Progress Report to the
Western Association of Fish and Wildlife Agencies
on
WAFWA YY Male Consortium Activities

Contract Period: July 1, 2023 to June 30, 2024

Authors:

Daniel J. Schill - Fisheries Management Solutions, Inc.

Elizabeth R. J. M. Mamer - ERJMM Research, LLC

and

Bruce A. McIntosh

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Introduction

Hamilton (1967) is typically credited with proposing that an invasive population could be eliminated by shifting the sex ratio completely to one sex. The idea that such a shift might be accomplished by aquaculture-induced sex reversal in fish first occurred to John Teem who hypothesized that sex reversal in a captive broodstock via use of exogenous sex hormones could be used to produce a genetically YY male broodstock whose progeny could be released into an undesired population (Mills 2009). The concept, dubbed the Trojan Y Chromosome or TYC approach, was formally explored first in a modeling paper evaluating the potential of the method for eradicating an invasive Nile Tilapia *Oreochromis niloticus* population (Gutierrez and Teem 2006). The authors noted that, for successful development of a TYC broodstock for a given species, it must be technically feasible to 1) develop an accurate genetic sex marker or test and 2) feminize a juvenile male fish via exogenous hormone exposure in a hatchery setting.

The development of a Trojan Y Chromosome broodstock for actual use in invasive fish control was first undertaken for the Brook Trout (*Salvelinus fontinalis)* in November 2008 by the Idaho Department of Fish and Game (IDFG) in November 2008 (Schill et al. 2016a). These authors utilized the indirect broodstock development approach (Beardmore et al. 2001) and their use of PIT-tagging, a sex marker, and other production methods reduced the time required for YY broodstock development from five generations (e.g., Mair et al. 1997) to three, a process that took about 5 years (Schill et al. 2016a). In addition, the Idaho authors changed the name of the TYC approach to YY Males because the latter term is more readily understood by the general public and decision-makers.

Having created a YY Male Brook Trout broodstock in Idaho, population simulations were needed to provide sideboards for field experiments and identify a range of likely stocking densities. Using Brook Trout data from Idaho and the time series dataset of McFadden et al. (1967), an age-structured stochastic matrix model was constructed (Schill et al. 2017). Findings suggested that, in streams, extirpation times of only 2 - 4 years were predicted, assuming good YY Male fitness similar to wild Brook Trout, but 5 - 15 years if supermale fitness was poor; only 20 % that of wild males. Because the stocking of YY Male fingerlings and manual suppression can readily be conducted at levels assumed in many of the simulations predicting complete eradication, the authors recommended full-scale field testing of YY Male stocking in both streams and lakes within an Integrated Pest Management or IPM program that includes manual suppression (Schill et al. 2017).

Concurrent with the above modeling exercises, a pilot study was conducted to determine if stocked YY Male Brook Trout can survive, emulate the spawn timing of wild fish, reproduce with wild fish, and produce only XY males (Kennedy et al. 2018). Approximately 500 YY Male Brook Trout (mean TL = 250 mm) were evenly dispersed along short reaches (1.9 - 2.6 km) in each of four pilot study streams in June 2014 with the expectation that some would survive until the fall spawning period and breed successfully with wild fish. YY Male fish comprised an estimated 3.1 % of all adult Brook Trout during spawning. The genetic

assignment tests indicated that an average of 3.7 % of fry were the progeny of stocked YY Males and all were XY males (Kennedy et al. 2018). These pilot study results confirmed that stocked YY Male fish can survive and spawn successfully with wild females and produce all-male progeny. Due to success and relative ease of creating the YY Male Brook Trout broodstock, in 2014 IDFG began undertaking the first steps to develop YY broodstocks for other non-native invasive species impacting Idaho sports fisheries, including Common Carp, Walleye, and Lake Trout.

IDFG first initiated a dialog with member states in 2016 at the annual WAFWA meeting via a presentation at the Fish Chiefs session. At that session a majority of the Fish Chiefs expressed interest in formation of a YY Male Consortium with the express purpose of expanding YY Male research efforts. The intent of the proposed approach was to integrate IDFG staff having the sex reversal and sex marker development experience with personnel from other state agencies having extensive fish culture expertise for species considered important gamefish in some states and yet invasive pests in others. In January 2018, Fish and Wildlife agency directors from WAFWA states approved a YY Male Consortium proposal. Thirteen states funded the associated budget with the overall goal of undertaking the creation of YY Male research broodstocks for five invasive species including the three begun earlier by IDFG (Common Carp, Walleye and Lake Trout) along with two new species, the Brown Trout and Northern Pike. Funding for the YY Male Consortium began on 1 July 2018, and funded over three fiscal years, FY19 - FY21. During July 2021, nine Fish Chiefs made the decision to continue funding the program with ten stated program objectives below for three years (FY22-FY24). An additional state (CA) returned to the Consortium during FY2024.

YY Male Consortium Program Objectives

DESCRIPTION OF SERVICES. Beginning on 1 July 2021, Contractor will begin/continue addressing the following objectives listed below.

1. Work with the Aquatic Animal Drug Approval Project ("AADAP"), the Food and Drug Administration ("FDA") and WAFWA partners to coordinate distribution of YY Male Brook Trout eggs.
2. Provide technical guidance on field evaluations of YY BK to WAFWA partners receiving eggs and continue with leadership/coordination of the YY Brook Trout Technical Team.
3. Continue to refine the program-derived sex markers for Brown Trout, Lake Trout and Common Carp and develop broadly functioning ones for the remaining two species (Walleye and Northern Pike).
4. Finalize existing program-derived sex reversal recipes for three species (Walleye, Brown Trout, and Common Carp) and develop effective ones for the remaining two species (Northern Pike and Lake Trout).
5. Continue to evaluate the likelihood of density-dependent sex change and document time to extirpation in field studies of YY Male Brook Trout on two Idaho streams.
6. Identify WAFWA partners or other collaborators willing to undertake creation of YY Male broodstocks for the above species as well as a backup broodstock for YY Male Brook Trout.
7. Communicate program objectives and findings verbally and in writing.
8. Work with AADAP, Novaeel Inc. and WAFWA partners to provide INAD coverage or Estradiol addition to FDA'S drug "Index", allowing for development of new YY Male broodstocks.
9. Assuming FDA approval is obtained; begin development of YY Male broodstocks for one candidate species by 2024.
10. Build in more emphasis on "outside" fundraising to allow for increased program expansion, particularly regarding drug approval and aquaculture aspects of YY Male broodstock production.

This report documents results of the main activities conducted during FY2024, the sixth program year to facilitate attainment of the above objectives.

FY24 YY Male Consortium Work

Brown Trout Sex Reversal and Spawning Research

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.

Background

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.

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. 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 regimes (10 mg 90 d) were 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 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 1), 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 1. 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 were reared in three separate troughs. Using the treatment feminization results from the previous year in Colorado, we sought to identify an effective Estradiol 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 2).

Table 2. 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. 2016a). 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. During the following spring on 19 March 2024, project personnel and LO 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 A) was systematically performed on every 6th netted fish (Health exam; 10 total per group). In addition, because Estradiol 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 2016a).

Results of FY24 Brown Trout Work

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 3). 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) 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 3. 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 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 3YO + 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 103DPH (78%), and also remained the poorest for the duration of the trial (Table 4). 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 4. 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.

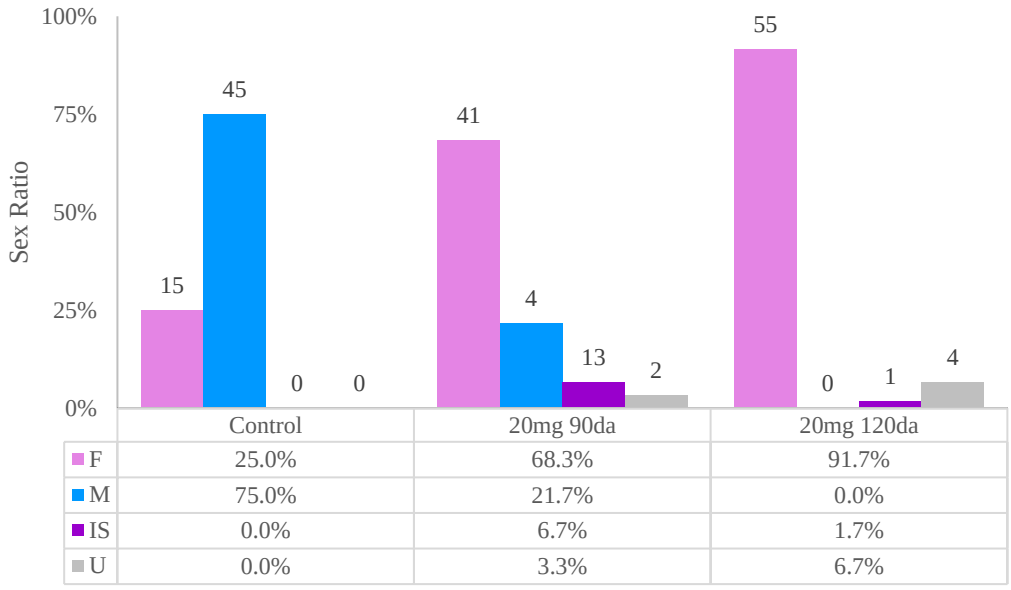
Treatment	Initial n	Survival 103 DPH	Survival 164 DPH	Survival 235 DPH	Survival 437 DPH
20 mg 90 d	641	78%	15%	14%	12%
20 mg 120 d	660	90%	23%	21%	20%
Control	645	87%	39%	27%	25%

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 1). 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 for creating and maintaining a YY Male broodstock (Figure 1). Further, no phenotypic males were observed with the 120-day duration while 21.7% were observed with the 90-day duration. Lastly, the intersex ratio was lower and thus more favorable for the 120-day treatment group.

Figure 1. Sex Ratio assessed by percent visual phenotype via necropsy by treatment type (n = 60) of Brown Trout at 437 DPH, following exposure to 20 mg treated feed of two durations versus Controls, Los Ojos Fish Hatchery, Los Ojos, NM, 19 Mar 2024. The parental cross was from 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

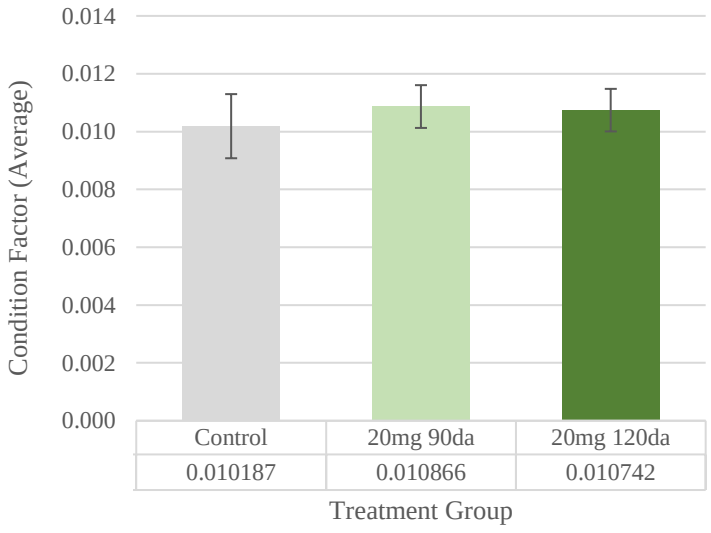
There are minor differences between treatment groups with respect to overall length and weight comparisons, in that for both parameters, the 20 mg 120 d treatment group had slightly lower values than either of the other two. However, none of these differences were statistically significant based on 95% confidence intervals (Table 5).

Table 5. Average lengths and weights by treatment group of Brown Trout at 437DPH, 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 2).

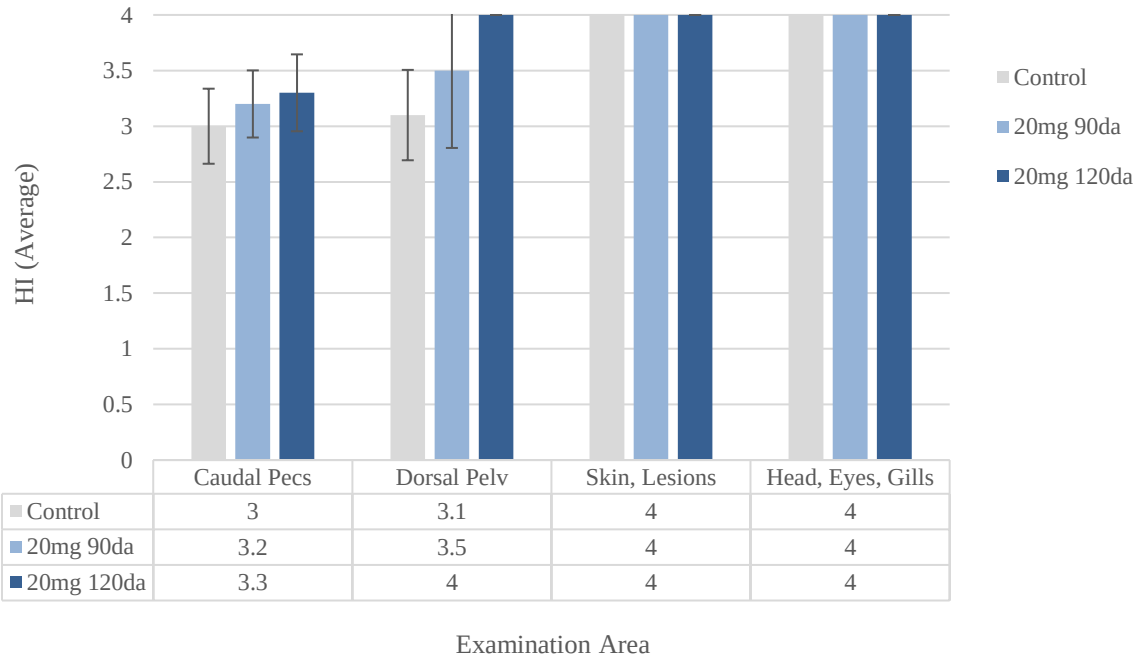
Figure 2. Average condition factor of Brown Trout following exposure to 20 mg treated feed for varying durations versus Controls, 437 DPH, Los Ojos Fish Hatchery, Los Ojos, NM, 19 Mar 2024. Bars represent 95% confidence intervals, n = 10 per group.



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 3). 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 3) and values for all fish in the three study groups received #4 rankings for all of these parameters.

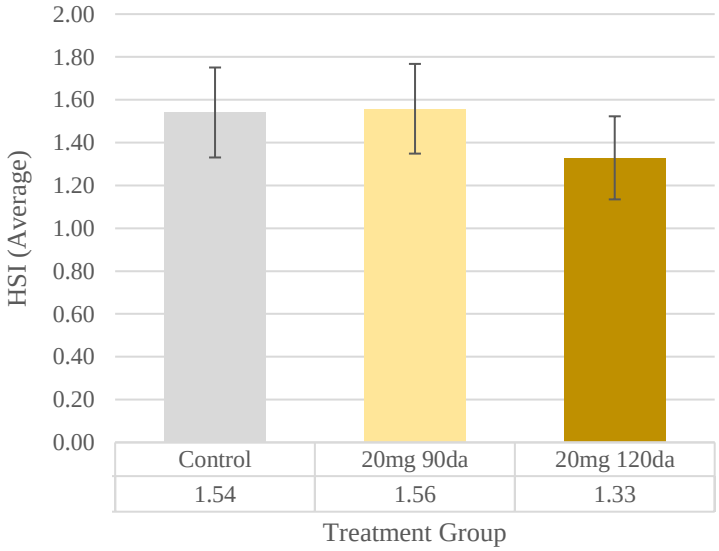
Figure 3. Health Index (HI) by treatment group from a subsample of Brown Trout exposed to 20 mg/kg Estradiol treated feed for two durations starting at first feeding compared to Controls, 437 DPH, Los Ojos Fish Hatchery, Los Ojos NM, 20 Mar 2024. See Appendix A - Fig 1 for Health Index description. Bars 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 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. 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 4. Hepatosomatic index (HSI) for Brown Trout following exposure to 20 mg treated feed for varying durations versus Controls, 437 DPH, 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.

Sex Markers

General Approach

In the field, mature adult fish of wild origin are collected from various populations, killed via anesthetic overdose, necropsied and visually sexed. Fin tissues are only taken from fish with clearly identifiable gonads and are placed on numbered Whatman filter paper sheets for storage. DNA is subsequently extracted from the fin tissue by IDFG's Eagle Fish Genetics Lab (EFGL) staff. To develop sex markers for species of interest to the WAFWA Consortium, EFGL uses existing Y-chromosome (sdY) DNA sequences already available or generates new DNA sequence data using Restriction site associated DNA sequencing (RADseq). These sequences can then be compared between phenotypic males and phenotypic females to find specific single nucleotide polymorphisms (SNPs) specific to each sex. The overarching goal of sex marker work in the program is initially to develop Y chromosome-linked markers that would permit the differentiation of XX and XY individuals but also eventually develop bi-allelic sex markers that would allow differentiation of XY and YY fish.

Common Carp

Background

Efforts to develop a sex marker for Common Carp were first initiated with some success by the Idaho Department of Fish and Game and the EFGL during 2014 with work focused on large samples from two Idaho populations. During FY2019 EFGL staff identified two candidate bi-allelic loci and screened one (Cca744444_87) on 800 fish from ten Common Carp populations, seven from Idaho and three from the Midwest, reporting an overall concordance rate between genetic and phenotypic sex of 93% (Schill and Mamer 2019). However, concordance varied considerably across populations, several samples had small n's or other limitations (e.g. hatchery feeder koi), and it was recommended that future work employ a second restriction enzyme that cuts the genome more frequently to identify additional candidate sex markers (Matt Campbell EFGL, pers. comm.). Funding for this subsequent work was obtained by YY Consortium staff with the encouragement and support of AWFWA under the MSCGP program and administered by the USFWS. Relative to the FY2019 work above, these samples were focused farther east to broaden the utility of the sex marker for the nation as a whole and to thus hopefully assist in securing additional Federal funding for future development of YY Common Carp.

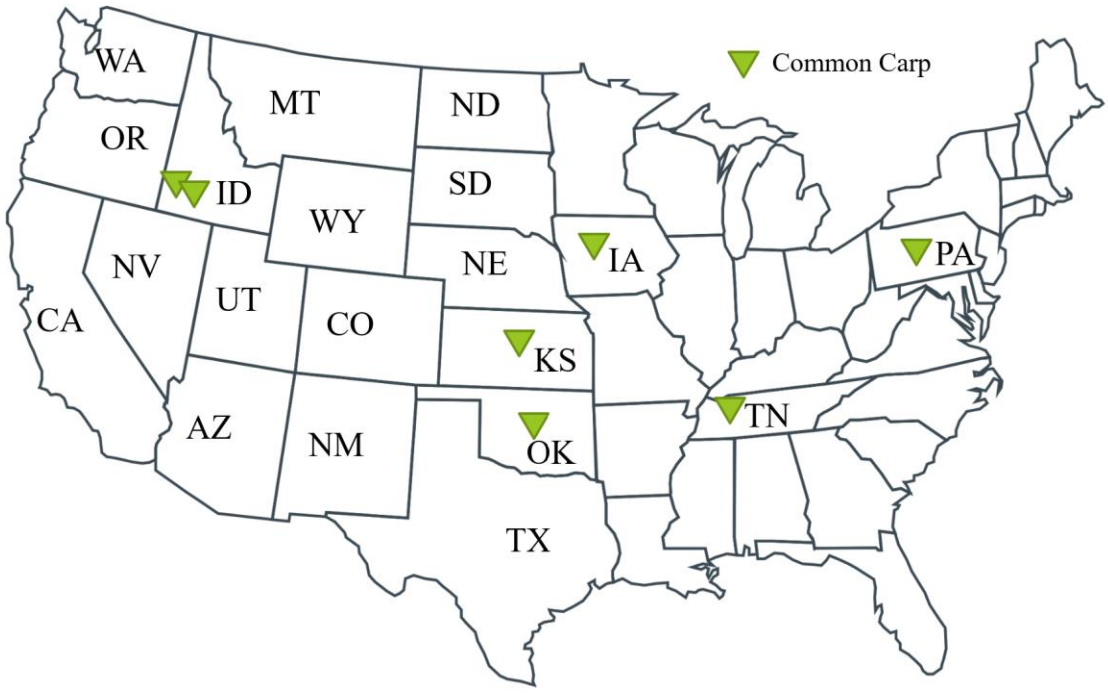
Accordingly, during FY2021, large DNA samples with associated known phenotype calls (target n = 200, 100 from each sex) were obtained from five new waters in PA, TN, IA, WA and OK . Results from this effort were encouraging with genotype-phenotype concordance averaging 93% and exceeding 96% for three of the five populations (Schill et al. 2022, Coykendall and Campbell, Appendix B). However, concordance for the two remaining populations were relatively low at 69 and 89%.

Additional work on the marker was therefore conducted in FY2023 under the MSCGP Grant. A detailed description of methods and results of FY2023 efforts is described in detail by EFGL staff in Schill et al. 2023, Coykendall and Campbell Appendix B1. Two new genetic assays were developed and EFGL staff ran the same samples from the five populations above using two new assays. This effort resulted in an overall mean concordance rate of 99% between genotype and phenotype for all samples across the five populations.

FY2024 Work

Funding for the MSCGP work in FY23 did not provide for inclusion of large existing samples from two Idaho Common Carp populations. Data from the Snake River and Lake Lowell populations were assayed using the final markers developed during FY2023. See Appendix B1 for a detailed description of methods and results for these two Idaho populations. Combining results for these two waters to the final FY23 effort provides an opportunity to evaluate the final Common Carp marker performance for seven populations across the U.S (Figure 5).

Figure 5. Geographically dispersed locations of Common Carp populations used for final evaluation of a two Common Carp genetic sex markers. All fish sampled by YY Consortium project staff with field assistance by local State Agency field staff.



Results

FY24 results for the two Idaho carp populations were encouraging, with a generally high level of concordance between genotypes at the two markers and observed phenotypic sex. In the Snake River population, overall concordance for the 744444_87 marker was 0.917, though concordance for males was lower at 0.768 (Coykendall and Campbell Appendix B1). For marker 1506016, concordance for both sexes was 1.0. However, while this marker exhibited improved concordance, a small percentage of YY genotypes were inexplicably observed. Further research is recommended to investigate whether the reported presence of YY genotypes is due to marker location, genotyping error or another mechanism.

At present, we recommend focusing on assay 744444_87 for the development of YY male carp broodstock. Combining results from this assay across all seven populations evaluated by the EFGL over the past two years (Figure 5) indicates that concordance exceeded 0.995 in five of the seven populations tested, without complications related to the unexplained YY genotypes. Continued investigation of both of these markers will likely assist researchers attempting to differentiate sex in this species across its geographically broad range.

Walleye

Background

During FY2019, FY2020, and FY2021 IDFG's Eagle Fish Genetics Laboratory staff made concerted efforts to develop a Walleye sex marker but unfortunately that prior work did not yield useful markers (Schill and Mamer 2019; Schill and Mamer 2020; Schill and Mamer 2021). Additional efforts were made by the lab in FY2022 by working with existing samples and the involvement of other U.S. and European collaborators with some success (Schill et al. 2022).

A focused effort was again made in FY23 by EFGL staff working in concert with their European collaborators. A detailed description of this effort along with subsequent results has been described in some detail (Schill and Mamer 2023, Campbell and Coykendall, Appendix B2). Briefly, the staff ran a walleye genotyping panel from 285 walleye fin clip samples (45 broodstock and 240 offspring with known phenotypic sex) collected during a prior YY Consortium sex reversal trial conducted at Garrison Dam National Fish Hatchery (Schill et al 2022). In these fish, genotypic sex matched phenotypic sex with a concordance rate of 95% (270 out of 285). These results by the EFGL lab further supported findings by their European collaborators and indicate that walleye have a ZW sex determination system (SDS) where females are the heterogametic sex (ZW) and males are homogametic (ZZ). Such a determination system in sportfish is not unheard of; for example, Muskellunge also have a ZZ-ZW system. However, these results were somewhat surprising in that a prior published study on Walleye by Malison et al. (1986) reported that Walleye had an XX-XY sex determination system.

FY 2024 Walleye Work

During the last year, EFGL staff applied the newly developed markers from FY23 to fin clips and known phenotype data collected from several Walleye populations located in IA, WY and ID (Schill and Mamer 2020). The objective was to confirm the ZZ-ZW system identified for the ND population in FY23 work (Schill et al. 2023, Campbell and Coykendall, Appendix B2) is the norm in several other U.S. Walleye populations, and also to report marker genotype-phenotype concordance.

Results

A summary of FY24 results (Appendix B2) indicates that the other Walleye populations sampled by Consortium staff also fit the ZZ-ZW sex determination model. Concordance between genotype and phenotype was 0.876, 0.910, and 0.930 for the Buffalo Bill, Lake Pend Oreille, and Rathbun Populations, respectively. Concordance for females exceeded 0.97 for females in all three populations but was consistently lower (0.84-0.88) for males. The lower concordance between phenotypic and genotypic sex, particularly for the males, could be indicative of a temperature override that acts upon otherwise genetically determined sex (Campbell and Coykendall Appendix B2). It was noted by EFGL staff (Appendix B2) that the samples from both Lake Pend Oreille and Buffalo Bill Reservoir were male-skewed. However, it is likely that much of this skew is behaviorally related. Male Walleye in both these waters are more readily captured via spring gillnetting since they lie in wait weeks for females to arrive on the spawning grounds, the latter typically spending only a day or two in spawning areas (Jason Burkhardt, Wyoming Game and Fish, pers. comm).

The above work confirming three Consortium-sampled populations conform to a ZZ-ZW species with possible temperature influences poses significant challenges to future development of a YY broodstock for Walleye. To begin, the use of the term YY Male would be incorrect for this species. While the term Trojan Sex Chromosome (TSC) Program (Teal et al. 2022) could be used to describe such a program, another more significant challenge remains. In a ZZ-ZW species, *all* fish to be stocked into the field must themselves be sex reversed (i.e exposed to hormones, as opposed to the sperm producing YY Brook Trout we currently stock, which are not), creating increased E2 containment issues, increased juvenile fish transport constraints, and additional food safety concerns by the FDA. While these latter issues do not completely eliminate a Consortium eradication program, the possibility that sex is not stable in this species, and may perhaps be influenced by temperature, would seem to rule out any further investment in broodstock development in the near term.

Northern Pike

At the onset of the YY Male Consortium in FY19, the Alaska Department of Fish and Game (ADFG) took the lead on sex marker development for Northern Pike. The agency genetics lab had some preliminary success in the effort during FY2020 by building a genome scaffold and identifying regions with high sex association (Chris Habicht, ADFG pers. comm.). In FY22, ADFG Division of Sport Fisheries provided

additional funding for the ADFG Gene Conservation Lab (GCL) to develop twelve 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 (Wei Cheng, ADFG, personal communication).

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 marker development.

Later in FY24, EFGL staff were asked to engage and develop a sex marker for WAFWA using the lower 48 samples. Unfortunately, Eagle staff discovered that genetic research published in the same year (Johnson et al 2024; and studies referenced within) suggest that the species is a poor candidate for Trojan 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 instead, the sex of Northern Pike in these areas is likely influenced strongly by environmental factors. While Northern Pike could be a candidate for Trojan YY technology in US regions where the amhby gene is present (Alaska), the absence of this gene in lower 48 populations, and an unstable sex determination system, limits the potential for widespread YY Male application. (Matt Campbell, EFGL, personal communication) In summary, Eagle Fish Genetics Laboratory staff responsible for development of at least 8 sex markers recommended against any further YY work on Northern Pike (Mathew Campbell, EFGL, pers communication), advice we feel is wise to take.

Brook Trout Density-dependent Sex Change

Background and Study Area

An unlikely, but important, issue that could ultimately affect the ability of YY males to completely eradicate invasive species relates to the stability of phenotype. Most freshwater fish species are gonochoristic, meaning that an individual fish can only become one of two distinct genetic sexes. However, it has been known for decades that phenotypic sex in some species can be environmentally changed (Reinboth 1980) although reports of such are infrequent in fish. A recent review of such literature suggests by far the most common form of such Environmental Sex Determination, or ESD, is known as Temperature-Dependent Sex Determination (TDSD), which invariably results in highly male-biased sex ratios (Ospina-Álvarez and Piferrer 2008). Such a form of phenotype change (female to male) is not a threat to the YY Male technique. However, Density-Dependent Sex Change (DDSC) has been suggested for both Sea Lamprey and Brook Lamprey (Docker 1992; Zerrenner and Marsden 2005) as well as American Eel (Krueger and Oliveira 1999). Sex

determination and differentiation in these two ancient species have heretofore been problematic to study and appear markedly different than that of the typical gonochore like Brook Trout. In the case of gonochores, such as those species currently being pursued for YY Male development, DDSC could be thought of as a possible density-related change in phenotypic sex. Lake Superior Lake Herring have been suggested as possibly capable of DDSC although this modeling study provided little empirical or genetic evidence for the assertion (Bowen et al. 1991). Regardless, the assumption that phenotype will remain stable in species that are vastly reduced in abundance is key to successful implementation of the YY Male technique (Schill et al. 2017).

There are several ways to test for such a possible density related phenomenon including the rearing of fish at very low densities in an aquaculture setting or the largescale suppression of wild populations (Docker 1992). In both cases, perhaps the best way to look for phenotypic shift is to examine gonads of fish rearing at low abundance and compare resultant observed phenotype for individuals at maturity to genotypic sex derived from sex markers. In this case the hoped-for result is 100 percent concordance between phenotype and genotype.

A field study of potential DDSC, initiated by IDFG and Bart Gamett of the United States Forest Service, was begun on two Idaho Brook Trout streams in 2016. Bear Creek and Willow Creek are two short, isolated streams containing only invasive Brook Trout. Both streams are small and have complete migration barriers at the bottom. Willow Creek is 2.9 km in length with a mean width of 0.8 m. Bear Creek is 2.6 km in length with a mean width of 2.6 km.

Methods

The entire length of both streams have been subjected to Pulsed DC electrofishing removal on two consecutive days in late June or early July for the past 9 years. All wild Brook Trout collected, as identified by an intact adipose fin, were killed and a tissue sample taken and stored on numbered Whatman sheets. Genetic sex was subsequently determined by staff at the Eagle Fish Genetics Lab (EFGL) for all fish killed during removal runs, using a sex marker (Schill et al. 2016b). Those fish deemed large enough to visually ascertain phenotypic sex based on prior sub-sampling efforts were placed in individual labeled bags, held on ice, and returned to the laboratory. Bagged fish were subsequently necropsied, and their phenotypic sex determined visually with the aid of microscopy when required. Phenotypic sex calls were recorded only on fish with clearly identifiable gonads, and the remainder were classified as unknown. Phenotype and genetic sex data were subsequently compared for concurrence and any discordance recorded.

Population Response to Suppression and Stocking

The ESD evaluation described above involved the annual genetic sexing of virtually every ad-fin intact Brook Trout handled during the study including YOY and wild adults. This enabled annual population abundance estimation for the main fish of interest in a YY Male field evaluation, the primary focus being the

remaining numbers of wild genetic females. We calculated 2-pass removal estimates (Seber and LeCren, 1967) of female population size for those years where capture probabilities on the two back-to-back removal days exceeded 50%. Population abundance was estimated using the MICROFISH software package (Van Deventer and Platts 1989). We estimated the proportion of the wild female population removed in each stream annually by dividing the total removed (sum of runs 1 and 2) by the associated population estimate. Similar 2-pass estimates and population removal proportions were also developed annually for wild genetic males using genetic sex identification and similar suppression removal data.

After three to four years of suppression with little sign of ESD, it was decided to initiate YY Male fingerling stocking in both streams to evaluate the extirpation potential of YY Males, and also speed examination of ESD at expected lower adult female population densities. Stocking was first begun on Willow Creek on 16 July 2018, immediately after completion of the second wild fish removal run. Bear Creek was delayed one year due to fish availability, with stocking initiated on the afternoon of the second removal run, 10 July 2019. Both streams have been stocked annually, always late in the afternoon on the day of the second removal run. The target stocking rate has been 50% of the initial Age 1+ population size in each stream before suppression began, with the fish being distributed along the entire reach of both streams. All YY Males are adipose fin-clipped prior to stocking, and, as of 2020, PIT-tagged as well, to facilitate easy field identification during subsequent suppression years. In addition, we calculated the same 2-pass population estimates as above for overwintering YY Males present in the stream that survived from prior stocking years.

Genetic Stock Identification, or GSI, was used to ascertain whether YY Males stocked in both streams subsequently spawned successfully. Genetic baselines were established for individuals from the YY BK broodstock residing at the Hayspur Hatchery, and from wild Brook Trout collected from the two study streams, before stocking was initiated. Enumeration of fish with genetic signatures intermediate between these two groups was used to identify YY Male progeny (Kennedy et al. 2018).

Results- Density-Dependent Sex Change

To date (2024) a total of 3763 wild Brook Trout in the two study streams have been visually sexed for phenotype and also successfully sexed genetically using a sex marker (Table 6). Of the 1180 and 473 fish examined in 2016 and 2018, respectively, no discordance between genotype and phenotype was detected. However, nine mismatches originally occurred in 2017 out of 772 fish (Schill and Mamer 2019) as well. Due to the occurrence of these incongruent phenotype-genotype calls, DNA samples for the year 2017 collections were re-evaluated in late 2019 using expanded RAD-sequenced sex marker panels (Matt Campbell, Eagle Fish Genetics Lab, Pers Comm).

Table 6. Phenotype and genotype for 3763 Age 1+ wild Brook Trout (>90 mm) collected from two Idaho isolated streams, Bear Creek and Willow Creek, during the ESD trial, 2016-2024. The data only includes those fish that were both successfully genotyped and phenotypically sexed by necropsy. A total of 58 wild Age 1+ fish collected in the study were thus excluded from this analysis.

			Genotype		
		Phenotype	**F**	**M**	**Total**
2016	**Bear Ck**	F	495	0	**929**
		M	0	434	
	Willow Ck	F	149	0	**251**
		M	0	102	
2017	**Bear Ck**	F	283	0	**594**
		M	1	310	
	Willow Ck	F	103	0	**179**
		M	0	76	
2018	**Bear Ck**	F	146	0	**264**
		M	0	118	
	Willow Ck	F	95	0	**209**
		M	0	114	
2019	**Bear Ck**	F	101	0	**189**
		M	0	88	
	Willow Ck	F	78	0	**148**
		M	0	70	
2020	**Bear Ck**	F	162	0	**286**
		M	0	124	
	Willow Ck	F	25	0	**57**
		M	0	32	
2021	**Bear Ck**	F	90	0	**208**
		M	0	118	
	Willow Ck	F	17	0	**74**
		M	0	57	
2022	**Bear Ck**	F	42	0	**112**
		M	0	70	
	Willow Ck	F	6	0	**38**
		M	0	32	
2023	**Bear Ck**	F	22	0	**128**
		M	0	106	
	Willow Ck	F	2	0	**3**
		M	0	1	
2024	**Bear Ck**	F	4	0	**80**
		M	0	76	
	Willow Ck	F	0	0	**14**
		M	0	14	
		Grand Total			**3763**

These analyses resulted in the clarification and resolution of all but one of the conflicted samples mentioned above. The remaining 2017 outlier was assigned as phenotypically M by visual call and genotyped as F two consecutive times. At 97 mm total length, this fish was borderline for being able to make a visual sex determination and as there were no residual frozen tissues available to reassess this call, no resolution was possible. Therefore, this discordance is unresolvable at this time, and it is possible it was due to actual phenotypic sex change, a mistake in necropsy sexing, recording error, or a genotyping error. However, we doubt it to be a case of phenotypic sex reversal given that no phenotype-genotype mismatches were observed before or since. Results from the latter sampling years when Brook Trout abundance in both streams was markedly lower than previously observed provide additional comfort that the single mismatch reported for 2017 was likely a visual phenotyping error. Based on the negative results to date on such a large sample, and so few wild fish of either genotype remaining in both study streams, we conclude that ESD in wild Brook Trout via DDSC has not occurred.

Results - Population Response to Suppression and Stocking

The genotyping of virtually all wild fish collected and killed during this study presented a unique opportunity to derive annual population estimates by genetic sex over the life of the project. Out of a total of 4495 wild Brook Trout collected and killed to date, less than 1.3% (n = 58) were unable to be genotyped successfully in the entire study. These fish were disregarded in the population estimates reported below.

Population abundance of wild females in both study streams has declined precipitously since the first year of suppression. The initial estimated Age 1+ female population in Bear Creek has been reduced from 542 fish in 2016 to only 4 fish in 2024 (Table 7). The latter estimate represents a 99% decrease from the first population estimate in 2016 (Table 7). Age 1+ wild Female Brook Trout appear to be completely eradicated in Willow Creek with no fish collected in either electrofishing pass in June 2024 (Table 7). The rapid decrease in female abundance is not surprising given that the observed removal rates resulting from two-pass electrofishing (percent population removed) has ranged from about 81 to 100% for female fish in both streams across the years (Table 7). These reported population removal rates are likely biased high as it has been estimated that 2-pass removal electrofishing underestimates true stream population estimates for trout by an average of 25% (Meyer and High 2011). In the case of Willow Creek in 2024, it is impossible to mathematically correct for bias of a zero-abundance value. However, there could theoretically be one or two females remaining, a possibility which will be evaluated in summer 2025. However, the strong downward trajectory towards zero in Willow Creek during the last three years (Table 7) strongly indicates full 2024 eradication.

Table 7. Results of electrofishing removal runs, resulting population estimates, and proportion of estimated population removed for Age1+ female Brook Trout in Bear and Willow creeks near Mackay Idaho, July 2016-2024. Population Estimates are 2-pass removal estimates (Seber and LeCren 1967) calculated when capture probabilities exceeded 50%. Stocking of YY BKT first occurred in July 2018 in Willow Ck and July 2019 in Bear Ck and annually thereafter.

	Removals						
	Day 1	Day 2	Total	Capture Probability	Population Estimate	95% Confidence Interval	Population Removed (%)
Bear Ck							
2016	411	100	511	0.759	542	524-560	94.3%
2017	180	127	307	0.304	-	-	-
2018	110	31	141	0.727	152	140-164	92.8%
2019*	76	35	111	0.561	137	108-166	81.0%
2020	128	34	162	0.743	173	161-185	93.6%
2021	68	24	92	0.667	103	89-117	89.3%
2022	29	13	42	0.609	49	35-63	85.7%
2023	18	4	22	0.846	22	20-24	100.0%
2024	3	1	4	0.800	4	2-6	100.0%
Willow Ck							
2016	117	33	150	0.732	161	149-173	93.2%
2017	92	18	110	0.821	113	107-119	97.3%
2018*	82	16	98	0.817	101	96-106	97.0%
2019	55	25	80	0.576	97	74-120	82.5%
2020	19	6	25	0.758	26	22-30	96.2%
2021	14	3	17	0.85	17	15-19	100.0%
2022	5	1	6	0.857	6	5-7	100.0%
2023	2	0	2	1	2	NA	100.0%
2024	0	0	0	NA	0	NA	NA

* Initial stocking year

Population estimates derived for Age 1+ XY male Brook Trout in both study streams has also declined markedly since the first year of suppression. The estimates in Bear Creek declined from 496 Age 1+ XY males in 2016 to 104 in 2024, a 79% decrease (Table 8). The decrease in Age 1+ XY males in Willow Creek was 88% across the same time period. These reductions in adult males are considerably lower than that reported above for adult females. However, it is important to recall that many of these XY males are the progeny of YY male fish.

Table 8. Results, by year, of annual removal efforts, population estimate, and proportion removed of Age 1+ XY male Brook Trout (including both wild and adult YY progeny) from two study streams involved in the YY Brook Trout evaluation in eastern Idaho, July 2016-2024. Estimates are from two-pass removals, and stocking of YY BKT first occurred in Fall 2018 in Willow Ck and Fall 2019 in Bear Ck.

	Removals						
	Day 1	**Day 2**	**Total**	**Capture Probability**	**Population Estimate**	**95% Confidence Interval**	**Pop % Removed**
Bear Ck							
2016	339	108	447	0.685	496	468-524	90.1%
2017	195	130	325	0.342	-	-	-
2018	93	20	113	0.801	117	111-123	96.6%
2019*	65	26	91	0.619	106	87-125	85.8%
2020	97	29	126	0.712	137	124-150	92.0%
2021	92	27	119	0.717	129	117-141	92.2%
2022	53	17	70	0.707	76	66-86	92.1%
2023	83	23	106	0.741	113	103.123	93.8%
2024	49	27	76	0.478	104	65-143	73.1%
Willow Ck							
2016	76	26	102	0.671	114	99-129	89.5%
2017	64	20	84	0.712	91	81-101	92.3%
2018*	80	29	109	0.657	123	107-139	88.6%
2019	56	16	72	0.735	77	69-85	93.5%
2020	28	4	32	0.889	32	30-34	100.0%
2021	44	14	58	0.707	63	54-72	92.1%
2022	27	5	32	0.865	32	30-34	100.0%
2023	4	1	5	0.833	5	4-6	100.0%
2024	13	1	14	0.933	14	13-15	100.0%

* Initial stocking year

Trends in Adult YY Male abundance for fish stocked in prior years differed between the two streams. YY Male abundance remained remarkably stable across years in Bear Creek, ranging from 265-308 fish (Table 9. In contrast, overwintering YY Male abundance in Willow Creek declined over 2-fold across years with available data. Reasons for this disparity between streams are unknown and unexpected since stocking numbers of fingerling fish remained constant across the years.

Table 9. Abundance estimates of YY Male Brook Trout (Age 1+) obtained by 2-pass electrofishing of overwintering fish present in Bear and Willow Creeks, late June or July 2021-2024. Stocking of YY BKT first occurred in Fall 2018 in Willow Ck and Fall 2019 in Bear Ck.

	Removals					
	Day 1	**Day 2**	**Total**	**Capture Probability**	**Population Estimate**	**95% Confidence Interval**
Bear Ck						
2021	216	64	280	0.711	305	286-324
2022	214	52	266	0.764	281	268-294
2023	224	35	259	0.846	265	258-272
2024	250	48	298	0.814	308	298-318
Willow Ck						
2021	93	34	127	0.651	144	126-162
2022	74	17	91	0.784	95	88-102
2023	59	12	71	0.816	73	68-78
2024	54	10	64	0.842	65	61-69

Along with the number of adult females remaining in a water, an equally important variable is the level of YOY recruitment. Salmonid fry do not lend themselves to accurate population estimation due to negative size selection associated with electrofishing gear (Meyer and High 2011). Despite this observation, large reductions in Age 0 recruitment in both streams is apparent when combining Day 1 and Day 2 YOY or fry catch (Table 10). On Bear Creek, total fry collected along the entire length of the stream decreased from 77 to 2 fish from 2016 to 2024. On Willow Creek, total fry collected decreased from 110 to 0 fish from 2016 to 2024 . We have not collected a single fry along the entire length of Willow Creek in two of the last three years during both electrofishing passes (Table 10).

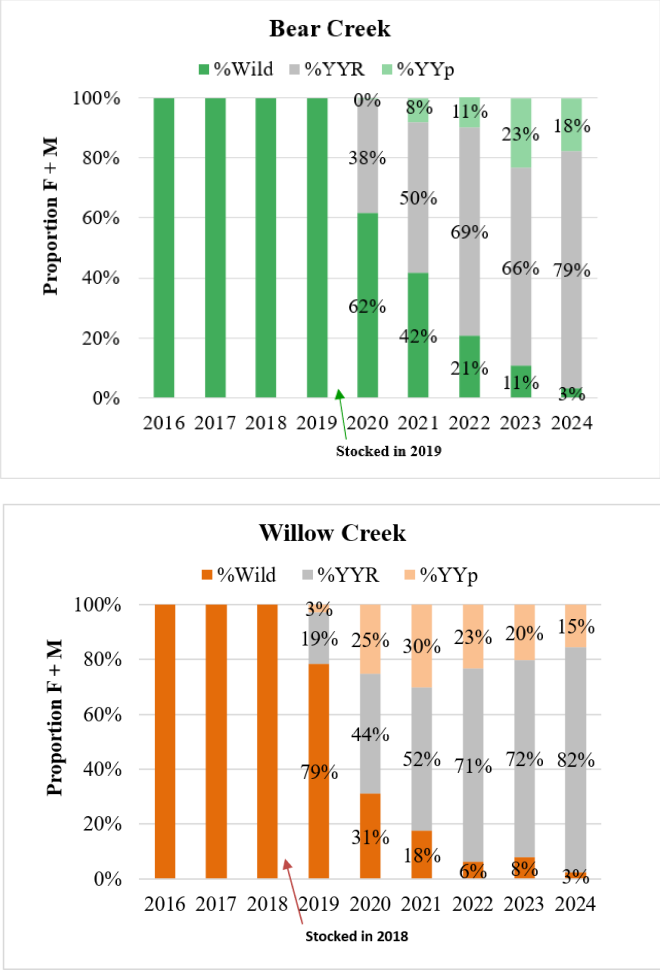
Table 10. Number of YOY Brook Trout collected during back-to-back electrofishing removal runs on consecutive days in Bear and Willow Creeks near Mackay Idaho, Summer 2016-2024.

Stream Name	Sample Date	2016	2017	2018	2019	2020	2021	2022	2023	2024
Bear Ck	7/5/16	22								
	7/6/16	55								
	7/5/17		13							
	7/6/17		22							
	7/5/18			3						
	7/6/18			12						
	7/9/19				6					
	7/10/19				8					
	7/7/20					10				
	7/8/20					8				
	7/6/21						32			
	7/7/21						32			
	7/6/22							6		
	7/7/22							2		
	7/5/23								4	
	7/6/23								1	
	6/25/24									2
	6/26/24									0
Bear Ck Total		77	35	15	14	18	64	8	5	2
Willow Ck	7/5/16	67								
	7/6/16	43								
	7/5/17		69							
	7/6/17		1							
	7/5/18			33						
	7/6/18			7						
	7/9/19				11					
	7/10/19				5					
	7/7/20					25				
	7/8/20					23				
	7/6/21						31			
	7/7/21						10			
	7/6/22							0		
	7/7/22							0		
	7/5/23								12	
	7/6/23								9	
	6/25/24									0
	6/26/24									0
Willow Ck Total		110	70	40	16	48	41	0	21	0

YY Male fish (n = 173) were stocked for the first time into Willow Creek in 2018 and GSI evidence indicates some of those fingerlings matured and spawned successfully that Fall. Based on fin clip observations and GSI screening of fish collected annually, after six years of YY male stocking, the Willow Creek population was composed of 3, 82, and 15% wild fish, stocked YY Males (YYR), and YY progeny (YYp), respectively (Figure 6). When comparing Bear Ck to Willow Creek, both after five years of stocking, the 2024 Bear Creek population was comprised of fewer wild fish (3% in Bear Ck vs 8% in Willow), a similar proportion of YY released from previous years (79 vs 72%), and virtually the same proportion of XY progeny

of stocked YY fish (18 vs 20%). At the end of the 2024 field effort, wild fish comprised 3% of both populations. In general, these two figures demonstrate the strong influence that YY Male stocking is having on both stream populations.

Figure 6. Proportion of both genetic female and male Brook Trout by origin collected during 2-pass electrofishing in Willow Creek and Bear Creek, Idaho, 2019-2024. Recaptured YY Males (YYR) were enumerated by observation of an adipose fin clip, while YY progeny (YYp) and Wild origin fish were ascertained via Genetic Stock Identification or GSI.



Examination of a length frequencies (even years only) over the project period reveals size-selective removal of the larger wild spawners from both the male and female populations over time and also the heavy impact that the combined IPM approach of electrofishing removal and YY stocking has on fry production (Figures 7 and 8).

Figure 7, Length frequency by stream of wild male Brook Trout collected during annual removal efforts 2016-2024. This includes genotypic wild males. Length frequency bins below graph.

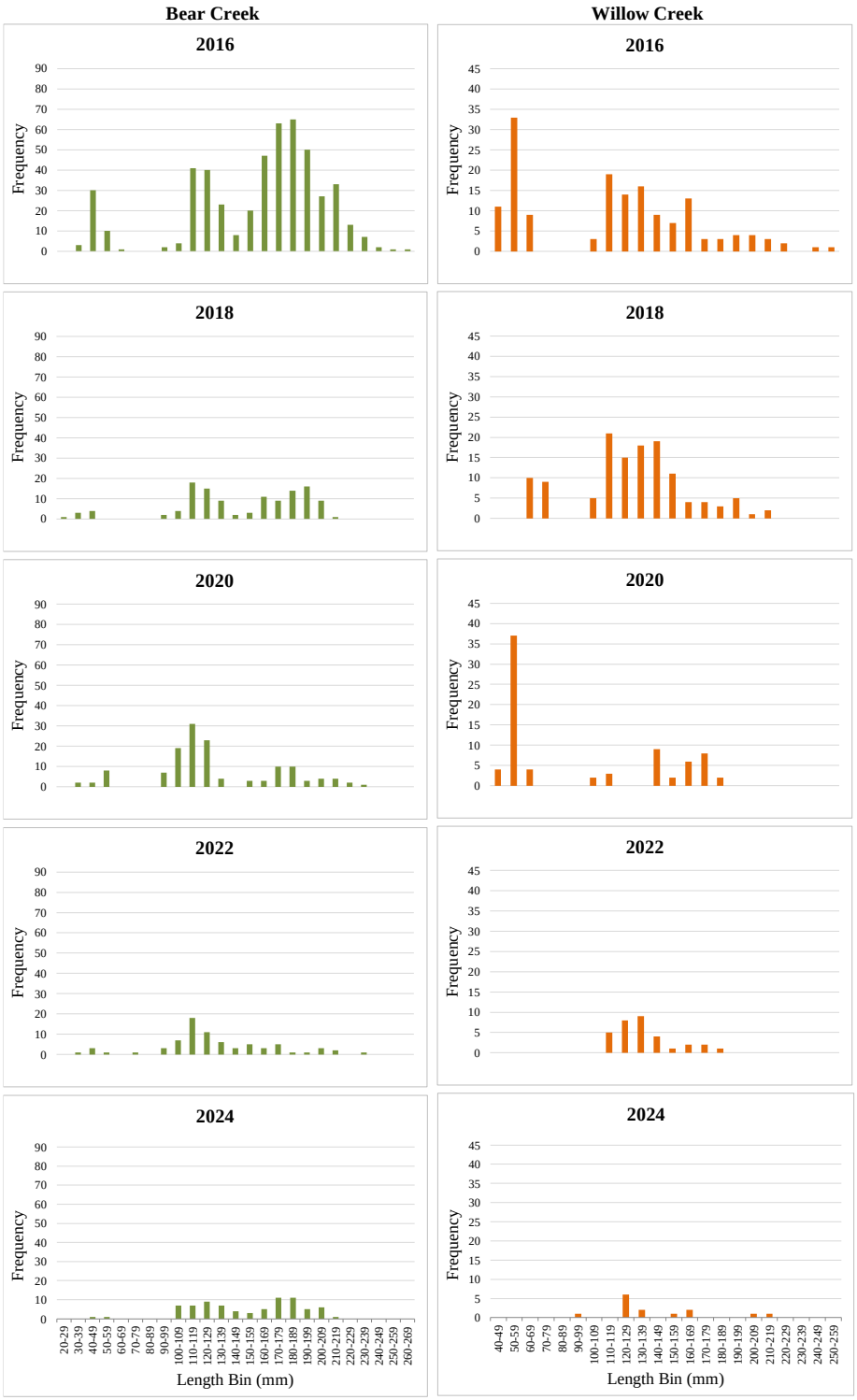
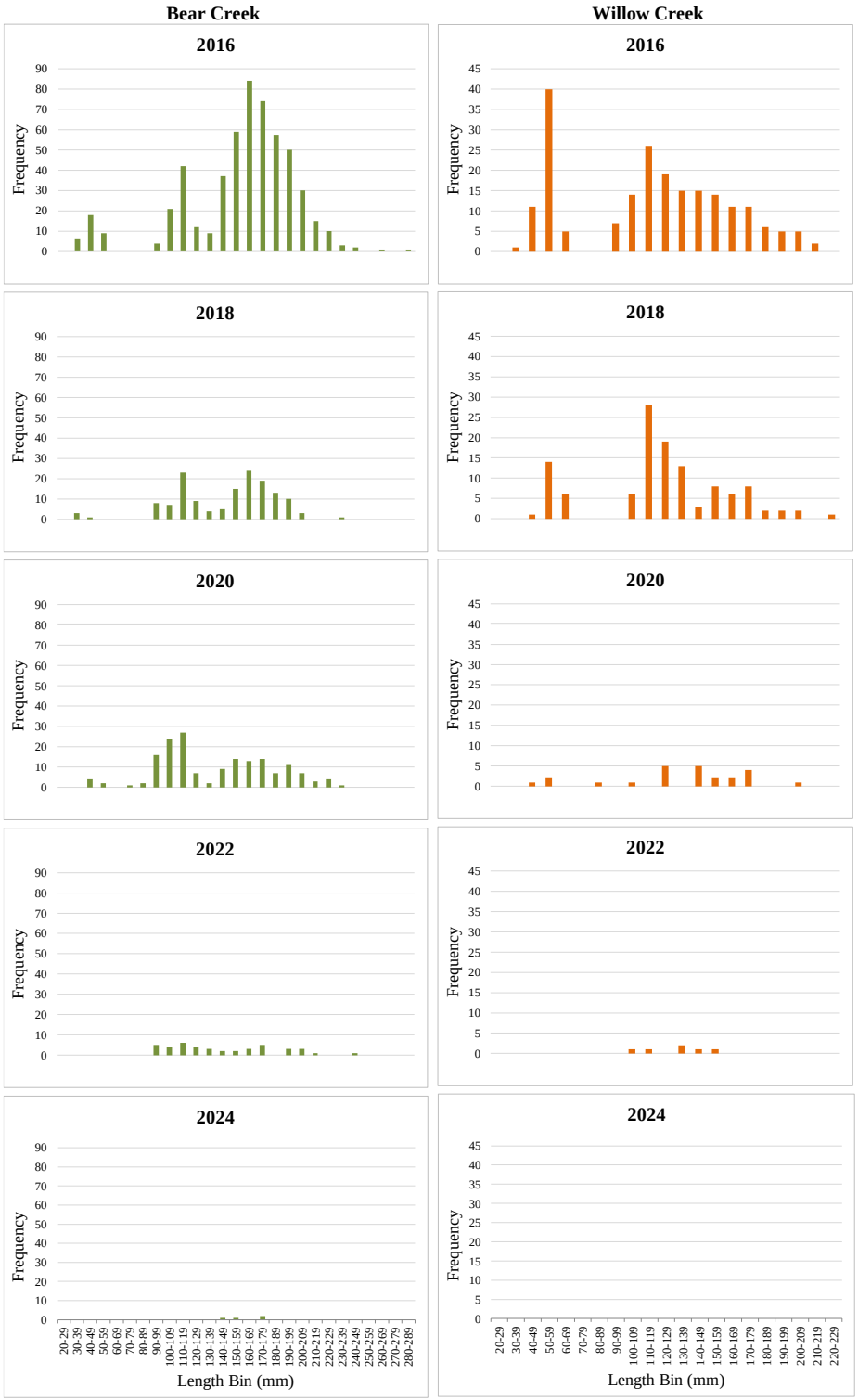
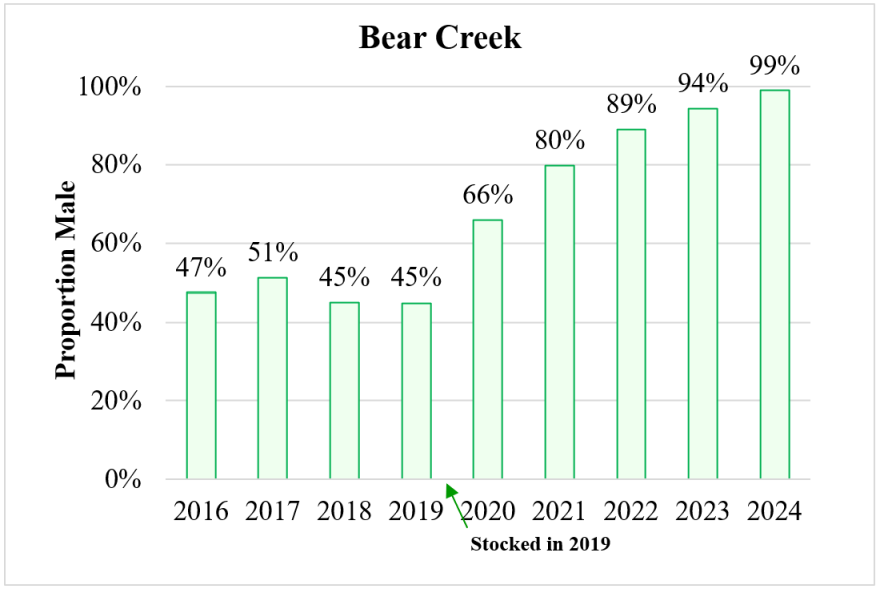


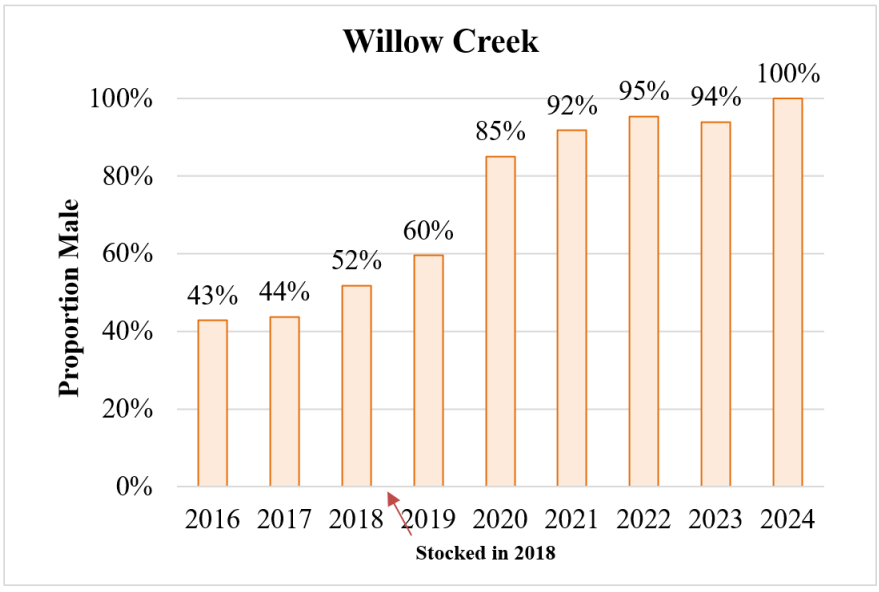
Figure 8 Length frequency by stream of wild female Brook Trout collected during annual removal efforts 2016-2024. Length frequency bins below graph.



A final population metric of interest for evaluating the Bear-Willow IPM effort is the overall population sex ratio. Prior to the initiation of YY Male stocking, the population genetic sex ratio averaged 46 and 47% male for Willow Creek and Bear Creek across suppression-only study years, respectively. In the five years since stocking in Bear Creek, the sex ratio for all fish sampled in the stream in mid-July increased to 99%. Six years post-stocking in Willow Creek, the male sex ratio reached the target, i.e. 100% (Figure 9).

Figure 9. Sex ratio of male Brook Trout (all parentages) from all fish collected in two Idaho streams, 2016-2024. Willow Creek was first stocked with YY fish in July 2018; Bear Creek first stocked in July 2019.





In summary, we are relatively confident that steep declines in fry recruitment, the 99-100% male sex ratios observed for the entire population (all fish sizes) in 2024, plus the complete or near complete lack of Age 1+ wild females remaining in the two populations, portend permanent Brook Trout eradication in both streams. Certainly, the lack of any females of any size class in Willow Creek indicates that full eradication has occurred, although follow-up confirmation sampling will be conducted for several years.

Project Communication

Three informational presentations were made by program staff during FY24. An in-person presentation of the Bear - Willow Brook Trout program results documenting near extirpation of female Brook Trout in Bear and Willow Creeks was made at the Idaho/Washington AFS meeting in Spokane Washington in April 2024. An in-person presentation was made to Great Basin National Park personnel (n = 7) on the status of the Bear-Willow eradication effort and possibility of getting YY Brook Trout fish for their future use. A discussion on the park providing additional YY Male funding for the Consortium followed. Lastly, at the request of Iowa DNR personnel, a zoom presentation on the use of YY fish and the Consortium was made at an Iowa statewide meeting of federal and state invasive fish biologists. Much interest at this latter session centered on the possibility of creating a YY Common Carp broodstock.

A two-day meeting during November 2023 was held in Phoenix AZ to address the possibility of Indexing the use of Estradiol, rather than continuing to attempt drug approval via the INAD program. The meeting included the AZ, NM, and ID Fish Chiefs, AADAP staff, Novaeel Inc. (President, Research Supervisor, CMC consultant), the president of Precision Science Inc, as well as YY program staff. A positive dialog resulted with the group expressing general belief that an Indexing for salmonids under the MUMS act should be undertaken as soon as possible. Results of this dialog proved decisive in YY project staff pursuing USFWS funding to attempt Indexing of salmonids during FY25 & FY26. Results of that funding attempt are discussed immediately below.

INAD/Index Coverage

Schill participated in both bi-annual INAD review zoom meetings held with AADAP each year to facilitate their interactions on the Brook Trout INAD with the FDA. Considerable progress was made towards the addition of Brown Trout to the Brook Trout INAD with AADAP's able assistance.

Progress Towards Indexing of Estradiol for Feminizing Salmonids.

Production of a YY broodstock for any species requires a feminizing hormone used to sex reverse genetic males. Estradiol is the drug currently being employed by WAFWA for this purpose. Such hormones

are considered drugs, and their use is regulated by the Food Drug Administration (FDA). Production of the existing YY Brook Trout broodstocks housed in Idaho and Colorado are covered in the FDA regulatory process by an INAD, or Investigational New Animal Drug, file currently held on WAFWA's behalf by AADAP in Bozeman MT. To date, AADAP and WAFWA have been unable to secure a drug sponsor for Estradiol in the arduous INAD process, but fieldwork using YY Brook Trout produced under the INAD is being conducted with the approval of the FDA.

Indexing, an alternative pathway for such drug uses, was created by the Minor Use/Minor Species (MUMS) Act of 2004, and is a more appropriate and less difficult pathway than the INAD/New Animal Drug Application (NADA) route. The indexing route enables the use of the drug without formal drug approval when risks are deemed minimal, such as the case where broodstock are treated with a drug but are not released. Indexing is a three-step process including 1) determination of eligibility by the Secretary of Health and Human Services, 2) an expert panel review of available information and subsequent acceptance (or rejection) of the panel recommendation by the Secretary, and 3) FDA addition of the drug to a list of Indexed drugs. A proposal was developed by program staff and submitted to the USFWS in FY24 under a federal NOFO funding invasive species eradication programs. The proposal was designed to specifically address items 1 and 2 above for the Indexing of Estradiol for feminization of multiple invasive salmonids.

The proposal was accepted by the USFWS with work commencing in FY25 by YY Consortium staff developing contracts with companies and consultants experienced in the submittal of Indexing eligibility applications, development of Chemistry, Manufacturing and Controls (CMC) data, feed development, and Environmental Assessments (EA). The associated work on Indexing is planned to occur over the next 2.5 years.

Impacts of Estradiol on Liver Tissue of Brown Trout – BY20 CO Health Wrap-up

Background

Impacts to fish health from exposure to a drug is an important aspect for consideration in the FDA drug approval or Indexing process. An E2 feminization trial was begun at the CO Research Hatchery in Fall 2020 and reported on in the subsequent year (Schill et al. 2022). Result of the effort were positive, with several recipes yielding high rates of feminization for genetically male fish. In general, health impacts of test fish from the three most successful treatment protocols appeared to show minimal fish health impacts based on fish growth and a series of external fish health ratings (Schill et al. 2022). Because E2 exposure has been shown to have potential impacts on rainbow trout livers in a past study, additional study focus was placed on this aspect of fish health. Values reported in Schill et al 2022 for HSI have been recalculated as an error was discovered in the above report. A new analysis is provided below.

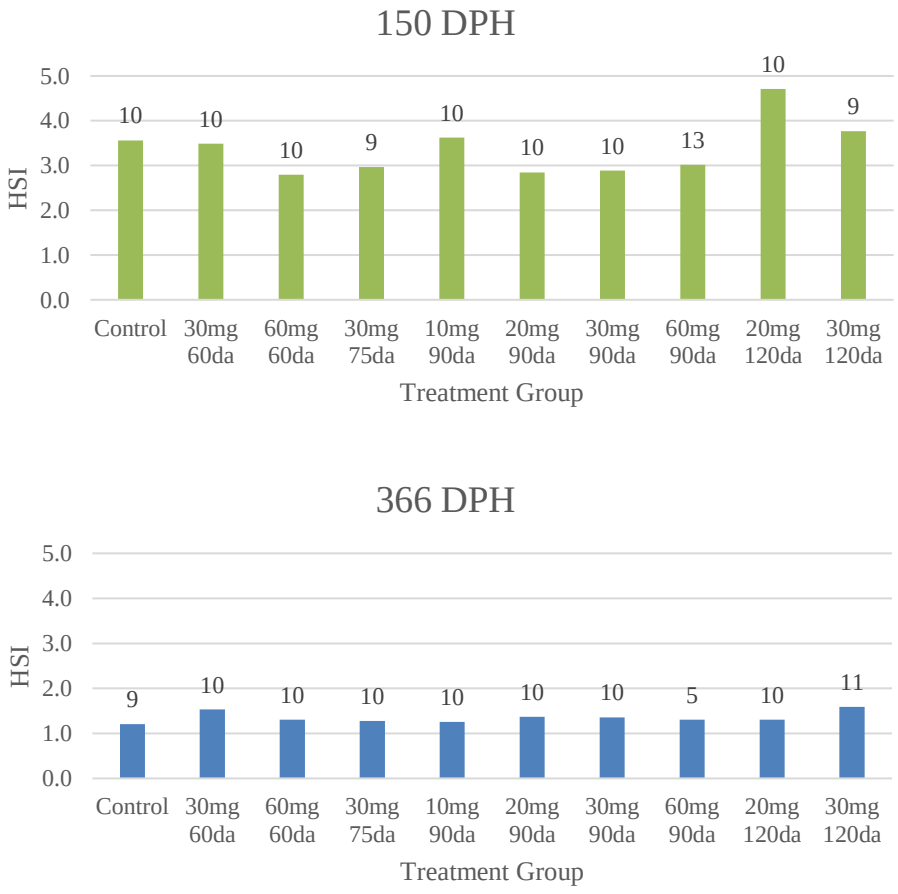
Methods

Livers were excised and weighed at two time intervals, including immediately after the completion of the longest E2 treatment at 150 DPH and at 366 DPH at the conclusion of the feminization trial. At both sampling events, 5 fish were randomly sampled from each treatment group tank. In addition to the collection of liver weights, liver tissues were stored in 10% neutral buffered formalin for further histological examination (see below).

Results

Based on Hepatosomatic Index (HSI) trends, at 150 DPH, although the 20 mg 120 d treatment group had the highest HSI, we saw no clear trend of E2 treatment intensity on HSI across the nine treatment regimens evaluated (Figure 10). At 366 DPH a similar lack of pattern in HSI was observed.

Figure 10. Hepatosomatic index (HSI) 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. Numbers above bars are n's for each treatment group.



FY2024 Follow-up Pathology Work on BY20 CO Trial Liver Tissue

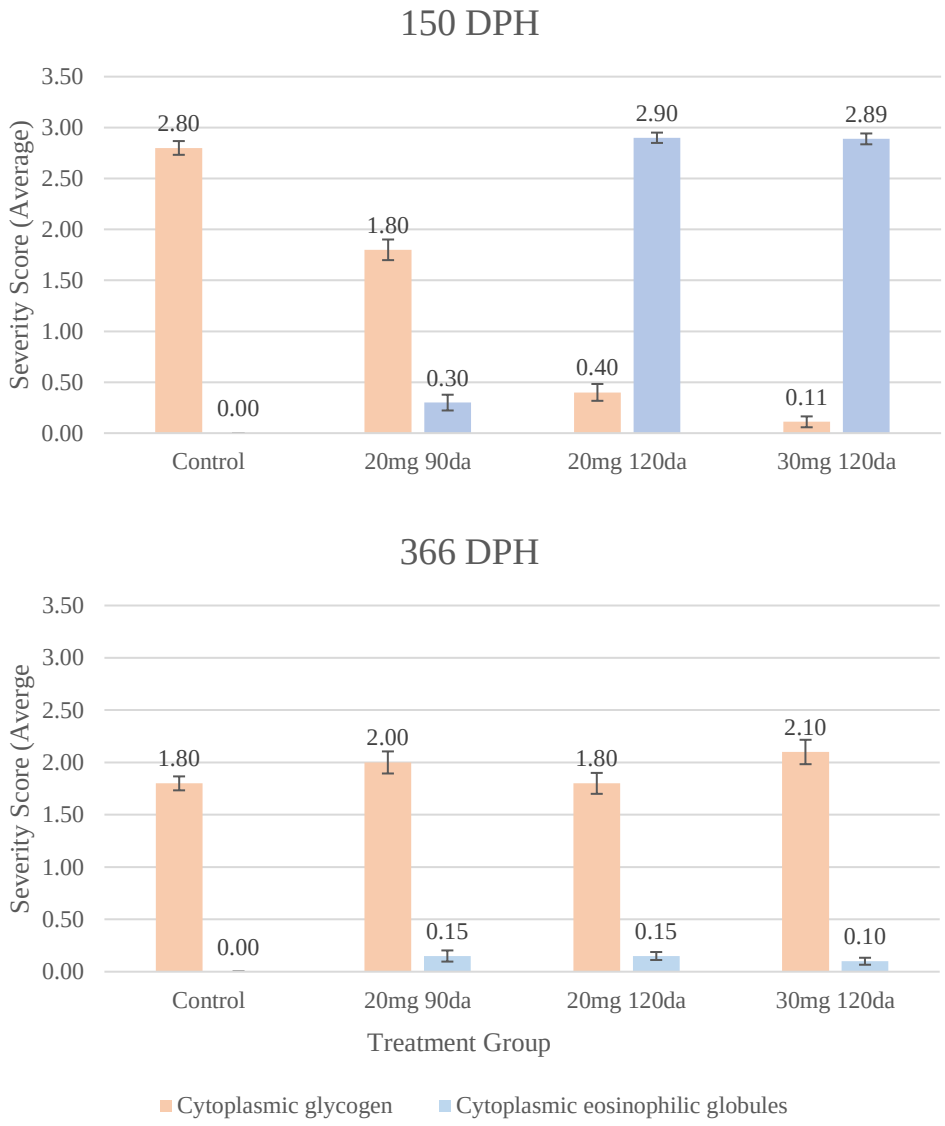
Background

Despite the lack of obvious liver impacts based on HSI as reported above in the BY20 CO trial, it was decided to have the preserved liver tissues examined by a certified pathologist. Given cost constraints, this follow-up work was focused solely on the three treatment doses and durations that demonstrated the most likely successful feminization recipes (20 mg 90 d, 20 mg 120 d, 30 mg 120 d) as well as Controls. Without prior knowledge of treatment dose or duration, a certified pathologist at Fish Head Laboratories evaluated the histology of the tissues for general morphology, inflammation, variation of cellular morphology and neoplasia.

Results

Lab personnel reported that there was liver degeneration in the high duration treatments but also indicated that livers can regenerate, and that healing should start within a week after treatment stopped (S. Fogelson, Fish Head Lab Pathologist, pers comm.). A temporal comparison of two hepatocellular cytoplasmic changes suggests that healing may have occurred. Cytoplasmic glycogen levels were reduced in all three treatment groups relative to Controls on the day after the longest duration treatment ended (150 DPH) with levels of the two 120 d treatments particularly affected. However, by 366 DPH, glycogen levels in the three treatment groups had recovered and were similar to that of Controls (Figure 11). Similarly, there were positive changes in eosinophilic globular material across the two time periods. Immediately after all three E2 treatments, eosinophils were elevated, particularly for the two 120 da durations. However, as with the glycogen levels reported above, by 366 DPH, eosinophils in all three treatment groups had declined precipitously to levels barely above those observed in Controls, suggesting healing had occurred. The elevated eosinophil in prolonged treatment groups (20 mg 120 d and 30 mg 120 d) is expected and is commonly characterized as transient. (N. Walrath, IDFG Eagle Fish Health Laboratory Veterinarian, pers. Comm.) Pathology reports for all liver sampling are digitally too large to include with this document and are readily available from the WAFWA YY Coordinator upon request.

Figure 11. Comparison of hepatocellular cytoplasmic changes (glycogen-type vacuolar change and eosinophilic globular material; with average severity scores and Standard Error) between treatment groups of BY20 CO Brown Trout at 150 and 366 DPH, having been exposed to differing doses and durations of Estradiol starting at first feeding. COFRH Bellvue, CO. Sample size for all estimates = 10.



Identify Additional YY Partners and Funding Opportunities

Numerous zoom presentations/discussions and phone conference calls were held by McIntosh and Schill with upper level USFS staff, various levels of the USFWS, including field and Washington DC staff, the National Park Service, and Iowa DNR. Considerable progress has been made, and continued YY funding from the USFS totaling $120,000 was garnered during FY24. A total of $540,000 in funding was also obtained from the USFWS for a project entitled "Rapid Response, Early Intervention and Eradication of Invasive Fish Using YY Male Technology". These funds were obtained by application for funds from the Biden Infrastructure Funding Bill. In May, an in-person YY Male funding dialog was held with personnel at a meeting in Great Basin National Park, where personnel reported preliminary success in their competition for a multi-year YY grant of $40-50,000 per year from a Las Vegas mitigation fund. A final decision for that funding should occur in FY25.

YY Brook Trout Technical Team

The goal of the team is to assist the other YY Brook Trout egg receiving entities in collectively planning their own research and monitoring activities. The members list varies slightly by year, having about 30 individuals across state and federal agencies who are copied on team emails, with a core group of roughly 20 individuals regularly involved, including the EFGL Manager, Matt Campbell, who provides guidance on field genetics sampling. In addition to an annual zoom meeting session, interactions occur between individual tech team members and the coordinator (Schill) throughout the year.

In Spring 2024 (18 April) the annual YY BK Tech Team zoom call took place with 26 participants from 8 states (AZ, CA, CO, ID, NM, NV, OR, WA). Participants provided updates on on-going YY BK project assessments and a presentation was given by UNM graduate student B. Graves regarding the comparison of the survival and growth of Age-0 Wild ($M_{XY} \times F_{XX}$) and Hybrid ($M_{YY} \times F_{XX}$) Brook Trout. Both ID and CO hatchery managers contributed to the important topic of condition and performance of stocked fingerling YY BK. Schill led a discussion on what appears to be working best and not so much across the various field efforts and informed the YY Tech Team of succession planning for the YY Consortium as L. Mamer will be retiring at the end of FY24 (30 June 2024) and D. Schill will be handing over the reigns on 31 Dec 2024 to a new program coordinator, as yet to be identified.

Group consensus was to continue with the annual meeting concept, but this will be up to the new coordinator. If that individual agrees, the next YY Tech Team meeting should again occur in Spring '25 to give participants time to work up their prior year's field data, but not impact on the upcoming field season.

Acknowledgements FY24

The YY Male Consortium effort is a multi-state team approach that would not be possible without the dedication and enthusiasm of a multitude of agencies and staff. While we will no doubt unintentionally fail to recognize some who have supported the consortium, please know we appreciate and respect the hard work and contributions provided by so many.

Sex Reversal & ESD Trials

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 and assisted and while we unfortunately neglected to note your names, we thank you all very much.

Brook Trout ESD field work on the two Mackay area streams continues to require extensive manpower coordination, contributions, and enthusiasm by B. Gamett (USFS) and staff , J. Vincent, K. Meyer and staff (IDFG), M. Campbell and many others on the IDFG EFGL staff including C. Coykendall, J. McCaine, J. Hargrove , as well as enthusiastic volunteer J. Dillon.

Thanks to C. Teal, and assistant C. Musser for driving the long haul from University of Utah to take stewardship of the voluminous Carp Feminization Trial materials in order to set up shop for the next round down in UT.

Sex Marker Development and Sampling

Many thanks go to the staffs at both CDOW Steamboat Springs (B. Atkinson, Lucas and Drew) and Hot Sulphur Springs regional offices (J. Ewert & H. Henderson) for collecting, holding and ably assisting with the thawing and handling of massive amounts of Northern Pike carcasses so that we may take additional tissue samples for genetic analysis. Your help was invaluable and greatly appreciated.

Without the expertise and support of M. Campbell, Eagle Fish Genetics Lab supervisor, Idaho's current broodstock for YY Brook Trout would not exist, nor would contemplation of the YY Male Consortium have been possible. K. Coykendall had the front-line responsibility of tackling the sex marker efforts on Common Carp and Walleye briefly chronicled in this report. They were assisted by a long list of lab technicians, particularly D. Eardley who does much of the bench work on these markers. Lastly, we thank the entire staff at the EFGL involved in DNA extraction etc., led by A. Boone and many others.

Last but certainly not least, J. McCane somehow keeps track of all the samples and data that come into the lab in relation to these projects, and also led field genetic data collection on Willow Creek, assisted there by J. Hargrove. We are grateful for his patience and ever efficient skillset.

Administrative support and assistance

Thank you to C. Campbell, from WAFWA, for assisting with submittal of the MSCGP grant as well as deftly stewarding the monthly fiscal administration of the WAFWA YY Male program. Without your monthly efforts, the project could not exist.

Financial Support

We thank the USFS for their substantial contribution to ongoing YY Consortium funding and their ongoing Brook Trout removal work with Consortium staff and IDFG near Mackay ID. Ten Fish Chiefs from the States of AZ, CA, CO, ID, KS, NM, NV, OR, WA, WY, and many of their program managers, were enthusiastic enough about the potential of YY Male fish that they contributed closely guarded funds from their budgets to support this FY24 work. The list of names supporting financial state contributions is too long to mention all here. We thank both the original and current funding Chiefs and program managers for their support. During FY24 three of the funding, Chiefs including L. Hebdon, J. Carter and K. Patten were asked to participate and provide guidance in a team effort to pursue the Indexing of salmonids and to lead the group of Chiefs in the hiring of a new YY Program Coordinator.

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Appendix A

Appendix A - 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				

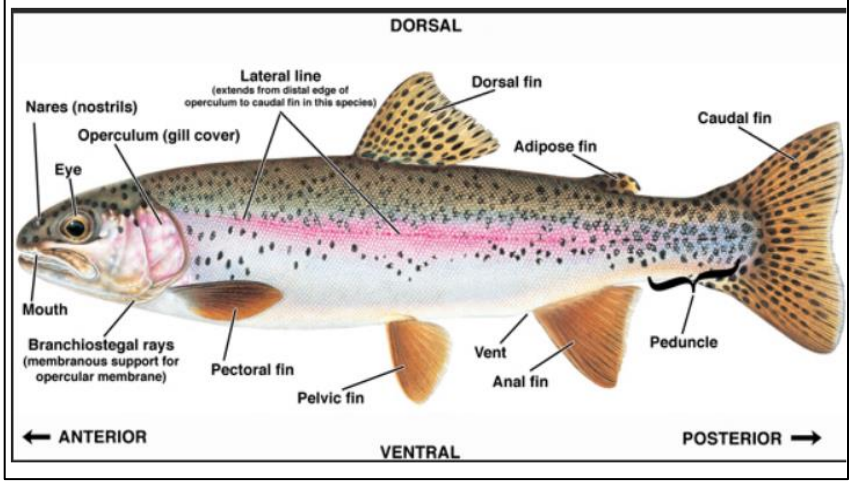
SCORING:
(*cumulative score could provide an early indication of arising health issues*):

15-16 – Very healthy

11-14 – Healthy

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

< 8 - Significant health concerns - requires action



Appendix B

Results of sex marker development efforts by the EFGL in FY2024

(K. Coykendall and M. Campbell)

B1 - Common Carp

At Eagle Fish Genetics Lab, we have two genetic sex markers that were discovered via reduced representation sequencing (RadSeq). We developed genotyping assays for both of them. Previously, the marker 744444_87 displayed high genotypic concordance with phenotypic sex in most populations tested. The phenotypic/genotypic concordance was low in a single population, which prompted the development of a second genetic sex marker and assay, 1506016. This marker displayed high phenotypic/genotypic concordance in all populations tested to date.

In FY2024, we ran both assays in two additional sampling groups: 153 samples from Lake Lowell, Idaho collected in 2016 and 220 samples from the Snake River collected in 2017. The 74444_87 genotyping assay is a Taqman assay (Thermofisher) and the conditions for amplification are as follows:

Each reaction contained 5 µl of TaqMan® Universal PCR Master Mix, 0.06 µl of the primer/probe mix, 1 µl DNA templated normalized to 10ng/ 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 54 cycles of 92 °C for 15 seconds (denature), and 62 °C for 1 minute (annealing), followed by a 4 °C hold for 10 minutes.

The 1506016 assay contains separate primers and probes and the conditions for amplification are as follows:

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 normalized to 10 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 5 minutes, and then 40 cycles of 95 °C for 15 seconds (denature), and 55 °C for 1 minute (annealing), followed by a 4 °C hold for 10 minutes. Both assays were run on a real-time PCR instrument (ABI 7500; Applied Biosystems).

Appendix B1 – Table 1. Concordance between genotypic and phenotypic sex in common carp from the Snake River, Idaho using the genotyping assay 744444_87. Under phenotype, the number of males (M) and females (F) are listed for each sampling group. Under genotype, the number of genotypic females (F) genotypic males (M), and the number that failed to genotype (NG).Below that are the concordance proportions parsed by phenotypic sex.

		Genotype			
		Male	Female	NG	Total
Phenotype	Male	43	13	1	57
	Female	5	156		161
	Total	48	169	1	

Male concordance proportion: 0.768

Female concordance proportion: 0.969

Total concordance: 0.917

Appendix B1 – Table 2. Concordance between genotypic and phenotypic sex in common carp from the Snake River, Idaho using the genotyping assay 1506016. Under phenotype, the number of males (M) and females (F) are listed for each sampling group. Under genotype, the number of genotypic females (F) genotypic males (M), and the number that failed to genotype (NG). Below that are the concordance proportions parsed by phenotypic sex.

		Genotype			
		Male	Female	MM	Total
Phenotype	Male	46	0	11	57
	Female	0	161		161
	Total	48	161	11	

Male concordance proportion: 1.000

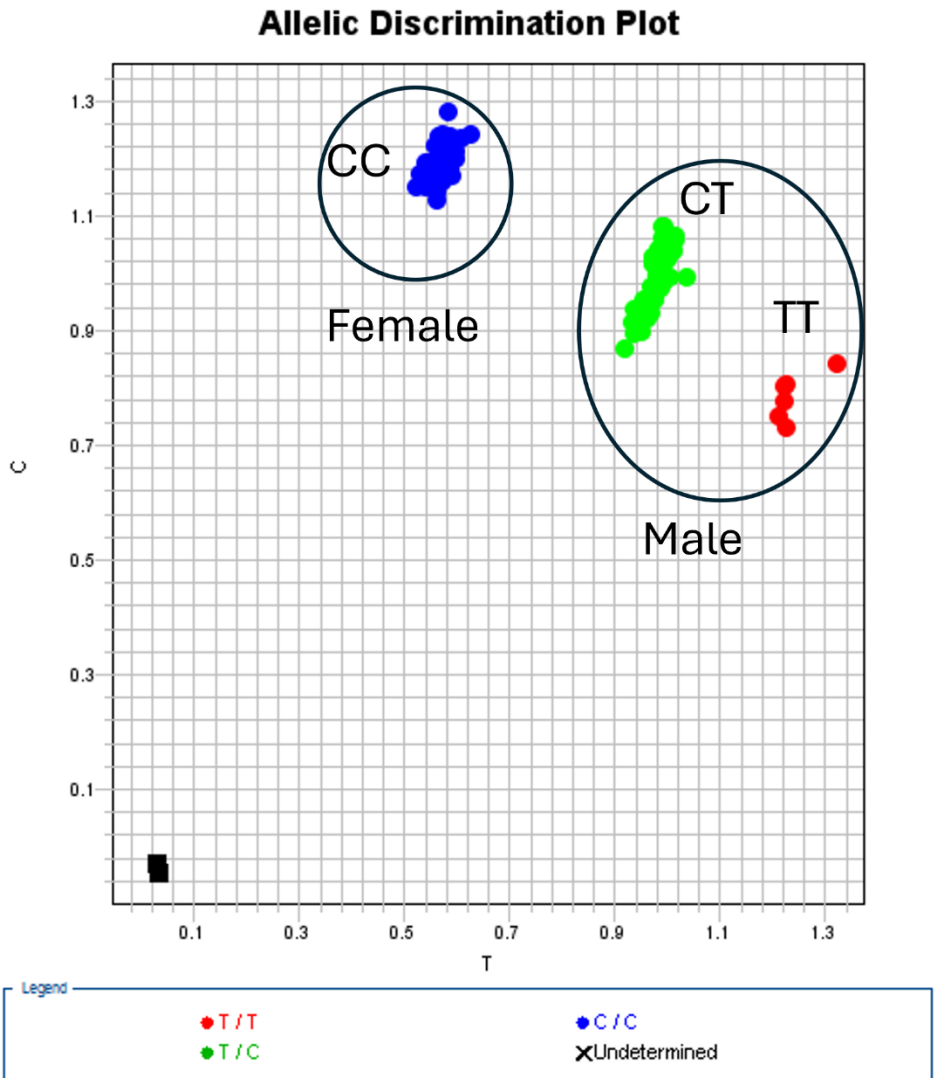
Female concordance proportion: 1.000

Total concordance: 1.000

In this case three genotype classes were observed instead of the expected two classes. This pattern was observed previously in the Guthrie City Lake, OK population (see 2023 report). The males with the TT

genotype are designated “MM” in Table 2. While this marker is an excellent tool to identify females and males in this population, it may not be a good candidate to discriminate between XY and YY fish produced during broodstock development.

Appendix B1 – Figure 1. Fluorescence plot for a subset of the Snake River common carp samples run with the 1506016 genotyping assay. Three distinct clusters are present representing the three possible genotypes at this marker.



Appendix B1 – Table 3. Concordance between genotypic and phenotypic sex in common carp from the Lake Lowell, Idaho using the genotyping assay 744444_87. Under phenotype, the number of males (M) and females (F) are listed for each sampling group. Under genotype, the number of genotypic females (F) genotypic males (M), and the number that failed to genotype (NG). Below that are the concordance proportions parsed by phenotypic sex.

		Genotype			
		Male	Female	NG	Total
Phenotype	Male	66	0	0	66
	Female	0	83	4	87
	Total	66	83	4	

Male concordance proportion: 1.000

Female concordance proportion: 1.000

Total concordance: 1.000

Appendix B1 – Table 4. Concordance between genotypic and phenotypic sex in common carp from the Lake Lowell, Idaho using the genotyping assay 1506016. Under phenotype, the number of males (M) and females (F) are listed for each sampling group. Under genotype, the number of genotypic females (F) genotypic males (M), and the number that failed to genotype (NG). Below that are the concordance proportions parsed by phenotypic sex.

		Genotype			
		Male	Female	NG	Total
Phenotype	Male	66	0	0	66
	Female	0	85	0	85
	Total	66	85	0	

Male concordance proportion: 1.000

Female concordance proportion: 1.000

Total concordance: 1.000

B2 - Walleye

Over the past two and a half years, the Eagle Fish Genetics Lab biologists have worked with several collaborators from University of Buffalo, Purdue University, University of Wisconsin-Steven's Point, Leibniz Institute of Freshwater Ecology and Inland Fisheries in Berlin, Germany, and the National Research Instititute for Agriculture, Food and Environment in Paris, France. Our collaborators have produced six annotated genomic assemblies, all high quality. They have confirmed the presence of the sex marker in every assembly and found genes nearby, including *foxl2a*, which is a transcription factor that regulates sex determination. The sex-linked pattern in the data is consistent with walleye having a ZZ/ZW sex determining system, where females are the heterogametic sex (ZW) and males are homogametic (ZZ).

Previously, we added the sex marker to our existing GTseq panel. The marker is a string of thymines (the T nucleotide of DNA). Females have 1 or 2 copies of a string of 8 T's. Males have a string of 9 T's. Note that this is different from our usual sex-linked markers where the two sexes are discriminated by a single nucleotide difference. We developed a genotyping script similar to the ones we use for genotyping other markers in our panels. We ran our walleye genotyping panel with the additional ZW sex marker on three sampling groups: 200 from Lake Pend Oreille, ID, 210 from Buffalo Bill Reservoir, WY and 100 from Rathbun Lake, IA. Note that the sample sizes reflect individuals that had a phenotypic sex call and a genotypic sex call. Below are tables containing results for each of the sampling groups

Appendix B2 – Table 1. Concordance between phenotypic and genotypic sex in Lake Pend Oreille, ID walleye. Samples were collected in 2017 and 2018.

		Genotype		
		Male	Female	Total
Phenotype	Male	106	19	125
	Female	0	85	85
	Total	106	104	210

Male concordance proportion: 0.848

Female concordance proportion: 1.000

Total concordance: 0.910

Appendix B2 - Table 2. Concordance between phenotypic and genotypic sex in Buffalo Bill Reservoir, WY walleye collected in 2020.

		Genotype		
		Male	Female	Total
Phenotype	Male	149	25	174
	Female	1	35	36
	Total	150	60	210

Male concordance proportion: 0.856

Female concordance proportion: 0.972

Total concordance: 0.876

Appendix B2 - Table 3. Concordance between phenotypic and genotypic sex in Rathbun Lake, IA. walleye collected in 2017.

		Genotype		
		Male	Female	Total
Phenotype	Male	44	6	50
	Female	1	49	50
	Total	45	55	100

Male concordance proportion: 0.880

Female concordance proportion: 0.980

Total concordance: 0.930

Concordance between phenotypic and genotypic sex varies between geographic populations. When results are parsed by phenotypic sex, concordance rates are higher in females than males. The lower concordance between phenotypic and genotypic sex could be indicative of a temperature override that acts upon an otherwise genetically-determined sex. Different fish taxa have been shown to have a genetically controlled sex determination system, an environmentally determined sex determination, or both (Ospina-Alvarez, Piferrer, 2008). The phenotypic sex ratios in the Lake Pend Oreille group and the Buffalo Bill Reservoir group skewed male (0.6 and 0.83 male, respectively). Sass et al (2022) reported differently skewed

sex ratios in walleye rearing ponds under different temperature conditions, providing further evidence of an environmental component to walleye's sex determining system. .

Ospina-Álvarez, N., Piferrer, F., 2008. Temperature-Dependent Sex Determination in Fish Revisited: Prevalence, a Single Sex Ratio Response Pattern, and Possible Effects of Climate Change. PLoS One 3, e2837. https://doi.org/10.1371/journal.pone.0002837

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