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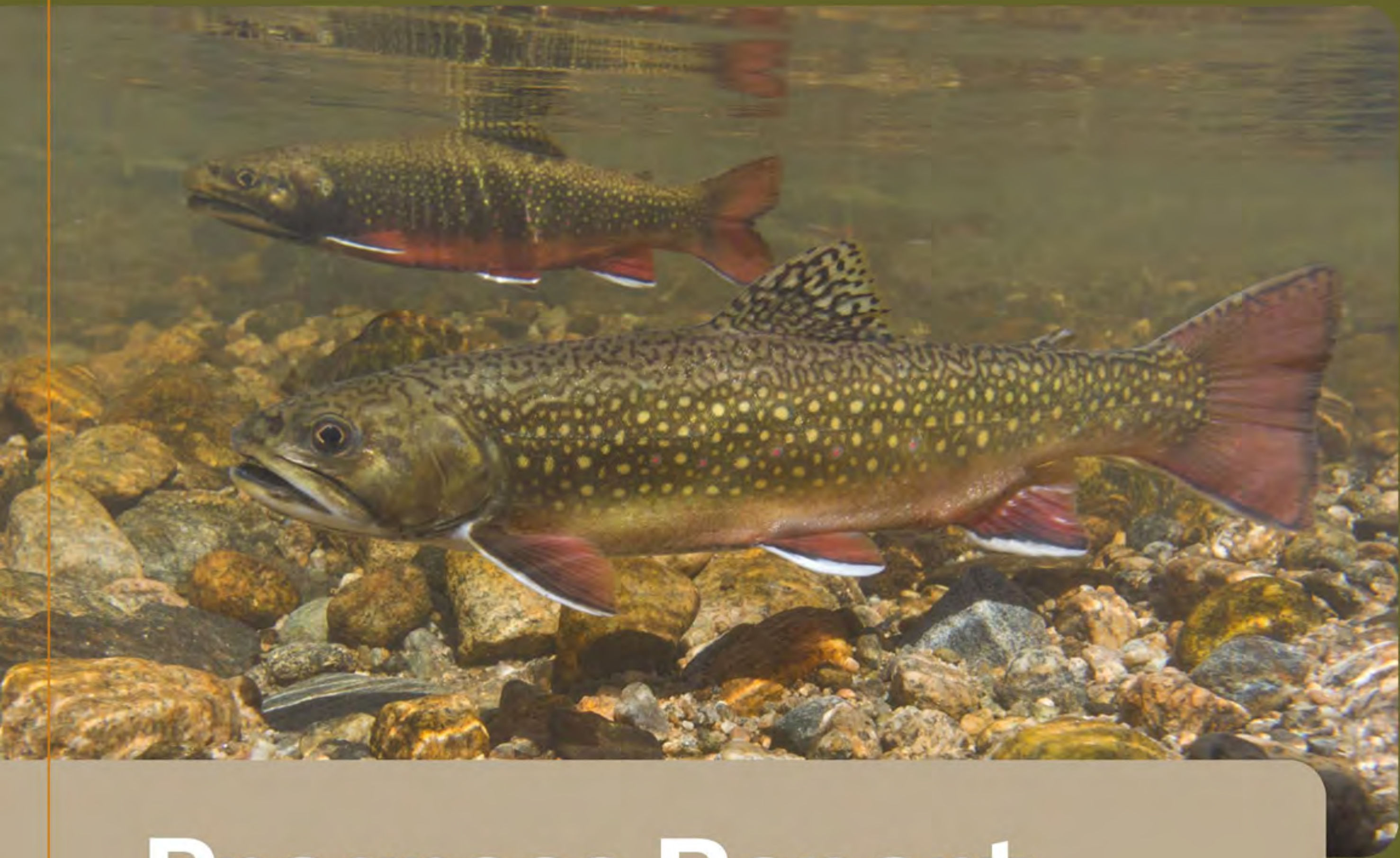

Progress Report

WAFWA Trojan (YY) Male Fish Consortium Activities

Prepared by

YY Fish Consortium Partners

Western Association of Fish and Wildlife Agencies
Progress Report
on
WAFWA Trojan (YY) Male Fish Consortium Activities

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Executive Summary:
Results to Date and Future Work

Results to Date

The Western Association of Fish and Wildlife Agencies (WAFWA) YY Male Fish Consortium has achieved significant progress across multiple invasive fish species. Key results include successful development of genetic sex markers for brown trout, lake trout, common carp, and walleye; confirmation of the first eradication cases of female wild brook trout in Idaho; substantial expansion of YY brook trout broodstock production; advances in brown trout and common carp feminization protocols; and completion of multi-state field trials demonstrating population declines in brook trout when YY males are stocked. Additionally, major regulatory, partnership, and funding milestones have strengthened the program's national implementation potential.

Future Work

Priority future efforts include advancing second-generation YY brown trout broodstock, finalizing common carp feminization protocols, expanding multi-state brook trout eradication projects, launching surrogate broodstock research for lake trout, evaluating feasibility of a WW-based biocontrol strategy for walleye, and continuing marker research for northern pike. Regulatory work will focus on FDA indexing of estradiol and expansion of INAD hatchery approvals. Additional goals include increasing hatchery capacity, strengthening cross-agency collaborations, and integrating YY fish deployment into large-scale invasive eradication plans across western states.

Contents

Introduction

Hamilton (1967) first proposed that eradication of an invasive population could be achieved by manipulating its sex ratio to produce exclusively one sex. The concept of applying this principle to fish through aquaculture-induced sex reversal was advanced by John Teem, who hypothesized that exogenous hormones could feminize captive male broodstock, enabling the production of genetically YY males. When released into target wild populations, these YY males would mate with wild females and produce only male offspring, progressively skewing the sex ratio toward all males and ultimately leading to population collapse (Mills 2009). Termed the Trojan Y Chromosome (TYC) strategy, this approach was formally evaluated through population modeling by Gutierrez and Teem (2006) for eradication of invasive Nile tilapia (*Oreochromis niloticus*). Their analysis identified two critical technical requirements for successful broodstock development in any target species: (1) availability of a reliable genetic sex marker or assay, and (2) effective feminization of juvenile males using exogenous hormones in a controlled hatchery environment.

The first implementation of a YY Male broodstock for invasive fish suppression targeted brook trout (*Salvelinus fontinalis*) and commenced in November 2008 under the leadership of the Idaho Department of Fish and Game (IDFG) (Schill et al. 2016). Utilizing the indirect broodstock development protocol (Beardmore et al. 2001) combined with PIT-tagging, genetic sex determination, and refined culture techniques, IDFG reduced the required timeline from five generations (as previously reported for tilapia; e.g., Mair et al. 1997) to three generations, achieving completion in approximately five years. To enhance accessibility for public and managerial audiences, the program adopted the term "YY Male" in preference to "Trojan Y Chromosome."

Subsequent population modeling was conducted to establish realistic expectations for field applications and to inform appropriate stocking densities. Schill et al. (2017) constructed an age-structured stochastic matrix model using Idaho-specific brook trout demographic data informed by the long-term time series data reported by McFadden et al. (1967). Under assumptions of YY Male fitness comparable to that of wild individuals, simulations predicted complete extirpation in stream environments within 2 to 4 years; under reduced fitness scenarios (e.g., 20% of wild male fitness), extirpation times extended to 5 to 15 years. Because YY Male fingerling stocking combined with manual suppression (e.g., electrofishing) could attain the densities incorporated in successful model runs, the authors advocated for large-scale field trials of YY Male releases integrated within an Integrated Pest Management (IPM; Kogan 1988) framework that includes manual suppression, applicable to both stream and lake settings.

In parallel, a pilot evaluation assessed the ecological viability of stocked YY Males. In June 2014, approximately 500 YY Male brook trout (mean total length 250 mm) were released across short stream reaches (1.9 to 2.6 km) in four Idaho study streams. During the ensuing spawning season, YY Males constituted an estimated 3.1% of spawning adults. Genetic assignment confirmed that an average of 3.7% of resulting fry were progeny of stocked YY Males, and all such progeny were XY males, thereby demonstrating post-stocking survival, synchrony with wild spawning timing, successful reproduction with wild females, and production of exclusively male offspring (Kennedy et al. 2018).

Encouraged by the technical feasibility of YY Male brook trout production and these promising pilot outcomes, IDFG initiated broodstock development in 2014 for additional invasive species of concern to Idaho fisheries, including common carp (*Cyprinus carpio*), walleye (*Sander vitreus*), and lake trout (*Salvelinus namaycush).*

To broaden the initiative, IDFG presented the approach at the 2016 Western Association of Fish and Wildlife Agencies (WAFWA) Fish Chiefs meeting, prompting strong interest and leading to the establishment of the YY Male Consortium. In January 2018, WAFWA agency directors approved a multi-state proposal, with initial funding secured from 13 states. The Consortium focused on developing YY Male research broodstocks for five priority invasive species: the three already in progress at IDFG, plus brown trout (*Salmo trutta*) and northern pike (*Esox lucius*).

During July 2021, nine Fish Chiefs approved continued funding for three additional years (FY22-FY24), with ten stated program objectives (see below). An additional state (CA) returned to the Consortium during FY2024. With the retirement of Dr. Dan Schill, YY Male Coordinator duties were transitioned to Dr. George Schisler on a part-time basis for 2025. The objectives of the project have remained largely the same, with some refinement of priorities based on knowledge gained during earlier phases of the program. Appendix A consists of the final report: “Executive Summary of YY Program New Species Work (2018 to present)” by Dr. Schill. This report is included to archive key findings and provide guidance for future work.

Notably, field monitoring in the primary long-term study streams (Bear Creek and Willow Creek) has demonstrated the first successful applications of YY Male technology for population-level extirpation when integrated with intensive manual suppression. In Willow Creek, no wild females were captured during 2024 electrofishing surveys (following progressive declines and zero captures in recent prior years). In Bear Creek, the estimated age 1+ wild female population declined by 99% from 542 individuals in 2016 to only 4 in 2024. Sampling conducted in 2025 in both streams has now validated the complete eradication of female brook trout, with no wild females detected despite comprehensive electrofishing efforts across the entire stream lengths. These outcomes align closely with earlier modeling predictions and represent initial confirmation of the YY Male approach as an effective, integrated tool for eradicating nonnative brook trout populations (WAFWA YY Male Consortium 2024 Progress Report; subsequent 2025 monitoring data).

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YY Male Fish Consortium Program Objectives

The YY Male Fish Consortium is focused on a number of objectives designed to advance the relevant science, address regulatory challenges, develop functional YY Male Fish broodstocks, and ultimately supply these fish to partner states for management of invasive fish species. Objectives include finalizing sex-reversal protocols and genetic sex markers for brown trout, including verifying the ability of the existing brown trout genetic sex markers to reliably distinguish XY from YY individuals. Another high priority near-term objective is finalizing the sex-reversal treatment protocol for common carp and the genetic sex markers for this species. Additional objectives are to facilitate discussions on the research direction for walleye, northern pike, and lake trout. Objectives related to YY brook trout are to conduct additional evaluations of these fish for management purposes and well as maintaining and enhancing the existing YY brook trout brood stocks. An emerging objective, based on interest from several member states, is the evaluation of mosquitofish as a YY fish candidate. Other ongoing objectives include project communication and fundraising, identification of suitable hatchery facilities to house both primary and backup YY male broodstocks, and to continue permitting efforts with the U.S. Food and Drug Administration (FDA) for the use of estradiol in YY broodstock production. This FY2025 progress report documents the principal activities conducted during the first program year under the new coordinator and evaluates progress toward attainment of the objectives outlined above.

FY25 YY Male Fish Consortium Work

Brown Trout Studies

Past Brown Trout Sex Reversal and Sex Determination Work

A summary of the work conducted on brown trout (*Salmo trutta*) sex reversal, sex marker determination, and health assessment studies for FY20 through FY24 was provided by Dr. Dan Schill at the beginning of this segment, and is included as Appendix B of this report.

Ongoing Brown Trout Broodstock Development

The Los Ojos State Fish Hatchery (LOFH), operated by the New Mexico Department of Game and Fish (NMDGF), has emerged as a cornerstone facility for YY Male Brown Trout (*Salmo trutta*) broodstock development within the WAFWA YY Male Consortium. New Mexico has assumed a leadership role in this species, leveraging the hatchery's experienced staff and infrastructure to advance sex reversal, spawning, and multi-generational production protocols.

Initial technology transfer and protocol validation occurred in 2023. In January 2023, LOFH received 183 two-year-old Brown Trout from the BY20 feminization trial conducted at the Colorado Research Hatchery. These fish, of known genetic sex, were reared to maturity to assess spawning performance of feminized XY males and to verify the efficacy of estradiol (E_2) treatments identified in earlier Colorado trials. Despite substantial pre-spawning mortality (reducing the cohort to 24 survivors), two feminized males from the 20 mg E_2/kg for 90-day treatment produced viable eggs that were successfully stripped and fertilized. Although survival to the eyed stage was low, likely due to handling stress and fungal infections, this represented the first documented spawning of feminized Brown Trout at the facility (Schill et al. 2024).

Concurrently, LOFH initiated its own BY22 sex reversal trial to produce the first YY Brown Trout. Eyed eggs from successful BY20 feminized XY male × normal XY male crosses were shipped to Los Ojos in December 2022. Fry were divided into treatment groups and exposed to dietary E_2 (20 mg/kg feed) for either 90 or 120 days beginning at first feeding. Sampling at 437 days post-hatch confirmed successful feminization and the initial production of YY individuals: the 120-day treatment achieved 91.7% phenotypic females with minimal intersex fish and no evidence of long-term liver hypertrophy, while untreated controls exhibited the expected 25% female: 75% male ratio (Schill et al. 2023, 2024).

YY Brown Trout broodstock development at Los Ojos continues to progress as anticipated. In early 2025, approximately 6,000 fish from the 2024 brood year (BY24) from Saratoga National Fish Hatchery entered feminization treatment, using the previously identified treatment regime of 20 mg/kg feed for 120 days, with approximately 3,000 fish remaining following treatment. An additional 6,000 fish from the 2025 brood year (BY25) are currently undergoing feminization with the same treatment regime. The BY24 cohort has been PIT-tagged and will be reared to a larger size. This will be followed by gonadal dissections to confirm phenotypic sex and genetic screening to cull XX individuals.

Assuming successful feminization of the BY24 cohort, these fish will be evaluated for sexual maturity in fall 2026 and spawned if ready. This spawning event will initiate the second generation of the YY Brown Trout broodstock and mark a significant step toward operational deployment of the technology in New Mexico and across the Consortium.

Brown Trout Sex Markers

A major milestone achieved over the past year has been the development of a brown trout Genotyping-in-Thousands (GT-seq) genotyping panel (Campbell et al. 2014) through collaboration among NMDGF, New Mexico State University, Idaho Department of Fish and Game, and GTSeek LLC, Twin Falls, ID. The panel is now fully operational and provides accurate genetic sex determination that is concordant with prior results from Idaho. Specifically, 207 fish collected from the control group (untreated fish) in the aforementioned BY22 sex reversal trial were previously classified with IDFG sex markers. However, the accuracy of that test for differentiating XY from YY fish was unknown. Four sexID loci were used by GTSeek to produce 'genotypes' of XX, YY, and XY among these fish. Concordance with the IDFG classifications was 97.98%. The results were 51 XX, 53 YY, and 103 XY (exactly 25%, 25%, and 50%). Critically, this demonstrates the ability to reliably distinguish putative YY individuals from XY fish, offering a powerful tool for future broodstock management and verification. Additional validation will be conducted by applying this panel to progeny from the planned crosses between feminized XY males and true XY males from the current Los Ojos trial.

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Common Carp Studies

Numerous calls with Dr. Chad Teal and Dr. Charlotte Musser of Utah State University were held to coordinate efforts on YY common carp (*Cyprinus carpio*) experiments (including January 10, February 26, April 22, May 19, September 12, and October 20). Substantial progress was made on common carp feminization trials, which combined with common carp sex markers, provides a promising outlook for development of a YY broodstock for this species. A summary of this work through December 31, 2025, is provided below.

Common Carp Feminization Trials

Overview

Common carp feminization with dietary estrogens alone [largely 17β-Estradiol (E_2)] has been attempted previously with largely suboptimal results reported (Bongers et al. 1991; Jiang et al. 2018; Komen et al. 1989; Rao and Rao 1983). We have been attempting to determine an ideal treatment regime for sex reversal of XY common carp. The first trial was performed in the winter/spring of 2025 but was limited by significant nutrition-related mortality. A second trial was performed in the summer of 2025, which resulted in higher survival through the use of a different diet. Results from the second trial will be available later this year.

Background

Invasive common carp (*Cyprinus carpio*) have major negative impacts on native ecosystems (Weber and Brown 2009). Traditional methods of removal include piscicides and physical capture, and large amounts of time and money have been dedicated to their removal without lasting success (Brown and Gilligan 2014; Rowe 2001; Weber et al. 2016). Novel methods for extirpation of invasive common carp populations are needed.

Modeling suggests that the TYC strategy combined with traditional removal methods may be successful at extirpating common carp populations. McCormick et al. (2021) suggested that the release of YY "supermale" common carp may be successful at extirpating invasive populations at a low population density in a small system. However, the release YY "superfemale" fish may be successful in the extirpation of more dense populations in larger systems, and on a faster time scale.

Production of YY fish requires feminization of XY fish to produce egg-bearing neofemales, which are then crossed with XY males (Gutierrez and Teem 2006). Our work has been focused on developing a method for the first feminization step for YY fish production.

Methods

Broodstock selection and spawning

Adult wild common carp were collected from Cutler Reservoir in Cache County, UT via electrofishing in fall of 2024. Male and female fish were maintained together in a recirculating system at 22-24 ̊C. The fish were fed a commercial koi diet to satiety once daily in addition to frozen/thawed shrimp and frozen/thawed or freeze-dried krill. For Trial 1, spawning was induced on December 5, 2024, with an intramuscular (IM) injection of carp pituitary extract at 0.3 mg/kg body weight. For Trial 2, spawning was induced on May 13, 2025, with Ovaprim at 0.5 mL/kg body weight IM. Spawning occurred on a shade cloth structure placed in the broodstock tanks to provide hiding places for the fish. Fry were reared in a separate recirculating system until three days prior to the start of treatment, at which time they were transferred to aquaria for acclimation.

Trial Methods

For both trials (Trial 1 and Trial 2), treatment occurred either from 30-120 DPH or 60-150 DPH. Each treatment replicate consisted of 60 fish housed in 76 L aquaria with the water temperature maintained at either 20-21 ̊C or 26-27 ̊C. During the treatment period, fish were fed diets treated with 200, 300, or 400 mg E_2 per kilogram diet two to four times daily either by hand or by a rotatory-type automatic feeder (see Table 1 for summary of treatment groups). Trial 1 fish were initially fed Zeigler Finfish Starter but were switched to Otohime Marine Larval and Grow-out Feed on 50 DPH. Trial 1 fish were fed approximately 10% of their body weight daily. Trial 2 fish were fed Otohime Marine Larval and Grow-out Feed prior to and for the full duration of the study. Trial 2 fish were fed to satiety for each feeding. During Trial 2, fish were randomly culled from 60 individuals to 20 individuals per tank at 80-82 DPH due to deteriorating water quality as density increased. Culling was not necessary during Trial 1 due to lower growth and survival. Trial 2 was a replication of Trial 1, with a higher quality diet used from the beginning of treatment.

Additionally, estradiol immersion was attempted from 50-150 DPH at a nominal concentration of 10 µg E_2 /L (Gimeno et al. 1998) in tandem with Trial 2. There were three replicates for fish exposed to estradiol and three immersion control replicates. Sixty fish were again housed in 76 L flow-through aquaria at 23-25 ̊C. The average flow rate was 163 mL/minute (range 108-297 mL/min). Estradiol in powder form was dissolved in 100% ethanol and the resulting solution was added to well water at a concentration of 1.25 µg/mL. A peristaltic pump was then used to continuously dose the E_2/ethanol/water solution to the tanks at a rate determined by the tank flow rate to maintain the nominal E_2 concentration. Control tanks had an equivalent ethanol/water solution delivered in the same manner without the addition of E_2. As with the fish receiving dietary E_2, fish were fed to satiety 2-4 times a day and were culled to 20 fish per tank at 80-82 DPH.

Dose (mg E2/kg diet)	Temperature (̊C)	Treatment Period (DPH)	Replicates
0 (control)	20-21	30-120	2
0 (control)	26-27	30-120	2
0 (control)	20-21	60-150	2
0 (control)	26-27	60-150	2
200	20-21	30-120	3
200	26-27	30-120	3
200	20-21	60-150	3
200	26-27	60-150	3
300	20-21	30-120	3
300	26-27	30-120	3
300	20-21	60-150	3
300	26-27	60-150	3
400	20-21	30-120	3
400	26-27	30-120	3
400	20-21	60-150	3
400	26-27	60-150	3

Table 1. Summary of treatment groups for Trial 1 and Trial 2.

At the end of Trial 1, all but eleven fish from each tank were euthanized and preserved in 10% neutral buffered formalin (NBF). Where fewer than six fish remained, all were euthanized. At the immediate end of the treatment period, the caudal peduncles of three euthanized fish per tank were removed and frozen at -20 ̊C. Seven and 14 days later, an additional three fish per tank were euthanized and the caudal peduncles collected and stored at -20 ̊C (where numbers permitted). The fish were then preserved in NBF. Five preserved fish per tank were then randomly selected and submitted for routine histology.

Following the treatment durations, water temperature was changed to 22-24 ̊C and fish were maintained at that temperature thereafter. Where numbers permitted, five fish per tank from Trial 1 and the remaining fish from Trial 2 and the immersion trial were PIT tagged and transferred to a recirculating system for grow-out. The fish were transitioned to a commercial koi diet (Mazuri) and fed to satiety once to twice daily. At eight months of age, Trial 1 fish were euthanized and dissected to determine phenotypic sex and to evaluate internal organs for gross pathology. Trial 2 fish will be euthanized at eight months post hatch for similar evaluation and for determination of gonadosomatic index.

Histopathologic data collection

Histology slides of Trial 1 fish were examined for pathologic changes. Macroscopic inspection of euthanized fish and initial evaluation of slides revealed changes to the kidneys of E_2-treated fish. Therefore, we are currently collecting histopathological data on kidneys to quantify the degree of renal hypertrophy and changes to the renal tubules and glomeruli present in these samples.

Tissue residue data collection

A commercial enzyme-linked immunosorbent assay (ELISA, Enzo) for 17B-estradiol was used to determine tissue E_2 concentrations at the end of treatment and seven days following the end of treatment. Caudal peduncles stored at -20°C were thawed, homogenized, and centrifuged. The homogenate was drawn off the centrifuged samples and combined with the kit's provided steroid displacement reagent and then analyzed according to the manufacturer's instructions.

Data analysis

Sex ratios

Sex ratios were obtained from euthanized Trial 1 fish submitted for histology at the end of the treatment period and at eight months post hatch. At the end of Trial 1, gonads were examined histologically and the sex of each individual identified, if possible. At eight months post hatch, gonads were examined grossly and a squash prep was performed if the sex was not identifiable grossly. A binomial generalized linear model was used to evaluate the degree of feminization at both time points, followed by a Type III ANOVA to assess the significance of fixed effects and estimated marginal means with a Tukey adjustment for post hoc pairwise comparisons.

Survival

Beta regression with a logit link was used to analyze survival using dose, temperature, and the treatment time period as factors. A Type III Wald chi-square test was used to further evaluate effects and pairwise comparisons were carried out using estimated marginal means with a Tukey adjustment.

Growth and Gonadosomatic Index

At the end of the treatment period (120 and 150 DPH), length and weight information was collected for surviving fish from Trial 1 and for 10 fish per tank for Trial 2. A linear mixed effects model was used to analyze log-transformed length and weight data using dose and temperature as fixed effects and tank as a random effect. A Type III ANOVA was used to assess the significance of fixed effects and estimated marginal means with a Tukey adjustment was used for post hoc pairwise comparisons.

Tissue E_2 residues

As for length and weight data, a linear mixed effects model was used to analyze log-transformed tissue E_2 concentration data using dose, temperature, and treatment time period as fixed effects and tank as a random effect. A Type III ANOVA was used to assess the significance of fixed effects and estimated marginal means with a Tukey adjustment was used for post hoc pairwise comparisons. This was performed at two different time points, immediately at the end of treatment and seven days later.

Immersion

Statistical analysis for length, weight, and survival were conducted as described for Trial 2.

Results to Date

Trial 1 Sex ratios

Gonads examined immediately at the end of treatment exhibited no significant difference in observed sex ratios regardless of dose ($p = 0.895$), time period ($p = 0.640$), or temperature ($p = 0.284$). Despite this lack of significance there were several treatment groups with high (80-100%) rates of feminization, including all 26-27 ˚C, 60-150 DPH E_2 treatment groups. In post hoc analysis, there was a significant increase in feminization rates at 60-150 DPH between the control groups and the 300 and 400 mg/kg groups ($p = 0.024$ and $p = 0.017$, respectively). This discrepancy is likely due to the small sample size of some treatment groups (e.g., the 300 mg/kg, 30-120 DPH, 26-27˚C treatment had a single tank out of three remaining for analysis due to high levels of mortality).

At eight months post hatch there was no significant difference in sex ratios regardless of dose, time period, or temperature (see Table 2).

Treatment	Duration (dph)	Temp (*C)	Number of female fish / number of fish assessed at end of treatment	Number of undifferentiated/ unidentified fish / number of fish assessed at end of treatment	Number of male fish / number of fish assessed at end of treatment	Mean % Mortality at end of treatment	Number of female fish / number of fish assessed at 8 months post hatch
Control	30-120	20-21	no data	no data	no data	100	no data
Control	30-120	26-27	4 / 10	0 / 10	6 / 10	36.5	5 / 8
Control	60-150	20-21	1 / 10	4 / 10	5 / 10	27.5	4 / 9
Control	60-150	26-27	3 / 8	2 / 8	3 / 8	23.5	6 / 9
200mg/kg	30-120	20-21	0 / 7	7 / 7	0 / 7	95.56	no data
200mg/kg	30-120	26-27	9 / 10	1 / 10	0 / 10	67.3	2 / 3
200mg/kg	60-150	20-21	4 / 15	11 / 15	0 / 15	45.6	10 / 17
200mg/kg	60-150	26-27	15 / 15	0 / 15	0 / 15	24.4	10 / 14
300mg/kg	30-120	20-21	no data	no data	no data	100	no data
300mg/kg	30-120	26-27	5 / 5	0 / 5	0 / 5	85	0 / 3
300mg/kg	60-150	20-21	7 / 16	9 / 16	0 / 16	57.2	9 / 13
300mg/kg	60-150	26-27	15 / 16	1 / 16	0 / 16	24	7 / 13
400mg/kg	30-120	20-21	no data	no data	no data	100	no data
400mg/kg	30-120	26-27	8 / 10	2 / 10	0 / 10	90.8	3 / 7
400mg/kg	60-150	20-21	9 / 16	7 / 16	0 / 16	53.9	9 / 11
400mg/kg	60-150	26-27	14 / 16	2 / 16	0 / 16	21.1	11 / 16

Table 2. Summary of Trial 1 sex counts and mortality.

Trial 1 Survival

Trial 1 fish were suspected to have a nutritional deficiency due to the onset of significant mortality and opportunistic pathogens starting around 30 DPH. Around 40 DPH, spinal malformations were noted and fish were transitioned to a different diet, after which mortality decreased markedly. Fish treated from 30-120 DPH had a very high mortality rate, likely due to synergistic effects of nutritional deficiency and E_2 exposure (see Table 2). Fish treated from 60-150 DPH had a significantly higher survival rate ($\chi^2_{1} = 106.13$, $p < 0.001$). For all Trial 1 fish, mortality increased with increasing E_2 dose ($\chi^2_{3} = 35.71$, $p = 0.015$) and lower temperature was a significant factor for increasing mortality ($\chi^2_{1} = 25.31$, $p < 0.001$).

Trial 1 Growth

For fish treated from 30-120 DPH, there was no significant effect of dose or temperature on length or weight, likely due to some replicates having very small numbers of fish that showed high levels of growth (see Figure 1). For fish treated from 60-150 DPH, there was a dose-dependent decrease in length and weight, with fish receiving higher doses of E_2 having lower lengths ($F_{3, 13.34} = 27.17$, $p < 0.001$) and weights ($F_{3, 535} = 48.78$, $p < 0.001$) (see Figure 2). Fish maintained at a higher temperature exhibited greater length ($F_{1, 13.36} = 18.26$, $p < 0.001$), but no change in weight compared to fish maintained at a lower temperature.

Trial 1 Pathology

Among all groups (including controls), fish were frequently noted to have spinal and skull abnormalities including kyphosis, scoliosis, lordosis, deviated or malformed mouths, and opercular shortening. Many E_2-treated fish had soft, distended coelomic cavities secondary to renal hypertrophy; this was subjectively more pronounced in fish receiving higher doses of E_2 and fish maintained at 20-21 ̊C.

Histopathologic evaluation of E_2-treated fish revealed primarily renal abnormalities. Representative slides were submitted to a board-certified veterinary pathologist for review. Renal tubular hypertrophy with tubular protein casts and Bowman's capsule reflux were noted in all treated fish but not in control fish.

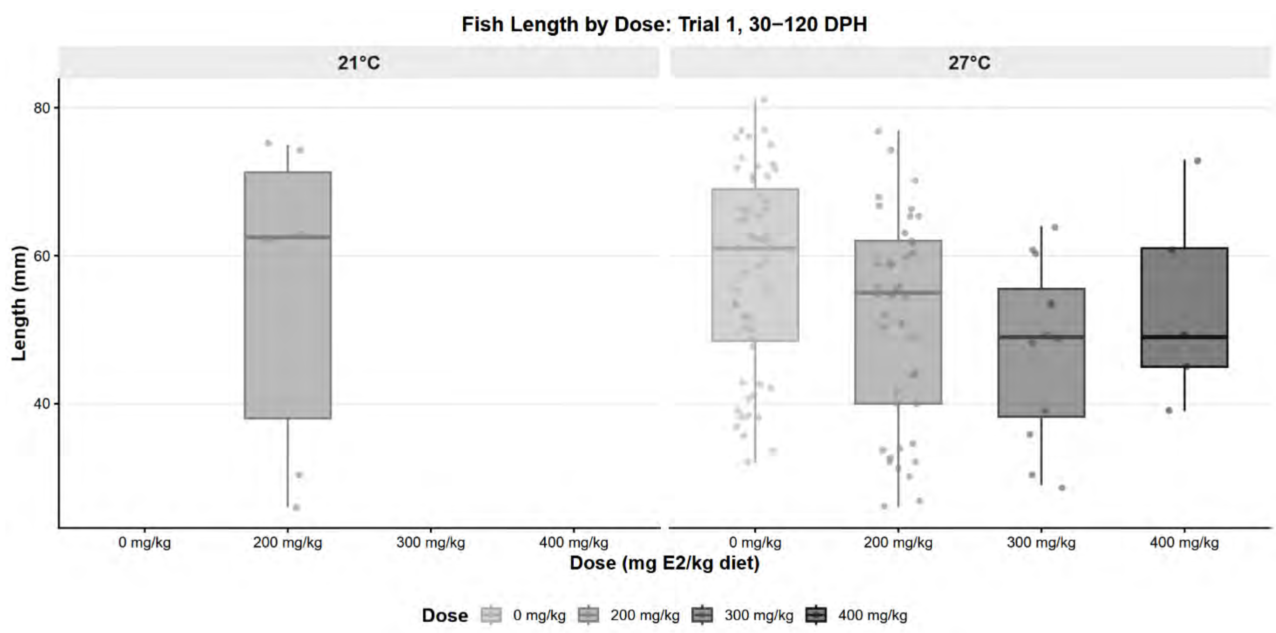

Figure 1. Lengths of Trial 1 groups treated with dietary E_2 from 30-120 days post-hatch at 120 DPH. Fish maintained at 21 ̊C are shown on the left, and fish at 27 ̊C on the right. For fish maintained at 21 ̊C, no lengths were recorded for 0 mg/kg, 300 mg/kg, or 400 mg/kg groups due to 100% mortality.

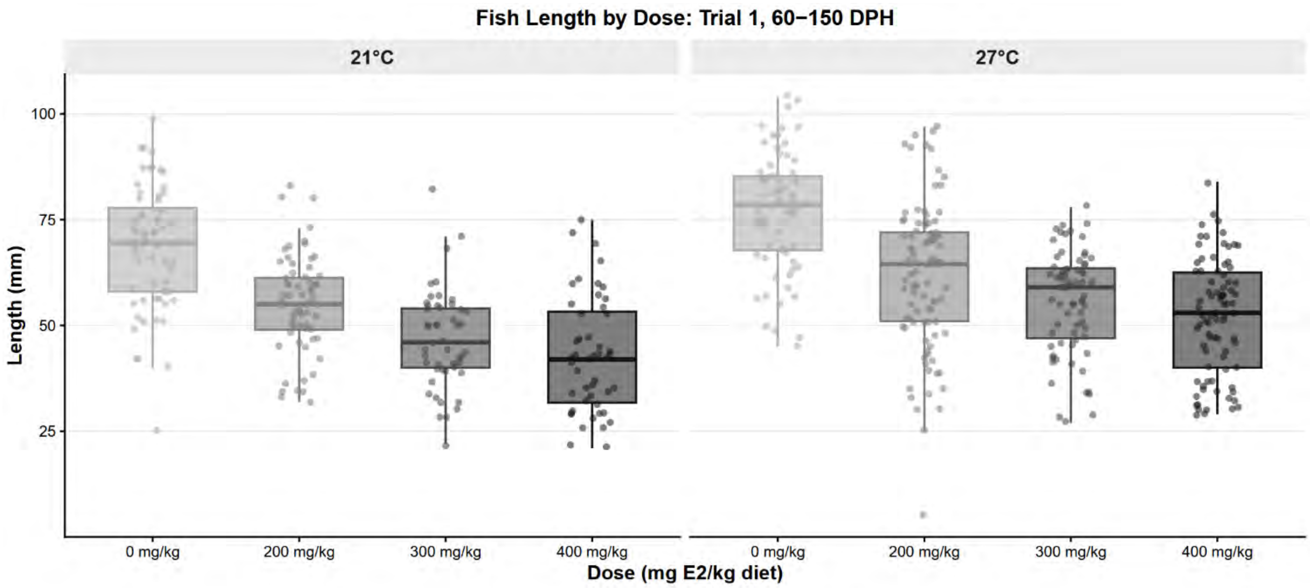

Figure 2. Lengths of Trial 1 groups treated with dietary E_2 from 60-150 days post-hatch at 150 DPH. Fish maintained at 21 ̊C are shown on the left, and fish at 27 ̊C on the right.

Additionally, three of four E_2-treated fish submitted had proteinaceous coelomic fluid present which could represent a fixation artifact or coelomic effusion. The 400 mg/kg fish submitted also had tubular ectasia and renal tubular necrosis present. Further evaluation of histopathologic changes is ongoing, including quantification of renal hypertrophy, tubular and glomerular dilation, and the presence or absence of tubular epithelial necrosis.

Trial 1 Tissue E_2 Residues

Immediately at the end of treatment, there was a significant increase in tissue E_2 levels with E_2 dose ($F_{3, 74} = 7.76$, $p < 0.001$). Fish treated at 60-150 DPH had significantly lower tissue E_2 levels than fish treated at 30-120 DPH ($F_{1, 74} = 5.96$, $p = 0.017$). No effect of temperature on tissue E_2 concentration was observed.

At one week following treatment, fish receiving 400 mg E_2/kg diet had a significantly elevated tissue E_2 concentration compared to controls ($p = 0.003$). Fish in the 300 mg/kg and 200 mg/kg groups did not have significantly different tissue E_2 concentrations from control fish. Temperature and treatment duration had no detectable effect on tissue E_2 concentration at this time.

Trial 2 Survival

Survival was generally high across all treatment groups with 37/44 tanks having greater than 90% survival (see Table 3). The 30-120 DPH, 20-21 ̊C, 400 mg/kg fish had the lowest survival of all treatments (mean survival 73.8%), which was lower than any other treatment groups (all $p < 0.047$). A primary driver of mortality in this group was clinical signs consistent with a gram-negative bacterial infection (hemorrhage of skin, fins, and internal organs), though no PCR validation was conducted to verify this diagnosis.

Treatment	Duration (dph)	Temp (*C)	Mean % Survival at end of treatment
Control	30-120	20-21	97.5
Control	30-120	26-27	97.5
Control	60-150	20-21	91.8
Control	60-150	26-27	100
200mg/kg	30-120	20-21	90.7
200mg/kg	30-120	26-27	95.2
200mg/kg	60-150	20-21	95.8
200mg/kg	60-150	26-27	98.3
300mg/kg	30-120	20-21	93.5
300mg/kg	30-120	26-27	94.5
300mg/kg	60-150	20-21	98.9
300mg/kg	60-150	26-27	96.7
400mg/kg	30-120	20-21	73.8
400mg/kg	30-120	26-27	95.6
400mg/kg	60-150	20-21	93.9
400mg/kg	60-150	26-27	97.8

Table 3. Summary of Trial 2 survival.

Trial 2 Growth

E_2 dose ($F_{3,212} = 107.7$, $p < 0.001$) and decreased temperature ($F_{1,212} = 5.15$, $p = 0.024$) both significantly decreased lengths and weights. At the end of treatment, all E_2 treatments resulted in reduced growth compared to the control groups, irrespective of rearing temperature (Figure 3, Figure 4). In addition, E_2-treated groups at lower temperatures were smaller than E_2 treated groups at higher temperatures. However, E_2-treated groups reared under the same temperature regimens did not exhibit difference in growth.

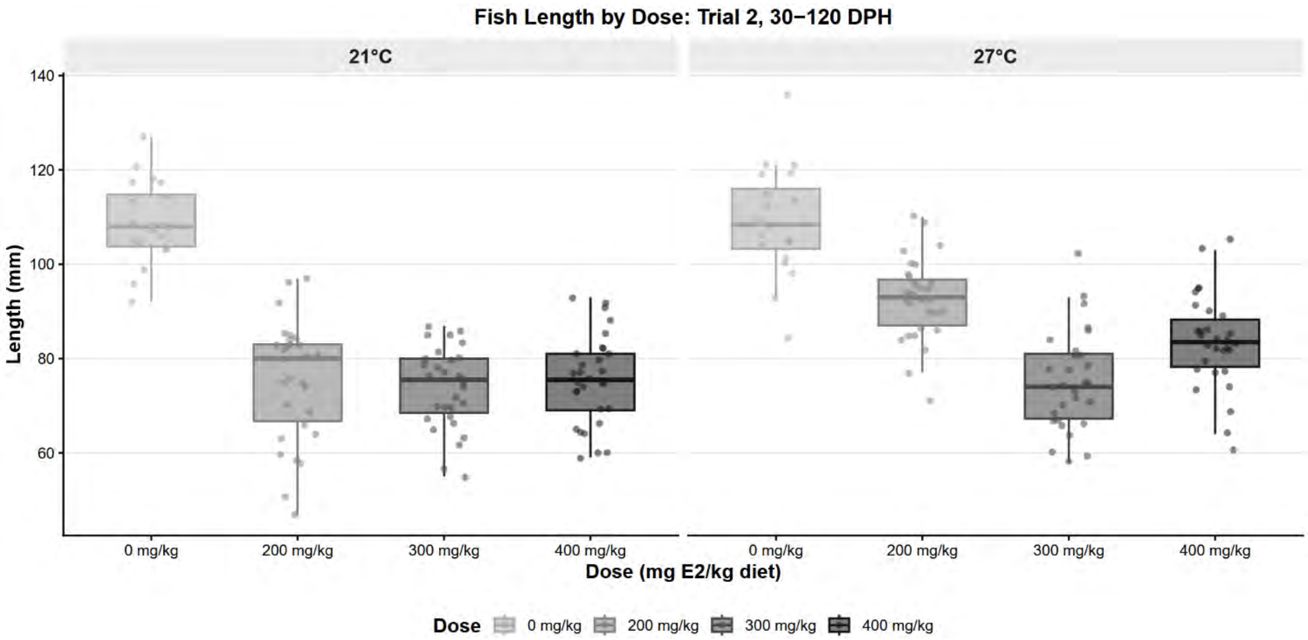

Figure 3. Length of Trial 2 fish treated from 30-120 DPH by dose at 120 DPH. Fish maintained at 21 ̊C are shown on the left, and fish at 27 ̊C on the right.

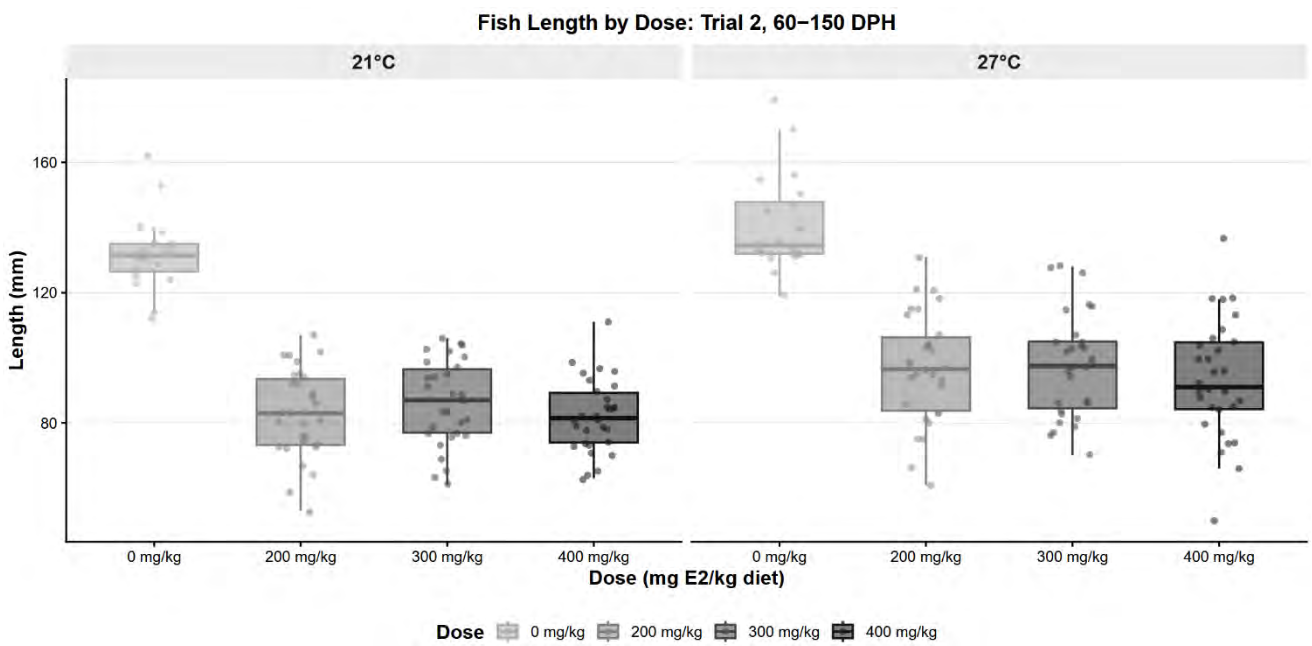

Figure 4. Length of Trial 2 fish treated from 60-150 DPH by dose at 150 DPH. Fish maintained at 21 ̊C are shown on the left, and fish at 27 ̊C on the right.

Trial 2 Pathology

Fish were not submitted for histology for Trial 2. Grossly, fewer deformities to the spine, mouth, and opercles were noted than with Trial 1 although occasional fish with lordosis, kyphosis, or deviated mouths were noted. Fish in the 20-21 ̊C treatment groups were again noted to have soft, distended coelomic cavities suspected due to renal hypertrophy.

Trial 2 Immersion

E_2-exposed fish had significantly lower lengths and weights than fish exposed to ethanol alone ($F_{1, 58} = 52.075$, $p < 0.001$). There was no significant difference in survival between E_2- and ethanol-exposed fish ($p = 0.378$).

Dose (µg/L)	Mean Weight (g)	Mean Length (mm)	Mean % survival
0 (control)	36.31	139	94.4
10	22.52	120	97.3

Table 4. Summary of immersion fish growth and survival.

Next Steps

Trial 2

Sex ratio and GSI data will be collected for Trial 2 fish at eight months post hatch (January 2026). From this data, we will determine if additional trials are needed for YY fish development.

Pathology quantification

Analysis of histology slides from Trial 1 is underway as described above.

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Common Carp Sex Markers

Development of a genetic sex marker for common carp began in 2014 at the Idaho Department of Fish and Game's Eagle Fish Genetics Laboratory (EFGL) using Restriction-site Associated DNA sequencing (RAD-seq) involving a large number of samples from two Idaho common carp populations. By FY2019, EFGL staff had identified two candidate bi-allelic single nucleotide polymorphism (SNP) loci. One marker (Cca744444_87) was screened across 800 individuals from ten populations (seven from Idaho and three from the Midwest), yielding an overall genotype–phenotype concordance of 93% (Schill and Mamer 2019). Concordance varied substantially among populations, and several collections were limited by small sample sizes or non-wild origins (e.g., hatchery feeder koi). Staff at the EFGL recommended additional marker discovery using a restriction enzyme that cuts the genome more frequently to increase marker density (K. Coykendall, EFGL, personal communication).

Subsequent funding was secured through the Multi-State Conservation Grant Program (MSCGP), administered by the U.S. Fish and Wildlife Service with strong support from the Western Association of Fish and Wildlife Agencies (WAFWA). This phase intentionally shifted sampling effort eastward to broaden the geographic applicability of the marker and strengthen the case for future federal investment in YY Male common carp. In FY2021, high-quality DNA samples with verified phenotypic sex (target n = 200 per population, 100 of each sex) were collected from five new water bodies in Pennsylvania, Tennessee, Iowa, Washington, and Oklahoma. Analysis of these samples produced encouraging results: mean concordance across the five populations was 93%, exceeding 96% in three populations (Schill et al. 2022; Coykendall and Campbell 2022, Appendix B). Concordance in the remaining two populations was lower (69% and 89%), prompting further refinement.

In FY2023, under continued MSCGP support, EFGL staff developed a new genetic assay (Cca_1506016) and re-analyzed the same five eastern populations. This work achieved a mean concordance of 99% across all samples and across all five populations (Schill et al. 2023; Coykendall and Campbell 2023, Appendix B1).

Funding from MSCGP in FY2023 did not include analysis of the two large, existing Idaho sample sets (Snake River and Lake Lowell). These samples were therefore assayed in FY2024 using the final markers developed the previous year (Coykendall and Campbell 2024, Appendix B1). Combining these Idaho data with the five eastern populations now provides performance metrics for the marker across seven widely distributed U.S. populations.

Results for the Idaho populations demonstrated high overall concordance, with some variation among markers and sexes. For marker Cca744444_87 in the Snake River population, overall concordance was 0.917, although male concordance was lower (0.768). Marker Cca_1506016 achieved perfect (1.0) concordance for both sexes in the Snake River; however, a small number of unexpected YY genotypes were observed and remain unexplained. In Lake Lowell, both markers performed well, with high concordance and no anomalous YY genotypes.

Across all seven populations evaluated with marker Cca744444_87, concordance exceeded 0.995 in five populations. Given its consistently high performance and the absence of unexplained YY genotypes, we recommend Cca744444_87 as the primary assay for future YY Male Common Carp broodstock development. Continued evaluation of both markers is warranted to support applications across the species' broad North American range and to investigate the occasional anomalous genotypes observed with marker 1506016.

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Walleye Studies

Walleye Past Work and Background

Walleye (*Sander vitreus*) were among the original five priority species targeted for YY Male broodstock development by the WAFWA YY Male Consortium. Early efforts focused on identifying a reliable genetic sex marker and optimizing hormonal feminization protocols to enable production of all-male progeny for invasive population control. Initial marker development at the Idaho Department of Fish and Game's Eagle Fish Genetics Laboratory (EFGL) from FY2019 to FY2021 produced no consistent results (Schill and Mamer 2019, 2020, 2021).

Subsequent collaborative work involving EFGL staff (Dr. Katharine Coykendall, EFGL/PSMFC), U.S. researchers (Dr. Peter Euclide, Purdue University, West Lafayette IN; and European partners (Dr. Heiner Kuhl; Leibniz Institute of Freshwater Ecology and Inland Fisheries, Berlin, Germany and Dr. Yann Guiguen; Institute for National Research for Agriculture, Food and Environment (INRAE), Rennes, France), using draft genomes and samples from Consortium sex-reversal trials yielded a significant advance. A genotyping panel applied to 285 fish (45 broodstock and 240 offspring of known phenotypic sex) achieved 95% overall concordance between genotypic and phenotypic sex, confirming that walleye possesses a ZZ/ZW sex determination system (females heterogametic ZW; males homogametic ZZ) rather than the previously assumed XX/XY system (Schill and Mamer 2023; Campbell and Coykendall 2023). Follow-up testing in FY2024 on additional populations (Lake Pend Oreille, Idaho; Buffalo Bill Reservoir, Wyoming; Rathbun Lake, Iowa) produced concordance rates of 0.876–0.930. Female concordance remained consistently high (>0.97), but male concordance was lower (0.84–0.88), suggesting possible temperature-dependent environmental influences on sex differentiation in some populations (Schill and Mamer 2024).

Parallel sex-reversal trials demonstrated that walleye males are highly responsive to dietary estradiol (E_2) feminization. Preliminary protocols (2017–2019) achieved 100% phenotypic females using 15 mg E_2/kg feed for 84 or 100 days (Schill and Mamer 2019). A more robust, replicated trial in 2021 at Garrison Dam National Fish Hatchery (BY2021 cohort) tested multiple doses and durations; optimal treatments (particularly 15 mg/kg for 60–84 days) consistently produced 100% females with negligible intersex individuals, while controls yielded the expected ~50% female ratio (Schill and Mamer 2022).

These trials not only refined feminization recipes but also generated the known-sex samples essential for marker validation. However, the confirmed ZZ/ZW system presents fundamental obstacles to traditional YY Male technology: all fish released for field suppression would require hormone exposure (unlike the untreated YY Brook Trout), raising regulatory, containment, transport, and food-safety concerns. In light of these challenges, along with evidence of potential environmental sex reversal, walleye YY broodstock development was deprioritized (Schill and Mamer 2024). However, State Fish Chiefs and Steering Committee members emphasized that this species remains a priority and expressed a desire to continue exploring potential applications of a sex chromosome manipulation strategy in walleye.

Potential for an All-Female (WW) Biocontrol Line Using Androgen-Induced Masculinization

The ZZ/ZW sex determination system in walleye opens a promising alternative biocontrol pathway, the production of an all-female (WW "superfemale") line, by inverting the Trojan sex chromosome approach to drive female-biased populations toward extinction. This strategy would employ androgen hormones, such as 17α-methyltestosterone (MT) or testosterone, to masculinize genetic females and generate neomales carrying the W chromosome. Existing literature demonstrates that walleye respond to androgen treatments for masculinization: gynogenetic diploid females (produced via UV-irradiated sperm and pressure shock) have been successfully masculinized with dietary MT to yield functional neomales, and preliminary trials have shown effective sex reversal in both directions (Dabrowski et al. 2020; NCRAC 2019). In a ZW system, masculinized ZW neomales produce both Z- and W-bearing sperm. When crossed with normal ZW females, approximately 25% of progeny are expected to be WW superfemales (W sperm × W egg). These WW individuals can then be masculinized with androgens to produce WW neomales that sire only W-bearing sperm.

Stocking such masculinized WW neomales into target populations would result in all-female offspring when they mate with wild ZW females (W sperm × Z egg = ZW female; W sperm × W egg = WW female), with half of the progeny carrying the WW genotype. Repeated stocking would progressively increase the frequency of WW superfemales, leading to increasingly female-biased sex ratios and, ultimately, population collapse through the absence of males, mirroring the demographic mechanism of the YY Male approach but in the opposite direction. This "Trojan W" strategy could be particularly advantageous for walleye because it leverages the species' demonstrated responsiveness to androgens and could be integrated with existing hatchery infrastructure and triploidy techniques developed for aquaculture all-female lines (NCRAC 2019). Although no Consortium trials have yet explored this pathway, the technical foundation exists, and pilot studies to optimize MT protocols, confirm WW viability, and model population outcomes would be logical next steps for adapting walleye biocontrol to its unique sex determination system.

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Northern Pike Studies

Northern Pike Past Work and Background

The sex determination system in northern pike (*Esox lucius*) shows pronounced geographic variation, particularly in North America, as elucidated by genomic analyses. In Eurasian populations (Europe and Asia) and certain Alaskan populations, northern pike exhibit a male-heterogametic XX/XY system. The master sex-determining (MSD) gene is a male-specific duplicate of the anti-Müllerian hormone gene (amhby), which originated from an ancient gene duplication event approximately 65–90 million years ago (Pan et al. 2019; Pan et al. 2021). This duplicate resides in a small (~140 kb) male-specific insertion on the Y chromosome (Linkage Group 24, sub-telomeric region), resulting in limited overall differentiation between the X and Y chromosomes despite the long-term sex linkage of the MSD gene (Pan et al. 2019).

In contrast, most continental North American populations (across Canada and the contiguous United States, excluding Alaska) have lost the ancestral MSD gene amhby and the associated Y-specific genomic region. Whole-genome resequencing studies reveal no detectable genome-wide sex-associated signals in these populations, suggesting either a recent loss of the Y chromosome and the ancestral sex determination mechanism or a shift to an alternative, possibly undetected genetic or environmental trigger (Pan et al. 2021; Johnson et al. 2024), potentially indicative of an Environmental Sex Determination (ESD) system.

This loss is consistent with post-glacial recolonization patterns from Alaskan refugia, where amhby persists in some instances, and is attributed to founder effects and bottlenecks during eastward expansion (Pan et al. 2021; Johnson et al. 2024). Phenotypically, the system remains male-heterogametic in most cases, but the absence of the conserved genetic trigger in continental populations complicates genetic-based approaches.

Sex marker development for northern pike in support of the YY Male Consortium, has been led by the Alaska Department of Fish and Game (ADFG). Efforts prioritize populations retaining amhby (e.g., Alaskan natives), as this enables reliable XX/XY-based markers critical for broodstock development and YY Male production.

Initial investigations (pre-Consortium and FY2020) employed restriction site-associated DNA sequencing (RADseq) to identify sex-associated regions and construct genome scaffolds. By FY2022, ADFG's Gene Conservation Lab developed 12 single nucleotide polymorphism (SNP) markers from RADseq data derived from invasive Southcentral Alaska samples (e.g., Tote Road Lakes, Threemile Lake, Alexander Lake). Five markers exhibited strong sex association within one genomic segment, with others showing weaker correlations. These markers were validated on approximately 1,000 individuals of known sex from both native and invasive populations to evaluate accuracy and transferability (Schill and Mamer 2022; Schill et al. 2023).

Consortium progress reports (FY2022–FY2023) document ongoing refinement of these markers, but challenges arise from the absence of amhby in lower 48 states' populations, rendering northern pike a limited candidate for broad YY Male application in those regions. In Alaskan contexts where amhby is present, markers demonstrate promise, however, unstable or alternative sex determination mechanisms elsewhere may constrain widespread utility. No fully validated, universally effective genetic sex marker has been reported as operational for achieving Consortium broodstock objectives, with efforts noted as deprioritized or constrained by these genetic complexities.

Northern Pike Samples and 2025 Testing

Fin clip and tissue samples from northern pike for sex marker development were collected from wild populations in three lower-48 states (Iowa, Arizona, and Washington) during FY2019–2020 and forwarded to the Idaho Department of Fish and Game's Eagle Fish Genetics Laboratory (EFGL) for analysis (Schill and Mamer 2020). In Iowa, 186 samples were obtained from Big Spirit Lake in April 2019. In Arizona, 87 samples were collected from Rainbow Lake in May 2019. In Washington, 107 samples were taken from Roosevelt Lake (also known as Lake Roosevelt) in January 2020.

These collections, coordinated by Consortium staff in partnership with state agency personnel, provided known-sex phenotypic data paired with fin clips from mature adults of wild origin. The samples were necropsied, visually sexed based on gonadal morphology, and preserved on Whatman filter paper (or in coin envelopes) for later DNA extraction. In July 2023, additional tissue samples for northern pike sex marker development were collected from two Colorado reservoirs and forwarded to the EFGL. At Catamount Reservoir (near Steamboat Springs), Colorado Parks and Wildlife staff collected and held mature fish in spring 2023, from which Consortium personnel obtained fin clips from 100 males and 100 females (n = 200 total). At Wolford Reservoir (near Hot Sulphur Springs), samples were collected from 61 males and 26 females (n = 87 total).

Revelations about the loss of amhby and the associated Y-specific genomic region in lower-48 populations of northern pike resulted in work on these samples being put on hold. However, after discussions with personnel at ADFG's Gene Conservation Lab in 2025, they agreed to run the Colorado samples against their marker set to assess whether these populations would have any concordance with the Alaska marker set.

A critical first step in implementing a Trojan YY program in northern pike is the development of reliable genetic markers to distinguish sex. Of the 12 candidate markers validated using Alaska northern pike with known phenotypic sex, the four best-performing markers achieved sex-prediction accuracies of 88–91% in Alaska populations. All 12 markers were run against the Colorado samples, and no consistent genotype–phenotype association was observed for any marker.

Results were conclusive in that the markers identified for the Alaska populations were completely non-predictive for the Colorado populations. Subsequent conversations with both the ADFG staff and other collaborators from Texas Christian University (Dr. Matt Hale) and the University of Sydney (Mathew "Mac' Campbell) suggest that a whole-genome analysis of the samples, along with other lower-48 samples, may be useful for identifying markers predictive for sex in these populations. Previous whole-genome sequencing has not been successful in this regard (Johnson et al. 2024), although this step may be useful for establishing comprehensive genomic baselines for these lower-48 populations relevant to management. Further discussions are in progress regarding this approach.

Potential Pathway to YY Northern Pike Broodstock

While the unconventional, non-uniform, sex determination systems and lack of universal sex markers for northern pike are likely difficult to overcome and would complicate development of a Trojan sex chromosome-based broodstock, other possible alternatives have not been completely ruled out. One possibility is that if the sex determination of populations with amhby and the associated Y-specific genomic region can override the apparent ESD of lower-48 northern pike populations, a broodstock created from populations retaining the amhby gene would be functional in populations with ESD systems. This would require crossing of male northern pike from Alaska populations that retain amhby, and conducting genetic tests on offspring to determine whether males from those crosses consistently retain the gene.

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Lake Trout Studies

Potential Pathway to YY Lake Trout Broodstock

The topic of lake trout (*Salvelinus namaycush*) as a Trojan Male candidate has been called into question (see Appendix A), due to the long maturation time of this species, and inconclusive results of feminization trials. Alternative methods for creating YY lake trout may need to be pursued in order for a broodstock of this species to be realistically created. One alternative may be the use of a “surrogate broodstock” approach. Surrogate broodstock technology, based on germ cell transplantation (GCT), provides an efficient means to accelerate development of a YY Male broodstock for lake trout by overcoming its extended maturation period (typically 5–10+ years) and large body size.

Brook trout are a closely related species in the genus Salvelinus, and could therefore serve as the surrogate recipient. Triploid female brook trout are produced by applying pressure or heat shock to suppress the second polar body shortly after fertilization, thereby rendering the recipients functionally sterile while preserving normal ovarian somatic development (Okutsu et al. 2007). Spermatogonial stem cells isolated from the testes of normal (non-sex-reversed) XY male lake trout donors are microinjected into the peritoneal cavity of newly hatched or larval triploid brook trout recipients. In the ovarian somatic environment of the female surrogate, these donor XY germ cells undergo oogenesis rather than spermatogenesis, colonizing the gonads and producing functional lake trout-derived eggs that carry either the X or Y chromosome in approximately equal proportions (Okutsu et al. 2006; Yoshizaki and Yazawa 2019). These surrogate-derived eggs are then fertilized with sperm from normal XY male lake trout, resulting in progeny in which approximately 25% are expected to be YY supermales (Y-bearing egg × Y-bearing sperm), 50% XY males, and 25% XX females. Once reliable genetic sex markers for lake trout become available, YY individuals can be identified and reared to establish a self-sustaining broodstock. This allogeneic approach dramatically shortens the generational timeline required for direct lake trout YY development, leverages existing brook trout infrastructure and expertise within the Consortium, and takes advantage of the well-documented plasticity of germ cell sexual differentiation in salmonids, wherein donor germ cells adopt the gametogenic pathway dictated by the recipient’s gonadal sex (Hattori et al. 2019; Okutsu et al. 2007).

Discussions with collaborators from Japan, including Dr. Yasunori Yamashita of the Japan Fisheries Research and Education Agency, are in progress, with the intent of pursuing this approach for lake trout broodstock development. Other Japanese collaborators have been involved in the YY brook trout projects described the “Expansion of YY Brook Trout Evaluations” portion of this report, including Dr. Yoichiro Kanno, at Colorado State University and Dr. Kaori Kochi, a visiting faculty member at Colorado State University, from Kindai University in Japan, who have been involved in a related YY brook trout food web study.

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Expansion of YY Brook Trout Evaluations

Investigations into alternative approaches with the use for YY brook trout have been undertaken in Colorado, to evaluate the utility of these fish in circumstances where wild brook trout populations have already been depleted to a large degree through conventional techniques. These are circumstances where YY brook trout stocking could be used to eliminate remaining wild brook trout in situations where final eradication would otherwise be difficult to achieve. Colorado Parks and Wildlife (CPW) staff have been key in these investigations, and enthusiastically initiated projects in these systems. Boyd Wright is CPW's Reclamation biologist, who has been a key staff member in these projects. Jon Ewert is the lead biologist and Kevin Rogers is the lead researcher on the Bobtail and Steelman Creek projects. Estevan Vigil is the lead biologist on the Rito Hondo project. Matt Campbell and the Eagle Fish Genetics Lab staff were critical to the YY brook trout genotyping for these projects.

Bobtail and Steelman Creeks

Introduction

The successful eradication of brook trout (*Salvelinus fontinalis*) from Bear and Willow creeks in Idaho in 2024 and 2025 has led to increased interest among other states in applying this approach in additional waters. With the establishment of a YY brook trout broodstock in Colorado, two sister streams, Bobtail and Steelman creeks, were identified as prime locations to further evaluate the approach in smaller systems containing valuable populations of native Colorado River cutthroat trout (*Oncorhynchus clarkii pleuriticus*), which had been invaded by brook trout. The use of YY brook trout provided an alternative management approach to the previously planned pesticide treatment, intended to remove brook trout and conserve remaining cutthroat trout populations. This project also provided an opportunity to evaluate the use of YY brook trout in systems with a history of intensive manual brook trout removal efforts and to assess the effectiveness of higher stocking-to-removal rates compared with those used in previous studies.

Study Area

Bobtail and Steelman creeks are tributary streams of the Williams Fork River in the Colorado River drainage of Colorado. Bobtail Creek includes 3.58 km (2.22 miles), and Steelman Creek includes 2.72 km (1.69 miles) of habitat occupied by the Uncompahgre lineage of Colorado River cutthroat trout (CRCT) and brook trout. Invasive nonnative brook trout were first recorded in these important conservation populations in 1981. Sporadic removal efforts in the late 1990s and early 2000s provided some relief, but by 2011, wild brook trout greatly outnumbered native cutthroat trout in both Bobtail (Figure 6) and Steelman (Figure 7) creeks. This led to the need for a more consistent, long-term removal effort to prevent further population declines of native cutthroat trout.

Subsequent annual removals have proven very beneficial in maintaining favorable ratios of native to nonnative trout. For example, in the most recent suppression effort, cutthroat trout outnumbered to brook trout 5:1 in Bobtail Creek, and 12:1 in Steelman Creek. These streams both include diversion structures operated by Denver Water that have historically served to limit brook trout immigration into the study reaches, but allowed some fish to pass during normal sluicing operations to clear sediment. Denver Water built a permanent barrier just downstream of their diversion structure in Bobtail Creek during the summer of 2024, and another in Steelman Creek in 2025. These barriers will enable Denver Water to operate their diversion structures as needed, without considerations for limiting upstream movement of nonnative trout.

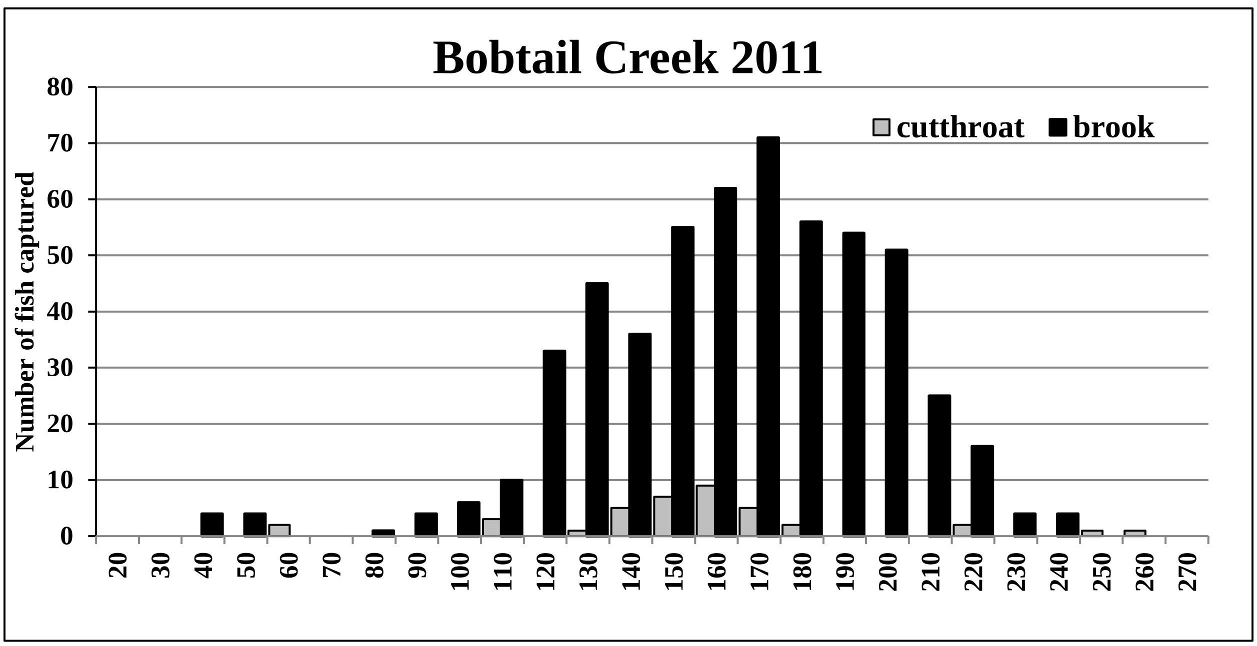

Figure 6. Historic Bobtail Creek brook and cutthroat trout length-frequencies.

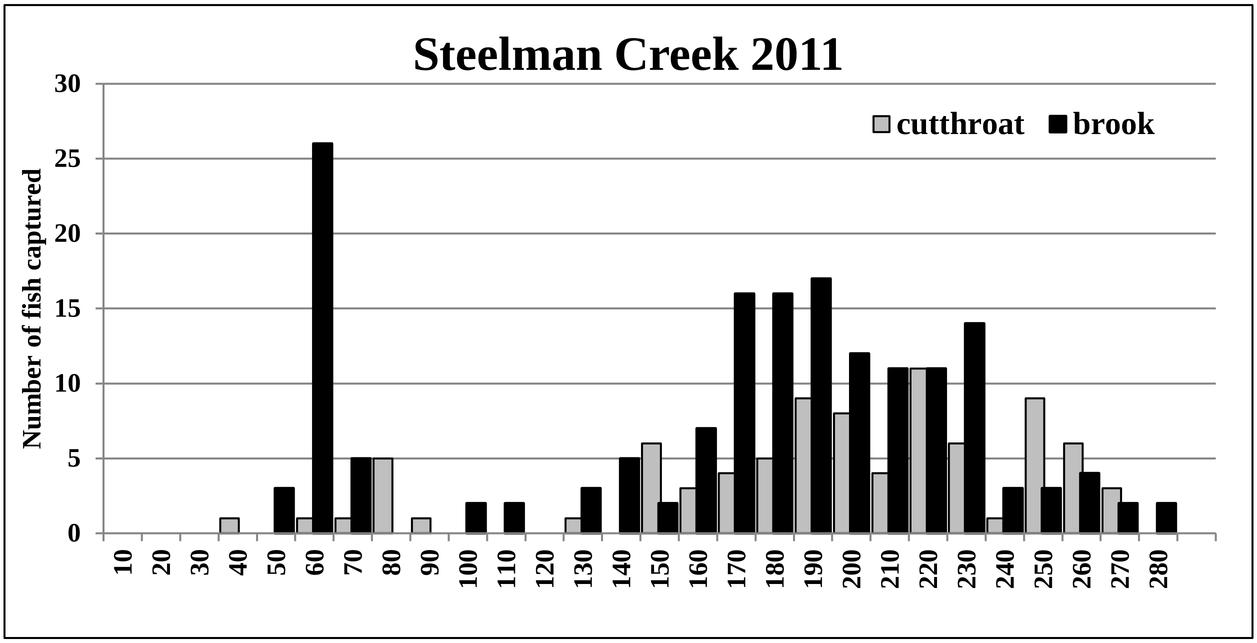

Figure 7. Historic Steelman Creek brook and cutthroat trout length-frequencies.

Methods

YY male brook trout stocking densities were determined from the most recent 5-year mean total population estimate for each stream. Standing crops of brook trout were calculated from the single pass removal data for each year spanning the entire occupied reaches, excluding YOY brook trout (determined from length-frequency plots) that had not yet recruited to the population (Young et al. 2005; Zeigler et al. 2019). These values were inflated to account for the stream-specific first pass capture probabilities calculated from extensive multi-pass removal surveys on the same streams two decades prior ($p = 0.70$ for Bobtail Creek and 0.88 for Steelman Creek). Using this approach, total brook trout population size in Bobtail Creek was estimated to be 343 fish (95% CI ± 59) occupying 3.58 km upstream of the Denver Water diversion structure that serves as a barrier to additional brook trout immigration. Annual suppression efforts in Steelman Creek have been more successful, with an average standing crop of age-1 or older brook trout over the last five years estimated at 38 fish (95% CI± 7) occupying 2.72 km of stream habitat above the diversion barrier to immigration.

2024 Sampling and Stocking

In 2024, backpack electrofishing was used to conduct intensive single-pass removals of wild brook trout from the entire length of both Bobtail and Steelman Creeks, starting at the downstream barriers at the lower end of the study reaches. In Bobtail Creek, an impassable waterfall 3.6 km upstream of the Denver Water diversion prevents any further upstream migration and is therefore fishless upstream. In Steelman

Creek, removals were conducted up to a steep cascade section far above where brook trout were last detected, 2.7 km upstream from the water diversion. Each of the target streams were divided into four sections, numbered 1-4 from downstream to upstream. These sections were sized to require roughly equal effort to complete brook trout removals across all sections. Crews consisting of two backpack electrofishers/netters, two additional netters, and one or two fish handlers were deployed to each of the four sections. In addition to the single-pass removals, a 100 m site located at the center-point within each section was designated for three-pass removals to obtain an estimate of capture probability and total population size (Seber and Whale 1970), to estimate total population size and removal efficiency for each of the sections. All size classes, including YOY fish, were used in the reported estimates. Because of low brook trout numbers in many of the sections, brook and cutthroat trout numbers were also pooled in the three-pass removal estimates to obtain total population estimates and capture probabilities.

Population estimates for each stream section were derived by applying the capture probability ($\hat{p}$) obtained from the three-pass depletion estimator conducted in the 100-m representative station to the single-pass catch across the entire section length:

$$\hat{N}_j = C_j / \hat{p}_j \text{ where}$$

C_j = number of fish captured during the single pass of section j

$\hat{p}_j$ = estimated capture probability for section j (from the three-pass removal estimator)

The total stream population estimate is the sum of the four section estimates:

$$\hat{N}\text{ total} = \Sigma_{j=1}^{4} \hat{N}_j$$

Variance of individual section estimates (Delta method approximation)

$$\hat{Var}(\hat{N}_j) \approx (C_j / \hat{p}_j^2)^2 \times \hat{Var}(\hat{p}_j)$$

Standard error:

$$SE(\hat{N}_j) = \sqrt{}\hat{Var}(\hat{N}_j)$$

Approximate 95% confidence interval (normal approximation):

$$\hat{N}_j \pm 1.96 \times SE(\hat{N}_j)$$

Because the four sections are sampled independently, variance of the total stream estimate:

$$\hat{Var}(\hat{N}\text{ total}) = \Sigma_{j=1}^{4} \hat{Var}(\hat{N}_j)$$

Schill et al (2017) suggested that wild brook trout removal rates of 50%, followed by stocking rates of 50% (removal to replacement rates of 1:1), could potentially result in wild brook trout extirpation in as few as four years. Previous removal efforts in Bobtail and Steelman creeks had reduced the number of wild brook trout to levels much lower than total carrying capacity, which allowed for a more flexible stocking rate without concerns for overstocking. The very low standing crop of wild brook trout in Steelman Creek provided an opportunity to stock equal numbers of YY brook trout into each of the two streams, and evaluate Bobtail Creek with a 1:1 removal to replacement rate, and Steelman Creek with a much higher rate (closer to 1:5).

A total of 240 YY brook trout were stocked into each stream. The stocked fish were spread out as much as possible, to account for potential lack of dispersal as observed by Miller (2024). The stocking concentration was designed to reflect longitudinal density of removed brook trout. All stocked YY brook trout was PIT (Passive Integrated Transponder) tagged, and each tag was tracked to a specific stocking location. Submersible PIT tag arrays were deployed at the downstream terminus of each stream for one-month post-stocking. Fin clips were taken from 40 brook trout in Bobtail Creek, and 25 brook trout in Steelman Creek, all positively identified by phenotypic sex, to serve as a baseline for comparison to sex marker panels genotyped by the Eagle Fish Genetics Lab (EFGL). These sex marker panels are run in a larger GT-seq SNP panel that will allow genetic sex determination of any offspring produced in these streams and to determine whether offspring were produced from stocked YY brook trout.

2025 Sampling

In 2025, similar removal efforts were undertaken, involving intensive single-pass removals conducted over the entire length of both streams. Four crews (two backpack electrofishing units each) conducted removals on each of four separate adjacent reaches. Population estimates were made using the same capture probabilities as in the 2024 sampling for each stream section and for the entire stream.

In addition to the wild brook trout removals, all YOY brook trout were collected and tissues preserved on Whatman sheets for genetic screening. Stocking was conducted with the same numbers and methods as in 2024, with the 240 fish stocked into each of the two streams distributed in equal ratios as the wild brook trout removed by stream segment.

Results

2024 Sampling

In 2024, 216 wild brook trout and 904 cutthroat trout were found in Bobtail Creek (Figure 8). Capture probabilities as determined by the three-pass removal estimates were 0.57, 0.77, 0.93, and 0.87 for stream reaches 1, 2, 3, and 4, respectively. This resulted in wild brook trout population estimates of 10.6 (95% CI = 6.5-14.7), 28.6 (95% CI = 23.4-33.8), 50.5 (95% CI = 45.4-55.7) and 161.4 (95% CI = 142.5-180.4). This also resulted in cutthroat trout population estimates of 357.0 (95% CI = 220.3-493.8), 413.6 (95% CI = 338.5-488.7), 250.5 (95% CI = 225.0-276.0), and 172.9 (95% CI = 152.6-193.2) for each of the four sections, respectively. Overall population estimates for the full stream were 251 (95% CI = 230.5-271.9) for wild brook trout and 1,194 (95% CI = 1,034.7-1,353.4) for cutthroat trout.

In Steelman Creek, 49 brook trout and 523 cutthroat trout were found during the sampling (Figure 9). Capture probabilities as determined by the three-pass removals were 0.91, 0.87, 0.82, and 0.72 for stream reaches 1, 2, 3, and 4, respectively. This resulted in brook trout population estimates of 3.3 (95% CI = 2.9-3.8), 13.7 (95% CI = 11.8-15.6), 31.8 (95% CI = 27.3-36.3), and 11.0 (95% CI = 7.7-14.3). These resulted in cutthroat trout population estimates of 87.1 (95% CI = 75.1-99.1), 138.4 (95% CI = 119.5-157.3), 232.3 (95% CI = 199.5-265.1), and 183.5 (95% CI = 128.8-238.2). Overall population estimates were 60 (95% CI = 54.0-65.8) wild brook trout and 641 (95% CI = 573.7-708.9) cutthroat trout.

All wild brook trout were removed during the surveys. In Bobtail Creek, the estimate of 252 brook trout minus the 216 wild brook trout removed, resulted in a remaining estimate of 36 (95% CI of 15-56) total brook trout remaining in the stream. The number removed closely adhered to the 1:1 removal-to-replacement rate set out at the beginning of the experiment for this creek. In Steelman Creek, with an estimate of 60 brook trout, and with 49 removed, this resulted in a remaining estimate of 11 (95% CI = 5-17) wild brook trout. This resulted in a close to a 5:1 replacement rate for Steelman Creek.

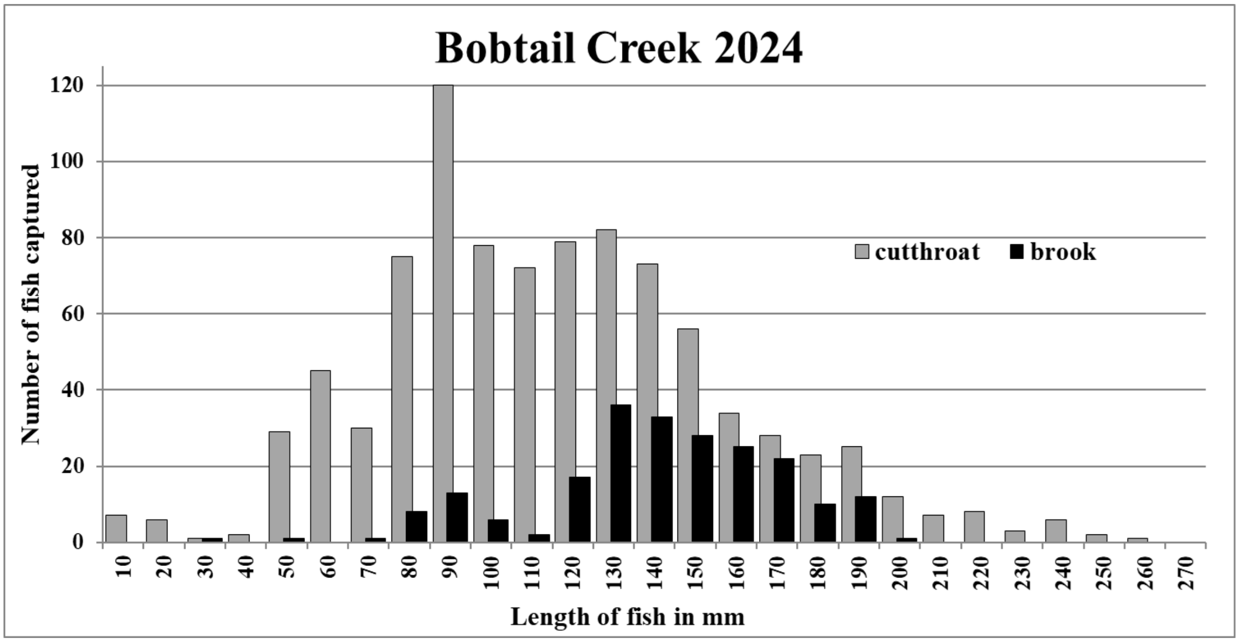

Figure 8. 2024 Bobtail Creek wild brook and cutthroat trout length-frequencies.

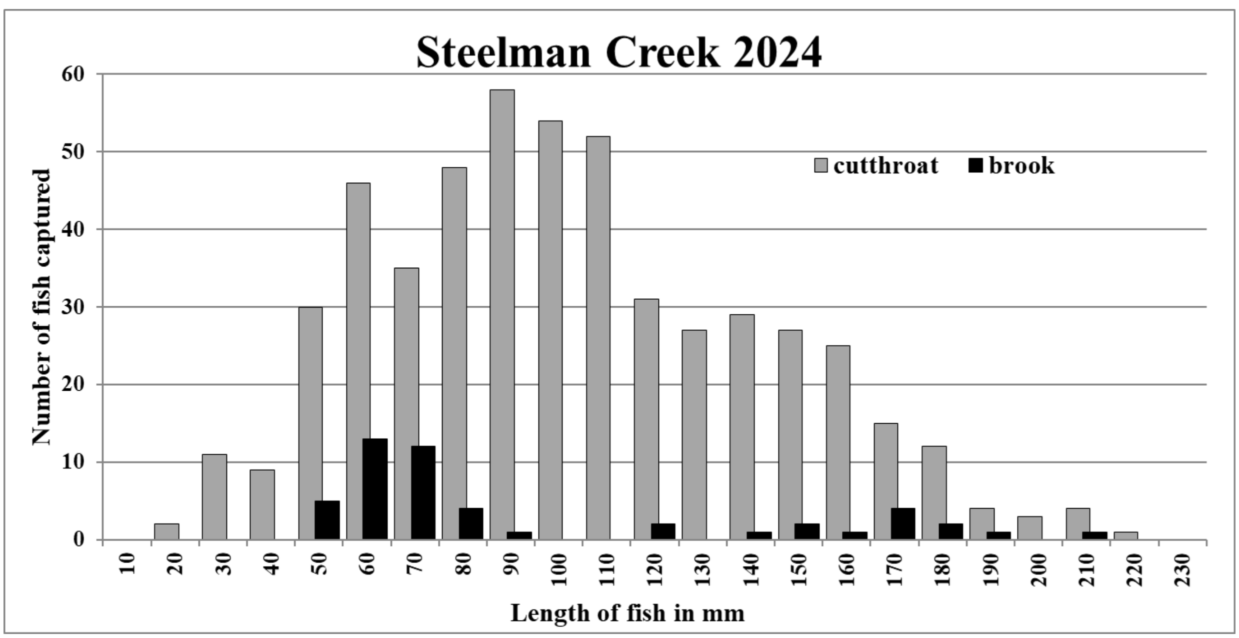

Figure 9. 2024 Steelman Creek wild brook and cutthroat trout length-frequencies.

2025 Sampling

In 2025, 819 cutthroat trout were captured in Bobtail Creek, and only 105 wild brook trout were captured (Figure 10). Survival of stocked YY brook trout was good, with 98 being captured during the survey. Capture probabilities for each of the four stream sections in Bobtail Creek from 2024 were applied to the single-pass removal captures in 2025 to estimate fish numbers per section and overall population size. This resulted in estimates of 126 (95% CI = 115.5-136.4) wild brook trout, 120 (95% CI = 109.5-131.2) YY brook trout, and 1,092 (95% CI = 940.6-1,243.9) cutthroat trout. The wild brook trout remaining in the study area were found primarily in the furthest upstream section, where 57 wild brook trout were removed, resulting in a population estimate of 65 (95% CI = 57.6-72.9) wild brook trout in that section. The lower-middle reach (Reach 2) contained the majority of the YOY fish captured during this effort, with 9 of the 20 total YOY captured in this reach. All 20 YOY brook trout collected and submitted for genotyping were found to be of wild origin. The estimated number of remaining wild brook trout after this year of removals was 21 fish.

In Steelman Creek, 484 cutthroat trout and only 33 wild brook trout were captured (Figure 11). All of the wild brook trout were again removed from the stream. Forty-one YY brook trout were captured, and were returned to the water after capture and processing. Applying the 2024 capture probabilities for each of the four sections to these capture data resulted in population estimates of 580 (95% CI = 527.3-632.8) cutthroat trout, 47 (95% CI = 42.9-52.1) YY brook trout, and 39 (95% CI = 36.0-43.0) wild brook trout. Estimated number of wild brook trout after this year of removals was only six fish (95% CI = 3-10). No brook trout YOY were found at all in this stream, and the length-frequency data demonstrate that this population has shifted primarily to age-1 and age-2 fish. The wild brook trout found in 2025 sampling exceeded the 95% confidence intervals of the estimated remaining brook trout after the 2024 removals (Table 5). This could be due to lower-than-predicted capture probabilities, resulting in an underestimate of total wild brook trout, and is likely driven by lower capture probabilities among YOY fish than age 1+ and larger fish, which were included in these estimates for simplicity at this stage. Further refinement of these estimates and population modeling are in progress.

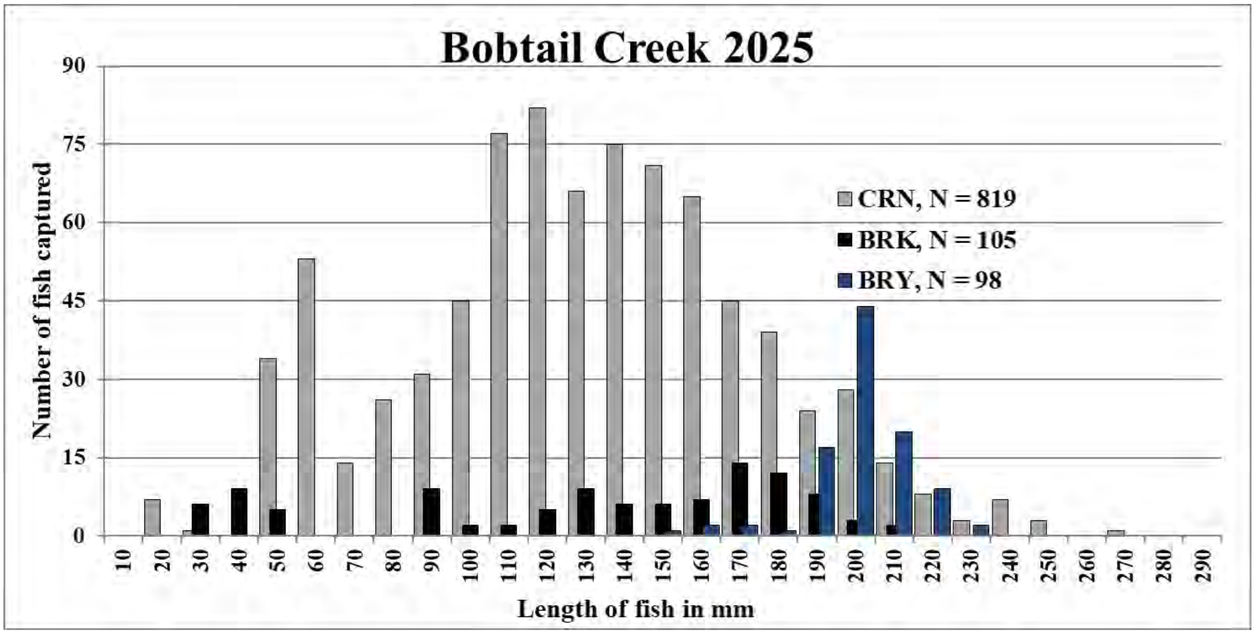

Figure 10. 2025 Bobtail Creek wild cutthroat trout (CRN), wild brook trout (BRK), and stocked YY brook trout (BRY) length-frequencies.

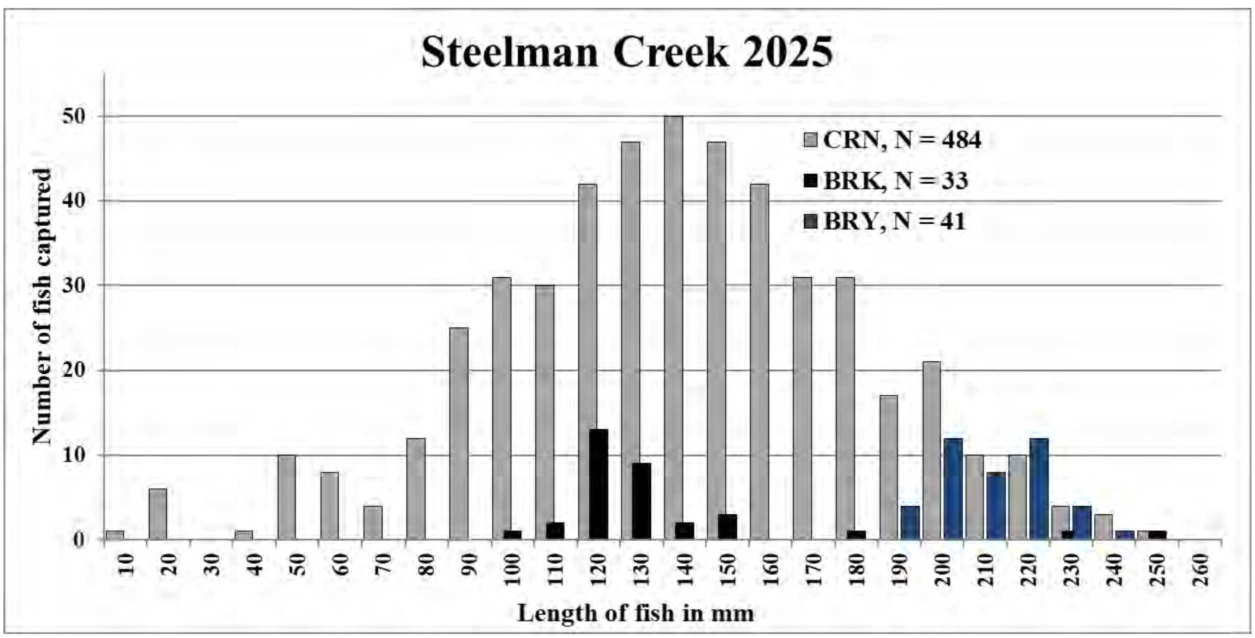

Figure 11. 2025 Steelman Creek wild cutthroat trout (CRN), wild brook trout (BRK), and stocked YY brook trout (BRY) length-frequencies.

	N̂ total (95% CI) Wild Brook Trout	No. Wild Brook Trout Removed	N̂ total (95% CI) Cutthroat Trout	N̂ total (95% CI) YY Brook Trout
Bobtail Creek				
2024	251 (230.5-271.9)	216	1,194 (1,034.7-1,353.4)	-
2025	126 (115.5-136.4)	105	1,092 (940.6 -1,243.9)	120 (109.5-131.2)
Steelman Creek				
2024	60 (54.0-65.8)	49	641 (573.7-708.9)	-
2025	39 (36.0-43.0)	33	580 (527.3-632.8)	47 (42.9-52.1)

Table 5. Total abundance estimates for wild brook trout, cutthroat trout, and YY Brook trout in Bobtail and Steelman creeks in 2024 and 2025, including the number of wild brook trout removed each year.

Discussion

While these results are preliminary, the use of YY brook trout in these streams appears to be on track for success, possibly occurring more rapidly than in other locations where this approach has been deployed, and consistent with modeling results described in Schill et al. (2017). With no observable reproduction in 2025 from the 2024 spawning year in Steelman Creek, it is expected that the wild brook trout population will be extirpated soon. The small number of wild YOY brook trout found in Bobtail Creek is also encouraging. Stocked YY brook trout may have interfered with normal spawning and recruitment of wild brook trout in these streams, occupying preferred habitat and potentially preying on juvenile wild brook trout. While none of the YOY collected in 2025 were offspring of stocked YY brook trout, the fall of 2025 represents the first year in which stocked YY brook trout would have reliably been able to spawn with wild brook trout in these systems. If a new year class of wild brook trout is produced from spawning in the fall 2025, it is expected to consist of a large proportion of offspring sired by stocked YY brook trout.

Rito Hondo Reservoir

Rapid response approach

Other ongoing projects in Colorado are assessing the use of YY brook trout in a different manner than has been implemented in the past, utilizing these fish as a rapid response measure to prevent small numbers of wild brook trout from establishing a viable population. One such project is Rito Hondo Creek and Rito Hondo Reservoir, located in the Rio Grande drainage of Colorado. This is a system where native Rio Grande cutthroat trout (*Oncorhynchus virginalis virginalis*) occur naturally, but have been invaded by non-native brook trout. The dam containing Rito Hondo Reservoir was scheduled for repairs in 2023, which provided an opportunity for a reclamation project in the reservoir basin and the upstream creek. Reclamation projects of this type, using piscicides such as rotenone, are not always 100% effective, and often require a second year of treatment to ensure all fish have been eradicated. In this case, because the reservoir was only drained for one year, a second treatment was not possible. Post-treatment physical sampling with backpack electrofishing units and eDNA testing in 2024 identified several locations in the drainage where wild brook trout survived the initial treatment. A total of three positive eDNA tests were found, as well as two individual wild brook trout. To reduce the risk of re-establishment by any remaining wild brook trout in this drainage, 500 YY brook trout were stocked, concentrated in areas where positive eDNA detections were found. This stocking was concurrent with Rio Grande cutthroat trout fry stocking to re-establish a native cutthroat trout population to this drainage. In addition to this creek stocking, an additional 500 YY brook trout were also stocked into the reservoir to provide additional assurance against wild brook trout establishment, and to provide a standing crop of brook trout for recreational angling and to deter illegal stocking. All YY brook trout were adipose clipped for easy identification in subsequent sampling, and were stocked at a large average size of 182 mm (7.12 inches).

Sampling throughout the drainage in 2025 with backpack electrofishers and gill nets did not reveal any wild brook trout reproduction, or any wild brook trout holdovers from the previous reclamation effort. The stocked YY brook trout exhibited rapid growth, particularly in the lake, and provided a bridge for angling opportunities while the native cutthroat trout population becomes established. This demonstrates how YY brook trout can be used as an effective management tool to reduce the risk of wild brook trout re-establishment.

Sex Marker Analysis for Colorado Brook Trout Populations

The IDFG EFGL genotypes a panel of markers designed to determine the genetic sex of brook trout. A panel of markers is used, because sex markers in brook trout have exhibited variation in phenotypic/genotypic concordance across populations collected from a wide geographic range. For this study, the marker that proved most robust and exhibited highest concordance with known-sex samples was Sfo_74221_58 (Table 6). As such, it was recommended as the best marker to use for the project monitoring moving forward. We observed 56% genetic females in Bobtail Creek (n = 39) and 48% genetic females in Steelman Creek (n= 25) from initial fin clip sampling collected during the first removal effort in 2024. Genetic diversity in the Bobtail Creek sample exhibited 34% heterozygosity, which is within the range seen in other brook trout populations. Steelman Creek samples exhibited slightly lower heterozygosity at 27%, although the sample size was smaller. Simulated F1 offspring between known YY Trojans and sample collections demonstrated that the genetic differentiation was sufficient to establish clear cutoffs for identifying F1 offspring using genetic assignment tests.

Pedigree	Individual Name	Pedigree name	Sfo_74221_58-A1	Sfo_74221_58-A2	Genetic Sex
SfoBOBT24C	SfoBOBT24C_0001	SfoBOBT24C	X	X	Female
SfoBOBT24C	SfoBOBT24C_0002	SfoBOBT24C	X	X	Female
SfoBOBT24C	SfoBOBT24C_0003	SfoBOBT24C	X	X	Female
SfoBOBT24C	SfoBOBT24C_0004	SfoBOBT24C	X	X	Female
SfoBOBT24C	SfoBOBT24C_0005	SfoBOBT24C	X	Y	Male
SfoBOBT24C	SfoBOBT24C_0006	SfoBOBT24C	X	X	Female
SfoBOBT24C	SfoBOBT24C_0007	SfoBOBT24C	X	X	Female
SfoBOBT24C	SfoBOBT24C_0008	SfoBOBT24C	X	X	Female
SfoBOBT24C	SfoBOBT24C_0009	SfoBOBT24C	X	X	Female
SfoBOBT24C	SfoBOBT24C_0010	SfoBOBT24C	X	Y	Male
SfoBOBT24C	SfoBOBT24C_0011	SfoBOBT24C	X	Y	Male
SfoBOBT24C	SfoBOBT24C_0012	SfoBOBT24C	X	Y	Male
SfoBOBT24C	SfoBOBT24C_0013	SfoBOBT24C	X	Y	Male
SfoBOBT24C	SfoBOBT24C_0014	SfoBOBT24C	X	X	Female
SfoBOBT24C	SfoBOBT24C_0015	SfoBOBT24C	X	X	Female
SfoBOBT24C	SfoBOBT24C_0016	SfoBOBT24C	X	Y	Male
SfoBOBT24C	SfoBOBT24C_0017	SfoBOBT24C	X	X	Female
SfoBOBT24C	SfoBOBT24C_0018	SfoBOBT24C	X	Y	Male
SfoBOBT24C	SfoBOBT24C_0019	SfoBOBT24C	X	Y	Male
SfoBOBT24C	SfoBOBT24C_0020	SfoBOBT24C	X	X	Female
SfoBOBT24C	SfoBOBT24C_0021	SfoBOBT24C	X	X	Female
SfoBOBT24C	SfoBOBT24C_0022	SfoBOBT24C	X	Y	Male
SfoBOBT24C	SfoBOBT24C_0023	SfoBOBT24C	X	Y	Male
SfoBOBT24C	SfoBOBT24C_0024	SfoBOBT24C	X	X	Female
SfoBOBT24C	SfoBOBT24C_0025	SfoBOBT24C	X	X	Female

SfoBOBT24C	SfoBOBT24C_0026	SfoBOBT24C	X	X	Female
SfoBOBT24C	SfoBOBT24C_0027	SfoBOBT24C	X	X	Female
SfoBOBT24C	SfoBOBT24C_0028	SfoBOBT24C	X	X	Female
SfoBOBT24C	SfoBOBT24C_0029	SfoBOBT24C	X	Y	Male
SfoBOBT24C	SfoBOBT24C_0030	SfoBOBT24C	X	X	Female
SfoBOBT24C	SfoBOBT24C_0031	SfoBOBT24C	X	Y	Male
SfoBOBT24C	SfoBOBT24C_0032	SfoBOBT24C	X	Y	Male
SfoBOBT24C	SfoBOBT24C_0033	SfoBOBT24C	X	X	Female
SfoBOBT24C	SfoBOBT24C_0034	SfoBOBT24C	X	X	Female
SfoBOBT24C	SfoBOBT24C_0035	SfoBOBT24C	X	Y	Male
SfoBOBT24C	SfoBOBT24C_0036	SfoBOBT24C	X	Y	Male
SfoBOBT24C	SfoBOBT24C_0037	SfoBOBT24C	X	Y	Male
SfoBOBT24C	SfoBOBT24C_0038	SfoBOBT24C	0	0	No Call
SfoBOBT24C	SfoBOBT24C_0039	SfoBOBT24C	X	X	Female
SfoBOBT24C	SfoBOBT24C_0040	SfoBOBT24C	X	Y	Male
SfoSTLM24C	SfoSTLM24C_0001	SfoSTLM24C	X	X	Female
SfoSTLM24C	SfoSTLM24C_0002	SfoSTLM24C	X	X	Female
SfoSTLM24C	SfoSTLM24C_0003	SfoSTLM24C	X	Y	Male
SfoSTLM24C	SfoSTLM24C_0004	SfoSTLM24C	X	Y	Male
SfoSTLM24C	SfoSTLM24C_0005	SfoSTLM24C	X	Y	Male
SfoSTLM24C	SfoSTLM24C_0006	SfoSTLM24C	X	X	Female
SfoSTLM24C	SfoSTLM24C_0007	SfoSTLM24C	X	Y	Male
SfoSTLM24C	SfoSTLM24C_0008	SfoSTLM24C	X	Y	Male
SfoSTLM24C	SfoSTLM24C_0009	SfoSTLM24C	X	X	Female
SfoSTLM24C	SfoSTLM24C_0010	SfoSTLM24C	X	X	Female
SfoSTLM24C	SfoSTLM24C_0011	SfoSTLM24C	X	X	Female
SfoSTLM24C	SfoSTLM24C_0012	SfoSTLM24C	X	Y	Male
SfoSTLM24C	SfoSTLM24C_0013	SfoSTLM24C	X	Y	Male
SfoSTLM24C	SfoSTLM24C_0014	SfoSTLM24C	X	Y	Male
SfoSTLM24C	SfoSTLM24C_0015	SfoSTLM24C	X	X	Female
SfoSTLM24C	SfoSTLM24C_0016	SfoSTLM24C	X	X	Female
SfoSTLM24C	SfoSTLM24C_0017	SfoSTLM24C	X	Y	Male
SfoSTLM24C	SfoSTLM24C_0018	SfoSTLM24C	X	Y	Male
SfoSTLM24C	SfoSTLM24C_0019	SfoSTLM24C	X	X	Female
SfoSTLM24C	SfoSTLM24C_0020	SfoSTLM24C	X	X	Female
SfoSTLM24C	SfoSTLM24C_0021	SfoSTLM24C	X	Y	Male
SfoSTLM24C	SfoSTLM24C_0022	SfoSTLM24C	X	Y	Male
SfoSTLM24C	SfoSTLM24C_0023	SfoSTLM24C	X	Y	Male
SfoSTLM24C	SfoSTLM24C_0024	SfoSTLM24C	X	X	Female
SfoSTLM24C	SfoSTLM24C_0025	SfoSTLM24C	X	X	Female

Table 6. Genotypic Sex classifications for original samples of Bobtail and Steelman Creek Brook Trout.

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YY Brook Trout Broodstock Production

Hayspur Hatchery Egg Production and Pick-off Numbers

YY brook trout egg production at the Hayspur hatchery continues to be robust, exceeding demand each year. In 2024, 508 females were spawned, and 328 females were spawned in 2025. Total egg production at the facility in 2024 was 901,365, not including eggs taken for replacement brood purposes. The production numbers were estimated to be 696,246 in 2025. This number does not include eggs taken for replacement brood purposes, and because of the excess production in 2025, the Hayspur staff did not pick and directly enumerate all eggs. Estimates for eye up rate in 2024 averaged 75% across four spawns (range = 70.2% - 79.0%). In 2025, eye-up was 73.2% in 3-year-olds and 76.5% in 2-year-olds. Pickoff numbers in 2024 averaged 25.0% across four spawns (range = 21.0% - 29.8%). In 2025, pickoff was 26.8% in 3-year-olds and 23.5% in 2-year-olds.

Bellvue Hatchery Egg Production and Pick-off Numbers

Production at the Bellvue Fish Research Hatchery (BFRH) has greatly improved over the past two years. Only 28 YY females were spawned in 2024, and 205 YY females were spawned in 2025. An additional ~29 females were stripped, and the eggs were not retained due to target numbers being reached at that time. Total egg production in 2024 was 65,161, and 303,216 in 2025. The 2025 figure number does not include eggs from 10 additional females that were used in the 2025 outbreeding experiment. Eye-up rate in 2024 was only 17%, but increased in 2025 to a range of 51-68%. Pick-off rates improved as well, with an 83% pickoff rate in 2024, decreasing to a range of 49-32% in 2025.

Maintenance and Improvement of YY Brook Trout Brood Stock

Some concern has arisen over the last several years about deformities found in the YY brook trout broodstock at both the Hayspur Hatchery in Idaho and the BFRH in Colorado. Although the prevalence and severity of deformities are low, it was considered important for the long-term health of the broodstock to take steps to understand and potentially eliminate these deformities. Fish health discussions and evaluation of these deformities, including lateral line issues, were addressed through a multiple-pronged research approach. This included investigations into lateral line pathology, water quality analysis, and genetic diversity in YY brook trout. These efforts involved many collaborators, including those from the Idaho Department of Fish and Game, Colorado State University, Alaska Department of Fish and Game, and the USFWS National Fish Hatchery system.

Lateral Line Dysplasia

One of the notable conditions identified in these broodstocks is Lateral Line Dysplasia, a condition that has a variety of proposed causes, but is not fully understood. Lateral Line Dysplasia occurs across a wide variety of fish species and is not unique to out YY brook trout broodstock. This condition has been reported in many states and Canadian provinces in recent years and has become sufficiently prevalent that a symposium was dedicated to this topic at the Western Division of the American Fisheries Society meeting in 2024. In affected fish, lateral line development is compromised, resulting in branching and tissue malformation along the lateral line. Dr. Paula Schaffer, Colorado State University veterinarian was recruited to help identify the cause of this condition in the YY Brook trout broodstock from a pathological perspective.

Water Quality Testing

One potential cause of Lateral Line Dysplasia is water quality or unknown conditions in water sources. Evidence supporting a potential role of water source includes cases observed in Alaska. Several species of fish reared at the Ruth Burnett Hatchery in Anchorage have exhibited this condition, where brood stock for these species are also held. Eggs produced at the Ruth Burnett Hatchery are also shipped to the Lee Martinez Hatchery in Fairbanks. Fish produced at that facility do not exhibit this condition, despite originating from the same brood stock as the fish reared at the Ruth Burnett Hatchery. Water sample test kits were developed for periodic testing of water quality parameters at hatcheries of interest, including the Hayspur, Idaho Hatchery, the BFRH in Colorado, the Ruth Burnett Hatchery in Anchorage, Alaska, and the Lee Matinez Hatchery in Fairbanks, Alaska. As of this writing, the water quality samples had not yet been analyzed, but collections will continue in 2026, and results will be reported when they become available.

Brook Trout Genetic Introgression Study

Introduction

Another potential source of high egg pick-off, hatch-to swim-up losses, and deformities could be due to genetic bottlenecking of the YY brook trout brood stock. While standard tests of heterozygosity have been conducted on this broodstock (M. Campbell, IDFG, personal communication), many of the deformities in the brood stock are characteristic of hatchery inbreeding, such as shortened opercles and fin deformities. A study was initiated to in order to evaluate whether or not increasing genetic diversity in the population would improve egg and fry survival, and reduce deformities.

Some context related to breeding in the original YY brook trout broodstock was provided by Matt Campbell (as follows). *When screening Trojan YY Brook Trout offspring (SfoHAYS15B) scheduled for spawning in the fall of 2017, they noticed that starting with fish SfoHAYS15B_0788, only one parent (female) was assigned during parentage testing. Fish in the SfoHAYS15B pedigree should have been assigned to two parents in the SfoHAYS15S pedigree. However, within SfoHAYS15S, adult samples 0148 to 0197 originated from Story Fish Hatchery males whose milt was cryopreserved and used to cross with feminized YY females. At the time, IDFG was bringing in new Story Hatchery males because the original adults (used to create the YY population in 2008) consisted of only eight individuals (four males and four females). When they performed a simple Leave-One-Out population assignment test, breaking the SfoHAYS15S pedigree into YY Hayspur parents (SfoHAYS15S) and XY putative male parents from Story Hatchery (SfoHAYS15S_01), they observed significant genetic differentiation (FST = 0.05) and clear separation between the two groups.*

When comparing the SfoHAYS15S_01 group to adults sampled from Story Hatchery in 2018, they saw no differentiation. This suggests that samples SfoHAYS15S_0148-0197 from Story Hatchery broodstock did not contribute to the offspring produced. This finding is indicative of gynogenesis, where all genetic material originates from the female parent (i.e., from the egg). In salmonids, gynogenesis is accomplished by fertilizing eggs with UV-irradiated sperm (Chourrout 1982; Ihssen et al. 1990; Johnstone and Stet 1995). While one might expect all loci to be homozygous, due to the separation of homologous chromosomes (carrying different alleles) during meiosis I and sister chromatids (carrying identical alleles) during meiosis II, some loci (closer to the telomeres) retain heterozygosity, while others (closer to the centromere) become homozygous.

Among the unique families assigned to a Trojan YY female but no male parent, average heterozygosity was ~30%, compared to ~88–96% heterozygosity in the parent populations. During the second year of introducing new genetics via Story Hatchery male milt, they observed the same pattern: offspring produced from feminized Trojan YY females crossed with Story Hatchery male milt were assigned to only the female parent. In the third year, IDFG stopped using cryopreserved Story Hatchery milt to increase the diversity of the YY population. Subsequently, all offspring were appropriately assigned to both parents.

Due to potential current or future inbreeding within this broodstock, we designed an experiment to outcross YY females from the existing YY brook trout broodstock at the Bellvue Fish Research Hatchery with fish from a completely unrelated brook trout broodstock. The objective was to evaluate eye-up and pick-off rates of outcrossed fish as an early indicator of improved viability. A domestic broodstock of brook trout at the Erwin National Fish Hatchery (ENFH), in Erwin, Tennessee, was identified as a favorable candidate for this cross. Disease inspection records and importation permits were obtained, and a trial shipment of milt containing both raw and extended samples was sent from ENFH to BFRH to validate that the shipping methods chosen would result in viable sperm samples for the experiment.

Methods

Milt was produced from 20 individual Sandwich strain male brook trout from ENFH on November 4, 2025. This milt was mixed with an in-house milt extender produced by Dr. Pete Cadmus of the Colorado Research Section Toxicological Lab at a ratio of 1:6 in 496 ml Tupperware containers, and transported to BFRH in a cooler with ice packs. To ensure equal treatment among groups, 20 Myy from the BFRH were also spawned, and their milt was mixed extender and held under refrigeration overnight to mimic transport conditions from ENFH. During the same sampling event, fin clips from 50 male and 50 female Sandwich strain brook trout were collected from the broodstock at ENFH to develop a panel to differentiate XY from YY offspring in the test groups.

On November 5, 2025, ten Fyy brook trout (created from Bellvue spawns in fall of 2023) were dry-spawned, and produced eggs split roughly equally into two groups. One group from each spawn was fertilized with extended milt from two Myy brook trout from the BFRH (created from Bellvue spawns in fall of 2024). The second group from each spawn were fertilized with extended milt from two normal males from ERFH. Each family lot was maintained separately in individually-labeled egg cups. The eggs were water-hardened and disinfected during the 40-minute transport (PVP Iodine, Western Chemical Inc., Ferndale, Washington; 50 ppm at a pH of 7) to the Poudre River Fish Hatchery. The egg cups were placed into Heath Incubators with a flow of 3 gpm at 45 °F. The eggs were fully eyed and first pick-off was conducted on December 15, 2025. Upon hatching, the fry were moved to individual rearing baskets inside of four hatchery troughs, with 2 gpm flow at 45 °F.

Results and Discussion

Eye-up and pickoff rates through December 31, 2025 showed that the outcrossed groups (ENFH) were much lower than the pure YY brook trout (BFRH), with percent pickoff numbers of 39.3% (95% CI = 9.1-38.7) for the ENFH groups versus 62.6 (95% CI = 11.4-48.7) for the BFRH groups (Figure 12). At the time of this writing, this trend was validated, in swim-up fry with much better overall survival in the ENFH families. Updated survival rates wil be provided in the 2026 report with outcomes of this experiment.

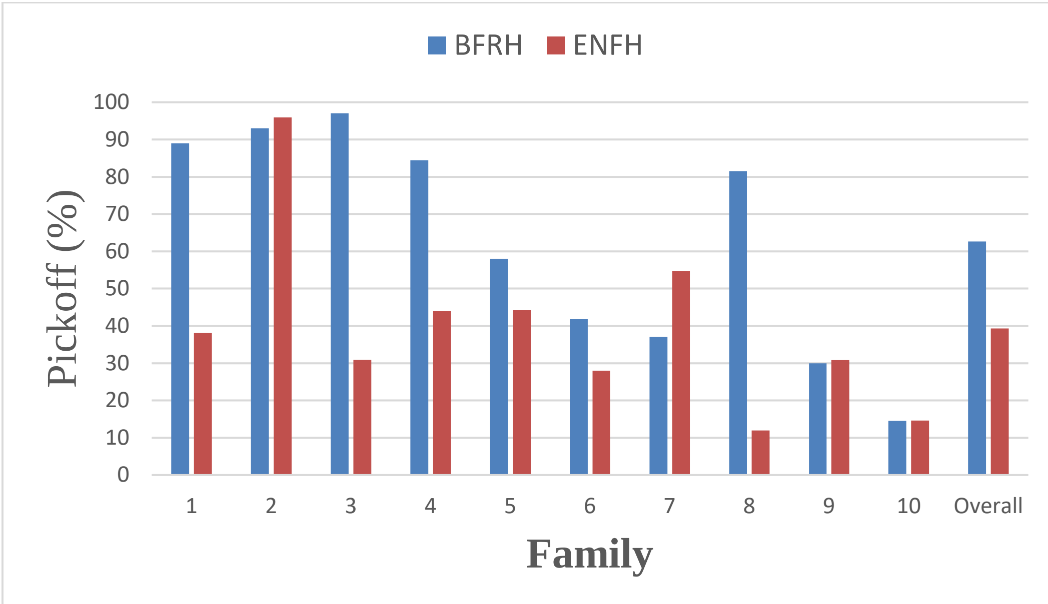

Figure 12. Percent pickoff numbers by family for each of the pure YY (BFRH) and outcrossed (ENFH) brook trout reared at the Poudre Rearing Unit, Colorado, through December 31, 2025

Mosquitofish Studies

Mosquitofish are another species of interest to the member states in the YY Consortium due to the widespread impacts of this species where it has been introduced outside its native range. Both eastern (*Gambusia holbrooki),* and western (*Gambusia affinis*) mosquitofish have been stocked in the past for mosquito control, and are arguably one of the most widespread and invasive freshwater fish species in the world (Pyke 2005, 2008). Several objectives were identified for these species, with the underlying goal of producing a Trojan Male broodstock that would be effective in eliminating unwanted Eastern Mosquitofish populations in participant states.

There is some lack of concurrence in the scientific community on the sex determination system of these two species. *G. affinis* is recognized as having a ZZ:WZ sex determination system, in which individuals possessing both a Z and W chromosome are females, and those possessing only Z chromosomes are males. *G. holbrooki* has been described as a XX:XY, in which individuals possessing only X chromosomes are female, and those possessing both an X and Y chromosomes are male. However, this characterization of *G. holbrooki* is not consistent. Some investigators believe that the sex determination system of certain populations of *G. holbrooki* are ZZ:WZ, such as in Australia (J. Patil, University of Tasmania, personal communication) which requires more investigation to clarify its implications for development of useful Trojan male broodstock.

G. holbrooki males tend to be larger than *G. affinis* males, and melanistic phenotypes of *G. holbrooki* males tend to also be much more aggressive than silver phenotypes. It has also been reported that crosses of *G. holbrooki* males with *G. affinis* females produce dead or deformed offspring (Black and Howell 1979), which may explain, in part, the relatively low heterozygosity in hybrid regions between the native ranges of the two species. These conditions may make melanistic phenotype YY male *G. holbrooki* good candidates for Trojan male broodstock, even for locations containing *G. affinis*. However, other investigators have reported viable offspring of *G. holbrooki* males with *G. affinis* females, and the reciprocal cross, to be viable (Scribner 1992, 1993). Validation of this is necessary to determine if YY *G. holbrooki* would be effective for *G. affinis* population control, or if a ZZ *G. affinis* broodstock would be preferable. Several pilot studies were undertaken to better understand these species and their potential for Trojan male fish management.

Objective 1. Evaluate inheritance of melanistic trait in a standard *G. holbrooki* strains.
Objective 2. Evaluate fertility and survival rates of *G. holbrooki* males crossed with *G. affinis* females.
Objective 3. Initiate sex-reversal trials in *G. holbrooki* fry and embryos.
Objective 4. Initiate sex-reversal trials in *G. affinis* fry and embryos.
Objective 5. Evaluation of sex-determination system for *G. affinis* and *G. holbrooki* populations maintained at Parvin Lake Research Station.

Objective 1

Pairings of five individual melanistic phenotype *G. holbrooki* males were made with five individual virgin silver phenotype *G. holbrooki* females, each in static 76 L aquariums, held at 80 °F. Fish were fed TetraMin flake feed with automatic feeders twice per day. Floating artificial aquatic plant mats (12 x 12 inches) were placed into each tank as refugia for offspring, which were collected and transferred to a community tank daily as they were produced. Currently, over 100 offspring have been produced, but they have not yet reached sexual maturity at the time of this report. Early indications are that the melanistic trait is not being universally inherited in the male offspring of this cross.

Objective 2

The initial stock of *G. affinis* for this project was collected from Running Deer Natural Area, near Fort Collins, Colorado. Virgin females are preferable to test this objective, because *G. affinis* can store sperm from previous matings for an extended period of time, which could confound the results of offspring produced in later matings. However, at the beginning of this segment, when virgin female *G. affinis* were not yet available, a pilot evaluation was conducted in which three individual *G. affinis* females, which were potentially bred prior to capture, were mated with individual *G. holbrooki* males, two of which were silver phenotypes, and one of melanistic phenotype. These paired groups were held in 76 L static aquariums, with floating artificial aquatic plant mats (4 x 3 inches), to evaluate reproduction of these matings. A large number (hundreds) of offspring were produced from these matings. However, because the females were not virgin at the time of introduction to the males it is entirely possible that the offspring were from earlier matings. Production of offspring from these females ended at roughly six months after the beginning of the experiment, which is reportedly the duration for which the females can store sperm from previous matings. If new offspring are produced from these pairs, they will be from subsequent matings with the *G. holbrooki* males.

Additional matings of five virgin *G. affinis* were made with five melanistic *G. holbrooki* males, in individual 76 L static aquariums, similar to the prior pilot evaluation, once virgin females were produced from the *G. affinis* culture. At the time of this writing, no offspring had been produced from these matings, despite a two-month duration of the pairing, which is adequate gestation time for these species. In the coming months of rearing, the viability of these crosses will become clear.

Objective 3

Five bred female *G. affinis* were held in breeding cages to capture and enumerate fry produced from each individual female. Upon production of fry, the fry cages were moved to a rearing aquarium where they were started on an E_2-treatment diet for 30 days at 40 mg/kg feed weight to produce feminization. Four of the five females produced broods, for which the E2 treatments are complete, and the offspring are currently being reared to a larger size to measure the success of the feminization.

The females were fed a normal diet until 14 days post parturition, at which time they were transitioned to an E_2-treatment diet of 200 mg/kg feed. Fry were produced by only one female after the feed-treatment for the adult females started. These offspring were held separately to evaluate feminization rates.

Objective 4

Five bred *G. holbrooki* females were similarly held in breeding cages to capture and enumerate fry. Four females produced offspring, which were moved to a rearing aquarium where they were fed an E_2-treatment diet for 30 days at 40 mg/kg feed weight to produce feminization. The four producing females were also transitioned to an E_2-treatment diet of 200 mg/kg feed at 14 days post parturition. All four of these females produced broods after the E_2 treatment. Those broods are also being held separately to assess phenotypic sex upon maturity.

Objective 5

In order to test the sex-determination system of the varieties of *G. affinis* and *G. holbrooki*, both known male and known female specimens of each species were sent to Dr. Jawahar Patil at the University of Tasmania for sex marker testing. Samples of five *G. affinis* males and five *G. affinis* females were sent to Matt Campbell at the Eagle Lab to do preliminary work on verifying sex markers identified in Lamatsch et al. (2015).

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Project Communication and Fundraising

Website and Public Outreach

The website for the YY (Trojan Male) Program at the Western Association of Fish and Wildlife Agencies (WAFWA) has been completed with assistance from Cortney Mycroft. It now features project summaries from all participating states. Program staff delivered multiple presentations throughout 2025. Public outreach was further expanded through popular media outlets, including feature articles published in *Colorado Outdoors*.

YY Steering Committee

The YY Steering Committee was initiated to provide State Fish Chiefs with regular updates on progress made by the YY Consortium, set priorities, and provide feedback for ongoing efforts. Steering Committee meetings were held on March 27, June 30, and October 3, 2025, with good attendance at each meeting.

YY Brook Trout Technical Team

The YY Brook Trout Technical Team assists egg-receiving entities in collectively planning research and monitoring activities. Membership includes approximately 30 individuals across state and federal agencies who receive team correspondence, with a core group of roughly 20 members actively engaged. This core group includes the Eagle Fish Genetics Laboratory (EFGL) Manager Matt Campbell, who provides technical guidance on field genetics sampling protocols.

In addition to regular year-round interactions between individual team members and the YY Coordinator (Schisler), the team held its annual meeting on May 1, 2025. The virtual session included 26 participants from nine states (Arizona, California, Colorado, Idaho, New Mexico, Nevada, Oregon, Washington, and Wyoming). All participating states delivered presentations on their ongoing YY projects.

Partnership Development and Funding Opportunities

Program staff (McIntosh and Schisler) conducted numerous Zoom presentations, discussions, and conference calls to identify and engage additional YY collaborators and funding partners.

A major achievement was the award of $540,000 under the Infrastructure Investment and Jobs Act (Public Law 117-58), Section 40804 (Ecosystem Restoration). The U.S. Fish and Wildlife Service (USFWS) issued the grant through Notice of Funding Opportunity F24AS00320 (issued March 26, 2024) for the nationwide project titled "Rapid Response, Early Intervention, and Eradication of Invasive Fish Using YY Male Technology." The project was one of only six selected from 76 applications seeking more than $34 million. Although the funding was placed on hold in February 2025, it was released for use in May 2025.

Key Collaborations

Several promising partnerships advanced during the reporting period:

- National Park Service (NPS): $140,000 allocated to the Consortium for Brook Trout eradication in the Great Basin National Park
- Bureau of Land Management: $30,000 in new funding was provided to the Consortium to continue on-going work
- US Forest Service: submitted a new proposal for $150,000 through USFS Region 6 to their FY26 IJA Solicitations for NFS Invasive Species Projects, would continue support for on-going work
- Malheur National Wildlife Refuge: continue to support implementing Common Carp eradication in the Malheur Basin. The Oregon Watershed Enhancement Board has allocated $300,000 from their Focused Investments Partnership Initiative in 2029 to support implementing a Common Carp eradication project
- Trout Unlimited (TU): Recent collaboration in Wyoming; TU has formally agreed to assist the Consortium in seeking new funding and has expressed strong support for the YY approach for invasive species control and eradication
- Avista Power: Potential collaboration targeting Common Carp in Washington
- Freshwater Conservation Canada and Alberta Fish and Wildlife: interested in joining the Consortium (funding) and implementing Brook Trout eradications
- Moore Charitable Foundation: Discussions regarding projects at Trinchera Ranch and Rio Grande Cutthroat Trout conservation

Particularly noteworthy is the developing collaboration at Trinchera Ranch (Colorado), the largest private ranch in the United States. In partnership with Colorado Parks and Wildlife, Trout Unlimited, the National Fish and Wildlife Foundation, New Mexico, the U.S. Forest Service (USFS), the USFWS, and others, the initial focus is large-scale eradication of Brook Trout using YY males in critical headwater habitats for Rio Grande Cutthroat Trout, including West Indian Creek. YY fish are already in production for this effort. The ranch has committed resources to repair a downstream barrier in spring 2026 to prevent re-invasion; wild Brook Trout removal operations will commence immediately thereafter.

Program Reporting

Quarterly reports continue to be provided to the USFS and biannual reports to the USFWS.

Aquaculture Considerations and Hatchery Identification

A YY Hatchery Team meeting was held on March 25th. Numerous meetings were held with Brad Neuschwanger of the Colorado Fish Research Hatchery and Jared Riemenschneider of the Idaho Hayspur Hatchery to discuss rearing topics, egg production, lateral line issues issue, as well as INAD and Indexing topics.

A series of meetings were held with California Fish and Game with respect to starting a new YY brook trout broodstock in California, paperwork and dialog to get California added to the YY brook trout INAD, and additional disease records and testing to satisfy California importation regulations. Investigators at Humbolt State University were also contacted to discuss their new project on Sacramento pikeminnow (*Ptychocheilus grandis)* and potential avenues to add their facility effluent tables to the INAD in order to use the fish potentially produced from their work for future broodstock.

Discussions were held with Nevada Department of Wildlife (NDOW) regarding the potential use of the Mead Lake Hatchery as a location for future broodstock. NDOW staff were briefed on the Trojan Male program, and various species fish culture needs for the program were described, in anticipation of an on-site discussion in January 2026.

Interest in YY rainbow trout (*Oncorhynchus mykiss*) resulted in a series of meetings to determine demand for this species, with key personnel in these discussions including Jacob Rash (NC Wildlife Resources Commission), Brad Fink (VA Department of Wildlife Resources), Doug Besler (NC Wildlife Resources Commission), Matt Kulp (Great Smoky Mountain National Park), and Tyler Hern (Erwin National Fish Hatchery) on March 4 and May 20), with follow-up discussions regarding a survey that was distributed among Eastern States. The results of that survey are provided in Table 7.

Agency	Using	Interest	Interest in	Contact
NC Wildlife Resources Commission	NO	**YES**	NO	Jacob Rash
AR Game and Fish Commission	NO	NO	NO	Christy Graham
SC Department of Natural Resources	.	.	.	Dan Rankin
TN Wildlife Resources Agency	.	.	.	Jim Habera
GA Department of Natural Resources	NO	**YES**	Spotted Bass	James Miles
VA Department of Wildlife Resources	NO	**YES**	.	Brad Fink
WV Department of Natural Resources	NO	NO	NO	David Thorne
MD Department of Natural Resources	NO	NO	NO	Matt Lawrence
Great Smoky National Park	NO	**YES**	NO	Matt Kulp
KY Department of Fish and Wildlife Resources	.	.	.	Nathan Hayes
PA Boat and Fish Commission	.	.	.	Jason Detar
Eastern Band of Cherokee Indians	NO	**YES**	UNCERTAIN	Mike LaVoie

Table 7. Potential interest from eastern states in YY program development.

Permitting and Regulatory Compliance

Production of YY broodstock for any fish species requires the use of a feminizing hormone to sex-reverse genetic males. The Western Association of Fish and Wildlife Agencies (WAFWA) currently employs estradiol (E_2) for this purpose, which is classified as a drug and is regulated by the U.S. Food and Drug Administration (FDA).

The existing YY brook trout broodstocks maintained in Idaho and Colorado operate under an Investigational New Animal Drug (INAD) authorization held on WAFWA's behalf by the Aquatic Animal Drug Approval Partnership (AADAP) program in Bozeman, Montana. All hatchery production and associated fieldwork using YY Brook Trout produced under this INAD have received FDA approval.

An alternative regulatory pathway, known as indexing, was established under the Minor Use/Minor Species (MUMS) Act of 2004. This route may prove more appropriate than the full INAD/New Animal Drug Application (NADA) process for cases where risks are minimal—such as when broodstock are treated but not released into natural waters. Indexing involves a three-step process: (1) eligibility determination by the Secretary of Health and Human Services, (2) expert panel review of available data with subsequent Secretary acceptance or rejection of recommendations, and (3) addition of the drug to the FDA's list of indexed compounds.

In FY24, program staff submitted a targeted proposal to the U.S. Fish and Wildlife Service (USFWS) under a federal Notice of Funding Opportunity (NOFO) supporting invasive species eradication programs. The proposal, which was accepted, addresses steps 1 and 2 for indexing E_2 use in feminizing multiple invasive salmonids. Work on this initiative began in FY25, with YY Consortium staff establishing contracts with experienced contractors (Novaeel, Donoghue, OCAMs, and Exponent) to develop eligibility applications, Chemistry, Manufacturing, and Controls (CMC) documentation, medicated feeds, and Environmental Assessments (EAs).

Progress on INAD Maintenance

Multiple meetings with AADAP and/or FDA staff advanced both INAD maintenance and E_2 indexing efforts, including the annual estradiol meeting and coordinating sessions held on January 22, March 25, April 1, May 16, June 24, July 14, July 31, and November 13. Regular correspondence also supported maintenance of the INAD and addition of receiving facilities for YY brook trout.

Brown trout have been successfully added to the INAD, and the Los Ojos Hatchery in New Mexico is currently in its second year of feminization efforts for this species. The state of California has been added to the list of approved receiving states. A major achievement during this period was the FDA's agreement that fish produced for experimental purposes may be retained for future broodstock development, provided that E_2 effluent tables are submitted through AADAP. Effluent tables were already established for red shiner (*Cyprinella lutrensis*; already enrolled in the INAD). New tables were submitted for common carp at Utah State University and for mosquitofish at the Parvin Lake Research Station in Colorado. The FDA, through AADAP, granted Categorical Exclusion authorization for these two facilities.

Progress on the Indexing Pathway

Contractor meetings for the indexing process were held on February 27, June 23, August 8, August 11, October 2, and December 2. Following discussions on scope, the initial submission was narrowed to brook trout and brown trout due to the availability of robust supporting literature and experimental data for these species only. Prior to submission of the request for eligibility, we were advised to complete the Environmental Assessment portion of this work.

Information regarding current E_2 usage and effluent treatment at the Hayspur, Bellvue, and Los Ojos hatcheries was provided to Exponent to support the Environmental Assessment. The next phase involved surveying hatchery rearing areas and effluent volumes across potential use regions. A survey was distributed on August 1st, 2025 to State Fisheries Chiefs requesting data from coldwater hatcheries on nursery areas used to raise salmonids from hatch to fingerling stages. This survey resulted in 35 responses for trout-rearing facilities throughout the United States. A follow-up survey was distributed to obtain additional information from respondents, which resulted in 15 responses, as well as six additional responses to the original survey. This information, along with the individual hatchery E_2 usage and effluent treatment already in hand from the INAD-approved facilities was used to generate a draft Environmental Assessment.

Additional advancements include completion of the required paperwork by Ann Donoghue to serve as WAFWA's representative in the indexing process. Conversations with OCAMs regarding medicated feed development remain on track for production once indexing approval is obtained.

Acknowledgements FY25

The work by Dr. Dan Schill and Liz Mamer have been critical to all of the background information and development of the YY Consortium to date. The scientific community and fisheries management agencies alike have greatly benefited from their efforts. We are all deeply grateful for their contributions.

Broodstock Maintenance and Development

Thanks to Jared Riemenschneider and the rest of the staff at the Hayspur Hatchery for their diligent work maintaining and improving the YY brook trout broodstock. Thanks to Brad Neuschwanger and the staff at the Bellvue Fish Research Hatchery for maintaining the backup YY brook trout broodstock and contribution to additional YY studies. Thanks to Andrew Delp and the staff at Los Ojos Fish Hatchery for taking on the YY brown trout broodstock development process at their facility. Thanks to Tyler Hern of the Erwin National Fish Hatchery for providing fin clips and milt for the YY brook trout outbreeding experiment. Also, thanks to Marde Hersch and staff at the Poudre Hatchery for rearing the fish in the outbreeding experiment. Thanks to Dr. Paula Schaffer and Dr. Pete Cadmus for their investigations into lateral line dysplasia and water quality. We again thank Dr. Cadmus for providing milt extender for the outbreeding experiment.

Feminization Trials

Thanks to Dr. Chad Teal and Dr. Charlotte Musser for their excellent efforts on the common carp and red shiner feminization projects, as well as moving the ball forward on other species such as channel catfish and bullfrog. Thanks to Liz Mamer for help with the dissections and sex identification work. Many special thanks to Sharon Schisler for assisting with the mosquitofish feminization trials, and for her support and patience throughout this year of new responsibilities.

Field Work

Thanks to all of the YY Tech Team members, conducting field work in each of their respective states, whose individual efforts are providing new pathways for native fish conservation. Thanks to Boyd Wright, Kevin Rogers, Jon Ewert, Estevan Vigil, Cory Nobel, and many other biologists at Colorado Parks and Wildlife for their work on Bobtail and Steelman creeks, Big Tooth Reservoir, and the Rito Hondo project. Thanks to Jenn Vincent and Kevin Meyer at Idaho Fish and Game for their hard work leading the way on YY brook trout and their willingness to provide additional presentations at key meetings.

Sex Marker Development and Analysis

Matt Campbell, Eagle Fish Genetics Lab supervisor, has played a key part in the Consortium success with his contribution of expertise and guidance. We also thank Katharine Coykendall for leading the sex marker efforts on common carp and walleye. We thank the entire staff at the Eagle Genetics Lab, particularly Jesse McCane, involved in sample prep, analysis, and record keeping. Thanks to Wei Chen of the Alaska Department of Fish and Game for her work on northern pike sex markers. Thanks to Mike Ruhl of New Mexico Department of Game and Fish, as well as Zach Klein and Kadie Heinle at New Mexico State University for facilitating the sex marker work for brown trout with GenSeq, and for their enthusiasm for moving the YY brown trout broodstock program forward.

Administrative Support and Assistance

Thank you to Cathy Campbell for assisting with grant tracking and monthly fiscal administration of the WAFWA YY program, and Cortney Mycroft for developing and maintaining the YY Consortium webpage on the WAFWA website. Thanks to Jenna Baxter for additional administrative assistance and design assistance.

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Leadership and Steering Committee

Thanks to Zach Lowe for his continued support of this program through WAFWA. We extend our sincere gratitude to members of the YY Steering Committee from the participating states for their invaluable support:

Julie Carter, Arizona Game and Fish Department, Aquatic Wildlife Branch Chief

Chris Crookshanks, Nevada Department of Wildlife, Fisheries Division Administrator

Christopher Donley, Washington Department of Fish and Wildlife, Region 1 Fish Program Manager

Mike Harrington, Oregon Department of Fish and Wildlife, Fish Division Administrator

Alexis Harrison, Oregon Department of Fish and Wildlife, Native Fish Investigations Manager

Jay Rowan, California Department of Fish and Game, Fisheries Branch Chief

Alan Osterland, Wyoming Game and Fish Department, Chief of Fisheries

Special thanks go to Lance Hebdon, Idaho Department of Fish and Game, Bureau Chief of Fisheries and YY Committee Chair, and Kirk Patten, New Mexico Department of Game and Fish, Fisheries Management Chief and YY Committee Vice-Chair, for their exemplary leadership.

We also acknowledge Matt Nicholl, Colorado Parks and Wildlife, Assistant Director of the Aquatic Wildlife Branch, for his participation on the Steering Committee and unwavering support for the program. Finally, we thank Jim Fredericks, Idaho Department of Fish and Game Director, for his continued commitment to the initiative's success.

Appendix A-

Executive Summary of YY Program New Species Work (2018 to present)
Schill 2025

1. Obtained functional sex markers for four of the five new target species (Brown Trout, Lake Trout, Common Carp and Walleye).
   - The sex marker for Northern Pike was undertaken by the Alaska Dept. of Fish and Game, with limited results to date (see below for additional details).
2. Obtained new, highly successful sex reversal treatment protocols for two of five species (Walleye and Brown Trout) with useful insight gained for future broodstock development on two more species (Common Carp and Lake Trout).
   - We initiated a sex reversal trial for Northern Pike, but unfortunately study protocols were accidently not followed by the study participant, so another trial will need to be conducted. However, given the lack of progress on a sex marker for Northern Pike, we concluded this work should be put on hold until this is resolved.
   - Given results on all five species, we focused on work that was likely to rapidly produce the next viable broodstock (i.e., Brown Trout)
3. Despite successful development of sex markers for the majority of species undertaken by the program, our work, along with the work of Dr. Chad Teal, has led us to conclude that environmental influences on phenotype (Environmental Sex Determination or ESD) may be more common than we originally expected.
   - This means environmental variables that are undetermined at this point likely play a large role in sex determination of non-salmonids. This will require additional research. See Walleye discussion below.
   - We recommend that future work on additional species be focused on the development of a viable sex marker before proceeding with broodstock development.
   - Salmonids are the current exception to this finding, as a universal sex marker exists for them.
4. On a number of occasions, we initiated studies at hatcheries where for a variety of reasons, they were unable to complete the work, thus leaving us just short of the finish line.
   - To prevent stranded investment, future development of sex reversal recipes for YY Male development should only be undertaken at facilities where there is an upfront commitment to subsequently develop a YY Male broodstock at that facility.
   - See detailed discussions below for Northern Pike and Walleye.

5. We recommend that when making decisions to move forward on future YY development for new YY species, a high premium should be placed on securing commitment for at least two separate facilities to enable backup of a developed broodstock.
    - This reduces the risk of program collapse for unforeseen circumstances (e.g., disease outbreaks, facility failure, etc.) and may expedite program development as both programs learn from each other as they go.

Status of the Sex Marker/Sex Reversal Work by Species
(2018 - present)

This report section is a summary of Consortium efforts over the past 6-year period to expand work on YY Brook Trout begun by the Idaho Department of Fish and Game and initiate development of broodstocks for other species of interest to the WAFWA Fish Committee. The intent of this brief summary is to provide the new program coordinator, and other interested parties with a brief record of what was done regarding the development of new YY Male broodstocks, exclusive of our extensive work on Brook Trout. What was accomplished, what worked, what didn't, and why? By design and as per contract, this document covers the scientific broodstock development results for new species work only. For a more detailed account of specific work done, along with methods and findings, 6 annual reports submitted by the program staff are available from WAFWA.

Background

The development of a Trojan Y Chromosome or YY Male Brook Trout broodstock was begun by the Idaho Department of Fish and Game (IDFG) in 2008. By 2014, IDFG hatchery staff at the Eagle Fish Genetics Laboratory had built a viable broodstock residing at the Hayspur Hatchery capable of producing small numbers of sperm-producing, genetically YY male fish for release (Schill et al. 2016). A simulation study published the following year predicted that stocking of M_{YY} fish could in fact extirpate an invasive Brook Trout population in time frames of interest to fisheries managers, particularly if concurrent manual suppression was conducted (Schill et al. 2017).

The impetus for development of the WAFWA YY Consortium began in 2016 with an IDFG presentation regarding its YY Brook Trout program and exploratory work being done on several other species (Lake Trout, Common Carp, and Walleye) to the WAFWA Fish Committee at the 2016 Annual meeting held in Wyoming. An additional presentation on YY Males was made at the 2017 WAFWA meeting in Colorado where the Fish Committee's interest in the Idaho program remained high. At the January 2018 WAFWA meeting in San Diego, California, WAFWA Directors approved a program for initiating a multi-state program with 13 states initially agreeing to fund the program via WAFWA. During several conference calls in Spring

2018, the funding Fish Chiefs agreed that the Consortium should continue the initial IDFG work on the above three species (Lake Trout, Common Carp and Walleye) and also prioritized two additional new species, Brown Trout and Northern Pike.

Various WAFWA and nearby states had aquaculture experience with rearing of the above species. The basic concept of the Consortium was that program staff would work with hatchery staff in some WAFWA or nearby states or Federal facilities to experiment in the development of YY fish. Those states that subsequently developed actual broodstocks would share eggs with other Consortium members. The egg sharing aspect of the program is exemplified by the currently running YY Brook Trout program where eggs from two broodstock hatcheries are presently being shared among eight Western states for field research evaluation.

New Species Development

Two basic science tasks are necessary for the development of YY Male broodstocks. The first task involves the feminization of male fish of the target invasive species. This is typically done by exposure of young fish to a natural or synthetic hormone during the period when they differentiate into males and females. The treatment duration and amount of the natural hormone 17β-Estradiol (E2) fed to fish in developing IDFG's Brook Trout broodstock was based on a published feminization study (Johnstone 1979) that called for feeding the hormone for 60 total days starting at first feeding. This paper did not specify a feeding rate although Hayspur Hatchery staff in Idaho eventually found that feeding their Brook Trout fry at 4% body weight per day for the above duration produced the best feminization rates. A "feminized" male is simply a genetic male that produces viable eggs (i.e. presents as a phenotypic female) that are subsequently crossed with regular sperm-producing males. Roughly one quarter of the progeny from such matings will be genetically YY fish. Once a feminization treatment protocol is established for a species, the next step necessary for eventual YY broodstock construction is having the ability to separate feminized XY males from standard XX genetic females and retain the former for subsequent spawning (see Sex Marker section immediately below). See Schill et al. (2016) for a diagram and verbal description of the entire process. There were no published successful feminization protocols for the five new species of interest to the WAFWA Chiefs, and a large portion of time and available Consortium fiscal resources, particularly during the first four years, was spent by program staff (Schill and Mamer) attempting to develop them with various levels of success.

The second science tool necessary for ready development of YY Broodstocks is the availability of a genetic sex test or sex marker. With one exception (see below) all sex marker work undertaken by the Consortium was conducted by Idaho Fish and Game staff at the Eagle Fish Genetics Laboratory (EFGL). The development of sex markers for each species of interest involves genetic sampling and DNA sequencing. First, mature adult fish of wild origin were collected by either WAFWA contractors or state agency field personnel, killed via anesthetic overdose, necropsied and visually sexed. Fin tissues were only taken from fish with clearly identifiable gonads and were placed on numbered Whatman filter paper sheets for storage. DNA was subsequently extracted from the fin tissue by EFGL personnel and cut into fragments using specific enzymes. Selected DNA fragments were then sequenced so that the exact order of nucleotides (i.e. A,C,T,G) was determined. These sequences were then compared between phenotypic males and females to find specific single nucleotide polymorphisms (SNPs) specific to each sex.

The general approach of the sex marker work in the YY program is initially to develop Y chromosome-linked markers that would initially permit the differentiation of XX and XY individuals (presence/absence markers) and eventually develop bi-allelic markers that would allow differentiation of XY and YY fish. The latter markers would subsequently be used to identify feminized YY Males in a later generation that had been successfully feminized in an Estradiol treatment protocol as described above.

Detailed Results by Species

For development of a YY broodstock for each of the five invasive species of interest, we required both a viable sex marker and a sex reversal recipe. The summary below briefly describes the success or failure of the Consortium to attain both components for the five targeted species. Challenges or unexpected results hindering YY development for a given target species are also noted and explained.

Lake Trout

Sex Marker results for this species were finalized by EFGL staff in FY21. Samples were collected from 2017-2020 in 6 lakes in MT, WY, OR, and ID (Combined N = 926). The overall concordance between phenotype and the marker-based genetic sex call was 93% and only one lake had concordance below 90%. In addition, EFGL staff also identified a bi-allelic sex marker so XY Lake Trout could be distinguished from YY Lake Trout. The results of the EFGL staff work indicate that markers exist that would enable development of YY Lake Trout Broodstock.

A Lake Trout feminization trial was initiated by IDFG in Jan 2017 before the Consortium was created but the fish subsequently proved so late maturing that the trial ran for 1000 days post hatch (DPH) until August 2019, the second FY of the Consortium. Unfortunately, we saw absolutely no evidence of feminization success for any of the various treatments employed, including several immersion recipes, standard top-coated feed recipes and a combination of the two methods. This was the first experimental feminization trial initiated by IDFG staff (IDFG had begun the YY Brook Trout broodstock development effort with a strong published feminization recipe) and their inexperience showed in that emphasis was placed only on treatment dosage and none on duration. The results eventually obtained suggest we did not treat the fish with Estradiol long enough. A follow-up sex differentiation study, initiated in Dec 2020 and completed in Dec 2021 suggests that lake trout would need to be treated with Estradiol starting as late as 150 DPH and continue through one year or perhaps slightly longer given that the final sample at 366 DPH still had males in early differentiation stages. While we have identified the likely sex differentiation period for Lake Trout reared at a specific temperature and facility, the long differentiation period may prove problematic for sex reversing the species.

Because we have a sex marker for this species, eventual YY broodstock development is still of interest but their slow maturation schedule makes it the least desirable of the five new species for developing a YY Male broodstock given how long it would take to build one using the standard 3 generation approach. It is possible that production of a broodstock could be shortened to one generation using an approach combining androgenesis and the sex marker, but technical challenges to this route certainly exist and the method remains untested.

There remains some interest in use of YY Males in the Lake Trout eradication arena due to the great cost associated with the two most prominent long-term Lake Trout manual suppression efforts ongoing in Yellowstone Lake and in Lake Pend Oreille. Up to $2,000,000 has been being spent annually on netting on these waters and there are other less high-profile lakes where a much cheaper alternative would be required. However, of the five species being considered for YY development by the consortium, YY Male Lake Trout would intuitively perform the slowest in attaining invasive eradication given their long generation time. A modeling effort combining netting and the YY approach to eradicate established populations has been discussed by IDFG and University of Idaho staff for many years but has not been developed. Given the lack of modeling on likely outcomes and the unusually long sex differentiation period for Lake Trout reported above, it has been the decision of the Consortium contractors to forestall further effort in development of a sex reversal recipe for Lake Trout until further modeling is conducted that predicts the probability of success with YY Males for this species. There has been discussion of a Lake Trout modeling effort for Yellowstone Lake to be done by a Great Lakes team. The new coordinator should check on possible progress in such a venture although it is also suggested that he should check with the outgoing coordinator (Schill) in regard to some nuances on the possible YNP simulation effort for YY Lake Trout.

Walleye

A paired sex reversal trial was initiated in May 2017 in Iowa (Rathbun Hatchery) and Kansas (Meade then Milford Hatcheries) with highly successful results. By March 2018, 100% of fish in two treatment groups across both states were 100% female, while sex ratios in Controls were near 50:50. By April 2019, at nearly two years of age, control group male and female gametes were flowing. However, none of the treatment fish retained had ripened gonads. It was likely that ripening of treated fish will be delayed a year, which often happens in fish exposed to sex reversing hormones. Unfortunately, due to space limitations, staff at the Milford Hatchery had to kill the fish before the end of year three so we were unable to verify successful egg production in treated females.

A follow-up study at the USFWS Garrison National Fish Hatchery was initiated June 2021. Two different durations were tried along with three different dosages, including one far lower than had been attempted in the successful IA and KS work. Results of this trial were again wildly successful with 98.8 to 100% of all Walleye in six different treatment groups being identified as phenotypic females. Unfortunately, the opportunity to grow these fish out to maturity was lost with the departure of a program supervisor, the hatchery manager, and the technician who had accomplished the first year of work. Further, direct confirmation that genetic males had been sex reversed in the trial was hindered by a lack of sex marker progress for the species (see below). Nonetheless, it appeared that Walleye were remarkably easy to sex reverse and high hopes were placed on successful development of a sex marker to initiate subsequent YY broodstock development.

In contrast to sex reversal, development of a sex marker for Walleye proved extremely challenging for the EFGL staff. In FY19 and FY20, EFGL initiated efforts on a Walleye sex marker, but the work did not yield useful results. In FY21, the EFGL team employed a newer technology known as pooled sequencing. However, after optimization, a number of marker assays were not concordant with phenotypic sex calls in study fish. After extensive work in FY22 and FY23, along with collaboration with a European genetics lab, EFGL has concluded that Walleye do not have an XX-XY sex determination system. This was surprising because a prior study by an experienced Walleye aquaculture researcher had concluded they had the commonly understood XX-XY system. Instead, EFGL and collaborators have concluded that Walleye have what is termed a ZZ-ZW sex determination system where the female gametes determine the sex of individuals. In addition, the European geneticists have also concluded that the species likely has an environmental component, making them a less genetically stable candidate for attempting the building of a broodstock. Further, while it is possible to construct a broodstock for eradicating invasive fish with a ZZ-ZW system, the broodstock in question cannot be referred to as a YY Male one. More problematically, *all* fish to be stocked in the field in such a program must themselves be sex reversed (as opposed to the sperm producing YY Brook Trout we currently stock), creating potential E2 containment issues, feminized live fish transport issues, and additional food safety concerns by the FDA.

In summary, the initial, and remarkably successful IDFG sex reversal work on Walleye with cooperating hatchery personnel from IA and KS, as well as the even more successful Consortium staff work at Garrison National, has been completely offset by the unexpected sex determination system with likely additional temperature/sex complications for this species. Despite the catastrophic effects of ongoing illegal walleye introductions by anglers, we recommend that, in the near term at least, this species be taken off the Consortium list for broodstock development. Perhaps if additional genetic work in the field can weed out individual fish subject to phenotype shift due to temperature, this species could be reconsidered for use as a renamed Trojan Sex Chromosome or TSC approach.

Northern Pike

A sex reversal trial was begun in May 2019 and concluded in June 2020, working with staff at Iowa DNR's Rathbun Hatchery. This was the same hatchery that conducted the first Walleye sex reversal trial noted above with outstanding feminization results. Unfortunately, due to an error by a DNR hatchery employee, the fish were only fed half the E2 dose laid out in the jointly developed study proposal. The error was discovered after E2 treatment ceased, but a decision was made to continue the trial in hopes that the level actually fed would prove adequate. The resulting phenotype data from June 2020 suggested that limited feminization of a small proportion of fish (chiefly intersex) occurred, but it was concluded that a follow-up trial would be needed. Given other priorities (chiefly extensive Brown Trout sex reversal work), a decision was made to forego a follow-up trial until a sex marker could be developed for the species.

At the onset of YY Male Consortium work in FY19, the Alaska Department of Fish and Game (ADFG) took the lead on sex marker development for Northern Pike. The agency's genetics lab had some preliminary success during FY2020, by building a genome scaffold and identifying regions with high sex association (Chris Habicht, ADFG personal communication). In FY22, ADFG Division of Sport Fisheries provided additional funding for the ADFG Gene Conservation Lab (GCL) to develop 12 potential markers with a high probability of differentiating sex in Northern Pike using (RADseq) techniques. The latest work by the ADFG Gene Conservation Lab (GCL) provided improved results but their work was only for Alaska populations.

In anticipation that AK would eventually get a useful sex marker, Consortium staff had worked with AZ, WA, IA staff (Schill and Mamer 2020) to sample three populations and with CO field staff in July of 2023 to collect the requisite phenotype data and fin clips for two additional populations. The five populations collectively provided excellent geographic spread for eventual western U.S. marker development as had been done for other WAFWA species of interest. However, marker progress at the Alaska lab remained slow.

In FY24, EFGL staff were asked to engage and develop a sex marker for WAFWA using the lower 48 population samples. Unfortunately, the lab manager (Matt Campbell) discovered that genetic research published in the same year (Johnson et al 2024; and studies referenced within) suggested that the species is a poor candidate for YY technology development in the United States. It appears that Northern Pike in parts of their range (Europe and Alaska) utilize an XX-XY male heterozygous sex determination system via a duplicated gene called amhby. Unfortunately, this sex determination system seems to have been lost in populations throughout much of North America, and the sex of Northern Pike in these areas is likely influenced strongly by environmental factors. While Northern Pike could be a candidate for YY Male technology in regions where the amhby gene is present (e.g., Alaska), the absence of this gene in other populations and an unstable sex determination system limits the potential for widespread YY Male application (Matt Campbell, EFGL, pers. comm.). In summary, Eagle Fish Genetics Laboratory staff responsible for development of numerous genetic sex markers, including for the five other YY Consortium species of interest (Brook Trout, Brown Trout, Common Carp, Walleye, and Lake Trout), recommended against any further YY work on Northern Pike (Mathew Campbell, EFGL, pers. comm.), advice we concur with.

Common Carp

Outstanding results on markers for Common Carp were obtained from work over a number of years by staff at EFGL. During FY2019, EFGL staff identified two candidate bi-allelic loci and screened one on samples from 10 populations reporting an overall concordance rate between genetic and phenotypic sex of 93%. However, concordance varied considerably across populations, with several samples having small n's or other limitations, we concluded additional work was necessary.

Funding for this subsequent work was obtained by YY Consortium staff with the encouragement and support of AWFWA under the Multi State Conservation Grants Program (MSCGP), administered by the USFWS. Relative to the FY2019 work above, these samples were focused farther east to broaden the utility of a sex marker for the nation as a whole and to potentially assist in securing future Federal funding for eventual development of YY Common Carp. Accordingly, during FY2021, large samples were obtained from five new waters in PA, TN, IA, WA and OK. Results from this effort were encouraging but concordance for two of these populations were relatively low and additional work on marker improvement was conducted in FY2023 with great success, including better concordance across all five populations. During FY24 work, samples from two large Idaho populations were added to the above analysis by EFGL staff using the latest markers. At present, we recommend focusing on assay 744444_87 for future development of a YY male carp broodstock (see FY24 report). Combining results from this assay across all seven populations evaluated by the EFGL over the past two years indicates that concordance exceeded 99.5% in five of the seven populations tested. Thus, locating Common Carp populations that could serve as solid egg sources for a future YY Male broodstock for the species should not prove difficult. Potential waters being considered as donor source for either a carp sex reversal trial or future YY broodstock development would ideally be screened in advance to ensure sex marker compatibility.

In regard to sex reversal trials, for commercial production, there have been several attempts to feminize Common Carp using top-coated feed, but the species has proven resistant to feminization. IDFG staff undertook a feminization trial at a private hatchery near Boise, ID in July 2017, which ran until May 2018 when fish were killed. Results indicated that a 140-day E2 treatment duration might be close to an effective treatment protocol but water temperature regulation issues at the hatchery killed many study fish and the resulting sample size was limited. A decision was made to conduct any further carp work with states or personnel that had extensive experience raising the species.

Funding for two paired sex reversal trials was obtained by Consortium staff under the MSCGP program noted above. The trials were undertaken by Consortium staff working with staff at two Midwest

WAFWA state hatcheries (OK initiated April 2021, TX initiated May 2021). In Oklahoma, we worked with Dr. William Shelton, an aquaculture professor with extensive experience rearing Common Carp. In Texas, work was initiated at a state facility that rears feeder koi carp. The trials included multiple dosages and E2 treatment concentrations. Unfortunately, poor growth and heavy mortality occurred at both facilities and there was little knowledge gained. We are thus left with the frustrating situation of having developed an outstanding sex marker for this especially destructive invasive species but being only slightly past square one in regard to sex reversal.

Common Carp are typically cited as the most destructive freshwater invasive worldwide and large amounts of money continue to be spent annually on their eradication or control in the U.S alone. Given their impacts, and the existence of a simulation paper suggesting YY Males could work in some scenarios for the species (but not large water bodies), we believe another effort to develop a feminization protocol for Common Carp is warranted. In FY25, WAFWA contracted with Dr. Chad Teal at the Utah Cooperative Fish and Wildlife Research Unit to undertake such an effort over the next three years.

Brown Trout

Work on a presence/absence sex marker for Brown Trout was initiated with some solid success by EFGL staff in FY2018 using samples collected from the South Fork Snake River in Idaho. Over the next several years fin clip samples with observed phenotype were obtained from populations in five states including ID, AZ, CO, NM and CA (n = 687 fish). Final EFGL analysis was conducted in FY20 with an excellent overall concordance result of 96%, so a solid presence-absence marker exists across WAFWA states. In addition, development of an effective and accurate bi-allelic sex marker for differentiating between XX, XY and YY male Brown Trout is ongoing at the Eagle Fish Genetics Laboratory and current results are promising with three distinct classification groups visible.

Sex reversal trials were begun in both CO and SD state hatcheries in Nov and Dec 2019, respectively. The standard recipe for Brook Trout (60 days from first feeding at 20mg E2/kg feed) was tried at both the Colorado Fish Research Hatchery and the McNenny Fish Hatchery in SD. Additional recipes using a shorter treatment duration and a higher dosage were tried at the CO and SD facilities, respectively. October 2020 results from the CO trial (a past recipe for Rainbow Trout) indicated that 20mg/kg E2 for 30 days had no observable feminizing effect while the 60-day duration treatment resulted in 74.3% phenotypic females, a statistical difference relative to controls. In the SD trial, the 20mg/kg dosage resulted in 84.2% females while the 30mg/kg group was comprised of only a few more females at 85.8%. Both of the SD treatment groups contained statistically greater proportions of females than the control groups, strongly suggesting feminization had occurred. The SD trial appeared to result in better feminization and SD staff opted to publish this work with Mamer and Schill (Voorhees et al 2023) providing a third published treatment protocol for feminizing salmonids.

The roughly 10% lower rate of females the CO fish relative to the SD study testing the same standard Brook Trout recipe (20mg/kg) was unexplained and CDOW staff volunteered to conduct a follow-up trial testing a broader range of dosages and durations to see if the existing results could be improved upon (See FY21 report for the expanded trial framework). The trial fish were examined for phenotype in Nov 2021 where a high percentage of all fish within each E2 treatment group were identified as phenotypic females (78.8-97.3%). Phenotypic females were then removed from the above analysis using a Brown Trout sex marker (see below) and resulting feminization rates of genetic males were greatest for two 120-day treatments. Thus, to attain high levels of feminization near that of the original published Brook Trout paper (Johnstone 1979), Brown Trout needed to be treated for twice as long as Brook Trout. Efforts were made to grow out subsamples of fish from this trial to 2 YO at the CO facility to check maturity with limited success due to small samples sizes (CO). High mortality of 3 YO fish in a second group of these trial fish transported to the Los Ojos Fish Hathery near Chama, NM, also experienced high mortality. Thus, we currently have what appears to be a highly efficacious treatment protocol (20 mg/kg for 120 days) but confirmation of viable egg production using this recipe still needs future confirmation.

A final sex reversal trial for Brown Trout was conducted in FY23 and 24 at Los Ojos Fish hatchery in NM. The objective of this effort was to give staff at Chama experience in the feminization of Brown Trout with Estradiol, confirm a previously developed 120 d treatment protocol will work at that facility, and to verify that putative YY male Brown Trout could be feminized. Results indicate that progeny of a cross between a feminized genetic male and a standard genetic male resulted in the successful creation and survival to Age 1 of the first YY male Brown Trout given an exact 75% sex ratio of male progeny in the study control group. In addition, 91.7% of fish in a 20 mg, 120 d treatment group were identified as females, suggesting that this feminizing recipe originally obtained during the CO study would work at the NM facility. Based on these results, a decision was reached by NMDGF to move forward on development of a YY Brown Trout broodstock at the Los Ojos facility and ongoing efforts are being made in concert with AADAP to obtain FDA authorization, which was received in Dec 2024.

In addition to the above summarization of feminization trial results for Brown Trout conducted at three state facilities over the years, we note the additional work we conducted documenting the effect of Estradiol treatment on fish health for the species. The protocol and variables monitored were developed in collaboration with Paul Smith, President of NovaEel Inc., a private corporation seeking FDA drug approval for use of Estradiol in the feminization of American Eels for commercial purposes. Such fish health data appears in reports across two years of CO work and the final year at Los Ojos in NM. In general, the data we collected does not show large and sustained impacts of Estradiol exposure to young Brown Trout. Although the data collected do not include the daunting level of rigor required in the FDA INAD approval process, it is hoped that this information will prove useful in future efforts to Index the species, along with other salmonids. Indexing is a much less rigorous process developed for approving drug use on broodstocks not released into the environment.

Lessons Learned in Hatchery Feminization Trials and Sex markers Development Over Six Years

1. Our efforts to meet the program goals of developing an effective sex reversal protocol AND a sex marker for 5 new species during this time period proved an overwhelming task that detracted from more aggressive work to move forward on a Brook Trout INAD and Indexing. Nonetheless, enough knowledge was obtained in these efforts to enable us to essentially rule out two of the five targeted species as viable candidates for development of YY Males due to unexpected genetic issues (Northern Pike and Walleye). Further, the extremely long sex differentiation period we documented in Lake Trout, along with the lack of anticipated population simulations also calls immediate additional work on this species into question, at least in the near term.
2. Another lesson that only became more apparent to us in the last two years of the 6-year period was that it may be unexpectedly difficult to obtain a viable sex marker for invasive species of interest free of other complicating factors. Indeed, Matt Campbell, the EFGL Lab Manager told us up front back in 2018 “there are no guarantees” in regard to certainty of obtaining a marker for a chosen species. One exception to this rule might be salmonids where the apparent universal salmonid sex marker Sd_y exists. For example, the EFGL developed a sex marker for Brown and Lake Trout as part of the present YY work and already had developed one for rainbow trout for general steelhead management purposes. However, as the relatively new science of genetic sex marker development moves forward there may be more species discovered where complicating environmental factors in sex determination make broodstock development problematic. Given such uncertainty, upfront investment in sex reversal could be fiscally unwise. Perhaps a more conservative approach in the future for those entities (including WAFWA and other State or Federal entities) considering pursuit of YY Males for a new species may be to conduct sex marker work first to ensure a straight forward “standard” sex determination system exists before attempting sex reversal.
3. Overall, our ability to conduct successful sex reversal trials at volunteer hatchery facilities was mixed. State agency hatchery staffs (and in one case a federal hatchery technician) experienced in rearing a specific Consortium target species proved capable of conducting several feminization trials (Brown Trout and Walleye) with planning and logistical assistance by Consortium staff. However, successful trials (in terms of high sex reversal rates) were not accomplished for the remaining three species. In a single case, negative results may have been due to a simple memory error by an experienced agency employee. In the

other two instances (Carp and Lake Trout), failure to develop may have been related to the suite of treatment protocols developed by Consortium staff. In one trial for Common Carp, an agency offered up a facility with a history of poor fish survival for over six months, unbeknownst to the Consortium staff prior to study initiation.

4. A general problem with the various trials completed was getting enough space allotted at the facilities to grow out fish past the initial feminization rate experiment to maturity. This was a problem at virtually all sites where we successfully feminized fish (SD, CO, IA, KS and USFWS ND). In all these cases, we simply had to take what space we could get in order to get the basic feminization work done. Thus, in almost all cases we had to sacrifice E2 treated fish leftover from the initial feminization experiment before they (and associated Control fish) had actually matured, a problematic result. For example, in the case of Brown Trout, we have results from several states indicating that a 20 mg/kg, 120 d treatment protocol will work quite well for feminization. However, due to limited rearing space (and in one instance excessive pre-spawning mortality of three YO fish) we were unable to clearly confirm that male fish treated with the above recipe will produce satisfactory numbers of eggs. Any state or federal facility initiating a YY Brown Trout broodstock will have to obtain this knowledge and adjust feed or rearing protocols as maturity data eventually becomes available for their specific location.
5. Another lesson learned over these years is that doing sex reversal trials in facilities of states unable or unwilling to subsequently develop the associated broodstock results in a stranded investment. By the end of their first sex reversal trial, several of the individuals we worked with were capable of moving on to broodstock development and many were enthusiastic, but their newfound skills were not destined for use. The lone exception to this situation is in Colorado where, with the support of his Bureau Chief, the hatchery manager overseeing two years of Brown Trout trials eventually agreed to take on the second Brook Trout broodstock. In any event, it is difficult to see how the stranded investment situation could have been avoided. As noted above, we were forced to work with how many months of time and space agencies volunteered for our initial sex reversal experiments. Hatchery capacity, expertise and commitment will continue to be a challenge as additional broodstocks are developed and implemented.
6. A final lesson learned is a familiar one to state and federal agencies, that being the brain drain that occurs when older staff retire. While not well published experts in the sex reversal literature, Mamer and Schill have amassed considerable knowledge in the feminization arena and that experience is now being lost. To offset this loss, future feminization trials conducted on behalf of the Consortium should be contracted out as is being done for the upcoming Common Carp feminization work recently begun in Utah by Dr. Chad Teal. Dr. Teal has experience in sex reversing trials in two other invasive fish species as a result of his non-WAFWA funded Doctoral research conducted in concert with his primary two advisors, Scott Bonar and Dan Schill.

Appendix B-

Summary Report

for

Western Association of Fish and Wildlife Agencies

on

WAFWA Brown Trout Program

Period: FY20 – FY24

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31 December 2024

APPENDIX B: Summary Report for WAFWA Brown Trout Program (FY20 – FY24)

[Please note Table & Figure numbering relates to this document specifically
and may differ from numbering in individual fiscal year reports]

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WAFWA Brown Trout Program FY20

FY20 Overview & Background

The Brown Trout is considered one of the 100 worst invasive species worldwide (Lowe et al. 2000) and use of moderate population suppression via electrofishing alone is unlikely to eradicate a population (Saunders et al. 2015). In addition, the species is famously less vulnerable to angling and associated overexploitation; thus, it represents a good possible target for employment of the YY Male approach. There has been a single published attempt to feminize Brown Trout, that being the early effort of Ashby (1957) who attempted immersion treatment at two concentrations. Unfortunately, there were few survivors in these two trials (N < 14) and no effects of E2 were observed. The effect of concentration and duration of Methyltestosterone treatment of feed at 0.5 and 3.0 mg/kg on masculinization rate has been evaluated (Chevassus and Krieg 1992) though again with poor results and small N's. Based on the recommendations of Feist et al. (1996) and successful results of a higher concentration of E2 in topcoated feed (20 mg/kg) in other salmonids (Simpson 1975; Johnstone et al. 1979; Schill et al. 2016) we designed trials at or near this concentration and also examined treatment duration in the present study.

FY20 Methods

BY19 Sex Reversal Trial

During November and December 2019 sex reversal trials using Estradiol (hereafter E2) on treatment groups of swim-up Brown Trout were initiated in hatcheries in Colorado and South Dakota. The first trial was conducted with G. Schisler, State Fish Research Supervisor, Colorado Parks and Wildlife, and B. Neuschwanger, Hatchery Manager, at the Colorado Fish Research Hatchery (COFRH), Bellvue, Colorado. In this trial, three test groups consisting of 1200 Control fish (four replicates of 300) and two 1200 treatment groups (four replicates of 300 for each group) were stocked and reared indoors in 75 L flow-through aquaria. Study fish were fed dry pelleted feed (Bio-Oregon) for the course of the study. Treatment group feed was topcoated with 20 mg/kg feed E2 solution diluted with non-denatured ethanol (EtOH), using a hand-held sprayer (Schill et al. 2016). Control feed was topcoated with pure EtOH at the same concentration as treatment groups. The treatment groups were fed E2 coated feed for either 30 or 60 days, beginning at first feeding (approximately 30 DPH). A sister study of similar design (four replicates per treatment group) was started at the McNenny Fish Hatchery (MFH), working with M. Barnes, Hatchery Manager, and J. Voorhees, Assistant Hatchery Manager, operated by South Dakota Game Fish & Parks in Spearfish, South Dakota. The dose of E2 applied to the feed varied at either 20 or 30 mg/kg feed for a fixed period of 60 days, beginning at first feeding (Table 1). At COFRH, eggs were hatched out in heath trays and fry counted into aquaria prior to swim-up. In South Dakota, eyed eggs were counted out and placed into 400 L flow-through circular tanks, where they then hatched out. However, on 27 February 2020 at 85 DPH, due to MFH facility operational needs, fish were relocated to 100 L flow-through circular tanks where, on 13 April 2020 at 129 DPH, they were culled to 200 fish per replicate. A sample for histological analysis of five random fish from each replicate was taken at this time.

I. Table 1. Sex reversal trial framework for Brown Trout receiving Estradiol (E2) via treated dry feed at two different facilities, initiated Winter 2019.

Facility		Dosage E2	Days on TX feed
CO Fish Research Lab	Short	20 mg/kg	30
	Long	20 mg/kg	60
	Control	0 mg/kg	60 (EtOH only)
McNenny Fish Hatchery	Low	20 mg/kg	60
	High	30 mg/kg	60
	Control	0 mg/kg	60 (EtOH only)

On 25 March 2020 at 139 DPH, staff at COFRH PIT-tagged, genetically clipped and collected biometric measurements from 75 fish from each replicate tank (300 per treatment, 900 total). An additional five random fish per tank were sampled for histology. For future preliminary sampling, 60 fish each from the 60-day exposure group and the Control group were differentially marked, using a pelvic clip and an Ad clip, respectively. All tagged and marked fish were relocated into indoor 300 L troughs for growout. On 16 June 2020 fish currently held at MFH were PIT-tagged, measured and a surrogate sample of fish clipped for preliminary sampling. They will be maintained in a common garden raceway until relocation to the D.C. Booth National Fish Hatchery, Spearfish, SD, for additional growout under the care of C. Martinez, Hatchery Superintendent, US Fish & Wildlife Service.

FY20 Sex Marker Work

Methods

To develop genetic sex tests or sex markers for the five species of initial interest to the WAFWA Consortium, IDFG's Eagle Fish Genetics Lab (EFGL) used existing Y-chromosome (sdY) DNA sequences available for salmonids or generated new DNA sequence data using Restriction site associated DNA sequencing (RADseq). For the RADseq work, mature adult fish of wild origin were collected by various personnel, killed via anesthetic overdose, necropsied and visually sexed. Fin tissues were only taken from fish with clearly identifiable gonads and were placed on numbered Whatman filter paper sheets for storage. DNA was subsequently extracted from the fin tissue of Brown Trout, Lake Trout, Common Carp and Walleye by EFGL personnel and cut into fragments using specific restriction enzymes. Selected DNA fragments were then sequenced so that the exact order of nucleotides (i.e. A, C, T, G) was determined. These sequences were then compared between phenotypic males and phenotypic females to find specific single nucleotide polymorphisms (SNPs) specific to each sex. For more detailed example descriptions of the procedures used by EFGL personnel in conducting species-specific analyses see Appendix A1.

Background and Sample Collections

The objective of genetics work in FY2019 was initially to develop Y chromosome-linked markers that would permit the differentiation of XX and XY individuals and secondarily, to initiate work to develop bi-allelic sex markers that would allow differentiation of XY and YY fish. In FY2020, the primary work objective was to refine developed sex markers and screen them against a sample of fish from 3-5 populations for each species to assess marker accuracy over a wider range of populations across WAFWA states (Figure 1).

In 2019, the EFGL developed an sdY presence/absence marker from sdY sequences on Genbank and demonstrated its accuracy by screening 53 phenotypic male and 42 phenotypic female Brown Trout from the South Fork Snake River (Mamer and Schill 2019, EFGL- Appendix A1). This marker (BrownT Sex) should assist in the future development of an YY Broodstock. In addition, a candidate bi-allelic sex marker was identified (Stru9767_37_15) through RADseq that successfully differentiated XY and YY individuals. In FY2020, our objective was to continue optimization of this marker and test its' accuracy on samples from 5 Brown Trout populations (Table 2).

II. Figure 1. Geographic location of Brown Trout, Lake Trout, Northern Pike, and Walleye populations sampled for final refinement of sex markers to be used in development of YY Male broodstocks.

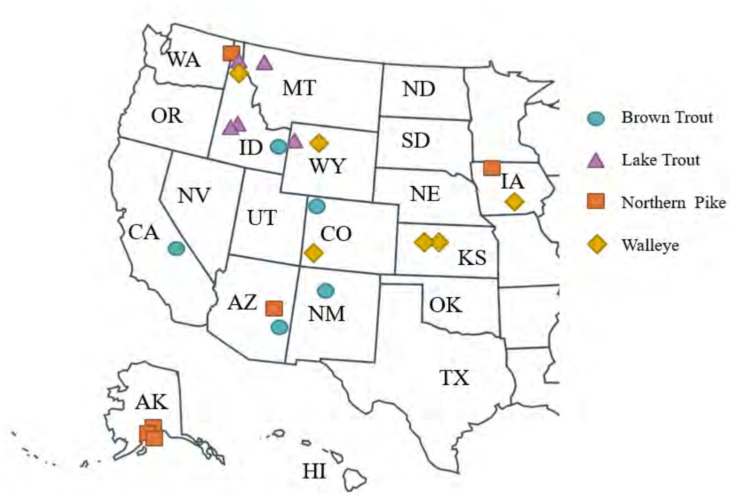
III. Table 2. Waters, state, sampling date and N's for individual populations of Brown Trout involved in sex marker development, 2018-2020.

Species	**Water**	**ST**	**Collection Date**	**n**
Brown Trout	WF Black River	AZ	Apr-18	198
Brown Trout	Owens River	CA	Nov-19	193
Brown Trout	Yampa River	CO	Sep-19	106
Brown Trout	SF Snake River	ID	Oct-18	200
Brown Trout	SF Snake River	ID	Oct-18	106
Brown Trout	Rio Cebolla	NM	Sep-19	146

FY20 Results and Discussion

BY19 Trial Results

Undertaking trials at two facilities allows for more recipe testing without overly burdening a given facility, and also provides a measure of safety in regard to unforeseen aquaculture hazards or miscues. The basic trial design involves a test of E2 treatment duration in CO (30 vs 60 days) and a test of dosage (20 vs 30 mg/kg) in SD. There is an overlapping treatment at both facilities (20 mg/kg for 60 days starting at first feeding), the best guess option based on prior salmonid studies including the successful Brook Trout recipe of Schill et al. (2016). Preliminary CO trial results indicate that both mean weight and length of the 30



d duration trials closely matched those of Controls (Table 3), while growth of the 60 d treatment fish was slightly depressed. The 60 d result is not necessarily cause for concern and may in fact be a sign of proper or optimal treatment levels as E2 sex reversed fish, or even exposed genetic females, often experience reduce initial growth. Preliminary SD results show a slightly different response with length and weight of both treatment groups experiencing slightly less growth than the Control group (Table 4). All fish measured in these preliminary samples were subsequently preserved in formalin for subsequent histology. Sampling of study fish to evaluate the efficacy of sex reversal recipes (sex ratios of treatment groups relative to Controls) will be conducted at both hatcheries later in 2020, while smaller subsamples of trial fish from each study group will be retained for growout to full maturity.

IV. Table 3. Preliminary length and weight results 138 DPH for a subsample of Brown Trout following 30- or 60-day E2 exposure versus Controls, Colorado Fish Research Hatchery, CO, 24 March 2020.

Treatment	Tank #	Ave L (mm)	Ave Wt (g)	n
30 Day	T5	78.6	5.3	5
	T6	80.6	6.0	5
	T7	80.6	5.9	5
	T8	81.4	5.7	5
30 Day Total		80.3	5.7	20
60 Day	T9	76.0	4.5	5
	T10	77.2	5.0	5
	T11	75.0	4.4	5
	T12	71.8	4.0	5
60 Day Total		75.0	4.5	20
Control	T1	83.6	6.9	5
	T2	78.0	5.1	5
	T3	78.0	5.1	5
	T4	81.6	5.5	5
Control Total		80.3	5.63	20

V. Table 4. Preliminary length and weight results 128 DPH for a subsample of Brown Trout following 20- or 30 mg/kg E2 exposure for 60 d versus Controls, McNenny State Fish Hatchery, SD, 12 April 2020.

Treatment	Tank #	Ave L (mm)	Ave Wt (g)	n
20 mg	5	64.7	2.7	5
	6	66.5	2.6	5
	7	61.0	2.2	5
	8	55.8	1.6	5
20 mg Total		62.0	2.3	20
30 mg	1	59.7	2.2	5
	2	59.5	2.2	5
	3	62.5	2.7	5
	4	62.1	2.5	5
30 mg Total		61.0	2.4	20
Control	9	62.9	2.5	5
	10	67.7	3.1	5
	11	64.9	2.9	5
	12	68.0	3.0	5
Control Total		65.9	2.9	20

APPENDIX B: Summary Report for WAFWA Brown Trout Program (FY20 – FY24)

Sex Marker Development

This year EFGL's work on Brown Trout was chiefly an effort to test the accuracy of the prior year's sex marker assay developed from a single population across a broader geographic group of WAFWA states (Figure 1). Details of the work including methods and more specific results are presented by EFGL staff in Appendix A. For all five populations combined, the presence/absence marker proved quite accurate with a 96% concordance rate between predicted sex and observed phenotype. Concordance ranged from 95 to 100% for four populations in Idaho, New Mexico, California and Arizona. On the Yampa River in Colorado, concordance was estimated to be 80% though the veracity of this estimate is limited by sample size (see details in Appendix A). Additional Yampa River samples would be desirable before concluding that the existing sex marker is measurably less accurate there than for the other populations. The sex marker easily appears accurate enough across a broad sweep of WAFWA states to permit straight-forward development of YY Male broodstocks for this species in most localities. Even in populations where the sex marker-phenotype is not 100 % concordant, we would only spawn concordant fish and subsequently would expect that the marker would correctly identify genetic sex for progeny from these crosses (M. Campbell, EFGL, personal communication).

In addition to assisting with YY Male broodstock development, the current brown trout sex marker is of sufficient accuracy to track population sex ratios for all age classes of Brown Trout, particularly fry and fingerlings. Such intensive sex ratio tracking through time including immature year classes is an important part of a monitoring effort evaluating a YY Male stocking effort (Curtis Roth, IDFG, personal communication). If state or federal entities get involved in such monitoring, but lack an internal genetics lab for their analysis, the situation is not overly troublesome. Costs for genetic sample processing have recently decreased in the commercial genetics lab market and should approach $5 U.S. per fish for a sex test, assuming assay parameters are provided to them (Mathew Campbell, personal communication).

The EFGL staff evaluated two potential biallelic SNP candidates for Brown Trout over the past year (see Appendix A for details). In short, neither candidate was successful. However, the lab identified four additional candidate biallelic markers which will be evaluated during FY2021.

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FY20 Appendix A – EGL Brown Trout Sex Marker Development

(K. Coykendall, T. Delomas; IDFG Eagle Fish Genetics Lab EFGL)

Assay Development

We have developed a presence/absence genetic sex marker assay for Brown Trout as part of work to develop YY male technology for this species (Project I180007). We are in the process of developing a bi-allelic assay as well. Two separate methods were used to develop both genetic sex marker assays:

1) We designed an assay within the sex determination gene on the Y chromosome (sdY), previously identified in salmonids (Yano et al. 2013). In its most complete form, this assay will quantitatively discriminate between females (XX; no amplification) and males (XY, YY; amplification). Details are below.
2) We completed a restriction site-associated DNA sequencing (RADseq) study to identify candidate biallelic markers to discriminate between XX, XY, and YY individuals future YY male experiments. We discuss these two approaches in more detail below.

Sex marker on the Y chromosome

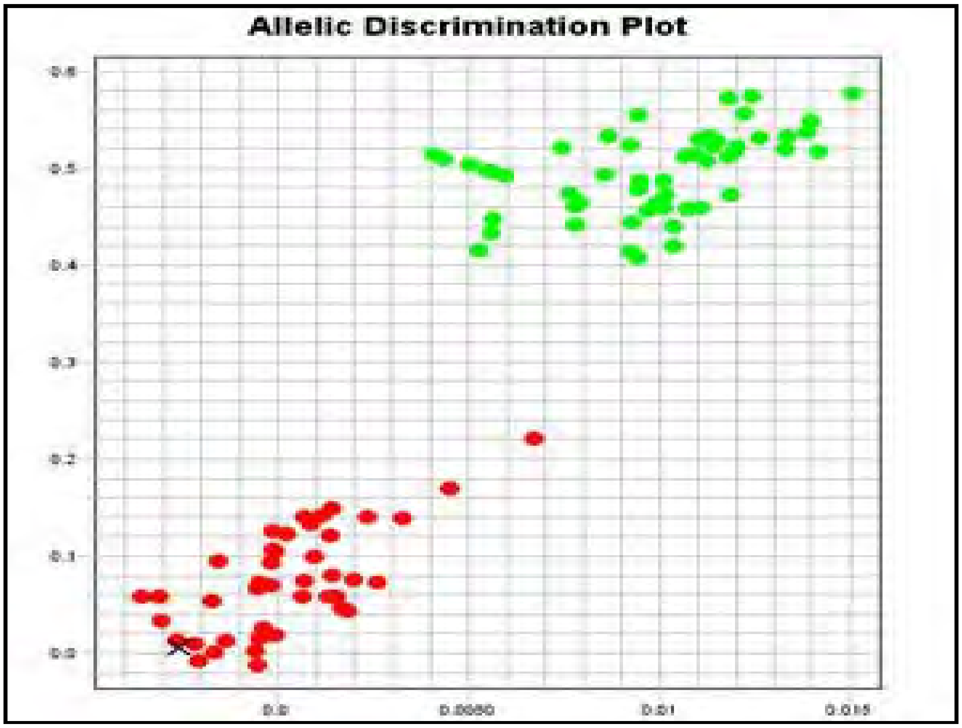
The sex determination gene, sexually dimorphic on the Y chromosome (sdY) has been found in a number of salmonids, including Brown Trout, *Salmo trutta* (Yano et al. 2013). To develop a genetic marker that interrogates the sdY gene in brown trout, we aligned sequences of sdY from Genbank and designed primer and probe sequences from this aligned sequence. The primer and probe sequences are as follows:

BrownT_sdY_F: 5'-TACTGCGAAGAGGAGGTGCT-3'
BrownT_sdY_R: 5'-GGTTGAACGGTCAGAGGAGA-3'
BrownT_sdY_P: 5'-AAGCCCTTCTCCCTGATGAT-3'

The probe is labeled with the fluorophore, FAM, on the 5' end. BrownT_sdY was amplified on a real-time PCR instrument (ABI 7500; Applied Biosystems). Each reaction contained 5 µL of TaqMan® Universal PCR Master Mix, 0.2 µM of forward and reverse primers, 0.15 µM of each probe 1 µL of genomic DNA (5 - 50 ng/ µL), and DNase-free water to bring the total volume to 10 µL. The PCR cycling conditions included an initial denature at 95 °C for 10 minutes, and then 30 - 55 cycles of 92 °C for 15 seconds (denature), and 59 °C for 1 minute (annealing), followed by a 4 °C hold for 10 minutes.

The Appendix A – Figure 1 shows the assay run on 42 phenotypic females (red) and 53 phenotypic males (green) from the South Fork Snake River, Idaho.

VI. Appendix A – Figure 1



The y-axis represents fluorescent signal from the FAM probe. In this case, we expect all fish with a Y-chromosome to have a strong signal along this axis. Fish lacking a Y-chromosome should correspond with low values on the y-axis. Because there is only a single probe in this assay (no probe for an X chromosome marker), there is low/no signal expected along the x-axis. This assay is designed to be qualitative. The next step will be to include a positive control - a marker roughly the same length as BrownT_sdY - that will amplify in both sexes equally. This will allow for discrimination between samples that failed to amplify (degraded DNA, laboratory error, etc.) versus those lacking a Y chromosome. Nevertheless, in this assay, we observed a clear separation between the two sexes. This coupled with no observations of phenotypic females with males or vice versa is a strong indication that this assay discriminates between genetic sexes in brown trout from the South Fork Snake River in Idaho.

To further explore whether this assay discriminates between sexes in other populations, we screened 687 samples of known phenotypic sex from four populations of Brown Trout: Owens River, California sampled in 2019 (StrOWEN19C); Rio Cebolla, New Mexico sampled in 2018 and 2019 (StrRIOC18C and StrRIOC19C); Yampa River, Colorado sampled in 2019 (StrYAMP19C); West Fork of the Black River, Arizona sampled in 2018 (StrWFBR18C); and the South Fork Snake River, Idaho sampled in 2019 (StrSFSN19C).

We used values of ΔRn, defined as the fluorescent signal from the FAM probe divided by the signal from the passive, reporter probe. Since there was no reporter probe present in our assay, the ΔRn value is not truly normalized. We used the supervised machine learning algorithm, knn (k nearest neighbors), to build a model that predicts sex from ΔRn values. We performed the analysis using the programming language R version 3.6.3 (R Core Team 2020) and R packages class (Venables et al. 2002), gmodels (Warnes et al. 2018), and caret (Kuhn, 2020). We split the dataset into a training set and a testing set. The algorithm uses the data from the training set to predict the sex within the testing set. When the model is predicting which group the unknown sample belongs to, it considers the closest points of known sex surrounding the unknown sample. The parameter, k, is the number of nearest neighbors the algorithm should take into account when predicting the value of the unknown. We typically chose the square root of the sample size rounded either up or down to the nearest odd number to avoid ties. In some cases, we tested different k-values to ascertain whether it had an effect on the accuracy of the model.

Individuals from each sampling group were analyzed together even though in some cases, they were run in separate assays. In most cases, data were split into training and testing datasets in an 80% (training) to 20% (testing) ratio with the exception of StrWFBR18C (90%/10%) and StrYAMP19C (75%/25%). Other caveats to note are the small sample sizes, in general, of the training and testing datasets within each analysis and the random draw component of the analysis. Each time the analysis was run, the training and testing datasets were drawn randomly from the dataset. Therefore, re-running the analysis will not necessarily yield the same accuracy. The results from several runs will fluctuate more with smaller sample sizes if there is variation in the dataset. Accuracies for predicting sex based on probe signal ranged from 0.8 to 1.0 (Table 1). The lowest accuracy resulted from the StrYAMP19C analysis. Note that this was also the sample group with the lowest sample size (57) and subsequently the lowest testing and training set sample sizes (42 and 15, respectively) and the most imbalanced sex ratios, which can lead to lower accuracies. Also, we assume that all phenotypic sex calls are accurate and so discrepancies between phenotypic sex and predicted sex result are due to a failure of the model. However, it could be that the phenotypic sex was misidentified, or the fish has intersex characteristics.

Overall, this assay performs very well for brown trout populations from Idaho, Arizona, New Mexico, and California. Although the accuracy drops to 80% for the Colorado population, greater samples sizes and more balanced sex ratios may increase the observed accuracy.

Future Directions

We are working on adding primers and probes to BrownT_sdY from non sex-linked markers. We have three options to test: CytB_Str_Capo, CytB_Carim, (both from the cytochrome b gene in the mitochondrial genome) and RNA Salmonid EF1-α (elongation factor 1 alpha subunit from the nuclear genome). This will allow for the assay software to assign “heterozygous” or “homozygous” calls to each sample based on fluorescent signal by providing a positive control to act as a baseline. The results of the knn analysis lead us to believe that refining the assay this way is a worthy undertaking.

RADseq for Bi-allelic sex marker identification

The presence/absence marker such as the one described above can discern between XX and XY samples. With known YY genotypes, we may be able to use quantitative PCR to discriminate XY and YY individuals in brook trout using this same marker (cite). However, a more straightforward approach would be to identify biallelic markers that interrogate both the X and Y chromosomes. This would allow unambiguous discrimination of XX, XY, and YY individuals for future YY Male broodstock development as well as allow improved field experiment monitoring.

To identify potential candidate bi-allellic SNP markers in Brown Trout, we used RADseq methods on brown trout samples of known sex captured from the South Fork Snake River, Idaho in 2018. RADseq was performed using methods adapted from Ali et al. (2016), using the restriction enzyme PstI. We attached a short, unique piece of DNA, i.e. barcode, to DNA library fragments from each sample so that DNA from several different samples could be pooled and sequenced simultaneously, then teased apart afterwards. A total of 10 females and 10 males were split into four separate libraries that were run on an Illumina NextSeq using mid-300 v2 output sequencing kits, with an expected output of 32-39 Gigabases. We took the resulting DNA sequences through the Stacks pipeline (Catchen et al. 2013), a tool that sorts the millions of DNA fragments from each library and each sample and puts them into bins that correspond to locations across the genome. These stacks of sequences are searched for single nucleotide polymorphisms (SNPs). The pipeline consists of several steps that sort reads into groups based on which sample they came from, then it looks for identical sequences within individuals and creates a catalog of these loci. Then it looks for these loci among all the individuals. We opted for a minimum depth of coverage (m) of 5, a maximum number of mismatches allowed between stacks (M) within samples varying from 2 to 8 in ustacks, and the number of mismatches allowed between sample loci (n) among samples from 1 to 9 in cstacks, depending on the M-value. The Stacks pipeline is run separately for each of the combinations of M- and n-values. In this case, there were 15 separate runs. The average number of paired end reads used in the stacks analyses was 208,856,270, average number of genotyped loci recovered was 1,254,750 with a mean coverage of reads per locus of 17 and a mean coverage of reads per sample of 173. The average number of loci that contained at least one SNP was 947,387.

The resultant VCF files were used as input into custom Python scripts that looked for patterns of SNP calls that were heterozygous in one sex, homozygous in the other sex, and lacking the third genotype class expected in normal autosomal chromosomal segregation. SNPs that were present in more than three of the 15 run results were ranked higher than those that did not. Candidates were further screened based on the number of individuals that were successfully genotyped at that locus, where the SNP occurred in the locus, and the number of SNPs nearby. Optimal assays of this type require 18 - 25 nucleotide primers that flank a stretch of DNA that should be 80 - 120 nucleotides long. The probes should be 20 - 25 nucleotide long and the SNP should fall as close to the center of the probe as possible. Initial genotyping assays were designed for the top two candidates using Primer3 v 0.4.0 (http://bioinfo.ut.ee/primer3-0.4.0/), but neither were successful.

SNP Biallelic Sex Markers

We have identified four additional candidate SNP biallelic sex markers. These were present in six to eleven of the 15 separate Stacks run and genotyped in 10 - 12 individuals. Primers and probes for these SNPs will be developed as described above for genotyping assays to be run on our ABI 7500 qPCR machine.

Appendix A - Table 1. Results of Presence/Absence sex marker panel results versus observed phenotype for five western USA Brown Trout populations. Sampled collected in 2018-2019.

Sample Collection	Pedigree	Females	Males	Undetermined	$Test_F$	$Test_M$	Accuracy
Owens River, CA (2019)	StrOWEN19C	100	93	0	17/17	21/22	0.97
Rio Cebolla, NM (2018)	StrRIOC18C	48	43	1*	10/10	9/9	1.00
Rio Cebolla, NM (2019)	StrRIOC19C	85	61	0	15/16	14/14	0.97
Yampa River, CO (2019)	StrYAMP19C	37	20	0	8/10	4/5	0.80
W.F. Black River, AZ (2018)	StrWFBR18C	100	78	0	8/8	10/10	1.00
S.F. Snake River, ID (2018)	StrSFSN18C	100	100	0	11/11	8/8	1.00
S.F. Snake River, ID (2019)	StrSFSN19C	50	50	0	11/12	10/10	0.95
	Total	370	317	1	80/84	76/78	0.96

$Test_F$ = the number of phenotypically-sexed females that were correctly assigned to female/ total number of females in the testing dataset

$Test_M$ = the number of phenotypically-sexed males that were correctly assigned to male/ total number of males in the testing dataset

* The single individual with an undetermined phenotypic sex was removed from the analysis.

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WAFWA Brown Trout Program FY21

FY21 Overview

The ability to feminize male fish for subsequent egg production is one of two requirements reported necessary for undertaking development of a Trojan Y Chromosome or YY Male broodstock (Cotton and Wedekind 2007). Much of the Consortium work on this topic during the workplan year involved fieldwork and summarization of results from sex reversal trials initiated on Brown Trout in Fall 2019 in South Dakota and Colorado, along with a follow-up trial at the Colorado facility begun in Fall 2020.

FY21 Methods

BY19 Sex Reversal Trial - Colorado

During November and December 2019 sex reversal trials using Estradiol (hereafter E2) on treatment groups of swim-up Brown Trout were initiated in hatcheries in Colorado and South Dakota. The first trial was conducted working with B. Neuschwanger, Hatchery Manager, at the Colorado Fish Research Hatchery (COFRH), Bellvue, Colorado and G. Schisler, State Fish Research Supervisor, Colorado Parks and Wildlife. In this trial, three test groups consisting of 800 Control fish (four replicates of 200) and two 800 fish treatment groups (four replicates of 200 for each group) were stocked and reared indoors in 75 L flow-through aquaria (Table 5). Trial eggs were hatched out in heath trays and fry counted into aquaria prior to swim-up. Mortality rate was documented daily. Treatment group feed was topcoated with a 20 mg/kg feed E2 solution diluted with non-denatured ethanol (EtOH), using a hand-held sprayer (Schill et al. 2016). Control feed was topcoated with pure EtOH at the same concentration as treatment groups. The treatment groups were fed E2 coated feed for either 30 or 60 days, beginning at first feeding (approximately 30 DPH).

On 25 March 2020 at 139 DPH, fish were PIT-tagged, fin clips taken for genetics, and total length and weight collected from 75 fish from each replicate tank (300 per treatment, 900 total). All tagged fish were then relocated into indoor 300 L troughs for continued growout. On 1 Oct 2020 at 329 DPH it was determined from a preliminary sample that fish were developed enough to perform the primary sampling event. Accordingly, on 27 Oct 2020 project personnel and COFRH staff sampled 716 fish at 355 DPH for total length, weight, visual phenotype, sexual maturity level, intersex observations and gonad weight. Gonad tissue for histology was taken from the first 30 fish measured in each tank.

The above sampling left 180 fish (average 15 fish per treatment group) for continued grow out to examine relative time to maturity for sex reversed versus Control fish and also possible crossing of sex reversed F_{XY} Males with standard XY Males to determine initial progeny survival and sex ratios. Initially these growout fish would be examined at 2 years of age, though it is possible or perhaps likely that feminized males may not mature until age 3.

VII. Table 5. Sex reversal trial framework for Brown Trout receiving Estradiol (E2) via treated dry feed at two different facilities, initiated Winter 2019.

Facility		Dosage E2	Days on TX feed
CO Fish Research Lab	Short	20 mg/kg	30
	Long	20 mg/kg	60
	Control	0 mg/kg	60 (EtOH only)
McNenny Fish Hatchery			
	Low	20 mg/kg	60
	High	30 mg/kg	60
	Control	0 mg/kg	60 (EtOH only)

BY19 Sex Reversal Trial - South Dakota

A sister study of similar design (four replicates per treatment group) was initiated at the McNenny Fish Hatchery (MFH), working with M. Barnes, Hatchery Manager, and J. Voorhees, Assistant Hatchery Manager, operated by South Dakota Game Fish & Parks in Spearfish, South Dakota. The dose of E2 to be applied to the feed varied at either 20 or 30 mg/kg feed for a fixed period of 60 days, beginning at first feeding (Table 5). Eyed eggs were counted out and placed into 400 L flow-through circular tanks, where the fish hatched out and were subsequently treated as above. On 29 February 2020 at 85 DPH, due to facility operational needs, fish were relocated to 100 L flow-through circular tanks where, on 13 April 2020 at 129 DPH, they were culled to 200 fish per replicate. Mortality rate was monitored daily.

On 14 June 2020 at 191 DPH fish were PIT-tagged (75 per tank, 300 per tx group), measured (total length, weight) and a surrogate sample of fish clipped for future preliminary sampling (“Canaries”; 10 per tank, 40 per tx group; Control Ad clip, Low tx – left pelvic, High tx – right pelvic). On 15 Jul at 222 DPH all trial BRT were transferred to the D.C. Booth National Fish Hatchery (DCB), Spearfish, SD, for additional growout under the care of C. Martinez, Hatchery Superintendent, US Fish & Wildlife Service. On 7 Oct 2020 at 306 DPH a preliminary sample of 27 fish representing some from each treatment group, was taken to assess development histologically as it was determined while sampling that phenotype could not be discerned visually. Given this finding, primary sampling was delayed until late March 2021 to allow for further development.

On 30 Mar 2021 at 480 DPH, project personnel assisted by staff at DCB performed the primary sample of 60 fish from each treatment group. Data collected included total length, weight, visual phenotype, sexual maturity level, intersex observations and gonad weight. Gonad tissue for histology was taken from the first 30 fish measured in each tank.

The above sampling left 122 fish (average 10 fish per treatment group) for continued grow out to examine relative time to maturity for sex reversed versus Control fish and also possible crossing of sex reversed F_{XY} Males with standard XY Males to determine initial progeny survival and sex ratios. Initially these growout fish would be examined at 2 years of age, though it is possible or perhaps likely that feminized males may mature not mature until age 3.

BY20 Sex Reversal Trial - Colorado

Based on the results from the BY 2019 efforts at both facilities (see results below), a follow-up trial was initiated at the Bellvue Colorado facility in Fall 2020. The main thrust of this effort was to extend the exposure duration in hopes of improving the feminization rates obtained in the prior year's trial. Based on input from P. Smith (Novaeel Inc.) we were also interested in whether a lower E2 dosage than those used in the BY2019 trial would be equally or more efficacious if combined with longer treatment duration and thus more acceptable in the FDA's approval process.

The 2020 trial consisted of 10 treatment groups experiencing various concentrations of E2 and exposure length, plus a Control group and a Control Interval group with 10 fish sampled every 30 days for histological preservation to document timing of gonadal differentiation (Table 6). To produce study fish, brood fish were genetically sampled then spawned on 6 Oct 2020 by Glenwood Springs Hatchery. Eggs were shipped to COFRH on 16 Nov 2020 where they were mixed and placed in heath trays for hatching. Prior to swim-up, hatched-out fry were counted into 75 L flow-through aquaria. The study fish were fed dry pelleted feed (Bio-Oregon, transitioning to Rangen and Skretting) for the course of the study. Treatment group feed was top-coated with E2 and EtOH as above. All treatment groups and the Control group feed were top-coated with the same volume of pure EtOH as that received by the highest dosage treatment group. Control Interval feed was not top coated with EtOH. The treatment groups (n = 100 fish) were fed E2 coated feed for varying durations of 60 to 120 days, beginning at first feeding (26 DPH) and mortality rate documented daily.

VIII. Table 6. Sex reversal trial framework for Brown Trout receiving Estradiol via treated dry feed at Colorado Fish Research Hatchery, Bellvue CO, initiated 29 Nov 2020. Numbers in matrix are replicates n's by treatment type (dosage and duration) for each treatment. Fry (n = 100) received either treated or Control feed beginning at swim-up (26 DPH).

		Dosage (mg E2/kg dry feed)					
E2 Duration Level	Duration (days)	10	20	30	60	None (EtOH only)	None (No EtOH)
Short	60			2	2		
Mid	75			2			
Long	90	2	2	2	1		
Max	120		2	2			
Control						2	
Control Interval							1

On 24 April 2021 at 150 DPH, project staff and COFRH personnel collected lengths, weights and genetic fin clips from 75 fish in each replicate tank and subsequently PIT tagged them (1500 total). No preliminary sampling "canary" fish were clipped, as rearing environments could not support any extra fish. At 198 DPH all tagged fish were grouped by treatment and relocated into indoor 300 L troughs for growout. This sex reversal trial is scheduled to be sampled for primary sex reversal results in early October of 2021.

FY21 Results and Discussion

Undertaking trials in two states (CO and SD) allowed for more recipe testing without overly burdening a given facility and also provides a measure of safety in regard to unforeseen aquaculture hazards or miscues. The basic trial design involved a test of E2 treatment duration in CO (30 vs 60 days) and a test of dosage (20 vs 30 mg/kg) for 60 days in SD. There was an overlapping treatment at both facilities (20 mg/kg for 60 days starting at first feeding), the best guess option based on prior salmonid studies including the successful Brook Trout recipe of Schill et al. (2016).

BY19 Trial Results - Colorado

Based on visual examination of gonads nearly 11 months after the trial started, sex ratios in the Control group (n = 239) approximated the expected 50:50 sex ratio at 48.5% female (Table 7). In the 30 d treatment exposure group (n = 238) the female ratio was exactly 50%, while the 60 d exposure group was comprised of 74.3% females. Confidence bounds around the difference between the mean Control versus 30 d treatment ratios (n = 4) were not statistically different (Figure 2). However, the difference between the mean Control and 60 d female ratios (n = 4) did not overlap zero indicating a statistically significant difference for the longer treatment group. Not surprisingly, there was also a statistical difference between the unsuccessful

30 d treatment and the 60 d treatment groups with the latter clearly being preferable (Figure 2). As expected, there were no intersex fish visually observed in the Control group and only two in the 30d treatment while 7 were observed in the 60 d group leading us to suspect that a longer treatment interval might improve on the feminization rate. In addition, our histological examination of 240 fish in the two E2 treatment groups yielded only 10 intersex fish that were not detected visually (4%). As examination of all fish histologically would have minimal effect on "true" feminization rates, we concluded that expensive histological examination of treated fish 11 months or older was unnecessary and thus did not pay for histological sampling of the 13-month-old fish at time of sampling in the South Dakota trial below.

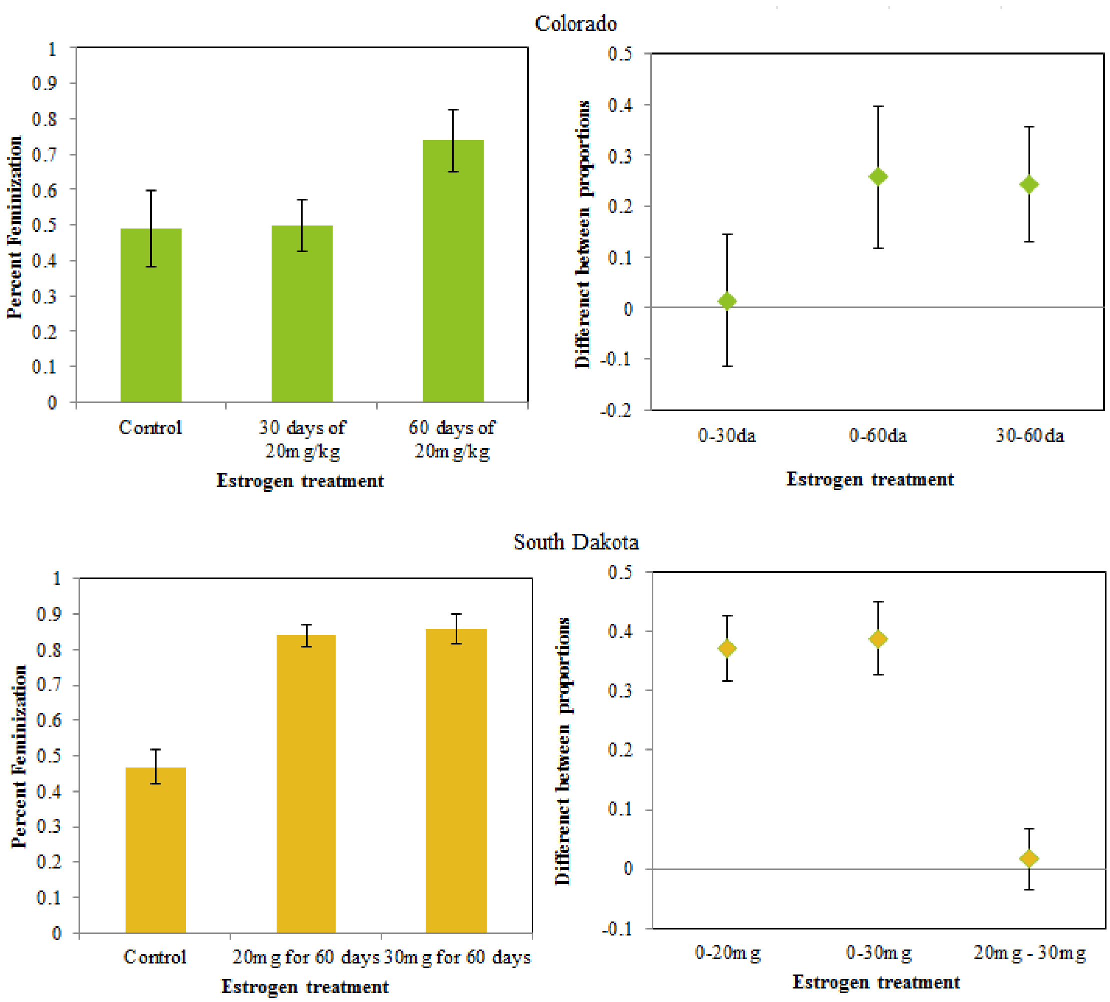
With one exception, mean lengths and weights by sex within study groups declined as the duration of E2 exposure increased from none (Control) to 60d (Table 7). This result is not necessarily cause for concern and may in fact be a sign of successful treatment levels because male fish sex reversed with E2, or even exposed genetic females, often experience reduce initial growth (Schill et al. 2016).

A total of 180 fish from this trial were retained for growout and will be examined this fall to examine maturity rates of E2 treated fish relative to Control fish at two years of age.

Table 7. Percent phenotype ascertained by visual observation of gonads from necropsied Brown Trout, aged 355 DPH, following 30- or 60-day exposure to 20 mg/kg E2 treated dry feed starting at first feeding versus Controls, Colorado Fish Research Hatchery, hatched Nov 2019 – sampled Oct 2020.

			Phenotype								
			Female			Male			Intersex		
Treatment	Tank #	% F	n	Ave L	Ave W	n	Ave L	Ave W	n	Ave L	Ave W
Control	CO 01	33.3%	20	181.8	69.4	40	186.2	81.4			
	CO 02	58.3%	35	181.1	68.7	25	184.5	76.2			
	CO 03	47.5%	28	182.0	70.0	31	178.5	70.2			
	CO 04	55.0%	33	183.3	70.5	27	187.7	81.7			
Control Total		48.5%	116	182.1	69.6	123	184.2	77.6			
30 Day	CO 05	49.2%	30	174.4	61.0	29	182.8	78.6	2	174.0	58.0
	CO 06	42.4%	25	177.2	63.5	34	180.4	71.3			
	CO 07	60.0%	36	177.6	63.5	24	256.2	75.5			
	CO 08	48.3%	29	173.6	59.8	31	178.5	69.8			
30 Day Total		50.0%	120	175.7	62.0	118	195.9	73.5	2	174.0	58.0
60 Day	CO 09	84.5%	49	169.9	57.0	7	179.4	72.9	2	181.0	75.0
	CO 10	68.9%	42	172.0	59.2	17	180.8	72.9	2	182.0	70.8
	CO 11	78.7%	48	166.5	54.5	12	174.3	67.3	1	180.0	68.1
	CO 12	64.9%	37	172.5	59.6	18	178.7	68.9	2	182.5	82.2
60 Day Total		74.3%	176	170.0	57.4	54	178.5	70.3	7	181.6	74.9

Figure 2. Mean proportions (± 95% confidence interval [CI]) of visually sexed female Brown Trout (left panels) following E2 treatment at two facilities; Colorado (hatched 7 Nov 2019, sampled 27 Oct 2020 at 355 DPH) and South Dakota (hatched 6 Dec 2019, sampled 30 Mar 2021 at 480 DPH). Differences between the female proportions (± 95% CI; right panels) were calculated as the mean proportion of the greater exposure groups minus the mean proportion for the lower exposure groups. Values above the zero line indicate that fish in the greater exposure groups contained larger proportions of female fish as determined via visual examination. Differences were considered statistically significant if the 95% CI did not overlap the zero line.



BY19 Trial Results - South Dakota

Based on visual examination of gonads nearly 15 months after the trial start, sex ratios in the Control group (n = 238) approximated the expected 50:50 sex ratio at 47.1% female (Table 8). In the 20 mg/kg (n = 224), the female ratio was 84.2% while the 30 mg/kg group was only slightly higher at 85.8% female. Mean difference in this proportion female (n = 4) between the Control versus 30 d treatment and between the Control and 60 d treatment groups were both statistically significant as their differences did not overlap zero (Figure 2). Sixteen intersex fish were visually observed in the 60 d group, again leading us to suspect that a longer treatment interval might improve on the feminization rate. Not surprisingly, less intersex fish (8) were observed in the higher 60 d, 30mg/kg E2 feed concentration group.

Table 8. Percent phenotype ascertained by visual observation of gonads from necropsied BY19 Brown Trout, aged 480 DPH, following 60-day exposure to either 20 or 30 mg/kg E2 treated dry feed starting at first feeding versus Controls, McNenny Fish Research Hatchery, Spearfish SD, hatched Dec 2019 – sampled Mar 2021.

			Phenotype								
			Female								
Treatment	Tank #	% F	n	Ave L	Ave W	n	Ave L	Ave W	n	Ave L	Ave W
Control	SD 09	54.2	32	189.6	77.0	27	186.8	67.1			
	SD 10	45.0	27	188.5	67.4	33	186.5	69.8			
	SD 11	44.1	26	190.0	70.1	33	183.0	64.4			
	SD 12	45.0	27	196.3	76.6	33	182.4	67.0			
Control Total		47.1	112	191.0	73.0	126	184.6	67.1			
20 mg	SD 05	83.3	50	194.8	75.3	6	182.8	62.7	4	208.3	93.6
	SD 06	86.4	51	228.8	84.8	4	187.8	69.7	4	194.0	76.9
	SD 07	80.0	48	191.1	71.4	7	190.6	74.5	5	194.6	81.9
	SD 08	86.9	53	193.3	90.2	5	196.2	76.9	3	183.0	62.6
20 mg Total		84.2	202	202.1	80.7	22	189.2	71.0	16	195.7	80.0
30 mg	SD 01	88.3	53	191.4	71.0	7	202.0	89.2			
	SD 02	85.2	52	192.4	76.0	7	181.7	64.8	2	177.0	63.4
	SD 03	89.7	52	192.6	72.8	4	187.5	70.6	2	202.0	75.0
	SD 04	80.3	49	189.3	69.8	8	173.3	55.2	4	195.5	78.0
30 mg Total		85.8	206	191.4	72.4	26	185.5	69.3	8	192.5	73.6

In terms of both length and weight, the relationship of growth among groups was slightly different than that observed in the Colorado trial. Fish exposed 20 mg/kg E2 in feed had slightly higher weights and lengths that either Control or fish in the 30 mg/kg group while fish in this latter group were remarkably similar in size to Control fish (Table 8). The reason for the disparity in these growth relationships between the CO and SD trials is unknown.

A total of 122 fish from this trial were retained for growout and will be examined this fall to examine maturity rates of E2 treated fish relative to Control fish.

FY21 Summary of BY19 Sex Reversal Trials

Taken collectively the sex reversal results of trials at both hatcheries yielded positive results. The 60d, 20 mg/kg treatment in CO yielded a significantly skewed female ratio of 74.3% while the same protocol resulted in an 84.2% ratio in SD. Reasons for the disparity in these results are unknown, but might have to do with differences in the rearing environment such as density or growth rates. The 60 d, 30 mg/kg treatment group in SD was only slightly more effective in feminization (85.8% female) but we observed half the number of intersex fish visually, so the additional 10mg/kg of the drug appeared to be worth it at that facility. The lower feminization rate in the CO 60 d exposure trial (74.3%) would still be adequate to build a broodstock because preliminary genetics sampling in this group suggests that 57 of 107 E2 treated genetic males (55%) sexed visually at the end of the trial produced ovaries. We stress this latter result is preliminary and the final proportion of sex reversed males along with the genetic methods used will be reported in detail in the FY22 report. Assuming these results hold, the sex marker reported in the prior report year (Schill and Mamer 2020) will enable hatchery workers in the future to select out the F_{XY} fish when building a YY Male broodstock as in Schill et al. 2016.

Given the above results and the possibility that the BY20 work could improve upon the already positive 2019 feminization results in this report, Brown Trout have become the best candidate for starting the next YY Male broodstock. Given this reality, additional focus will be paid in FY2022, working in concert with Novaeel Inc., to obtaining FDA authorization to proceed on a YY Male Brown Trout. The most likely route for authorization to start a broodstock will be working in concert with Novaeel Inc. *via* the Indexing process currently undergoing review by FDA's Center for Veterinary Medicine.

FY21 Acknowledgements

For the Brown Trout Feminization trial trials, we give thanks to B. Neuschwanger, G. Schisler, E. Fetherman, T. Davis, and A. Perkins for their nuanced stewardship at COFRH, and to M. Barnes, and J. Voorhees (McNenny FH) and C. Martinez, M. Adams and staff (DC Booth Nat'l FH) for providing vigilant oversight of the product of this multi-facility venture.

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WAFWA Brown Trout Program FY22

FY22 Overview

Much of the Consortium work on this topic during the workplan year involved hatchery fieldwork and summarization of results from sex reversal trials initiated on Brown Trout in Fall 2019 in South Dakota and Colorado along with a follow-up trial at the Colorado facility begun in Fall 2020. Results from the BY19 trial demonstrated successful feminization results but at levels well below those reported for other salmonids. We therefore conducted a follow-up trial in BY20 that included higher dosages and longer durations than used in the BY19 effort.

FY22 Methods

BY19 Sex Reversal Trial - Colorado

During November and December 2019 sex reversal trials using E2 on treatment groups of swim-up Brown Trout were initiated in hatcheries in Colorado and South Dakota. The first trial was conducted working with B. Neuschwanger, Hatchery Manager, at the Colorado Fish Research Hatchery (COFRH), Bellvue, Colorado and G. Schisler, State Fish Research Supervisor, Colorado Parks and Wildlife. In this trial, three test groups consisting of 800 Control fish (four replicates of 200) and two 800 fish treatment groups (four replicates of 200 for each group) were stocked and reared indoors in 75 L flow-through aquaria (Table 8). For detailed methods and reporting of trial feminization rates, see Schill and Mamer (2021). Additional work conducted in 2021 on this trial during the contract period is described below.

IX. Table 8. Sex reversal trial framework for Brown Trout receiving Estradiol (E2) via treated dry feed at two different facilities, initiated Winter 2019.

Facility		Dosage E2	Days on TX feed
CO Fish Research Lab	Short	20 mg/kg	30
	Long	20 mg/kg	60
	Control	0 mg/kg	60 (EtOH only)
McNenny Fish Hatchery			
	Low	20 mg/kg	60
	High	30 mg/kg	60
	Control	0 mg/kg	60 (EtOH only)

Maturity Monitoring

In October 2021, due to drought-limited water availability at COFRH, a decision was made to follow only the Control and long duration exposure study fish through Fall for maturity monitoring. After culling all of the 20mg 30d treatment fish, 97 fish remained (55 Controls, 42 20 mg 60 d) which were sorted by visual phenotype into separate raceways in anticipation of maturation, isolating any feminized genotypic males from accidental spawning with normal males. Female fish were examined for maturity weekly from 11 Nov – 2 Dec 2021. Individuals were scanned for a PIT tag, palpated for ripeness and stripped completely if flowing eggs were present. Any shed PIT tags were reinserted or a new one inserted. After stripping, fish were returned to a common raceway for another year's growth and possible spawning in Fall 2022. Results of 2021 maturity monitoring effort are reported below.

BY19 Sex Reversal Trial – South Dakota

A sister study of similar design was initiated at the McNenny Fish Hatchery (MFH), working with M. Barnes, Hatchery Manager, and J. Voorhees, Assistant Hatchery Manager, operated by South Dakota Game Fish & Parks in Spearfish, South Dakota. The design was the same as the Colorado effort described above except that the two treatment regimes examined differed in the amount of Estradiol fed to the fish (Table 9). For detailed methods and reporting of trial feminization rates, see Schill and Mamer (2021).

Maturity Monitoring

The above sampling effort left 122 fish (average 10 fish per treatment group) for continued growout to examine relative time to maturity for sex reversed versus Control fish. Due to space limitations at McNenny Hatchery, trial fish were relocated to D.C. Booth National Fish Hatchery, C. Martinez, Hatchery Supervisor, in Spearfish, SD, for further growout. Fish were examined by hatchery staff a single time in Oct 2021 (679 DPH) for external sex characteristics (coloration, kype, ovipositor) and the presence, upon palpation, of milt or ovarian fluid. Few ripe fish were observed. Given the maturity results observed in the concurrently running Colorado trial (see below) as well as time constraints for Booth Hatchery staff, it was decided to delay a full maturity monitoring effort for this work until this Fall 2022 at which time all fish will be examined for visual phenotype and gonadal development.

BY20 Sex Reversal Trial - Colorado

Based on the results from the BY 2019 efforts at both facilities which resulted in 74.4 - 85.8% females across treatment groups (Schill and Mamer 2021), a follow-up trial was initiated at the COFRH facility in Fall 2020. The main thrust of this effort was to extend the exposure duration in hopes of improving the feminization rates obtained in the prior trial. In addition, based on input from P. Smith (Novaeel Inc.), we were also interested in whether a lower E2 dosage than those used in the BY19 trial would be equally or more efficacious if combined with longer treatment duration and thus more acceptable in the FDA's approval process.

The 2020 trial consisted of 9 treatment groups experiencing various concentrations of E2 and exposure duration, plus a Control group, and an additional Control Interval group consisting of 10 fish sampled every 30 days for histological preservation to document timing of gonadal differentiation (Table 9). This latter group were sampled in case the BY20 feminization results were poor and future E2 exposure work was needed. To produce study fish, brood fish were genetically sampled then spawned on 6 Oct 2020 by Glenwood Springs Hatchery. Eggs were shipped to COFRH on 16 Nov 2020 where they were mixed and placed in heath trays for hatching. Prior to swim-up, hatched-out fry were counted into 75 L flow-through aquaria in an indoor, photoperiod-controlled lab. The study fish were fed dry pelleted feed (Bio-Oregon, transitioning to Rangen and Skretting) for the course of the study. Treatment group feed was top-coated with E2 and EtOH as above. All treatment groups and the Control group feed were top-coated with the same volume of pure EtOH as that received by the highest dosage treatment group. Control Interval feed was not top coated with EtOH. The treatment groups (n = 100 fish) were fed E2 coated feed for varying durations of 60 to 120 days, beginning at first feeding (26 DPH).

Table 9. Sex reversal trial framework for Brown Trout receiving Estradiol via treated dry feed at Colorado Fish Research Hatchery, Bellvue CO, hatched 29 Nov 2020. Numbers in matrix are replicates n's by treatment type (dosage and duration) for each treatment. Fry (n = 100) received either treated or Control feed beginning at swim-up (26 DPH).

		Dosage (mg E2/kg dry feed)					
E2 Duration Level	Duration (days)	10	20	30	60	None (EtOH only)	None (No EtOH)
Short	60			2	2		
Mid	75			2			
Long	90	2	2	2	1		
Max	120		2	2			
Control						2	
Control Interval							1

On 24 April 2021 at 150 DPH, project personnel and COFRH staff collected lengths, weights and genetic fin clips from 75 fish in each replicate tank and subsequently PIT tagged them (1500 total). Anticipating the possibility that Indexing would soon be more readily allowed by the FDA for Minor Use Species like Brown Trout, we initiated a health inspection based upon a protocol adapted from Novaeel Inc. (P. Smith, Novaeel Inc., pers comm), that provided a suite of health observations to be used to assess possible deleterious effects on fish from exposure to E2. Ten fish per treatment group received an exam to determine Health Index values, from which 5 fish also had length (mm), weight (g) liver weight (g) and tissues taken for

histology (Table 10, Appendix B - Figure 1). A random number table was used in advance to assign which fish netted in sequence from each treatment group would be used in the health exam work. After selection, fish within the separate groups were examined "blind" by project staff in an adjoining room with no knowledge of treatment group when conducting the assessments.

X. Table 10. Health Index matrix with parameters, scoring scale and focused areas of examination used on Brown Trout receiving Estradiol via treated dry feed at Colorado Fish Research Hatchery, Bellvue CO.

Parameter	None	Minor erosion	Medium erosion	Severe erosion
Pectoral fin erosion	4	3	2	1
Anal/caudal/dorsal fin	4	3	2	1
Head and gills	4	3	2	1
Body (lesions/bites)	4	3	2	1
SUM SCORE				

SCORING:
4 – no erosion 3 – minor erosion 2- medium erosion 1 – severe erosion
(*cumulative score could provide an early indication of arising health issues*):
15-16 – Very healthy
11-14 – Healthy
8-11 – Some health concerns – requires further investigation/ obs.
< 8 – Significant health concerns – requires action

After each 75 fish study group was tagged and health study fish examined, all fish remaining in each treatment tank were culled. At 198 DPH all tagged fish were grouped by treatment and relocated by Bellvue staff into separate indoor 300 L troughs for growout. On 6 October 2021 at 312 DPH it was determined from a preliminary sample that fish were not yet developed enough to perform the primary sampling event, and additional time was allowed for growth.

Main Sampling - 366 DPH

On 29 Nov 2021 project personnel returned to the facility and working with COFRH staff, conducted the main sex reversal trial sampling. Data collected during necropsy from 1133 fish at virtually one year (366 DPH) included total length, weight, visual phenotype and intersex observations. Genetic sex of these fish was subsequently assessed by the Eagle Fish Genetics Lab (EFGL) using Brown Trout sex markers developed and field tested against five western U.S. populations with an overall accuracy/concordance rate of 96% (Schill and Mamer 2020).

Health Sampling

Health sample fish, (10 fish per tank) were selected randomly, PIT-tagged, and then placed in a communal raceway to be held until all treatment groups had been sorted. After that time, health exams were performed without prior knowledge of treatment type (as during the sampling at 150 DPH) and the fact that all fish from the 11 study groups were comingled further minimized any potential observer bias. All 10 health sample fish received a health exam (including total length, weight, visual phenotype, sexual maturity level, intersex observations and gonad weight), of which liver weights and tissue taken from five and the remaining fish (5) were preserved whole for possible pathological exam.

FY22 Results and Discussion

BY19 Sex Reversal Trial - Colorado

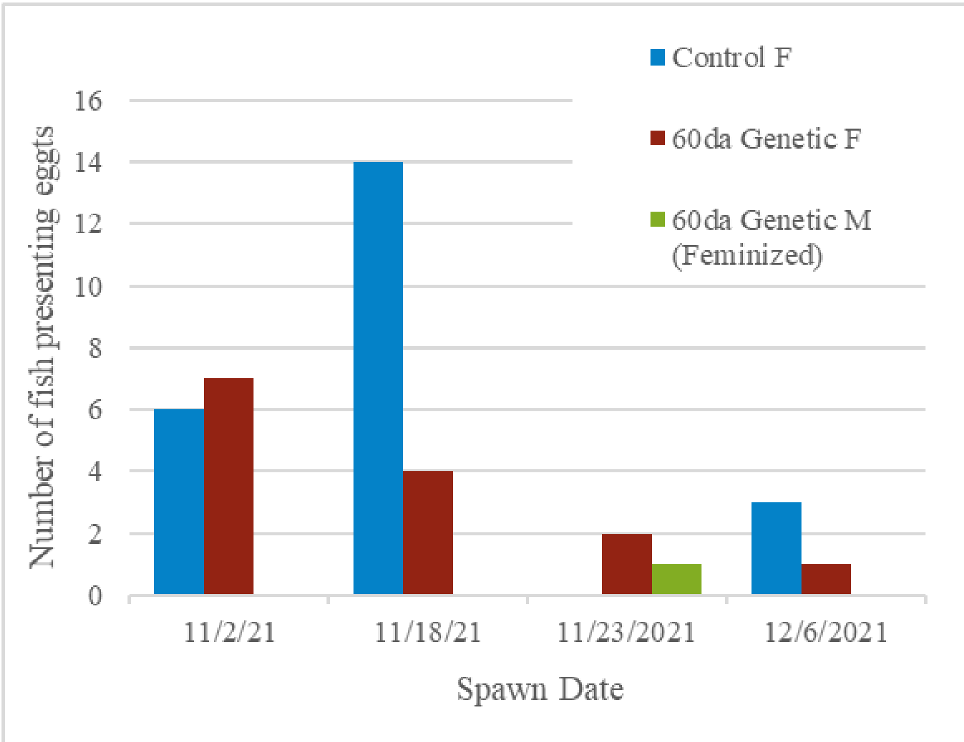
Maturity Monitoring

Of the 41 genetic females examined weekly from 2 Nov – 6 Dec 2021 (age during stripping being 726 – 760 DPH), COFRH staff observed successful maturation of all Control females (n = 23; 100%), and noticeably lower genetic female maturation ratio in the 20 mg 60 d treatment group (n = 18; 77.8%). On the third observation date, towards the end of the monitoring period, a single feminized genetic male (4.8%) matured adequately enough to enable egg stripping. The remaining 20 potential feminized genetic males and 4 genetic females did not produce eggs at any time during monitoring. (Table 11, Figure 3). The fact that feminized males did not mature as quickly as Control or treated females is not surprising based on other species (Schill et al. 2016). These fish are being reared a final year to ascertain what proportion of feminized males successfully produce eggs, whether the eggs are viable and can subsequently be spawned to produce viable YY Brown Trout offspring.

Table 11. Rate of maturation of Brown Trout by treatment type, as demonstrated by the presentation of eggs, respective to phenotype and genotype, when monitored for one month (2 Nov – 6 Dec 2021; 726-760 DPH), rearing at Colorado Fish Research Hatchery.

Treatment	Phenotype	Genotype	n	Presented eggs
Control	F	F	23	23 (100%)
20mg 60da		F	18	14 (77.8%)
		M	21	1 (4.8%)

Figure 3. Maturation timing by treatment type of Brown Trout at 726-760 DPH, as related to genotype and phenotype, Colorado Fish Research Hatchery, Nov 2021.



BY20 Sex Reversal Trial - Colorado

Based on sex marker results, the genetic sex of all trial fish combined (n = 1099) closely approximated 50:50 at 50.4% male. There was a small amount of variation in the percent genotypic males across the 10 treatment groups, ranging from 42.9 to 55.0 % male for combined replicates (Table 12). For those treatments with replicates, we observed variation in phenotypic sex ratios but, overall, there was good concordance in phenotypes and true feminization rates, with minor differences across those treatments (Appendix B - Table 1). Necropsies conducted at 366 DPH revealed that a relatively high proportion of all fish within each E2 treatment group were identified as phenotypic females when replicates were combined, ranging from 78.8 - 97.3 % (Appendix B - Table 1).

Table 12. Percent genotype by treatment type of 1099 Brown Trout, following various E2 exposures and durations, versus Controls, 366 DPH, Colorado Fish Research Hatchery, 29 Nov 2021.

			Genotype (%)	
Treatment	Tank #	n	F	M
Control	15	60	58.3	41.7
	16	59	44.1	55.9
	Total	119	51.3	48.7
30 mg 60 d	8	59	57.6	42.4
	9	58	56.7	43.3
	Total	117	57.1	42.9
60 mg 60 d	11	60	53.3	46.7
	12	59	39.0	61.0
	Total	119	46.2	53.8
30 mg 75 d	2	60	48.3	51.7
	3	58	48.3	51.7
	Total	118	48.3	51.7
10 mg 90 d	19	58	48.3	51.7
	20	60	41.7	58.3
	Total	118	45.0	55.0
20 mg 90 d	13	59	50.0	50.0
	14	55	53.4	46.6
	Total	114	51.7	48.3
30 mg 90 d	6	56	48.3	51.7
	7	57	46.7	53.3
	Total	113	47.5	52.5
60 mg 90 d	10	57	49.2	50.8
20 mg 120 d	17	53	54.5	45.5
	18	58	39.7	60.3
	Total	111	46.9	53.1
30 mg 120 d	4	57	51.7	48.3
	5	56	54.4	45.6
	Total	113	53.0	47.0
Grand Total		1099	49.6	50.4

Consideration of phenotype solely for genetic males reveals true rates of feminization within each treatment (Table 13). Feminization of males ranged from 63.1% for the 10 mg 90 d treatment where the amount of drug used was clearly insufficient, to over 94% for the two 120-day treatments at either drug amount (20 or 30 mg). The third highest feminization rate of true males (81.8 %) was produced by the 20 mg 90 d treatment. Treatment duration thus appeared the more important of the two factors tested in the study. As expected, there were no intersex (IS) fish observed in the Control group. However, the IS condition was common in some treatment groups, particularly in the two trials involving the highest dose of E2 at 60 mg, fed for either 60 or 90 days where the IS rate in genetic males was 17.2 and 20.7 %, respectively. Overall, based on treatment efficacy alone, the two superior treatments were the two 120 d exposure groups which resulted in the highest feminization and lowest IS rates (Table 13).

Table 13. Percent visual phenotype by treatment type of 566 genetically male Brown Trout, following various E2 exposures and durations versus Controls, 366 DPH, Colorado Fish Research Hatchery, CO, 29 November 2021. When available, data from replicates were combined for this summary.

		Phenotype (%)	
Treatment	n	F	IS
Control	58		
30 mg 60 d	51	63.3	10.2
60 mg 60 d	64	64.1	17.2
30 mg 75 d	62	73.3	13.3
10 mg 90 d	66	63.1	13.8
20 mg 90 d	57	81.8	7.3
30 mg 90 d	63	75.4	16.4
60 mg 90 d	30	75.9	20.7
20 mg 120 d	60	94.9	3.4
30 mg 120 d	55	94.4	0.0

We observed an anomalous finding in the study, that being the detection of nine genetic females that presented as phenotypic males at the study conclusion (Table 14). These findings are indicative of either low levels of genotyping error or possibly Environmental Sex Determination (ESD) which can occur in crowded hatchery settings in some species (Degani and Kushnirov 1992). In those situations, females of some species sometimes become phenotypic male due to their inability to produce adequate levels of aromatase which is required to synthesize testosterone into estrogens that subsequently bathe developing PGC cells in genetic females (Luckenbach et al. 2009). The data do not allow for a clear identification of either genetic error or ESD as the cause of the observed data. Low levels of genotype-phenotype discordance (mean = 4%) has been reported by this project for a large sample of Brown Trout across five western U.S. populations (Schill and Mamer 2020). However, the fact that none of these nine anomalies occurred within the four most intense treatments in terms of dose and duration might suggest that fish in those test groups were all able to overcome a possible ESD effect due to additional levels of E2 in their feed.

Table 14. Genotype by visual phenotype of 1099 Brown Trout, following various E2 exposures and durations, versus Controls, 366 DPH, sampled 29 Nov 2021, Colorado Fish Research Hatchery.

		Visual Phenotype		
Treatment	Genotype	F	IS	M
Control	F	58		*3*
	M			58
30 mg 60 d	F	65		3
	M	31	5	13
60 mg 60 d	F	53	2	
	M	41	11	12
30 mg 75 d	F	58		
	M	44	8	8
10 mg 90 d	F	52		1
	M	41	9	15
20 mg 90 d	F	57		2
	M	45	4	6
30 mg 90 d	F	52		
	M	46	10	5
60 mg 90 d	F	28		
	M	22	6	1
20 mg 120 d	F	52		
	M	56	2	1
30 mg 120 d	F	59		
	M	51		3

As expected, growth of test fish exposed to the various hormone treatments was slower than in Control groups. Mean lengths and weights within study groups at both marking (150 DPH) and at one year (366 DPH) were reduced relative to Controls. For example, fish treated at 20 mg for 90 days experienced a 10 % and 26 % decrease in growth in length and weight by the 150 DPH marking event (Table 15). However, by 366 DPH, growth in the same study group of fish trailed Controls by only 1 and 6 % for length and weight, respectively. Similar improvements in growth between the two measurement periods were observed in the longest-running treatments as well (e.g. the 20 mg 120 d group) Thus the prevalence of "catch-up" growth in E2 treated fish was observed as in previous trials (Schill and Mamer 2021) and may in fact be a sign of successful treatment levels because male fish sex reversed with E2, or even exposed genetic females, often experience reduce initial growth (Schill et al. 2016).

Table 15. Comparison of length and weight from date of PIT-tagging (150 DPH) to the final sample (366 DPH), and the resulting percent relative gain when compared to Controls at the end of this time period, of Brown Trout having been exposed to various doses and durations of E2, Colorado Fish Research Hatchery, CO, 2021.

		150 DPH		366 DPH	
Treatment	n	L(mm)	Wt (g)	L(mm)	Wt (g)
Control	119	85.4	6.9	157.9	42.4
30 mg 60 d	117	80.3	5.6	157.4	42.2
60 mg 60 d	119	79.3	5.4	153.8	40.0
30 mg 75 d	118	78.1	5.1	157.8	43.0
10 mg 90 d	118	79.3	5.5	153.2	38.0
20 mg 90 d	114	76.8	5.1	155.0	39.8
30 mg 90 d	113	74.2	4.5	150.4	37.0
60 mg 90 d	57	70.8	4.0	151.6	38.8
20 mg 120 d	113	72.2	4.7	149.0	36.4
30 mg 120 d	111	67.8	3.6	145.3	34.6

Based on Hepatosomatic Index (HI) trends, we saw no evidence of long-term impact of exposure to E2 on liver weight from the treatment regimens evaluated. Control fish HI's averaged 0.23 at 150 DPH, only 5 days after treatment in the longest duration group had ceased, while HI's of treated fish, when compared to Controls, were depressed slightly in most (but not all) treatment groups (Table 16). However, one year into the experiment (366 DPH), HI's had all increased, but were generally quite homogenous, across both treated and Control groups. Indeed, the HI for the most intense E2 treatment group (30 mg 120 d) was identical to Controls at 0.36.

Table 16. Hepatosomatic index of from Brown Trout having been exposed to various doses and durations of E2, 150 and 366 DPH, Colorado Fish Research Hatchery, CO, 29 Nov 2021.

Treatment	DPH	n	HI
Control	150	20	0.23
	366	19	0.36
30 mg 60 d	150	20	0.21
	366	18	0.42
60 mg 60 d	150	20	0.16
	366	20	0.40
30 mg 75 d	150	19	0.18
	366	20	0.36
10 mg 90 d	150	17	0.26
	366	20	0.30
20 mg 90 d	150	20	0.17
	366	20	0.38
30 mg 90 d	150	20	0.16
	366	20	0.30
60 mg 90 d	150	18	0.14
	366	10	0.33
2 0mg 120 d	150	20	0.26
	366	20	0.32
30 mg 120 d	150	18	0.15
	366	20	0.36

In terms of the other health factors examined, in the treated groups, there were generally more downward rankings for the two fin rankings than for the body and head/gills/eye variables. There were very minor reductions in 3 of 9 pectoral fin ranking for treated fish at 150 DPH, but more sizeable reductions were evident at 366 DPH, particularly for longer treatment regimens where rankings ranged from 3.1 to 3.5 relative to the 3.8 rank for Controls (Table 17). Other fin rankings for treated fish were all below that of Controls at 150 DPH, but this was not the case at 366 DPH where fins for two treatment regimens nearly met or exceeded Control rankings, suggesting a reduction in long-term E2 effects. At 366 DPH there were no reductions in head/gill/body rankings with the exception of the 20 mg 120 d group which experienced a slight but likely insignificant reduction (Table 17). Body health rankings showed no ill effects due to E2 exposure.

Table 17. Health Index by treatment from a subsample of Brown Trout having been exposed to various doses and durations of E2, when compared to Controls, 366 DPH, Colorado Fish Research Hatchery, CO, 29 Nov 2021. See Appendix B - Fig 1 for Health Index description.

Treatment	n	Pectoral Fins	Other Fins	Head/Gills/Eyes	Body
Control	19	3.8	3.1	3.8	4.0
30 mg 60 d	18	3.9	3.3	3.8	4.0
60 mg 60 d	18	3.3	2.6	3.9	4.0
30 mg 75 d	19	3.7	2.6	3.9	4.0
10 mg 90 d	17	3.5	2.0	3.9	3.9
20 mg 90 d	18	3.4	2.7	3.8	4.0
30 mg 90 d	19	3.4	2.8	3.9	4.0
60 mg 90 d	10	3.3	2.8	3.9	4.0
20 mg 120 d	20	3.5	2.4	3.6	4.0
30 mg 120 d	20	3.1	3.0	3.9	4.0

Maturity status observations demonstrated that few Brown Trout will be mature by 1 YO. Of the fish that were mature at time of 366 DPH Health sampling (7%, n = 11), all exhibited the male phenotype, and the majority were from the Control or shorter duration treatment groups (Table 18). It is worth noting that while females may differentiate earlier than males, it appears that the masculine maturation process proceeds much more quickly as expected given the salmonid literature.

Table 18. Gonad maturation level of 178 Brown Trout, 366 DPH, after exposure to varying levels of Estradiol, at the time of Health Index examination, Colorado Fish Research Hatchery, 29 Nov 2021.

Matura-tion Status	Phenotype	Control	30 mg 60 d	60 mg 60 d	30 mg 75 da	10 mg 90 d	20 mg 90 d	30 mg 90 d	60 mg 90 d	20 mg 120 d	30 mg 120 d
IMM	F	7	16	16	16	16	17	18	10	19	18
	IS			2	1	3	2	1			
	M	6			1	1	1	1		1	1
MAT	M	6	2	2	2						1

FY22 Summary- BY20 BRT Sex Reversal Trial

Based on feminization of genetic males, our results demonstrate that Brown Trout need longer exposure to E2 than Brook Trout to attain high rates of feminization. The two 120-day treatment periods resulted > 94% feminization of genetic males with < 3.4% intersex. The next best feminization and intersex results were observed in the 20 mg 90 d trial at 81.8% and 7.3%, respectively. However, ascertaining the best of these protocols will ultimately depend on growout. A total of 285 fish from the various treatment tanks remained alive at the cessation of the sex reversal trial. These tagged fish are being reared in a communal raceway and will be examined in Fall 2022 and 2023 to ascertain long-term survival, growth and time to maturity. The best treatment protocol will depend, in large part, upon survival and maturity schedules of fish from the above three groups.

Given the above results, the fact that the BY20 work did improve upon the already positive feminization results of the BY19 trial (Schill and Mamer 2021), and the availability of a broadly functioning sex marker for the species (Schill and Mamer 2020), Brown Trout have become the best candidate for undertaking the next YY Male broodstock. Given this reality, additional focus will be paid in FY22 and FY23, working in concert with Novaeel Inc., to obtaining FDA authorization to proceed on a YY Male Brown Trout. The most likely route for authorization to start a broodstock will be *via* the Indexing process which has recently undergone several positive changes by the FDA's Center for Veterinary Medicine.

FY22 Acknowledgements

Again, as in FY21, we give thanks to B. Neuschwanger, G. Schisler, E. Fetherman, T. Davis, and A. Perkins for their continued vigilant stewardship at COFRH, and to M. Barnes, and J. Voorhees (McNenny FH) and C. Martinez, M. Adams and staff (DC Booth Nat'l FH) for helping maintain these important studies.

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FY22 Appendix B

Appendix B - Table 1. Genotype and visual phenotype of 1099 Brown Trout following various E2 exposures and durations versus Controls, and subsequent demonstration of phenotypic shift of genetic males, 366 DPH, Colorado Fish Research Hatchery, CO, 29 November 2021.

			Genotype (%)		Visual Phenotype (%)			Feminization of Genotypic Males (%)			
Treatment	Tank #	n	F	M	F	IS	M	n	F	IS	M
Control	15	60	58.3	41.7	53.3	0.0	46.7	25			100
	16	59	44.1	55.9	44.1	0.0	55.9	33			100
	Total	119	51.3	48.7	48.7	0.0	51.3	58			100
30mg 60da	8	59	57.6	42.4	84.7	3.4	11.9	25	72.0	8.0	20.0
	9	58	56.7	43.3	79.3	5.2	15.5	26	54.2	12.5	33.3
	Total	117	57.1	42.9	82.1	4.3	13.7	51	63.3	10.2	26.5
60mg 60da	11	60	53.3	46.7	81.7	8.3	10.0	28	64.3	14.3	21.4
	12	59	39.0	61.0	76.3	13.6	10.2	36	63.9	19.4	16.7
	Total	119	46.2	53.8	79.0	10.9	10.1	64	64.1	17.2	18.8
30mg 75da	2	60	48.3	51.7	90.0	6.7	3.3	31	80.6	12.9	6.5
	3	58	48.3	51.7	82.8	6.9	10.3	31	65.5	13.8	20.7
	Total	118	48.3	51.7	86.4	6.8	6.8	62	73.3	13.3	13.3
10mg 90da	19	58	48.3	51.7	79.3	8.6	12.1	31	63.3	16.7	20.0
	20	60	41.7	58.3	78.3	6.7	15.0	35	62.9	11.4	25.7
	Total	118	45.0	55.0	78.8	7.6	13.6	66	63.1	13.8	23.1
20mg 90da	13	59	50.0	50.0	84.7	3.4	11.9	30	76.7	6.7	16.7
	14	55	53.4	46.6	94.5	3.6	1.8	27	88.0	8.0	4.0
	Total	114	51.7	48.3	89.5	3.5	7.0	57	81.8	7.3	10.9
30mg 90da	6	56	48.3	51.7	89.3	7.1	3.6	31	80.0	13.3	6.7
	7	57	46.7	53.3	84.2	10.5	5.3	32	71.0	19.4	9.7
	Total	113	47.5	52.5	86.7	8.8	4.4	63	75.4	16.4	8.2
60mg 90da	10	57	49.2	50.8	87.7	10.5	1.8	30	75.9	20.7	3.4
20mg 120da	17	53	54.5	45.5	96.2	1.9	1.9	25	91.7	4.2	4.2
	18	58	39.7	60.3	98.3	1.7	0.0	35	97.1	2.9	0.0
	Total	111	46.9	53.1	97.3	1.8	0.9	60	94.9	3.4	1.7
30mg 120da	4	57	51.7	48.3	98.2	0.0	1.8	29	96.6	0.0	3.4
	5	56	54.4	45.6	96.4	0.0	3.6	26	92.0	0.0	8.0
	Total	113	53.0	47.0	97.3	0.0	2.7	55	94.4	0.0	5.6
Grand Total		1099	49.6	50.4	82.9	5.2	11.9	566	68.1	9.9	22.0

Appendix B - Figure 1. Health Index for fish condition

Health Index (HI):

Scale

4 - no erosion 3 - minor erosion 2- medium erosion 1 - severe erosion

Parameter	None	Minor erosion	Medium erosion	Severe erosion
Pectoral fin erosion	4	3	2	1
Anal/caudal/dorsal fin	4	3	2	1
Head and gills	4	3	2	1
Body (lesions/bites)	4	3	2	1
SUM SCORE				

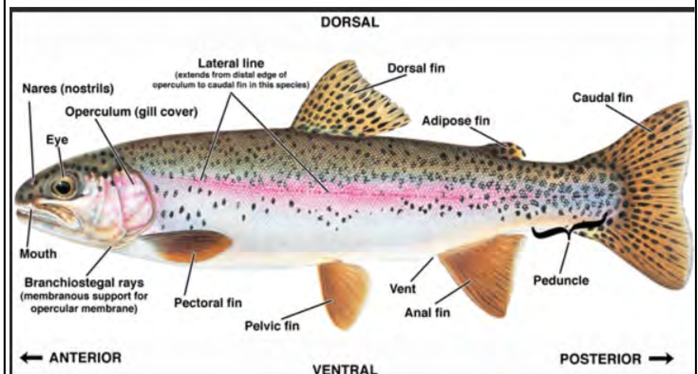
SCORING:
(*cumulative score could provide an early indication of arising health issues*):

15-16 – Very healthy

11-14 – Healthy

8-11 – Some health concerns – requires further investigation/ obs.

< 8 - Significant health concerns - requires action



WAFWA Brown Trout Program FY23

FY23 Overview

The ability to feminize male fish for subsequent egg production is one of two requirements reported necessary for undertaking development of a Trojan Y Chromosome or YY Male broodstock (Cotton and Wedekind 2007). Consortium work on sex reversal during FY22 involved hatchery fieldwork and summarization of final spawning results from sex reversal trials initiated on Brown Trout in Fall 2020 at the Colorado Research Hatchery (COFRH) in Bellvue CO, along with a follow-up trial begun in Los Ojos, NM in Winter 2023.

FY23 Methods

Based on the recommendations of Feist et al. (1996) and successful results of a higher concentration of E2 in topcoated feed (20 mg/kg) in other salmonids (Simpson 1976; Johnstone et al. 1979; Schill et al. 2016) we designed trials in BY19 at or near this concentration and also examined several typical treatment durations. These trials were conducted at the McNenny Fish Hatchery (MFH) in South Dakota and at the Colorado Fish Research Hatchery (COFRH), Bellvue, Colorado. Results from the BY19 trial demonstrated statistically greater proportions of phenotypic females in Treatment vs Control groups at both facilities. However, apparent feminization rates of treatment groups at both facilities were well below those reported for other salmonids (Schill and Mamer 2021; Voorhees et al. 2023). We therefore initiated a follow-up trial at CO in BY20 that included higher dosages and longer durations than used in the BY19 efforts. The main thrust of this effort was to extend the exposure duration in hopes of improving the feminization rates obtained in the BY19 trial. In addition, based on input from P. Smith (Novaeel Inc.), we were also interested in whether a lower E2 dosage than those used in the BY19 trial would be equally or more efficacious if combined with longer treatment duration and thus more acceptable in the FDA's approval process

Feminization rate and spawning/maturity observations for sex reversed 2-year-old fish in the BY19 trial are summarized in Schill et al. (2022). In this report we summarize results for BY19 study fish at 2.9 years of age only, in addition to similar observations on the now 2YO BY20 fish.

BY19 – Colorado

Maturity Monitoring at 2.9 Years Old

The BY2019 Brown Trout feminization trial was begun in Winter 2019 (Schill & Mamer, 2021), evaluating two E2 treatment regimes along with a Control group (Table 19). In October 2021, due to drought-limited water availability at COFRH, a decision was made to follow only the Control and long duration (20 mg for 60 days) exposure study fish through Fall of 2022 for maturity monitoring. Beginning on 11 October 2022, eighty-one approximately 3-year-old fish were examined by CO Bellvue hatchery staff, sorted to phenotype and held separately, being examined weekly from 1 – 17 Nov 2022 for maturation status. Morphological and maturational characteristics were noted, specifically looking for the ability of both Control and feminized males to express eggs, and, if presented, egg quality and volume. Should a phenotypic female present with eggs, a fecundity was calculated. From these, a sub-sample of 4 ripe females (three Control F_{XX}'s and a single F_{XY}), were stripped and an attempt made to fertilize these eggs with fresh sperm collected that day from ripe BY20 Control group XY males on station. Families were kept separate so that, once fertilized, egg-to-fry performance could be monitored. At 25 DPH, tissues (fin clips, n = 100 per family) were collected to potentially confirm the successful production and survival of YY Brown Trout to 25 DPH. Identification of successful YY production would be evidenced by 75% of progeny in the single XY by FXY cross being genetically identified as male. The genetic results of this effort are not yet available.

Table 19. Timeframe of sampling as related to Days Post Hatch (DPH), maturation monitoring and spawning efforts performed on the BY2019 Brown Trout Feminization Trial fish held at CO Fish Research Hatchery, Bellvue, CO. Initial examinations for maturity were made at approximately 2 Years Old (YO) and 2.9 YO.

Event	Date	DPH
Hatch Date	11/07/19	
PIT tagged & Preliminary Sampling	03/24/20	138
Sampling at ~1YO	10/27/20	355
Maturity Monitoring First Sort to Phenotype at ~2 YO	10/06/21	699
Maturity Monitoring and Spawning Period Start	11/02/21	726
Maturity Monitoring and Spawning Period End	12/06/21	760
Maturity Monitoring Census at ~ 2.9 YO	10/11/22	1069
First stripping	11/01/22	1090
Final sampling	11/17/22	1106

Growth & Health Indices Sampling

On 17 November 2022, after the conclusion of maturity monitoring and spawning (see above), the remaining 3 YO fish (n = 81) of this trial were examined. Total lengths taken, fish were necropsied, and a visual health inspection performed, following the Health Index protocol established in Schill et al (2022). All fish were subsequently culled, and the trial ended.

BY20 – Colorado

A similar approach to that of the BY19 trial fish, maturation monitoring and egg performance was undertaken by COFRH staff during Winter 2023. In review, the 2020 trial consisted of 9 treatment groups experiencing various concentrations of E2 and exposure duration, plus a Control group (Table 20). For a detailed summary of methods and results obtained from this trial during FY21 and FY22 see Schill and Mamer (2021) and Schill et al. (2022). Briefly, three highly efficacious treatment protocols were identified with two such treatments approaching 95% sex reversal of genetic male fish. The key remaining question from this trial then becomes how well sex reversed Brown Trout produced during the most efficacious treatment regimes (as measured at 366 DPH) mature and what will their fecundities be?

Table 20. Sex reversal trial framework for Brown Trout receiving Estradiol via treated dry feed at Colorado Fish Research Hatchery, Bellvue CO, hatched 29 Nov 2020. Numbers in matrix are replicate n's by treatment type (dosage and duration) for each treatment. Fry (n = 100) received either treated or Control feed beginning at swim-up (26 DPH).

		Dosage (mg E2/kg dry feed)					
E2 Duration Level	Duration (days)	10	20	30	60	None (EtOH only)	None (No EtOH)
Short	60			2	2		
Mid	75			2			
Long	90	2	2	2	1		
Max	120		2	2			
Control						2	

Maturity Monitoring at 1.9 Years Old

Beginning on 11 October 2022, 183 ~2-year-old fish were sorted to phenotype and held separately, being examined weekly from 1 – 28 Nov 2022 for maturation status by CO Bellvue hatchery staff (Table 21). Morphological and maturational characteristics were noted, specifically looking for adequate egg expression, and if observed, egg quality and volume. Should a phenotypic female present with eggs, a fecundity was calculated and, from these a sub-sample of nine ripe females (three Control F_{XX}'s and six F_{XY}'s) were stripped and an attempt made to fertilize with fresh sperm collected that day from ripe BY19 Control group XY males on station. Families were kept separately so that, once fertilized, egg-to-fry performance could be monitored by hatchery staff. At 25 DPH, tissues from four families (fin clips, n = 100 per family) were collected to quantify production of YY Males. Eggs from the remaining five F_{XY} families were then reared to the 'strong eye' stage, pooled and shipped to Los Ojos State Fish Hatchery, (New Mexico dept of Game and Fish, A. Delp, Hatchery Manager) for the beginning of a new feminization trial (see below).

Table 21. Timeframe of sampling, maturation monitoring and spawning efforts performed on the BY2020 Brown Trout Feminization Trial fish held at CO Fish Research Hatchery, Bellvue, CO. Initial examinations for maturity were made at approximately 2 Years Old (YO), spawning undertaken, followed by transfer to receiving hatchery, Los Ojos Fish Hatchery, Los Ojos, NM.

Event	Date	DPH

Hatch Date	11/29/2020	0
First day on Feed	12/24/2020	25
PIT tag & Preliminary Sampling	4/27/2021	150
Additional Preliminary sampling	10/5/2021	311
Main Sampling	11/29/2021	366
Maturity Monitoring Period Start at ~ 2 YO	10/11/2022	682
Spawning Begins	11/2/2022	704
Visual Health Inspection	11/18/2022	720
Spawning Event for NM Trial	11/28/2022	730
Final Maturity Monitoring	12/12/2022	744
Shipped Trial Fish to NM	1/13/2023	776

Growth & Health Indices Sampling

After a visual examination for external health indices (Schill et al 2022), 183 BY20 trial fish at 1.9 years old were relocated to Los Ojos State Fish Hatchery, (New Mexico Dept of Game and Fish, A. Delp, Hatchery Manager) on 18 Jan 2023 for future maturity/spawning evaluation by NM state personnel.

BY20 Brown Trout Spawning and BY22 Sex Reversal Trial – New Mexico

Background

New Mexico has expressed strong interest in leading the development of a YY Brown Trout Broodstock in their state. The intent of this effort was to familiarize New Mexico hatchery staff with the use of E2 for feminizing the species, develop experience in the methods for spawning feminized male salmonids, and to confirm the performance of two efficacious treatments identified in the CO BY20 trial in a NM hatchery facility under different rearing conditions.

Methods

BY20 Spawning – New Mexico

On 13 Jan 2023 Los Ojos Fish Hatchery staff drove to COFRH to receive and bring to New Mexico 183 2YO fish from the BY20 Brown Trout feminization trial. These fish are to be reared for maturity and spawning evaluation in Fall 2023 by NMDGF personnel. The fish sent to NM are from treatment groups that span the BY20 Feminization Trial framework, and of all phenotype-genotype combinations (Table 22). Those fish with unknown phenotypes (U_{XX}, U_{XY}) that did not display definitive external sexual characteristics at 2 YO will be examined in Fall 2023 for phenotype confirmation. Spawning efforts will be focused on the three most efficacious treatment groups in terms of sex reversal in CO (20 mg 90 d, 20 mg 120 d and 30 mg 120 d) while the lowest treatment regime (10 mg 90 d) will be likewise monitored and examined to document low E2 dose effects and Health Index documentation. Maturation schedules, spawning performance, fecundities and egg performance of feminized XY males will be compared to that of Control (untreated, XX) females.

Table 22. Treatment groups and Pheno-Geno condition of Brown Trout transported to Los Ojos Fish Hatchery, Los Ojos, NM, from BY20 BRT CO Feminization Trial. The fish of primary spawning interest include genetic females (F_{XX}) and feminized genetic males (F_{XY}).

	Pheno-Geno Condition (number of fish)					
Treatment	F_{XX}	F_{XY}	M_{XY}	U_{XX}	U_{XY}	Total by Treatment
Control	8		12			20
20mg 90da	10	11	2			23
20mg 120da	6	4	1	2	1	14
30mg 120da	1	3			1	5
10mg 90da	8	5	3		1	17
30mg 60da	13	6	3	2		24
30mg 75da	8	9	7	1	3	28
30mg 90da	10	7	1	1	3	22
60mg 60da	9	5	5		3	22
60mg 90da	4	2		1	1	8
Grand Total	77	52	34	7	13	183

BY22 Sex Reversal Trial – New Mexico

As a result of successful spawning of five BY20 BRT feminized CO males with BY19 normal males, as mentioned above, on 18 Jan 2023, eggs at 20 days post fertilization were shipped overnight to the Los Ojos Fish Hatchery in Los Ojos, NM, to identify the most effective treatment for Brown Trout feminization at this facility. Given New Mexico space and staff time availability, only two of the three most efficacious E2 treatments from the BY20 CO trial were chosen for evaluation (Table 23). Eggs of these families were pooled then split between MacDonald jars for hatching and released at 20 DPH into troughs for growout.

Table 23. Sex reversal trial framework for Brown Trout receiving Estradiol via treated dry feed at Los Ojos Fish Hatchery, Los Ojos, NM, hatched 8 Jan 2023. Numbers in matrix are replicates n's by treatment type (dosage and duration) for each treatment. Fry (n = 688 per tank) received either treated or untreated feed beginning at swim-up (40 DPH).

		Feed Treatment Dosage	
E2 Duration Level	Duration (days)	20 mg/kg E2	None

Short	90	1	
Long	120	1	
Control			1

In this trial, two feed treatment groups of different durations, 90- or 120-days exposure, and one Control group were reared in three rectangular rearing troughs (15.5’ x2 2’ x 9”, approximately 154 gal) with flow through water (6 gpm, 49 - 50 °F well water), covered with insulating lids to inhibit jumping once fish were able. Study fish were fed dry pelleted feed (Bio Oregon) for the course of the treatment period. Treatment group feed was topcoated with 20 mg/kg feed E2 solution diluted with non-denatured ethanol (EtOH), using a hand-held sprayer (Schill et al. 2016). The treatment groups were fed E2 coated feed (to satiation, 4 – 6 times daily) for either 90 or 120 days, beginning at first feeding (approximately 40 DPH). Troughs were inventoried at 103, 164 and 235 DPH.

FY23 Results and Discussion

BY19 – Colorado

Maturity Monitoring

Of the 36 genetic females examined weekly from 1– 17 Nov 2022 (age during stripping ~ 3 YO), COFRH staff observed (Table 24) successful maturation of all but one of the Control females (n = 20; 95.7%), and noticeably lower genetic female maturation ratio in the 2 0mg 60 d treatment group (n = 8; 50%). Feminized genetic males were maturing during this same time period though only a single fish matured adequately enough to enable egg stripping (n = 8; 12.5 %).

Table 24. Functional spawning morphology as ~ 3 YO, as demonstrated by the expression of eggs, of BY19 Brown Trout by treatment type, respective to phenotype and genotype, when monitored for two weeks (1-17 Nov 2022; 1090 -1106 DPH), rearing at Colorado Fish Research Hatchery.

Pheno Geno Condition	Treatment	Maturity Condition			Grand Total
		Gave good eggs	Difficult or eggs bad	Didn’t give eggs	
F_{XX}	Control	19		1	20
	60 Day	4		4	8
F_{XY}	60 Day	1	4	3	8

IS_{XY}	60 Day			1	1
Grand Total		24	4	9	37

Functional Morphology and Fecundity

BY19 Control group females had higher fecundities than the feminized males. Three-year-old XX Control females produced an average of 2377 eggs, while feminized XY males produced 28% fewer eggs (mean = 1719), however only 1 F_{XY} produced eggs that were viable (Table 25).

All but four of the ~3YO BY19 fish from which fecundities were calculated had eggs expressed naturally. Those four F XY's that were difficult to express, eggs were removed via vivisection and spawning attempted, but fertilization and further egg survival was not successful.

Despite the apparent inability to release eggs successfully, the rate of maturation indicated by the presence of flowing, or upon dissection of abdominal wall, loose, well-developed eggs inside the body cavity, was high across all F_{XX} and F_{XY} pheno-genotypes (98 %, n = 44). The lone exception was the single intersex fish (IS_{XY}) that was genetically male but presented with both a mature male teste and a female ovary that was immature as indicated by a tighter skein with smaller eggs.

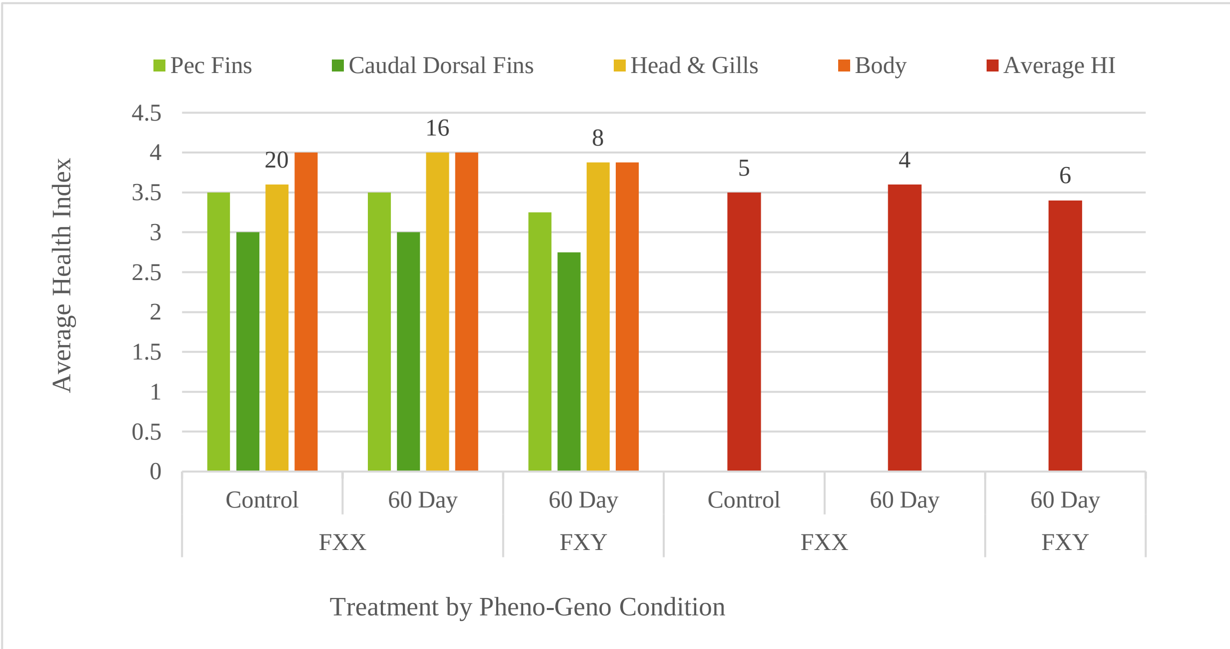
Table 25. BY19 3 YO Fecundity, frequency and results of spawning attempts by true females (F XX) and feminized males (F XY) and treatment group.

Treatment	Fecundity		Strip date and outcome 2022	Gave eggs in 2021
		F XY		
Control	2381		11/2/2022 stripped	yes
	2709		11/2/2022 stripped	yes
	2744		11/2/2022 stripped	yes
	2457		11/2/2022 stripped	yes
	2212		11/2/2022 stripped	yes
	1832		11/2/2022 stripped	yes
	2079		11/2/2022 spawned	yes
	2296		11/2/2022 spawned	yes
	2975		11/2/2022 spawned	yes
	2081		11/2/2022 stripped	yes
Average	2377			
20mg 60da		1785	11/2/2022 spawned	yes
		2440	11/16/22; Killed to spawn; eggs bad	no
		1566	11/16/22; Killed to spawn; eggs bad	no
		1436	11/17/22; Killed to spawn; eggs bad	no
		1370	11/16/22; Killed to spawn; eggs bad	no
Average		1719		

Health Indices

In regard to fish health rankings (FY23 Appendix C), there was a slight reduction in observed fin quality for F_{XY} fish relative to both Control and treated XX females although this effect may have been simply due to the tendency for normal genetic males to be more aggressive than females. It is unknown if such male-centric behaviors would be diminished in feminized males or if the decline is, in fact, related to feminization. Head and gill scores of Control fish were slightly lower relative to the other groups (Figure 4). In terms of overall health scores, there was a very small decrease in the overall ratings of F_{XY} fish, but the difference was minor.

Figure 4. Health Indices for four specific examination areas overall average HI score of BY19 Brown Trout at 2.9 YO, at time of final examination post maturity monitoring at COFRH, Bellvue CO, 17 Nov 2022.



BY20 - Colorado

Maturity Monitoring

On 12 Oct 2022, COFRH staff performed the initial inspection and sort to phenotype of the BY20 BRT, placing all phenotypic females together, separately from males, with any that didn't yet express an identifiable phenotype held with the females. Maturity monitoring began in earnest on 2 Nov and occurred weekly until 28 Nov 2023 (age during stripping ~ 2 YO). Successful maturation was observed in all the Control females (100 %, n = 9), whereas 51 feminized genetic males were maturing during this same time period, with more than half of the F XY's being capable of expressing eggs (51 %, n = 26) of apparent good quality (Table 26).

Table 26. BY20 2 YO Functional spawning morphology and egg quality as observed during maturity monitoring on 1-17 Nov 2023 at COFRH, Bellvue, CO.

	Pheno-Geno Condition									
	F XX					F XY				
Treatment	n	Gave good eggs	Eggs difficult to strip or bad	Didn't give eggs	% gave eggs	n	Gave good eggs	Eggs difficult to strip or bad	Didn't give eggs	% gave eggs
Control	9	8	1		88.9%	-	-	-	-	-
30 mg 60 d						6	4	2		66.7%
60 mg 60 d						5	2	2	1	40.0%
30 mg 75 d						9	4	4	1	44.4%
10 mg 90 d						5	3	1	1	60.0%
20 mg 90 d						11	7	4		63.6%
30 mg 90 d						7	2	3	2	28.6%
60 mg 90 d						2	2			100.0%
20 mg 120 d						4	2	1	1	50.0%
30 mg 120 d						2		2		0.0%
Total	9	8	1	2		51	26	19	6	

BY22 Sex Reversal Trial – New Mexico

A total of 2064 eggs were used in initiating the Los Ojos feminization trial begun in mid-December 2022. Eggs in all study groups hatched on the same date and experienced similar survival to hatch (Table 27) resulting in starting n's of 641, 660 and 645 fish for the Short, Long and Control groups respectively. The study fish went on feed at 40 DPH. Survival of fish in the 20mg, 90-day E2 treatment group was the lowest of the three groups at 103 DPH (78 %), and also remained the poorest at 164 DPH, four days after E2-infused feed use ended in the long trial. The Long treatment group (20 mg 120 d) group survival was intermediate between the Short and Control group mortality at both 164 DPH and 235 DPH. Control survival was well above that in either treatment groups at these same DPH.

Table 27. BY22 hatch and survival rates of eggs received from COFRH on 21 Dec 2022 that were the blended product of five families of BY20 F_{XY} Brown trout crossed with traditional BY19 Control M_{XY} males.

Treatment	Initial n	Date put in jars	Date Hatch	Dead eggs	% Hatch by trough	Survival 103 DPH	Survival 164 DPH	Survival 235 DPH
SHORT 20 mg 90 d	688	12/21/2022	1/8/2023	47	93.2%	78%	15%	14%
LONG 20 mg 120 d	688	12/21/2022	1/8/2023	28	95.9%	90%	23%	21%
Control	688	12/21/2022	1/8/2023	43	93.8%	87%	39%	27%

FY23 Summary - BY19 & BY20 Sex Reversal Trials

Maturation, Spawning, and Viability

BY19 - In general, the ability to obtain viable eggs from feminized three-year old Brown trout from the BY19 trial group was poor at 15 % (n = 8). Half of these fish were indeed mature, but only a single F_{XY} had viable eggs. In retrospect, the COFRH staff suspected that they might not have adequately stripped out these fish in 2021, possibly contributing to the blockages and poor results observed as 3 YO fish.

BY20 - Armed with the knowledge that fish feminized males might require slightly more pressure for spawning, the COFRH staff successfully spawned about half of the fish in the trial (51 %, n = 51) including sizeable numbers from treatment groups (Table 8) with relatively poor initial feminization rates at one year as reported by Schill et al. (2022). It is well worth noting that the staff was able to strip 19 more of these fish although they considered them either "difficult" to strip or had bad eggs. Only 6 of these putative F_{XY}'s (12 %) did not give eggs, again, despite the fact that many of them were from low feminization success groups based solely on the trial results at about 1 year. For this reason, we suggest that Los Ojos NM staff consider examining all CO BY20 F_{XY} fish surviving until three years this fall carefully, either by stripping of the high feminization groups reported by Schill et al (2022) or at a minimum, autopsying and examining gonads of all fish in the remaining groups, paying close attention to note egg appearance and the presence or absence of testes and/or the intersex condition. It is possible that the relatively long 120 d treatment regimes apparently necessary to induce sex reversal rates approaching 95 % (Schill et al. 2022) may not result in large numbers of surviving mature F_{XY} fish. In that case, one of the less efficient treatment regimens may prove more useful for the development of a future broodstock.

FY23 Acknowledgments

For the Brown Trout Feminization trial trials, we give again thanks to B. Neuschwanger, G. Schisler, T. Davis, and N. Yates for their dedicated and attentive stewardship of our multiple year classes of trials they have managed at COFRH. They set the research and good humor standard exceedingly high for all of us.

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FY23 Appendix C

Appendix C - Figure 1. Health Index for fish condition

Health Index (HI):

Scale

4 - no erosion 3 - minor erosion 2- medium erosion 1 - severe erosion

Parameter	None	Minor erosion	Medium erosion	Severe erosion
Pectoral fin erosion	4	3	2	1
Anal/caudal/dorsal fin	4	3	2	1
Head and gills	4	3	2	1
Body (lesions/bites)	4	3	2	1
SUM SCORE				

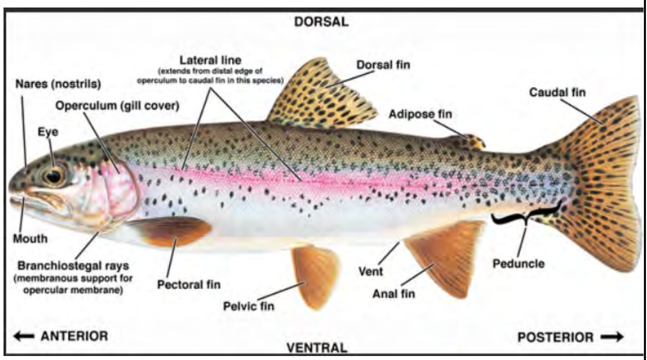
SCORING:
(*cumulative score could provide an early indication of arising health issues*):

15-16 – Very healthy

11-14 – Healthy

8-11 – Some health concerns – requires further investigation/ obs.

< 8 - Significant health concerns - requires action



WAFWA Brown Trout Program FY24

FY24 Overview

The ability to feminize male fish for subsequent egg production is one of two requirements reported necessary for undertaking development of a Trojan Y Chromosome or YY Male broodstock (Cotton and Wedekind 2007). Consortium work on sex reversal during FY23 and FY24 involved hatchery fieldwork and summarization of final spawning results from sex reversal trials initiated on Brown Trout in Fall 2020 at the Colorado Research Hatchery (COFRH) in Bellvue CO, along with a follow-up trial begun in Los Ojos, NM in Winter 2023.

New Mexico has expressed strong interest in leading the development of a YY Brown Trout Broodstock in their state. The intent of the below effort was to familiarize hatchery staff at the Los Ojos State Fish Hatchery, Los Ojos, New Mexico, with the use of E2 for feminizing the species, develop experience in the methods for spawning feminized male salmonids, and to confirm the performance of two efficacious feminization treatments identified in the CO BY20 trial in a NM hatchery facility under different rearing conditions. In addition, the rearing of maturing feminized BRT broodstock from the previous BY20 trial in Colorado is expected to glean more information regarding maturation and spawning from these treatment group fish.

Feminization rate and spawning/maturity observations for sex reversed 2-year-old fish in the BY20 CO trial are summarized in Schill et al. (2023). In the current report, we summarize the work performed at Los Ojos Hatchery in New Mexico. This includes 1) spawning/maturity results for BY20 CO BRT study fish at 3.0 years of age (YO) held at Los Ojos Hatchery, as well as 2) results of rearing progeny from BY20 CO feminized XY male x standard XY male crosses, creating what we anticipated to be the first YY Brown Trout, and 3) the results of the first ever attempted feminization of putative YY male Brown Trout.

FY24 Methods

BY20 CO at NM Maturation Monitoring and Spawning Efforts

On 13 Jan 2023 Los Ojos Fish Hatchery (LOFH) staff assumed care of 183 2YO BY20 CO BRT remaining from the BY20 Brown Trout feminization trial. The genetic sex of these fish was known from prior sex marker work (Schill et al. 2022). The fish were held separately by observed phenotype, ascertained by visual appearance and abdominal palpation, and reared to maturation, and spawning was attempted in Fall 2023 by NMDGF personnel. As reported in FY22 (Schill et al, 2022), the fish holding at LOFH are from treatment groups that span the BY20 Feminization Trial framework, and of all phenotype-genotype combinations. However, for efficiency and space utilization, maturity monitoring and spawning efforts focused on the Control group and the three most efficacious treatment groups in terms of sex reversal in CO (20 mg 90 d, 20 mg 120 d and 30 mg 120 d) while the lowest treatment regime (10 mg 90 d) was utilized for health sampling as required by the State of New Mexico. The intent was to monitor maturation schedules, spawning performance, fecundities, and egg performance of feminized genetic XY males compared to that of Control (untreated, genetic XX) females.

Grow-Out and Maturity Monitoring of BY20 CO at NM at 2.9 Years Old

When maintaining a population of fish in any New Mexico state hatchery, a requirement of the program is to provide to the NM State Health Lab individuals for lethal sampling to assess disease risk at the facility. Due to space limitations as the BY20 fish grew, on 20 Jun 2023 (934 DPH), treated XY females were used to provide these 2023 health sample fish, which also allowed for more tank space for growth in preparation for maturation in Fall 2023. Beginning on 17 October 2023 phenotypic females from the four treatment groups identified above were retained and any remaining fish culled. All retained fish were examined two times for maturation status by LOFH hatchery staff, and morphological and maturational characteristics noted, specifically looking for adequate egg expression, i.e. a functioning oviduct (Table 28), and, if observed, egg quality and volume. Should a phenotypic female present with viable eggs, a fecundity would be calculated, and an attempt made to fertilize with fresh sperm from Saratoga Fish Hatchery, Saratoga WY.

Table 28. Time frames of maturation monitoring and spawning efforts performed on the BY20 Brown Trout Feminization Trial fish held at Los Ojos Fish Hatchery, Los Ojos, NM. Initial examinations for maturity were made at approximately 3 YO.

Event	Date	DPH
Hatch Date	11/29/2020	0
Shipped Trial Fish to NM	1/13/2023	776
First sort for Maturity	10/17/2023	1053
First attempt to strip	11/21/2023	1088
Final stripping attempt	11/28/2023	1095
Final stripping attempt	12/5/2023	1102
Trial ended	12/15/2023	1112

Final Sampling

On 15 December 2023, after the conclusion of maturity monitoring and spawning (see above), the remaining 3 YO fish (n = 7) of this trial were culled. Fish were held frozen until the closure of the BY22 Trial the following Spring 2024 where a brief inspection for oviduct development was performed at that time.

BY22 Sex Reversal Trial – New Mexico

As a result of successful spawning of five BY20 BRT CO feminized genetic XY males with BY19 CO normal XY males by Colorado Fish Research Hatchery in Fall 2022, the eggs of 5 families were pooled and shipped overnight at 23 days post fertilization (strong eye-up stage) to Los Ojos Fish Hatchery (see Schill et al. 2022 for more details). At Los Ojos, eggs were split equally and placed in three MacDonald jars, and survivors reared in three separate troughs. Using the treatment feminization results from the previous year in Colorado, we sought to identify an effective E2 treatment protocol for Brown Trout feminization at the New Mexico facility. At the same time, we expected to verify the creation of YY Brown Trout and attempt their feminization. Our feminization effort was focused on two of the three most efficacious E2 treatments from the BY20 CO trial being chosen for evaluation (Table 29).

Table 29. Sex reversal trial framework for Brown Trout exposed to 20 mg treated feed of varying durations at Los Ojos Fish Hatchery, Los Ojos, NM, hatched 8 Jan 2023. There was a single study group for each treatment and the Controls. Fry (n = 688 per tank) received either treated or untreated feed beginning at first feeding (40 DPH).

		Feed Treatment Dosage	
E2 Duration Level	Duration (days)	20 mg/kg E2	None
Short	90	1	
Long	120	1	
Control			1

Two treatment groups of the same dose of E2 (20 mg) and of different durations, 90- or 120-days exposure, and one Control group, were reared in three rectangular rearing troughs (15.5’ x 2 2’ x 9”, approximately 154 gal) with flow-through water (6 gpm, 8.9 - 9.4 °C well water), covered with insulating lids to inhibit jumping once fish were able. Study fish were fed dry pelleted feed (Bio-Oregon) for the course of the treatment period. Treatment group feed was topcoated with 20 mg/kg feed E2 solution diluted with non-denatured ethanol (EtOH), using a hand-held sprayer (Schill et al. 2016). The treatment groups were fed E2 coated feed (by hand, to satiation, 4 – 6 times daily) for either 90 or 120 days, beginning at first feeding (40 DPH).

Growth and survival of trial fish were tracked and gonadal development first assessed at 1YO to assess differentiation and development as needed for visual inspection to discern phenotype. Based on a few initial necropsies, the decision was made to allow further growout as gonads were too underdeveloped for easy phenotypic identification. On 19 March 2024, project personnel and LOFH staff collected lengths, weights and genetic fin clips from 60 fish from each of the three groups. A visual examination for external health indices (see Appendix D) was systematically performed on every 6th netted fish (Health exam; 10 total per group). In addition, because E2 treatment has sometimes caused liver hypotrophy, liver weights were taken, and a Hepatosomatic index (HSI) calculated using the formula liver weight divided by the fish weight multiplied by 100.

The remaining trial fish surviving to the end of the trial (n = 218) were shipped as fingerlings to University of New Mexico for further grow out and eventual assessment of visual phenotypic and back crossing to confirm production of YY fish (Schill et al 2016).

FY24 Brown Trout Results and Discussion

Maturation and Spawning Efforts – BY20 CO at NM

Due to heavy pre-spawning mortality, likely caused by infections of saprolegnia and stresses related to tank size (M. Ruhl, pers. comm.), only 24 of the 183 fish transferred from CO to NM survived to 3 YO when spawning would be attempted. Checking for ripening occurred on 17 Oct 2023 and monitoring for spawnable fish occurred three times (21 Nov, 28 Nov and 5 Dec) at which point it was halted. All putative feminized males were green during the first monitoring event, and only two had eggs that were strippable, one each at the second and third events (Table 30). The sole Control female that survived was not stripped successfully, as the eggs were poor quality and clumpy. The eggs from the feminized males, both from the 20 mg 90 d treatment group, appeared to be in good condition, so fecundities were calculated (1002 and 2085 respectively) and crosses attempted using traditional male sperm provided. Unfortunately, both families had zero eye-up. The cause of poor eye-up is unknown at this time, but may perhaps be due to stresses related to the adult rearing environment noted above.

Table 30. BY20 3 YO Brown Trout functional spawning morphology and egg quality as observed in those fish that survived to maturity monitoring (21 Nov – 5 Dec 2023) at Los Ojos Fish Hatchery, Los Ojos, NM.

	Pheno-Geno Condition									
	F XX					F XY				
Treatment	n	Gave good eggs	Eggs difficult to strip or bad	Didn't give eggs	% gave good eggs	n	Gave good eggs	Eggs difficult to strip or bad	Didn't give eggs	% gave good eggs
Control	1		1							
20 mg 90 d						5	2		3	40%
20 mg 120 d						1			1	
30 mg 120 d						2			2	
Total	1		1			8				

A cursory examination of oviduct integrity was performed on the carcasses of 24 BY20 Age 3YO+ adult Brown Trout that died near the time of 2023 spawning efforts or shortly thereafter. These fish were held frozen by LOFH staff until WAFWA personnel could be onsite. On 19 Mar 2024 fish were thawed and an attempt made to strip eggs from phenotypic females. It was interesting to note that while these observations are subjective at best, it appeared that of the 20 fish where confident phenotype calls could be made, 11 expressed somewhat easily, 7 were difficult, and 2 appeared blocked. Of the four remaining fish, 2 were immature and 2 appeared spent from previous spawning attempts. In all but one case, the expressed eggs appeared to have developed normally. This observation lends credence to the presence of a functioning oviduct and ovary in these Age 3 YO + feminized BY20 Brown Trout, but a final judgement will obviously require the stripping of live fish in the future.

YY Rearing and Feminization Trial - BY22 NM

Survival of fish in the 20 mg 90 d E2 treatment group was the lowest of the three groups at 103 DPH (78%), and also remained the poorest for the duration of the trial (Table 31). The 20 mg 120 d treatment group survival was consistently intermediate between the other groups after 164 DPH. Control survival was well above that in either treatment groups at 164 DPH and remained moderately greater thereafter.

Table 31. BY22 hatch and survival rates of eggs that were the blended product of five families of BY20 F_{XY} Brown trout crossed with traditional BY19 Control M_{XY} males, received on 21 Dec 2022 from COFRH and reared at Los Ojos Fish Hatchery, Los Ojos NM.

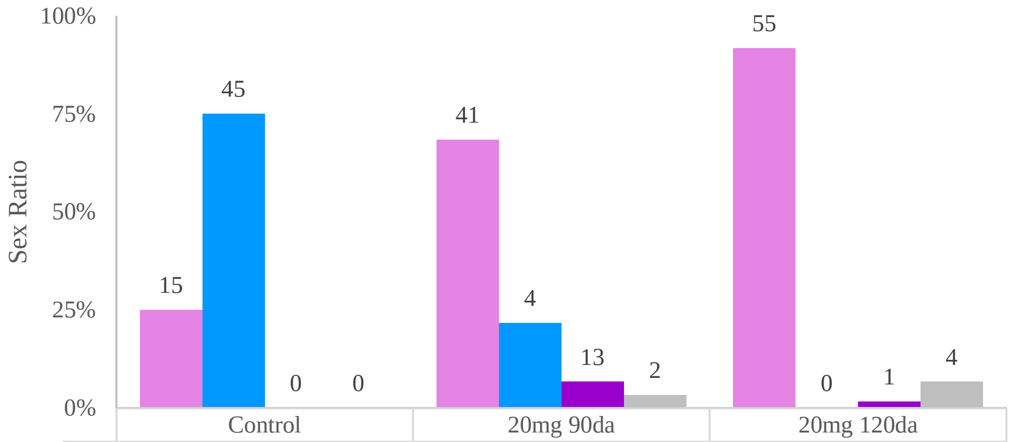
Treatment	Initial n	Survival 103 DPH	Survival 164 DPH	Survival 235 DPH	Survival 437 DPH
20 mg 90 d	641	78%	15%	14%	12%
20 mg 120 d	660	90%	23%	21%	20%
Control	645	87%	39%	27%	25%

Creation of YY Brown Trout and feminization

Visual phenotype was used to calculate sex ratios for each of the treatment groups. For the main sampling event on 20 Mar 2024, 60 fish from each group were necropsied, and gonads examined. The Control group had a female-to-male ratio of 25 % (15 females to 45 males; Figure 5). This ratio was exactly as expected, indicating that these progeny of a cross between a feminized genetic male and a traditional genetic male almost certainly resulted in the successful creation and survival to Age 1 of the first YY male Brown Trout.

In regard to feminization, of the two E2 treatment groups, the female phenotypic sex ratio for the 20 mg 90 d group was considerably lower than for the 20 mg 120 d exposure (68.3 % to 91.7 %), suggesting that at this facility and environs, the longer duration would be the most likely candidate for creating and maintaining a YY Male broodstock (Figure 5). Further, no phenotypic males were observed with the 120-day duration, while 21.7% were observed in the 90-day duration. Lastly, the intersex ratio was lower, and thus more favorable, for the 120-day treatment group.

Figure 5. Sex Ratio assessed by percent visual phenotype via necropsy by treatment type (n = 60) of Brown Trout at 437 DPH, following exposure to 20mg treated feed of two durations versus Controls, Los Ojos Fish Hatchery, Los Ojos, NM, 19 Mar 2024. The parental cross was between a feminized genetic male and a standard sperm producing male. Numbers above bars are n's for each phenotype.



	Control	20mg 90da	20mg 120da
F	25.0%	68.3%	91.7%
M	75.0%	21.7%	0.0%
IS	0.0%	6.7%	1.7%
U	0.0%	3.3%	6.7%

Growth and Health Indices

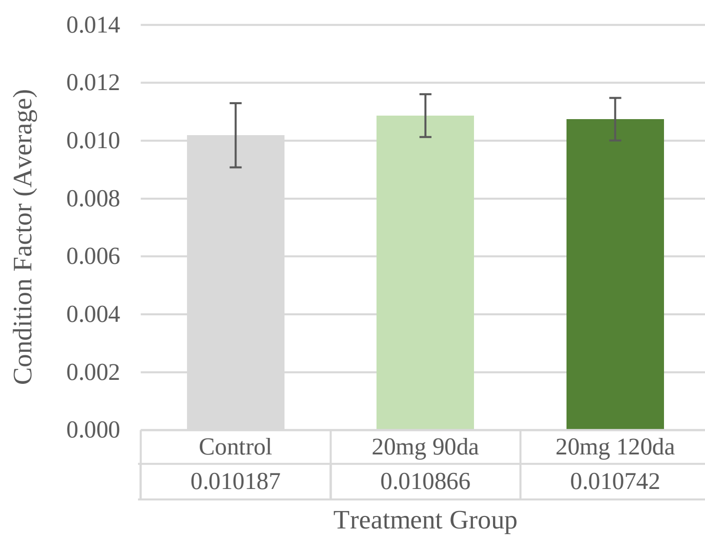
There are minor differences between treatment groups with respect to overall length and weight comparisons, in that for both parameters, the 20 mg 120 d treatment group had slightly lower values than either of the other two. However, none of these differences were statistically significant based on 95 % confidence intervals (Table 32).

Table 32. Average lengths and weights by treatment group of Brown Trout at 437 DPH, after exposure to 20 mg treated feed for varying durations starting at first feeding, when compared to Controls, Los Ojos Fish Hatchery, Los Ojos NM, 20 Mar 2024. Values in parentheses are 95% Confidence Intervals.

Treatment Group	n	Length (mm)		Weight (g)	
Control	60	156.7	(4.9)	43.5	(4.7)
20 mg 90 d	60	158.2	(5.5)	45.2	(4.8)
20 mg 120 d	60	155.7	(5.0)	42.9	(4.1)

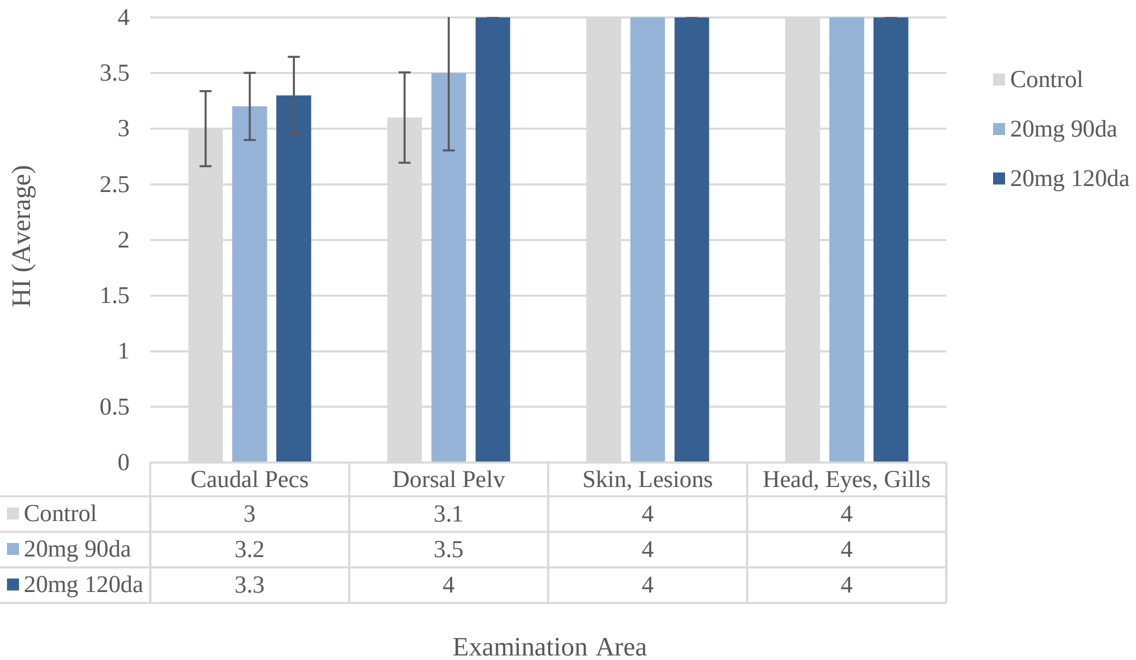
Given the similarity of lengths and weights across the three study groups noted above it is not surprising that condition factors across the treatment groups are also similar and not statistically different (Figure 6).

Figure 6. Average condition factor of Brown Trout at 437 DPH, following exposure to 20 mg treated feed for varying durations versus Controls, Los Ojos Fish Hatchery, Los Ojos, NM, 19 Mar 2024. Bars represent 95% Confidence Intervals, n = 10 per group.



As is very common in fish rearing in hatchery environments, some erosion of fins is expected and the appearance of erosion across all feminization trial study groups was normal with no significant differences across the treatments (Figure 7). Other external health metrics involving the physical appearance of the head, eyes, and gills, as well as general skin condition, show no treatment effect across all groups (Figure 7) and values for all fish in the three study groups received the highest ranking (value: 4) for all of these parameters.

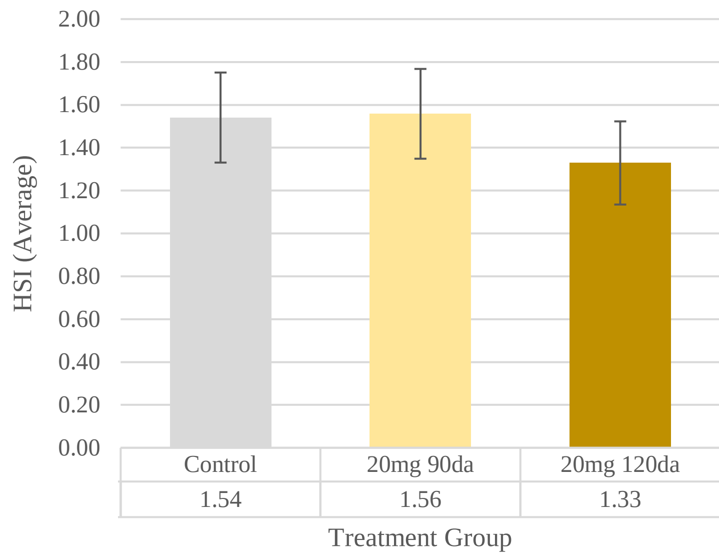
Figure 7. Health Index (HI) by treatment group from a subsample of Brown Trout at 437 DPH, exposed to 20 mg/kg Estradiol treated feed for two durations starting at first feeding compared to Controls, Los Ojos Fish Hatchery, Los Ojos NM, 20 Mar 2024. See Appendix D - Fig 1 for Health Index description. Bars are ± 95 % Confidence Intervals, n = 10 per group.



	Caudal Pecs	Dorsal Pelv	Skin, Lesions	Head, Eyes, Gills
Control	3	3.1	4	4
20mg 90da	3.2	3.5	4	4
20mg 120da	3.3	4	4	4

Based on Hepatosomatic Index (HSI) trends, we saw no evidence of long-term liver hypertrophy in Brown Trout from the two treatment regimens evaluated. Instead, Control and 20 mg 90 d fish HSI's averaged 1.5 and 1.6 respectively at 437 DPH, while HSI's of treated fish in the longest treatment group were lower at 1.3, and all treatment groups confidence limits overlapped (Figure 8). Although our sample sizes were small, those fish receiving the longest treatment had the lowest HSI, results inconsistent with liver hypotrophy due to heavy E2 exposure. For those interested in fish health work associated with the feminization of Brown Trout, additional FY24 work was conducted on fish from a previous trial conducted at COFRH. For a description of that work and results, see the INAD/Index coverage portion of this annual report.

Figure 8. Hepatosomatic index (HSI) for Brown Trout at 437 DPH, following exposure to 20 mg treated feed for varying durations versus Controls, Los Ojos Fish Hatchery, Los Ojos, NM, 19 Mar 2024. Bars represent 95 % Confidence Intervals, n = 10 per group.



Summary - FY24 New Mexico work

Insights from the maturity/spawning work on 3YO BY20 BRT conducted at LOFH in FY23 were limited due to the relatively small n's per treatment groups available to ship from the prior CO work, plus heavy mortality that occurred at the NM facility during handling prior to spawning efforts. After several years of attempting to spawn low numbers of maturing feminized XY males at both the CO and NM facilities, there is still some uncertainty about how well fish from the most desired feminization recipe (20 mg 120 d) will eventually mature and produce eggs. This question will have to be addressed by Los Ojos staff moving forward, and results might require fine tuning of the program feeding regime as has occurred at the Hayspur Hatchery for Brook Trout. On a positive note, rearing of Control progeny from a genetic XY by feminized XY cross resulted in the exact 75:25 % male-to-female ratio expected if YY Males were produced. Moving forward, future Consortium or NMDGF staff might work with K. Coykendall of the EFGL to apply her currently developing XY:YY discerning sex marker protocol to available genetic samples from these Control fish.

In general, E2 exposure in Rainbow Trout has been shown to result in increased liver size, at least in the short term (Herman and Kincaid 1988; Krisfalusi and Cloud 1996). However, our study included fish reared far longer than these studies (437 DPH), and results suggest that if hypertrophy occurred in Brown Trout, it had dissipated in fish held for 277 days or more post-E2 treatment.

FY24 Acknowledgments

Many thanks are to be given to the staff at Los Ojos Fish Hatchery for their taking on of the YY Male BRT feminization trial so willingly. A. Delp, M. Gordan, K. Erickson rose to the call and reared the world's first feminized YY Brown Trout in tight quarters with much enthusiasm. Multiple technicians came through with assistance, and while we unfortunately neglected to note your names, we thank you all very much.

FY24 Literature Cited

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FY24 Appendix D

Appendix D - Figure 1. Health Index for fish condition

Health Index (HI):

Scale

4 - no erosion 3 - minor erosion 2- medium erosion 1 - severe erosion

Parameter	None	Minor erosion	Medium erosion	Severe erosion
Pectoral fin erosion	4	3	2	1
Anal/caudal/dorsal fin	4	3	2	1
Head and gills	4	3	2	1
Body (lesions/bites)	4	3	2	1
SUM SCORE				

SCORING:
(*cumulative score could provide an early indication of arising health issues*):

15-16 – Very healthy

11-14 – Healthy

8-11 – Some health concerns – requires further investigation/ obs.

< 8 - Significant health concerns - requires action