Using Molecular Techniques to Determine Effective Number of Breeders (N_b) for Razorback Sucker and Colorado Pikeminnow in the San Juan River

Fiscal Year 2022 Scope of Work

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Introduction & Justification

Recent advances in population-level genetic analyses are increasingly helping biologists monitor and adaptively manage the recovery of endangered populations (Hartl & Clark 2007). Recovery plans for many endangered fishes include the production and release of hatchery-reared individuals to augment populations with two major objectives: increase population size and promote genetic diversity (Miller & Kapuscinski, 2003). Survival, reproduction, and recruitment of wild and augmented individuals must occur at a sustainable scale in order to meet these recovery criteria. However, factors that limit success in achieving self-sustaining populations can be difficult to identify. This frequently requires long-term datasets to provide insight into population responses that might hinder recovery. Long-terms datasets include utilizing genetic monitoring to understand complex ecological, demographic, and genetic factors that can impede the establishment of self-sustaining populations (Schwartz et al. 2006; De Barba et al. 2010). Continued and consistent genetic monitoring in conjunction with habitat and demographic monitoring is vital to species conservation in the San Juan River. Examining relationships between environmental conditions and changes in annual genetic contributions can reveal patterns (like reduced contributions in low flow years) that can be used to mitigate bottlenecks in spawning and retention in the system. Continued genetic monitoring will also help determine the forces behind hybridization seen in Razorback Suckers so that effective strategies can be determined to minimize future hybridization (e.g., where in the system the hybrid larvae occur, or if both male and female Razorbacks are contributing to hybridization).

Augmentation of endangered fish populations in the San Juan River using captive-reared Razorback Sucker (*Xyrauchen texanus*) and Colorado Pikeminnow (*Ptychocheilus lucius*) began in the mid-1990s and continues as a recovery action (USFWS 2005; USFWS 2015). Annual monitoring of survival, reproduction, and recruitment of these populations has been supported through the San Juan River Basin Recovery Implementation Program (SJRRIP). Mark-recapture data on PIT tagged individuals have enabled estimation of survival for both stocked species (Franssen & Durst *unpublished data*; Clark et al. 2018), which in turn has prompted additional research investigating ways to increase survival. Larval fish surveys have documented successful reproduction of both species in the river (Farrington et al. 2015), but recruitment to the adult stage is extremely limited. Successful recovery requires a significant portion of the reestablished population to reproduce annually to both increase population sizes and ensure maintenance of genetic diversity. Therefore, quantifying the number of individuals that reproduce annually

provides data to make informed management decisions to aid in the reestablishment of self-sustaining populations.

Population-level spawning success (i.e., number of reproducing adults) is innately difficult to quantify from field studies, especially for highly fecund species where few individuals can produce a large number of offspring. Furthermore, there is substantial evidence that shows reproductive output can depend on environmental conditions and age- or size-related factors (Lauer et al. 2005; Lambert 2008). Both temporal and spatial variation in spawning effort has been detected for Razorback Sucker. Adults may vacate a spawning area early in the season and return to spawn again later that year (Marsh et al. 2015), with individuals visiting multiple sites during the same spawning period (Modde & Irving 1998). Such reproductive strategies further compound the difficulty in determining individual contribution to cohorts over a reproductive season; however, this question can be addressed by estimating the effective number of breeders (N_b) using genetic analyses. Colorado Pikeminnow and Razorback Sucker have defined seasonal reproductive bouts, thus making N_b an extremely useful metric for understanding population-level spawning success despite these species having life history traits (i.e., long-lived, highly fecund, and iteroparous with overlapping generations) that typically confound similar analyses (Waples et al. 2013; Waples et al. 2014). Single cohort N_b estimates can reliably quantify the number of individuals that contribute to a given cohort (Waples et al. 2014). Obtaining annual N_b estimates for the endangered fishes of the San Juan River provides insight into how management activities (i.e., increasing passage, managing flows) affect population-level reproduction, thus, improving the opportunity to manage the San Juan River to favor spawning success.

Estimating N_b for Razorback Sucker is especially challenging due to the potential for hybridization with Flannelmouth Sucker. Hybridization is a major factor influencing the status of all native catostomids in the Colorado River basin (Quist et al. 2009; Bangs et al. 2018). Genetic analyses have demonstrated that even fish identified as non-hybrids in a field setting may exhibit hybrid ancestry (Flannelmouth and White Sucker; Quist et al. 2009, Mandeville et al. 2015). Recent genetic results from wild young-of-year recruits in the San Juan River indicate that hybridization of Razorback and Flannelmouth sucker is not only occurring, but accounts for all young-of-year fish exhibiting Razorback traits (Mussmann & Saltzgiver *unpublished data*). Despite morphometries indicating that these young wild recruits were Razorback Sucker, genetic testing revealed that 100% had hybrid ancestry, with 55 of 62 fish (89%) collected in 2018-2019 being first-generation Razorback by Flannelmouth Sucker hybrids. This is especially concerning because very few hybrids have been identified at larval life stages (Diver et al. 2021), indicating a potential shift in cohort composition as fish transition from one life stage to the next. Therefore, we need to investigate hybridization in both larval and juvenile life stages to provide baseline data that will inform decision-making processes and allow for management of the San Juan to improve survival of non-hybrids from one life stage to the next.

Gathering insight to the origins of hybridization is crucial to Razorback Sucker recovery. Past genetic studies have indicated that Razorback and Flannelmouth Sucker are capable of reproducing (Douglas & Marsh, 1998; Dowling et al. 2012). However, reduced fitness can result when genes that have coevolved within a species to form beneficial relationships are mixed with homologous genes from a second species during a hybridization event (Coyne & Orr 2004). Unfortunately, little is understood about the number and genome-wide distribution of genetic incompatibilities separating species (Schumer et al. 2014). However, these incompatibilities have been shown to differ based on sex-specific parental combinations (Susuki & Nachman 2015). For example, the combination of Species A eggs and Species B sperm may produce viable offspring, but the reverse combination (i.e., Species B eggs and Species A sperm) may not (Gibeaux et al. 2018). Information specific to Razorback and Flannelmouth Sucker hybrids was deficit in this regard until Wolters et al. (2019) compared hatch success and larval

survivability of Razorback by Flannelmouth Sucker hybrids based on the sex of the parents. Their study concluded all parental combinations can produce viable offspring; however, first-generation hybrids with Razorback Sucker mothers exhibited markedly higher post-hatch survival rates (94.3% to 94.8% per year) relative to those with Flannelmouth Sucker mothers (survival = 40% to 64.5% per year). Therefore, we propose to examine parental contributions to hybrid fish collected from the San Juan using maternally-inherited mitochondrial DNA. This will clarify if hybridization is disproportionally represented by certain parental species-sex combinations.

Objectives:

1. Continue N_b monitoring for both Razorback Sucker (N = 120) and Colorado Pikeminnow (N = 120) collected in the mainstem San Juan River during 2022.
2. Genotype larvae identified as 'Catostomidae' collected from 2018 to 2021 (N=120) using ddRAD sequencing to evaluate hybrid status.
3. Genotype additional young-of-year Razorback Sucker collected from 2018 to 2021 (N=300) using ddRAD sequencing to evaluate hybrid status.
4. Sequence a portion of the mitochondrial genome for all larvae and young-of-year identified as hybrids from 2018 to 2021 (N = 500) to identify maternal linage.

Methods

Larval fish surveys are conducted annually along a 140-mile section of the San Juan River between Shiprock, NM, and Clay Hills, UT. Up to 120 larval samples per species representing the spatial and temporal distribution of these sampling efforts will be examined to calculate 2022 N_b estimates. Additional larval fish identified as 'Catostomidae' (N=120) and young-of-year Razorback Sucker (N=300) collected from this section of river from 2018 through 2021 will be also be sampled. Tissue subsamples will be taken from the posterior portion of all Razorback Sucker and Colorado Pikeminnow larvae. The anterior portion of all specimens will be saved for otolith studies. Rare collections will be targeted while sites with high larval densities will be proportionally reflected in samples in order to ensure N_b estimates are not artificially lowered due to limited spatial representation of samples. Larval Razorback Sucker are collected during much of the sampling season. Early larval stages (e.g., protolarvae to mesolarvae) will be targeted throughout larval collections under the assumption that these individuals are from recent spawning events; thus ensuring sampling is representative of the temporal spawning season. Conversely, Colorado Pikeminnow are collected later in the sampling season, making it relatively easy to ensure captured individuals reflect the entire seasonal spawning period of adults.

Previous N_b estimates have been obtained using multi-locus microsatellite markers (Diver & Wilson 2018); however, the field of population genetics has shifted to utilize massively parallel "next-generation sequencing" (NGS) methods. This technology provides a cost-effective and efficient means for sampling large quantities of genetic data from individuals through "reduced representation" genomic sequencing (Campbell et al. 2018). Past samples collected for microsatellite analysis can be used for NGS as long as they have been properly stored in 95% ethanol or frozen to avoid tissue degradation. These NGS methods sequence a small fraction of an organism's genome to identify thousands of single nucleotide polymorphisms (SNPs) that are analogous to microsatellite markers for conservation genetic applications. However, SNPs quantify an order of magnitude more loci compared to microsatellites (i.e., SNPs = hundreds or thousands of loci, microsatellites = 10 – 30 loci) which will functionally increase our resolution of genetic variation among individual genomes. Application of SNP-based methods for

determining relatedness and N_b estimates has increased as microsatellite-based studies have reduced in number (Flanagan & Jones 2019). SNPs have been successful in reconstructing multi-generational pedigrees in the absence of known parental relationships (Levine et al. 2019). Comparative studies have found that SNP-based methods provide improved confidence intervals and equal or greater power for determining relationships among individuals (Thrasher et al. 2018; Lemopoulos et al. 2019; Galla et al. 2020). SNP data also promote other analyses of interest in aquatic conservation, including, but not limited to, the assessment of hybridization in complex systems (Chafin et al. 2019). Finally, SNPs offer benefits over traditional microsatellite methods due to lower error rates and broader genome coverage (Smouse 2010; Hauser et al. 2011). Therefore, N_b estimates for 2022 will continue to be collected using these methods.

Genomic DNA will be extracted from tissues following standard protocols used at Southwestern ARRC. The mitochondrial gene Cytochrome c Oxidase Subunit I (COI) will first be amplified in all larval and juvenile Razorback Sucker collected from 2018 through 2021 to determine their maternal lineage. Amplification will occur in 20 µl polymerase chain reactions (PCR) that contain the following: 1 µl DNA, 8 µl Qiagen Multiplex Master Mix® (Qiagen, Valencia, CA, USA), 0.2 µl each of 10 µM forward and reverse primers, and 10.6 µl of nuclease-free water. Amplification for all samples will consist of an initial denaturing step at 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, 55°C for 90 s, and 72°C for 60 s, with a final extension of 30 min at 70°C. Amplified products will be Sanger sequenced on an ABI 3500XL Genetic Analyzer. Raw sequences will be examined and trimmed in Sequencher v5.1 (Gene Codes Corporation, Ann Arbor, MI USA). Geneious version 11.1.5 (Kearse et al. 2012) will be used to identify unique sequences that will be aligned to the National Center for Biotechnology Information (NCBI) GenBank nucleotide collection database via BLAST (Altschul et al. 1990) for species confirmation. COI was chosen for analysis due to its widespread usage for species identification. It is used for most DNA barcoding studies of fishes (Hubert et al. 2008), and has extensive applications for the animal kingdom as a whole (Pentinsaari et al. 2016).

Next-generation sequence data will be prepared using double digest Restriction-Site Associated DNA (ddRAD) libraries (Peterson et al. 2012). Restriction digest of 1µg genomic DNA/sample will be performed in 50µl reactions containing 5µl New England BioLabs CutSmart Buffer and 20 units each PstI and MspI. Samples will be digested at 37°C for 18-24 hours then purified using Agencourt AMPure XP beads (Beckman Coulter, Inc.). Barcoded samples (100 ng DNA each) will be pooled in sets of 48 following Illumina adapter ligation, then size-selected using the Pippin Prep System (Sage Science) to retrieve DNA fragments between 350 and 400 bp in length for Razorback Sucker and 325 to 425 for Colorado Pikeminnow (Bangs et al. 2018; Chafin et al. 2018). Size-selected DNA will be subjected to 12 cycles of PCR amplification using Phusion high-fidelity DNA polymerase (New England Bioscience), according to manufacturer protocols. Four indexed libraries (192 samples) will be pooled per lane for 150bp single-end sequencing on an Illumina NextSeq 500 (Midwest Fisheries Center; Onalaska, WI). Data will be de-multiplexed and filtered in STACKS 2 (Rochette et al. 2019) to discard reads with uncalled bases or low Phred quality scores (<10), while simultaneously attempting to recover those reads with ambiguous barcodes (=1 mismatched nucleotide). The *de novo* assembly of ddRAD loci will be accomplished in STACKS 2 (Rochette et al. 2019) with clustering parameters being determined by the methods of Rochette & Catchen (2017). Only loci appearing in 90% of individuals will be retained for analysis. Data will be filtered to retain one SNP per ddRAD locus. A second researcher will perform a 10% quality assurance/quality control of samples to ensure accuracy.

Hybridization Assessment

Samples will first be processed to determine species ancestry. A Maximum Likelihood approach in ADMIXTURE will be utilized to assess the contributions of Bluehead, Flannelmouth, and Razorback Sucker to the genomic composition of each sample (Alexander et al. 2009; Alexander and Lange 2011). These analyses will involve 20 replicates in ADMIXPIPE (Mussmann et al. 2020) using a clustering (*K*) value of 3. Assignments will also confirmed using the variational Bayesian framework of FASTSTRUCTURE (Raj et al. 2014) with *K*=3. Results from these programs will be used to identify potential introgression of Bluehead Sucker alleles into Flannelmouth and Razorback Sucker populations and classify any misidentified Bluehead Sucker existing in the dataset. Analyses will use non-admixed adult Bluehead, Flannelmouth, and Razorback Sucker collected from the San Juan River during 2019 and 2020 as reference samples.

Hybrid classifications will be performed in NEWHYBRIDS v2.0 (Anderson and Thompson 2002). This program explicitly classifies individuals as 1) non-admixed Flannelmouth Sucker, 2) non-admixed Razorback Sucker, 3) first generation (F1) hybrids, 4) second generation (F2) hybrids, 5) F1 by Flannelmouth Sucker hybrid (Flannelmouth Backcross), and 6) F1 by Razorback Sucker hybrids (Razorback backcross). Our SNP data will be reduced to 200 loci showing the greatest among-population differentiation (F_{ST}) and lowest linkage disequilibrium ($r^2 < 0.2$) as determined through GENEPOPEDIT (Stanley et al. 2017). A power analysis will be conducted using the HYBRIDDETECTIVE workflow (Wringe et al. 2017) following the methods of Chafin et al. (2019) to ensure the 200-locus panel can adequately identify different hybrid classes. Non-admixed adult Flannelmouth and Razorback collected from the San Juan River during 2019 and 2020 will be utilized as reference samples.

Our second approach to hybrid analysis will be to calculate a hybrid index (Buerkle, 2005) using the est.h function in the INTROGRESS R package (Gompert & Buerkle, 2010). Data will be filtered so as to acquire only SNPs representing fixed differences between parental species (i.e., Flannelmouth Sucker and Razorback Sucker). Interspecific heterozygosity will be calculated using the calc.intersp.het function in INTROGRESS, and results will be visualized using the triangle.plot function.

Effective Breeders Estimates

Any hybrid individuals or misidentified Bluehead Sucker will be removed from the dataset prior to N_b estimation. SNPs will be evaluated for linkage disequilibrium and Hardy-Weinberg Equilibrium (Raymond & Rousset 1995; Purcell et al. 2007). Pairwise relatedness of all larvae will be estimated using the sibship-assignment (SA) method in COLONY version 2.0.4.0 (Jones & Wang 2010). This software uses a maximum likelihood method to estimate relationships among offspring belonging to a single cohort by identifying full and half-sibling families while considering genotyping error rates and allowing for inferences related to the mating strategy of the organism. For both species, male and female polygamy will be assumed and parameter settings (i.e., dioecious, diploid, inbreeding, medium run length, full-likelihood with medium likelihood precision, no sibship prior, and updated allele frequencies) will be maintained across years. N_b will be estimated using the linkage disequilibrium method (Waples & Do 2008) in NeEstimator 2.1 (Do et al. 2014). Confidence intervals will be calculated using the jackknife method of NeEstimator, and rare alleles ($P_{crit} < 0.02$) will be excluded from analysis following recommendations of Waples & Do (2010). Analyses will be conducted separately for each year to estimate N_b, the number of adults that contributed at least one offspring, number of sampled offspring produced by each parent, and the number of parental pairs.

Schedule:

Completion of genetic analysis March 30, 2023

Final Report June 30, 2023

Deliverables:
Dissemination of the results will include a final report and presentation of project results at the San Juan Researcher's meeting.

Increased Funding Justification:

Recent genetic analysis indicated hybridization between Flannelmouth and Razorback Sucker in the San Juan River. Due to this development we are proposing a broader Scope of Work that encompasses calculating not only the N_b of pure Razorback but examining the origins of hybrid larvae and young-of-year recruits. Analyzing these data will help estimate how large the hybridization problem is and elucidate the patterns driving it. Some of the increased cost of this scope ($5,000) was requested through Grand Junction FWCO in previous years as part of their demographic monitoring efforts (FY 2021 SOW 19b), and consequently does not represent an overall increase in cost to the SJRRIP relative to our previous SOW submission (FY 2021 SOW 2). The remainder of the budget difference relative to FY 2021 SOW 2 ($33,738.61) is primarily a one-time cost to genotype potential hybrids collected across four sampling years (2018 through 2021). Therefore, we predict that the cost for genotyping hybrids will decrease substantially in future years if the SJRRIP would like this work to continue for samples collected beyond 2021.

FY22 - Detailed Spending Plan for Southwestern ARRC

1. PERSONNEL	
A. Laboratory Work	
1 Bio/Geneticist (GS-11-2; 520 hours – 6 pay periods) @ $45.77/hr	$23,800.40
B. Data Analyses and Report Writing	
1 Bio/Geneticist (GS-11-3; 480 hours - 4 pay periods) @ $47.25/hr	$22,680.00
C. Travel	
2 Trips to San Juan Meeting (4 days each; with travel) @ $664.50/trip	$1,329.00
1 Trip to Museum of Southwestern Biology to obtain larval samples (4 days; including travel) @ $576.50/trip	$576.50
Subtotal Personnel	**$48,385.90**
2. MATERIALS/SUPPLIES/SEQUENCING	
A. Extractions	$2,852.14
B. Library Prep	$3,499.40
C. Library Quantification	$1,168.26
D. ddRAD Sequencing	$9,475.00
E. mtDNA Sequencing	$5,046.48
F. Other (tubes, tips, etc.)	$5,216.21
Subtotal Supplies	**$27,257.49**
Southwestern ARRC Utilities	
-Electrical, (approx. 4,259 KW/h @ 0.34569 per KW/h) =	$1,472.29
Total	**$77,115.68**
Administrative and Overhead Costs Regional Office @ 3%	$2,313.47
Project Total FY2022	**$79,429.15**

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Responses to Comments:

Program Office

How can the technical aspects of this SOW be improved?

Due to the new techniques in genetic analyses (i.e., next generation sequencing), is there any concern with holding specimens over several years before being analyzed?

Response:

Thank you for your question. There should be no problem running next generation sequencing methods on older samples as long as the specimens were stored properly (such as storage in a freezer or 95% ethanol which will minimize tissue degradation). Any past San Juan collections, such as those archived at the Museum of Southwest Biology, should be useable as long as they were not stored in formalin.

Program Office

What is this SOW's contribution to recovery?

We think this is a great tool to quantify reproductive output of our adult endangered fishes. Additionally, an annual assessment of number of breeders may help identify environmental factors or population parameters limiting reproductive output. The addition of work assessing hybridization will be important given recent results of the true identify of putative wild juvenile razorback sucker.

Response:

Thank you for your comments. We hope that using next generation sequencing in conjunction with mitochondrial analysis will help further the understanding of the forces driving reproduction and hybridization in the San Juan River.