NATIVE THREE-SPECIES RESEARCH

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The results of the research investigations contained in this report represent work of the authors and may or may not have been implemented as Colorado Parks & Wildlife policy by the Director or the Wildlife Commission.

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Three-species tributary use and spawning investigations

Period Covered: March 1, 2022 to November 31, 2022

RESEARCH PRIORITY:

Test a resistance board weir as a means of controlling the entire Roubideau Creek spawning run, allowing for the selective exclusion and removal of non-native and hybridized suckers. *This priority is an ongoing project that has been covered in prior reports. Genomics data associated with this project was delivered to CPW during the covered period and is thus presented here for all previous years of study under this priority.*

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OBJECTIVES:

I) Test the functionality and operability of a resistance board weir located near the mouth of Roubideau Creek.

II) Evaluate the effect of the weir on the species composition of the larvae produced in the Roubideau drainage by sampling larvae and genetically assessing their species identity.

III) Compare the extent of tributary use between native and non-native suckers via longitudinal larval sucker sampling and genetic identification.

INTRODUCTION

Hybridization - Non-native species are a leading threat to biodiversity. Introduction of non-native species can lead to competition [1], predation [2], disease [3], and hybridization [4]; resulting in the loss of the imperiled native species and global biodiversity [5]. Hybridization can be described as the mating between genetically distinct species. Allendorf et al. (2001) broke down hybridization into six different types, starting with a distinction between natural and anthropogenic hybridization. Natural hybridization occurs when species that are historically sympatric hybridize without human influence. This can result in the formation of a natural hybrid taxon, natural introgression, or a natural hybrid zone. Alternatively, when humans produce an influence on the environment or species which leads to hybridization, we designate this as anthropogenic hybridization. When anthropogenic hybridization occurs with only sterile F1 individuals being created, this is called hybridization without introgression. However, when we have hybridization with resulting offspring producing offspring of their own and backcrossing

with parental species, we call this widespread introgression. The last type of anthropogenic hybridization is complete admixture. Complete admixture occurs when hybridization is so extensive that we can no longer identify any individuals within the population that are not hybrids [6].

When native and non-native species interbreed, the resulting hybridization can put the native population at risk through both genetic and demographic swamping [7–9]. When hybrid genotypes replace native parental genotypes, we describe this as genetic swamping. Concerns of genetic swamping affecting species persistence have been described in fish [10, 11], plants [12], and mammals [13, 14]. Alternatively, demographic swamping refers to hybrids physically replacing native species within the population, as occurs when hybridization produces lower-fitness or infertile offspring and also results in a lost opportunity for conspecific reproduction [8]. Both genetic and demographic swamping can be deleterious and lead to the extinction of native species.

There are many known examples of hybridization occurring as a result of human activity. For example, domestically-raised mallards released into the wild for hunting mate extensively with natural populations of mallards. Hybridization was detected in 65% of samples, with 12% of detected instances being early generation hybrids. The authors concluded that although hybridization was previously thought to be contained to eastern populations of mallards, this was not the case and hybridization is much more widespread across North America [15]. Another example of human-influenced hybridization is among species of Alosa fish. Sympatric populations of anadromous alewife (*Alosa pseudoharengus*) and blueback herring (*Alosa aestivalis*) were forced into the same lake after a dam was constructed that stopped their ability migrate back down the rivers. As a result, these previously sympatric species of fish began to hybridize. It was found that every landlocked individual was a hybrid, whereas in comparison, the anadromous populations have a rate of 0-8% hybridization [16]. Similarly, the endangered Java warty pig (*Sus verrucosus*) is losing genetic isolation through habitat destruction and hunting causing them to hybridize with the common Indonesian banded pig (*Sus scrofa vittatus*). The authors found that zoos and captive breeding centres were able to maintain unadmixed populations of Java warty pig, however, wild population were much more admixed [14]. These are just a few examples of how species introductions, habitat alteration, and habitat destruction through human-mediated changes can result in hybridization. Anthropogenic hybridization is a problem that remains widespread across the globe with detrimental outcomes for many species, including Catostomus suckers, the focal group for this study.

Catostomus sucker system - *Catostomus* suckers are a genus of freshwater fish that are widely distributed across North America. These fish live in both rivers and lakes, and can be easily identified by their characteristic mouth morphology, which allows them to feed on benthic algae and invertebrates. This genus of fishes exhibit tremendous diversity and an intricate evolutionary history. Throughout the history of *Catostomus* fishes they have hybridized extensively. There are plenty of recorded instances in the literature of hybridization between sucker species [17–23]. This family of fishes, Catostomidae, is thought to be the result of a whole genome duplication event approximately 50 million years ago after species hybridized [24]. This allopolyploidization

event resulted in the species being historically tetraploid. Within the Catostomidae family, it is seen that most species have a diploid complement of approximately 100 chromosomes [25].

Catostomus suckers in the Upper Colorado River basin are known to hybridize extensively with one another, leading to concerns about the persistence of the native and endemic sucker species [20, 26, 27]. The native bluehead and flannelmouth suckers (*C. discobolus*, BHS and *C. latipinnis*, FMS) are put at risk by hybridizing with the non-native white suckers (*C. commersonii*, WHS). Previous evidence has shown that hybridization is highly variable in this system with hybrid crosses ranging in ancestry from first generation hybrids to advanced back-crosses [22, 28]. Roubideau Creek, a tributary within the Gunnison River Basin, contributes substantially to the mainstem Gunnison sucker population. Each year, thousands of adult sucker fish move up Roubideau Creek to spawn when snow melt fills these intermittent tributaries.

Fish barriers and weirs - Fish barriers have been thought of as a way to reduce hybridization among fish. The use of fish barriers has been successful for invasive species management in the past. For example, barriers have had success within the Great Lakes at reducing invasive sea lamprey movement [29]. However, concerns are raised about the increased fragmentation of waterways through the use of barriers and the inability for native species movement. It has been found that a multitude of non-target species are sensitive to barrier interventions [30]. Barriers, more specifically weirs, are also used as a method for understanding population size and other important parameters in fish. By monitoring these weirs and counting migrating fish caught within them, scientists can make estimates on population size and makeup. For example, salmon counts have been recorded for over 20 years at two weir sites in Alaska, allowing for an extensive evaluation of population trends [31]. However, weirs pose problems of their own as rigid weirs are vulnerable to high flow events and debris in the flow, which can cause them to wash out of their place or become ineffective at blocking passage. The Resistance Board Weir (RBW) has been thought of as a flood resistant alternative to other traditional weirs. This design was originally conceived for use in Alaskan salmon runs, and is amenable to streams much deeper and wider than rigid designs can survive in.

In this study, we investigated the ability of a resistance board weir to restrict nonnative sucker access into spawning habitat of Roubideau Creek, in the Gunnison River Basin (Colorado, USA). We investigated how well selective removal of non- native WHS and longnose suckers and their hybrids at the weir translates to a decrease in non-native sucker ancestry within the sampled larval populations. If selective removal of non-native suckers was successful, we would expect lower proportional genetic contributions of WHS to larval fish cohorts in years with restricted access to Roubideau Creek (Figure. 1). In addition, we evaluated the relative genetic contribution of the three species of interest at different spawning locations upstream to understand the longitudinal ancestry trends within the spawning habitat, to better assess spatial separation of spawning adults in this tributary system. Based on prior knowledge of this system, native suckers were believed to use upstream reaches of the spawning habitat in Roubideau Creek, while WHS were expected to remain farther downstream, closer to the weir. We accomplished both objectives through genomic analysis of larval fish specimens.

METHODS

Site location and sampling - This study took place within the Gunnison River Basin of Colorado (USA). A resistance board weir (RBW) was used as a fish barrier to restrict access to spawning habitat during years of intervention (Figure 2). The RBW was set up across Roubideau Creek, a tributary of the Gunnison River. While Roubideau Creek is relatively small, and dries seasonally, it is known to be an important tributary for spawning suckers. In March through May each year, spring runoff makes this creek habitable for adult catostomids, which run upstream to spawn. Larval fish develop in Roubideau Creek, and then exit the tributary to the mainstem of the Gunnison River.

From 2019 to 2022 a subset a spawning adults (fin clips) and their progeny (whole larval fish) were collected from the weir and various sites upstream of the RBW. The RBW consists of PVC picket panels that are anchored on the upstream end to the substrate along a rail upon which they pivot, and are held above the water's surface downstream by hydraulic lift generated by large

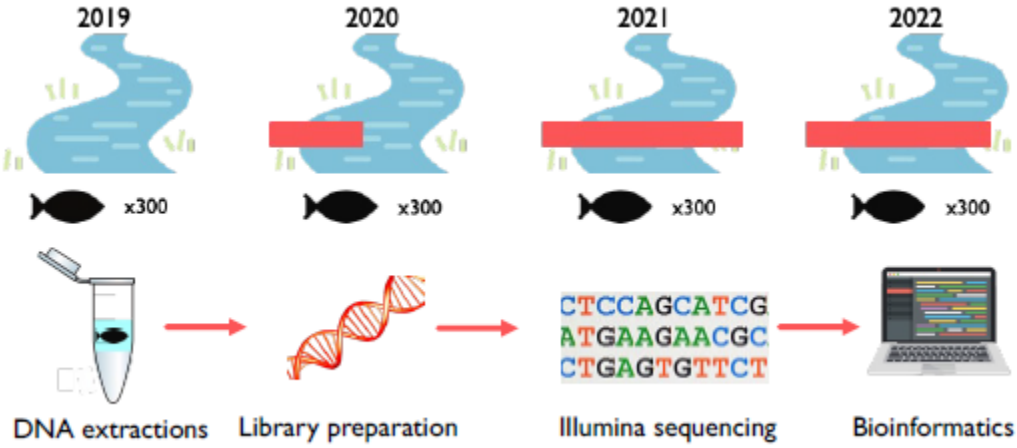

Figure 3. A visual schematic summarizing the degree of restricted access to Roubideau Creek in the four years of this study (top row), as well as the laboratory and bioinformatic workflow pursued for this project (bottom row).

boards attached to the underside of the panels (Figure 3). One panel has an "entry chute," essentially a tunnel of PVC pickets that allows fish to pass through the weir and a fyke into a large (6 x 10 x 5 foot) cage. All fish captured were identified to species, and those that were deemed pure native suckers (and RTC) were passed upstream to continue their spawning migration, while non-native and hybrid suckers were removed from the population. Additionally, length and weight data were taken on a daily subset of each species, and annually several thousand native suckers and RTC were PIT tagged.

Each year corresponded to a different degree of attempted control over the entrance of spawning adult fish to upstream reaches of Roubideau Creek (Figure 1, Figure 3). 2019 represents the year where there was no intervention present. 2020 represented a partially controlled year as the weir was pulled early in the season due to the COVID-19 pandemic. During both these years 240 larval fish were collected after the spawning season. In 2021, the RBW intervention was installed

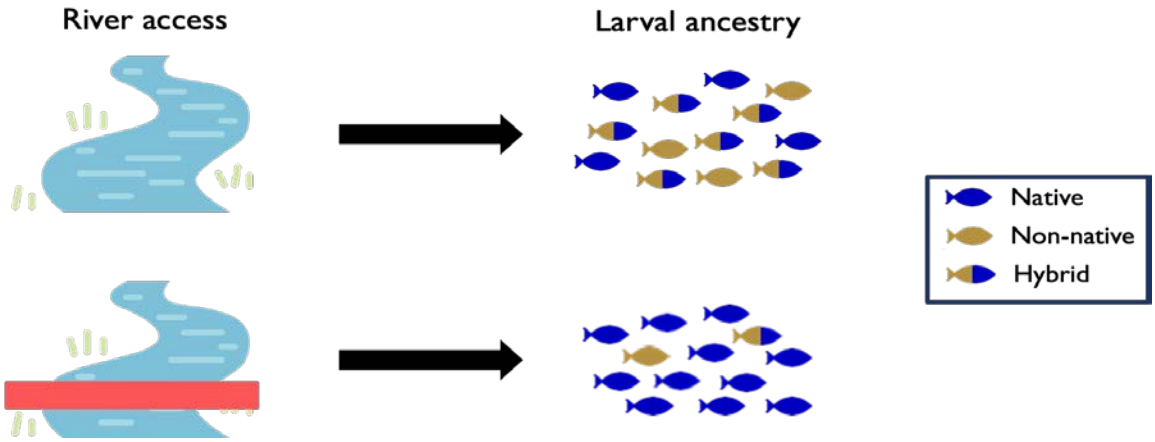

Figure 1. We hypothesized that if a resistance board weir and selective fish passage was successful at preventing white sucker spawning, there would be less white sucker ancestry in larval fish cohorts in years with the weir in place.

Figure 2. The fully assembled resistance board weir (RBW) in Roubideau Creek. The photo faces upstream. The PVC pickets in foreground are anchored on the upstream end to a substrate rail, but can pivot freely with water level. The only passage through which fish can pass is the PVC chute leading into the aluminum cage. The wall-tent in the back ground houses the fish-working station.

and all fish identified phenotypically as being non-native to the system or as a hybrid involving a non-native fish were removed from the spawning population moving upstream. Native sucker fish were allowed to pass the RBW. Three hundred fifty five larval fish were collected after spawning. Additionally, 203 random adult fin clips were taken to confirm proper fish identification by field technicians. In 2022, the RBW was installed for an additional year of

intervention. Unfortunately, rapid warming in the spring lead to fast melting of snow off the mountains and therefore increased flows within the river system. This caused the weir to be pulled from the creek as it could not withstand the debris build up and increased flows. Therefore, 2022 represents a partially controlled year. In total, 472 larval fish were collected after spawning and 133 random adult fin clip samples were collected at the weir. Overall, the weir was installed 29 days in 2020, 78 days in 2021, and 48 days in 2022. Table 1 summarizes sampling effort across years.

Table 1: Summary of number of days with the Resistance Board Weir being actively installed, larval fish sampled, and adult fish sampled per year.

Year	Active Weir Days	Adult Fish Sampled	Larval Fish Sampled
2019	0	0	240
2020	29	0	240
2021	78	203	355
2022	48	133	472

Genomic data preparation - DNA was extracted from all samples using DNeasy Blood & Tissue Kits (Qiagen, Inc.). For each sample, approximately half a larval fish or half a fin clip was used. Extracted DNA was quantified using a Nanodrop spectrophotometer. The extracted DNA was then used to create highly-multiplexed genomic libraries for high-throughput sequencing. These libraries were prepared following methods from Parchman et al. (2012) [32]. This process involved restriction digest of the prepared samples followed by ligation of adaptors, including unique barcodes assigned to each individual sample. The restriction/ligation product was then amplified using two rounds of PCR. The resulting genomic libraries were size selected at a targeted range of 275 base pair fragments using a PippinPrep machine. The final genomic libraries were then sent to SickKids Toronto for Illumina sequencing on the NovaSeq 6000 at The Centre for Applied Genomics (TCAG). Sequencing yielded large amounts of raw genomic data which was then assessed using bioinformatics.

Filtering and variant calling - To begin, the raw genomic libraries were first demultiplexed using sabre (https://github.com/najoshi/sabre). This process involved matching 8-10 base pair barcodes with samples using text files containing each unique barcode and paired sample ID. The output FASTQ files were then aligned to a reference FMS genome (Mandeville, unpublished) using Burrows-Wheeler alignment [33, 34, bwa mem, version 0.7.17]. Single nucleotide variants (SNPs) were called using samtools version 1.9 and bcftools version 1.9 [35]. Variant calling was completed on the output bam files, followed by filtering of the new vcf file [36, VCFtools]. In total 682 individuals were retained after filtering. For the purpose of this study we want sites that are polymorphic in multiple populations. Therefore, sites with a minor allele frequency of less than 5% were filtered out. Additionally, sites with more than two alleles were removed and only one variable site per contig was randomly selected. In total, 35,437 SNPs were retained in .vcf format. We further filtered out paralogous loci using vcftools version

0.1.16, as these fish resulted from a whole genome duplication. This filtering step removed an additional 6,180 SNPs. The remaining 29,257 SNPs were used for all analyses going forward.

Ancestry determination using entropy - Ancestry of individual larval and adult fish was estimated using the program entropy [37]. The program entropy is a hierarchical Bayesian model which creates estimates of individual ancestry while accounting for genotype uncertainty. Values for q and Q provide information on ancestry from the different parental species, where q evaluates the proportion of an individual's ancestry that comes from each parental species. A q value of 0.5 would suggest a first or second generation hybrid (F1 or F2), or potentially advanced generation hybrids beyond F2. A value of 0.25 or 0.75 would suggest the individual is a back-crossed hybrid with one of the parental species. Q evaluates the proportion of loci in an individual that have ancestry from both parental species, which is a measure of recombination and a proxy for number of generations of hybridization. Q is expected to equal 1.0 for a first generation hybrid and 0.5 for a second generation hybrid or back-crossed individual. With these two values combined, we can determine whether a fish is an F1 hybrid, F2 hybrid, back-crossed individual or unadmixed parental species from the species of interest.

For our run of entropy the number of Markov chain Monte Carlo (MCMC) steps was set to 50,000 and the number of burn-in steps was set to 25,000. After the burn-in was complete, every 25th step was stored. An appropriate starting value for the program was estimated using a custom bash script. Program entropy was run for values of K=1-6, with each value of K having three replicate runs. The optimal K value was determined by running an entropy command on the output hdf5 files which evaluates the deviance of the model. The lowest number suggests the K value with best fit for the data.

RESULTS

RBW operation and adult catch – Please see previous annual reports for 2020 and 2021 RBW operation and catch results. In 2022, we deployed the weir and trap on March 3rd, and effectively operated the weir through April 18th. However, flows rose substantially during the week of April 11th and it became exceedingly difficult to keep the weir operating correctly. The biggest challenge during this period was removing the abundant grasses and plant roots that would wrap around pickets. Because they wrapped around the pickets, they would not self-flush, even when the weir submerged. Therefore, these materials had to be constantly removed as the flows rose, to keep the weir emergent and maintain closure of Roubideau Creek. Simply maintaining functionality of the weir required at least two staff performing sub-hourly cleaning passes around the clock. During this period, fish movement was substantial, so a full fish working crew was also required during afternoon-late, evening, and much of the morning. While difficult, these conditions were manageable up until the early morning of April 19th, when stream level at the weir rose an additional 6-8 inches between midnight and 3:00 AM. At that point, velocity and depth became too great to physically cross weir panels to continue the cleanings, and it became unsafe to enter the cage due to entrapment potential. We opened the cage doors and removed as much loose infrastructure from the water as possible at this point in hopes of minimizing equipment loss and fish mortality. During the night of April 21st, the cage was rolled by the

creek, and on April 22nd, we used winches to roll the cage up onto shore to prevent it from sustaining further damage and from damaging the submerged weir panels.

We gauge flow near the weir site with an Onset U22 HOBO water-level logger, and based on our rating estimates, discharge rose from 253 cfs at midnight, to 338 cfs at 3:00 AM (Figure 4). Discharge continued to rise over the next several weeks, peaking at an estimated 900 cfs on the evening of April 22nd, and staying above 300 cfs until May 14th. Due to these high flows, and the resulting damage to the weir and sediment deposition onto the weir panels, we were unable to resume operation again during this season. During the week of June 6th, we were able to unbury the weir panels and remove it from the creek. Relatively little damage actually occurred, and was reparable.

Snowpack is the primary factor we can look at for estimating peak flows on Roubideau Creek. During 2022, snowpack on March 3rd, when we installed the weir, was 124% of the seasonal median. There was not a substantial amount of additional accumulation past March, but temperatures warmed rapidly in April, causing a more rapid runoff than expected, leading to the exceptional flows that occurred. In 2021 we noted that Roubideau Creek above Buttermilk Creek was not flowing at a fish-passable level until April 22nd, but in 2022, it began flowing before the weir was installed, and was passable by March 18th at the latest.

During the 47 total nights of operation (March 3rd through April 17th), the weir captured numerous fish. In total 10,333 fish were captured, of which 10,188 were suckers. Of the suckers, 9,229 were identified as only having native characteristics (2,644 BHS, 6,388 FMS, and 197 FXB), and 959 were identified as having non-native characteristics (154 WHS, and 805 non-native hybrids). Overall, 10.4% of suckers captured were identified as having non-native traits.

We captured fish beginning on March 24th, which coincided with the first pulse of irrigation water from Buttermilk Creek, which has repeatedly been the trigger for immigration in previous years. Bluehead Sucker, FMS, and non-native sucker catch followed similar patterns, with FMS consistently being the most abundant of the species (Figure 4). Discharge alone did not appear to explain variations in catch.

Sequencing and filtering - Our sequencing effort resulted in a total of 3,302,373 raw reads. After filtering and variant calling we ended up with a total of 29,257 SNPs and 682 individuals. This resulted in a mean coverage of 17.97 reads per locus per individual. These loci were used for all results going forward. Unfortunately, the majority of samples (adult and larval) collected in 2022 did not align well to reference genomes, and we suspect ethanol preservation was at fault – likely due to overly dilute ethanol shipped from the manufacturer. Additionally, unavoidable library preparation issues of 2019 and 2020 samples led to substantially reduced sample sizes (n=79 in 2019; n=132 in 2020 of 240 collected each year). The 2019 and 2020 samples can be re-sequenced at a future date, but many of the 2022 samples are likely unrecoverable as the majority of DNA sequenced was associated with bacteria.

Validation of adult suckers field identification - Adult suckers collected in 2021 were used to verify correct phenotypic identification in the field by confirming with genetic data. 160 adult samples from 2021 were retained after filtering and used to check identification. Overall, 55 fish were identified in the field as pure FMS, 53 as pure BHS, and 50 as pure WHS. Additionally, 2 fish were identified in the field as White x Bluehead sucker hybrids (WXB), and no White x Flannelmouth sucker (WXF) or Flannelmouth x Bluehead sucker (FXB) hybrids were observed in the field. Through genetic analyses we determined that 39 individuals were pure FMS, 48 were pure BHS, and 31 were pure WHS. We identified 17 WXB, 8 WXF, and 12 FXB hybrids. Overall, 50 fish were incorrectly identified in the field and 110 were correctly identified in the field. Table 2 breaks down how species were identified in the field versus how they were identified through genetics work. Most incorrect identifications in the field were hybrid fish being identified as pure suckers. Only two misidentified fish were field-identified as one pure species, but genetically identified as another pure species – these are likely due to clerical mistakes. The rest of the misidentified fish were identified as pure in the field, but hybrids genetically. Looking at these individuals, on average the dominant species accounted for 91.6% of the individuals' ancestry indicating that overall, it is easy to misidentify heavily back crossed species. Still, there were at least 10 individuals that genetically appeared to be close to F1 hybrids, including both field-identified pure natives and pure non-natives that were genetically

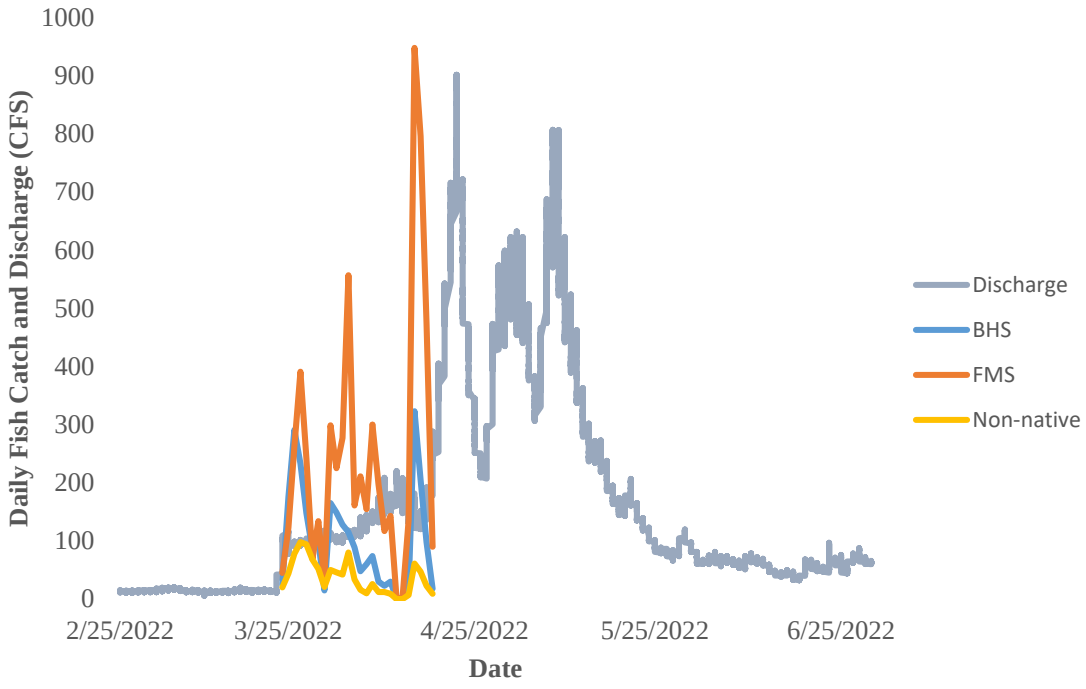

Figure 4. Comparison of daily catch (number per species) at the Roubideau Creek resistance board weir, and stream discharge (CFS) during the 2022 sucker migration and spawning period. BHS = Bluehead Sucker; FMS = Flannelmouth Sucker; Non-Native = any sucker with any suspected White or Longnose sucker genetics.

identified to be hybridized. In total, there were 10 fish identified in the filed as pure that contained non-native genetics.

Table 2: Summary of expected (field) vs actual (genetic) identification of adult Catostomus species.

Ancestry	Expected ID (field identification)	Actual ID (genetic identification)
WHS	50	31
BHS	53	48
FMS	55	39
WXB	2	17
WXF	0	8
FXB	0	12
Other	0	5

Efficacy of the RBW at reducing the incidence of non-native suckers and their hybrids - In total, 514 larval fish samples were used to analyze the efficacy of the weir at reducing WHS and hybrids with WHS ancestry from spawning. The year 2019 (n=79 larval samples) represented the non-manipulated year with no weir days, and 2020 (n=132) and 2021 (n=303) represented years where the weir was active, for varying degrees of time. In 2020, there were 29 active weir days, and in 2021 there were 78 active weir days. Estimates of ancestry with entropy revealed that WHS and their hybrids comprised only a small portion of the sampled larvae in any sampled year, including 2019, where access to the spawning tributary was not restricted by a RBW (Figure 5). The most abundant species sampled was FMS, followed by BHS. Hybrids between these species were sampled in all years. As expected, hybrids included crosses between the native species and introduced species; both WXB and WXF hybrids were sampled in all three years. Hybrids between the two native species (FXB) were also sampled in all years. A small number of hybrids with ancestry from all three parental species were sampled as well. Proportion WHS ancestry was similar in 2019 and 2020, at 11.39% and 11.37% summed across all individuals, respectively (Figure 5). In 2021, WHS ancestry represented only 6.6% summed across all individuals. This decrease is substantial, but both hybrid individuals with WHS ancestry and individuals with 100% WHS ancestry were still observed. Some of these hybrids were individuals with intermediate ancestry between the two parental species, likely F1 hybrids. Proportion of BHS ancestry increased across the three sampling years.

Longitudinal larval ancestry trends within the spawning habitat - Ancestry of larval suckers was analyzed relative to distance upstream of the RBW. Prior work by Colorado Parks and Wildlife biologists suggested that species might partially separate spatially across upstream reaches of Roubideau Creek. Sampling sites for larval fish were located up to 37 km upstream of the site where the RBW restricted fish passage. We plotted individual larval fish ancestry in each of three genetic clusters as a function of distance upstream from weir site (Figure 6). We ran linear models to assess longitudinal trends in ancestry. Flannelmouth Sucker ancestry was highest at the weir, and significantly declined towards upstream reaches. Conversely, BHS ancestry was

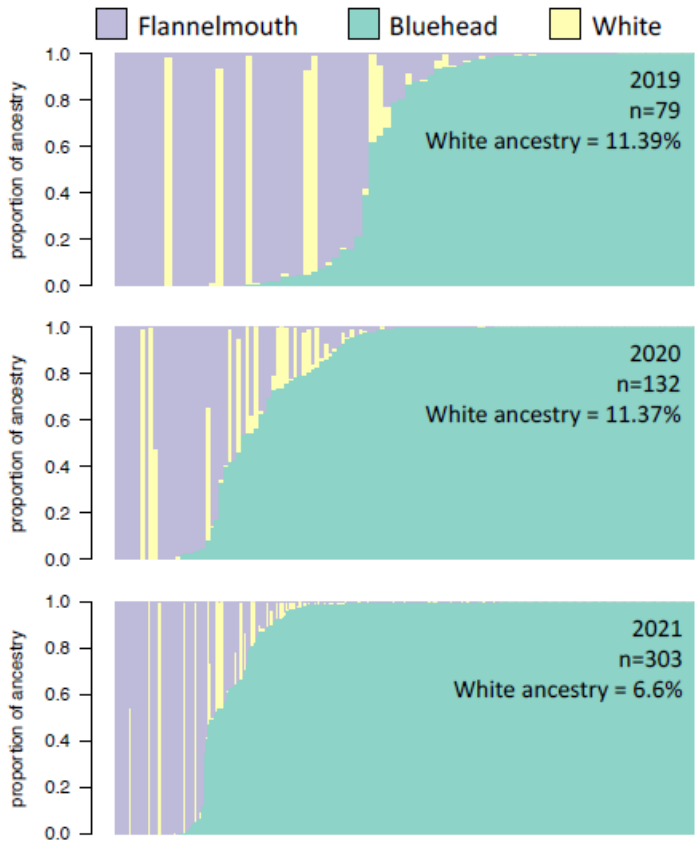

Figure 5. Ancestry of larval fish collected in 2019–2021, estimated using entropy. Each vertical bar represents one individual fish; colors correspond to proportional ancestry in each of three possible parental species.

highest farther upstream, and lowest at the weir. These trends were statistically significant ($p<0.001$), but had fairly low R^2 values (0.075 for FMS, 0.091 for BHS). For WHS, there was a slight decrease in proportional ancestry from the weir site towards the upstream reaches. The trend in WHS ancestry was significant ($p<0.05$), but with a very low R^2 (0.0091).

DISCUSSION

We used a resistance board weir to manipulate the Catostomid spawning migration in Roubideau Creek, and we then used genomic data to assess genetic contributions of three species - BHS, FMS, and WHS - to three cohorts of larval fish in Roubideau Creek, Colorado. Larval fish were sampled prior to intervention (2019), in a year with partially restricted tributary access (2020), and following successful implementation of a resistance board weir intervention (2021) to prevent non-native WHS from accessing important spawning areas. Additional samples representing mostly-successful RBW implementation were collected in 2022 and DNA sequencing was attempted, but due to tissue preservation issues, these samples sequenced poorly,

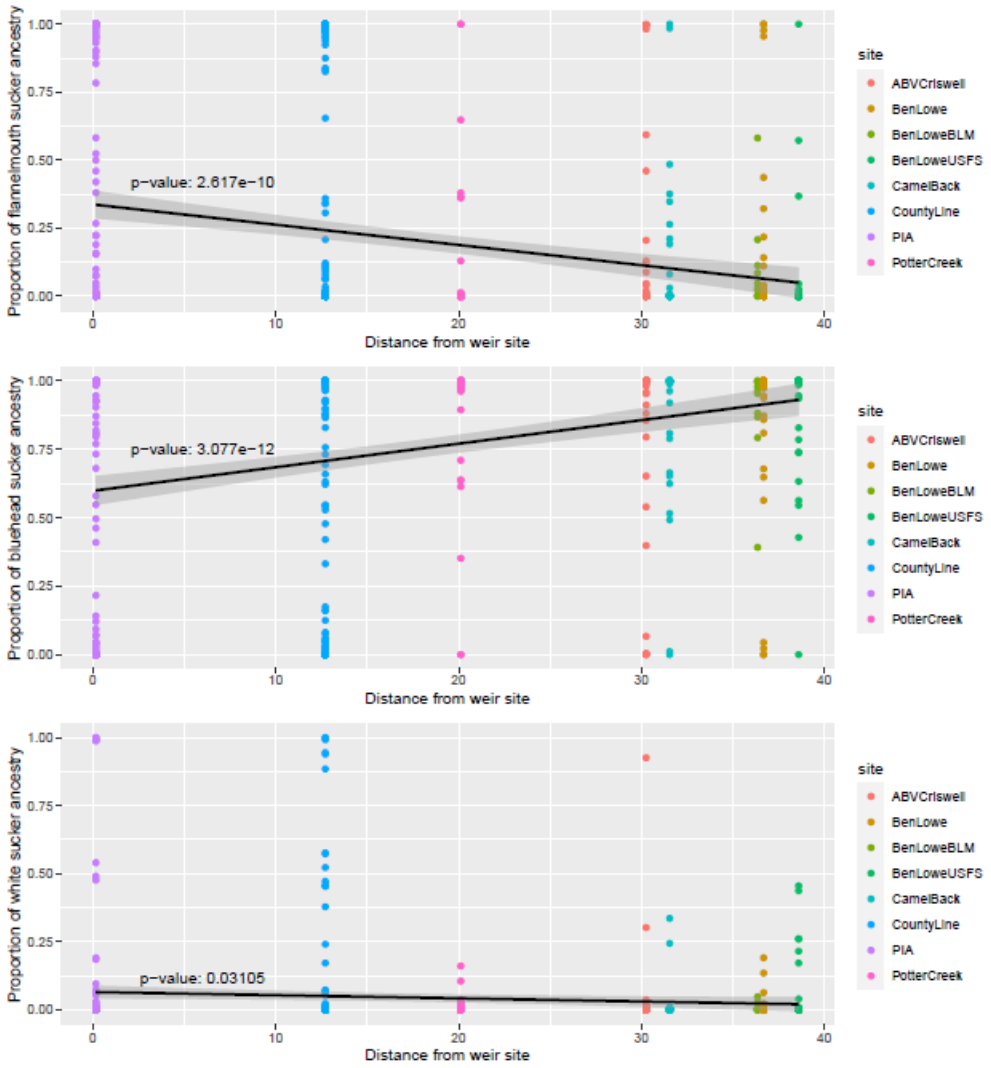

Figure 6. Proportional ancestry in each of three species for all sampled larval fish 2019–2021, plotted as a function of distance upstream from the weir site (km).

and results are not discussed here.) If successful, this sort of intervention could be used in important spawning tributaries to bolster populations of native Catostomus suckers and preserve the native fish biodiversity of the Upper Colorado River basin.

RBW operation and catch – In 2022, high flows prevented the full exclusion of non-native catostomids, as we were only able to operate the weir during the beginning of the migration. While high discharge and associated debris loads prevented us from completing our objective in 2022, it did provide insight on operable conditions for the RBW. We lost containment of the weir at roughly 340 cfs, but struggled to maintain functionality at flows above 250 cfs, so we likely can-not operate the weir at extend flows above 250-300 cfs in Roubideau Creek. The weir is likely operable at much higher flows in other systems with a less confined channel and with less

fine and flexible grass-like debris in the drift. In the Roubideau Creek drainage, it may be difficult to predict which years will be suitable for weir deployment. In 2022, the March 1st seasonal median snowpack of 124% did not indicate to us that the drainage would experience the extreme discharge levels that occurred. A number of variables besides late winter snowpack can affect runoff levels in the basin, including April and May precipitation, warming rates, and soil moisture levels. Therefore, there is risk to the weir even in years where snowpack is fairly average at the beginning of March, when the decision to deploy the weir is made. We therefore recommend that this weir only be used in average or below average snowpack years, with the caveat that saturated soils, or heavy spring snow may cause periods of flow too high to operate the weir in or may even warrant early removal of the weir to prevent equipment loss or damage. Removal of the weir under flows much above base flow (20 cfs) is not likely feasible, so the decision to remove likely needs to be made before the irrigation network is activated and Buttermilk Creek begins flowing heavily – usually around mid - late March.

In 2022, we caught many more FMS than BHS. In previous years we caught many more BHS than FMS, especially early in the run. We do not know why this major shift occurred in 2022. We also saw a higher non-native presence in 2022 at 10.4% of the total sucker catch. This was up from 8.3% in 2021. However, as we sampled only a portion of the run, this may not be a meaningful change. We do caution that repeated operation of the weir may deter individuals from returning over time, so migration demographics should be monitored closely over future years to make sure the intervention doesn't diminish the spawning event.

Efficacy of the RBW intervention - Our results suggest that the RBW intervention was successful in reducing WHS ancestry in 2021, the only year where full control was achieved and DNA sequencing was successful for larval fish. Proportional ancestry contributed by WHS was 6.6% in 2021, compared to 11.39% and 11.37% in 2019 and 2020 respectively (Fig. 4). In all years, both unadmixed WHS larval fish and hybrid larval fish with WHS ancestry were sampled. It is important to note that although the RBW was installed in spring 2020, it was removed prior to the peak of catostomid spawning due to restrictions on fieldwork associated with the COVID-19 pandemic. Our genetic results suggest that no meaningful reduction in non-native sucker ancestry in larval fish was achieved by the partial control in 2020, which is not unexpected given the limited period of RBW operation in 2020. It is unclear how completely upstream access for spawning adults needs to be controlled to substantially reduce hybridization and reproduction of WHS. In 2022, when the RBW was operational, a period of high flows in April necessitated the early cessation of weir operations and this likely allowed numerous WHS to pass upstream of the weir. Unfortunately, due to issues with larval fish tissue preservation, results from 2022 cannot be directly compared to the 2019–2021 data at this time, but new analyses may be able to clarify how successful exclusion of WHS was in 2022 with partial RBW usage. It is also possible that effects of subsequent years of WHS exclusion from spawning in Roubideau Creek could have additive effects, and removal of WHS year after year and lower production of WHS offspring could decrease the number of adult WHS returning to Roubideau Creek to spawn. The degree of sucker spawning site fidelity (i.e., degree to which adult fish return to spawn in the location where they were born) in tributaries of the Gunnison River in western Colorado appears high, so

suppression of WHS in Roubideau Creek and exclusion of WHS from spawning areas could have desirable long term effects through repeated intervention.

One of the biggest challenges associated with this type of manipulation is the correct identification of individuals in the field based on morphology. Overall, we misidentified 31.25% of adults sampled. Several of these mistakes are likely clerical in nature, but the majority suggest field identification will not always be accurate. The biggest issue appeared to be the identification of hybrids that were heavily backcrossed, with only a small proportion of ancestry attributable to one of the parental species. The greatest concern with misidentification is that non-native or hybrids are mistakenly passed allowing for their participation in the spawn. In our sample of 160 adults, 10 (6.25%) were incorrectly allowed to pass the weir despite having non-native genetics present. This reflects the unfortunate reality that when sorting thousands of fish, some mistakes are inevitable. These mistakes may be limited through additional training of fish-working staff, but some undesired fish will be passed accidentally when sorting based on phenotypic identification. Even with up imperfect sorting, we would hope that a reduction in non-natives in the spawning run should routinely improve the genetics of larvae produced in the system.

Longitudinal distribution of sucker ancestry in Roubideau Creek - Previous observations of sucker habitat use and spawning in Roubideau Creek upstream of the RBW site have suggested potential for spatial separation of sucker species. It was previously hypothesized that native suckers preferentially used upstream reaches, while WHS tended to use farther downstream reaches closer to the weir. However, previous work on these Catostomus species at another location in the Upper Colorado River basin has suggested complete overlap of spawning timing and location [38]. Our results support a statistically significant but weak relationship between position upstream of the weir and ancestry for BHS, FMS, and WHS ancestry. Bluehead Sucker and FMS exhibit opposite patterns, with increasing BHS ancestry with greater distance upstream of weir, and reduced FMS ancestry with greater distance upstream of the weir (Fig. 5). These opposing patterns could point to historical differentiation of spawning area for FMS and BHS, albeit with lots of individual-level variation. The presence of FXB hybrids in the larval fish sampled in all years support some overlap in spawning between the native species, however. White suckers also exhibited a negative relationship with distance upstream of the weir, but given the exceptionally shallow slope of this relationship it is likely that although this is statistically significant, it is probably not biologically meaningful.

CONCLUSIONS

Genomic analyses of larval fish have provided new insights into the spawning biology and hybridization dynamics of suckers in Roubideau Creek, Colorado. Our results suggest that while the resistance board weir intervention to exclude WHS from spawning areas was successful at reducing non-native WHS ancestry in Roubideau Creek, WHS ancestry was initially relatively low among larval fish even before the implementation of the RBW (<12% white sucker ancestry in both 2019 and 2020). The RBW reduced WHS ancestry to 6.6% in 2021; results from 2022 remain unclear. It is unclear how distribution of ancestry might change as the fish age and grow, and as selection through differential survival has an effect on this cohort of larval fish.

Additionally, it is still unknown to what extent effects of WHS exclusion from the spawning tributary might be compounded across years for a greater long term effect. While it appears that there is some spatial separation between species along the spawning tributary, this trend is not strong or consistent enough within a species to result in strong reproductive isolation between species. It also appears that there is not a strong trend of WHS usage along the spawning tributary. Taken together, the results of this work will hopefully help support successful management of Catostomus sucker spawning habitat.

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RESEARCH PRIORITY

Identify tributary fidelity rates and spawning movement patterns in Three-Species fishes as well as non-native suckers in the Roubideau Creek drainage.

OBJECTIVES:

Determine annual spawning tributary fidelity of PIT-tagged Three-Species fishes.

INTRODUCTION

Information is relatively sparse on whether individual BHS and FMS suckers tend to select specific tributaries and locations for spawning repeatedly or if they stray among tributaries. If they do exhibit high rates of spawning tributary fidelity, efforts to limit hybridization in tributaries such as those described in the following research priority are more likely to result in decreased hybridization in the basin over the long term. In this scenario, a higher proportion of natives are likely to return to controlled tributaries as genetically pure fish recruit to the spawning population following control measures, even if hybridization continues to increase in uncontrolled portions of a basin. Alternatively, if fish stray from tributary to tributary among years, we would expect to see a long-term increase in hybridized fish in a controlled tributary, reflecting the basin wide continued increase in hybridization incidence. Therefore, in conjunction with testing the feasibility of spawning run control measures (see previous Research Priority), we deemed it important to simultaneously evaluate tributary fidelity among the Three-Species fishes. In recent years we identified high tributary fidelity rates in Gunnison River tributaries. We are continuing to monitor these movement patterns to see if these patterns are affected by the highly variable climatic and hydrographic conditions typical of the Colorado River basin.

METHODS

Since 2014, CPW and partners have been PIT-tagging Three-Species fishes in the Lower Gunnison basin. Many of those have been tagged in the Roubideau Creek drainage. In 2015, we installed a PIT-tag detecting, passive interrogation array (PIA) at the mouth of Roubideau Creek. The PIA has been operated continuously since 2015, and in 2016, we began deploying portable, submersible PIT-Tag readers (SPRs) in various locations in Roubideau Creek and its tributaries. We have used redetections of PIT-tagged fish on the PIA and SPRs to determine fidelity to the Roubideau drainage as a whole (via PIA detections), and to specific tributaries within the drainage (via SPR detections). We have estimated short term fidelity rates as simply the proportion of fish detected in a given year that return in the following year. More detailed methodology on this Research Priority (through 2018 sampling) can be found in the publication referenced in the results and discussion section below. In 2022 specifically, we tagged new individuals of all three species, and continued to monitor redetections with the Roubideau PIA, and SPRs. We also installed SPR's in new locations – the North Fork of the Gunnison and the Dry Fork of Escalante Creek - to determine movement and fidelity patterns in previously unstudied tributaries.

RESULTS AND DISCUSSION

This Research Priority is partially complete. Our 2019 technical report including this project can be referenced for detailed methodology and results through 2018.

- Thompson, K. G., and Z. E. Hooley-Underwood. 2019. Present Distribution of Three Colorado River Basin Native Non-game Fishes, and Their Use of Tributary Streams. Colorado Parks and Wildlife Technical Publication 52.

Results from 2019- 2021 are available in our previous annual reports. During 2022, 1,970 additional PIT tags were deployed in the Gunnison Drainage and detection data was collected. Detections of PIT tagged fish at the PIA give us the best indication of tributary fidelity as antenna efficiency is typically 100%. In previous years, we saw a high proportion of individuals of both BHS, FMS, and RTC returning to spawn from one year to the next, and this trend held true for fish detected in the system in 2021 returning in 2022 (Figure 7). In 2022, species-specific return rates (proportion of fish detected in 2021 redetected in 2022) were 76.7, and 84.6 for BHS, and FMS, respectively. These rates are similar to those observed annually since 2016, but were slightly higher for all three species. Interestingly, we also observed a very high return rate for RTC of 90.8%. Given that some mortality is expected from one year to the next (previously estimated at up to 10% for this system), in 2022 it appears that nearly all fish detected in 2021 returned to Roubideau Creek. We suspect that abundant and early flows, as described in the previous research priority, provided optimal conditions for fishes to access the tributary system, and few fish sought other spawning locations, which we believe they do under less optimal conditions.

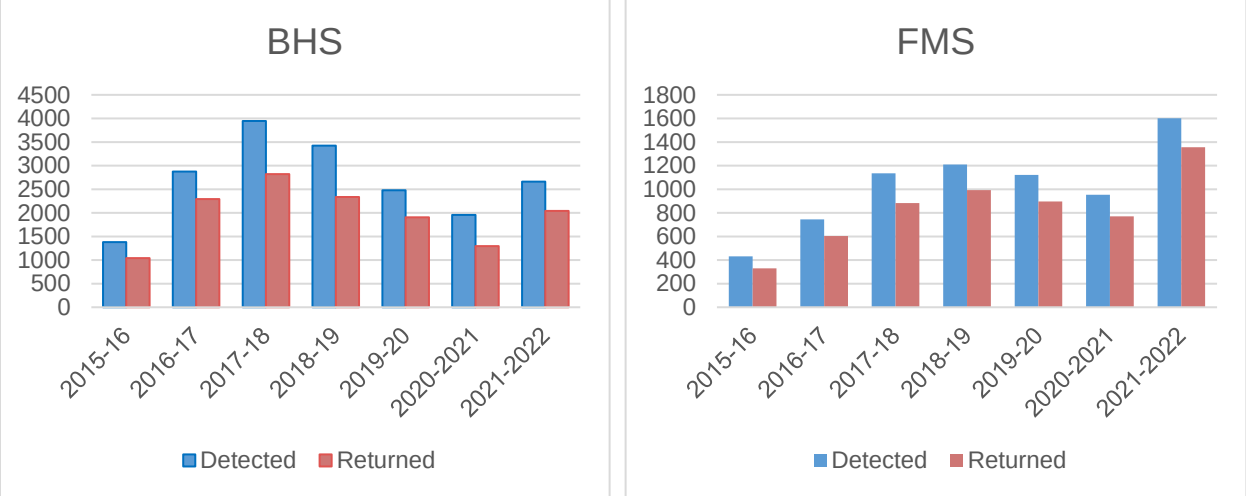

Figure 7. One-year fidelity rates for PIT-tagged BHS and FMS based on Roubideau Creek PIA detections. Bar-pairs represent the number of individual fish detected in $year_i$ (Blue) and redetected in $year_{i+1}$ (Red). The difference between annual pairs represents the number of individuals that did not return. Reduced tagging efforts between 2017 and 2020 are responsible for the overall declines in detections in more recent years.

We struggled to maintain SPRs under the high flow conditions, and there was approximately one month during which we were unable to collect data from SPRs. Therefore, we were unable to estimate tributary specific returns. However, we did detect PIT-tagged fish accessing Cottonwood, Buttermilk, Potter, and Upper Roubideau creeks in abundance. Unfortunately, the new SPR deployments in the North Fork of the Gunnison (North Fork) and in the Dry Fork of Escalante Creek (Dry Fork) were both unsuccessful. The North Fork SPR battery charger was misprogrammed resulting in a two week battery life as opposed to a 40-60 day battery life. The North Fork is a larger river, so battery changes are impossible until runoff recedes. During the very beginning of the SPR's deployment, two fish (1 FMS and 1 WXF) were detected, but the battery died before the migration truly began. In the Dry Fork, rapidly rising flow and mobile substrates led to the scouring of the SPR anchors. The SPR was transported 0.5 mi downstream where we relocated it as runoff receded. The battery was disconnected during the incident so no data was recorded.

We have now monitored fidelity rates and tributary usage during multiple years of extreme flows, extreme drought, as well as more average conditions. Across these years, Roubideau Creek fidelity rates have remained in the 60 to 90% range for all three species, and we have seen immigration into the smaller tributaries annually, as long as they actually flow.

ACKNOWLEDGMENTS
Former Aquatic Researcher Kevin Thompson initiated and conducted the bulk of the work described here. Dozens of people over the years PIT-tagged the suckers and chub used and re-detected in this priority.

RESEARCH PRIORITY:
Monitoring "perennial island" Three-Species populations in an intermittent stream-scape.

OBJECTIVES:

I) Identify perennial segments of intermittent streams that support the Three-Species.

II) Monitor population demographics within the perennial segments across seasons and assess mobility of fish within these populations.

INTRODUCTION
The biological and physical benefits of intermittent and ephemeral streams are not given nearly the consideration of perennial streams due to their short wetted periods. In fact, these streams are often not even afforded the protections of regulatory laws such as the Clean Water Act in the United States. Intermittent streams are those that flow continuously only at certain times of year,

while ephemeral streams are those that flow only briefly in direct response to local precipitation. Both are often overlooked with respect to aquatic organisms. In the arid, lower elevations of the Colorado River basin, the majority of waterways are intermittent or ephemeral. Recently, researchers and biologists have gathered a wealth of data showing that intermittent streams are important for Three-Species fishes when it comes to fulfilling certain life-history components. In prior research priorities, we have identified heavy use of intermittent tributaries by the Three-Species for spawning and early larval rearing. Many of the streams we have studied closely, including Cottonwood Creek, Roubideau Creek, and other streams draining the Uncompahgre Plateau, flow during April and May, when spawning, hatch-out, and larval drift occur, and then dry up in June. Therefore, we've viewed the streams as only important seasonally. However in March 2020, CPW aquatic technician Chase Garvey observed small suckers in pools in a short flowing segment of the Dry Fork of Escalante Creek prior to runoff while on a recreational hike. The observation was significant, as the stream flows less regularly than Cottonwood Creek, and is partially isolated from the mainstem of Escalante Creek by a derelict irrigation diversion. The fact that fish were present in these pools following a record dry summer and fall was surprising, suggesting that these fish had survived at the location through at least one significant drought year. More so, the presence of the barrier downstream probably means that these fish survived in isolation in the short perennial reach for many years. We sampled the segment and another that we subsequently identified, and confirmed the suckers were BHS, and that SPD were also present. Following this discovery, we prioritized identifying other perennial islands of occupied habitat among the largely intermittent streams of the area. Our goal was to identify locations that may have perennial flow due to groundwater input, and then sample those areas to determine if they were occupied. For a subset of occupied habitats, we will monitor population dynamics over several seasons, and look at movement between populations. The presence of these populations increases the amount of occupied stream length in the state. Studying these habitats will better our understanding of the diversity of habitats that Three-Species fishes use, will allow us to increase the precision of the range-wide database, and will further inform management and conservation practices for intermittent desert streams.

METHODS

We initiated this priority in summer 2021 and continued to monitor fish populations and stream conditions in 2022. First, we used Google Earth aerial imagery to search drainages on the east slope of the Uncompahgre Plateau for stream segments that were potentially perennially wet (Figure 8). We compared imagery from different dates to assess wet and dry conditions through time. Imagery captured on August 8, 2019 was particularly helpful, as the quality was good, and summer of 2019 was very dry, so visible water was scarce. What was visible in the images was likely perennial, and reliably so even under moderately to extremely dry years. We selected a subset of reaches to confirm whether water was present, and if so, sample for fish. Fish sampling was conducted with one LR24 backpack electrofisher (Smith-Root®), as all segments were very narrow and relatively shallow. If fish were present on the first pass, a second pass was completed. All fish were counted, identified to species, weighed, and measured. Any native suckers and chub captured over 120 mm TL were implanted with a 12mm PIT tag. At a subset of occupied sites, we established a "wet" sampling reach and upstream and downstream "dry" sampling reaches, based on water and fish presence at the time of the survey. Features such as

Figure 8. Aerial imagery (A) of Cottonwood Creek captured August 25, 2019 used to identify a potential perennial reach. The reach begins at the marked “spring” and flows north for approximately 800 feet. Downstream of the indicated spring (B) water was present and fish were abundant in October 2021. Upstream (C) the stream bed was dry, and abundant plant growth indicated substantial flow has been absent for a large amount of time. Below the spring, there was active flow (D). In the center of image D, our temperature logger is visible, anchored to a boulder with a rock-climbing bolt and chain.

short drops or shallow riffles (expected under runoff conditions) were used to demarcate reach termini, and lengths were held between 300 and 500 stream-feet. Within each reach, we placed an Onset MX2203 temperature logger to record temperature throughout the season. Additionally, these loggers have the ability to log whether they are in water or air, so they will be able to collect data on whether reaches remain wet or dry throughout seasons. We attempted to place loggers in locations that would best indicate whether water was flowing if they were wet (we avoided deep pools that would remain wet well after flow stopped, and shallow riffles that may register as dry if only a rivulet of flow existed. Loggers will be downloaded annually.

A survey of each reach (assuming they are wet) will be completed prior to runoff, on the descending limb of runoff, and in fall. We will conduct depletion sampling using multiple passes (at least two) to achieve adequate depletion to estimate abundance. We will track population size and demographics over time for a total of three years. Additionally, we will scan all fish captured

for a PIT tag, and use recapture data to determine whether there is movement among reaches and analyze annual survival.

RESULTS AND DISCUSSION

From satellite imagery, we identified over 13 candidate locations for further investigation. In September – November 2021, we visited seven suspected perennial reaches in the Roubideau and Escalante creek drainages. Above-average precipitation occurred during summer 2021, but all intermittent streams we visited were in fact completely dry for most of their lengths. Six of the seven specific sites visited were wet and active flow was observed. We established one wet and two dry sampling reaches on Cottonwood Creek, and three wet and three dry reaches on the Dry Fork of Escalante Creek (Table X).

Table 3: Sampling sites in Dry Fork Escalante and Cottonwood creeks. Both creeks are primarily intermittent but have short reaches of perennial flow. Baseflow conditions indicate whether the site has flowing water during summer baseflow conditions. In 2022, sites were sampled two to three times. For each sampling occurrence, condition indicates whether flowing water was present, and detected species are listed under SPP present.

	Reach	Baseflow condition	Pre-Runoff (March)		Post-Runoff (June)		Fall (November)	
			Condition	SPP present	Condition	SPP present	Condition	SPP present
Dry Fork Escalante	DED1	Dry	Dry	-	Wet	None	Wet	None
	DEW1	Wet	Wet	SPD	Wet	SPD	Wet	None
	DEW2	Wet	Wet	BHS, SPD	Wet	BHS, SPD	Wet	None
	DED2	Dry	Dry	-	Wet	SPD	Wet*	-
	DEW3	Wet	Wet	BHS, SPD	Wet	BHS, SPD	Wet	BHS, SPD
	DED3	Dry	Dry	-	Wet	None	Dry	-
Cottonwood	CCD1	Dry	Wet	None	-	-	Dry	-
	CCW1	Wet	Wet	BHS, FMS, FXB, SPD	-	-	Wet	BHS, FMS, SPD
	CCD2	Dry	Dry	-	-	-	Dry	-

In 2022, we visited all nine sites at least twice. All sites were visited before spring runoff, and in the fall. Additionally, all Dry Fork sites were visited during the receding limb of runoff, but heavy rains made the Cottonwood sites inaccessible due to the steep, off-trail hike. In Dry Fork, only BHS, and SPD were found as in past years sampling. In Cottonwood Creek, FMS and a FXB were also found. Pre-runoff, all site flow conditions (flowing vs. dry) matched our original characterization of condition at base summer flow, with the exception of CCD1 which was flowing. Post-runoff, all Dry Fork sites were flowing as expected. In fall, many of the typically dry sites were flowing, likely due to summer monsoons elevating groundwater levels. Pre and

post-runoff, sites characterized as wet at base levels were all occupied by fish, the DEW1 only had SPD. Post-runoff, SPD had moved into DED2, though BHS were not detected. The other "dry" sites were not occupied by fish. In fall, we observed fewer fish in general in Dry Fork, and only DEW3 was occupied. There had been heavy sedimentation of the stream below Tatum Draw which flows into Dry Fork at DED2. This occurred due to a flash flood resulting from monsoonal rain. Much of the previously occupied pool habitat was completely filled with fine silt. After another year of sampling, we will analyze population size shifts, individual movement, and site intermittency.

ACKNOWLEDGMENTS
CPW aquatic technicians John Fesenmaier and Gwen Harris assisted with sampling, exploration, and logger deployment.

TECHNICAL ASSISTANCE AND COLLABORATIONS

- Collaborated with CPW Research Scientist/Toxicologist Tawni Riepe on the design and implementation of a thermal tolerance study on larval BHS. Collected wild spawn for this project, and participated in manuscript preparation.
- Provided data to Sophia Bonjour at Kansas State University, and participated in the preparation of a manuscript comparing FMS migration timing at different locations.
- Collected streamflow data in the Roubideau Creek drainage and shared with Colorado Water Conservation Board and CPW's Water Section to aid in instream flow studies. Shared local knowledge of flow timing, biological connections, and field work travel routes.
- Participated in the planning process for the eventual construction of a permanent Fish sorting structure on Roubideau Creek based on information learned during the RBW study.
- Dolores River investigations:
    - Assisted Dan Cammack (CPW Aquatic Conservation Biologist) with PIT tagging Three-Species in the Dolores and San Miguel rivers for ongoing movement studies.
    - Assisted Dan Cammack, Eric Gardunio, and BLM biologist Russ Japuntich with investigations into Smallmouth Bass removal options. Attempted to estimate abundance and conduct localized removals with electrofishing, netting, trapping, and angling.
- Participated in non-native fish removals on the White River with Jenn Logan.
- Maintained stream temperature loggers at tributary and mainstem sites in the Dolores, Gunnison, and White river basins to continue the long-term dataset that has been collected at those sites. Data from these loggers is used by CDPHE to support updates to the 303(d) Impaired Waters List.
- Sampled Milk Creek (Yampa drainage) for larval sucker, and provided sampling advice to BLM.
- Participated in Rio Grande sucker and chub surveys in the San Luis Valley with CPW and the US Fish and Wildlife Service.

- Provided an SPR and helped deploy it in Deep Creek (San Miguel drainage) to show that a recently constructed culvert intended to be a fish barrier was not preventing brown trout from entering cutthroat habitat.
- Worked with Trout Unlimited (TU) to monitor the fishery and physical conditions of the warm water section of Escalante Creek. Continued to develop a plan to assess effects of the improvements.
- Discussed North Fork Gunnison River fish passage projects aimed at improving the Three-Species fishery. Provided data, and developed a movement monitoring plan.
- Provided updates on CPW's Three-Species research progress to the Desert Fishes Council and Three-Species working group.

APPENDIX:

Distribution of genetic variation and hybrids of Catostomid fishes among sub-basins of the Colorado River

Prior to Aquatic Research Scientist Kevin Thompson's retirement in 2021, he and Kevin B. Rogers collaborated on a manuscript headed up by Evan Carson of University of New Mexico, Biology Department and Museum of Southwestern Biology and seven other authors. This study used nuclear (microsatellite) and mitochondrial (ND2) DNA to explore genetic variation in Catostomid populations across the Colorado River basin. Manuscript submission fulfilled contract obligations, but the paper was not accepted despite containing valuable genetic information. The associate editor for Transactions of the American Fisheries Society suggested (among other things) that authors would need to *revise this manuscript to defend the genetic approaches taken, especially with regard to the sensitivity of markers. In revising the manuscript, the authors need to comment on the fact that the genetic data used (6 microsatellites) had much lower resolution (vs. >10,000 SNPs) than that used in other recent studies.*

Shortly after receiving these recommendations for revision, the first three authors accepted new jobs outside the area or retired. Since it is uncertain that the paper will be resubmitted in its current form – particularly now that new molecular tools with much higher resolution for nuclear markers are available, we felt it important to include the following with this Three-Species Annual Report. It is likely that this topic will be revisited with more informative genetic tools in the future, but until then, these results may still be useful to fisheries managers. We advise that these results likely underestimate diversity among populations, and that should be carefully considered before actions are taken that could influence established population genetics.

The following has been prepared for this report by Kevin Rogers, and Zachary Hooley-Underwood.

Distribution of genetic variation and hybrids of Catostomid fishes among sub-basins of the Colorado River

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Abstract – Conservation of catostomid fishes of the Colorado River basin is complicated because it is necessary to retain genetic diversity and population genetic structure of native species while simultaneously eliminating hybridization and introgression from nonnatives. We used mitochondrial DNA sequences and microsatellite DNA markers to evaluate population structure and incidence of hybridization among native (Bluehead Sucker *Catostomus discobolus*, Flannelmouth Sucker *C. latipinnis*, Mountain Sucker *C. platyrhynchus*, and Razorback Sucker *Xyrauchen texanus*) and non-native (White Sucker *C. commersonii* and Longnose Sucker *C. catostomus*) suckers in six drainages (Colorado, Dolores, Gunnison, San Juan, White, and Yampa) of the Western Slope of Colorado. Six microsatellite loci differentiated species and identified interspecific hybrids, though accurate assignments to specific crosses (e.g., Bluehead x Flannelmouth) were limited to F_1 hybrids. Analyses of landscape-scale distribution of genetic variation in Bluehead and Flannelmouth suckers revealed significant population genetic structure among sub-basins for mtDNA and low but significant levels of differentiation for microsatellites. These findings are important for establishment of broodstocks from wild populations for future hatchery propagation. Because backcross hybrids can appear indistinguishable morphologically from parental species, genetic screening will be required to manage against inclusion of cryptic hybrids in hatchery stocks. Conservation and management of these native suckers also will benefit from fine-scale assessment of population genetic variation within and among West Slope drainages of the Colorado River basin.

INTRODUCTION

Management of imperiled fishes of western North America often is focused on a combined problem of habitat loss and interaction between native and non-native species. Extensive changes that transform riverscapes for agricultural, urban, and other uses also cause fragmentation and degradation of habitat, which has resulted in reduced connectivity of populations, declines in population size and range, extirpation of populations, and extinction (Platania and Altenbach 1998; Bestgen and Platania 1991; Hopken et al. 2013; Perkin et al. 2015a and 2015b; Gido et al. 2015). Threats from introduced species include competition and predation (Karam and Marsh 2010), as well as hybridization and introgression (Rhymer and Simberloff 1996). Altered environmental conditions associated with climate change (Hopken et al. 2013) might further disrupt natural interactions, including the possible evolutionary importance of introgressive hybridization (Dowling and Secor 1997; Muhlfeld et al. 2017).

Catostomid suckers are a diverse group fishes that have a long history of introgressive hybridization between native forms, many of which are currently threatened or endangered (Unmack et al. 2014; Dowling et al. 2016). With the widespread introduction of non-native suckers to some river systems in western North America, hybridization between native and non-native species also occurs. In the Colorado River basin of western Colorado, hybridization occurs sporadically among native species, including Bluehead Sucker *Catostomus discobolus* (BHS), Flannelmouth Sucker *C. latipinnis* (FMS), Mountain Sucker *C. platyrhynchus* (MOS), and Razorback Sucker *Xyrauchen texanus* (RBS), and between native suckers and the non-native White Sucker *C. commersonii* (WHS) and Longnose Sucker *C. catostomus* (LNS). Because advanced backcrosses and other hybrids (e.g., F_2, F_3, etc.) often are similar morphologically to parental species, identification of these hybrids is difficult in the field, where most identifications are made. Consequently, little is known about the extent introgressive hybridization in these species. Molecular phylogenetic and population genetic based analyses can resolve much of this uncertainty. For selection of broodstock for hatchery-based supplementation of populations subject to hybridization in the wild, such genetic assessment is crucial for maintaining evolutionary potential (genetic diversity) of populations and for minimizing inclusion of hybrids in the captive populations.

We used mtDNA sequences and microsatellite DNA markers to assess the distribution of genetic variation and the incidence of hybridization among native (Bluehead, Flannelmouth, and Mountain) and non-native (Longnose and White) suckers in six Western Slope drainages of Colorado. By evaluating introgressive hybridization within phylogenetic and landscape-scale contexts, this study advances management of wild and broodstock populations of native suckers of the Colorado River basin of western Colorado.

METHODS

Field sampling

A total of 834 catostomid suckers were collected, including Bluehead Sucker (n = 274), Desert Sucker *C. clarkii* (DES; n = 20), Flannelmouth Sucker (n = 241), Longnose Sucker (n = 51), Mountain Sucker (n = 83), Utah Sucker *C. ardens* (UTS; n = 10), White Sucker (n = 96), and hybrids (n =59). Collection sites for Bluehead and Flannelmouth suckers included six tributaries (Colorado, Dolores, *Gunnison*, San Juan, White, and Yampa) of the Western Slope of Colorado.

Samples of Longnose and White suckers were obtained from a combination of sites in Eastern Slope (native range) and Western Slope (non-native range) drainages of Colorado. Desert Sucker samples were obtained from the native range of the species in Utah and Nevada, and those for Utah Sucker from the native range in Utah. Samples were obtained by excising a small portion of tissue from the upper lobe of the caudal fin and preserving the sample in a 4-mL screw-cap vial filled with 80% EtOH. Each vial was labeled with a unique identification code at the time of collection. After collection of each specimen, scissors were dipped in alcohol and flamed to prevent foreign DNA from contaminating the next specimen. After collection, specimens were maintained in a refrigerator until shipped to a genetics laboratory (Pisces Molecular, Boulder, Colorado) for processing. Genomic DNA was isolated from fin clips by using the entire fin clip or an approximate 3 mm^2 subsample. Total DNA was extracted from all samples by using a spin-column DNA purification procedure (Qiagen DNeasy Blood and Tissue Kit Cat# 69506) according to the manufacturer's instructions.

Characterization of mtDNA

Mitochondrial DNA sequence variation was assessed at a 648 bp fragment of the mitochondrial ND2 gene. Polymerase chain reaction (PCR) amplifications were conducted using 20 µl reaction volumes that included 0.2 µl (5 U/µL) AmpliTaq® DNA Polymerase (Cat# N808-0156 Applied Biosystems™); 2 µl GeneAmp® 10X PCR Buffer II (Cat# N808-0156Applied Biosystems™); 1.2 µL (25 µM) $MgCl_2$; 0.5 µL (0.25 µM), each, of forward and reverse primers; 1.6 µL (800uM) dNTPs; 12 µL ddH2O; and 2-µL (1-4 ng) DNA. Thermal parameters were 94°C for 2 min followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 75 s.

Phylogenetic relationships of haplotypes were established in MEGA7 (Kumar et al. 2016) using a maximum likelihood approach (Tamura and Nei 1993); six in-group species (Bluehead, Desert, Flannelmouth, Mountain, Utah, and White suckers) and one outgroup species (Longnose Sucker) were included. Evolutionary distance over sequence pairs between groups was calculated with a Maximum Composite Likelihood model (Tamura et al. 2004) as implemented in MEGA7. For collections of Bluehead and Flannelmouth suckers, number and diversity of mtDNA haplotypes were determined with Arlequin version 3.5 (Excoffier and Lischer 2010); to correct for differences in sample size among collections, number of haplotypes was adjusted by rarefaction (N_{HR}), as implemented in Analytic Rarefaction 1.3 (Holland 2003). Homogeneity of haplotype distributions was evaluated with exact tests and analysis of molecular variance (AMOVA), as implemented in Arlequin. Pairwise estimates of Φ_{ST}, an analogue of F_{ST}, were generated using Arlequin and with significance determined by exact tests (Raymond and Rousset 1995; Goudet et al. 1996). Tests of significance were adjusted using the B-Y method (Narum 2006).

Characterization of microsatellites

Genetic variation was surveyed at six tetra-nucleotide microsatellite loci developed by Tranah et al. (2001; *Dlu409, Dlu456, Dlu482, Dlu4184, Dlu4235,* and *Dlu4300*). Assorted PCR conditions and thermal parameters were used for the six loci, with a two-step protocol used for *Dlu456, Dlu482,* and *Dlu4184* and a three-step protocol used for *Dlu409, Dlu4235, and Dlu4300*. The thermal parameters and annealing temperatures for the two-step protocol were as follows: 98°C for 30 s, 35 cycles of 98°C for 8 s, (71°C for 15 s for *Dlu4184*) or (69°C for 15 s for *Dlu482* and *Dlu456*), followed by 72°C for 10 min. The three-step protocol was as follows: 98°C for 30s, 35 cycles of 98°C for 8s, (64°C for 15s for *Dlu409*), or (66°C for 15s for *Dlu4235*), or (56°C for 15s

for *Dlu4300*), 72°C for 15 s, then followed by 72°C for 10 min. Amplifications were conducted in 20 µl PCR reactions that included 0.2 µl (2 U/µL) Phusion Hot Start II® DNA Polymerase (Cat#F549L New England BioLabs); and 4 µl 5x Phusion HF Buffer (Cat#F549L New England BioLabs); 0.5 µL [0.25 µM] M13- Oligo (FAM, HEX or NED); 1 µL [0.5 µM], each, of forward and reverse primers; 1 µL [500uM] dNTPs; 10.3 µL ddH2O; and 2-µL [1-4 ng] DNA.

For each of the six loci, the 5' end of the forward primer was extended with a florescent (FAM, HEX, or NED) M13 tail; sequencing runs were in triplex (two panels). All PCR products, each labeled with a different fluorescent dye, were diluted into Molecular Biology grade H_2O to normalize the fluorescence signals across three dyes in the two dilution panels and sequence runs: Panel A1= *Dlu409*-FAM, *Dlu4235*-HEX, *Dlu4184*-NED, and Panel A2= *Dlu482*-FAM, *Dlu456*-HEX, *Dlu4300*-NED. Each panel was run on an ABI 3130 Genetic Analyzer, using a 36cm array, and POP7 polymer. Sequencing capillary-electrophoresis was performed on each panel solution of 1 ul of the combined post-amplification reaction dilutions (three loci; FAM, HEX, and NED), 0.5 µL ROX500 size standards (Gel Company, San Francisco, CA), and 8.95 ul Hi-Di™ Formamide (Applied Biosystems™). Genotypes were scored using GENEIOUS v6.1.8, microsatellite plug-in, v1.4.0 (Biomatters Ltd, Auckland, NZ). Fragment sizes were normalized (binned) across all samples, as implemented in Tandem (Matschiner and Salzburger 2009).
The program STRUCTURE 2.3.4 (Pritchard et al. 2000; Falush et al. 2003; Hubisz et al. 2009) was used to evaluate genetic distinctiveness and incidence of hybridization among catostomid species. The following parameters were used: 200,000 iterations plus a 25% burn-in, correlated allele frequencies, and species information included as priors. This analysis used five genetic markers, as one locus, *Dlu4300*, failed to amplify for Longnose Sucker. The number of genetic clusters (*K*) was set to seven to reveal distinctness among species and permit identification of putative hybrids. Individuals were considered as genetic hybrids if >10% of allelic composition was from each of two or more species (Dakin et al. 2015).

Genetic variation within populations of Bluehead and Flannelmouth suckers was evaluated using average number of alleles per locus (N_A), gene diversity (H_E, expected heterozygosity), observed heterozygosity (H_O), and F (inbreeding coefficient), as calculated in GENALEX 6.5 (Peakall and Smouse 2006; 2012), and allelic richness (A_R), as calculated in FSTAT version 2.9.3.1 (Goudet 1995). Homogeneity of allelic variation among drainages was evaluated using global and pairwise estimates of F_{ST}, as implemented in ARELEQUIN; tests of significance were adjusted using the B-Y method (Narum 2006). Population structure was evaluated using AMOVA, as implemented in ARELEQUIN, and STRUCTURE. For analyses in STRUCTURE, parameters were as described above except the number of genetic clusters was evaluated following the method of Evanno et al. (2005). The low level of allelic variation among basins required use of individuals from an outgroup to appropriately calculate ΔK, the number the genetic clusters. For analysis of population structure of Bluehead Sucker, Flannelmouth Sucker served as the outgroup, and vice versa.

RESULTS

Characterization of mtDNA

Phylogenetic relationships of haplotypes (Figure A-1) and genetic distances between species-pairs (Table 1) delineated species into six lineages, of which five were represented by single

species (Desert, Flannelmouth, Longnose, Utah, White suckers) and one was represented by two species (Bluehead and Mountain suckers). Haplotypes of Bluehead and Mountain suckers were indistinguishable; for the purposes of this study, these were considered as haplotypes of Bluehead Sucker.

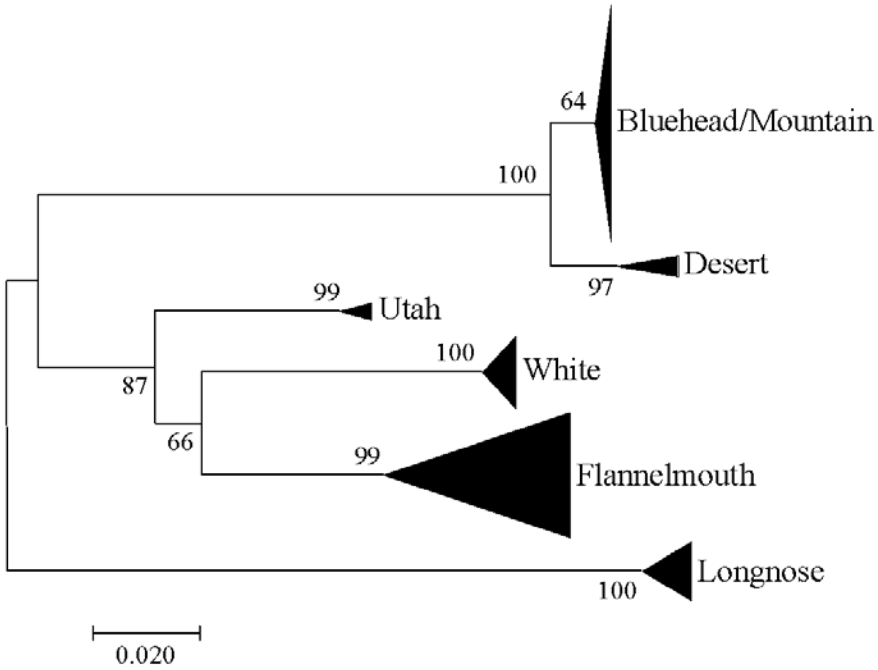


Figure A-1. Maximum Likelihood tree based on 150 nucleotide sequences covering 648 base pairs of the mitochondrial ND2 gene in suckers from across the southern Rocky Mountains. The tree with the superior log-likelihood is shown with branch lengths measured in the number of substitutions per site. Percent branch support was evaluated with 1000 bootstrap pseudo-replicates.

Table A-1. Pairwise sequence divergence in the mitochondrial ND2 gene for Bluehead (BHS), Flannelmouth (FMS), Longnose (LNS), Mountain (MOS), and White (WHS) sucker. Haplotypes from Bluehead and Mountain suckers were not distinguishable, and were therefore combined.

Species	BHS/MOS	DES	FMS	LNS	UTS
BHS/MOS					
DES	0.039				
FMS	0.209	0.215			
LNS	0.243	0.264	0.215		
UTS	0.175	0.192	0.106	0.189	
WHS	0.176	0.185	0.113	0.208	0.098

Maternal lineages (Figure A-1) of samples were generally consistent with field-based morphological identification for Bluehead, Flannelmouth, and hybrid suckers. Among 274 fish identified by phenotype as Bluehead Sucker, 56 haplotypes were observed, including 54 from the Bluehead Sucker mitochondrial lineage and 1 each from the Desert Sucker and White Sucker

lineages. Of the 241 fish identified by phenotype as Flannelmouth Sucker, 35 haplotypes were observed, including 31 from the Flannelmouth Sucker mitochondrial lineage, one from the Bluehead Sucker lineage, and three from the White Sucker lineage. All 59 suckers identified by phenotype as hybrid also were identified by genotype as hybrid. In approximately 70% (n = 41) of these, morphological identification to parental cross (e.g., BHS x FMS) was identical to that based on assignment from microsatellites and was consistent with the maternal lineage (ND2). The remaining 18 exhibited mismatches between phenotype and genotype, including 12 in microsatellite genotype included a parental species that differed from that indicated by phenotype or mtDNA lineage; four that carried an mtDNA lineage that was consistent one parental species identified by microsatellite genotype but inconsistent with identification based on phenotype; one had a mtDNA lineage that was consistent with putative parents based on phenotype but inconsistent with putative parents based on microsatellites; and one had a mtDNA lineage that was inconsistent with putative parents based on morphology and microsatellites. Most hybrids carried a White Sucker mitochondrial lineage (n = 31), followed by Flannelmouth (n = 13), Bluehead (n = 9), Longnose (n = 4), and Desert (n = 2) sucker lineages.

Additionally, 21 suckers that were identified to species based on phenotype were genetically hybrid based on microsatellite genotype, mtDNA haplotype, or both. Of these, one was identified by phenotype and microsatellite genotype as Flannelmouth Sucker but carried a White Sucker mtDNA haplotype. The remaining 20 hybrids were identified by analysis of microsatellite genotypes in STRUCTURE. Seven of these possessed microsatellite genotypes that were consistent with hybrids between the parental species identified by phenotype and the parental species identified by mtDNA haplotype, including two F1 crosses and five post-F1 hybrid ancestry; all but two possessed mtDNA of White Sucker origin. Eight of the 20 specimens were identified as post-F1 hybrid (microsatellite genotype) but possessed mtDNA haplotypes that were concordant with phenotype (e.g., Bluehead Sucker phenotype and mtDNA lineage). The remaining five individuals were identified as multispecies hybrids but included putative parental species identified by phenotype and mtDNA haplotype; difficultly in discriminating hybrid cross may result from the low number of number of loci available for study rather than errant identification of hybrid individuals.

Summary statistics for variation in ND2 sequences of BHS and FMS are shown by drainage in Table 2. For BHS, the number of haplotypes ranged from 8 (White River) to 22 (Dolores and San Juan rivers); haplotype diversity from 0.789 (White River) to 0.931 (Dolores River). Corrected number of haplotypes, N_{HR}, ranged from 7.8 (White River) to 14.5 (Dolores River). For FMS, the number of haplotypes ranged from 7.0 (San Juan River) to 14 (Colorado River) and haplotype diversity ranged from 0.695 (San Juan River) to 0.883 (White River); N_{HR} ranged from 5.7 (San Juan River) to 11.2 (White River). Global Φ_{ST} was significant in BHS (Φ_{ST} = 0.073; $P < 0.0001$) and in FMS (Φ_{ST} = 0.025; P = 0.001). Before correction for simultaneous tests, pairwise estimates of Φ_{ST} were significant in 10 of 15 comparisons for BHS and in 8 of 15 comparisons for FMS. After B-Y correction, pairwise estimates of Φ_{ST} were significant in 8 comparisons for Bluehead Sucker (Table 3) and in 2 comparisons in Flannelmouth Sucker (Table 3).

Table A-2. Summary statistics for six populations of Bluehead Sucker and Flannelmouth Sucker, where N is number of samples, N_H is number of haplotypes, N_{HR} is number of haplotypes corrected, by rarefaction, for differences in sample size among collections, and H_D is haplotype diversity. The 95% confidence intervals of N_{HR} are shown in parentheses.

Bluehead Sucker	N	N_H	N_{HR}	H_D
Colorado	59	19	11.7 (8.5-14.8)	0.873
Dolores	56	22	14.5 (11.4-17.6)	0.931
Gunnison	25	11	11	0.830
San Juan	74	22	12.8 (9.5-16.1)	0.887
White	27	8	7.8 (7.1-8.6)	0.789
Yampa	33	11	10.1 (8.5-11.7)	0.886

Flannelmouth Sucker	N	N_H	N_{HR}	H_D
Colorado	58	14	9.6 (6.8-12.4)	0.817
Dolores	61	13	8.8 (6.1-11.5)	0.697
Gunnison	30	11	11	0.782
San Juan	31	7	6.9 (6.3-7.5)	0.695
White	30	12	12	0.883
Yampa	31	9	9.0 (8.6-9.3)	0.841

Table A-3. Estimates of pairwise Φ_{ST} for populations of Bluehead Sucker and Flannelmouth Sucker from six major drainages. Values shown in bold indicate statistical significance after B-Y correction ($\alpha \leq 0.05$).

Bluehead Sucker	Colorado	Dolores	Gunnison	San Juan	White	Yampa
Colorado						
Dolores	**0.0306**					
Gunnison	-0.0049	0.0259				
San Juan	0.0145	**0.0358**	0.0146			
White	**0.0972**	0.0431	**0.1676**	**0.1600**		
Yampa	0.0270	0.0243	**0.0597**	**0.0331**	**0.1226**	

Flannelmouth Sucker	Colorado	Dolores	Gunnison	San Juan	White	Yampa
Colorado						
Dolores	0.0062					
Gunnison	-0.0159	-0.0082				
San Juan	0.0128	-0.0108	-0.0026			
White	0.0218	0.0266	0.0185	**0.1053**		
Yampa	0.0435	0.0463	0.0042	**0.0666**	0.0585	

Characterization of microsatellites

Summary statistics of variation among six microsatellite DNA loci are presented in Table A-4. For Bluehead Sucker, mean number of alleles (N_A) over 6 loci ranged from 17.2 to 24.8 and allelic richness (A_R) ranged from 16.7 to 21.1 (Table 4). Gene diversity (H_E) for BHS was similar

across all subpopulations (mean = 0.939). Mean observed heterozygosity (H_O) was slightly lower at 0.869, which indicated fewer heterozygotes than expected per population. For Flannelmouth Sucker, mean number of alleles ranged from N_A = 15.8 to 21.3. After adjustment for sample size, diversity was similar across all subpopulations (A_R = 15.6 to 17.6). Observed and expected heterozygosity were similar across all subpopulations (mean H_O = 0.852 and mean H_E = 0.869).

Table A-4. Summary statistics for Bluehead Sucker and Flannelmouth Sucker populations in six major drainages, where N is mean of samples across loci, N_A is mean number of alleles per locus, A_R is allelic richness, H_O is observed heterozygosity, H_E is expected heterozygosity (gene diversity), and F is the fixation index.

Bluehead Sucker	N	N_A	A_R	H_O	H_E	F
Colorado	58.7	24.8	19.7	0.850	0.943	0.100
Dolores	56.0	23.8	18.8	0.869	0.938	0.074
Gunnison	25.0	20.8	20.8	0.867	0.943	0.082
San Juan	73.8	24.5	18.8	0.889	0.938	0.053
White	27.0	17.2	16.7	0.877	0.923	0.051
Yampa	31.8	23.3	21.1	0.861	0.939	0.097

Flannelmouth Sucker	N	N_A	A_R	H_O	H_E	F
Colorado	56.8	19.7	16.3	0.861	0.872	0.013
Dolores	59.7	21.3	17.2	0.848	0.876	0.033
Gunnison	30.0	17.8	17.6	0.867	0.876	0.011
San Juan	30.8	15.8	15.6	0.822	0.837	0.017
White	29.8	17.0	16.9	0.877	0.877	-0.006
Yampa	30.7	16.3	16.0	0.838	0.875	0.045

In general, Bayesian estimation of genetic composition was congruent with initial identification based on morphological characteristics (Figure A-2). Twenty-five (<3%) individuals identified from morphology as one species were identified by genotype as hybrid between that species and a second species. Complete mismatch of identification from morphology and from genotype occurred in two specimens, which were identified as Mountain Sucker, based on phenotype, and as Flannelmouth x White sucker, based on genotype. These mismatches represented <0.02% of the sample and possibly were recorded in error during collection. Among fish identified in the field as hybrids, all (n = 59) exhibited genetic characteristics consistent with hybridization. Most (n = 41) had a genotype that was consistent with an F_1 cross between the parental species indicated by morphology of the specimen. Genotypes of the 18 remaining hybrids indicated that these individuals possibly were backcrosses.

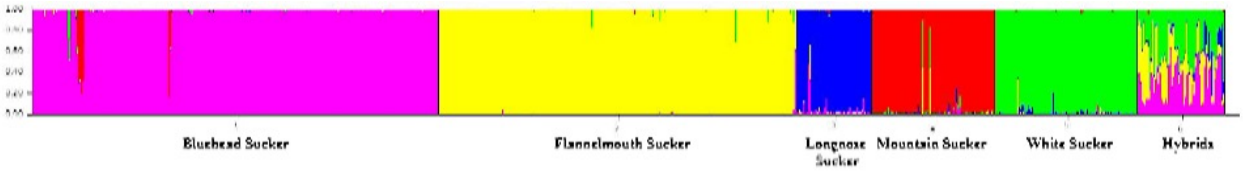
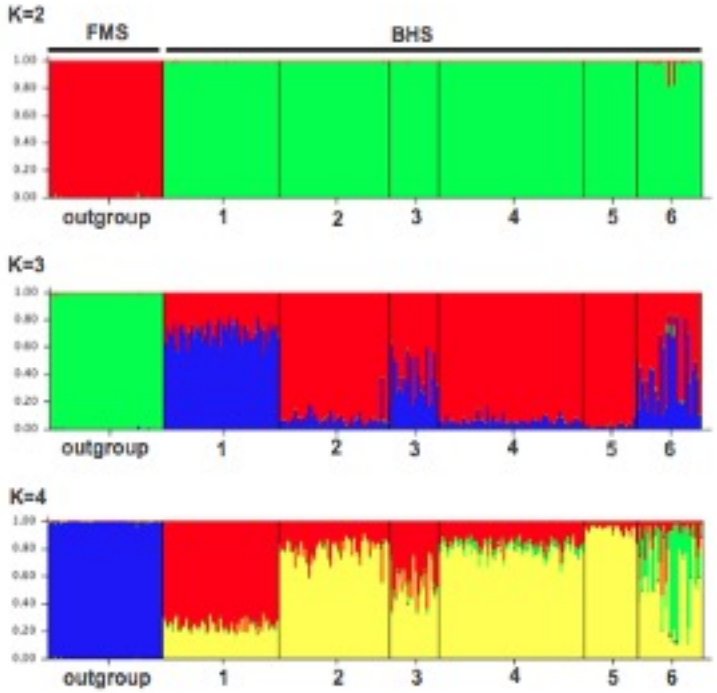


Figure A-2. Genetic clustering among five catostomid suckers (Bluehead, Flannelmouth, Longnose, Mountain, and White) and their hybrids from across the Colorado River basin. Species assignments based on morphological characteristics and genetic assignments (clustering) based on analysis, in STRUCTURE, of allelic variation at five microsatellite DNA loci.

STRUCTURE revealed little differentiation among populations of Bluehead and Flannelmouth suckers from the six drainages (Figure A-3, A-4). Using the method of Evanno et al. (2005), the relative change in likelihood scores (ΔK) recovered a single genetic cluster within each species. Differences in allele frequency were evident among drainages, however, as suggested by results for successive values of genetic clusters (i.e., K = 3; Figure A-4 and K = 4; Figure A-5). Global F_{ST} was significant in BHS (F_{ST} = 0.073; P < 0.0001) and in FMS (F_{ST} = 0.025; P = 0.001). Before correction for simultaneous tests, pairwise estimates of F_{ST} were significant in 13 of 15 comparisons for BHS and in 13 of 15 comparisons for FMS. After B-Y correction, pairwise estimates of F_{ST} were significant in 12 comparisons for BHS (Table A-5) and in 11 comparisons



in **Figure A-3.** STRUCTURE results indicating population structure for K = 2, 3, or 4 genetic clusters for six populations of Bluehead Sucker (BHS) in: 1, Colorado; 2, Dolores; 3, Gunnison; 4, San Juan; 5, White; and 6, Yampa rivers; Flannelmouth Sucker (FMS) was used as the outgroup.

FMS (Table 5). Similarly, results from AMOVA revealed low but significant ($P < 0.001$) levels of genetic divergence among subpopulations for both species. Global estimates for F_{ST} were 0.009 for Bluehead Sucker and 0.012 for Flannelmouth Sucker, with most of the variation distributed within populations in both species.

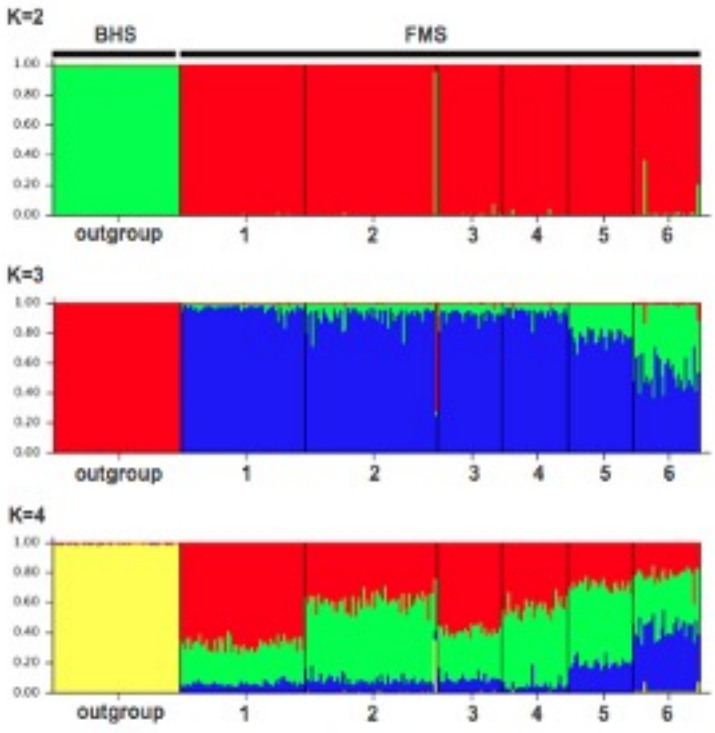


Figure A-4. STRUCTURE results indicating population structure for $K = 2$, 3, or 4 genetic clusters for six populations of Flannelmouth Sucker (FMS) in: 1, Colorado; 2, Dolores; 3, Gunnison; 4, San Juan; 5, White; and 6, Yampa rivers; Bluehead Sucker (BHS) was used as the outgroup.

Table A-5. Estimates of pairwise F_{ST} for six populations of Bluehead Sucker and Flannelmouth Sucker. Values in bold indicate statistical significance after B-Y correction ($\alpha \leq 0.05$).

Bluehead Sucker	Colorado	Dolores	Gunnison	San Juan	White	Yampa
Colorado						
Dolores	**0.0093**					
Gunnison	0.0058	**0.0076**				
San Juan	**0.0127**	**0.0065**	**0.0079**			
White	**0.0191**	**0.0096**	**0.0150**	**0.0166**		
Yampa	**0.0075**	0.0018	0.0022	**0.0060**	**0.0120**	

Flannelmouth Sucker	Colorado	Dolores	Gunnison	San Juan	White	Yampa
Colorado						
Dolores	**0.0102**					
Gunnison	0.0007	**0.0080**				
San Juan	**0.0188**	**0.0057**	**0.0165**			
White	**0.0103**	0.0062	0.0034	**0.0169**		
Yampa	**0.0183**	**0.0173**	**0.0206**	**0.0267**	0.0080	

DISCUSSION

Conservation management of catostomid fishes aims to maintain the genetic diversity, evolutionary potential, and distinctiveness of native species and populations. Because catostomids have a propensity to hybridize (Hubbs 1955), conservation efforts for these species must account for natural hybridization dynamics of native suckers and mitigate hybridization with non-native suckers. This concern is heightened when management efforts include hatchery-origin supplementation of wild populations, as occasionally occurs for native suckers of the West Slope of Colorado. In these cases, detection of hybrid individuals is crucial to avoid populating hatchery stocks with introgressed individuals, especially with ones between native and non-native species. The difficulty of using field-based morphological assessments to detect, with certainty, all advanced generation hybrids means that genetic screening often is required to distinguish these individuals from parental species. Our study used mitochondrial and microsatellite DNA markers to evaluate landscape-scale distribution of genetic variation in Bluehead and Flannelmouth suckers, and to identify hybrids from crosses involving native (Bluehead, Flannelmouth, and Mountain) and non-native (Longnose and White) suckers in six Western Slope drainages of Colorado.

At the landscape-scale (i.e., among rivers), the distribution of genetic variation in Bluehead and Flannelmouth suckers was consistent with findings of Shiozawa et al. (2003). Significant differentiation at mtDNA (global Φ_{ST}) and microsatellites (global F_{ST}) was observed for both species. For Bluehead Sucker, most pairwise (between rivers) comparisons were significant for Φ_{ST} and F_{ST}, whereas, for Flannelmouth Sucker, this was true only for F_{ST}. This difference is

consistent with the relatively high level of mtDNA diversity and greater number of unique (to river) haplotypes observed in samples of Bluehead Sucker. The low but significant differentiation observed at microsatellites indicates that consideration of population structure is important to management of both species. A more fine-scaled assessment of population genetic diversity and structure is warranted but will require additional loci, larger sample sizes, and collections designed to test for micro-geographic structure within drainages.

With respect to hybrids, five microsatellite loci were sufficient to differentiate among five sucker species and to identify interspecific hybrids, including F_1 and backcross individuals. Individuals that were identified as hybrid based on morphology also were identified as hybrid based on genotype, and F_1 genotypes were congruent with cross as determined from morphology (e.g., Bluehead x Flannelmouth). Some individuals identified genetically as hybrid were, however, identified to species (e.g., Bluehead) based on morphology. Most of these individuals exhibited genotypes that were consistent with backcrossing. These specimens exemplify the challenges of identifying hybrids based on morphology alone because some hybrids, particularly advanced backcrosses, can appear indistinguishable morphologically from parental species (Arnold 1997). This can occur, in part, because mixing of genes can decouple morphology and neutral markers (Gerber et al. 2001).

This study provides important information for managers to mitigate the negative effects of introgressive hybridization in native Bluehead and Flannelmouth suckers of the Upper Colorado River basin. Although the extent and outcomes of hybridization among these species are known to differ substantially among locations (Mandeville et al. 2017), introgression is of increasing concern, particularly with respect to introgression from the introduced White Sucker (Mandeville et al. 2015). This threat also is not limited to direct effects of hybridization in the wild, as inclusion of hybrids in hatchery broodstocks could, through supplementation, amplify introgression in wild populations. This risk to wild populations, while focused on increases in hybrids between native and non-native species (Mandeville et al. 2015), also could have unintended effects on the natural dynamics of hybridization between Bluehead and Flannelmouth suckers if hybrids between these native species are included in broodstocks. To maintain genetic diversity and evolutionary potential of native suckers, hatchery-based supplementation programs must include genetic screening to manage against unintended inclusion of hybrids in captive broodstocks.

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