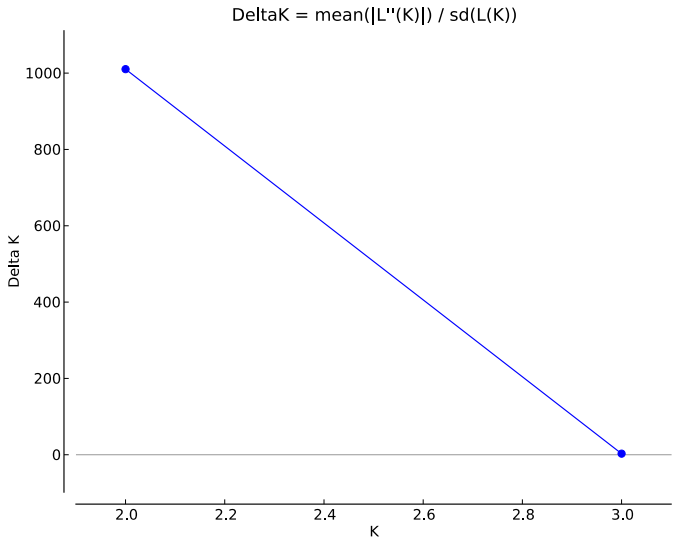
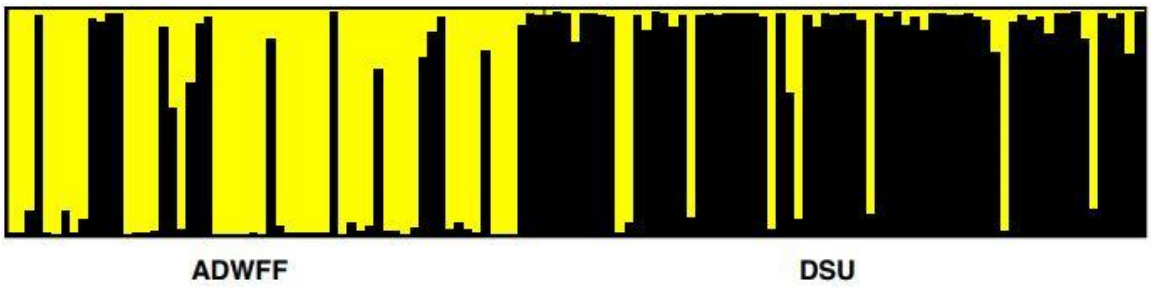


Figure 3.2 *ΔK*



Both the Δ*K* analysis of the STRUCTURE results and the log likelihood scores suggested that there were two genetic groups (**Figure 3.1, Figure 3.2**). I used fish collected in fall on the spawning grounds as a reference in designating the genetic groups as representing either the fall or spring spawning runs. An F_{ST} value of 0.12 between the two indicated that there is a substantial amount of genetic differentiation present. Individuals were designated as either fall or spring spawn if their admixture score (q) was > 0.7, although most (88%) of the individuals had q scores > 0.9. Of the 102 fish analyzed, 47 were assigned as spring fish, 76 fall fish, and 3 were admixed fish with q scores between 0.3 and 0.7 (**Figure 3.3**). The average q score for the fall groups was 0.969 (ranging from 0.994 to 0.881), the q score for the spring groups was 0.958 (ranging from 0.995 to 0.743), and for admixed individuals their average spring group q score was 0.628 (ranging from 0.678 to 0.568; **Table 3.2**).

Figure 3.3 *Gulf Sturgeon Fall/Spring membership coefficients*



Bar plots of membership coefficients of Gulf Sturgeon from STRUCTURE analyses of fin clip microsatellite data, Yellow: Fall, Black: Spring

Two of the 7 ADWFF fish caught in the spring season were genetically identified as spring fish and 5 were fall fish. The remaining 16 fish making up the spring spawn group were sampled in the fall. A total of 35 fish caught in the fall were identified as fall spawn group (**Table 3.3**). During ADWFF sampling in late summer and fall, 17 fish were running ripe and their sexes were documented. Of those 17 fish, 15 were genetically determined to be fall fish (3 males, 12 females). The remaining 2 consisted of a spring female (q = 0.907, 8/12/2021) and an admixed spring male (q = 0.678, 10/10/2019).

Table 3.2 *Summary statistics for admixture (q) scores*

	ADWFF	DSU	All
Fall			
N	40	7	47
Avg q	0.975	0.933	0.969
SE	0.004	0.014	0.005
Range	0.994-0.886	0.989-0.881	0.994-0.881
Spring			
N	18	58	76
Avg q	0.930	0.966	0.958
SE	0.018	0.005	0.006
Range	0.994-0.743	0.995-0.810	0.995-0.743
Admixed			
N	2	1	3
Avg q	0.623	0.638	0.628
SE	0.055	NA	0.032
Range	0.678-0.568	NA	0.678-0.568

Summary statistics were calculated for each season group and each collection authority and feature abundance (N), average q score, standard error (SE) and range

Table 3.3 *Distribution of fish in spawn groupings according to season of sampling*

Spring Caught	ADWFF	DSU
Spring Spawn Group	2	0
Fall Spawn Group	5	0
Admix Group	0	0
Total	7	0

Fall Caught	ADWFF	DSU
Spring Spawn Group	16	58
Fall Spawn Group	35	7
Admix Group	2	1
Total	53	66

All DSU fish were collected in October and in non-spawning reaches of the river. A total of 58 fish were identified as spring spawners, 7 as fall spawners, and 1 admixed individual. A total of 14 fish which were included in STRUCTURE analysis had their sex determined, resulting in 7 males and 7 females. Only 1 female was grouped as a fall spawner.

Genetic diversity metrics between the fall and spring groups did not significantly differ. Kruskal-Wallis test results for observed heterozygosity, expected heterozygosity, and allelic richness were as follows: H_o – H(1) = 0.0005, P = 0.98; H_e – H(1) = 0.26, P = 0.61; A_R – H(1) = 0.008, P = 0.93 (**Table 3.4)**.

Table 3.4 *Kruskal-Wallis test summary statistics*

Ho	N	Mean	SD	Min	Median	Max
Fall	14	0.533	0.239	0.122	0.647	0.889
Spring	14	0.526	0.232	0.063	0.566	0.797

He	N	Mean	SD	Min	Median	Max
Fall	14	0.585	0.270	0.116	0.688	0.893
Spring	14	0.563	0.246	0.061	0.683	0.830

Ar	N	Mean	SD	Min	Median	Max
Fall	14	6.802	3.839	2.894	5.652	14.583
Spring	14	6.751	3.649	2.293	5.664	14.208

Observed heterozygosity (Ho), Expected heterozygosity (He), Allelic richness (Ar), Number of loci (N), Standard deviation (SD)

3.4 Discussion

The purpose of this study was to investigate the suspected presence of genetically distinct Gulf Sturgeon spawning groups in the Choctawhatchee River, with respect to season of spawning. It was hypothesized that at least two distinct spawning groups would be identified representing spring fish and fall fish. Adult sturgeon sampling by the ADWFF and DSU only took place in the summer and fall seasons. ADWFF sampling

intended to capture individuals in upper river reaches, where it could be cautiously assumed any fish sampled were actively spawning or migrating to spawn. However, some of the fish sampled could represent wandering individuals or immature fish learning the spawning run (S. Rider, pers comm). This data set was used to define the fall and spring groups, where the more populous STRUCTURE grouping of this data set would be perceived to be the fall group, and the smaller group would be assumed to represent the spring group.

While most individuals clearly assigned to one of the two genetic groups, there was not always congruence between the genetic group and the season in which the fish was collected. While most of the ADWFF fish were collected on the spawning grounds in the fall, 30% (18 of 60) of ADWFF were genetically grouped as spring fish. Only 11% of ADWFF individuals caught in the summer and assumed to be spring spawners were genetically identified as such, whereas 89% assigned as fall individuals. Each genetic group consisted of individuals which were caught as early as June 30th and as late as October 10th. The spring genetic group also had one fish sampled October 17, 2019. The DSU samples were collected in the lower reaches of the Choctawhatchee River outside of the spawning grounds during the fall. Correspondingly, most of the DSU samples were identified as spring fish. Gulf Sturgeon have been documented as straying between rivers (Dugo et al., 2004), but my results indicate that straying can also occur between seasons. While fish may travel upriver in the "wrong" season, what also is important is whether or not they are moving to reproduce.

The presence of three admixed fish indicates that spawning does occur across spawning groups. Missing loci did not appear to have a significant effect on the

classification of these admixed individuals. Farrae et al. (2017) also found evidence of admixed fish between the two spawning seasons in Atlantic Sturgeon from the Edisto River. The documentation of ripe fish at time of sampling further supports that fishes do not always adhere to their genetic spawning season, as a ripe spring female and a male which trended towards spring ancestry (q = 0.678) were sampled in the spawning reaches during the fall. While fish might stray between seasons it must not be common or we would not detect the presence of strongly differentiated genetic groups (based on F_{ST}).

USFWS abundance estimates concluded the Choctawhatchee hosts the 2nd largest population of Gulf Sturgeon, and we now know this is the product of two different spawning groups. About 40% of the fish in my study assigned to the fall spawn group, although admittedly some of the adult sampling was conducted to target fall spawning fish. However, this ratio is consistent with what Kreiser et al. (unpublished data) reported for two genetic groups in the Choctawhatchee from a larger sample of adults. I found that genetic diversity metrics were similar between the two groups. While this is not a strict measure of abundance, it does suggest that the fall group has not experienced a dramatic population bottleneck that would have reduced the amount of genetic diversity present in that group (Hartl and Clark, 2007).

Sex ratios were near equal when including individuals with lower confidence assignments. While this data did not impact this study, it will prove useful when paired with telemetry data on the seasonal movements of individuals throughout the river. Presumably the males move upriver to the spawning grounds first (Fox et al., 2000, Dovel and Berggren, 1983), but with the molecular identification of sex we will be able to validate these patterns.

As this is the first study to confirm a fall spawn with eggs, it serves as a first step for significantly more research which can delve into the complexities and unique attributes of distinct spawning groups. Denison et al. (2023) described differential cues of spawning runs between Spring and Fall Atlantic Sturgeon. This evidence, along with anectdotal evidence of water conditions during fall sampling, highlight the need for additional research to learn more about this newly describe paradigm (S. Rider, pers. comm). Future studies can further utilize sex determination, telemetry techniques, population dynamics and genetics in order to evaluate the significance of a dual spawn and the specifics of the fall spawn. This study presents evidence that adjustments to management practices or instream projects may be justified. Dams, flow regime, and other anthropological impacts of Gulf Sturgeon spawning are managed only for the spring spawn. In contrast to five of the other natal rivers, the Choctawhatchee River does not possess any dams or hydroelectric plants, operations which tend to alter natural flow regimes, especially concerning hydroelectric plants and the technique of hydropeaking. With additional research in other natal drainages, additional fall spawns may be identified, and the management of summer and fall dam and hydroelectric plant water control may need to be revised. Another issue of concern is that across the Gulf states water usage is not decreasing. The Choctawhatchee, Pea and Yellow Rivers Comprehensive Watershed Management Plan (Hinson et al., 2015) notes that surface and groundwater usage increased 86% and 67% respectively between 1970 and 2005, and predicts state usage will continue to increase, where estimates are for a 36-40% increase between 2010 and 2050. As individuals were being collected from the spawning grounds through summer into the fall, increased water demands could certainly impact the

availability of necessary flow parameters for spawning. Most of this water is used for irrigation and other agricultural practices. Mitigation strategies may include public water conservation campaigns to educate the public or regulations on water usage. Dredging can have negative impacts on Gulf Sturgeon, either killing them directly, or limiting migration and reproductive potential by altering habitat and siltation. Denison et al. (2023) found that migration cues and extents varied between spring and fall spawns of Atlantic Sturgeon. As we don't currently know the specifics of when or where the fall spawn takes place, it is too early to take definitive action. However, regulating when dredges can operate to accommodate both spawns, or utilizing relocation trawling may be the best strategy (Kaeser and Hueblein, 2022). Contamination and pollution can also be a significant factor that can be considered within management plans. Education, monitoring, and regulation of point and nonpoint source pollution and nutrient loading can help to maintain a sufficient water quality to allow for the best possible spawning conditions.

The end goal of all threatened and endangered species is to be delisted. With the findings of two spawning groups and limited admixture between the two, the Choctawhatchee Gulf Sturgeon population may be more resilient than previously thought. However, the definition of management units may need to be altered, and management practices may need to be adjusted to accommodate this newly characterized fall spawn population in order to maximize the species' recovery potential.

APPENDIX A – ASSOCIATED DATA

Table A.1 *Sequenced egg haplotypes with ID, season spawned, and year*

Egg ID	Season	Year	Haplotype
AL_GS_19_01_01	spring	2019	C
AL_GS_19_02_01	spring	2019	C
AL_GS_19_02_02	spring	2019	C
AL_GS_19_03_01	spring	2019	C
AL_GS_19_03_02	spring	2019	D
AL_GS_19_03_03	spring	2019	C
AL_GS_19_03_04	spring	2019	C
AL_GS_19_03_05	spring	2019	C
AL_GS_19_04_01	spring	2019	C
AL_GS_19_05_01	spring	2019	C
AL_GS_19_07_01	spring	2019	D
AL_GS_19_07_02	spring	2019	D
AL_GS_19_08_01	spring	2019	C
AL_GS_19_08_02	spring	2019	C
AL_GS_19_08_03	spring	2019	D
AL_GS_19_08_05	spring	2019	C
AL_GS_19_08_07	spring	2019	C
AL_GS_19_08_08	spring	2019	C
AL_GS_19_08_17	spring	2019	C
AL_GS_19_08_18	spring	2019	C
AL_GS_19_08_25	spring	2019	C
AL_GS_19_09_01	fall	2019	D
AL_GS_19_10_01	fall	2019	D
AL_GS_19_10_02	fall	2019	D
AL_GS_19_10_06	fall	2019	C
AL_GS_19_10_07	fall	2019	C
AL_GS_19_11_01	fall	2019	D
AL_GS_19_12_01	fall	2019	D
AL_GS_19_12_10	fall	2019	C
AL_GS_19_12_11	fall	2019	C
AL_GS_19_12_12	fall	2019	C
AL_GS_19_12_13	fall	2019	D
AL_GS_19_12_14	fall	2019	D
AL_GS_19_13_01	fall	2019	F.1
AL_GS_19_14_01	fall	2019	C

AL_GS_19_16_02	fall	2019	D
AL_GS_20_01_01	spring	2020	C
AL_GS_20_01_02	spring	2020	C
AL_GS_20_01_03	spring	2020	C
AL_GS_20_01_06	spring	2020	C
AL_GS_20_01_07	spring	2020	C
AL_GS_20_01_10	spring	2020	C
AL_GS_20_01_13	spring	2020	C
AL_GS_20_01_15	spring	2020	C
AL_GS_20_01_17	spring	2020	C
AL_GS_20_02_02	spring	2020	C
AL_GS_20_02_03	spring	2020	C
AL_GS_20_02_04	spring	2020	C
AL_GS_20_02_05	spring	2020	C
AL_GS_20_02_06	spring	2020	C
AL_GS_20_02_07	spring	2020	C.1
AL_GS_20_03_03	spring	2020	C
AL_GS_20_04_01	spring	2020	B
AL_GS_20_04_02	spring	2020	B
AL_GS_20_04_03	spring	2020	B
AL_GS_20_04_04	spring	2020	B
AL_GS_20_05_01	spring	2020	C
AL_GS_20_06_01	fall	2020	D
AL_GS_20_06_02	fall	2020	D
AL_GS_20_06_03	fall	2020	D
AL_GS_20_06_04	fall	2020	D
AL_GS_20_06_05	fall	2020	D.2
AL_GS_20_06_08	fall	2020	D
AL_GS_20_06_09	fall	2020	D
AL_GS_20_06_11	fall	2020	D
AL_GS_20_06_34	fall	2020	D.1
AL_GS_20_07_01	fall	2020	D
AL_GS_20_07_03	fall	2020	D
AL_GS_20_07_04	fall	2020	D
AL_GS_20_07_05	fall	2020	D
AL_GS_20_07_07	fall	2020	D
AL_GS_20_07_08	fall	2020	D
AL_GS_20_07_101	fall	2020	D.1
AL_GS_20_07_125	fall	2020	D

Table A.2 *Gulf Sturgeon STRUCTURE analysis results*

Unique identification of individual fish (ID), source where ADWFF refers to fish sampled by Steve Rider and Alabama Division of Wildlife and Freshwater Fishers and DSU20xx refers to Dewayne Fox and Delaware State University sampled fish and year of collection, the number of loci which lacked scoring, q values for both fall and spring groups (Fall, Spring), the call of which genetic group the individual belongs to with a threshold of q > 0.7, genetic sex assignment, and whether the fish was ripe at sampling (Threshold > 0.7)

ID	Source	# Missing Loci	Fall	Spring	Threshold > 0.7	Sex	Ripe?
GS17043	ADWFF	5	0.984	0.016	Fall	F	No
GS17044	ADWFF	4	0.983	0.017	Fall	M	No
GS17045	ADWFF	0	0.886	0.114	Fall	M	No
GS17046	ADWFF	3	0.018	0.982	Spring	F	No
GS17047	ADWFF	0	0.983	0.017	Fall	F	No
GS17048	ADWFF	0	0.994	0.006	Fall	M	No
GS17049	ADWFF	0	0.889	0.111	Fall	M	No
GS17050	ADWFF	3	0.989	0.011	Fall	F?	No
GS17051	ADWFF	0	0.926	0.074	Fall	F	No
GS17052	ADWFF	0	0.033	0.967	Spring	F	No
GS17053	ADWFF	0	0.046	0.954	Spring	M	No
GS17054	ADWFF	0	0.009	0.991	Spring	M	No
GS17055	ADWFF	0	0.008	0.992	Spring	M	No
GS17056	ADWFF	1	0.991	0.009	Fall	M	No
GS17057	ADWFF	2	0.990	0.010	Fall	F	No
GS17058	ADWFF	3	0.988	0.012	Fall	M?	No
GS17059	ADWFF	0	0.979	0.021	Fall	M	Yes
GS17060	ADWFF	0	0.073	0.927	Spring	M	No
GS17061	ADWFF	0	0.432	0.568	Admixed	M	No
GS17062	ADWFF	0	0.974	0.027	Fall	M	No
GS17063	ADWFF	0	0.322	0.678	Admixed	M	Yes
GS17064	ADWFF	0	0.059	0.941	Spring	M?	No
GS17065	ADWFF	0	0.030	0.970	Spring	F	No
GS17066	ADWFF	0	0.992	0.008	Fall	F	Yes
GS17067	ADWFF	2	0.993	0.007	Fall	F	Yes
GS17068	ADWFF	2	0.991	0.009	Fall	F	Yes
GS17069	ADWFF	0	0.991	0.009	Fall	F	No

GS17070	ADWFF	2	0.989	0.011	Fall	F	No
GS17071	ADWFF	0	0.993	0.007	Fall	M	Yes
GS17072	ADWFF	1	0.126	0.874	Spring	F	No
GS17073	ADWFF	0	0.955	0.045	Fall	M	No
GS17074	ADWFF	4	0.983	0.017	Fall	M	No
GS17075	ADWFF	0	0.989	0.011	Fall	M	No
GS17076	ADWFF	1	0.990	0.010	Fall	M	No
GS17077	ADWFF	0	0.990	0.010	Fall	F?	No
GS17078	ADWFF	0	0.985	0.015	Fall	F	Yes
GS17079	ADWFF	0	0.007	0.994	Spring	F	No
GS17080	ADWFF	0	0.992	0.008	Fall	F	Yes
GS17081	ADWFF	0	0.940	0.060	Fall	F	Yes
GS17082	ADWFF	0	0.976	0.024	Fall	F	No
GSRS_2101	ADWFF	2	0.956	0.045	Fall	M	No
GSRS_2103	ADWFF	4	0.257	0.743	Spring	F?	No
GSRS_2104	ADWFF	2	0.978	0.022	Fall	F	Yes
GSRS_2105	ADWFF	3	0.979	0.021	Fall	F?	Yes
GSRS_2106	ADWFF	1	0.992	0.008	Fall	F	Yes
GSRS_2107	ADWFF	5	0.963	0.037	Fall	M	Yes
GSRS_2108	ADWFF	1	0.206	0.794	Spring	M	No
GSRS_2109	ADWFF	3	0.093	0.907	Spring	F	Yes
GSRS_2110	ADWFF	2	0.027	0.973	Spring	F	No
GSRS_2111	ADWFF	0	0.973	0.027	Fall	F	Yes
GSRS_2113	ADWFF	1	0.943	0.057	Fall	M	No
GSRS_2115	ADWFF	0	0.968	0.032	Fall	M	No
GSRS_2116	ADWFF	0	0.987	0.013	Fall	M	No
GSRS_2117	ADWFF	1	0.178	0.822	Spring	M	No
GSRS_2118	ADWFF	2	0.992	0.008	Fall	F	Yes
GSRS_2119	ADWFF	4	0.993	0.007	Fall	F?	Yes
GSRS_2120	ADWFF	3	0.991	0.009	Fall	F?	No
GSRS_2121	ADWFF	0	0.062	0.938	Spring	M	No
GSRS_2122	ADWFF	3	0.009	0.991	Spring	F?	No
GSRS_2123	ADWFF	0	0.020	0.980	Spring	F	No
GS-18-025	DSU2018	4	0.016	0.984	Spring	M	
GS-18-026	DSU2018	3	0.006	0.994	Spring	M	
GS-18-030	DSU2018	3	0.009	0.991	Spring	F	
GS-18-031	DSU2018	2	0.141	0.859	Spring		
GS-18-032	DSU2018	3	0.010	0.990	Spring	F	

GS-18-033	DSU2018	4	0.009	0.991	Spring	M
GS-18-034	DSU2018	3	0.017	0.984	Spring	F
GS-18-035	DSU2018	2	0.025	0.975	Spring	F
GS-18-036	DSU2018	2	0.989	0.011	Fall	
GS-18-037	DSU2018	4	0.944	0.056	Fall	
GS-18-038	DSU2018	3	0.020	0.980	Spring	M
GS-18-039	DSU2018	4	0.089	0.911	Spring	M
GS-18-045	DSU2018	5	0.007	0.993	Spring	F
GS-18-049	DSU2018	3	0.008	0.992	Spring	M
GS-18-051	DSU2018	2.5	0.075	0.925	Spring	M
GS-18-058	DSU2018	2	0.020	0.980	Spring	F
GS-18-060	DSU2018	4	0.919	0.081	Fall	F?
GS-19-001	DSU2019	1	0.019	0.981	Spring	
GS-19-003	DSU2019	0	0.012	0.988	Spring	
GS-19-004	DSU2019	0	0.017	0.983	Spring	
GS-19-005	DSU2019	0	0.012	0.988	Spring	
GS-19-006	DSU2019	0	0.011	0.989	Spring	
GS-19-008	DSU2019	0	0.010	0.990	Spring	
GS-19-007	DSU2019	0	0.015	0.986	Spring	
GS-19-009	DSU2019	0	0.030	0.971	Spring	
GS-19-010	DSU2019	1	0.972	0.028	Fall	
GS-19-011	DSU2019	0	0.011	0.989	Spring	
GS-19-012	DSU2019	0	0.362	0.638	Admixed	
GS-19-013	DSU2019	2	0.925	0.075	Fall	
GS-19-014	DSU2019	0	0.019	0.981	Spring	
GS-19-015	DSU2019	1	0.070	0.930	Spring	
GS-19-016	DSU2019	0	0.011	0.989	Spring	
GS-19-017	DSU2019	0	0.016	0.984	Spring	
GS-19-018	DSU2019	1	0.013	0.987	Spring	
GS-19-019	DSU2019	1	0.011	0.989	Spring	
GS-19-020	DSU2019	1	0.039	0.961	Spring	
GS-19-021	DSU2019	1	0.902	0.098	Fall	
GS-19-022	DSU2019	0	0.010	0.990	Spring	
GS-19-023	DSU2019	1	0.026	0.974	Spring	
GS-19-024	DSU2019	4	0.005	0.995	Spring	
GS-19-025	DSU2019	1	0.066	0.935	Spring	
GS-19-026	DSU2019	0	0.027	0.973	Spring	
GS-19-027	DSU2019	0	0.085	0.915	Spring	

GS-19-028	DSU2019	1	0.009	0.991	Spring
GS-19-029	DSU2019	0	0.012	0.988	Spring
GS-19-030	DSU2019	1	0.021	0.979	Spring
GS-19-031	DSU2019	1	0.015	0.985	Spring
GS-19-032	DSU2019	1	0.010	0.990	Spring
GS-19-033	DSU2019	0	0.027	0.973	Spring
GS-19-034	DSU2019	3	0.042	0.958	Spring
GS-19-035.1	DSU2019	1	0.183	0.817	Spring
GS-19-036	DSU2019	1	0.048	0.952	Spring
GS-19-038	DSU2019	1	0.019	0.981	Spring
GS-19-039	DSU2019	1	0.044	0.957	Spring
GS-19-040	DSU2019	2	0.029	0.971	Spring
GS-19-041	DSU2019	1	0.104	0.896	Spring
GS-19-042	DSU2019	1	0.012	0.988	Spring
GS-19-043	DSU2019	1	0.015	0.985	Spring
GS-19-044	DSU2019	2	0.007	0.993	Spring
GS-19-045	DSU2019	1	0.125	0.875	Spring
GS-19-046	DSU2019	1	0.881	0.119	Fall
GS-19-047	DSU2019	1	0.009	0.991	Spring
GS-19-048	DSU2019	4	0.034	0.966	Spring
GS-21-009	DSU2021	5	0.010	0.990	Spring
GS-21-023	DSU2021	2	0.190	0.810	Spring
GS-21-026	DSU2021	4	0.007	0.993	Spring

APPENDIX B –IACUC

THE UNIVERSITY OF
SOUTHERN MISSISSIPPI.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

118 College Drive #5116 | Hattiesburg, MS 39406-0001
Phone: 601.266.5997 | Fax: 601.266.4377 | iacuc@usm.edu | www.usm.edu/iacuc

NOTICE OF COMMITTEE ACTION

The proposal noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER:	**17101202.3**
PROJECT TITLE:	**Population Genetics and Systematics of Freshwater Fishes and Herps**
PROPOSED PROJECT DATES:	**5/2023 – 09/2025**
PROJECT TYPE:	**Renewal**
PRINCIPAL INVESTIGATOR(S):	**Brian Kreiser**
DEPARTMENT:	**Biological Sciences**
FUNDING AGENCY/SPONSOR:	**N/A**
IACUC COMMITTEE ACTION:	**Designated Review Approval**
PROTOCOL EXPIRATON DATE:	**September 30, 2025**

May 17, 2023

Jake Schaefer, PhD
IACUC Chair

Date

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