Sport Fish Research Studies

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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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BELLVUE FISH RESEARCH HATCHERY PRODUCTION AND RESEARCH UPDATES

The Hofer (GR or HOF; used interchangeably throughout) strain of Rainbow Trout *Oncorhynchus mykiss* is resistant to whirling disease (*Myxobolus cerebralis*), and has been incorporated into Colorado's hatchery program for both stocking into recreational fisheries and for crossing with other wild strains of Rainbow Trout to increase *M. cerebralis* resistance. A GR brood stock is maintained at the Colorado Parks and Wildlife (CPW) Bellvue Fish Research Hatchery (BFRH; Bellvue, Colorado) for both research and stocking purposes. The BFRH also rears and distributes other Rainbow Trout strains, species of salmonids, and Boreal Toads *Anaxyrus boreas* for research and management purposes as needs arise. Additional sport fish research projects are conducted at the BFRH annually.

WHIRLING DISEASE RESISTANT RAINBOW TROUT BROOD STOCK PRODUCTION

The *M. cerebralis*-resistant Rainbow Trout brood stocks reared at the BFRH are unique, and each requires physical isolation to avoid unintentional mixing of stocks. Extreme caution is used during on-site spawning operations and throughout the rearing process to ensure complete separation of these different brood stocks. All lots of fish are uniquely fin-clipped and most stocks are individually marked with Passive Integrated Transponder (PIT) and/or Visible Implant Elastomer (VIE) tags before leaving the main hatchery. This allows for definitive identification before the fish are subsequently used for spawning.

Starting in mid-November 2023, BFRH personnel checked all of the two- and three-year-old GR brood fish weekly for ripeness. Eggs or milt flowing freely when slight pressure was applied to the abdomen of the fish indicated maturation. The first females usually maturated two to four weeks after the first group of males. As males were identified, they were moved into a separate section of the raceway to reduce handling and fighting injuries. On November 7, 2023, the first group of GR females were ripe and ready to spawn.

Before each fish was spawned, it was examined for the proper identification (fin clip, PIT, or VIE tag), a procedure that was repeated for each fish throughout the winter. Fish were spawned using the wet spawning method, where eggs from the female were stripped into a bowl along with the ovarian fluid. After collecting the eggs, milt from several males was added to the bowl. Water was poured into the bowl to activate the milt, and the bowl of eggs and milt was covered and left undisturbed for several minutes while the fertilization process took place. Next, the eggs were rinsed with fresh water to expel old sperm, feces, egg shells, and dead eggs. Eggs were poured into an insulated cooler with iodine to water harden for approximately one hour.

Water-hardened fertilized (green) eggs were moved to the BFRH main hatchery building. Upon reaching the hatchery, green eggs were tempered and disinfected (PVP Iodine, Western Chemical Inc., Ferndale, Washington; 100 ppm for 10 min at a pH of 7). Eggs were then put into vertical incubators (Heath Tray, Mari Source, Tacoma, Washington) with five gallons per minute (gpm) of 12.2ºC (54ºF) flow-through well water. The total number of eggs was calculated using number of eggs per ounce (Von Bayer trough count minus 10%) multiplied by the total ounces of eggs. Subsequent daily egg-takes were put into separate trays and

recorded. To control fungus, eggs received a prophylactic flow-through treatment of formalin (1,667 ppm for 15 minutes) every other day until eye-up.

Eggs reached the eyed stage of development after 16 days in the incubator. The eyed eggs were removed from the trays and physically shocked to detect dead eggs, which turn white when disturbed. Dead eggs were removed both by hand and with a Van Gaalen fish egg sorter (VMG Industries, Longmont, Colorado) for two days following physical shock. The total number of good eyed eggs was calculated using the number of eggs per ounce multiplied by total ounces.

The on-site Rainbow Trout production spawn started on November 7, 2023, with the last group of GR females spawned December 5, 2023. A total of 100 females were spawned over this time period. The goal was to produce 1,000 green GR eggs from which 850 eyed eggs would be retained for brood stock replacement purposes, and this goal was met. No eggs were shipped from the BFRH in 2023/2024. All eggs were kept at the BFRH for brood stock purposes.

As of 2022, the BFRH no longer maintains a Harrison Lake (HL) Rainbow Trout brood stock or produces HL eggs. The transfer of all three year classes of HL brood fish to the CPW Poudre Rearing Unit occurred on June 21, 2022. The purpose of the transfer was two-fold: 1) create space at the BFRH for incoming YY Brook Trout *Salvelinus fontinalis*, and 2) increase the Poudre Rearing Unit production using *M. cerebralis*-resistant fish. The two primary brood stocks now maintained at the BFRH are the GR strain and YY Brook Trout (see below).

Annual Disease Testing

The BFRH annual disease inspection was conducted on March 4, 2024. Both the GR and YY Brook Trout brood stocks were tested. All fish were negative for all diseases for which they were tested, including *Renibacterium salmoninarum*, the bacteria causing Bacterial Kidney Disease, which had been present on the unit in 2016 through 2020.

YY Brook Trout Brood Stock Production

The use of supermales, male fish that have two Y-chromosomes, shows promise as an alternative for eradication via mechanical removal or pesticides of nonnative or undesirable wild fish populations (Schill et al. 2017). Development of a YY Brook Trout brood stock has been successful at producing large numbers of fish for stocking (Schill et al. 2016). Simulations suggest that at a 50% annual stocking rate of the age-0 density, combined with a 50% annual selective suppression rate, wild Brook Trout could be extirpated in only 2-4 years, and in most populations, eradication could occur in less than 10 years regardless of the suppression rate (Schill et al. 2017).

Our research hatchery staff works on a number of collaborative projects with hatcheries and researchers throughout the country. One recent project was the development of M_{YY} Brown Trout. Briefly, the female sex chromosomes are XX whereas the male are XY. When spawned as normal, this creates a typical population of roughly 50:50 male and female fish. However, in this process, the fish are exposed to estradiol top-coated feed to get XY males to produce eggs. When spawned with normal XY males, this produces some fish that are YY. Finally, genetic

testing is used to determine which males and females are YY and spawn those fish together. This ultimately results in a monosex male YY broodstock. Working with the YY consortium, we determined the amount of estradiol, and number of days over which to feed the estradiol, to produce XY females used to develop the M_{YY} Brown Trout brood stock (Fetherman et al. 2020, 2021, 2022). This project has been handed over to the New Mexico Game and Fish to allow the BFRH to acquire YY Brook Trout from Idaho and utilize the YY Brook Trout in Colorado.

The first year class of YY Brook Trout was imported on June 27, 2022, and a second year class was imported on August 8, 2023, from the Hayspur Hatchery in Idaho. These two year classes of YY Brook Trout were utilized to spawn the first ever YY Brook Trout progeny for Colorado. These fish will be utilized for production and research projects in Colorado, Arizona, and Wyoming in 2024.

Starting on October 2, 2023, BFRH personnel checked all of the 2022 and 2023 YY Brook Trout brood stock weekly for ripeness. Maturation was indicated by eggs or milt flowing freely when slight pressure was applied to the abdomen of the fish. The majority of the 2022 female YY Brook Trout showed very poor egg quality. Poor egg quality included blood, blood clots, clumps, and non-flowing eggs. Males and females were kept separately during the rearing to keep fighting and female harassment to a minimum.

On October 25, 2023, the first group of YY Brook Trout females were ripe and ready to spawn. We were able to find some 2023 males that gave just enough milt to allow a spawn take to happen, however, we were only able to pair six 2022 YY females with six 2023 YY males. On October 31, 2023, while stripping the 2022 YY females of poor eggs, we were able to find five more free flowing, good egg quality females. There were no more 2023 YY males to pull milt from, so we utilized the 2022 YY males to produce a spawn take. These eggs will only be used for field use and not for any brood stock at BFRH.

Table 1.1. Bellvue Fish Research Hatchery on-site spawning information for the YY Brook Trout (BKT) during the 2023 spawning season.

Strain	Date Spawned	No. Spawned Females	No. Green Eggs	No. Eyed Eggs	Destination
YY BKT	10/25/23	6	13,740	8,894	BFRH, AZ, WY
YY BKT	10/31/23	5	11,452	7,998	BFRH, AZ, WY
Total	10/25/23-10/31/23	11	25,192	16,892	

All eggs were eyed and hatched to be utilized for brood stock and research purposes. (Table 1.1). Fish have been reared to a subcatchable size ranging from 20 to 30 fish per lb. At the time of writing, Arizona will receive 5,000, Wyoming will receive 3,950, and Colorado will receive 480 YY Brook Trout for research purposes.

The replacement brood stock was taken from the first spawn on October 25, 2023, and 1,000 eyed eggs were separated into two batches of 500 each. Egg numbers were retained equally from all parents to represent our M_{YY} and F_{YY} brood stock. The M_{YY} brood stock were fed a regular diet of Bio-Oregon starter fish meal. The F_{YY} brood stock were fed 20 mg/kg estradiol

coated feed for 60 days before being switched to a normal diet containing no estradiol. The amount of estradiol coated feed that was used to create our F_{YY} brood stock was 175.88 g. These brood fish will be held at the BFRH and utilized for future spawn takes once they mature.

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Boreal Toad Production

Over the past decade, the BFRH has performed research projects and helped biologists produce thousands of Boreal Toad tadpoles for reintroduction. In 2023, we helped produce 13,789 tadpoles for release in Rocky Mountain National Park. The primary release site was Boulder Brook Pond, which received 9,118 tadpoles. This site will receive tadpoles again in 2024, and toadlets from the previous release have already been sighted in this location. Two other sites in Rocky Mountain National Park, the Wind River Ponds and Abandoned Beaver Ponds, received excess tadpoles in 2023, and were added to the list of prospective relocation sites in the park. The Wind River Ponds received 2,264 tadpoles and the Abandoned Beaver Ponds received 2,407 tadpoles. In 2024, we continue to produce tadpoles for Rocky Mountain National Park, as well as some sites in Colorado for CPW.

Sport Fish Research Project Updates

Upper Colorado River Salmonid Population Monitoring

Whirling disease (*Myxobolus cerebralis*) caused significant declines in Rainbow Trout populations throughout Colorado following its accidental introduction and establishment in the late 1980s. *M. cerebralis*-resistant Rainbow Trout have been developed by CPW and are currently stocked in a large number of locations across Colorado in an attempt to recover lost populations and create self-sustaining Rainbow Trout populations. The success of *M. cerebralis*-resistant Rainbow Trout introductions is highly variable, dependent on a number of factors

including flow, temperature, stream type, habitat availability for different size classes, Brown Trout *Salmo trutta* densities, prey availability, the size at which the Rainbow Trout are stocked, and strain type. Post-stocking evaluations conducted throughout Colorado allow comparisons of different management options to increase post-stocking survival, recruitment, and the potential to produce self-sustaining populations of *M. cerebralis*-resistant Rainbow Trout. Management actions, including stocking strategies, predator/competitor manipulations, habitat improvements, and increased river connectivity, continue to be evaluated in ongoing field experiments in the Colorado, Fraser, and Yampa rivers. Results from experiments conducted in the upper Colorado River within the last reporting cycle are presented below.

2023 Salmonid Fry Population Estimates

Rainbow Trout fry stocking evaluations began in the upper Colorado River in 2013. In 2013, 2014, and 2015, the 3.9-mile stretch of the upper Colorado River between Hitching Post Bridge on the Chimney Rock Ranch and the Sheriff Ranch (Figure 2.1) was stocked with 100,000 to 250,000 Hofer (HOF) by Colorado River Rainbow Trout (H×C) fry annually. Due to the detection of *Renibacterium salmoninarum* at the CPW Glenwood Springs Hatchery in late 2015, H×C fry were not available for stocking in 2016. Previous studies conducted in collaboration with Colorado State University showed that the HOF survived just as well as the H×C when stocked as fry into small streams (Avila et al. 2018), but the survival of the HOF had not been evaluated in a large river. As such, approximately 60,000 to 70,000 HOF fry were stocked into the upper Colorado River in 2016, 2017, and 2018. Once *M. cerebralis*-resistance evaluations of the HOF by Gunnison River Rainbow Trout (H×G) were completed (Fetherman et al. 2018), survival evaluations of stocked H×G fry began in 2019 (Fetherman et al. 2020, 2021, 2022, 2023). Although the Rainbow Trout fry evaluation study begun in 2013 was completed following the fry estimates conducted in 2021, given the results of these evaluations, H×G fry continue to be stocked above and below Byers Canyon in an effort to increase the adult Rainbow Trout populations in these sections.

On July 11, 2023, approximately 150,000 H×G fry were stocked into the upper Colorado River between Hitching Post Bridge on the Chimney Rock Ranch and the Sheriff Ranch (Figure 2.1). Half of the Rainbow Trout fry were loaded into large coolers supplied with a constant flow of oxygen on a stocking raft at the Hitching Post Bridge. Rainbow Trout were stocked in the margins on both sides of the river in the 1.2-mile stretch between Hitching Post Bridge and Red Barn at the confluence of Drowsy Water Creek. The second half of the Rainbow Trout fry were loaded onto the raft at Red Barn, and fry were similarly stocked on both sides of the river from this point to the Sheriff Ranch (2.7 miles).

Pre-stocking fry population estimates were conducted at eight sites in the upper Colorado River the day prior to fry stocking, and post-stocking fry population estimates were conducted at these same eight sites at the end of July, August, September, and October 2023. Fry estimates completed prior to H×G stocking provided information on the number of Rainbow Trout and Brown Trout fry occurring from natural reproduction, whereas the estimates completed at the end of July, August, September, and October provided information regarding the post-stocking survival of the H×G fry and survival of wild Rainbow Trout and Brown Trout fry. Sampling sites (n = 4) in the Chimney Rock/Sheriff Ranch study section included the Sheriff Ranch, Lower

and Upper Red Barn, and the Hitching Post Bridge (Figure 2.1), which are historical sites used to evaluate fry production and survival in this section of the Colorado River. Four reference sites below Byers Canyon were used to compare survival of stocked H×G fry above and below Byers Canyon. Sampling sites (n = 4) below Byers Canyon included sites in the Kemp-Breeze and Paul Gilbert State Wildlife Areas. The Parshall Island site was added in the Kemp-Breeze State Wildlife Area in 2019 to provide pre-construction fry estimates at multiple locations prior to habitat enhancement work that occurred on the State Wildlife Area in fall 2022 (Figure 2.1). The Colorado River below Byers Canyon had been stocked with H×C fry between 2010 and 2015, not stocked between 2016 and 2019 to allow evaluation of natural reproduction and determine if there was evidence for a self-sustaining Rainbow Trout population, and stocked with H×G fry in between 2020 and 2023 to increase Rainbow Trout recruitment in this section of the river.

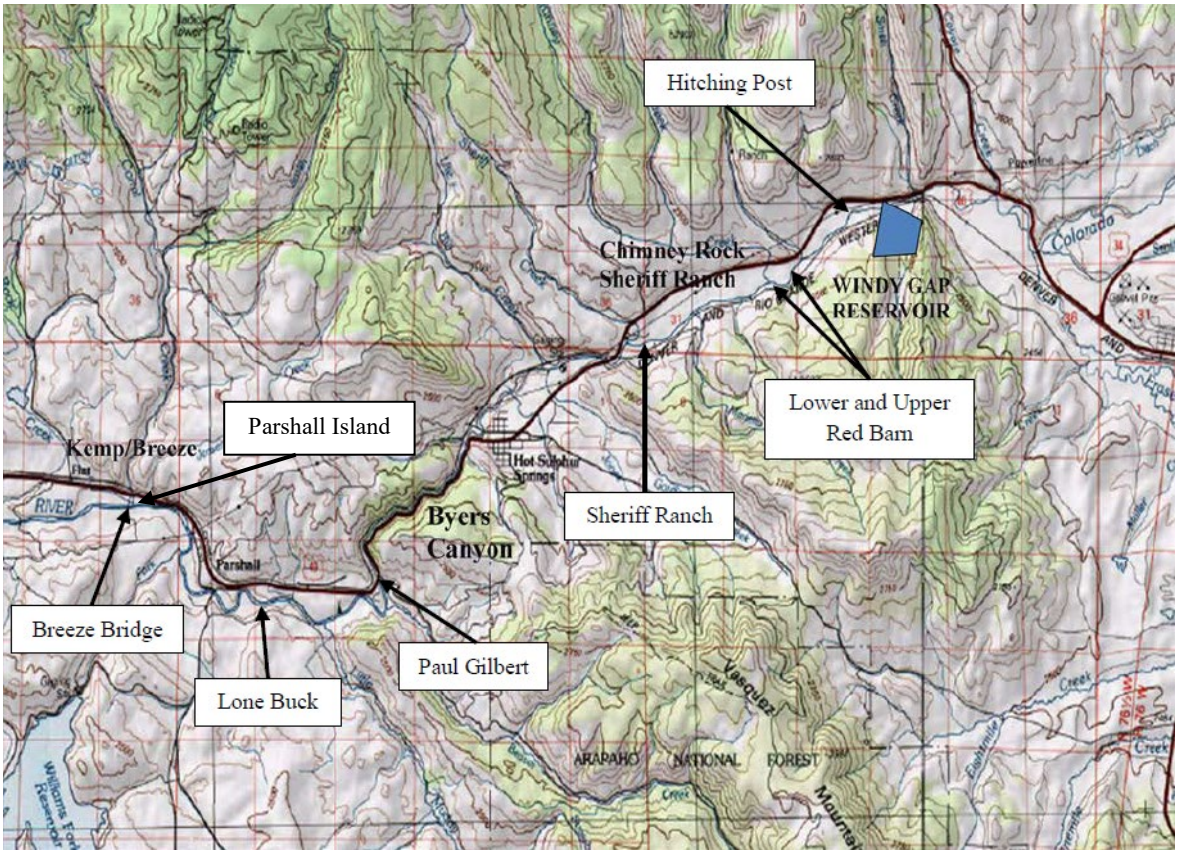

Figure 2.1. Upper Colorado River study area showing the eight sites at which salmonid fry population estimates were conducted in July, August, September, and October 2023.

Salmonid fry abundance estimates were obtained using two Smith-Root LR-24 backpack electrofishing units running side-by-side to cover available fry habitat. Three passes were completed through each of the 50-foot study sites, and fry were removed on each pass. All salmonid fry encountered were identified to species, measured (mm), and returned to the site. Fry density estimates were calculated using the three-pass removal equations of Seber and Whale (1970). In October 2023, up to ten Brown Trout and ten Rainbow Trout were collected from each of the eight fry sites to obtain myxospore counts. Myxospore enumeration was completed at the CPW Aquatic Animal Health Laboratory (Brush, Colorado).

Brown Trout fry were only encountered in and collected for myxospore enumeration from the four sites below Byers Canyon in October 2023 (see fry estimate results below). Overall, Brown Trout averaged 6,865 (± 2,408) myxospores per fish, significantly lower than myxospore counts in 2022 (Fetherman et al. 2023). Two Brown Trout of the ten collected exhibited signs of whirling disease, one fish with spinal deformities at the Breeze Bridge site, and one fish with spinal deformities and blacktail at the Parshall Island site (myxospore counts of 4,044 and 22,233, respectively). Rainbow Trout fry, which were encountered in and collected from all but the Parshall Island fry site in October 2023, averaged 645 (± 400) myxospores per fish, similar to myxospore counts observed in 2022 (Fetherman et al. 2023). Three Rainbow Trout fry of the 26 collected exhibited signs of whirling disease, one fish with spinal deformities at Breeze Bridge, and two fish with caudal peduncle deformities at the Lower Red Barn site. However, myxospores were not found in any of the three fish exhibiting signs of disease suggesting that the deformities may have been genetic defects or a result of exposure to a different pathogen such as *Flavobacterium psychrophilum* in the hatchery prior to being stocked.

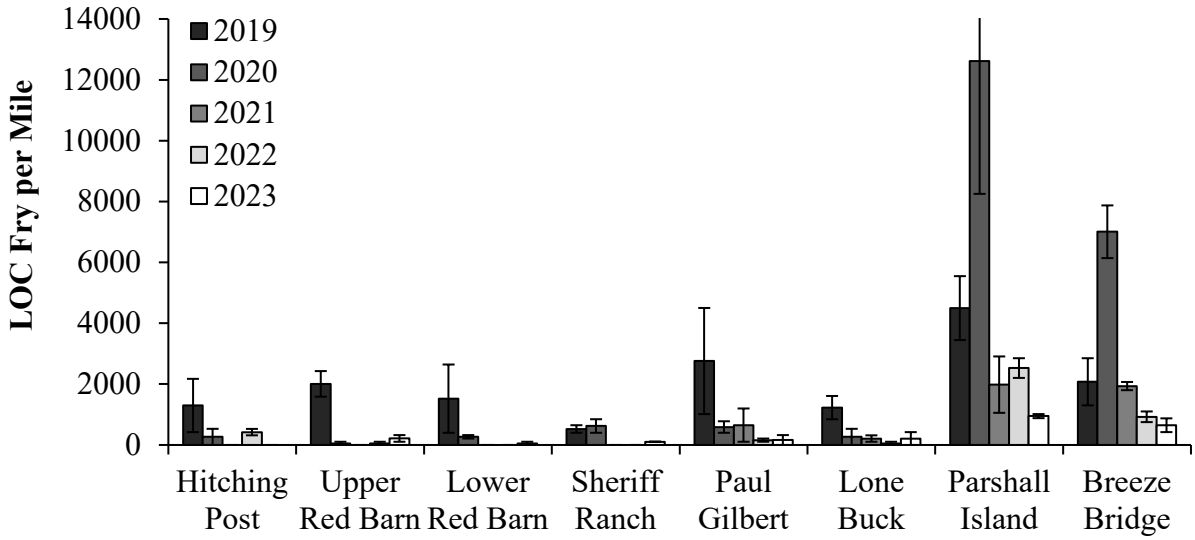

Figure 2.2. Average Brown Trout (LOC) fry abundance (fry per mile; SE bars) in 2019 (typical), and 2020, 2021, 2022, and 2023 (atypical) between Hitching Post Bridge, the furthest upstream site, and Breeze Bridge, the furthest downstream site, in the Colorado River.

Brown Trout fry have historically been evenly distributed throughout the eight fry sites, with the patterns in abundance from upstream to downstream observed in 2019 (Figure 2.2) being representative of the distribution occurring over the course of the study from 2013 to 2019. In 2020, a different pattern in abundance was observed, with significant decreases in fry abundance in the sites located upstream of the Williams Fork confluence, and increases in abundance in the two sites located below the Williams Fork confluence. Although the cause of the change in Brown Trout fry abundances is unknown, draining of Windy Gap Reservoir for survey work in late 2019 may have played a role (Fetherman et al. 2021). Fine sediment from construction activities at Windy Gap Reservoir, as well as ash and sediment from major rain events occurring over the extent of the East Troublesome burn area, continued to be deposited in the Colorado River below Windy Gap Reservoir in 2021 and 2022. As a result, Brown Trout fry numbers

were lower in almost all sites, including those below the Williams Fork confluence in 2023 (Figure 2.2). Overall, this has resulted in a nearly complete loss of the 2021, 2022, and 2023 age classes of Brown Trout within the study site, and a reduction in the adult Brown Trout population as a result of this lack of recruitment was observed in the Chimney Rock Ranch study section in 2023 (Fetherman et al. 2023). This could potentially result in lower recruitment to the adult population below Byers Canyon as well. Runoff reached a higher peak in spring 2023, and a lower, though still higher than typical peak in 2024. These flows may have helped clear out the fine sediment prior to the Brown Trout spawn in 2023, and may continue to keep interstitial spaces clear in 2024. Although the potential benefits of these flushing flows have yet to be observed, we could potentially see higher Brown Trout fry abundances in all sites in 2024 as a result. The 2024 fry abundance results will be available in the next reporting cycle.

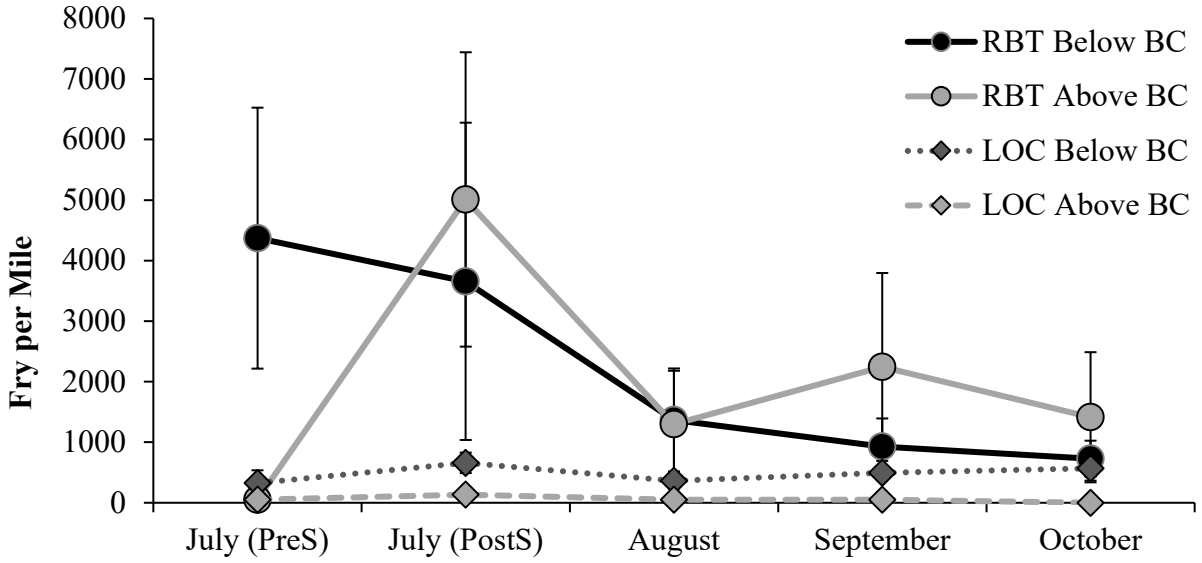

Figure 2.3. Average Rainbow Trout (RBT) and Brown Trout (LOC) fry abundance (fry per mile; SE bars) in the sites below Byers Canyon (BC; Breeze Bridge, Parshall Island, and Lower and Upper Paul Gilbert) and above Byers Canyon (Sheriff Ranch, Lower and Upper Red Barn, and Hitching Post Bridge) in 2023. H×G fry were stocked into all eight fry sites in July 2023.

Very few wild Rainbow Trout fry were encountered in the sites above Byers Canyon in 2023. It is suspected that the fine sediment observed in the river in 2022 and prior to runoff in 2023 affected success of the Rainbow Trout spawn in 2023 similar to the Brown Trout spawn in 2022. H×G fry stocking occurred Below Byers Canyon prior to the first fry estimates conducted in July, so the July pre-stocking estimates below Byers Canyon actually represent the stocked fry abundance, and wild Rainbow Trout production could not be accurately evaluated below Byers Canyon in 2023. Rainbow trout fry abundances below Byers canyon exhibited a significant decline between July and August before leveling out at under 1,000 fry per mile in September and October. Rainbow Trout fry abundance was significantly increased by stocking H×G fry above Byers Canyon. There was a significant reduction in H×G fry in the Chimney Rock/Sheriff Ranch study sites between July and August, but fry abundance remained steady between August and October with close to 700 more fry per mile above Byers Canyon relative to below Byers Canyon in October 2023 (Figure 2.3). There were also significantly more H×G fry present

above Byers Canyon in October 2023 compared to October 2022 (Fetherman et al. 2023). Reduced fine sediment and greater access to interstitial spaces that resulted from the spring 2023 runoff appeared to benefit H×G fry survival in the Chimney Rock/Sheriff Ranch Study section. Additionally, increased interstitial spaces likely increased prey sources and availability, and along with reduced competition due to the lack of Brown Trout fry, resulted in increased post-stocking growth compared to previous years. Although average length in October was fairly similar among the previous three years (2021: 79 ± 1.5 mm; 2022: 83 ± 3.0 mm; 2023: 84 ± 1.7 mm), the proportion of the fry population greater than the three year average of 83.2 ± 0.7 mm has increased from 0.36 in 2021 to 0.5 in 2023, even with more fry having been captured in 2023 than in previous years, concurrent with the reductions in Brown Trout fry abundances and increase in interstitial spaces. Anecdotal data from the 2024 adult population estimates, during which some H×G fry stocked in 2023 were captured, show that fish continue to grow, with these 2023 H×Gs ranging in size between 89 and 150 mm after less than one year in the river. Future adult population estimates will provide more information regarding whether these larger fish are recruiting to the adult population at a higher rate, and if this increased growth continues through subsequent age classes as competition with Brown Trout remains low.

Results obtained from the H×G fry stocking evaluations conducted between 2019 and 2021 suggested that the H×G fry appear to both survive and recruit well after being stocked in the upper Colorado River (Fetherman et al. 2020, 2021, 2022), and this continues to be the case for fry stocked after the fry evaluation study was concluded in 2022 (Fetherman et al. 2023) and 2023. The results from all of these evaluations suggest that H×Gs should continue to be used to reestablish self-sustaining Rainbow Trout populations in the Colorado River and throughout the state. Although the reduced Brown Trout fry abundances continue to be a concern, the low number of Brown Trout fry and adults has likely reduced competition for the stocked H×G fry, and provided space for recruitment into the adult Rainbow Trout population. Given the current river conditions and past performance of the H×G, H×G fry will continue to be stocked and evaluated as part of the monitoring plan associated with the Upper Colorado River fish movement study through its completion in 2026.

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2024 Adult Salmonid Population Estimates

An adult salmonid population estimate was conducted in the 3.9-mile Chimney Rock/Sheriff Ranch study section of the upper Colorado River in May 2024, with the mark run occurring on May 14 and the recapture run occurring on May 16. The estimates were conducted at the highest flows since spring sampling began in this section in 2009, with flows ranging between 1,280 and 1,920 cfs on the mark and recapture runs, respectively. High flows resulted in a slight change to the typical reach, as the short section between just upstream of the Hitching Post Bridge and the Hitching Post stationary antenna station was unable to be sampled due to safety. To adjust for this missing short section and maintain the same length of river sampled, the end of the study section was extended downstream from the standard station end at the top of the Sheriff Ranch island to the raft pullout. Two raft-mounted, fixed-boom electrofishing units were used to conduct the population estimates. Fish work up and release sites were systematically chosen based on the ability to safely pull the boats into slack water as close to previously established release sites as possible. This resulted in fewer release sites on both days than typically used during previous estimates.

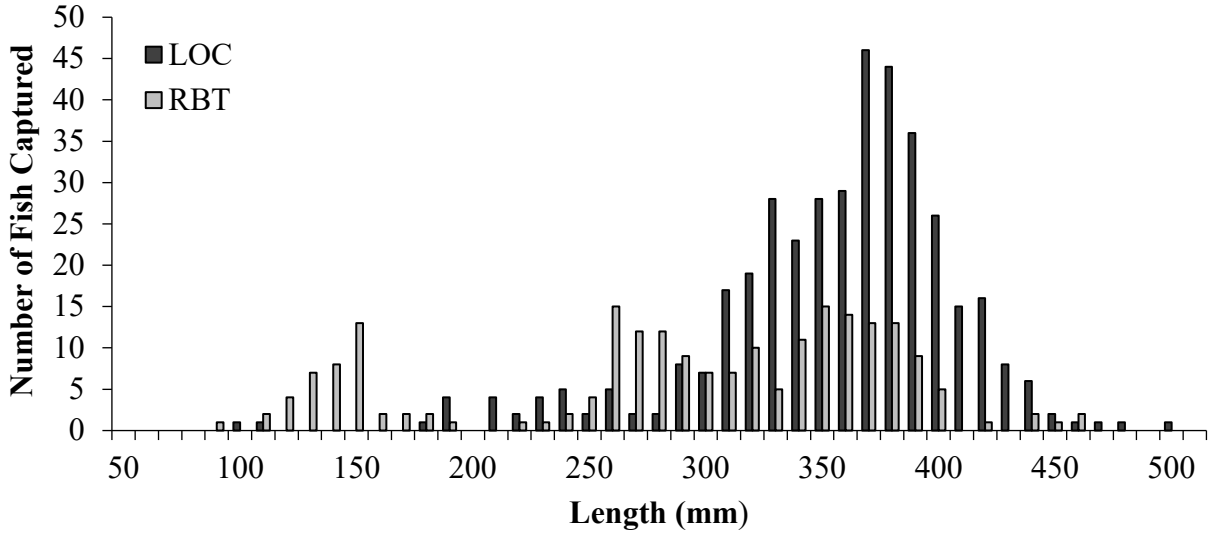

Figure 2.4. Number of Brown Trout (LOC) and Rainbow Trout (RBT) captured by total length (mm) during the 2024 adult salmonid population estimates in the Chimney Rock/Sheriff Ranch study section of the upper Colorado River.

All fish captured on the mark run were given a caudal fin punch for identification during the recapture run, scanned for a passive integrated transponder (PIT) tag, measured (mm), and returned to the river. On the recapture run, fish were examined for the presence of a caudal fin punch and PIT tag, measured, and weighed (g). On both runs, Brown Trout and Rainbow Trout that had been previously PIT tagged but had lost their tag, indicated by an adipose clip but no PIT tag upon scanning, were re-tagged, measured, and weighed. Population estimates were calculated using the Lincoln-Peterson estimator with a Bailey (1951) modification, which accounted for fish being returned to the population following examination of marks on the recapture run, making them potentially available for subsequent recapture. Due to the high flows, only 241 Brown Trout and 103 Rainbow Trout were caudal punched during the mark run. On the recapture run, 180 Brown Trout were handled, of which 28 were caudal fin punched (recaptured), and 82 Rainbow Trout were handled, of which five were recaptured.

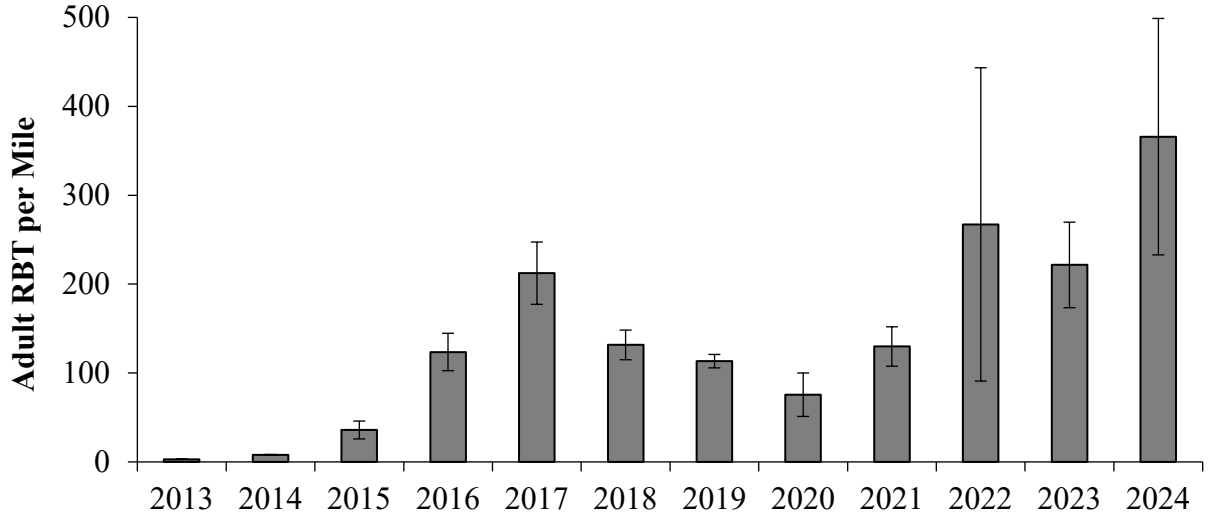

Figure 2.5. Estimated number of adult Rainbow Trout (RBT) per mile (SE bars) in the Chimney Rock/Sheriff Ranch study section of the upper Colorado River between 2013 and 2024.

An estimated 1,504 (± 251) adult Brown Trout were present in the Chimney Rock/Sheriff Ranch study section in 2024, approximately 300 less than in 2023 (Fetherman et al. 2023). Overall, only 386 (± 65) Brown Trout were present per mile in the study section. Although a small number of all age classes of Brown Trout ≥ 150 mm TL were represented in the sample, the majority of the Brown Trout captured were age 3+ (≥ 310 mm TL; Figure 2.4). Adult Brown trout averaged 342 (± 51) mm TL and 445 (± 158) g. The average size of adult Brown Trout was larger than in 2023 as a result of the higher number of older fish in the system and lack of younger, smaller age classes. Numbers of Brown Trout fry and juveniles captured during the population estimates remained low, reflecting the trends observed in fry estimates conducted in 2020 through 2023. This estimate represents the lowest Brown Trout abundance within the study reach since 1981. The cause of the reduction in the population is likely a lack of recruitment over the previous four years due to the draining of Windy Gap Reservoir and release of fine sediment in the fall during the Brown Trout spawning period, continued upstream construction activities, and additions of ash and fine sediment from the 2020 East Troublesome fire. Adult numbers are currently below what we suspect is needed to maintain natural recruitment in this section of the river (about 600 per mile), but it is unknown what the natural

reproduction in the section will be until fry estimates can be conducted in summer 2024. The recent completion of the Colorado River Connectivity Channel may allow more fish to move into the section from upstream of Windy Gap Reservoir, which could help maintain, and potentially increase, the Brown Trout abundance in the coming years.

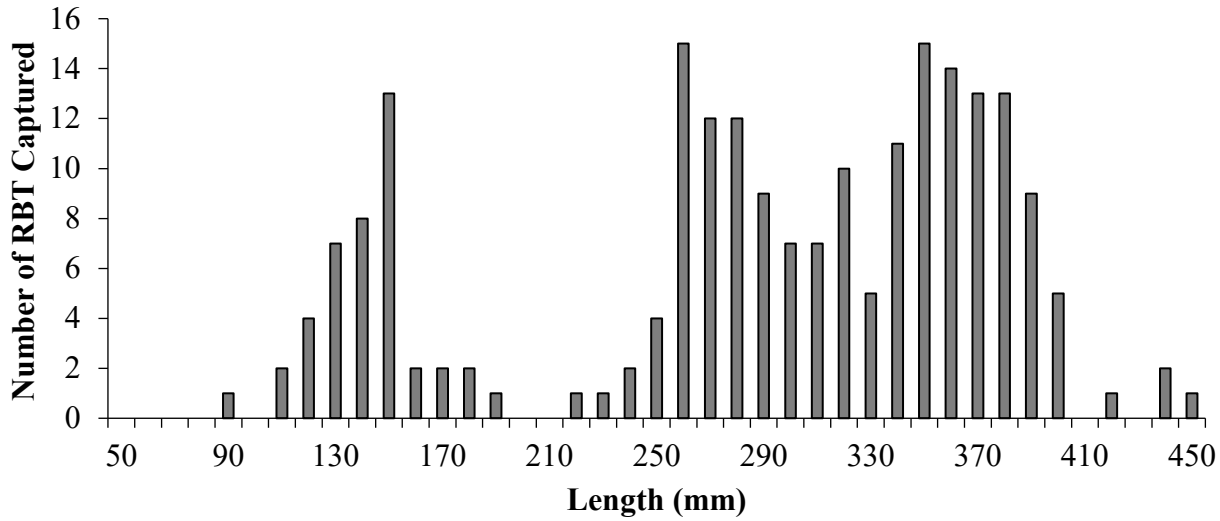

Figure 2.6. Number of Rainbow Trout (RBT) captured by total length (mm) during the 2024 adult salmonid population estimates in the Chimney Rock/Sheriff Ranch study section of the upper Colorado River.

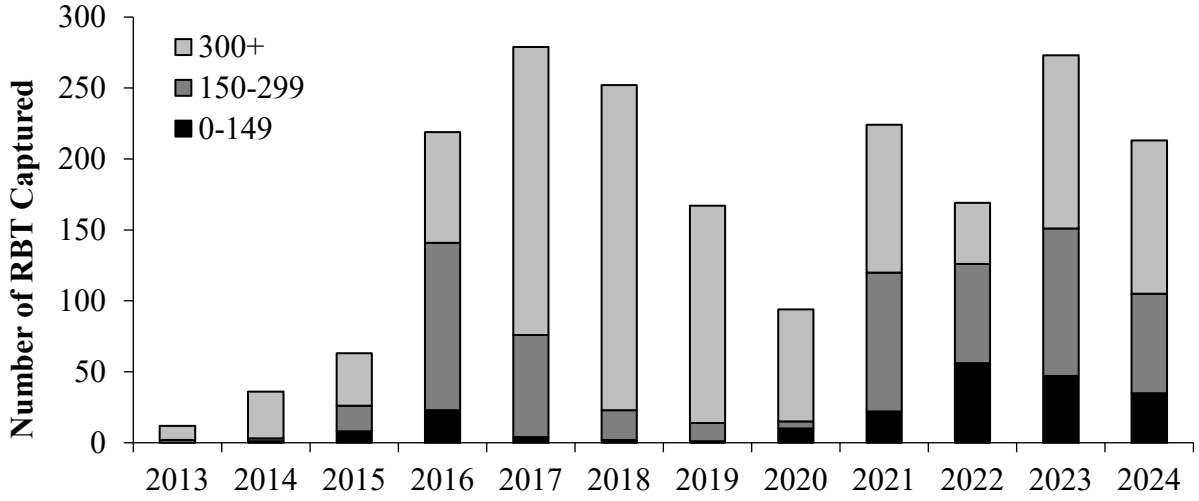

Figure 2.7. Number of age-1 (≤ 149 mm TL), age-2 (150-299 mm TL) and age-3+ (≥ 300 mm TL) Rainbow Trout (RBT) captured in the Chimney Rock/Sheriff Ranch study section of the upper Colorado River between 2013 and 2024.

Rainbow Trout abundance increased, but within the range of error, between 2023 and 2024, with an estimated 864 (± 188) adult Rainbow Trout present in 2023 (Fetherman et al. 2023), and 1,425 (± 519) present in 2024. This resulted in an estimated 365 (± 133) adult Rainbow Trout

per mile in the Chimney Rock/Sheriff Ranch study site (Figure 2.5). Adult Rainbow Trout averaged 315 (± 61) mm TL and 389 (± 167) g, and all age classes of Rainbow Trout were well represented in the sample despite the higher flows reducing the total number of fish captured (Figure 2.6). Although not recruited to the gear used, a number of age-1 Rainbow Trout were captured during the population estimates, suggesting that fry remaining in October 2023 had overwintered well. Several fish stocked as fry in 2023 had already recruited to the adult population (i.e., were ≥ 150 mm TL). Higher flows resulted in lower capture efficiency, and fewer age-2 Rainbow Trout were captured than in the year previous. However, the numbers of age-3+ fish remained similar to that of 2023, suggesting that survival of the H×Gs was high once they recruited to the adult population (Figure 2.7), potentially a result of relaxed competition and predation due to the low adult Brown Trout abundance.

In 2021, the adult Rainbow Trout population in the upper Colorado River exhibited an increase in abundance for the first time since 2017, and has continued to increase through 2024. Survival of the H×Gs appears to be high once the fry have recruited beyond age one. With fewer Brown Trout present within the study reach, relaxed competition and predation should lead to higher survival in 2024. The 2024 adult population estimates represent the highest abundance of Rainbow Trout observed in the upper Colorado River since 1994. Additionally, with the reduced Brown Trout abundances, Rainbow Trout now constitute nearly half of the total salmonid population in the upper Colorado River, a ratio that has not been observed since prior to the establishment of whirling disease in the mid-1990s. However, the abundances have not reached the level we expect is needed for natural recruitment to result in a self-sustaining population. Therefore, H×G stocking will continue through the next couple of years to help bolster the population. The recent connection of the Colorado River to the Colorado and Fraser rivers upstream of Windy Gap Reservoir may also help increase the adult population throughout the system as a whole, as the Fraser River Ranch contains some of the best spawning habitat for Rainbow Trout. Rainbow Trout from below Windy Gap Reservoir had access to this habitat for the first time since the early 1980s in spring 2024. Fry produced in this spawning section are expected to distribute throughout the Colorado River Connectivity Channel and into the Colorado River downstream of Windy Gap Reservoir as they recruit to the adult population. Adult population sampling will continue through the completion of the Upper Colorado River Fish Movement Study in 2026, and results from the spring 2025 population estimates will be available in the next reporting cycle.

Bailey, N. T. J. 1951. On estimating the size of mobile populations from recapture data. Biometrika 38:293-306.

Fetherman, E. R., B. Neuschwanger, and R. E. McDevitt. 2023. Sport Fish Research Studies. Annual Report. Colorado Parks and Wildlife, Aquatic Research Section. Fort Collins, Colorado.

Upper Colorado River Fish Movement Study

The Upper Colorado River Fish Movement Study is being conducted in conjunction with and as a part of the Upper Colorado River Headwaters Projects Monitoring Plan. The fish movement study focuses specifically on fish use of the connectivity channel constructed around Windy Gap

Reservoir, reconnecting the Colorado and Fraser rivers upstream of the reservoir with the Colorado River downstream of the reservoir for the first time in decades. Experimental design and timelines for the study were approved by all interested parties involved in the Upper Colorado River Headwaters Monitoring Plan in 2019, and the final draft of the study proposal can be found in Fetherman et al. (2020). The following describes the steps taken to implement the Upper Colorado River Fish Movement Study within the last year.

Fetherman, E. R., B. Neuschwanger, B. W. Avila, and T. B. Riepe. 2020. Sport Fish Research Studies. Annual Report. Colorado Parks and Wildlife, Aquatic Research Section. Fort Collins, Colorado.

Population Estimates and Tag Releases in the Fraser and Colorado Rivers

Two-pass removal population estimates were conducted in the Fraser River on the Fraser River Ranch and in Kaibab Park on September 5 and 6, and in two stations on the City of Granby property at River Run in the Colorado River on October 3, 2022. A bank electrofishing unit was used to complete the surveys in each location. Fish were held in separate net pens by pass, measured (mm), weighed (g), and a large portion of untagged fish were tagged with a 32 or 12 mm tag, dependent upon size. Fish were anesthetized prior to tagging using AQUI-S 20E, administered with permission and oversight from the US Fish and Wildlife Service Investigational New Animal Drug (INAD) program and CPW aquatic veterinarian Colby Wells. PIT tags were inserted posterior of the pectoral fin through the midventral body wall into the peritoneal cavity via a hypodermic needle (Prentice et al. 1990; Acolas et al. 2007). All fish were adipose clipped to indicate they had been tagged as part of the fish movement study, and to identify and quantify tag loss. Fin-clipped fish from previous tagging events were measured and weighed, and PIT tag numbers were recorded. If a recaptured fish lost their tag, they received a new tag prior to release. Fish were given time to recover in the net pens before being returned to the river. Any mortalities that occurred from the tagging procedure were scanned for a PIT tag number and removed from the released fish dataset. Population estimates were calculated using the two-pass removal equations of Seber and Whale (1970).

The 677-foot station located about 0.25 miles upstream of the railroad crossing at the lower end of the Fraser River Ranch contained 141 Brown Trout, 92 adults (≥ 150 mm total length [TL]) and 49 fry/juveniles (< 150 mm TL; Figure 2.8). An estimated 1,153 ± 32 Brown Trout per mile were present on the ranch, 770 ± 36 adult Brown Trout per mile and 390 ± 10 Brown Trout fry/juveniles per mile. Fewer Brown Trout were present in 2023 compared to 2022 (Fetherman et al. 2023). Adult Brown Trout averaged 283 ± 9 mm TL and 294 ± 25 g, with the largest measuring 483 mm TL and weighing 1,048 g. Brown Trout outnumbered Rainbow Trout in the total catch, with 28 Rainbow Trout captured, 25 adults and 3 fry/juveniles (Figure 2.8). An estimated 236 ± 21 Rainbow Trout per mile were present on the ranch, 208 ± 17 adult Rainbow Trout per mile and 31 ± 27 Rainbow Trout fry/juveniles per mile. Many fewer fry/juvenile Rainbow Trout were present in 2023 compared to 2022 (Fetherman et al. 2023), though the reason for the reduction is unknown. Flows were higher and more sustained in spring 2023 relative to the years prior, which may have affected the Rainbow Trout spawn or fry emergence and survival within the sampling station. Adult Rainbow Trout averaged 310 ± 26 mm TL and 268 ± 42 g, with the largest measuring 655 mm TL and weighing 847 g. Fourteen Mottled

Sculpin *Cottus bairdii* were captured in the site, however, because four were captured on the first pass and 10 were captured on the second, the abundance of Mottle Sculpin on the Fraser River Ranch could not be estimated in 2023. Mottled Sculpin averaged 103 ± 7 mm TL and 17 ± 4 g. Longnose Sucker *Catostomus catostomus* (364 ± 32 per mile), Creek Chub *Semotilus atromaculatus*, Speckled Dace *Rhinichthys osculus* (796 ± 59 per mile), Iowa Darter *Etheostoma exile*, and Fathead Minnow *Pimephales promelas* (94 ± 0 per mile) were also captured on the Fraser River Ranch.

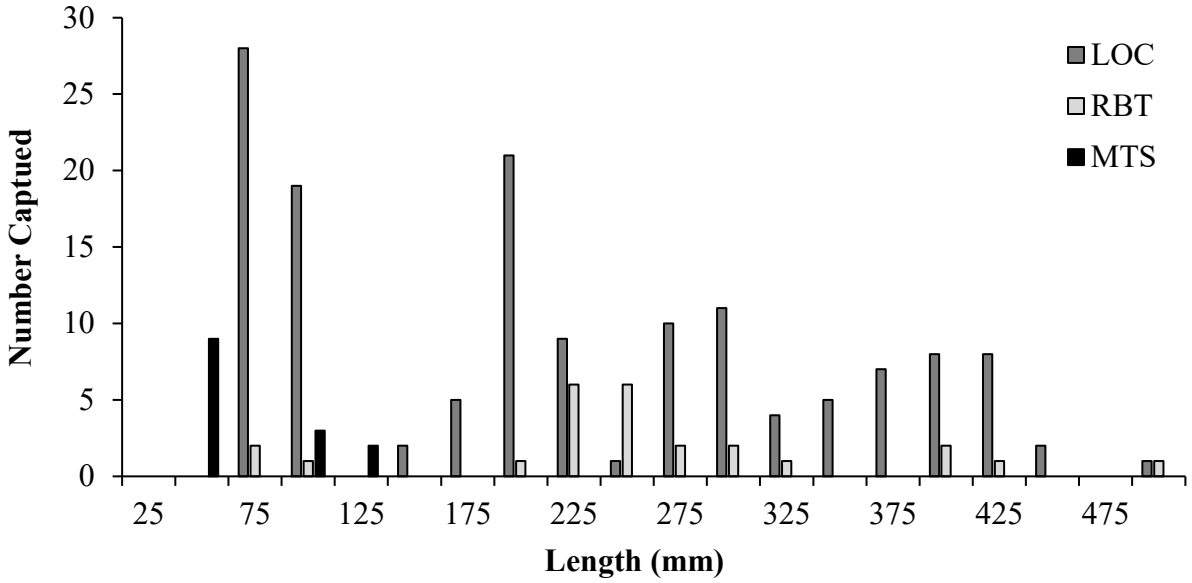

Figure 2.8. Number of Brown Trout (LOC), Rainbow Trout (RBT), and Mottled Sculpin (MTS) captured by total length (mm) during the Fraser River Ranch 2023 population estimate.

Twenty-one Brown Trout and five Rainbow Trout were recaptured on the Fraser River Ranch in 2023. This represented a recapture rate of 9.4% and 4.8% of the Brown Trout and Rainbow Trout released on the ranch between 2020 and 2022, and 1.2% and 0.3% of the total 1,716 fish (all species) released above Windy Gap Reservoir between 2020 and 2022. Of those, four Brown Trout and one Rainbow Trout had lost their tags, a tag loss rate of 19% and 20% for the two species, respectively, and were retagged prior to release. Ten Brown Trout and one Rainbow Trout recaptured in 2023 had been released in the site in 2022. Two other Brown Trout had been released in the site in 2020 and 2021, but were recaptured for the first time in 2023. Two Brown Trout and one Rainbow Trout had been released in the site in 2020, and were recaptured in 2021, 2022, and 2023. Five Brown Trout and one Rainbow Trout had been released in the site in 2021 and were recaptured in 2022 and 2023. One Rainbow Trout was a tag loss that had been retagged and released in the site in 2022 and recaptured in 2023. In 2022, we recaptured a Brown Trout that had been released on the Chimney Rock Ranch in May 2021 and passed through Windy Gap Dam prior to being recaptured on the Fraser River Ranch in 2022; this fish was recaptured within the section again in 2023. Brown Trout grew an average of 53 ± 10 mm TL and 177 ± 34 g since their last recapture, while Rainbow Trout grew an average of 32 ± 15 mm TL and 107 ± 68 g. A total of 93 new fish were PIT tagged in the Fraser River Ranch, 71 Brown Trout, 17 Rainbow Trout, and 5 Mottled Sculpin. Mottled Sculpin (89 to 122 mm TL)

were tagged with 12 mm PIT tags, whereas the Brown Trout (137 to 415 mm TL) and Rainbow Trout (196 to 562 mm TL) were tagged with 32 mm PIT tags.

A total of 255 Brown Trout, 160 adults and 95 fry/juveniles, were captured in the 643-foot electrofishing station in Kaibab Park. The majority of the Brown Trout captured were age-0 and age-1 fish (Figure 2.9). Kaibab Park contained an estimated 2,283 ± 74 Brown Trout per mile, 1,401 ± 46 adult Brown Trout per mile and 894 ± 69 Brown Trout fry/juveniles per mile. The abundance of Brown Trout in Kaibab Park was higher in 2023 than in 2022 (Fetherman et al. 2023), continuing to show a recovery from the disturbances caused by construction activities conducted in 2021 immediately upstream of the site to make the Highway 40 diversion more fish passable. Adult Brown Trout averaged 201 ± 5 mm TL and 101 ± 8 g, with the largest measuring 385 mm TL and weighing 542 g. Fewer Rainbow Trout were captured in Kaibab Park than on the Fraser River Ranch, with only ten Rainbow Trout captured (Figure 2.9). An estimated 87 ± 11 Rainbow Trout per mile were present in Kaibab Park, 75 ± 4 adult Rainbow Trout per mile and 8 ± 0 Rainbow Trout fry/juveniles per mile. Adult Rainbow Trout averaged 176 ± 4 mm TL and 57 ± 4 g, with the largest measuring 192 mm TL and weighing 75 g. Ninety-one Mottled Sculpin were captured in Kaibab Park, providing an estimate of 2,281 ± 1,983 Mottled Sculpin per mile. Mottled Sculpin averaged 95 ± 2 mm TL and 13 ± 1 g. Brook Trout *Salvelinus fontinalis* (8 ± 0), Longnose Sucker (1,591 ± 154 per mile), White Sucker *C. commersonii* (8 ± 0), Speckled Dace (432 ± 78 per mile), Fathead Minnow, and Iowa Darter were also captured in Kaibab Park.

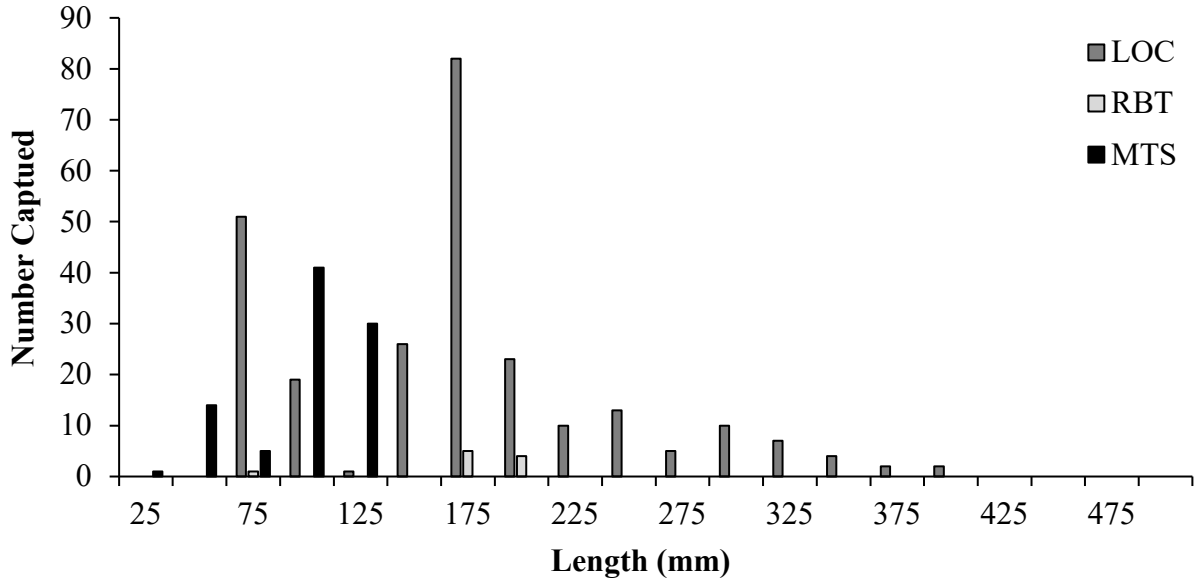

Figure 2.9. Number of Brown Trout (LOC), Rainbow Trout (RBT), and Mottled Sculpin (MTS) captured by total length (mm) during the Kaibab Park 2023 population estimate.

One hundred forty-six new fish were PIT tagged in Kaibab Park, 71 Brown Trout, nine Rainbow Trout, and 66 Mottled Sculpin. Mottled Sculpin (74 to 122 mm TL) were tagged with 12 mm PIT tags, whereas the Brown Trout (144 to 364 mm TL) and Rainbow Trout (157 to 192 mm TL) were tagged with 32 mm PIT tags. No Rainbow Trout were recaptured, although thirty-four Brown Trout and six Mottled Sculpin were recaptured in Kaibab Park in 2023. This represents a

recapture rate of 11.7% and 2.3% of the Brown Trout and Mottled Sculpin released in Kaibab Park between 2020 and 2022, and 2% and 0.3% of the 1,716 fish released above Windy Gap Reservoir between 2020 and 2022. Of those, six Brown Trout had lost their tags, a tag loss rate of 17.6%, and were retagged prior to release. One Brown Trout had been released in the Fraser River Ranch section in 2022 and was recaptured in Kaibab Park in 2023. Four Brown Trout were released in Kaibab Park in 2020 and recaptured every year since. Similarly, one Mottled Sculpin and six Brown Trout were released in 2021 and recaptured every year since. Two Brown Trout released in 2020 were recaptured in both 2021 and 2023, whereas two others released in 2020 were recaptured in both 2022 and 2023. Two Brown Trout released and one retagged in 2021 were recaptured in 2023. All other Brown Trout (n = 10) and Mottled Sculpin (n = 5) recaptured in Kaibab Park in 2023 had been released there the year prior. Brown Trout grew an average of 53 ± 5 mm TL and 92 ± 10 g since their last recapture, while Mottled Sculpin grew an average of 18 ± 6 mm TL and 7 ± 2 g.

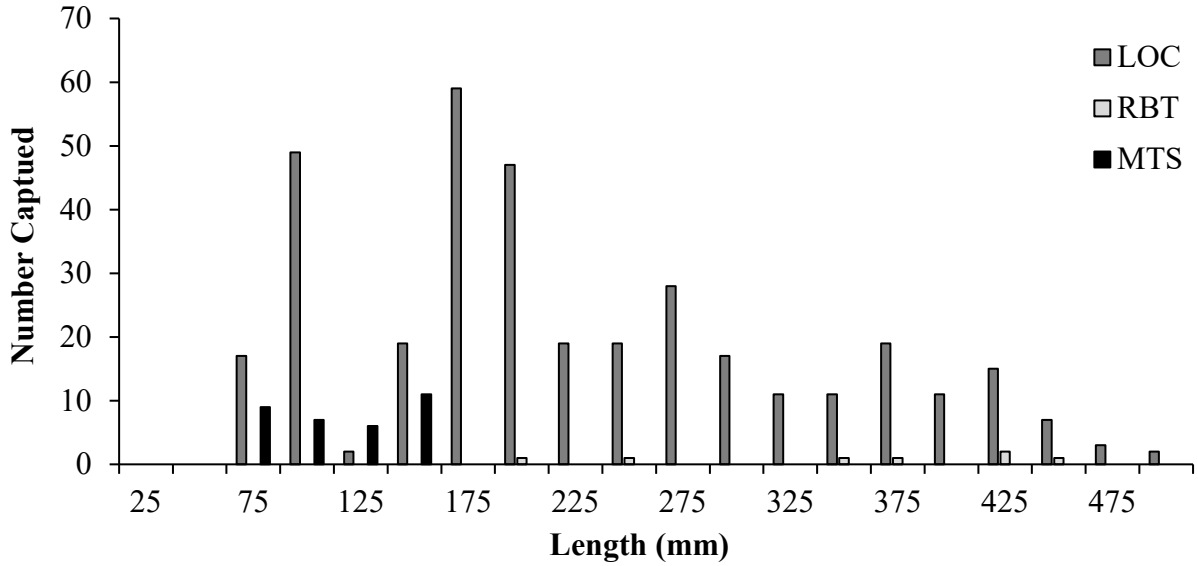

Figure 2.10. Number of Brown Trout (LOC), Rainbow Trout (RBT), and Mottled Sculpin (MTS) captured by total length (mm) during the 2023 River Run population estimates.

Two sites were sampled in the River Run section of the Colorado River, a 630 foot site located downstream of the River Run bridge, and a 493 foot site located upstream of the bridge. The data from both sites were combined for the purposes of this summary. The River Run sites contained 356 Brown Trout, 270 adults and 86 fry/juveniles (Figure 2.10). An estimated 1,818 ± 85 Brown Trout per mile were present in River Run, 1,370 ± 74 adult Brown Trout per mile and 450 ± 44 Brown Trout fry/juveniles per mile. The average number of Brown Trout per mile remained similar between 2022 and 2023 (Fetherman et al. 2023). Adult Brown Trout averaged 259 ± 6 mm TL and 246 ± 16 g, with the largest measuring 525 mm TL and weighing 1,527 g. Only seven adult Rainbow Trout were captured in 2022 (Figure 2.10), for an estimated 34 ± 0 adult Rainbow Trout per mile. Adult Rainbow Trout averaged 344 ± 35 mm TL and 505 ± 121 g, with the largest measuring 450 mm TL and weighing 923 g. Thirty-three Mottled Sculpin were captured within the two sites, but in both sites, more sculpin were captured on the second pass than the first pass, and therefore the abundance of Mottled Sculpin could not be estimated

for the River Run section in 2023. Mottled Sculpin averaged 102 ± 5 mm TL and 24 ± 2 g. Longnose Sucker, White Sucker, Speckled Dace (457 ± 77 per mile), Fathead Minnow, and Iowa Darter (21 ± 0 per mile) were also captured in River Run.

Thirty-three Brown Trout and six Mottled Sculpin were recaptured in the River Run reach of the Colorado River in 2023, a recapture rate of 5.7% of the Brown Trout and 4.8% of Mottled Sculpin released in River Run between 2020 and 2022. This equated to 1.9% and 0.4%, respectively, of the 1,716 fish released above Windy Gap Reservoir between 2020 and 2022. No Rainbow Trout were recaptured in the River Run reach in 2023. Of the fish recaptured, ten Brown Trout had lost their tags, a tag loss rate of 30.3%, and were retagged prior to release. Fifteen Brown Trout released or retagged in 2022 were recaptured in 2023. Two Brown Trout had been released or retagged in 2021 and recaptured in 2023. Five other Brown Trout had been released in 2020, with two recaptured for the first time in 2023, one recaptured in 2021, one recaptured in both 2022 and 2023, and one recaptured in all years sampled after its release. Of the six Mottled Sculpin recaptured, one was released in 2020 and five were released in 2021, and all were recaptured for the first time in 2023. Tagged fish in River Run exhibit extreme site fidelity. All of the fish recaptured in the Lower River Run site had been previously released in the lower site, and same with the fish recaptured in the upper site, despite there being only about a half mile of river between the two sites, and in several cases, many years between release and recapture events. Brown Trout grew an average of 65 ± 10 mm TL and 210 ± 34 g since their last recapture, while Mottled Sculpin grew and average of 5 ± 2 mm TL and 2 ± 2 g. A total of 163 new fish were PIT tagged in River Run, 140 Brown Trout, six Rainbow Trout, and 17 Mottled Sculpin. Mottled Sculpin (72 to 146 mm TL) were tagged with 12 mm PIT tags, whereas the Brown Trout (143 to 499 mm TL) and Rainbow Trout (193 to 450 mm TL) were tagged with 32 mm PIT tags.

In summary, 402 fish were PIT tagged in the Fraser and Colorado rivers above Windy Gap Reservoir in 2023. The goal had been to release a minimum of 250 tagged fish of each species. We met this goal for Brown Trout with 282 tagged, but did not meet this goal with Mottled Sculpin or Rainbow Trout, with only 32 and 88 tagged of each, respectively. All Rainbow Trout and Mottled Sculpin captured in the four sampling sites were tagged; the inability to meet this goal was subject to their availability in the sites in 2023. Since the beginning of the study, 2,118 fish have been tagged in the Fraser and Colorado rivers above Windy Gap Reservoir.

Eighty-five new fish were PIT tagged with 32 mm tags immediately downstream of Windy Gap Reservoir in October 2023, 64 Brown Trout (170 to 494 mm TL) and 21 Rainbow Trout (171 to 510 mm TL). Eighteen Brown Trout were recaptures, a recapture rate of 5.4% of the fish released in the site between 2020 and 2022, and 0.7% of the 2,617 fish (all species) released below Windy Gap Reservoir. Of those, six had lost their tag, a tag loss rate of 33%, and were retagged prior to release. Three of the recaptured Brown Trout had been released in the site in 2022. The remainder of the fish had previously been released or retagged at other sites below Windy Gap Dam, as far down as Kinney Creek, between 2020 and spring 2023. Four of these had been handled after their release but prior to this recapture event, with two recaptured in the site in 2022, one recaptured near the Hitching Post antenna in 2022, and one recaptured near the Red Barn antenna in spring 2023. Recaptured Brown Trout grew an average of 55 ± 15 mm TL and 193 ± 41 g since their last recapture.

Twenty-three Brown Trout and 18 Rainbow Trout were recaptured during the adult salmonid population estimates conducted in the Chimney Rock/Sheriff Ranch study section of the Colorado River below Windy Gap Reservoir in May 2024. This represents a recapture rate of 1.1% of Brown Trout and 3.2% of Rainbow Trout previously released below Windy Gap Dam. Of the fish captured, 12 Brown Trout and three Rainbow Trout had lost their tags, a tag loss rate of 52% and 17%, respectively, and were retagged prior to release. Two recaptured fish had been tagged in 2020, four in 2021, seven in 2022, and 13 in 2023. Similar to what was observed in the River Run section of the Colorado River, fish show site fidelity, being captured either in the same site or within a site or two of where they had been initially released. Brown Trout grew an average of 47 ± 14 mm TL and 59 ± 135 g since their last recapture, while Rainbow Trout grew and average of 56 ± 11 mm TL and 167 ± 46 g. Given that 2024 is considered a construction year, and is not part of the post-construction monitoring period, no new tagged fish were released in the section in spring 2024.

In summary, 85 fish were PIT tagged in the Colorado River below Windy Gap Reservoir in fall 2023, bringing the total number of tagged fish released below the reservoir since the beginning of the study to 2,702. No additional tagged fish were released below Windy Gap Dam during the spring 2024 population estimates conducted in the Chimney Rock/Sheriff Ranch study section.

The pre-construction phase of the fish movement study was completed following the release of PIT tagged fish below Windy Gap Dam in October 2022. Initial construction of Colorado River Connectivity Channel (CRCC) was completed in fall 2023, with water run through the channel for the first time in October 2023. However, a few impediments to unobstructed fish movement remain in the channel and construction activities will continue through fall 2024. Therefore, the post-construction phase of the study will begin with the release of PIT tagged fish in the Fraser River in September 2024. From there, PIT tag releases will follow a similar pattern to those that occurred during the pre-construction phase of the study. More information regarding these PIT tag releases will be available in the next reporting cycle.

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Stationary Antenna Installations, Performance Evaluations, and Data Collection

Stationary antennas used to detect the movements of PIT-tagged fish were installed at three sites in the Colorado River in 2020: 1) immediately downstream of the confluence of the Fraser and Colorado rivers above Windy Gap Reservoir on Northern Water property (Confluence; CF), 2) just downstream of the Hitching Post (CR 57) bridge on the Chimney Rock Ranch (Hitching Post; HP), and 3) in the Red Barn area of the Chimney Rock Ranch upstream of the Red Barn diversion structure (Red Barn; RB; Figure 2.11). With a few exceptions, these antennas have been operating continuously since they were installed, collecting data on PIT-tagged fish movements 24 hours a day, 365 days a year. In 2022, trees and large woody debris damaged the downstream antenna (CF5) at the confluence site, requiring it to be repaired in August 2022 (Fetherman et al. 2023). During high spring flows in spring 2023, a large cottonwood tree got lodged on the upstream antenna (HP4) at the Hitching Post site, requiring replacement in September 2023. Repair of the antenna required disconnection from the antenna tuner boxes and complete removal of the river spanning PVC housing around the antenna wire. Similar to the installation of antennas at the beginning of the study in 2020 (Fetherman et al. 2021), the PVC housing was repaired on-shore, reusing as much of the original unbroken structure as possible. Antenna wire was run through the PVC prior to gluing all of the new pieces together. Following completion of the repairs, a crew of 14 people was deployed to lift and support the antenna as it was taken back into the river and reinstalled in its original location. The crew utilized as many of the duckbill anchors holding down the original antenna as possible, and new anchors were driven in where they had broken or additional support was needed to hold the antenna closer to the substrate. The antenna was secured to the duckbill anchors and substrate using both river and ratchet straps. After reconnecting the wire to the tuning boxes and readers, the antenna was tuned to optimize read range.

In an attempt to prevent further damage and ensure continuous future operation, additional duckbill anchors and ratchet straps were added to antennas at all three antenna stations. This included ratchet straps across the crossbeams maintaining antenna shape, as well as anchors and straps at five foot intervals between the crossbeams. Large rocks were used to fill gaps between the PVC and the substrate to prevent debris from catching on the antenna as it passed. At the Confluence antenna station, CPW was able to enlist the assistance of one of the crews working on the construction of CRCC to help move additional substrate to upstream, between, and downstream of the confluence antennas. Using hand tools, CPW personnel leveled this additional substrate around the antennas, essentially burying them just below the substrate surface. Although it did not damage the antennas themselves, ice caused several smaller duckbill anchors to break at the Hitching Post site over the winter. Damaged duckbill anchors were replaced in March 2024 prior to runoff. At the time of writing, spring flows had not yet receded enough to evaluate antenna integrity following the 2024 runoff, but no large debris had been observed hung up on any of the antennas and data loggers did not show any loss of operation during the high flow period.

In September 2023, three additional antenna stations were added in the newly constructed CRCC (Figure 2.11). Two stations, side channel (CS) and downstream connectivity channel (CD) were installed at the downstream end of the connectivity channel, and were located close enough to each other that the components for the two stations could be housed in the same job box, and run

off the same battery bank and solar panels. The two stations are being used to determine whether fish are using the main channel, which exits just below Windy Gap Dam, or the side channel, which exits about a half mile downstream, to enter and move through the CRCC from downstream of Windy Gap. Differential use of the two channels could be a result of the amount of flow in each channel at any given time (flows are generally higher in the main channel than the side channel), attraction flows in the Colorado River where the channels exit, or location of the exits relative to fish location in the Colorado River at certain times of year (e.g., fish moving up to spawn may approach the dam, bypassing the side channel and using the main channel to enter the CRCC). A third station, upstream connectivity channel (CU) was installed about a quarter mile downstream of where water enters the channel above Windy Gap Reservoir. Antennas were constructed and installed while the CRCC was dry (Figure 2.12), and the same materials and processes were used as for the stationary antenna stations installed in the Colorado River in 2020 (Fetherman et al. 2021). Installation prior to running water through the channel ensured that the antenna stations were operational and able to detect fish moving through the CRCC as soon as water was run through the channel in October 2023.

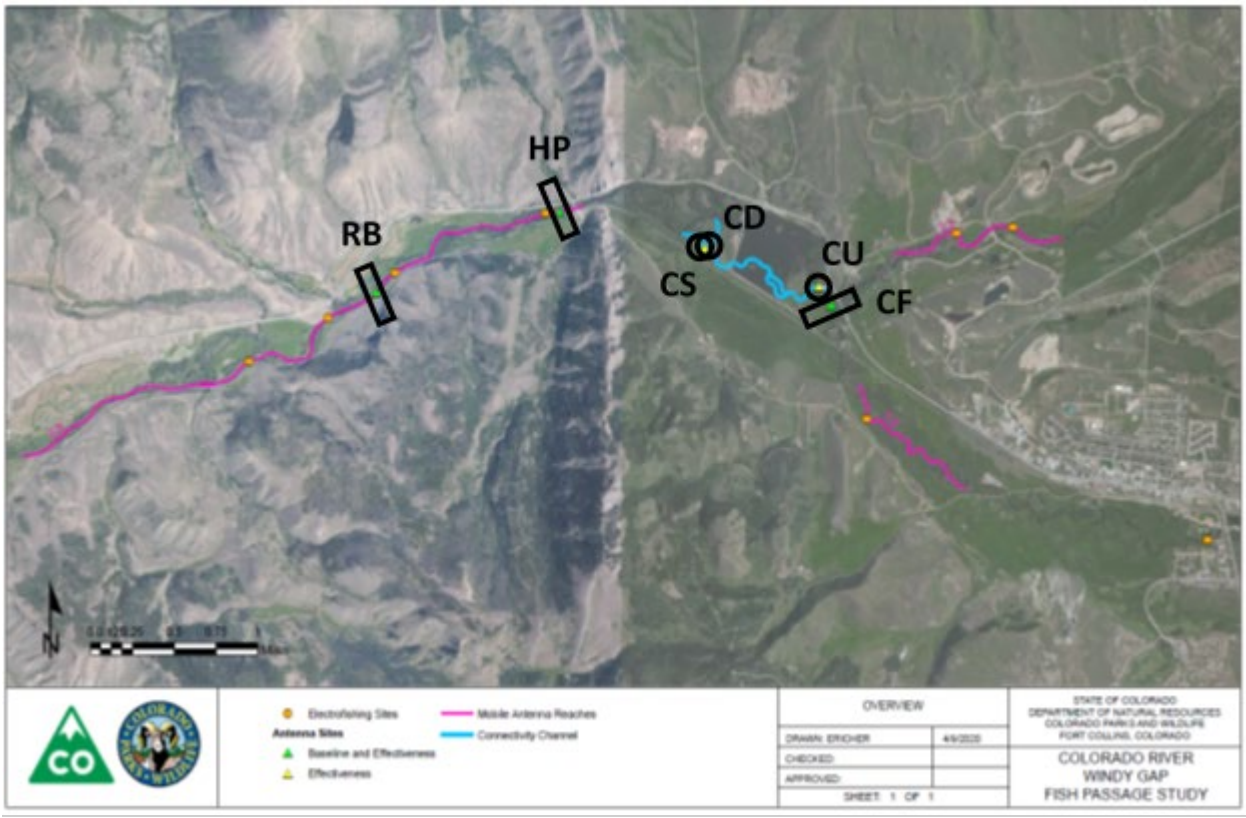

Figure 2.11. Locations of the Red Barn (RB), Hitching Post (HP), and Confluence (CF) stationary antenna stations installed in 2020 (rectangles), and the side channel (CS), downstream connectivity channel (CD), and upstream connectivity channel (CU) antenna stations installed in 2023 (circles).

Antenna detection distances were measured at all six stationary antenna sites in fall 2023 following the installation of antennas in the CRCC to determine if vertical detection distances

exceeded average water depth. Note that detection distance measurements were taken at the CS, CD, and CU stations within the connectivity channel prior to when water was run through the channel in late-October 2023. Therefore, velocity and water depth data are only presented for the Red Barn, Hitching Post, and Confluence sites. Detection distances were measured using a 32 or 12 mm PIT tag on a PVC stick run perpendicular to the antenna wire (optimal tag orientation and most likely orientation of a fish crossing the antenna). The tag was raised from the antenna until an audible beep from the reader, indicating detection, was no longer heard. The tag was then lowered back down towards the antenna until beeping resumed. The distance from the antenna to the tag was measured (tenths of feet), and a measurement of 0.2 feet was added to account for the distance from the top of the PVC to where the wire sat on the bottom of the pipe. Previous work had showed that antenna detection distances did not differ between the upstream and downstream sides of the antenna (Fetherman et al. 2020), so detection distances, along with water depth and velocity measurements, were taken every ten feet along the upstream side of each antenna only.

Figure 2.12. Construction of the side channel (CS) and downstream connectivity channel (CD) antennas (upper left), construction of the upstream connectivity channel (CU) antennas (Upper right), CS antennas buried in the substrate (lower left), and aquatic research scientist Eric Richer installing the electrical components for the CD antenna station (lower right).

Velocities in fall 2023 averaged 0.39 ± 0.15, 0.34 ± 0.18, and 0.28 ± 0.16 m/s at the Red Barn, Hitching Post, and Confluence sites, respectively. On average, velocities did not exceed 0.50 m/s, the maximum velocity measured by Fetherman (2013) at which detection probability remained 1.0. Detection distances for a 32 mm PIT tag in fall 2023 ranged between an average of 1.27 and 2.40 feet, and for a 12 mm PIT tag between 0.43 and 0.90 feet. Detection distances for the newly installed antennas within the connectivity channel (CS7, CS8, CD9, CD10, CU11, and CU12) were similar between the two antennas within a site, as well as among sites, and in general, where greater than those of the existing sites within the Colorado River. On average, the paired antennas at the Red Barn, Hitching Post, and Confluence sites had similar detection distances. However, there was discrepancy in detection distances within sites, with the upstream antenna at Red Barn having a greater detection distance than the downstream antenna, and the downstream antenna at the Hitching Post and Confluence sites having greater detection distances than the upstream antennas. Detection distance for the 32 mm PIT tags exceeded the average water depth at each of the three Colorado River antenna sites, suggesting full coverage of the water column. Read ranges for the 12 mm PIT tags was similar to the water depth at the Red Barn and Hitching Post sites, but significantly lower than the water depth at the Confluence site (Figure 2.13). However, 12 mm PIT tags were primarily used to tag Mottled Sculpin. Given their sedentary nature and the likelihood that movement occurs near the substrate, 12 mm PIT tag detection distances should be sufficient for detecting Mottled Sculpin movements at all sites. Overall, detection distance results continue to suggest that detection probability should be high for both salmonids and Mottled Sculpin at all antennas. A more formal analysis of detection probability will be completed using the long-term tagged fish detection data upon completion of the fish movement study.

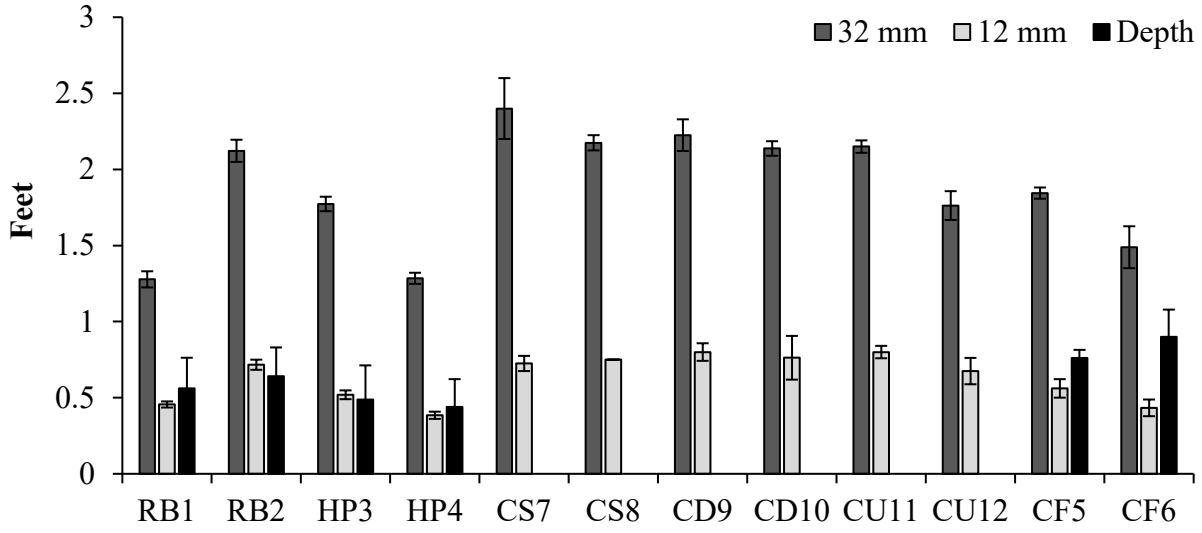

Figure 2.13. Detection distances (feet; 2 SE bars) for 32 mm and 12 mm PIT tags for the paired antennas located at Red Barn (RB), Hitching Post (HP), side channel (CS), downstream connectivity channel (CD), upstream connectivity channel (CU), and Confluence (CF) sites, and water depths (feet; 2 SE bars) measured at the RB, HP, and CF sites in fall 2023.

With the exceptions mentioned above, antennas have been operating continuously since late August 2020, and have collected tens of thousands of data points from moving fish. Additional

data points have been obtained from marker tags located at each antenna, which reveal a tag with a known number every 15 minutes. These marker tag detections allow researchers to determine if there are gaps in operation and tag detection (e.g., due to power failure) in the time between visits to the stations. Data are downloaded from the readers once a month, at which time antennas are visually inspected and cleared of debris, ratchet straps are tightened as needed, and read ranges are checked to ensure the antennas continue to function as designed. Antennas are also retuned as needed visit to optimize read range for the current flows, temperatures, and environmental conditions. Data are being stored in a large PIT tag database developed for the fish movement study, and an R script has been written to provide visual summaries of the data.

Fetherman et al. (2023) showed that Brown Trout exhibit an increase in movement during the fall spawning period, Rainbow Trout show a spike in activity following ice-off and during the spring spawning period, and that Mottled Sculpin are generally sedentary and do not move far from their release locations. Additionally, diurnal movement patterns of all fish species suggest that movement occurs primarily at night when fish are less likely to encounter predators (Fetherman et al. 2023). A review of the database showed that these patterns of movement continued through 2024.

The PIT tag database was queried in June 2024 to determine the number of tagged fish that have passed through the connectivity channel in either direction, as determined by the order of detections occurring at CS, CD, and CU for any given fish, since water started flowing through the channel in fall 2023. Since October 2023, 16 Rainbow Trout and 33 Brown Trout have been detected moving through the channel; no Mottled Sculpin had been observed on any of the antenna sites in the connectivity channel as of when this analysis was conducted. Release lengths of Brown Trout averaged 320 ± 78 mm TL and weights averaged 356 ± 230 g, whereas Rainbow Trout averaged 323 ± 93 mm TL and 420 ± 352 g at the time of release. Many of these fish have likely grown since the last time they were handled, and these sizes may not reflect the current size of the fish that moved through the CRCC.

Five fish that moved through the CRCC had been tagged and released in 2020, five fish in 2021, ten fish in 2022, 28 fish in 2023, and one fish had been retagged during the adult population estimates conducted on the Colorado River below Windy Gap Reservoir in May 2024. Three fish had been released in the Fraser River upstream of Windy Gap Reservoir (one at Kaibab Park, two at the Fraser River Ranch), seven had been released in the River Run section of the Colorado River above Windy Gap Dam, and the remainder had been released in the Colorado River below Windy Gap Dam. Although most of those fish from below the reservoir had been released in the site immediately below Windy Gap Dam, several came from other locations within the Chimney Rock/Sheriff Ranch study section as far downstream as the Sheriff Ranch. Thirty-four fish had successfully traversed the channel moving upstream from below Windy Gap Reservoir to above, while the remaining 15, two Rainbow Trout and 13 Brown trout, moved downstream through the channel. Of the 34 fish that moved upstream, 14 were Rainbow Trout and 20 were Brown Trout. Upstream movement around Windy Gap Reservoir was the primary goal of the CRCC. Fish that move upstream through the CRCC now have access to 235 additional river miles of connectivity in the Colorado River, Fraser River, and their tributaries, up to the Moffat Collection System in the Fraser River headwaters (Figure 2.14).

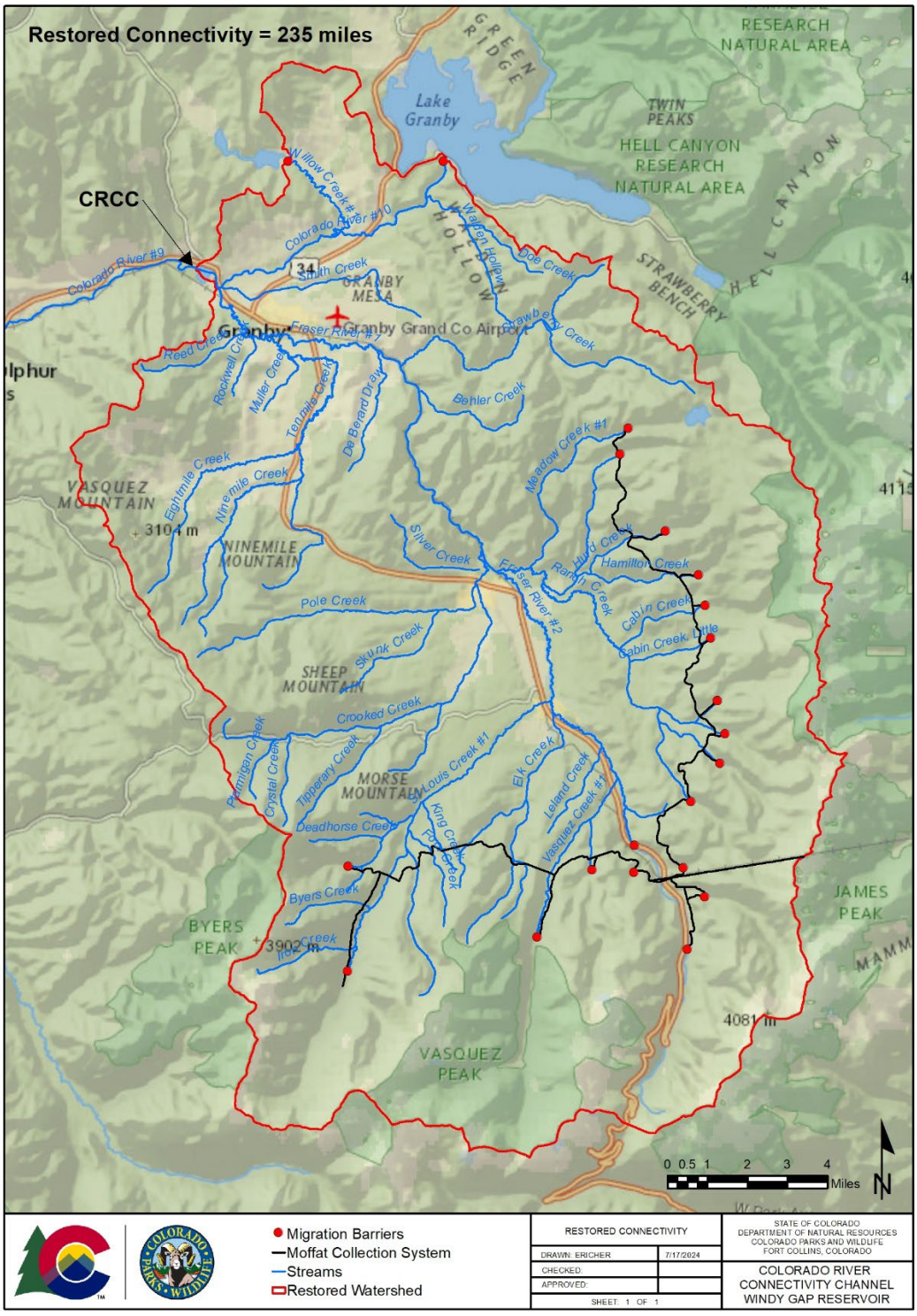

Figure 2.14. Map showing how the completion of the Colorado River Connectivity Channel (CRCC) resulted in 235 miles of restored connectivity up to the Moffat Collection System in the Colorado River, Fraser River, and their tributaries above Windy Gap Reservoir.

Stationary antenna stations will continue to remain operational as conditions allow through the remainder of the fish movement study, which will conclude in fall 2026. Movement data collected in 2023 and 2024 may be affected by construction activities associated with completion of the CRCC. Therefore, data collected in these years will only be used to determine use of the CRCC in its first year of operation, and to inform individual fish locations at the start of the post-construction monitoring phase. The post-construction monitoring phase of the study will begin with the release of PIT tagged fish in the Fraser and Colorado Rivers in fall 2024 and continue through 2026. Data collected during the post-construction monitoring phase will be compared to the baseline movement data collected during the pre-construction phase to determine how the CRCC and restored connectivity around and above Windy Gap Reservoir changed fish abundance, movements and survival throughout the system. Until the full analysis can be completed in 2026, summaries of movements through the CRCC and throughout the Colorado and Fraser rivers will continue to be presented in future reporting cycles.

Fetherman, E. R. 2013. Introduction and management of *Myxobolus cerebralis*-resistant rainbow trout in Colorado. Ph.D. dissertation. Department of Fish, Wildlife and Conservation Biology, Colorado State University, Fort Collins, CO.

Fetherman, E. R., B. Neuschwanger, B. W. Avila, and T. B. Riepe. 2020. Sport Fish Research Studies. Annual Report. Colorado Parks and Wildlife, Aquatic Research Section. Fort Collins, Colorado.

Fetherman, E. R., B. Neuschwanger, and R. E. McDevitt. 2023. Sport Fish Research Studies. Annual Report. Colorado Parks and Wildlife, Aquatic Research Section. Fort Collins, Colorado.

Fetherman, E. R., B. Neuschwanger, T. B. Riepe, and B. W. Avila. 2021. Sport Fish Research Studies. Annual Report. Colorado Parks and Wildlife, Aquatic Research Section. Fort Collins, Colorado.

Mobile Antenna Deployments and Data Collection

Mobile antennas (Figure 2.15) are being deployed as part of the fish movement study to supplement movement and detection data obtained from the stationary antennas. Data obtained from mobile antenna deployments will be used to adjust estimates of movement and survival probabilities when conducting the full analysis of the fish movement data. GPS locations of detected fish from the mobile antennas can be used to inform distance moved by tagged fish, especially those never detected at a stationary antenna station. This will allow for a more accurate estimation of tagged fish movement and survival probabilities, particularly for those fish that remained at or near the location they were released.

Repeat detections in the same location by the mobile antennas can help identify ghost tags, PIT tags that are no longer inside the fish due to tag expulsion or mortality (Richer et al. 2017). Failure to account for ghost tags can lead to incorrect interpretations regarding fish location and fate (Fetherman et al. 2014). Using the number of times a tag had been detected by the mobile antennas as a starting point and then evaluating the encounter histories of each tag to see if it had

not moved the previous three times that it had been detected, Fetherman et al. (2023) determined that there were 232 ghost tags in the system as of spring 2023. An estimated date on which the tag became a ghost tag was assigned to each, as some tags were retained in fish for months or years before being lost during spawning or due to mortality. The assignment of date and determination that the tag is a ghost tag will allow data obtained prior to the ghost tag date to be included as part of the movement and survival analyses, and data after the ghost tag date to be omitted such that it no longer is incorporated into estimates of movement or survival. Tags that became ghost tags during the study will be assigned to a "Ghost" state, such that the probability that a tag becomes a ghost tag will be one of the movement parameters estimated as part of the Program MARK modelling, but unlike other states evaluated using tags known to be in fish, the ghost tag will not be able to re-enter the analysis in a different state. This same evaluation of tags within the database will be repeated at the end of the study prior to conducting the full Program MARK analysis covering the duration of the study from 2020 to 2026.

Figure 2.15. (A) Antenna wire encased in PVC to maintain rigidity, shape, and tuning during deployment. (B) Pelican box, containing batteries, reader, tuner box, and Campbell Scientific datalogger, and external GPS unit for marking the location of detected PIT tags. (C) Portable antennas set to deploy on the Fraser River Ranch.

Mobile antennas have previously been deployed in three reaches: 1) the River Run reach in the Colorado River upstream of Windy Gap Reservoir (1.2 miles), 2) the Fraser River Ranch reach in the Fraser River upstream of Windy Gap Reservoir (1.0 miles), and 3) the Chimney Rock reach in the Colorado River downstream of Windy Gap Reservoir (4.5 miles; Figure 2.16). Two rafts were used to complete a single pass, one running the left side of the river and one running the right, to provide the greatest detection coverage. Rafts remained about 100 yards apart during deployment to prevent reader interference. The starting location for deployment through River Run was located on the River Run property just downstream of the Miller Ranch, and the reach included portions of the Colorado River through River Run, the Horn Ranch, and Northern Water property. Upon reaching the confluence of the Colorado and Fraser rivers, the rafts were walked upstream in the Fraser River to the pullout located downstream of the Fraser River gauge. The starting location for the Fraser River Ranch was at the upstream-most end of the

property, just downstream of the Granby water treatment plant. The Fraser River splits just downstream of the starting location. The north channel was run to avoid beaver dams and a water diversion structure located on the south channel, and the rafts were pulled out upstream of the Fraser River gauge. Rafts were deployed in the Colorado River immediately below Windy Gap Reservoir, covering the river section from immediately below the dam downstream through the Chimney Rock Ranch, and rafts were pulled out at the Sheriff Ranch. Each reach was run on a separate day, and two passes were completed in the same day in all three reaches. Mobile antennas were not deployed in fall 2023 or spring and summer 2024 due to ongoing construction activities in the CRCC.

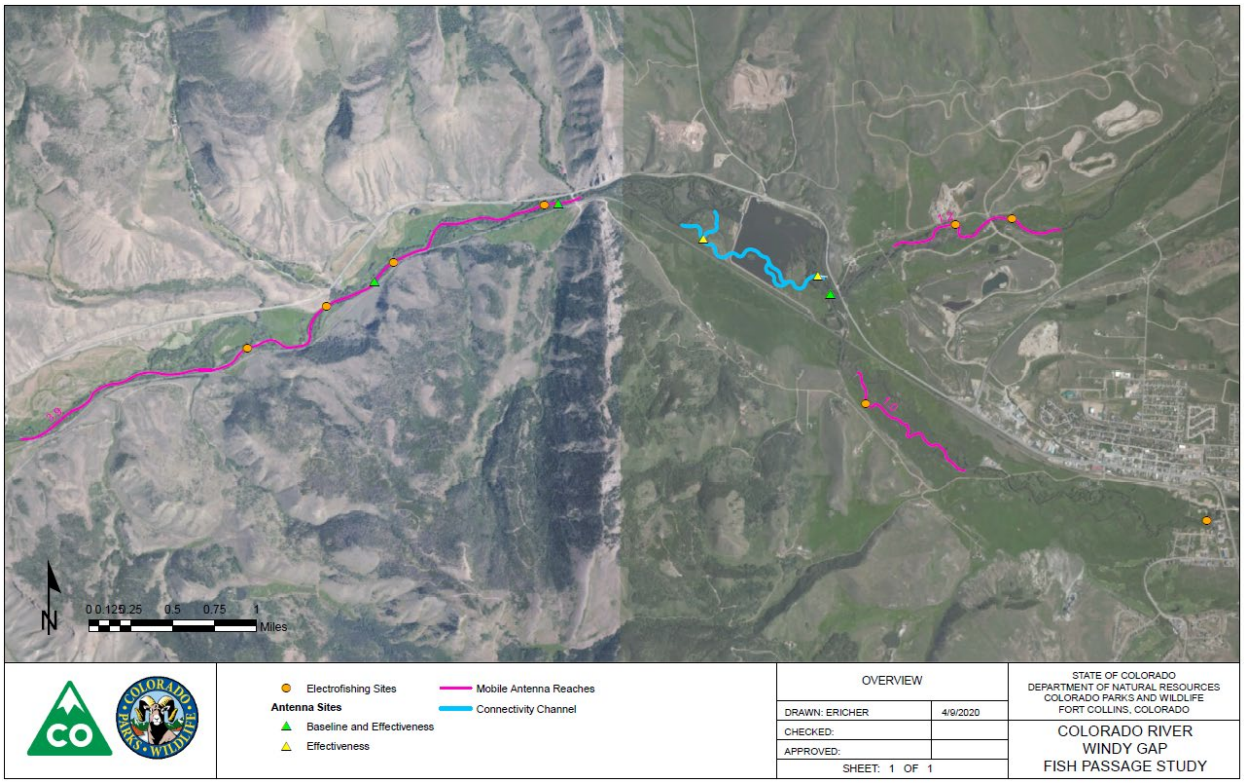

Figure 2.16. Mobile antenna reaches in the Colorado and Fraser rivers above Windy Gap Reservoir and the Colorado River below Windy Gap Reservoir (red lines). The blue line represents a mobile antenna reach that will be utilized in 2024 following completion of construction activities in the Colorado River Connectivity Channel.

With the completion of the CRCC, mobile antenna reaches could change during the post-construction evaluation phase of the study as there would be no need to pull the rafts out above Windy Gap Reservoir. Therefore, it may be an option to run two reaches over two days: 1) River Run through the CRCC down to Sheriff Ranch, and 2) Fraser River Ranch through the CRCC down to Sherriff Ranch. A potential drawback to this approach would be that only one run would be completed through the River Run and Fraser River Ranch reaches, whereas the CRCC and Colorado River below the CRCC would be run twice. To determine how much information may be lost by running these two sections only once in any given sampling period, we conducted an analysis of data collected from the spring, summer, and fall runs in the three reaches in 2021.

Three parameters were investigated. The first examined the number of tagged fish detected when two passes were run through each reach compared to the average number of fish detected on only one pass (average of the first and second passes). The other two parameters, detection probability and abundance, were estimated using a Program MARK analysis of the encounter histories generated by the mobile antennas. Encounter histories for the two pass data were constructed using the data from both rafts on each pass such that the pass one encounters were the total number of fish detected by both rafts on the first pass, and the pass two encounters were the total number of fish detected by both rafts on the second pass. In comparison, the single pass encounter histories were constructed from the first pass data only, treating the data from each raft as a pass (e.g., raft one = pass one, raft two = pass two). Fish detected on either pass were coded with a "1", whereas fish not detected on either pass were coded with a "0", which resulted in three potential encounter histories for any given individual: 1) 10 = fish detected on the first pass, but not on the second; 2) 01 = fish detected on the second pass, but not on the first; and, 3) 11 = fish detected on both passes. An encounter history of 00 was not possible because a fish had to be detected on one of the two passes to be included in the analysis.

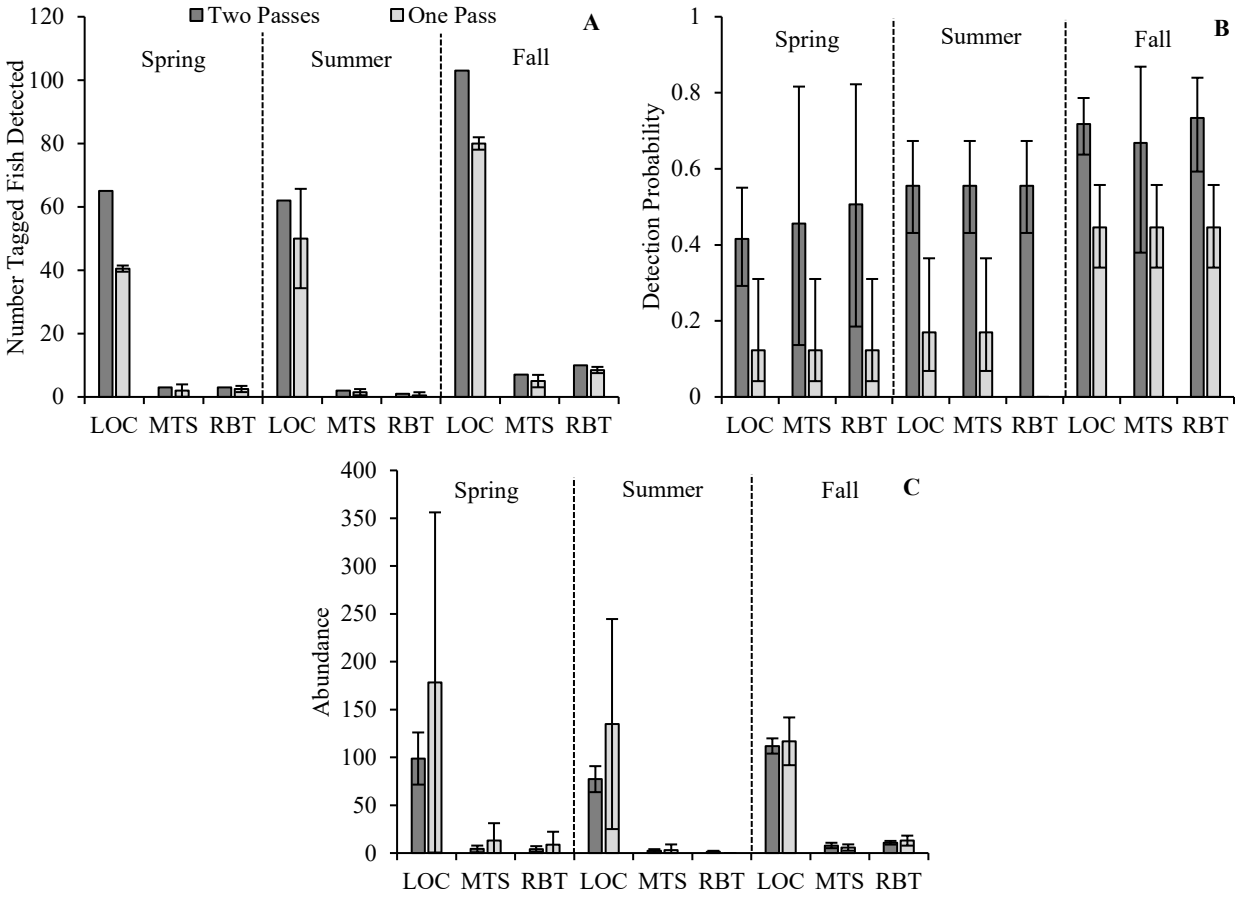

Figure 2.17. Number of tagged fish detected (A), detection probability (B), and abundance (C; SE bars) estimated for Brown Trout (LOC), Mottled Sculpin (MTS), and Rainbow Trout (RBT) when detected using two passes with both rafts versus one pass with both rafts in the spring, summer, and fall of 2021 in the River Run reach of the Colorado River.

Model sets were constructed for each set of data (i.e., two pass versus one pass in each reach and each season) and contained three constructs for detection probability: 1) an intercept model; 2) a model in which detection probability differed by species (Brown Trout, Rainbow Trout, and Mottled Sculpin; and, 3) a model in which detection probability differed between salmonid species as a group versus Mottled Sculpin (reflecting the difference in potential detection distances due to PIT tag size, 12 mm for Mottled Sculpin and 32 mm for salmonids). Note that the salmonid model set could only be constructed for mobile antenna datasets from River Run and the Fraser River Ranch as Mottled Sculpin are not currently found below Windy Gap Reservoir. For all models, the recapture probability (c) was set equal to the detection probability (p) as these were not expected to differ among passes. Detection probability estimates were obtained from the model sets as a real parameter, whereas the abundance (N) estimate was a derived parameter, and the model averaged estimates for these two parameters are presented.

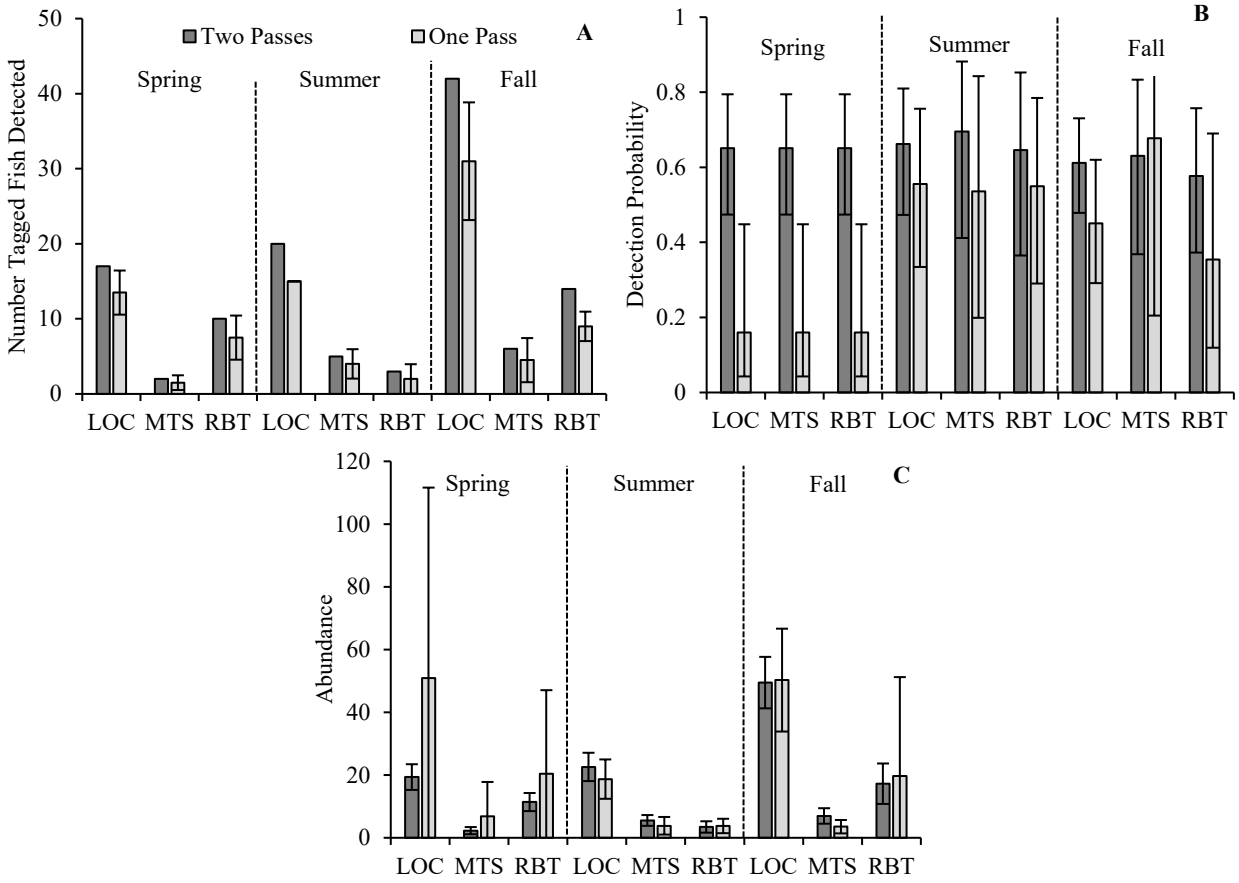

Figure 2.18. Number of tagged fish detected (A), detection probability (B), and abundance (C; SE bars) estimated for Brown Trout (LOC), Mottled Sculpin (MTS), and Rainbow Trout (RBT) when detected using two passes with both rafts versus one pass with both rafts in the spring, summer, and fall of 2021 in the Fraser River Ranch reach of the Fraser River.

In the River Run reach of the Colorado River, the number of tagged fish and detection probability increased, and, at least for Brown Trout, abundance estimates and associated error

decreased as flows decreased from spring through fall (Figure 2.17). For Brown Trout, the number of tagged fish detected was lower when one pass versus two passes were completed through the reach in the spring and the fall, but not in the summer; the difference in unique number of tagged fish detected on one versus two passes was around 25 fish. The number of tagged fish did not differ between one and two passes for Mottled Sculpin or Rainbow Trout in any season. Detection probabilities for all three species were similar in the spring and for Mottled Sculpin in the fall, but were significantly lower with one pass versus two passes for all species in the summer, and for Brown Trout and Rainbow Trout in the fall. Rainbow Trout were only detected when two passes were completed in the summer. Overall, abundance estimates of tagged fish in the reach did not differ between one and two passes for any species in any season (Figure 2.17).

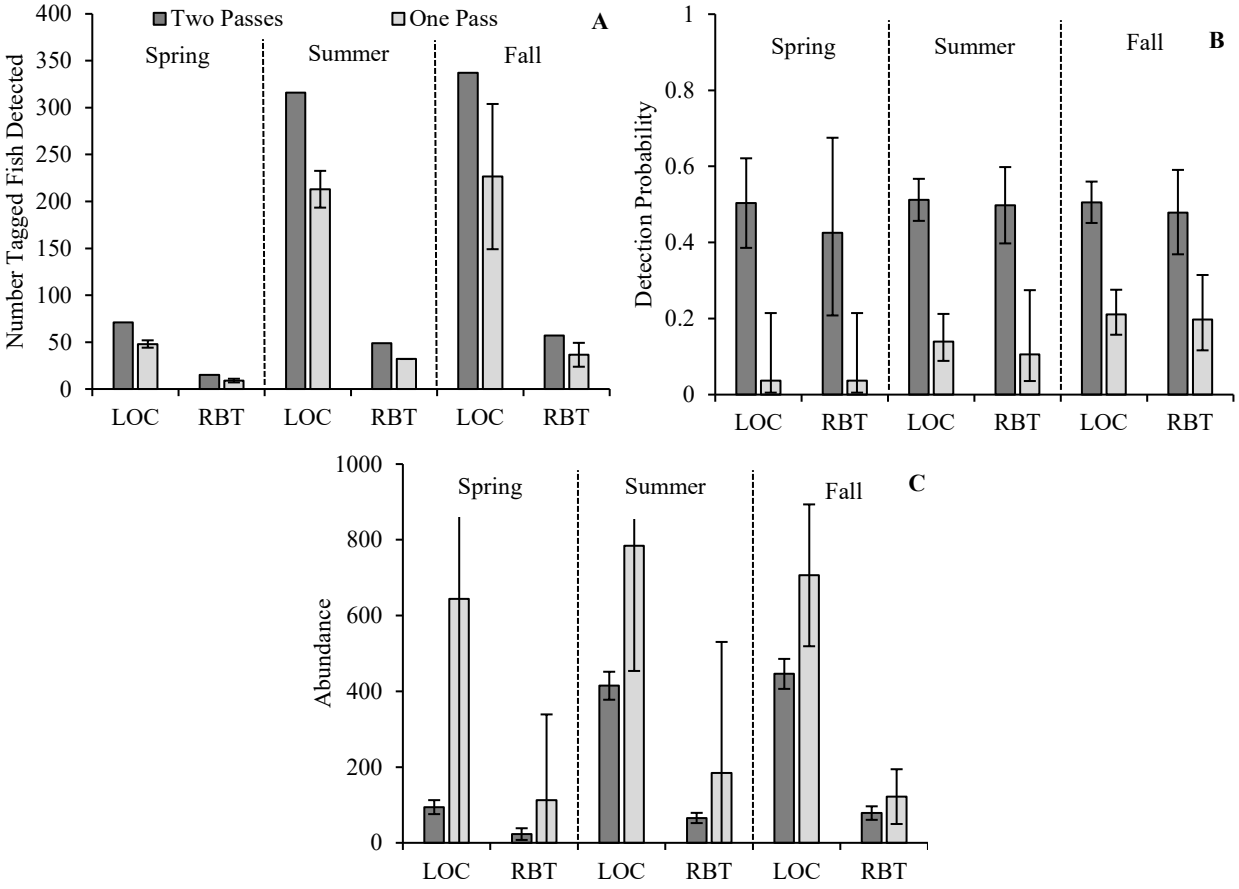

Figure 2.19. Number of tagged fish detected (A), detection probability (B), and abundance (C; SE bars) estimated for Brown Trout (LOC) and Rainbow Trout (RBT) when detected using two passes with both rafts versus one pass with both rafts in the spring, summer, and fall of 2021 in the Chimney Rock reach of the Colorado River downstream of Windy Gap Reservoir.

Similar to what was observed in the River Run reach, the number of tagged fish detected in the Fraser River Ranch reach increased as flows decreased from spring through fall (Figure 2.18). The number of tagged Brown Trout detected in all seasons and the number of tagged Rainbow Trout detected in the fall were lower with one versus two passes, but the difference in unique

number of tagged fish detected was less than five fish. Detection probability was significantly lower using one pass for all species in the spring when flows were highest. However, across seasons and species, detection probabilities were relatively constant between one pass and two passes through the reach. Overall, abundance estimates of tagged fish in the reach did not differ between one and two passes for any species in any season (Figure 2.18).

The number of tagged fish detected in the Chimney Rock Reach below Windy Gap Reservoir increased with a decrease in flows from spring through fall (Figure 2.19). In all seasons, the number of tagged fish detected was greater with two passes versus one pass, and the unique number of tagged Brown Trout detected differed in the summer and fall by up to 100 fish. Although fairly consistent among seasons, detection probability was significantly lower when using only one pass compared to two passes. Even when using two passes, detection probabilities in the Chimney Rock reach were lower than those observed in the River Run or Fraser River Ranch reaches. Abundance estimates were lower using two passes compared to one pass, however, the two pass estimates had a much smaller range of error and were likely a more accurate reflection of the tagged fish abundance than those estimates obtained from a single pass through the reach (Figure 2.19).

The results of this analysis suggest that, with the exception of a relatively small number of tagged fish detected, little information is lost by conducting one pass versus two passes in the River Run and Fraser River Ranch reaches. In contrast, many more tagged fish are detected using two passes versus one pass, detection probability is much lower using a single pass, and abundance estimates are more accurate using two passes through the Chimney Rock Ranch reach below Windy Gap Reservoir. Therefore, moving forward, we plan to deploy the mobile antennas over two days, one day covering the River Run reach through the CRCC and the Chimney Rock Ranch reach, and the second day covering the Fraser River Ranch reach through the CRCC and the Chimney Rock Ranch reach, with both reaches being about 8 miles long. Mobile runs for the post-construction phase of the Fish Movement Study will begin in October 2024, and continue through the springs, summers, and falls of 2025 and 2026.

Fetherman, E. R., B. W. Avila, and D. L. Winkelman. 2014. Raft and floating radio frequency identification (RFID) antenna systems for detecting and estimating abundance of PIT-tagged fish in rivers. North American Journal of Fisheries Management 34:1065-1077.

Fetherman, E. R., B. Neuschwanger, and R. E. McDevitt. 2023. Sport Fish Research Studies. Annual Report. Colorado Parks and Wildlife, Aquatic Research Section. Fort Collins, Colorado.

Richer, E. E., E. R. Fetherman, M. C. Kondratieff, and T. A. Barnes. 2017. Incorporating GPS and mobile radio frequency identification to detect PIT-tagged fish and evaluate habitat utilization in streams. North American Journal of Fisheries Management 37(6):1249-1264.

Water Filtrations for Triactinomyxon Quantification

Whirling disease is established in the upper Colorado River, and Windy Gap Reservoir is one of the primary sources of triactinomyxon (TAM) production in the system. With the construction

of the CRCC, water now bypasses Windy Gap Reservoir for the majority of the year, potentially reducing TAM production and overall infection prevalence in the system. In addition, recent high spring runoff events in 2023 and 2024 have cleared out sediment and worm habitat from the Colorado River downstream of Windy Gap Reservoir. To monitor the potential change in TAM production before, during, and after the construction of the CRCC, we began taking water samples in 2020 to quantify the amount of TAMs in the water column at multiple times of year.

Table 2.1. Number of triactinomyxons per liter (TAMs/L; T) and number of wells that fluoresced (F) for each sample collected on six dates and from five locations (Hitching Post, HP; upper Red Barn, URB; lower Red Barn, LRB; below the Red Barn diversion, BRBD; Sheriff Ranch, SR) in the upper Colorado River in 2022. A "-" indicates that samples were not collected from a given location/date.

Site	5/5/22		7/8/22		7/26/22		8/31/22		9/27/22		10/20/22	
	T	F	T	F	T	F	T	F	T	F	T	F
HP1	0	0	1.2	3	0	1	0.1	1	0	1	0.3	3
HP2	0	0	3.1	3	0	1	0	0	0	0	0	0
HP3	0	0	3.1	3	2.2	3	0	0	0	0	0.4	2
URB1	-	-	0	1	0	0	0	0	0	0	1.4	3
URB2	-	-	0	2	0.6	3	0	0	0	0	0	0
URB3	-	-	0.2	3	0.1	2	0.1	3	0.1	2	1.7	3
LRB1	0	0	0.1	2	0	1	0.1	2	0	0	1.5	3
LRB2	0	0	0.7	3	0	1	0	0	0	0	0	0
LRB3	0	0	0.2	3	0	0	0	0	0	2	0	0
BRBD1	0	0	-	-	-	-	-	-	-	-	-	-
BRBD2	0.6	3	-	-	-	-	-	-	-	-	-	-
BRBD3	0	0	-	-	-	-	-	-	-	-	-	-
SR1	0	0	0	0	0	0	0	1	0	0	1.2	3
SR2	0	0	0.1	1	0.7	3	0	0	0	0	0.1	2
SR3	0	0	0.5	2	0	1	0	0	0	0	0	0

Water samples were taken at four locations in the Chimney Rock/Sheriff Ranch study section during the adult population estimates in May 2022 and June 2023, and during fry stocking in July 2023: 1) Hitching Post, 2) lower Red Barn, 3) below the Red Barn diversion, and 4) Sheriff Ranch. Samples were also collected from each of four fry sites at Hitching Post, upper Red Barn, lower Red Barn, and Sheriff Ranch during each fry sampling occasion in July, August, September, and October 2022 and 2023 to determine if TAM counts were correlated with myxospore counts in salmonid fry. At each location and on each sampling occasion, four consecutive 1-L samples were collected by placing the sample bottle at 0.67 the depth of the water column and removing the lid to quickly fill the bottle. Samples were kept on ice until filtering could occur. Water was vacuum filtered through 5 μm filters, one to three per 1-L sample depending on turbidity. The entire filters were folded, placed in a 2-ml tube with several

drops of 100% ethanol to stabilize the sample, and frozen. Samples were sent to Sascha Hallett and Steven Atkinson at Oregon State University (OSU) for TAM quantification.

Table 2.2. Number of triactinomyxons per liter (TAMs/L; T) and number of wells that fluoresced (F) for each sample collected on six dates and from five locations (Hitching Post, HP; upper Red Barn, URB; lower Red Barn, LRB; below the Red Barn diversion, BRBD; Sheriff Ranch, SR) in the upper Colorado River in 2023. A "-" indicates that samples were not collected from a given location/date.

Site	6/7/23		7/11/23		7/25/23		8/29/23		9/26/23		10/24/23	
	T	F	T	F	T	F	T	F	T	F	T	F
HP1	0	0	0.1	3	0.2	1	0	0	0	0	0.7	3
HP2	0.4	3	1.2	3	0	0	0.1	3	0.2	3	0	0
HP3	0	1	1.9	3	0	0	0	0	0.4	3	0	0
HP4	0	1	2.7	3	0	0	0	0	0.9	3	0	0
URB1	-	-	-	-	0	1	0	1	1.8	3	0	0
URB2	-	-	-	-	0	0	0	0	0	0	1.3	3
URB3	-	-	-	-	0	1	0	1	1.4	3	0	0
URB4	-	-	-	-	0	1	0	0	0	0	0	0
LRB1	0.9	3	0	2	0.1	3	0	0	0	0	0	1
LRB2	0	3	0	3	0	1	0.1	2	0.9	3	0.1	2
LRB3	0.8	2	0	0	0	0	0.1	1	0.3	3	0	0
LRB4	0.3	3	0.2	3	0	1	0	0	0	0	0.3	3
BRBD1	0	0	0.7	3	-	-	-	-	-	-	-	-
BRBD2	0	0	0.6	3	-	-	-	-	-	-	-	-
BRBD3	0	0	2.0	3	-	-	-	-	-	-	-	-
BRBD4	0	0	0	0	-	-	-	-	-	-	-	-
SR1	0	0	1.9	3	0	2	0	0	1.9	3	0	0
SR2	0	0	1.5	3	0	0	0	0	1.8	3	0	0
SR3	0	0	0	1	0	0	0	0	0.2	2	1.7	3
SR4	1.4	3	0	0	0	0	1.0	3	0	0	0.1	3

In 2022, three of the four samples collected from each site and date were processed by OSU (Table 2.1), with the fourth retained for processing if inhibition occurred during analysis. All four samples collected from each site were processed by OSU in 2023 (Table 2.2). Filters were extracted and total *M. cerebralis* DNA purified according to the method of processing environmental samples presented in Hallett et al. (2012). The amount of *M. cerebralis* DNA was assayed by qPCR (Kelley et al. 2004), with modifications in chemistry and machine programing consistent with current technology. For calibration purposes, reference control samples were prepared from in-house cultures of *M. cerebralis*. Replicates of five TAMs were counted and added to filter papers, and non-target carrier DNA was added. Control samples were then extracted using the same protocol as used with the environmental water samples. A second

positive control reference of diluted *M. cerebralis*-infected Rainbow Trout was also used on some plates. All samples were diluted 1:10 in Qiagen buffer AE prior to running qPCR to reduce the effect of environmental PCR inhibitors that may have co-purified with the *M. cerebralis* DNA. Each sample was run in triplicate through qPCR (technical replicates). Samples were considered positive when two or three wells fluoresced, and considered "not detected" (i.e., 0 TAMs/L) when zero or one well fluoresced.

Examination of the data from 2022 and 2023 reveals some interesting patterns. First, TAMs seem to be unevenly distributed in the water column, both within sites, and among sites, leading to a lot of variability in TAMs/L and number of wells fluoresced in individual one liter samples. This could, in part, account for the variability observed in individual fish myxospore counts throughout the reach, both within and across sites (in addition to individual variability in resistance, immune system function, size or age at exposure, etc.). In 2023, the Sheriff Ranch generally had higher TAM counts throughout the sampling period, which was unexpected given its distance downstream from Windy Gap Reservoir. A high runoff and large flows in Drowsy Water Creek created a large alluvial fan just above the Red Barn diversion structure which may have created additional worm habitat downstream of the other three sites at which samples were collected. Continued construction activities on the ranch to repair the damage to the road into the Red Barn area caused by these high spring flows way have perpetuated disturbance to these worm populations throughout 2023, resulting in higher TAM releases and counts downstream of this location as worms release more TAMs following disturbance than they typically do in an undisturbed state. There appears to be a spike in TAM production in July, and TAM counts per liter are generally highest at the Hitching Post site, suggesting that Windy Gap Reservoir continued to be a point source for TAM production in the CRCC pre-construction monitoring period. This may be a result of disturbance experienced by the worms during runoff where more water is run through Windy Gap Reservoir than other times of the year. However, these July spikes in TAM releases appear to be short lived, with many fewer TAMs per liter observed in the samples by the end of July and August. Increased TAM counts in the samples in September and October were likely a result of disturbance to the worms from reservoir draining (2022) or construction activities associated with completion of the CRCC (2023).

TAM releases appear to be relatively consistent across years in July and August (Figure 2.19). High TAM counts in May 2021 were primarily observed in samples taken below the Red Barn Diversion, suggesting a disturbance potentially caused by runoff or spring rains in Drowsy Water Creek, and high TAM counts in September and October 2021 were likely a result of draining the reservoir in mid-September (Fetherman et al. 2022). As a result of these spikes, the average annual TAM counts in 2021 (1.02 ± 0.21) were higher than those in 2022 (0.30 ± 0.12) or 2023 (0.34 ± 0.12). However, in general, TAM releases in May, September, or October are inconsequential to the myxospore counts obtained from fry in the Colorado River. Brown Trout may have hatched, but have not likely emerged from the gravel during any spikes in TAM production in early May, making it unlikely that TAM production during this time would affect infection severity of the Brown Trout fry. Although they could still contribute to additional infection in age-1+ fish, spikes in TAM production in September or October would not contribute to the myxospore counts of the fry collected in October as there would not be enough degree-days to allow for myxospore development prior to collection.

The TAM counts during the July fry stocking occasions in each year therefore likely drive the infection rates for both salmonid fry naturally produced in the system and the stocked fry. By this time of year, Brown Trout fry have emerged, but are still small and young enough that their skeleton has not yet ossified. Rainbow Trout fry produced in the river are usually emerging during this time. The H×G fry stocked into the Colorado River in 2021 through 2023 were stocked on the day these water samples were collected. The number of TAMs per liter collected in early July is consistent across all three years, suggesting little variation in exposure rates. We see a similar pattern in Rainbow Trout myxospore counts, with no difference in average spore counts across these three years (2021: 414 ± 266 myxospores per fish; 2022: 1,152 ± 602 myxospores per fish; 2023: 557 ± 324 myxospores per fish). Brown Trout were only collected from one site, Hitching Post, within the Chimney Rock Ranch study section in 2022 due to the low natural reproduction occurring in the system in recent years. Brown Trout averaged 23,450 ± 23,450 myxospores per fish, higher than those of the Rainbow Trout. This pattern in infection severity is consistent with Rainbow Trout and Brown Trout myxospore counts from fry collected below Byers Canyon in 2022 as well (Brown Trout: 31,822 ± 18,261; Rainbow Trout: 806 ± 467). These results suggest that despite the same exposure rates during the same time of year, the Rainbow Trout being stocked in the Colorado River are more resistant on average than the Brown Trout being produced in this system.

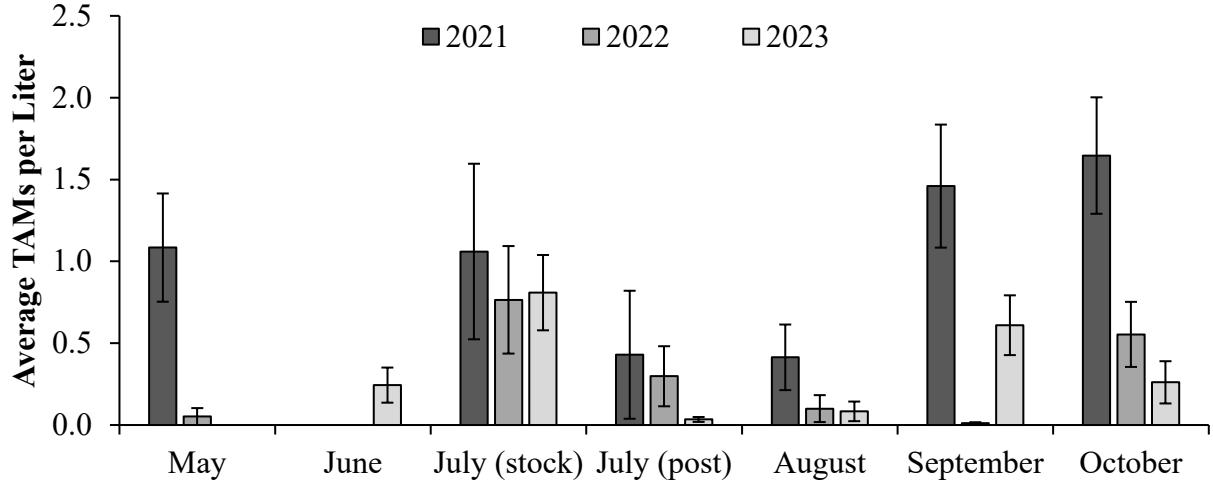

Figure 2.19. Average TAMs per liter (SE bars) by month in 2021, 2022, and 2023. Two sampling occasions occurred in July, the first associated with stocking H×G fry, and the second as a post-stocking evaluation of survival two to three weeks later.

Unfortunately, none of the years in which water samples have been collected (2020-2023) can be considered "normal" years in which there were not atypical disturbances occurring in the system due to the construction activities at Windy Gap Reservoir. Continued collection of samples through the CRCC post-construction years (2025-2026) should show what the new normal for this system will be in terms of TAM production when the majority of the water is bypassing Windy Gap Reservoir. Timing of releases from the reservoir will be dependent upon the magnitude and timing of runoff, and whether or not water is being pumped out of versus passed through Windy Gap during this time. Our results suggest that, assuming Windy Gap Reservoir is the primary source of TAM releases, if the reservoir is no longer releasing water in July, this

could significantly reduce TAM counts and exposure levels in the river, increasing the survival of the wild and stocked fry.

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YAMPA RIVER RAINBOW TROUT BROOD STOCK

In April 2019, the wild Rainbow Trout brood stock in Harrison Creek and Lake Catamount (Steamboat Springs, Colorado) tested positive for *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease (BKD), during a routine annual health inspection. Since then, fish in this location have gone through periods of testing positive and negative for the presence of *R. salmoninarum*. Adult fish spawned in Harrison Creek in 2020 tested positive for *R. salmoninarum*, but fish collected from Lake Catamount in 2021 and both Lake Catamount and Harrison Creek in 2022 tested negative for the bacteria. The negative result in 2022 spurred additional testing per the state regulations that a previously positive site must test negative three times within a year, with tests occurring at least three months apart and 12 months between the initial and final negative test, in order for the site to return to a negative status. Fish from this location tested negative for a second time in September 2022, but on the third and final test for this protocol in May 2023, the fish once again tested positive for *R. salmoninarum*. On April 30, 2024, 60 fish were collected from Lake Catamount to maintain the disease history of this location and ascertain the status of *R. salmoninarum*. All 60 fish tested negative for any pathogens of concern, including *R. salmoninrum*, restarting the above protocol to obtain three negative tests in a one year period. The next collection of fish from Lake Catamount as part of this protocol will occur in September 2024.

The CPW Crystal River Hatchery is interested in receiving wild eggs to supplement the H×H brood stock maintained by the hatchery. Taking eggs from an *M. cerebralis* positive wild brood stock would incorporate more wild-type genetics and potentially increase the whirling disease resistance of the brood stock. Researchers, biologists, and hatchery managers have developed a plan for taking eggs and bringing them in the hatchery when that occurs in the future (Fetherman et al. 2023). However, given the history of *R. salmoninarum* in Lake Catamount and Harrison Creek, alternative locations for a wild spawn take within the Yampa River were investigated.

With other pathogens such as *Myxobolus cerebralis*, once established in a portion of a connected system, the entire system is considered positive for that pathogen. However, recent field experiments and risk mapping suggest that *R. salmoninarum* may not have a continuous

distribution within a system depending on species distributions, spawning locations, habitat, and water flows and temperatures. One of the goals of the Yampa River post-stocking survival experiment was to establish H×H and increase Hofer *M. cerebralis*-resistance characteristics throughout the entire Yampa River between Stagecoach Reservoir and Lake Catamount (Fetherman et al. 2018), which would allow egg takes to occur anywhere in this section of the river. Although Harrison Creek, Lake Catamount, and the Yampa River are connected, it is possible that fish positive for *R. salmoninarum* remain in the lake or spawn in Harrison Creek only, but do not move up the Yampa River to spawn. If fish do move up into the Yampa River from Lake Catamount, there are 6.5 miles of river between Lake Catamount and the Stagecoach Reservoir tailwater, which could prevent fish that are stressed and infected with *R. salmoninarum* from moving this far to spawn. Additionally, three tributaries, Green Creek, Sarvis Creek, and Morrison Creek, are encountered along the way and are known spawning locations for salmonids, potentially diverting fish before reaching the tailwater.

In 2021, the first group of 60 fish, 30 from the Wellar Ranch above Morrison Creek and 30 from the upper Stagecoach Tailwater above the habitat project reach, were collected from the Yampa River and tested for the presence of *R. salmoninarum* only. All of the Rainbow Trout collected from the Yampa River tested negative for *R. salmoninarum* by DFAT, suggesting that *R. salmoninarum* may not be distributed throughout the entire system and egg takes from the Wellar Ranch or Stagecoach Tailwater may be possible in the future. The Yampa River between Lake Catamount and Stagecoach Reservoir is a different water code than Lake Catamount and Harrison Creek. Therefore, a full disease inspection and three-year disease history is needed for this site before taking eggs. Groups of 60 fish were collected from the same locations as those in 2021 in 2022, 2023, and 2024. All fish tested negative for all pathogens of concern, including *R. salmoninarum*. Therefore, a spawn take could occur in the Yampa River in 2025 if CPW chooses to move forward with supplementation of the Crystal River brood stock with wild eggs.

Another important consideration for the wild egg take is the genetic resistance of the Yampa River population to whirling disease. Most recently, the resistance of individual fish has been evaluated using the presence of the WDRES-9 quantitative trait loci (QTL; Fetherman et al. 2020, 2022). During future spawn takes, eggs will initially be retained from families of which both parents have two copies of the WDRES-9 QTL (classified as resistant-resistant; RR), but may also be kept from families of which one or both parents have only one copy of the WDRES-9 QTL (classified as resistant-susceptible; RS). No families in which one or both parents do not have a copy of the WDRES-9 QTL (classified as susceptible-susceptible; SS) will be retained to maintain higher genetic resistance to *M. cerebralis* in the hatchery brood stock. Once genetic analyses have been obtained and eggs from SS parents have been culled, the remaining resistant fish will be sent to the Crystal River Hatchery for incorporation into their brood stock program.

Genetic samples collected during the 2023 disease testing were sent to UC Davis to identify the presence of the WDRES-9 QTL in the fish from both Lake Catamount and the Yampa River. The results suggested eggs should be taken from Lake Catamount versus the Stagecoach Tailwater to maintain a higher percentage of *M. cerebralis*-resistant fish for brood stock supplementation. More families would need to be spawned and egg groups maintained separately until genetic results informed which families to retain due to their higher resistance (Fetherman et al. 2023). However, given the current *R. salmoninarum*-positive status of Lake

Catamount, the Stagecoach Tailwater will be the only location from which eggs can be taken for the foreseeable future. Selection pressures and continued exposure to *M. cerebralis* could result in a change in genetics over time in both Lake Catamount and the Yampa River. Therefore, 96 samples collected from Lake Catamount and the Yampa River during the disease testing in 2024 were sent for evaluation of the presence of the WDRES-9 QTL in individual fish from these two locations. This sample included 68 fish of known sex (26 males, 42 females), representing the current spawning population, and 28 smaller wild fish for which the sex was unknown, representing the future spawning generation. Fourteen of the samples (15%) did not amplify, and therefore, the presence of the WDRES-QTL could not be assessed. The results for those fish that did amplify are presented below.

Table 2.3. Total number of fish for which genetic results were obtained from Lake Catamount and the Yampa River, and number of fish that tested genetically RR, RS, or SS.

Location	Status	Total	RR	RS	SS
Lake Catamount	Sex known	21	16	4	1
Lake Catamount	Sex unknown	10	8	2	0
Yampa River	Sex known	38	16	18	4
Yampa River	Sex unknown	13	5	4	4

The proportion of the population in Lake Catamount that has at least one copy of the WDRES-9 QTL remains high in both the current spawning population (95% of the fish tested) and the next spawning generation (100% of fish tested; Table 2.3). More fish in the Yampa River were considered susceptible, i.e., they did not have the WDRES-9 QTL. However, the proportions of spawning fish with at least one copy of the WDRES-9 QTL was still relatively high (89% of fish tested). This proportion dropped in the next generation of spawning fish to 69% of the fish tested, but this was a smaller sample size relative to the spawning population.

Table 2.4. Total number of arbitrarily paired spawns in 2024 for which genetic results were obtained from Lake Catamount and the Stagecoach Tailwater, and number of families for which the parents would be genetically RR-RR, RR-RS, RR-SS, RS-RS, RS-SS, or SS-SS.

Location	Total	RR-RR	RR-RS	RR-SS	RS-RS	RS-SS	SS-SS
Lake Catamount	8	3	4	1	0	0	0
Yampa River	10	2	4	2	1	1	0

To determine what might have occurred genetically after a theoretical spawn of the fish in both locations, fish were arbitrarily paired by sex using a random number generator, similar to random selection from a tank of fish, for a preliminary examination of how many families would have originated from parents with the various possible combinations of resistant and susceptible genetic markers. Eight females, one in Lake Catamount and seven from the Yampa River, were removed from this analysis prior to pairing as they were already spent and would not have been available for use during the spawn. This resulted in eight pairs in Lake Catamount and 10 pairs in the Yampa River (Table 2.4). The eggs from only one family (12.5%) in Lake Catamount would have been discarded due to one of the parents being classified as SS. In contrast, eggs from three families (30%) would not have been retained from the Yampa River due to one parent

being classified as SS, and an additional family (10%) would have been removed due to both parents being classified as RS.

Taken together, the genetic results continue to suggest that eggs should be taken from Lake Catamount versus the Stagecoach Tailwater to maintain a higher percentage of *M. cerebralis*-resistant fish for brood stock supplementation. However, given the current *R. salmoninarum*-positive status of Lake Catamount, the Yampa River will be the only location from which eggs can be taken for the foreseeable future. Sixty percent of the families collected from the Yampa River had the correct combination of parental genetic characteristics to be retained, higher than what was observed in 2023 (Fetherman et al. 2023). The goal in future years will be to collect and retain roughly 30,000 eggs for brood stock supplementation purposes from either or both locations. Assuming a conservative estimate of 1,000 eggs per female, had eggs been collected this year, only 6,000 eggs would have been retained from the Yampa River spawns. Therefore, if eggs are collected only from the Yampa River in the future, and genetic proportions in the Yampa River do not move towards more resistant fish, approximately 50 families will need to be spawned and maintained separately to meet the egg request; an additional eight families should be spawned to account for the potential that up to 15% of the samples will not amplify and the presence of the WDRES-9 QTL cannot be assessed. Genetic samples will continue to be collected from the Stagecoach Tailwater to monitor changes in resistance and adjust spawning family projections as needed.

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Borns Lake Rainbow Trout Evaluations

Borns Lake is a privately owned lake in Pagosa Springs, Colorado, which contains a unique Rainbow Trout population that was originally stocked in the lake around 1901 and never restocked. Jim White, CPW aquatic biologist based in Durango, contacted aquatic researchers to see if there was interest in evaluating this population for both their genetic origin and performance or use in other locations, and it was determined that this population warranted further evaluation.

Thirty fin clips were collected from Rainbow Trout captured from the lake using hook-and-line sampling on October 17, 2023. Aquatic researchers will determine the appropriate genetic technique for using these genetic samples to ascertain the potential strain or origin of these fish. In addition, historical data and stocking records will be used to match with genetic results to help determine location of origin. A quick search through some historical records in the CPW library showed that approximately 17,000 Rainbow Trout were stocked in the state of Colorado in 1901, all from federal hatcheries, although some may have been stocked by state hatcheries as well (unknown). Numbers of stocked Rainbow Trout from state and federal sources vary between 1900 and 1903 (Wiltzius 1981). The Leadville Hatchery was in operation during this time and could be a potential source of the federal fish. Additionally, at that time, fish were still being distributed from California, with the primary source in the late 1800s being the McCloud River (Halverson 2010), so this could also be a potential origin for these fish. If the strain or origin can be identified, the state of origin will be contacted to see if they are interested in getting these fish back as they likely represent a unique genetic lineage that has not largely changed over the last century.

A wild spawn take occurred on June 4 and June 17, 2024. Rainbow Trout were captured from the spawning channel using backpack electrofishing and held in a large tank until spawning occurred. Fish were spawned using the wet spawning method, where eggs from the female were stripped into a bowl along with the ovarian fluid. After collecting the eggs, milt from a single male was added to the bowl. Water was poured into the bowl to activate the milt, and the bowl of eggs and milt was covered and left undisturbed for several minutes while the fertilization process took place. Next, the eggs were rinsed with fresh water to expel old sperm, feces, egg shells, and dead eggs. Eggs were poured into a two gallon bucket with 50 ppm iodine to water harden for approximately one hour. After the water hardening period, the eggs were rinsed to remove the iodine, placed in a cooler with freshwater from the spawning channel, and transported to the BFRH. Upon arrival at the BFRH, fish were treated again with a 100 ppm iodine solution for ten minutes to disinfect the surface of the eggs before being brought into the hatchery. The first spawn on June 4 produced 11 ounces of eggs with a count of 261 eggs per ounce for a total of 2,871 eggs collected. The second spawn on June 17 produced 21 ounces of eggs for a total of 5,481 eggs collected.

Because these are wild fish that have not been reared in a hatchery setting for over a century, it will be important to keep a good record of the hatchery performance of these fish during the rearing process. To maintain replication, eggs from both spawns were distributed into three Heath trays to eye up. Eggs were treated with 1,667 ppm formalin every other day to prevent fungal infection. Upon eye-up, the eggs from each tray were moved to a tank in FR1 to hatch. Replication will be maintained through the entirety of the evaluations. Metrics including percent pick-off, percent eyed eggs, and number of cripples or fish removed because they didn't swim up are being collected separately for each of the replicates. At the time of writing, fish were just beginning to hatch.

After swim-up, fish will be reared in a minimum of three tanks until a subset are removed for whirling disease evaluations, and the remainder are stocked into Parvin Lake. Fish will be separated into additional hatchery troughs as they grow and as needed, but groups from the original tanks will not be mixed if it can be avoided to maintain specific records for each

replicate throughout the entirety of the rearing process. Fish will be fed using current hatchery feeding protocols and percent body weight per day to promote growth. Metrics including post-hatch survival and growth will be collected separately for each of the replicates. Hatchery staff will also keep notes on observed performance, such as how the fish take to first feeding, if fish are not feeding well, other behaviors observed (e.g., afraid of or acclimated to feeder), etc. These metrics and notes will not only be used for reporting, but could also be important if this strain was found to be as useful for incorporating into Colorado's hatchery program at some point in the future.

Given that the Borns Lake Rainbow Trout have been isolated in a system not known to contain *Myxobolus cerebralis*, it is unlikely that the population shows any resistance to the parasite. However, knowing the resistance or lack thereof of these fish will be important if this population were to be utilized for stocking in other locations in the future. Although infection prevalence and severity information could potentially come from the Parvin Lake stocking evaluations since the lake is positive for the parasite, recent stocking experiments have experienced a low number of recaptures from the lake. Therefore, a lab exposure experiment will be used as the primary evaluation of whirling disease resistance if fry swim-up timing and availability of triactinomyxons coincide.

Ten age-1+ Brown Trout were collected from the Colorado River during the adult population estimates on May 7, 2024, transported back to Fort Collins, and held in the lab refrigerator. The last week of May, these fish were taken up to the CPW Parvin Lake Research Station, homogenized, and put into the *Tubifex tubifex* worm cultures maintained at the station to allow consumption of myxospores and induce triactinomyxon production.

Exposure evaluations will be conducted in the CPW Salmonid Disease and Sport Fish Research Lab in Fort Collins, Colorado. At about six to seven weeks post-hatch, or around the week of August 12, fish to be used in the exposure experiment will be moved from the BFRH to the lab in Fort Collins. Fish will be given one to two weeks to acclimate to lab conditions prior to exposure. The experiment will consist of four tanks, each containing 25 fish, one control tank, and three exposure tanks, for a total of 100 fish. Fish will be exposed to 2,000 triactinomyxons per individual (produced by worm populations at the Parvin Lake Research Station) at roughly eight weeks (~600 degree days) post-hatch, and reared for 2,000 degree days to allow full development of myxospores. Upon reaching 2,000 degree days, fish will be euthanized and sent to the CPW Aquatic Animal Health Lab for myxospore enumeration.

At three to four months post-hatch, all remaining fish will be stocked into Parvin Lake to evaluate survival, growth, and whirling disease development. Fish will be adipose clipped prior to being stocked so they can be identified during recapture events. Recapture events will occur in late October or early November 2024 to evaluate survival and growth after a month or two in the lake, and again in the summer of 2025 to evaluate overwinter survival and growth. Heads will be collected from up to 40 individuals during these sampling events and sent to the CPW Aquatic Animal Health Lab for myxospore enumeration. Updates for this project will be included in the 2025 Sport Fish Research Studies Annual Report.

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COLLABORATIVE RESEARCH PROJECTS WITH COLORADO STATE UNIVERSITY

Collaborations with graduate students at Colorado State University (CSU) provide an opportunity to expand on management and research questions of interest to the State of Colorado. One such project focused on bacterial kidney disease and conducted in conjunction with sport fish research was initiated in 2022. Bacterial kidney disease, caused by *Renibacterium salmoninarum*, is a major disease of concern for Colorado hatcheries. As a regulated pathogen in Colorado, current regulations prevent the transfer or stocking of infected eggs or fish. Additionally, *R. salmoninarum* can be transmitted in two ways, presenting challenges for prevention and management. Understanding the rate of vertical and horizontal transmission in Colorado hatcheries, the role these transmission routes play in maintaining infection prevalence, and determining the optimal tissues for detecting *R. salmoninarum* infections can help with management and regulatory decisions for this pathogen. Additionally, bacterial strain and virulence differences may affect mortality rates of fish infected with *R. salmoninarum* in both wild and hatchery environments, and this is the focus of the current study.

BACTERIAL KIDNEY DISEASE RESEARCH

Project Collaborators: Rebecca E. McDevitt, M.S. candidate, and Dana L. Winkelman, Ph.D.

The Susceptibility of Oncorhynchus Species to Two Strains of Renibacterium salmoninarum

The following is the full proposal for the project being conducted by M.S. candidate Rebecca McDevitt at Colorado State University entitled "The Susceptibility of *Onchorhynchus* Species to Two Strains of *Renibcacterium salmoninarum*", and approved by her committee (Advisor Dana Winkelman and Committee Members Eric Fetherman, Caitlin Wells, and Paula Schaffer) in October 2023:

Disease outbreaks pose a significant threat to both hatchery stocks and wild fisheries, leading to substantial economic losses and environmental damage, and disease prevention and management are critical for sustainable aquaculture and fisheries management (FAO 2022). In general, epidemics require a susceptible host, a favorable environment for both the host and pathogen, and an infective and virulent pathogen. Virulence is the ability of a pathogen to cause disease in a host organism and can be measured by metrics such as host survival or pathogen numbers infecting the host. Host susceptibility is influenced by many factors intrinsic to the host but can generally be measured by a pathogen's effect on host survival (Van Seventer and Hochberg 2017). Environmental factors influence disease transmission, and include abiotic variables such as temperature and biotic interactions such as host density. Understanding each of these factors is necessary for understanding disease outbreaks and developing effective disease control strategies.

Renibacterium salmoninarum is a gram-positive, intracellular parasite that causes bacterial kidney disease (BKD) and primarily infects members of the Salmonidae family, with varying levels of susceptibility among salmonid species. Bacterial kidney disease has been documented globally since its initial detection in Atlantic Salmon *Salmo salar* in Scotland in the 1930s and has continued to cause major issues in North America. In 1935, Brook Trout *Salvelinus fontinalis*, Brown Trout *Salmo trutta*, and Rainbow Trout *Oncorhynchus mykiss* in North America showed similar signs to fish in Scotland (Belding and Merrill 1935; Fryer and Sanders 1981; Eissa and Elsayed 2006; Matejusova et al. 2013), and Belding and Merrill (1935) identified a gram-positive bacterium from kidney tissue that was similar to previous research (Belding and Merrill 1935; Fryer and Sanders 1981; Eissa and Elsayed 2006; Matejusova et al. 2013). In 1936, Bacterial Kidney Disease was first reported on the Pacific coast of the United States (Fryer and Lannan 1993). Since then, it has been observed in numerous regions where salmonids are raised, including North America (MacLean and Yoder 1970; Mitchum et al. 1979), Canada (Evelyn et al. 1973; Paterson et al. 1979), Europe (Pfeil-Putzien et al. 1985; Jansson et al. 1996), Iceland (Gudmundsdottir et al. 1991), Asia (Sakai and Kobayashi 1992; Nagai and lida 2002), and South America (Sanders and Barros 1986). Since BKD is widespread and can cause significant losses in wild and hatchery settings, it is considered a regulated pathogen (USFWS and AFS-FHS 2014; Fetherman et al. 2020). For example, up to 80% mortality of Pacific salmon stocks and 40% mortality of Atlantic Salmon stocks were documented in BKD outbreaks (Evenden et al. 1993; Weins 2011). *R. salmoninarum* has several unique properties which make it ubiquitous and difficult to manage. This bacterium grows slowly which may cause infected hosts to show no clinical signs of disease (Fryer and Sanders 1981; Woo and Cipriano 2017), utilizes two modes of transmission (Evelyn 1984; Evenden et al. 1993; Balfry et al. 1996), can survive intracellularly, and can survive in macrophages (Grayson et al. 2002).

Host susceptibility to *R. salmoninarum* varies among salmonid species and among life stages or life stage transitions. Sockeye Salmon *O. nerka*, Chinook Salmon *O. tshawytscha*, Coho Salmon *O. kisutch*, Atlantic Salmon, and Chum Salmon *O. keta* display higher susceptibility to BKD, whereas Lake Trout *S. namaycush*, Brown Trout, Bull Trout *S. confluentus*, Brook Trout, Rainbow Trout, and steelhead exhibit lower susceptibility (Starliper et al. 1997; Rhodes and Mimeault 2019). Specific life stages of salmonids also exhibit varying levels of susceptibility to infection and disease, with heightened vulnerabilities observed during migration to seawater and the return to freshwater for spawning. The vulnerability of salmonids to disease development during the smoltification process or upon entering seawater shows considerable variation. The transition from sexual maturation to spawning represents another distinct phase of heightened susceptibility in salmon life history. Since infection can occur at any stage preceding spawning and disease progression may occur gradually, spawning individuals carry a greater risk of disease development and vertical transmission (Rhodes and Mimeault 2019).

Pathogen virulence is a crucial factor in understanding disease outbreaks. Disease severity is a function of physiological or genetic factors and transmission dynamics. Virulence factors are the specific traits or mechanisms that allow a pathogen to infect and cause disease in a host organism. *R. salmoninarum* can be a highly virulent bacterium that can cause high mortality (Pascho et al. 2002). Infections in salmonid fish are caused by bacterial adhesion to host cells through the interaction of microbial surface components and the invasion of host cells, which is facilitated by various virulence factors (Delghandi et al. 2020). *R. salmoninarum* can survive

phagocytosis, which is the ingestion of bacteria, and replicate within a variety of cells such as macrophages (Gonzalez et al. 1999). The primary virulence factor in *R. salmoninarum* is p57 (57-kDa protein), which is encoded on three copies of the *msa* gene (*msa*1, *msa*2, and *msa*3) . The p57 protein is present on the bacterial surface and secreted into the surrounding fish tissues. For full virulence, two copies of the *msa* gene (*msa*1 and *msa*2) need to be expressed (Coady et al. 2006), while the presence of a third *msa* gene (*msa*3) may be correlated to increased virulence and mortality (Rhodes et al. 2004). The increased number of *msa* copies can lead to an increased expression of the p57 protein which can result in greater virulence under the right conditions (Aguilar et al. 2023).

R. salmoninarum can be transmitted through vertical (from adult to egg; Evelyn 1984) and horizontal routes (direct and indirect contact; Evenden et al. 1993; Balfry et al. 1996). Important sources of horizontal transmission include contaminated feed and water, cannibalism, and the ingestion of feces from infected fish (Balfry et al. 1996). Horizontal transmission through water is limited but can still occur farm-to-farm or from wild fish (Evenden et al. 1993). Bacteria can be shed and spread horizontally by both asymptomatic and symptomatic fish (Balfry et al. 1996). Some non-salmonid species such as the Three-spined Stickleback *Gasterosteus aculeatus* and minnows can act as reservoirs for the *R. salmoninarum* bacterium (Rhodes and Mimeault 2019). Vertical transmission occurs through coelomic fluid and egg yolk during fertilization (Balfry et al. 1996; Industry 2010; Murray et al. 2012; Woo and Cipriano 2017).

Environmental factors influencing BKD outbreaks include pollution, low levels of dissolved oxygen, decreased availability of food, presence of harmful plankton, and increased stress during handling and transfer of fish or from changes in temperature (Yildiz et al. 2017; Boerlage et al. 2018; Larson et al. 2020). High fish density may favor horizontal transmission due to increased contact among individuals and increased exposure to feces or water borne bacteria (Murray et al. 2012). While BKD has been reported in wild fish populations, many instances occur in hatcheries (Mitchum and Sherman 1981). For instance among wild fish, significant prevalence of infection has been reported in smolts and returning adult salmon (Banner et al. 1986; Rhodes and Mimeault 2019). In the aquaculture industry, consistently high rearing densities could increase horizontal transmission and may reduce the reliance of bacterial reproductive success on host survival, thereby promoting the emergence of more virulent strains (Dale et al. 1997). Water temperature also plays a critical role in BKD outbreaks. Temperatures ranging from 10-18°C are suitable for bacterial viability (Pascho et al. 2002). Rozas-Serri et al. (2020) indicated that colder water accelerates the progression of BKD leading to an increase in the shedding of bacteria, potentially facilitating the spread of the disease to other fish. Lower temperatures can cause higher mortality rates and longer periods of time till mortality occurs (Purcell et al. 2016; Yildiz and Secer 2017; Boerlage et al. 2018; Larson et al. 2020).

Even though *R. salmoninarum* is widespread across Colorado, strain type, genetic variation, and virulence across the landscape is still largely unknown. *R. salmoninarum* is common and widespread throughout Colorado's wild trout fisheries, but active infections are rare and clinical BKD is uncommon (Kowalski et al. 2022). Typically, low levels of bacterial antigens are common in non-anadromous trout and observations of clinical signs of BKD are rare and usually seen in spawning Brook Trout in small high elevation streams. Although *R. salmoninarum* is widespread in Colorado, BKD was primarily viewed as a hatchery problem. BKD was detected

causing disease outbreaks in Colorado hatcheries in the 1950s and 60s (Kowalski 2021). In federal or state hatcheries, *R. salmoninarum* was detected at least 16 times during fish health inspections between 1970 and 1997 (Kowalski 2018). *R. salmoninarum* wasn't detected again for 18 years until 2015 (Fetherman et al. 2020), where six hatcheries and a wild broodstock tested positive. Since 2016, two clinical outbreaks have occurred in Colorado hatcheries (Kowalski et al. 2022). The financial impact of a single outbreak resulted in expenditures exceeding $2.1 million for depopulation and disinfection measures (Kowalski et al. 2021). This outbreak had far-reaching consequences for fish management across the state, leading to the loss of more than 675,000 sport fish (Kowalski 2021). Drastic depopulation measures may not be necessary given that the distribution and prevalence of *R. salmoninarum* in Colorado is high but clinical disease is rare and bacterial load is low (Kowalski 2018). Low bacterial loads and the absence of evidence for clinical signs of BKD (Kowalski et al. 2022) may be due to host resistance or the presence of an attenuated virulent strain of the bacterium (Riepe 2022).

I propose to evaluate the consequences of *R. salmoninarum* infection in Chinook Salmon and Cutthroat Trout. My proposed approach has two major objectives. The first is to determine the infection intensity of bacteria in each fish and determine relative percent survival of two salmonid species exposed to two different *R. salmoninarum* strains. We expect to see greater mortality in fish injected with the highly virulent strain and in fish characterized as more susceptible such as Pacific salmon. The second is to identify the strain isolated from Rainbow Trout at the BFRH through next generation sequencing. From previous work, limited mortality events in the wild, and the knowledge that *R. salmoninarum* is a highly conserved and a slow growing bacterium, we believe we have an attenuated virulent strain in Colorado.

I will conduct proposed research at Colorado State University in conjunction with the CPW Hatchery Section and Aquatic Research Section. Research will begin in 2022 and continue through 2024. I have divided my proposed research into 2 major objectives; (1) an endpoint mortality experiment and (2) genetically characterizing the unknown strain of *R. salmoninarum* isolated from the BFRH.

Objective 1: Conduct an experiment comparing end-point mortality between an unknown strain of *R. salmoninarum* found in Colorado and a virulent strain of *R. salmoninarum* found in the Pacific Northwest using various species and subspecies of salmonids. Objective 1, exposing Chinook Salmon to *R. salmoninarum*, has been partially completed as of March 2023. The remainder of objective 1 will begin in October 2023 and will be completed in December 2023.

Justification

Mortality from *R. salmoninarum* is a function of the host species susceptibility and the virulence of the bacterial strain. It is necessary to understand the relationship between susceptibility and virulence to properly inform management and conservation efforts. Previous work examined the auto-agglutination property for a strain of *R. salmoninarum* isolated from Rainbow Trout at the BFRH compared to that of a highly virulent strain. These results suggested that the isolated strain displayed a non-agglutinating property in contrast to the virulent strain. Non-agglutination suggests that the bacterial strain encountered in the BFRH is of lower virulence, potentially accounting for the reduced detection rates observed with DFAT in comparison to PCR methods.

These reduced detection rates could also be explained by the low incidence of disease (Riepe 2022). To validate the agglutination experiment, I propose to conduct an end-point mortality experiment to compare the susceptibility of various species and subspecies of salmonids when exposed to a possible attenuated strain of *R. salmoninarum* from Colorado with a known virulent strain from the Pacific Northwest.

Experiment 1, conducted using Chinook Salmon, was completed in March 2023 and consisted of 4 treatments: 1) no exposure (control), 2) mock saline injection (control), 3) exposure to the Unknown (Colorado) strain, and 4) exposure to the ATCC 33209 (Pacific Northwest) strain. Treatment one and two each contained two replicate tanks, and treatments three and four each contained ten replicate tanks (Figure 3.1). Each of 24 tanks contained 10 fish, for a total of 240 Chinook Salmon used in this experiment.

Experiment 2 will contain 8 treatments: 1) No exposure (control), 2) Mock saline injection (control), 3) Colorado River Cutthroat Trout Nanita exposed to the Unknown strain, 4) Colorado River Cutthroat Trout Nanita exposed to the ATCC 33209 strain, 5) Yellowstone Cutthroat Trout exposed to the Unknown strain, 6) Yellowstone Cutthroat Trout exposed to the ATCC 33209 strain, 7) Trappers Lake Cutthroat Trout exposed to the Unknown strain, and 8) Trappers Lake Cutthroat Trout exposed to the ATCC 33209 strain. Treatment one and two will each contain two replicate tanks, and treatments three through eight will each contain ten replicate tanks. Each of 72 tanks will contain 10 fish, for a total of 720 fish used in the experiment.

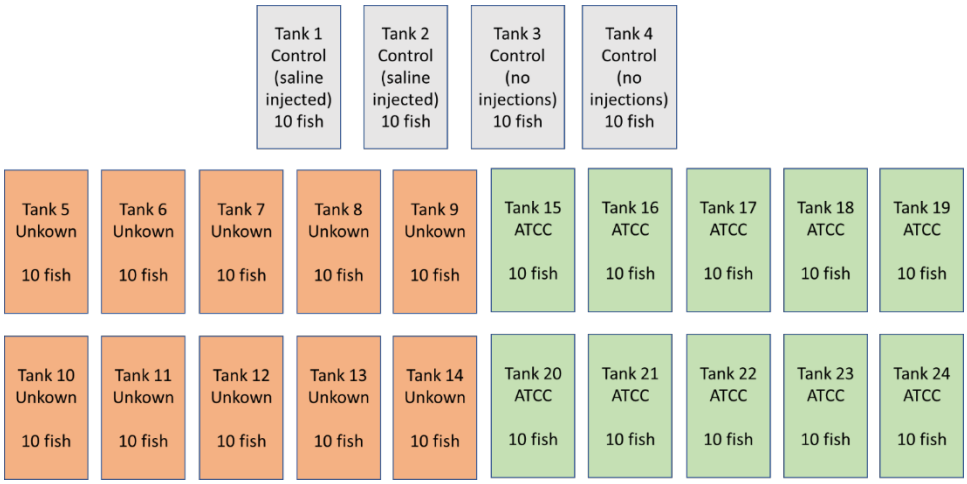

Figure 3.1. Experimental set up in 20-gallon flow through tanks comparing fish exposed to two different strains of *R. salmoninarum*. Orange represents the Unknown *R. salmoninarum* strain and green represents the ATCC *R. salmoninarum* strain. The experimental setup will be replicated for each species/strain of fish being exposed.

Task 1 – Selection and collection of fish species

My proposed research will test four species of salmonids that are susceptible to *R. salmoninarum*: Chinook Salmon, Colorado River Cutthroat Trout Nanita *O. clarkii pleuiticus*, Yellowstone Cutthroat Trout *O. clarkii bouvieri* originating form LeHardy Rapids, and Trappers Lake Cutthroat Trout (Colorado River x Yellowstone). Chinook Salmon are considered a

susceptible species to *R. salmoninarum* and were selected to provide a baseline for comparison to Cutthroat Trout. Little is known about Cutthroat Trout susceptibility to *R. salmoninarum*, and more research is needed; however, there seems to be higher resistance among inland trout (Starliper et al. 1997; Elliott et al. 2014; Jones et al. 2007). In the state of Colorado, there are three distinct subspecies of native Cutthroat Trout, and among them is the Colorado River Cutthroat Trout that is considered a species of special concern. Trappers Lake Cutthroat Trout are a hybrid between Colorado River and Yellowstone Cutthroat Trout and was home to Colorado's renowned wild Colorado River Cutthroat Trout fishery. Hybridization between the species and previous exposure to *R. salmoninarum* may have had an influence on their resistance to disease. Yellowstone Cutthroat Trout are a non-native species to Colorado but may vary in susceptibility to *R. salmoninarum*.

Three hundred age-1 (hatched in December 2021) Chinook Salmon were collected in November 2022 from Pahsimeroi Fish Hatchery (May, Idaho) and transported to Colorado. The fish were held in ponds beginning in June 2022 that were fed by river water ranging in temperature between 46 and 64°F. The fish were transported in tanks with a steady flow of oxygen and temperature controlled to maintain a temperature range of 8-14°C during transport. Fish were moved from the hatchery truck tanks to the wet laboratory located on the CSU main campus in the basement of the Anatomy/Zoology building.

Colorado River Cutthroat Trout (Nanita), Yellowstone Cutthroat Trout (YCT), and the Trappers Lake Cutthroat Trout (Colorado River x Yellowstone; TLC) were collected and are being reared at the BFRH until the start of the experiment in October 2023. These fish will be transported from the BFRH to the basement of the Anatomy/Zoology building prior to the start of the experiment. Upon arrival to the wet laboratory, fish will be acclimated to the water temperatures and placed into tanks resulting in a total of 10 fish per tank. Each tank will be batch weighed prior to the start of the experiment to determine the correct amount of feed (% body weight per day). Fish will be treated twice with a static bath of 150 ppm formalin for 15 minutes when they first arrive from the hatchery to clear any external parasites that could affect mortality prior to or during the exposure experiment.

Task 2 – *Renibacterum salmoninarum* culture, isolation, and quantification

I obtained a known reference strain of *R. salmoninarum* referred to as American Type Culture Collection (ATCC) 33209D-5 and an Unknown strain isolated from the BFRH. Both strains were stored at -80°C. The ATCC strain was cultured after five rounds of reinoculation at 1.56 x 10^9 bacteria/mL. The Unknown strain that was previously stored had gone through six rounds of reinoculation. Thus, I had to reinfect Cutthroat Trout with the unknown strain to maintain the virulence. To do this, I injected fish intraperitoneally with this strain. Each fish was injected with 20 µL of bacteria and received an injection boost after two weeks. After five weeks, each fish was euthanized, and bacteria was cultured in a specialized medium for *R. salmoninarum* known as selective kidney disease medium 2 (SKDM2). I followed the standard operating procedure (SOP) from the U.S Geological Survey when making the culture plates using peptone, yeast extract, cysteine-HCL, deionized water, nurse medium, agar, fetal bovine serum, cyclohexamide, D-cycloserine, polymyxin-B sulfate, and oxolinic acid (Evelyn 1977; Evelyn et al. 1990; Jansson et al. 1996).

Bacteria collected from the kidneys of the Cutthroat Trout using inoculation loops was streaked on SKDM2 (mentioned above). These plates were wrapped with parafilm and placed in an incubator at 15°C. Growth took place anywhere from 1 to 3 weeks after. Inoculation continued until there were pure colonies of *R. salmoninarum* on the agar plates. Direct Fluorescent Antibody Test (DFAT), performed following the protocols of Bullock et al. (1980) and Pascho et al. (1991), and gram stains were used to confirm the growth and purity of the *R. salmoninarum* culture. *R. salmoninarum* isolates were then inoculated in a kidney disease medium (KDM) broth to increase the number of bacteria. This broth contains peptone, yeast extract, cysteine-HCL, deionized water, and fetal bovine serum (Evelyn 1977; Evelyn et al. 1990; Jansson et al. 1996). The broth was placed in an incubator at 15°C with constant stirring for 7-8 days (Elliott et al. 2013). Reinoculation occurred four more times on the selective kidney disease medium broth until the Unknown strain reached purity with 5 passes. Serial dilutions were created with each flask to determine the number of bacteria in 1 mL of broth in a 10-7 dilution (Riepe 2022). I followed protocols from Elliott and Barila (1987) and Elliott and McKibben (1997) to perform a Membrane-filtration Fluorescent Antibody Test (MF-FAT) to count the total number of bacteria in 1 mL of broth. I replicated this process eight times with the same broth, averaging 227.625 ± 79.52 cells. From this, I determined the total number of bacteria in the starting flask by back calculation. The total number of bacteria in the sample was 2.28×10^9 bacterial/mL. This was used for the injections described below.

Task 3 – IP injections of ATCC and unknown strains

Prior to the start of the experiment, food will be withheld for 24 hours. I will be exposing fish to the American Type Culture Collection (ATCC) 33209D-5 strain of *R. salmoninarum*, as well as the Unknown strain of *R. salmoninarum*. Fish will be exposed by intraperitoneal injection of 6.5×10^6 bacteria/mL (Murray et al. 1992; McKibben and Pascho 1999; O'Farrell et al. 2000). The fish will be anesthetized with MS-222 and injected intraperitoneally anterior to the pelvic fin with 20 uL of the ATCC strain of *R. salmoninarum* in one treatment group, while the other treatment group will receive the same injection with the Unknown strain of *R. salmoninarum*. Fish in the mock injection tanks will be injected with 20 µL of saline. After injections, fish will be monitored in 5-gallon buckets with fresh water and aeration until they recover. We anticipate fish will recover within 3-5 minutes based on previous experiments in our lab. Fish will be placed into designated, and randomly assigned, 20-gallon flow-through tanks (Figure 3.1) with aeration for the duration of the experiment.

Task 4 – Monitoring and collection

Feed will be withheld 24 hours after injection exposures and subsequently fish will be fed once a day with Biolife Biovita fish feed (2.5 mm) at the manufacturer's recommendation of 3% body weight per day. Fish will be monitored every day (twice a day) after injections. Daily temperatures will be recorded. Dead fish in each tank will be removed twice daily and recorded for up to 10 weeks to determine the timing of mortality after injection. All fish remaining at the end of the experiment (10 weeks) will be euthanized. Euthanasia will be accomplished with an overdose of MS-222 (250 mg/L buffered to a pH of 7.0). We will follow standard AVMA guidelines for euthanasia with MS-222, keeping the fish in the solution for 10 minutes following loss of rhythmic opercular movement. The deceased fish will be assigned a unique number, and the length, weight, and notes about any internal or external gross signs will be taken. The liver, kidney, and spleen will also be collected from dead fish, placed into the same sterile whirl-pak

bag but separated by individual fish identity, and stored in a -20°C freezer immediately after dissection. These will later be processed in Wagar 203 lab.

Task 5 – Testing methods

All fish tissues collected during the experiment will be screened for *R. salmoninarum* with DFAT as a presumptive test and quantitative polymerase chain reaction (qPCR) as a confirmatory test. Both a molecular and immunological assay should be used to determine the different stages of the infection and the presence/absence of *R. salmoninarum* (AFS-FHS 2016).

The DNA extraction process adheres to the protocols established by Chase and Pascho (1998) and Chase et al. (2006). To complete all DNA extractions from tissues, I will be using the Qiagen DNeasy Blood and Tissue Kit. To begin, work areas and tools will be disinfected before starting. Fish tissues will be thawed and homogenized using a sterile rolling pin. Approximately 25 mg of homogenized tissue will be placed into a sterile 1.5-mL microcentrifuge tube containing 180 µL of ATL Buffer and 20 µL of Proteinase K solution. The sample will be vortexed and incubated overnight at 37°C which allows the tissue to be completely lysed. Following incubation, samples will be vortexed and 200 µL of Buffer AL will be added. I will briefly vortex the sample and add 200 µL of 100% ethanol. The entire volume will be transferred into a spin column placed in a 2-mL collection tube. The mixture will be centrifuged at 6000 x g for 1 minute. The spin column will then be placed into a new collection tube and 500 µL of Buffer AW1 will be added and centrifuged at 6000 x g for 1 minute. The spin column will be placed into another collection tube and 500 µL of Buffer AW2 will be added and centrifuged at 20,000 x g for 3 minutes. After centrifuging, the spin column will be placed into a 2-mL microcentrifuge tube and 400 µL of Buffer AE will be added and centrifuged at 6000 x g for 1 minute. Following Elliot et al. (2013), 200 µL of Buffer AE were eluted to increase the concentration of the DNA sample. Final DNA product will be stored at 4°C until qPCR analysis.

The qPCR procedure follows the protocols from Chase et al. (2006). This procedure targets and amplifies the 69-bp region of the major soluble antigen (*msa*) gene (encoding the p57 protein). Specific primers and probes will be used to target this region. The forward primer used is RS1238 5’ - GTG ACC AAC ACC CAG ATA TCC A - 3’ and the reverse primer used is RS1307 5’ - TCG CCA GAG CCA CCA TTT ACC - 3’. The internal probe used will be the RS1262 6FAM - 5’ CAC CAG ATG GAG CAA C - 3’ with a 3’ MGBNFQ Quencher. Using the reagents from table 3.1, I will be able to make a working reaction mix.

Table 3.1. Modified working reagents from Chase et al. 2006 and Riepe 2022.

PCR Reagent	Vol. Per Reaction
GenEx Master Mix	12.5 µL
Forward Primer	2.25 µL
Reverse Primer	2.25 µL
TaqMan Probe	0.625 µL
dH_2O	2.375 µL
Total	**20 µL**

20 µL of the working reaction will be aliquoted to each well on a 96-well fast-clear reaction plate with 25 µL of the total sample in each well after adding DNA. 5 µL of water will be added to the template controls, 5 µL of extracted DNA from the tissue samples will be added to each well, and 5 µL of extracted DNA from serially diluted positive controls will be added to the last few wells. All samples will have duplicates on the plate. I will centrifuge the plate for 3 minutes at 1500 x g. Using the StepOnePlus System with the loaded samples on the plate, I will begin with an initial incubation of 50°C for 2 min, followed by a 95°C incubation for 10 minutes, then 40 cycles following denaturing at 95°C for 15 seconds and annealing at 60°C for 60 seconds (Chase et al. 2006). Riepe (2022) determined the standard curves for quantification by creating ten-fold serial dilutions of *R. salmoninarum*. The positive control samples spanned from 1.1×10^5 to 1.1 × 10 bacterial cells/mL and Riepe (2022) determined 37.75 to be an appropriate qPCR cut-off C_q value. The final analysis of the qPCR output provides the output load of bacteria per reaction which then can be used to calculate the number of bacteria per gram of tissue.

The DFAT procedure follows the protocols of Bullock et al. (1980) and Pascho et al (1991). Using the homogenized tissue from the whirl-paks, I will use a sterile cotton swab to smear tissue onto a 10-well, 6-mm diameter well slide. Each sample will be duplicated using two wells on the slide. Once tissues are smeared onto the slides, I will allow them to air dry completely and then heat fix the slide. Once the slides are prepped, they will be fixed in acetone for 5 minutes. 50 µL of the FITC conjugated antibody (Fluorescein-labeled affinity purified antibody) diluted 1:40 in phosphate buffed solution (PBS) will be added to each well and then placed in a dark humidified chamber for 1 hour. Slides will be rinsed with PBS and then counterstained with a 1:60 (w/v) solution of Eriochrome Black T, leaving it for about 10 seconds. Slides will be allowed to air dry completely. All slides will be analyzed using a Nikon compound microscope fitted with a 420 nm fluorescent emission high-pressure mercury lamp and examined at 1000X magnification. Positive bacterial cells will fluorescence strongly, apple green in color, and exhibit the same cell morphology and size as the positive control. These cells are about 0.3–1.0 µm × 1.0–1.5 µm in size.

Task 6 – Data analysis

I will calculate the survival of infected fish over time of exposure (days) with the survival package in Rstudio version 4.2.1. The package will allow us to use the Kaplan Meier Survival Curve to estimate cumulative survival of the fish infected with the ATCC and Unknown strain of bacterium. Specifically, I will model and plot the Kaplan-Meier estimates of probability of survival over time for each bacterial strain. I plan to compute the bacterial load among individual fish using Cq values provided by qPCR results (Riepe 2022). With these counts, I will quantify the bacterial load among natural mortalities vs euthanized fish within treatments and between assays (qPCR and DFAT).

Outcomes

Data analysis from the first exposure experiment with Chinook Salmon has been completed. At the start of the experiment, all fish in the four treatments (control, saline, ATCC, Unknown) were approximately the same weight (Figure 3.2). Therefore, fish size-at-exposure was not considered to be a factor affecting mortality during the exposure experiment.

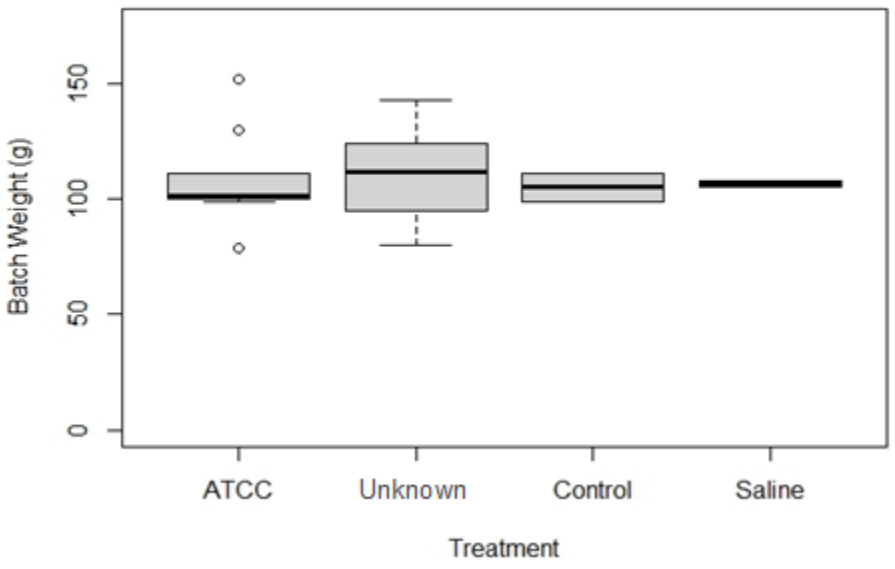

Figure 3.2. Chinook Salmon batch weights (g) by treatment (ATCC, Unknown, Control, Saline) at time of injection with *R. salmoninarum*.

There were no mortalities in our control or saline injected tanks, indicating that all mortality in the injection tanks occurred as a result of *R. salmoninarum* infection. However, we detected *R. salmoninarum* in both the control and saline injected fish with qPCR and DFAT, but bacterial loads were low compared to fish injected with the ATCC and Unknown *R. salmoninarum* strains (Table 3.2). We had anticipated that control fish would have a mild infection because they came from an *R. salmoninarum* positive hatchery at which they had been previously treated for the bacteria, but the infection loads were so low that they didn't affect the experiment. Many fewer control and saline injected fish were determined to be infected with *R. salmoninarum* when using the DFAT compared to the qPCR assay (Table 3.2).

Table 3.2. Summary of qPCR and DFAT results from each of the four treatments. Data include the percent of fish that died during the experiment (Mortality) or were euthanized at ten weeks post-injection (Survived) that were infected with *R. salmoninarum* (% Infect), and the average *R. salmoninarum* cells g^{-1} in the homogenized tissue samples of the two groups, estimated from the qPCR standard curve.

Treatment	qPCR				DFAT	
	Mortality (% Infect)	Mortality (cells g^{-1})	Survived (% Infect)	Survived (cells g^{-1})	Mortality (% Infect)	Survived (% Infect)
Control	NA	NA	100	22	NA	25
Saline	NA	NA	100	8	NA	10
ATCC	100	765,108	100	351,187	100	100
Unknown	100	683,463	100	716,880	100	100

The exposure experiment lasted 71 days. Fish injected with the Unknown strain started dying from an *R. salmoninarum* infection at 31 days post-injection, whereas fish injected with the ATCC strain started dying from an *R. salmoninarum* infection at 46 days post-injection. Overall, only 1% of the fish injected with the Unknown strain compared to 31% of the fish injected with the ATCC strain survived to the end of the experiment (Figure 3.3). Fish injected with either the ATCC or Unknown strain that died during the experiment had a similar number of bacterial cells g^{-1} as determined by the qPCR standard curve. However, fish injected with the ATCC strain that survived to the end of the experiment had fewer bacterial cells g^{-1} than did those injected with the Unknown strain, suggesting that Chinook Salmon were able to clear the ATCC strain infection (Table 3.2). Taken together, the results of this experiment suggest that 1) location-of-origin of the bacterial strain and exposed fish has an effect on both mortality and the ability of exposed fish to clear an infection, and 2) that the Unknown strain isolated from Colorado may not be less virulent than the ATCC strain isolated from the Pacific Northwest when fish from outside the strains range are exposed to the bacteria.

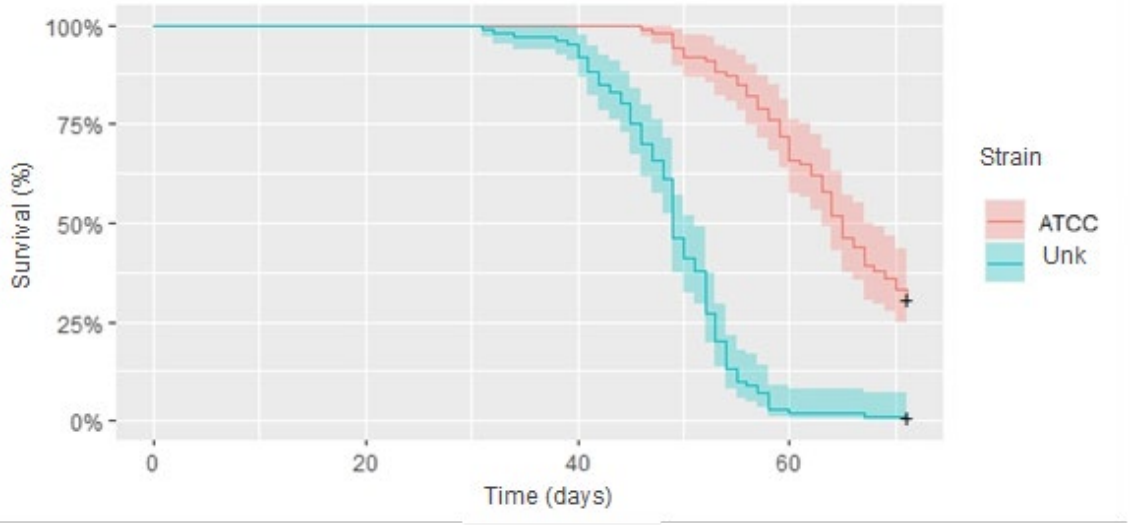

Figure 3.3. Kaplan-Meier cumulative percent survival of Chinook Salmon injected with the ATCC strain (red) and the Unknown strain (blue), plotted daily over the 71-day exposure experiment.

Initially, I expected that mortality would be higher in fish exposed to the ATCC strain, compared to the Unknown strain of *R. salmoninarum*, due to the hypothesized attenuated nature of the Unknown strain. However, in the first experiment, Chinook Salmon mortality was higher in the Unknown strain. We could be mistaken about our inferences regarding the Unknown strain or higher mortality in Chinook Salmon could be due to an interaction between the host susceptibility and pathogen virulence.

Taking the Chinook Salmon results into consideration, two general outcomes are possible from the Cutthroat Trout exposure experiment. One, mortality of Cutthroat Trout will parallel the Chinook Salmon experiment and will be higher for the Unknown strain. That result would indicate that our inferences regarding the virulence of the Unknown strain are incorrect. Two, mortality of Cutthroat Trout exposed to the Unknown strain will be lower than that of fish

exposed to the ATCC strain. This result could potentially indicate that there is an interaction of host susceptibility with pathogen virulence, indicating potential co-evolution with the pathogen.

Objective 2: Identify the unknown strain isolated from a Colorado hatchery. Objective 2, task 1 has been partially completed.

Justification

From previous work completed in Colorado, we believe we have an attenuated virulent strain of *R. salmoninarum* due to the auto-agglutination testing (Riepe 2022). Sequencing this *R. salmoninarum* strain would be useful to identify the strain type and could aid in understanding its effects on inland trout populations.

Task 1 – Strain identification

Pure bacterial colonies of the Unknown strain and the ATCC strain were sent to Azenta Life Sciences to be sequenced. Through this service, I requested short-read non-human whole genome sequencing (WGS) with the next generation sequencing service line. DNA sequencing was run on the Illumina MiSeq platform configured 2x150bp.

Riepe (2022) tested the agglutination property expressed by *R. salmoninarum* of the Unknown strain. Strains that exhibit auto-agglutination are identified as hydrophobic and are deemed highly virulent. These virulent strains can invade the host, multiply, and intensify infections in the host. Reduced virulence is expressed in non-agglutinating strains (Bruno 1988). Riepe (2022) tested this property for ATCC strain and the Unknown strain. The ATCC strain exhibited the auto-agglutination property whereas the Unknown strain was seen as non-agglutinating. These results suggest the Unknown strain to be less virulent. I expect the sequencing results to confirm these findings that the Unknown strain is an attenuated virulent strain.

Relevance of Research

Disease outbreaks can lead to significant economic losses and have a negative impact on aquaculture sustainability. In addition, hatchery stocking may introduce a pathogen to a previously unexposed population or introduce a novel disease that wild fish may not be resistant to, especially when pathogenic effects are not well characterized for wild populations (Fenichel et al. 2009). As such, disease prevention and management are critical for sustainable aquaculture and fisheries management, with research focusing on developing effective disease control strategies and improving our understanding of the underlying causes of disease outbreaks.

My research will provide information to CPW regarding the identity and virulence of the strain found in Colorado, the susceptibility of species to each strain, and future management approaches for the various bacterial strains when they appear in hatchery or wild populations. The results will directly inform management of the pathogen in Colorado.

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Response of Chinook Salmon and Cutthroat Trout to Exposure to Two Renibacterium salmoninarum Strains

Renibacterium salmoninarum, the causative agent of bacterial kidney disease (BKD), is a gram-positive, intracellular bacteria that primarily infects members of the family Salmonidae, with varying levels of susceptibility among species. Infection with *R. salmoninarum* is known to cause high mortality in both wild and cultured salmonids, especially Coho Salmon *Oncorhynchus kisutch* and Chinook Salmon *O. tshawytscha*. *R. salmoninarum* is found in salmonid populations throughout the U.S. and has been detected within several of Colorado's wild trout fisheries and hatcheries (Fetherman et al. 2020; Kowalski et al. 2022; Riepe et al. 2023). Previous research has shown that Sockeye Salmon *O. nerka*, Chinook Salmon, Coho Salmon, Atlantic Salmon *Salmo salar*, and Chum Salmon *O. keta* are more susceptible to an *R. salmoninarum* infection compared to some inland trout species such as Lake Trout *Salvelinus namaycush*, Brown Trout *S. trutta*, Bull Trout *S. confluentus*, Brook Trout *S. fontinalis*, and Rainbow Trout and steelhead *O. mykiss* (Rhodes and Mimeault 2019; Starliper et al. 1997). Susceptibility to infection may depend on whether the pathogen and host originate from the same location. If the host and pathogen are not of the same origin, the pathogen may have a greater effect on the survival of the host (Johnson et al. 2020).

Chinook Salmon are one of the most susceptible species to *R. salmoninarum* infection, and are typically infected with a virulent strain found in the Pacific Northwest (ATCC 33209) that causes high mortality rates. Little is known about Cutthroat Trout susceptibility to *R. salmoninarum,* and more research is needed; however, there seems to be higher resistance among inland trout (Starliper et al. 1997; Elliott et al. 2014; Jones et al. 2007). The susceptibility of these species to other strains, such as the one found in Colorado, is unknown. Therefore, we evaluated the response of Chinook Salmon and Colorado River Cutthroat Trout (*Oncorhynchus clarkii*

pleuiticus) to two strains of *R. salmoninarum*, the ATCC 33209 strain isolated from the Pacific Northwest (hereafter the ATCC strain) and a suspected attenuated virulent strain (Riepe 2022) that was isolated from a Colorado hatchery (hereafter the Unknown strain). Our goal was to infect Chinook Salmon and Colorado River Cutthroat Trout with the two bacterial strains and evaluate the cumulative percent survival over a 10 week period following exposure. We predicted that the Chinook Salmon would exhibit lower survival following exposure to the Unknown strain compared to the ATCC strain given that Unknown strain is suspected to originate from outside of the native range of the Chinook Salmon. However, if the Unknown strain is an attenuated virulent strain, the Chinook Salmon exposed to the Unknown strain would be expected to show similar or higher survival rates compared to those exposed to the potentially more virulent ATCC strain. We predicted that the Colorado River Cutthroat Trout would exhibit higher survival rates following exposure to the Unknown strain compared to the ATCC strain given that the Unknown strain is suspected to originate from the native range of Colorado River Cutthroat Trout.

In November 2022, we collected and transported about 300 age-1 Chinook Salmon from the Pahsimeroi Fish Hatchery (May, Idaho). The fish were transported in tanks with a steady flow of oxygen and temperature controlled to maintain a temperature range of 8-14°C. Upon arrival, ten fish each were placed into 24 tanks located in a wet lab in the basement of the Anatomy/Zoology building on the CSU main campus. Fish in each tank were treated twice with 10% formalin after they arrived to clear any external parasites that could affect mortality prior to or during the exposure experiment. In October 2023, we collected and transported about 300 age-1 Colorado River Cutthroat Trout from Daniel Fish Hatchery (Daniel, Wyoming) following the same procedure described above.

Our exposure experiments were completed separately for the two different fish species. Each exposure experiment followed the same set up, which consisted of ten tanks (replicates) per bacterial strain, with ten fish in each tank (200 fish total: 100 fish for the ATCC strain, 100 fish for the Unknown strain). All ten fish in each tank were injected with the bacteria strain assigned to that tank. The experiment additionally contained two tanks in which fish received a mock saline injection, and two tanks serving as naïve controls (no injection). Using ten fish per tank allowed for an assessment of mortality in each tank and comparison to other studies that had used similar exposure evaluation methods.

The dosage necessary for injection (6.5×10^6 bacterial cells/mL) was determined from a previous study (Mckibben et al. 1999). We used the American Type Culture Collection (ATCC) 33209D-5 isolate of live *R. salmoninarum* for ATCC injection exposures to 100 fish, while injection exposures to the other 100 fish used the Unknown strain that was previously isolated in Colorado. Using the well-known ATCC strain provided for consistency in comparisons of mortality rates with previous laboratory studies using injections for inoculation. The bacteria were cultured and prepared in diluted phosphate buffered solution at CSU prior to injection. Fish were anesthetized with tricane methanesulfonate (MS-222) and injected in the intraperitoneal cavity anterior to the pelvic fin with 200 µL of the ATCC strain of *R. salmoninarum* in one treatment group, while the other treatment group received the same injection with the Unknown strain of *R. salmoninarum.* Fish in the mock injection tanks were injected with saline. After injections, fish were monitored in 5-gallon buckets containing fresh water and aeration until they

recovered. Fish were then placed back into their designated, and randomly assigned 20-gallon flow-through tanks with aeration for the duration of the experiment.

Fish were fed once a day with size 3 BioOregon feed at the manufactures recommendation of 3% body weight per day. Every two weeks, fish were batch weighed to determine the amount of feed to be distributed to each tank. Fish were monitored twice to three times a day after injections. Moribund and dead fish were removed from each tank twice daily, lengths and weights were recorded, and internal liver, kidney, and spleen tissues were collected from all fish to determine the timing of mortality and infection severity after injection. All fish remaining at ten weeks post-injection were euthanized, and lengths, weights, and tissues were collected to test for *R. salmoninarum* using DFAT and qPCR to determine if clearance of the infection occurred among the surviving fish.

Following Riepe (2022), tissues were thawed, homogenized together, and prepared for DFAT and qPCR analyses. Specifically, DFAT samples were smeared on a 12-well slide in duplicate, stained with an FITC-conjugated *R. salmoninarum* antisera, and counter-stained using Eriochrome Black (USFWS and AFS-FHS 2014). All slides were analyzed using a Nikon compound microscope fitted with a 420 nm fluorescent emission high-pressure mercury lamp and examined at 1000X magnification (Fetherman et al. 2020). Any detection of *R. salmoninarum* was considered infected tissue. DNA extractions were completed with Qiagen DNeasy Blood and Tissue Kits with an additional elution step (Elliot et al. 2013). We used previously determined primer and probe sets (RS 1238 F, RS 1307 R, and RS 1262 MGB probe; Chase et al. 2006; Elliott et al. 2012) to complete the qPCR analyses. Samples were determined positive when the Cq values were less than 37.75 (Riepe 2022). A previously developed standard curve was used to determine the number of bacteria in each sample.

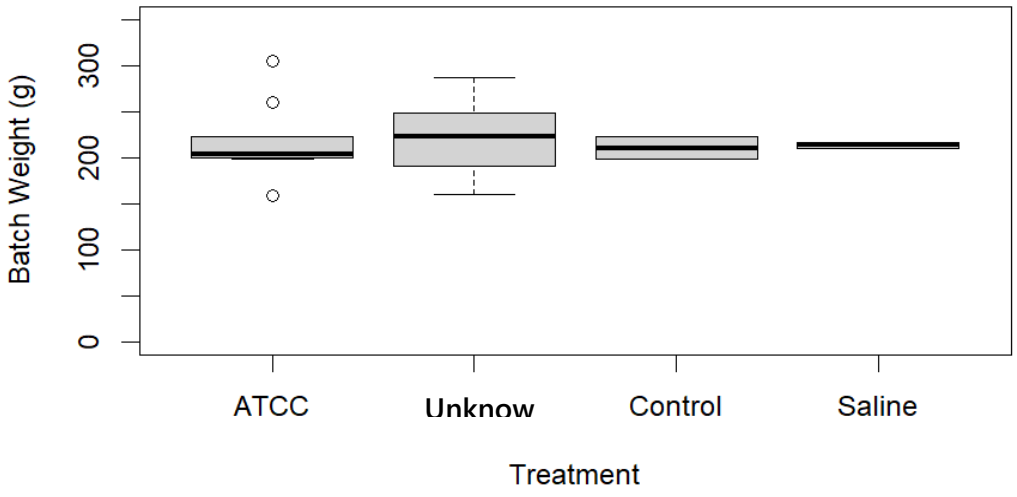

Figure 3.4. Chinook Salmon batch weights (g) by treatment (ATCC, Unknown, Control, Saline) at time of injection with *R. salmoninarum.*

We calculated the survival of infected fish over time of exposure (days) with the *survival* package in Rstudio version 4.2.1. The package allowed us to use the Kaplan-Meier Survival Curve to estimate cumulative survival of the ATCC and Unknown infected fish. Specifically, we modeled and plotted Kaplan-Meier estimates of probability of survival over time for each bacterial strain.

All fish used for both exposures in the four treatments (control, saline, ATCC, Unknown) were approximately the same weight at the start of the experiment (Figure 3.4, Figure 3.5). Therefore, fish size-at-exposure was not considered to be a factor affecting mortality during the exposure experiment.

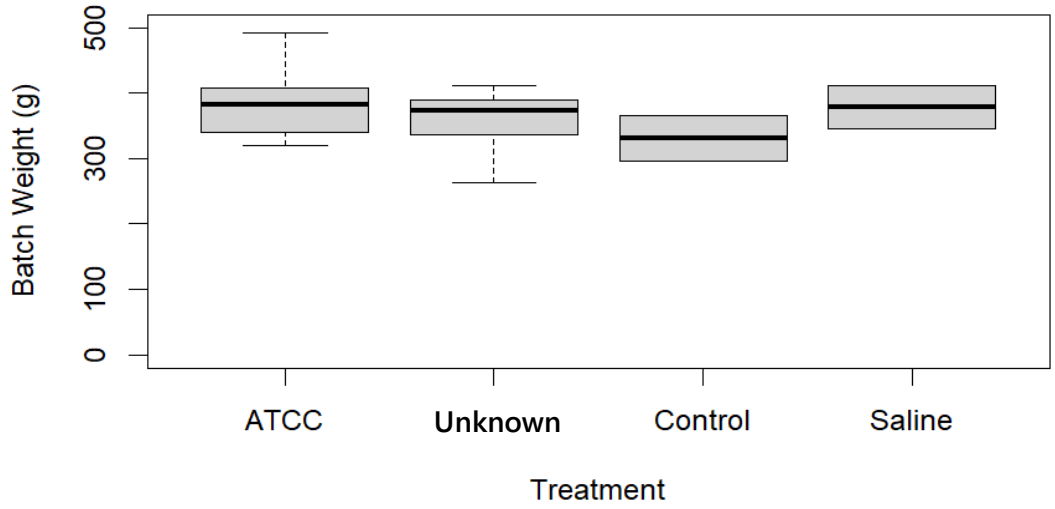

Figure 3.5. Colorado River Cutthroat Trout batch weights (g) by treatment (ATCC, Unknown, Control, Saline) at time of injection with *R. salmoninarum.*

Table 3.3. Summary of qPCR and DFAT results from each of the four treatments for Chinook Salmon. Data include the percent of fish that died during the experiment (Mortality) or were euthanized at ten weeks post-injection (Survived) that were infected with *R. salmoninarum* (% Infect), and the average *R. salmoninarum* cells g^{-1} in the homogenized tissue samples of the two groups, estimated from the qPCR standard curve.

Treatment	qPCR				DFAT	
	Mortality (% Infect)	Mortality (cells g^{-1})	Survived (% Infect)	Survived (cells g^{-1})	Mortality (% Infect)	Survived (% Infect)
Control	NA	NA	100	22	NA	25
Saline	NA	NA	100	8	NA	10
ATCC	100	765,108	100	351,187	100	100
Unknown	100	683,463	100	716,880	100	100

For both species, there were no mortalities in our control or saline injected tanks, indicating that all mortality in the injection tanks occurred as a result of *R. salmoninarum* infection. We detected *R. salmoninarum* in both the control and saline injected Chinook Salmon and Colorado River Cutthroat Trout with qPCR and DFAT, but bacterial loads were low compared to fish injected with the ATCC and Unknown *R. salmoninarum* strains (Table 3.3, Table 3.4). We had anticipated that the Chinook Salmon controls would have a mild infection because they came from an *R. salmoninarum* positive hatchery at which they had been previously treated for the bacteria, but the bacterial loads were so low that they didn't affect the experiment. However, we did not anticipate the Colorado River Cutthroat Trout to be infected since they had previously tested negative by DFAT during routine health inspections at the source hatchery, but the bacterial loads were still very low. Many fewer control and saline injected fish were determined to be infected with *R. salmoninarum* when using the DFAT compared to the qPCR assay (Table 3.3). Between the two species, Chinook Salmon have a greater bacterial load than the Colorado River Cutthroat Trout.

Table 3.4. Summary of qPCR results from each of the four treatments for Colorado River Cutthroat Trout. Data include the percent of fish that died during the experiment (Mortality) or were euthanized at ten weeks post-injection (Survived) that were infected with *R. salmoninarum* (% Infect), and the average *R. salmoninarum* cells g^{-1} in the homogenized tissue samples of the two groups, estimated from the qPCR standard curve. DFAT values are blank because the samples are still being analyzed.

Treatment	qPCR				DFAT	
	Mortality (% Infect)	Mortality (cells g^{-1})	Survived (% Infect)	Survived (cells g^{-1})	Mortality (% Infect)	Survived (% Infect)
Control	NA	NA	85	11		
Saline	NA	NA	45	2		
ATCC	100	49,205	94.4	2,976		
Unknown	100	260,473	100	16,330		

The Chinook Salmon exposure experiment lasted 71 days. Fish injected with the Unknown strain started dying from an *R. salmoninarum* infection at 31 days post-injection, whereas fish injected with the ATCC strain started dying from an *R. salmoninarum* infection at 46 days post-injection. Overall, only 1% of the fish injected with the Unknown strain compared to 31% of the fish injected with the ATCC strain survived to the end of the experiment (Figure 3.6). Fish injected with either the ATCC or Unknown strain that died during the experiment had a similar number of bacterial cells g^{-1} as determined by the qPCR standard curve. However, fish injected with the ATCC strain that survived to the end of the experiment had fewer bacterial cells g^{-1} than did those injected with the Unknown strain, suggesting that Chinook Salmon were able to clear the ATCC strain infection (Table 3.3).

The Colorado River Cutthroat Trout exposure experiment lasted 71 days. Fish injected with the Unknown strain started dying from an *R. salmoninarum* infection at 22 days post-injection, whereas fish injected with the ATCC strain started dying from an *R. salmoninarum* infection at 27 days post-injection. Overall, only 2% of the fish injected with the Unknown strain compared to 90% of the fish injected with the ATCC strain survived to the end of the experiment (Figure

3.6). Fish injected with the ATCC strain had a lower number of bacterial cells g^{-1} in both mortalities and those that survived compared to the Unknown strain.

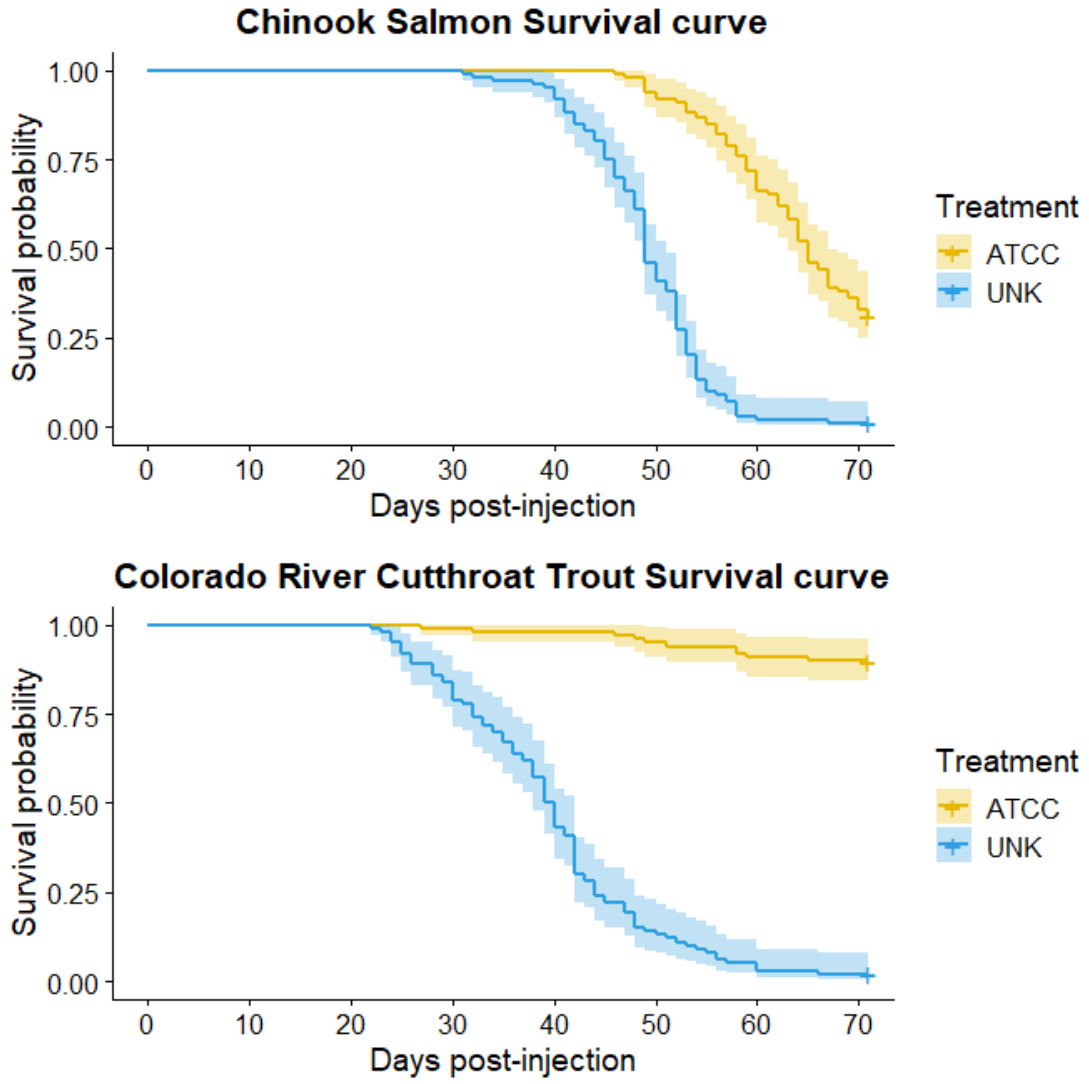

Figure 3.6. Kaplan-Meier cumulative percent survival of Chinook Salmon and Colorado River Cutthroat Trout injected with the ATCC strain (yellow) and the Unknown strain (blue), plotted daily over the 71-day exposure experiment.

Taken together, the results of these exposure experiments suggest that 1) location-of-origin of the bacterial strain and exposed fish has an effect on both mortality and the ability of exposed fish to clear an infection, 2) that the Unknown strain isolated from Colorado may not be less virulent than the ATCC strain isolated from the Pacific Northwest when fish from outside the strains range are exposed to the bacteria, 3) the Unknown bacterial strain replicates faster over a shorter duration whereas the ATCC bacterial strain replicates slower in that same amount of time, and 4) the Colorado River Cutthroat Trout were previously exposed to *R. salmoninarum* (strain to

which they had been exposed is still being investigated) and therefore produced an immune response to the infection when exposed to the ATCC strain, but not the Unknown strain.

The next step is to start another exposure experiment in which Harrison Lake Rainbow Trout, which have had prior exposure to the Unknown strain, will be injected with the two bacterial strains to understand cumulative survival. We predict that injection with the ATCC strain may cause lower survival than injection with the Unknown strain because the fish most likely have not been previously exposed to the ATCC strain. Results from this injection experiment and DFAT results from the Colorado River Cutthroat Trout will be available when completed.

Chase, D. M., D. G. Elliott, and R. Pascho. 2006. Detection and quantification of *Renibacterium salmoninarum* DNA in salmonid tissues by real-time quantitative polymerase chain reaction analysis. Journal of Veterinary Diagnostic Investigation 18(4):375-380.

Elliott, D. G. 2012. Bacterial kidney disease. *In* USFWS and AFS-FHS. FHS blue book: suggested procedures for the detection and identification of certain finfish and shellfish pathogens, 2020 edition. Accessed at: https://units.fisheries.org/fhs/fish-health-section-blue-book-2020/ (February 2021).

Elliott, D. G., L. J. Applegate, A. L. Murray, M. K. Purcell, and C. L. McKibben. 2013. Bench-top validation testing of selected immunological and molecular *Renibacterium salmoninarum* diagnostic assays by comparison with quantitative bacteriological culture. Journal of Fish Diseases 36(9):779-809.

Fetherman, E. R., B. Neuschwanger, B. W. Avila, and T. B. Riepe. 2020. Sport Fish Research Studies. Annual Report. Colorado Parks and Wildlife, Aquatic Research Section. Fort Collins, Colorado.

Johnson, P., D. M. Calhoun, W. E. Moss, T. McDevitt-Galles, T. B. Riepe, J. M. Hallas, T. L. Parchman, C. R. Feldman, T. J. Achatz, V. V. Tkach, J. Cropanzano, J. Bowerman, and J. Koprivnikar. 2020. The cost of travel: How dispersal ability limits local adaptation in host–parasite interactions. Journal of Evolutionary Biology 34(3):512-524.

Kowalski, D. A., R. J. Cordes, T. B. Riepe, J. D. Drennan, and A. J. Treble. 2022. Prevalence and distribution of *Renibacterium salmoninarum*, causative agent of bacterial kidney disease, in wild trout fisheries in Colorado. Diseases of Aquatic Organisms 149:109-120.

McKibben, C., and R. Pascho. 1999. Shedding of *Renibacterium salmoninarum* by infected Chinook Salmon *Oncorhynchus tschawytscha*. Diseases of Aquatic Organisms 38:75-79.

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Rhodes, L. D., and C. Mimeault. 2019. Characterization of *Renibacterium salmoninarum* and bacterial kidney disease to inform pathogen transfer risk assessments in British Columbia. DFO Canadian Science Advisory Section Research Document 2019/018. 46 p.

Riepe, T. B. 2022. Detection and transmission of *Renibacterium salmoninarum* in Colorado inland trout. Dissertation. Colorado State University, Fort Collins, Colorado.

Riepe, T. B., E. R. Fetherman, B. Neuschwanger, T. Davis, A. Perkins, and D. L. Winkelman. 2023. Vertical transmission of *Renibacterium salmoninarum* in Cutthroat Trout (*Oncorhynchus clarkii*). Journal of Fish Diseases 46(4):309-319.

Starliper, C. E., D. R. Smith, and T. Shatzer. 1997. Virulence of *Renibacterium salmoninarum* to salmonids. Journal of Aquatic Animal Health 9(1):1-7.

USFWS and AFS-FHS (U.S. Fish and Wildlife Service and American Fisheries Society-Fish Health Section). 2014. Standard procedures for aquatic animal health inspections. *In* AFS-FHS. FHS blue book: suggested procedures for the detection and identification of certain finfish and shellfish pathogens, 2020 edition. Accessed at: https://units.fisheries.org/fhs/fish-health-section-blue-book-2020/ (February 2021).

Susceptibility of Harrison Lake Rainbow Trout to Renibacterium salmoninarum

Rebecca McDevitt (MS candidate, Colorado State University) has recently completed two *Renibacterium salmoninarum* exposure experiments to investigate strain- and species-specific susceptibility to infection. Using an autoagglutination test, Riepe (2022) showed that the Colorado strain of *R. salmoninarum* isolated from Rainbow Trout at a Colorado hatchery may be an attenuated virulent strain relative to the ATCC strain isolated from salmonids in the Pacific Northwest. To test this, Rebecca exposed a salmonid species from the Pacific Northwest, Chinook Salmon, and an inland trout species, Colorado River Cutthroat Trout, to the ATCC and Colorado strains of *R. salmoninarum*. Experimental endpoints for these exposure experiments included mortality, detection of the bacteria via DFAT and qPCR, and quantification of the number of bacteria per gram of tissue in combined liver and kidney tissues.

In addition to potential differences in virulence of the two strains of bacteria, susceptibility may depend on whether the pathogen and host originate from the same location. Given this, we predicted two potential outcomes for the first experiment conducted with Chinook Salmon. We predicted that the Chinook Salmon would exhibit lower survival following exposure to the Colorado strain compared to the ATCC strain given that the Colorado strain originates from outside of the native range of the Chinook Salmon. However, if the Colorado strain is an attenuated virulent strain, the Chinook Salmon exposed to the Colorado strain would be expected to show similar or higher survival rates compared to those exposed to the potentially more virulent ATCC strain. Results indicated that mortality of Chinook Salmon began 15 days sooner in tanks with fish exposed to the Colorado strain compared to those exposed to the ATCC strain. Additionally, only 1% of fish exposed to the Colorado strain survived to the end of the 71-day experiment compared to 31% of fish exposed to the ATCC strain. Overall, the results of this experiment suggest that 1) location-of-origin of the bacterial strain and exposed fish has an effect on both mortality and the ability of exposed fish to clear an infection, and 2) that the Colorado strain may not be less virulent than the ATCC strain isolated from the Pacific Northwest when fish from outside the strains range are exposed to the bacteria (Fetherman et al. 2023).

Given the results of the Chinook Salmon experiment, we predict that Colorado River Cutthroat Trout would exhibit higher mortality when exposed to the ATCC strain versus Colorado strain of *R. salmoninarum* based on the origin of both the bacterial strain and fish species being exposed, but that was not the case. There was also a question as to whether the species of fish from which the bacteria was isolated can also affect the virulence of the specific bacterial strain. Bacterial virulence has been known to shift to a more optimal virulence after the successful infection of a new species. The Colorado strain of *R. salmoninarum* was isolated from Rainbow Trout. Therefore, if species from which the bacterial strain was isolated plays a role in bacterial virulence, we could expect to see higher than expected mortality in the Cutthroat Trout, i.e., similar to that of the Chinook Salmon, but lower mortality in Rainbow Trout. Cutthroat Trout mortality was indeed similar to that of the Chinook Salmon when exposed to the Colorado starin, but it is unknown if this was due to the virulence of the Colorado strain or potential previous exposure to *R. salmoninarum* that allowed them to fight infection by the ATCC strain more effectively.

This third study will investigate the susceptibility of Harrison Lake Rainbow Trout to infection by *R. salmoninarum*. Harrison Lake Rainbow Trout have been previously exposed to the bacteria at both the Bellvue Fish Research Hatchery, during which time the Colorado strain was isolated from the facility, and the Poudre Rearing Unit. Fish for this experiment were spawned at the Poudre Rearing Unit in May 2023 and hatched in July 2023. Four hundred age-1 individuals were avialbe for use in this experiment at the beginning of July 2024, and on July 17, 164 were moved from the Poudre Rearing Unit to Fort Collins for experimentation.

The Harrison Lake injection experiment will take place in the Salmonid Disease and Sport Fish Research Laboratory in Fort Collins, Colorado. Fourteen 10-gallon tanks are being used for the experiment: two pure controls, two saline injection controls, and five tanks each of fish injected with the Colorado and ATCC strains of *R. salmoninarum* (injected with the same dose of 6.5×10^6 *R. salmoninarum* cells/mL of each bacterial strain). Tanks contain 10 fish each, for a total of 140 fish used in the experiment; 24 fish are being held in an extra tank for use if mortality occurs in any tank prior to injections. The Rainbow Trout currently average approximately 125 mm total length and 8 grams. Fish will be injected with the bacteria on August 5, 2024.

Fish will be monitored twice a day after injection, following the same experimental procedures as those used for the Chinook Salmon and Cutthroat Trout exposure experiments conducted at CSU (Fetherman et al. 2023 and sections above). Moribund and dead fish will be removed from each tank twice daily, lengths and weights will be recorded, and liver, kidney, and spleen tissues will be collected from all fish removed to determine the timing of mortality and infection severity after injection. All fish remaining at ten weeks post-injection will be euthanized, and lengths, weights, and tissues will be collected to test for *R. salmoninarum* using DFAT and qPCR to determine if clearance of the infection occurred among the surviving fish.

Following Riepe (2022), tissues will be thawed, homogenized together, and prepared for DFAT and qPCR analyses. Specifically, DFAT samples will be smeared on a 12-well slide in duplicate, stained with an FITC-conjugated *R. salmoninarum* antisera, and counter-stained using Eriochrome Black (USFWS and AFS-FHS 2014). All slides will be analyzed using a Nikon

compound microscope fitted with a 420 nm fluorescent emission high-pressure mercury lamp, examined at 500X magnification, and confirmed at 1000X magnification (Fetherman et al. 2020). Any detection of *R. salmoninarum* will be considered infected tissue. DNA extractions will be completed with Qiagen DNeasy Blood and Tissue Kits with an additional elution step (Elliott et al. 2013). We will use previously determined primer and probe sets (RS 1238 F, RS 1307 R, and RS 1262 MGB probe; Chase et al. 2006; Elliott et al. 2012) with TaqMan Gene Expression Master Mix to complete the qPCR analyses. Samples will be determined positive when the Cq values are less than 37.75 (Riepe 2022). A previously developed standard curve will be used to determine the number of bacteria in each sample.

We will calculate the survival of infected fish over time since exposure (days) with the survival package in Rstudio. The package allows us to use the Kaplan-Meier Survival Curve to estimate cumulative survival of the ATCC and Unknown infected fish. Specifically, we will model and plot Kaplan-Meier estimates of probability of survival over time for each bacterial strain. A log-rank test will be used to test for differences between the survival curves of the infected fish.

Upon completion of the analyses, the results from the Harrison Lake Rainbow Trout exposure experiment will be compared to those obtained from the Chinook Salmon and Cutthroat Trout exposure experiments. Results will be presented in the 2025 Sport Fish Research Studies Annual Report. In addition, results may be combined with the Chinook Salmon and Cutthroat Trout exposure results for publication, if not already published, or submitted as a Management Brief to an appropriate journal.

Chase, D. M., D. G. Elliott, and R. Pascho. 2006. Detection and quantification of *Renibacterium salmoninarum* DNA in salmonid tissues by real-time quantitative polymerase chain reaction analysis. Journal of Veterinary Diagnostic Investigation 18(4):375-380.

Elliott, D. G. 2012. Bacterial kidney disease. In USFWS and AFS-FHS. FHS blue book: suggested procedures for the detection and identification of certain finfish and shellfish pathogens, 2020 edition. Accessed at: https://units.fisheries.org/fhs/fish-health-section-bluebook-2020/ (February 2021).

Elliott, D. G., L. J. Applegate, A. L. Murray, M. K. Purcell, and C. L. McKibben. 2013. Benchtop validation testing of selected immunological and molecular *Renibacterium salmoninarum* diagnostic assays by comparison with quantitative bacteriological culture. Journal of Fish Diseases 36(9):779-809.

Fetherman, E. R., B. Neuschwanger, B. W. Avila, and T. B. Riepe. 2020. Sport Fish Research Studies. Annual Report. Colorado Parks and Wildlife, Aquatic Research Section. Fort Collins, Colorado.

Fetherman, E. R., B. Neuschwanger, and R. E. McDevitt. 2023. Sport Fish Research Studies. Annual Report. Colorado Parks and Wildlife, Aquatic Research Section. Fort Collins, Colorado.

Riepe, T. B. 2022. Detection and transmission of *Renibacterium salmoninarum* in Colorado inland trout. Dissertation. Colorado State University, Fort Collins, Colorado.

USFWS and AFS-FHS (U.S. Fish and Wildlife Service and American Fisheries Society-Fish Health Section). 2014. Standard procedures for aquatic animal health inspections. In AFS-FHS. FHS blue book: suggested procedures for the detection and identification of certain finfish and shellfish pathogens, 2020 edition. Accessed at: https://units.fisheries.org/fhs/fishhealth-section-blue-book-2020/ (February 2021).

TECHNICAL ASSISTANCE

Effective communication between researchers, fishery managers and hatchery supervisors is essential to the management of fish populations in Colorado and across the globe. The objective of technical assistance is to provide information on impacts of fish disease on wild trout populations to the Aquatic Section of CPW and other resource agencies through publications, presentations, and research collaborations, as well as contribute editorial assistance to professional journals and other organizations upon request.

Internal presentations to CPW staff were used to update managers on current research and inform management decisions in Colorado. One presentation was given at the CPW Aquatic Section Meeting:

- Fetherman, E. R. 2024. Aquatic research project updates. Colorado Parks and Wildlife Aquatic Section Meeting. Mount Princeton, Colorado. February 6, 2024.

External presentations provided an opportunity to give research updates to managers both within and outside Colorado. In addition, participation in webinars and presentations to early career professionals and students provided an opportunity to talk about careers with CPW and provide advice on how to get a job with a state agency in the future. Sport fish personnel participated in one webinar hosted by the Western Division of AFS Early Career Professionals Committee, presented research as an invited plenary speaker at the annual meeting of the Montana Chapter of the American Fisheries Society, presented three talks at the annual meeting of the Colorado/Wyoming Chapter of the American Fisheries Society, and presented an invited talk to the Colorado State University Subunit of the American Fisheries Society:

- Fetherman, E. R. 2024. Getting a job with a state agency, navigating the experience needed to do so, and the role of AFS in obtaining that experience. Panel member. Western Division of the American Fisheries Society Early Career Professionals Committee Winter Webinar. Virtual Webinar. January 16, 2024.
- Fetherman, E. R. 2024. Improving operational, rearing, and production efficiency through research in Colorado hatcheries. 2024 Annual Meeting of the Montana Chapter of the American Fisheries Society. Lewistown, Montana. February 21, 2024.
- Avila, B., E. Fetherman, M. Kondratieff, E. Richer, D. Kowalski, and J. Spohn. 2024. Red spots, black spots, Brown Trout. 2024 Annual Meeting of the Colorado/Wyoming Chapter of the American Fisheries Society. Laramie, Wyoming. February 28, 2024.

- McDevitt, R., T. Riepe, E. Fetherman, and D. Winkelman. 2024. The susceptibility of Chinook Salmon to two strains of *Renibacterium salmoninarum*. 2024 Annual Meeting of the Colorado/Wyoming Chapter of the American Fisheries Society. Laramie, Wyoming. February 29, 2024.
- Dils, R., T. Riepe, P. Schaffer, D. Winkelman, and E. Fetherman. 2024. Pathogenesis of *Renibacterium salmoninarum* in Chinook Salmon following intraperitoneal injection: Description of disease progression by qPCR and histopathology. 2024 Annual Meeting of the Colorado/Wyoming Chapter of the American Fisheries Society. Laramie, Wyoming. February 29, 2024.
- Fetherman, E. R. 2024. Salmonid disease and sport fish research in Colorado. Colorado State University Subunit of the American Fisheries Society. Fort Collins, Colorado. April 18, 2024.

In addition to public and professional meeting presentations, two presentations were given to the fisheries management class at Front Range Community College in Fort Collins, Colorado. The first, an informal presentation/laboratory, was presented at the BFRH. During this lab, students learned about the various tagging methods used in research and management across Colorado, and were given a chance to try the tagging methods on live fish. The second, a formal presentation, was given to the class in March 2024.

- Fetherman, E. R. 2024. Salmonid disease research in Colorado. Guest lecture, Introduction to Fisheries. Front Range Community College. Fort Collins, Colorado. March 21, 2024.

Manuscripts published in peer-reviewed scientific journals help to inform fisheries management decisions locally, nationally, and internationally. Three manuscripts were submitted for publication in peer-reviewed scientific journals:

- Kopack, C. J., R. L. Moran, E. D. Broder, C. McDonald, E. R. Fetherman, K. L. Hoke, and L. M. Angeloni. *In review*. The effect of environmental enrichment on whole-brain expression in an imperiled fish. Submitted to Molecular Ecology.
- Avila, B. W., D. L. Winkelman, and E. R. Fetherman. *In review*. Hatchery rearing density affects post-stocking survival. Submitted to Aquaculture Journal.
- Riepe, T. B., E. R. Fetherman, K. P. Huyvaert, J. D. Drennan, R. E. McDevitt, B. Yeatts, and D. L. Winkelman. *In review*. Leveraging detection uncertainty to estimate *Renibacterium salmoninarum* infection status among multiple tissue assays. Submitted to PLoS ONE.

Eric Fetherman is also coauthor on Nutrition and Feeding chapter of Piper Redux, the third edition of Fish Hatchery Management being published by the American Fisheries Society, and primary author on two chapters in the Fishes of Colorado book being published by CPW:

- Palm, B., E. R. Fetherman, J. T. Trushenski, and W. Sealey. *In revision*. Nutrition and feeding. *In* Piper Redux. American Fisheries Society.
- Fetherman, E. R., and G. J. Schisler. *In press*. Rainbow Trout *Oncorhynchus mykiss*. Fishes of Colorado. Colorado Parks and Wildlife.
- Fetherman, E. R., G. J. Schisler, and R. Streater. *In press*. Cutbow (Cutthroat Trout X Rainbow Trout) *Oncorhynchus clarkii x O. mykiss*. Fishes of Colorado. Colorado Parks and Wildlife.

Through his role as President of the Western Division of the American Fisheries Society and Vice Chair of the American Fisheries Society Nominating Committee, Eric Fetherman also published three articles in *Fisheries*:

- Zipp, K., J. Kopaska, A. Croxton, S. Decker, L. Dorsey, L. Earley, E. Fetherman, P. Kusnierz, B. Missildine, B. Nerbonne, and J. Whittier. 2024. Future-focused leadership: 2024 AFS Second Vice President nominations. Fisheries 49(3):135-136. DOI: https://doi.org/10.1002/fsh.11059.
- Fetherman, E., T. Grabowski, C. Jennings, and J. Carter. 2024. Top 10 reasons to attend the annual meeting in Honolulu, Hawai'i. Fisheries 49(4):153-155. DOI: https://doi.org/10.1002/fsh.11082.
- Cannon, S., E. M. Dean, L. Guo, and E. Fetherman. 2024. From recognition to action: Strengthening AFS's commitments to indigenous peoples. Fisheries 49(6):249-251. DOI: https://doi.org/10.1002/fsh.11097.

Technical assistance milestones included consultation and assistance with experimental design, data collection, and analysis on projects being conducted by CPW researchers and biologists:

- Assisted with PIT tagging tiger muskie at Wray hatchery.
- Discussed next steps for investigating diploid testing, and temperature and egg volume effects on triploid walleye induction rates.
- Developed ideas for new explanatory variables for residual pool depth and factors affecting fish abundance and biomass in pools with and without toe wood.
- Assisted with plumbing sucker temperature trailer.
- Continued discussions and providing information regarding use of quaternary ammonium compounds for disinfection purposes.
- Assisted with sampling Northern Pike in College Lake.
- Prepared lab and kept oxytetracycline-marked walleye in tanks to allow growth for evaluation of mark retention in otoliths.

Sport Fish Research staff supported research projects using AQUI-S 20E under the Investigational New Animal Drug Program as program administrator:

- Fetherman et al. 2023. PIT tagging fish in Fraser River. August 2023-November 2023.
- Fetherman et al. 2023. PIT tagging fish in Colorado River. September 2023-November 2023.
- Riepe 2023. Rainbow Trout ECG pilot study. November 2023-January 2024.
- Riepe 2024. Rainbow Trout ECG pilot study number 2. January 2024-March 2024.
- Riepe 2024. ECG and temperature tolerance in suckers and chub. May 2024-current.

Technical assistance included peer review of manuscripts submitted to scientific journals:

- James, C. T., and S. Simmons. *In review.* Can anglers help identify trout populations impacted by whirling disease? A case study on the Bow River, AB, Canada. Submitted to North American Journal of Fisheries Management.
- Rahman, M. Z., A. B. M. Baki, H. Ghamry, and C. Katopodis. *In review*. Fish spawning habitat suitability for local bed change around instream boulders: Experimental investigation – II. Submitted to Journal of Ecohydraulics.

- Honda, K., K. Hasegawa, M. Ban, Y. Yano, and Y. Ogura. *In review*. Massive stocking of chum salmon (*Oncorhynchus keta*) fry fattens exotic brown trout (*Salmo trutta*) in Hokkaido, Japan. Submitted to PLOS ONE.
- Monnolo, A., M. T. Clausi, F. Del Piano, M. Santoro, M. L. Fiorentino, L. Barca, G. Fusco, B. degli Uberti, L. Ferrante, R. Mercogliano, and M. C. Ferrante. *In review*. Do organochlorine contaminants modulate the parasitic infection degree in Mediterranean trout (*Salmo trutta*)? Submitted to Animals.
- Rummel, S. M., A. Lutz, J. Tomlinson, E. Buck, and A. Wolfe. *In review*. Evaluation of aquatic organism passage at road-stream crossing improvement projects in Pennsylvania. Submitted to North American Journal of Fisheries Management.
- Baker, S. M., and S. M. Sammons. *In review*. Predation of stocked Rainbow Trout in an Alabama tailwater. Submitted to North American Journal of Fisheries Management.

Service outside of CPW:

- Member of the North American Journal of Aquaculture subcommittee of the AFS Publication Awards Committee. 2023-2024.
- Editor of abstracts submitted to CO/WY AFS meeting for AFS style. 2024.
- Member of the Fish Culture Section Hall of Fame Review Committee. 2024.
- President of the Western Division of the American Fisheries Society. 2023-2024.
  - Member of the WDAFS Financial Sustainability Committee.
  - Member of the WDAFS Diversity and Inclusion Committee.
  - Member of the AFS Management Committee.
  - Member of the AFS Governing Board.
  - Vice-Chair of the AFS Nominating Committee.
  - AFS Honolulu 2024 General Meeting Co-chair.
  - Co-chair of the AFS Honolulu Local Planning Team.
  - Member of the AFS Honolulu Program Committee.
  - Member of the AFS Respectful Meetings Working Group.

C O L O R A D O P A R K S & W I L D L I F E

Brown Trout Life History

EVALUATING BROWN TROUT POPULATION STRUCTURE AND LIFE HISTORY IN THE SOUTH PLATTE RIVER

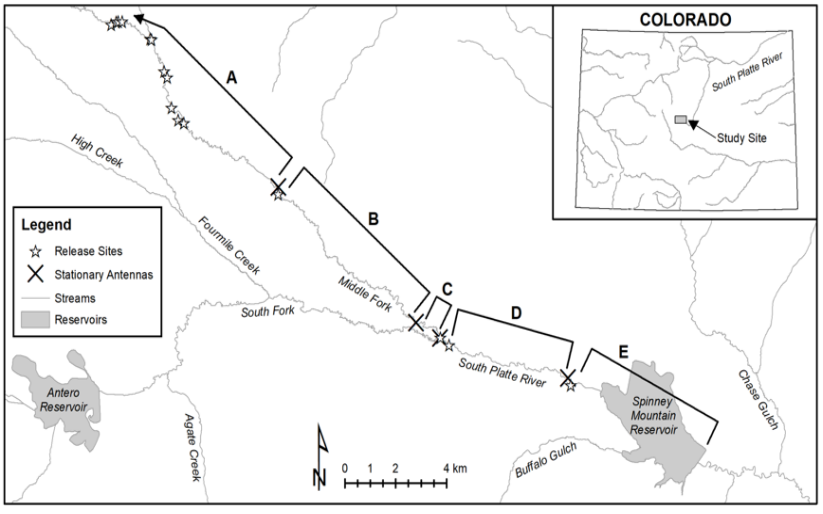
Brown Trout Ecology

Black (top), intermediate (middle), and red (bottom) spotting patterns of Brown Trout from the Middle fork of the South Platte River.

Brown Trout are native to Europe, Western Asia, and Northern Africa, and have been introduced to every continent except Antarctica. Across their native and introduced ranges, Brown Trout show a large diversity in life-history traits. For example, some Brown Trout stay within a small section of stream their entire life. Some populations consist of fish that spawn only once, while in other populations, fish spawn multiple times over their lifetimes. There are also populations that are anadromous, where fish move up freshwater rivers from the ocean to spawn, as well as adfluvial populations, where fish migrate between rivers and lakes. Along with these differences in life history, the physical appearance of Brown Trout varies greatly, which could be related to genetics, habitat use, diet, and fish age, among other factors. However, the connection between life-history traits, factors affecting physical appearance, and management in North America is not well studied.

Brown Trout were first introduced to the United States in 1883, and subsequently introduced into Colorado in 1890. Different source populations were used to establish these wild Brown Trout fisheries, including lake populations from Scotland and river populations from Germany. Brown trout populations in Colorado are generally considered a mix of these two, and exhibit physical and behavioral characteristics of both as part of their life histories. Understanding Brown Trout life history is an important aspect of managing these populations throughout the state.

Brown Trout in the South Platte River



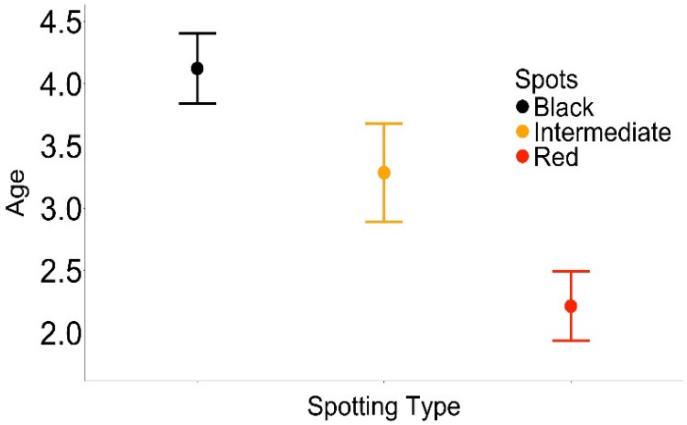
The Brown Trout population in the Middle Fork of the South Platte River near Hartsel is of interest to both anglers and mangers due to the unique angling opportunities provided by both Spinney Mountain Reservoir and the state wildlife areas along the South Platte River. Between 2006 and 2011, a stream restoration project was completed in a small section of the Middle Fork of the South Platte to create deeper pools for overwinter habitat and to hold big fish throughout the rest of the year. Between 2013 and 2016, CPW biologists and researchers conducted a tagging study to investigate habitat use by Brown Trout throughout the river. Fish were tagged with passive integrated transponder (PIT) tags containing a unique identification number that can be used to track the location of fish through physical recaptures and scanning during population estimate, and by river-spanning

antennas placed in the river that can detect the tags when the fish move past their location. These antennas record the date and time when movements are made year-round. Over the course of the study, three Brown Trout spotting patterns were identified: those with only black spots, those with red spots, and an intermediate spotting pattern between the two. To determine if spotting pattern explained observed movement patterns or the life history of the Brown Trout population in the Middle Fork of the South Platte River, additional data was collected in 2016, including stable isotopes, a technique used to identify chemical signatures in the fish tissue and characterize their diets and where feeding occurred, scales for aging fish, and genetic samples.

South Platte River Brown Trout Population Structure and Life History

A total of 1,259 Brown Trout were PIT-tagged over the three year study. Brown Trout with typically black spots and larger sizes moved longer distances, crossing all four of the stationary antennae at least once during the study. Although some PIT-tagged fish made long-distance movements, most moved less than 9 miles, with the majority of the fish moving 1 mile or less, suggesting many were river residents. Larger movements were made in the fall (September through October) as well as the spring (March through May). Fall movements occurred during the Brown Trout spawning period, whereas spring movements occurred when the ice was melting. Brown Trout primarily used the section of river at the upstream end of the study area, and Spinney Mountain Reservoir. When Brown Trout chose to move, they generally moved between these two locations fairly quickly, bypassing the habitat in between, including the deep pools in the restoration section, to reach favorable spawning areas or overwinter habitat in the reservoir.

Brown Trout with black spots were older than Brown Trout with red spots, and fish with an intermediate spotting pattern fell between the two, suggesting spotting pattern changed as fish got older. Isotope data indicated that black spotted fish and red spotted fish each eat different foods and reside in different parts of the system. Black spotted fish spend most of their time within the reservoir, entering the river to spawn in the fall, and the red spotted fish live primarily within the river. Fish with an intermediate spotting pattern are likely transitioning from life in the river to life in the reservoir as they get older. Genetic testing indicated that the Brown Trout with black spots, red spots, and the intermediate spotting pattern were related and form one adfluvial Brown Trout population. Brown Trout in the Middle Fork of the South Platte River rely on both the river and the reservoir to complete parts of their life history (e.g., spawning and juvenile rearing versus overwinter habitat and growth), and change spotting patterns as the fish age and use these different habitats.



Average age (95% confidence intervals) of Brown Trout with black, intermediate, and red spotting patterns from the Middle Fork of the South Platte River.

Management of Brown Trout

This study allowed biologists to better understand the life history of Brown Trout in the South Platte River. Based on the suite of data collected, these Brown Trout represent one population with an adfluvial life history incorporating characteristics of lake and river source populations historically used to establish Brown Trout in Colorado and North America. The connection between the reservoir and the upstream spawning habitat is important to the life history of the Brown Trout in this system, providing habitats needed to complete different parts of their life cycle. This information will help inform management of the fish in both the Middle Fork of the South Platte River and Spinney Mountain Reservoir, promoting their persistence, and ultimately providing more angling opportunities in both systems. Additionally, knowledge of the life history can be used to guide future river restoration activities in this and other rivers around the state to maximize their success.

Associated Literature

Avila, B. W., E. R. Fetherman, M. C. Kondratieff, E. E. Richer, D. A. Kowalski, M. R. Baerwald, A. Goodbla, and J. Spohn. *In preparation*. Brown Trout population structure and life history in the South Platte River, Colorado.