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

Contract Period: July 1, 2021 to June 30, 2022

Authors:

Daniel J. Schill - Fisheries Management Solutions, Inc.

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

and

Bruce A. McIntosh

Sept 30, 2022

Table of Contents

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 in invasive fish control was first undertaken for the Brook Trout (*Salvelinus fontinalis)* in November 2008 by the Idaho Department of Fish and Game (hereafter 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.

Based on both the positive population simulation and pilot study results, IDFG subsequently expanded YY Male Brook Trout research efforts to full-scale field evaluations involving 13 waters including six alpine lakes and seven streams. The design includes a test of stocking both fingerling and catchable-sized YY Male fish as well as suppression versus no suppression of the resident wild populations. These studies thus comprise full-scale tests of the IPM concept for two different stocked fish sizes and suppression strategies. The initial results of this research effort are just beginning to be documented (Kennedy et al. 2018). In 2014, due to the success and relative ease of creating the YY Male Brook Trout broodstock, 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.

In 2017, IDFG initiated a dialog with member states in WAFWA, the Western Association of Fish and Wildlife Agencies, regarding the 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 with the requisite 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 July 1, 2018 with nine stated program objectives and funded over three fiscal years, FY19-FY21. During July 2021, a decision made by 10 Fish Chiefs to continue funding the program for another three years (FY22-FY24).

YY Male Consortium Program Objectives

DESCRIPTION OF SERVICES. Beginning on July 1, 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 in regard to drug approval and aquaculture aspects of YY Male broodstock production.

This report documents results of the activities conducted during the fourth program year to enable attainment of those objectives. Appendix A lists tasks to be undertaken during the second three-year funding phase for attainment of program objectives. The pages below summarize results of efforts in FY2022 to address program objectives.

Background and Methods

Sex Reversal Trials

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 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. Additional FY22 hatchery fieldwork and results evaluation was completed for sex reversal trials initiated in Spring 2021in North Dakota for Walleye and for Common Carp at hatcheries in Texas and Oklahoma.

Brown Trout Trial 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 less vulnerable to angling and associated overexploitation so little help can be expected from anglers in terms of population reduction. The species thus 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 Estradiol (hereafter E2) were observed. The effect of concentration and duration of Methyl testosterone 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 1976; Johnstone et al. 1979; Schill et al. 2016a) we designed trials in BY19 at or near this concentration and also examined several typical treatment durations. 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.

BY2019 Trials

Colorado Trial

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 Brad Neuschwanger, Hatchery Manager, at the Colorado Fish Research Hatchery (COFRH), Bellvue, Colorado and George 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 1). 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.

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)

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 20mg 60da) 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.

South Dakota Trial

A sister study of similar design was initiated at the McNenny Fish Hatchery (MFH), working with Mike Barnes, Hatchery Manager, and Jill 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 regimens examined differed in the amount of Estradiol fed to the fish (Table 2). 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, Carlos 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.

BY2020 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 Paul 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 2). 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 2. 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. (Paul 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 3, 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.

Table 3. 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 Trial 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 were 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 done 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 mixed up together 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.

Common Carp Trials - BY2021

During April and May 2021 sex reversal trials using E2 on treatment groups of Common Carp were initiated in hatcheries in Oklahoma and Texas.

Oklahoma Trial

The first trial was started with Dr. William Shelton, University of Oklahoma (Emeritus) and Richard Snow, Fisheries Research Supervisor, Oklahoma Dept. of Wildlife Conservation, stationed at the Oklahoma Fishery Research Laboratory, Oklahoma City, OK. In this trial, begun on 24 March 2021, three test groups consisting of various concentrations of E2 and varying exposure lengths, plus a Control group. (Table 4). Broodstock were collected from Lake Thunderbird, OK, and allowed to free spawn in two hapas. Study fish were hatched in these tanks, one reared on solely natural food, one supplemented with dry feed (Otohime) until 21 DPH. Trial tanks were then stocked, n = 500, from the Otohime supplemented tank into indoor 400 l circular tanks, on flow-through well water (19 - 20 °C) influenced by ambient air temps, and fed dry pelleted feed (Otohime then transitioning to Rangen) for the remaining course of the study. All treatment and Control group feed was 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 any EtOH. The treatment groups were fed E2 coated feed beginning at either 25 or 60 DPH, until 150 DPH (Table 4). At spawning, genetic samples were collected from broodstock, and 100 genetic fin clips were taken at the time of stocking to document trial starting sex ratio. Mortality rate was documented daily. Control Interval samples were taken when the first tanks went on treated feed (25 DPH) and continued every 3 weeks until Sep 2021.

Table 4. Sex reversal trial framework for Common Carp receiving Estradiol via treated dry feed at the Oklahoma Fish Research Hatchery, Norman OK, initiated 24 Apr 2021. Numbers in matrix are replicates n's by treatment type with dosage, initiation and duration for each treatment group.

			Dosage (mg E2/kg dry feed)		
E2 Dose	Age at Initiation (DPH)	Duration (days)	200	300	None (EtOH only)
Short	60	90	2		
Mid	25	125		2	
Long	25	125		2	
Control					1

Main Trial Sampling - 151 DPH

Working with OFRL staff, project personnel sampled the fish remaining in this trial. Water quality issues appear to have had a large impact on trial populations and very few fish were left by the end of the

longest treatment duration, those remaining being quite undersized compared to previous carp studies growth rates. Data collected during necropsy from 212 fish at 151 DPH included total length, weight, and visual phenotype where possible, though inadequate fish size required the majority of these fish to be preserved whole for later histological examination (see results below).

Texas Trial

A companion study was initiated with Carl Kittel, Hatchery Supervisor, and Mike Matthews, Hatchery Manager, Texas Parks and Wildlife Department, at the A. E. Woods Hatchery, San Marcos, TX. In this trial, five E2 test groups were initiated at 25 DPH with E2 exposures and durations varying from 25 - 200 mg/kg and 90 - 150 days, plus Control and Control Interval groups (Table 5). An important feature of this trial was the inclusion of lower treatment levels for longer durations than that recently reported (200 mg/kg) to successfully feminize roughly half of male Common Carp (Jiang 2020). Using AE Woods Koi maintained on station as feeder fish, broodstock were hand spawned, eggs hatched on 22 May 2021, and reared to scalation in outside ponds on natural feed. Fry were counted into indoor 14' x 3' raceways screened into 12 individual sections (n = 500) fed by 22 - 24 °C river run water and trained on dry feed (Rangen), starting at 12% BW, reducing by 2% every two weeks until reaching 4% BW as a maintenance diet. Genetic samples of broodstock and 100 fry from communal stocking tank (for starting sex ratio) were collected prior to initiation of trial. Mortality rate was documented daily. A Control Interval sample was taken at first day on treated feed (25 DPH) and continued every 3 weeks until all groups were off treated feed in Nov 2021.

Table 5. Sex reversal trial framework for Common Carp (Koi) receiving Estradiol via treated dry feed at A. E. Woods State Fish Hatchery, San Marcos, TX, initiated 22 May 2021. Numbers in matrix are replicates n's by treatment type (dosage and duration) for each treatment group. All fry received Treated and Control feed beginning at 25DPH.

		Dosage (mg E2/kg dry feed)					
E2 Level	Duration (days)	25	50	100	200	None (EtOH only)	None (No EtOH)
Very Low	150	2					
Low	150		1				
Moderate	120			2			
Moderate	150			2			
High	90				2		
Control						2	
Control Interval							1

Main Trial Sampling

Initial sample – 331 DPH

On 24 April 2022 project personnel and AEWFH staff conducted what was anticipated to be the main sex reversal sampling effort. Biometrics was taken from fish in each replicate tank (target n of 60 fish per tank) for a total n of 577 fish. However, unusually large fat deposits and difficulty locating gonads of sufficient size to pick made our usual sampling approach of preserving whole gonads problematic. Mortality was continuing to occur and the trial fish should have readily differentiated by 331 DPH. A decision was thus made to remove heads and tails of all fish sampled, cut open the body cavity and preserve the remainder of whole fish bodies for subsequent gonad histology evaluation. Unfortunately, we made a mistake in not making a large enough insertion into the body cavity of fish this large and when the samples arrived at the histology lab all but the smallest had undergone lysis and were not capable of being used for histological examination. Remaining fish in each tank had been pooled by treatment group for further growout. Given the lysing incident, these fish were subsequently used for feminization evaluation as described immediately below.

Follow-up sample – 380 DPH

On 6 Jun 2022 project personnel returned to the facility and working with AEWFH staff, again conducted sex reversal sampling, this time on 252 remaining fish from the various treatment groups originally intended for growout to 2 years of age. Fish were deeply chilled via ice prior to sampling to improve discernment of gonads. Data collected during necropsy at just over one year (380 DPH) included total length, weight, visual phenotype and intersex observations. As replicates had been pooled at the time of initial sampling, data was collected by treatment type only. There was an exceptionally large range of fish sizes at time of sampling which resulted in gonads being dissected from most fish above 110 mm, and any fish below that size preserved whole after excising abdominal wall tissue to provide adequate exposure to formalin. As done for the initial sample, these tissues were sent to the histology lab for processing and imaging for gonad examination. Project personnel (Mamer) interpreted the histology images after consultation and training with S. Fogelson, a certified pathologist (Fishhead Labs, Stuart FL).

Walleye Trial - BY2021

Prior sex reversal work on the species, initiated in 2017 by Idaho Fish in Game in cooperation with the States of Iowa and Kansas, and subsequently reported on by Consortium project staff (Schill and Mamer 2019) yielded two highly efficacious recipes (100% feminization rates) for two different treatment protocols (15 mg/kg for 84 or 100 days). However, due to predation losses late in the trial, sample sizes were small for individual trial groups including Controls (n = 23 - 35) and the design did not permit replication at either facility. In May 2021 we initiated a follow-up sex reversal trial for Walleye with our USFWS partners at the

Garrison Dam National Fish Hatchery, Riverdale, ND, working with hatchery staff Rob Holm (now retired) and Ben Oldenburg. The framework for the design of this trial involves four different dose/duration combinations along with Control and a Control Interval group (Table 6). An important improvement of the current effort relative to the 2017 trial, is the employment of replicates in 84d treatment and Control regimens (n = 3). This addition should enable us to confirm that the positive results noted above for the 2017 study (100% feminization) is on target. Due to the need to begin exposing fish to treated feed exposure prior to scalation, a period before which Walleye are extremely susceptible to disease and mortality from handling, a bulk rearing design was utilized that allowed fry to hatch out and rear at similar densities while receiving the appropriate treated feed and then being split out into terminal replicate tanks post-scalation at approximately 42 mm in size.

Table 6. Sex reversal trial framework for Walleye receiving Estradiol via treated dry feed at Garrison Dam National Fish Hatchery, Riverdale, ND, initiated 4 Jun 2021. Number of replicates by treatment type (dosage and duration) for each treatment. Fry received Treated and Control feed beginning at approximately 20 mm in size.

		Dosage (mg E2/kg dry feed)				
E2 Duration Level	Duration (days)	5	15	75	None (EtOH only)	None (No EtOH)
Low	60		1			
Low	84	1				
High	84		3	1		
Control					3	
Control Interval						1

On 5 May 2020, broodstock collected from Garrison Reservoir were hand spawned and eggs measured volumetrically to provide an effective initial density (31 fry/l per Alan Johnson IDNR, pers communication) and reared in McDonald jars which hatched into the bulk rearing tanks. Once established in the terminal tanks, fry were reared at 1.7 fish/l to continue the course of the trial. Study fish were fed dry pelleted feed, top-coated with the appropriate mg/kg feed E2 solution diluted with non-denatured ethanol (EtOH), using a hand-held sprayer (Schill et al. 2016a). 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 any EtOH. The treatment groups were fed E2 coated feed for varying durations of 60 to 84 days, beginning at first feeding (22 DPH, 22mm average L). Genetic fin clip samples were collected from broodstock at spawning, and 100 fry genetic samples were taken at the time of post-scalation tank splitting to document early sex ratio if a sex marker for Walleye is eventually found (see below).

At 64 DPH, tank populations were beginning to show a widening span in length frequency which led us to suspect cannibalism which can be problematic in Walleye larvaculture (Alan Johnson, IADNR, pers communication). As a result, Garrison staff culled down all tanks to the same density (0.08 fish/L) and selectively removed the largest putative predatorial fish from each tank to avoid cannibalization and disease brought on by biting.

Main Trial Sampling - 279 DPH

On 10 Mar 2021, project personnel working with GNFH staff conducted the main sex reversal trial sampling. Data were collected during necropsy from 1133 fish (279 DPH) and included total length, weight, visual phenotype, sexual maturity level, and intersex observations.

Fish Health Sampling - 279 DPH

Health sample fish, (10 fish per tank) were selected randomly, PITtagged, and placed in a communal raceway to be held until all treatment groups had been sorted. Health exams were thus done without prior knowledge of treatment, and the fact that fish from the 9 study groups were mixed up together during exam further "blinded" the lone reviewer, minimizing any potential observer bias. Each health sample fish received an external exam to quantify relative external appearance as in the Brown Trout work described above (Table 3, Appendix B - Figure 1). The sampled fish were also measured for total length (mm), weight (g) and underwent a necropsy exam that included visual phenotype, sexual maturity level, intersex observations and measurement of gonad weight. In addition, for those treatment groups that had replicates (15mg 84 d & Controls) 5 of these fish also had liver weight taken (g) and liver tissue preserved for histology The remaining fish within a treatment group (n = 5) were preserved whole for possible pathological exam. The two remaining non-replicate treatment groups (15mg 60 d & 5mg 84 d) had liver data collected from 8 and 10 fish respectively.

Lake Trout Differentiation Study - BY2020

Given our own unsuccessful E2 sex reversal efforts on Lake Trout (Schill and Mamer 2020) and those of Wenstrom (1975) and Herman and Kincaid (1991) with similar poor results, it was determined that the full window of gonadal differentiation needed better documentation at the IDFG Lake Trout hatchery and within those specific environmental conditions. In November 2020, IDFG's Grace Fish Hatchery (GFH) staff began rearing a year class of LKT for serial sampling and associated histological preservation. The intent of this sampling is to observe the onset of anatomical differentiation and completion of cytological differentiation. Ideally, developing male LKT fry should be exposed to E2 over this entire time period to ensure full sex reversal. Developing fry were sampled at two-week intervals beginning at 14 DPH to identify the initial period where sex differentiation began for both sexes.

Fertilized eggs were received 16 Nov 2020 from Story Fish Hatchery, WY, hatched, and bi-weekly sampling performed from 7 Dec 2020 at 14 DPH (677 CTU) to 6 Dec 2021 at 364 DPH (5118 CTU). Sampled fish were placed in 10% neutral buffered formalin. As the fish developed temporally, samples were submitted periodically for histological examination (Fishhead Labs, Stuart, FL). Fish were subsequently cut along the transverse axis and digitally imaged at 4 - 40x magnification. The work ended when complete (terminal) cytological differentiation could readily be observed in a strong majority of both sexes.

Sex Markers

General Approach

To develop genetic sex tests or sex markers for species of initial interest to the WAFWA Consortium, EFGL uses existing Y-chromosome (sdY) DNA sequences available for or generate new DNA sequence data using Restriction site associated DNA sequencing (RADseq). For the RADseq work, mature adult fish of wild origin are collected by project personnel, 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 and cut into fragments using specific restriction enzymes. Selected DNA fragments are then sequenced so that the exact order of nucleotides (i.e. A,C,T,G) can be determined. These sequences can then be compared between phenotypic males and phenotypic females to find specific single nucleotide polymorphisms (SNPs) specific to each sex.

Background and Sample Collections

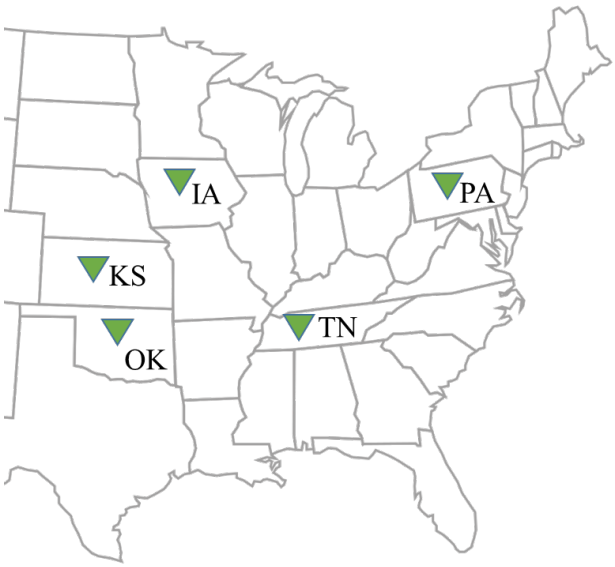
The overarching goal of sex marker work in the program is initially to develop Y chromosome-linked markers that would permit the differentiation of XX and XY individuals and subsequently, if possible, develop bi-allelic sex markers that would allow differentiation of XY and YY fish.

Common Carp

During FY2019 EFGL staff identified two candidate bi-allelic loci and screened one (Cca744444_87) on 800 samples from 11 samples, reporting an overall concordance rate between genetic and phenotypic sex of 93 %, values considered adequate for development of a YY Male broodstock (Schill and Mamer 2019). However, concordance varied considerably across populations and it was recommended that future work employ a second restriction enzyme that cuts the genome more frequently to identify additional candidate sex markers (Schill and Mamer 2019, Matt Campbell EFGL - Appendix B2). Accordingly, during FY2021, large samples (target n = 200, 100 from each sex) from five new waters in PA, TN, IA, WA and OK. 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 (Figure 1). Funding for this work was obtained with the support of AWFWA

under the MSCGP program and administered by the USFWS. A detailed description of methods and results of this effort is described in detail in Appendix C1.

Figure 1. Location of Common Carp populations sampled by project staff in Spring 2021 for sex marker expansion under the Multi-State DJ Grant program (MSCGP).



Walleye

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. The approaches attempted by EFGL staff and partners along with subsequent results are described in detail in Appendix C2.

Northern Pike

At the onset of the YY Male Consortium, the Alaska Department of Fish and Game (AKFG) 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, AKFG personal communication). In FY22, AKFG Division of Sport Fisheries provided additional funding for the AKFG Gene Conservation Lab (GCL) to develop 12 potential markers with a high probability of differentiating sex in northern pike using (RADseq) techniques. The first five markers are associated with a section of the genome highly correlated with sex and the remaining seven markers are associated with other sections of the genome correlated with sex at lower levels. To further assess the

effectiveness of these markers to differentiate sex in pike, the 12 markers were applied to about 1000 new individual Northern Pike of known sex which included both native and invasive populations (Wei Cheng, AKF&G, personal communication). Data analysis and a draft report is planned for Spring 2023.

Density-dependent Sex Change

Background and Overview

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 can be environmentally changed (Reinboth 1980). 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, 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. 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 a 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 ESD 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.

IDFG has undertaken three ESD evaluations including two in the hatchery setting and one *in situ* with wild Brook Trout. A small pilot trial for Common Carp was undertaken in a hatchery setting, and the null finding (no ESD observed) was reported previously in Schill and Mamer (2019). A larger effort working on Lake Trout was conducted with hatchery managers of the Grace Fish Hatchery (GFH) including Malia Gallagher, Eric Pankau, and Wayne Fowler. Of fish raised at five different initial density environments (5, 10, 20, 50 & 100 fish per 14 L pot, at hatch through 300 DPH), 100% were found to have matching pheno-

genotypes and there were zero instances of intersex observed in any of the histological samples. Thus, assuming the sex marker was accurate in this population, we observed no evidence of density-dependent sex change in this hatchery Lake Trout study (Schill and Mamer 2021).

Brook Trout ESD

A larger field study of potential ESD, initiated by IDFG and Bart Gamett of the United States Forest Service, was initiated 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 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. The entire lengths of both streams have been subjected to Pulsed DC electrofishing removal on two consecutive days in early July for the past 7 years. All wild Brook Trout collected were killed and a fin clip 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 labelled bags, frozen on dry ice, and returned to the laboratory. Bagged fish were subsequently defrosted, 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 mature individuals and any discordance recorded.

Population Response to Suppression and Stocking

The ESD evaluation described above involved the annual genetic sexing of virtually every wild Brook Trout handled during the study including YOY and adults. This enabled annual population abundance estimation for the main fish of interest in a YY Male field evaluation, that being 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 (VanDeventer 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 several years of suppression, it was decided to initiate YY Male fingerling stocking in both streams to maximize wild Brook Trout population reductions and thus speed examination of ESD at desired low 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 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.

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 and Discussion

Because male sex reversal and sex marker development are the two primary hurdles to YY Male broodstock development for a given species and comprise the initial main thrust of the YY Male Consortium program, we present a combination of those results by species below when work was conducted on both aspects. The remaining results in this report are presented under separate topical headings.

Brown Trout

BY2019 Sex Reversal Trial

Colorado

Maturity Monitoring

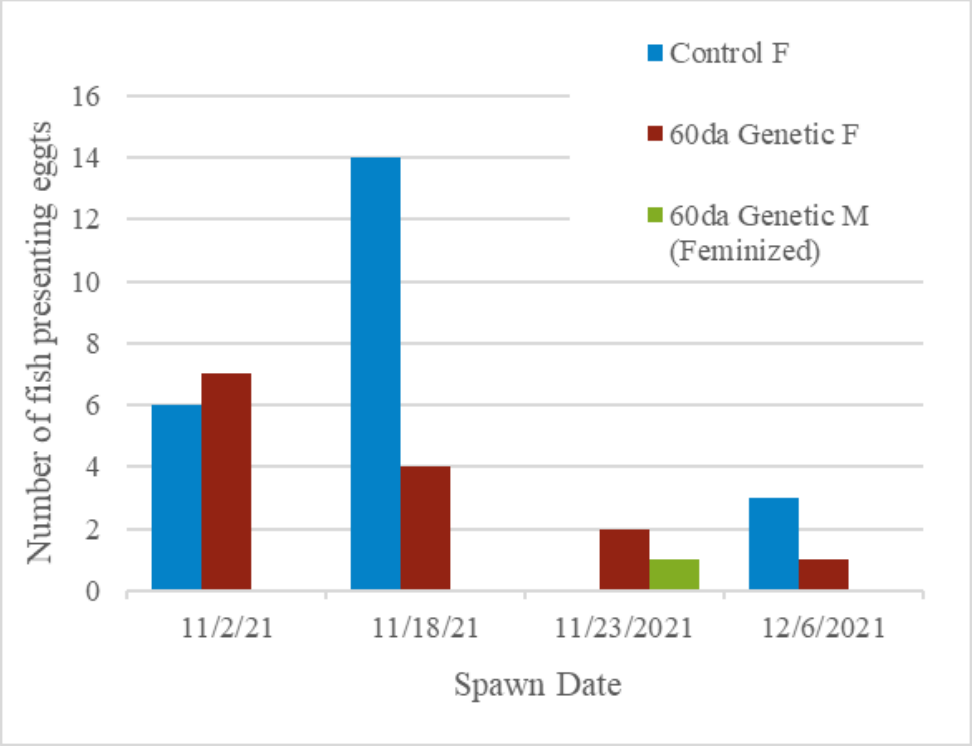
Of the 41 genetic females examined weekly from 2 Nov – 6 Dec 2021 (age during stripping being 726 – 760 DPH), COFRH staff observed successful maturation of all Control females (n = 23; 100%), and noticeably lower genetic female maturation ratio in the 20mg 60d treatment group (n = 18; 77.8%). On the 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 7, Figure 2). 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. 2016a). 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 7. 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 2. Maturation timing by treatment type of Brown Trout at 726-760 DPH, as related to genotype and phenotype, Colorado Fish Research Hatchery, Nov 2021.



BY2020 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 8). 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 8. 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
30mg 60da	8	59	57.6	42.4
	9	58	56.7	43.3
	Total	117	57.1	42.9
60mg 60da	11	60	53.3	46.7
	12	59	39.0	61.0
	Total	119	46.2	53.8
30mg 75da	2	60	48.3	51.7
	3	58	48.3	51.7
	Total	118	48.3	51.7
10mg 90da	19	58	48.3	51.7
	20	60	41.7	58.3
	Total	118	45.0	55.0
20mg 90da	13	59	50.0	50.0
	14	55	53.4	46.6
	Total	114	51.7	48.3
30mg 90da	6	56	48.3	51.7
	7	57	46.7	53.3
	Total	113	47.5	52.5
60mg 90da	10	57	49.2	50.8
20mg 120da	17	53	54.5	45.5
	18	58	39.7	60.3
	Total	111	46.9	53.1
30mg 120da	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 9). Feminization of males ranged from 63.1% for the 10mg 90day 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 30mg). The third highest feminization rate of true males (81.8%) was produced by the 20mg 90day 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 60mg, 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 day exposure groups which resulted in the highest feminization and lowest IS rates (Table 9).

Table 9. 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		
30mg 60da	51	63.3	10.2
60mg 60da	64	64.1	17.2
30mg 75da	62	73.3	13.3
10mg 90da	66	63.1	13.8
20mg 90da	57	81.8	7.3
30mg 90da	63	75.4	16.4
60mg 90da	30	75.9	20.7
20mg 120da	60	94.9	3.4
30mg 120da	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 10). 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 10. 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
30mg 60da	F	65		3
	M	31	5	13
60mg 60da	F	53	2	
	M	41	11	12
30mg 75da	F	58		
	M	44	8	8
10mg 90da	F	52		1
	M	41	9	15
20mg 90da	F	57		2
	M	45	4	6
30mg 90da	F	52		
	M	46	10	5
60mg 90da	F	28		
	M	22	6	1
20mg 120da	F	52		
	M	56	2	1
30mg 120da	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 11). However, by 366DPH, 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 20mg 120d 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. 2016a).

Table 11. 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
30mg 60da	117	80.3	5.6	157.4	42.2
60mg 60da	119	79.3	5.4	153.8	40.0
30mg 75da	118	78.1	5.1	157.8	43.0
10mg 90da	118	79.3	5.5	153.2	38.0
20mg 90da	114	76.8	5.1	155.0	39.8
30mg 90da	113	74.2	4.5	150.4	37.0
60mg 90da	57	70.8	4.0	151.6	38.8
20mg 120da	113	72.2	4.7	149.0	36.4
30mg 120da	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 12). 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 (30mg 120da) was identical to Controls at 0.36.

Table 12. 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
30mg 60da	150	20	0.21
	366	18	0.42
60mg 60da	150	20	0.16
	366	20	0.40
30mg 75da	150	19	0.18
	366	20	0.36
10mg 90da	150	17	0.26
	366	20	0.30
20mg 90da	150	20	0.17
	366	20	0.38
30mg 90da	150	20	0.16
	366	20	0.30
60mg 90da	150	18	0.14
	366	10	0.33
20mg 120da	150	20	0.26
	366	20	0.32
30mg 120da	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 13). 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 20mg 120d group which experienced a slight but likely insignificant reduction (Table 13). Body health rankings showed no ill effects due to E2 exposure.

Table 13. 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
30mg 60da	18	3.9	3.3	3.8	4.0
60mg 60da	18	3.3	2.6	3.9	4.0
30mg 75da	19	3.7	2.6	3.9	4.0
10mg 90da	17	3.5	2.0	3.9	3.9
20mg 90da	18	3.4	2.7	3.8	4.0
30mg 90da	19	3.4	2.8	3.9	4.0
60mg 90da	10	3.3	2.8	3.9	4.0
20mg 120da	20	3.5	2.4	3.6	4.0
30mg 120da	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 14). 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 14. 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.

Maturation Status	Phenotype	Control	30mg 60da	60mg 60da	30mg 75da	10mg 90da	20mg 90da	30mg 90da	60mg 90da	20mg 120da	30mg 120da
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

Summary- BY2020 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 20mg 90d 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 FY2022 and FY2023, 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.

Common Carp

BY2021 Sex Reversal Trial

Oklahoma

At 150 DPH, the conclusion of this trial, the remaining 212 fish were sampled and the trial terminated. While specimens were preserved whole for histology, it was later determined from a subsample that few of the fish had sexually differentiated, precluding our ability to draw any conclusions from the effects of E2 exposure on phenotype. This result was not surprising. We realized at the time of sampling that the fish were likely too small for discriminating phenotype. However, the ongoing high levels of mortality in all study groups forced our hand and we sacrificed the remaining trial fish in hopes that some useful phenotypic observations could be made. Unfortunately this proved not to be the case.

Some generalizations on growth can be made however, though their usefulness for future trials is unclear. Paradoxically, those fish in the Control tanks performed poorly in terms of growth compared to the majority of those in other treated groups (Table 15). Density varied across all tanks and, theoretically could explain some of the variation. However, because of extreme levels of mortality in all test groups throughout the experiment, actual rearing densities were extremely low in all the study tanks. Thus density should have played no role in the results and the relatively poor control fish growth relative to treated fish remains unexplained. Growth ranged widely, both in length and weight, across treatments, again, paradoxically,

moderate treatment of 200mg 125d having both the largest and heaviest individuals. Those in the 300mg 125d exposure group appeared to have the highest condition factor, however all groups were effectively equivalent.

An unanticipated limitation of conducting the sex reversal trial at the particular Oklahoma facility was that of water quality. Surprisingly, after nearly two years of extensive long-distance planning with facility staff which culminated in our travel to the site for project initiation, a local staff biologist suggested this aspect could have an impact on trial fish survival and/or fish growth. Unfortunately, at that juncture we were committed and unable to search for a more suitable facility given MSCGP contract timeline requirements. Subsequently, water quality or some other facility-specific factor clearly confounded study results beyond utility. Had the possibility of this situation been made apparent to us early on, we would have declined to conduct a study at that location.

Table 15. Lengths, weights and Condition Factor (W/L^3x10^4) of 212 Common Carp sampled at 150 DPH after having been exposed to varying doses and durations of Estradiol, while rearing at Oklahoma Fish Research Hatchery, Norman OK, Summer 2021.

			Length (mm)			Weight (g)			K		
Treatment	Tank #	n	Ave	Min	Max	Ave	Min	Max	Ave	Min	Max
Control	OK 4	70	47.9	32.0	72.0	1.6	0.5	5.0	0.134	0.062	0.187
200mg 90d	OK 3	25	56.0	26.0	81.0	2.8	0.2	8.0	0.136	0.063	0.168
	OK 3A	76	49.3	34.0	73.0	1.8	0.4	5.0	0.138	0.067	0.223
200mg 90d Total		101	51.0	26.0	81.0	2.0	0.2	8.0	0.137	0.063	0.223
200mg 125d	OK 2	5	76.6	50.0	118.0	8.5	1.9	20.8	0.142	0.127	0.154
	OK 2A	13	55.4	27.0	95.0	3.2	0.2	11.2	0.126	0.102	0.149
200mg 125d Total		18	61.3	27.0	118.0	4.7	0.2	20.8	0.130	0.102	0.154
300mg 125d	OK 1	15	56.1	32.0	70.0	3.2	0.5	7.6	0.149	0.088	0.222
	OK 1A	8	59.6	34.0	88.0	4.1	0.6	9.0	0.148	0.131	0.164
300mg 125d Total		23	57.3	32.0	88.0	3.5	0.5	9.0	0.148	0.088	0.222

We'd like to acknowledge at this time the passing of Dr. William Shelton with whom we had the pleasure and honor of working with on this trial. His guidance and mentorship has been most valuable, though pales in comparison to the massive contribution of his lengthy aquaculture and fish management career. We sampled this trial in mid-September, 2021, and Dr. Shelton passed away unexpectedly two weeks later, amidst shock and sadness from family, friends and colleagues. While we were not able to perform a successful carp feminization trial with him during this time, his knowledge and experience will continue to guide us as we move forward to find a successful carp feminization treatment regime.

Texas

The loss of a large number of samples at 331 DPH due to improper preservation techniques was unfortunate but this did not impact the overall results gleaned from the trial. Necropsy results of samples collected 49 days later at 389 DPH indicated that a large proportion of trial fish gonads were not identifiable to phenotype (see below). Thus, had we been able to use the 331 DPH samples, the proportion of undifferentiated fish that confounded the study would likely have been even greater.

Summarization of the data collected at 380 DPH yielded results that were perplexing. Control fish (n = 52) had an unexpected high proportion of undifferentiated gonads at 15% and an intersex fish was observed as well (Table 16). At 380 DPH, these fish were well beyond the normal time for observable sexual differentiation based on the literature (Shelton et al. 1995) and our own prior work (Schill and Mamer 2019). On the positive side, phenotypic females outnumbered males by about 2-fold across all study groups, but the proportion of undifferentiated fish was high, averaging 20% across all treated groups relative to the 15% value noted above for Controls. The number of intersex fish observed histologically averaged over 6-fold greater in treated groups than that observed in controls, with more skewing towards the higher treatment groups (Table 16). Taken collectively, the above results suggest that the various E2 treatments were indeed having an impact on phenotype but, overall, the proportion of definitive females was poor. Additional work will be needed to develop an effective sex reversal protocol for the Common Carp.

Growth of fish in this experiment was unusually variable and slow although condition remained largely constant across fish in the different treatment regimes relative to Control fish (Table 17). The fastest growth in the trial in terms of both length and weight was the 50mg 150 day treatment group. Control fish growth was intermediate, while fish receiving the two highest dose treatment grew more slowly than others, particularly the 200 mg group. Within all study groups there was an unusually large range in both length and weight. For example, in the Control group, fish weights ranged from 1.3 to 407.8 grams, and large size ranges within treated groups were common (Table 17). There were a few large "jumpers" (those few fish much larger than the general population of the tank) in each of the rearing tanks, however, numbers of especially small fish were much more common. Comparatively, mean growth in length and weight to 337 DPH in a prior Common Carp sex reversal trial (Schill and Mamer 2019) were over two and four-fold greater than respective averages in the current trial at 380 DPH.

The reason for the poor growth of fish in the BY21 trial is uncertain, although it was clearly not treatment with E2 as the Control fish also grew quite slowly. The study fish did become infected with KHV which likely caused considerable mortality across the experiment and could also have impacted growth (Carl Kittel TPWD, pers communication). In addition, although koi are indeed Common Carp, the broodstock which was used to produce our trial fish are used at the AE Woods hatchery to produce feeder koi for gamefish production. For this reason, the genetics of the broodstock are not tightly monitored and the fish themselves may be genetically limited to substandard growth relative to a "wilder" carp source. Although the staff at AE

Woods Hatchery are keen to attempt a follow-up carp sex reversal trial in the future using a less domesticated broodstock source, the hurdle of KHV at the facility would seem problematic unless fish there could be reared exclusively on virus-free water until necropsy and subsequent phenotype sampling.

Table 16. Percent visual phenotype by treatment type of 238 Common Carp, following various E2 exposures and durations versus Controls, 380 DPH, A.E. Woods State Fish Hatchery, San Marcos, TX, 2021-2022.

		% Phenotype			
Treatment	n	Female	Male	IS	Undifferentiated
Control	52	35%	48%	2%	15%
25mg 50d	52	46%	27%	8%	19%
100mg 120d	34	50%	21%	9%	21%
100mg 150d	32	47%	22%	16%	16%
50mg 150d	30	33%	23%	23%	20%
200mg 90d	38	50%	16%	11%	24%

Table 17. Lengths, weights and Condition Factor ($W/L^3 \times 10^4$) of 238 Common Carp (koi) sampled at 380 DPH after having been exposed to varying doses and durations of Estradiol, while rearing at A.E. Woods State Fish Hatchery, San Marcos, TX, 2021-2022.

			Length (mm)			Weight (g)			Condition Factor		
Treatment	Tank #	n	Ave	Min	Max	Ave	Min	Max	Ave	Min	Max
Control	10 11 & 12	52	121	47	265	54.1	1.3	407.8	0.172	0.118	0.248
25mg 150d	8 & 9	52	119	55	244	45.2	2.2	266.6	0.180	0.107	0.299
50mg 150d	7	30	131	55	226	58.9	2.8	232.0	0.188	0.118	0.244
100mg 120d	3 & 4	34	120	51	246	45.9	2.1	293.7	0.193	0.005	0.274
100mg 150d	5 & 6	32	116	49	197	40.8	1.7	165.6	0.188	0.144	0.240
200mg 90d	1 & 2	38	111	55	205	37.2	3.0	152.7	0.185	0.131	0.229

Summary- BY2020 CC Sex Reversal Trial

Slow growth of fish throughout the TX trial likely contributed to the lack of readily discernable feminization. It has been shown experimentally that gametogenesis in Common Carp is initiated sooner in fast growing populations but generally at larger sizes than in slower growing populations (Shelton et al. 1995).

The current trial resulted in a few large fish and unusually slow growth in the remainder. It would be helpful to peer into individual fish and ascertain whether any of the phenotypic females observed were, in fact, sex reversed genetic males as we were able to do in the Brown Trout trial above. Unfortunately, the sex marker functioning well for wild Common Carp stocks across much of the nation did not work for the domestic koi broodstock at AE Woods (see below).

In conclusion, further work is required for developing a highly effective sex reversal protocol for the Common Carp. Male carp have proven resistant to feminization (Komen et al. 1989; Teem and Gutierrez 2010) although Bongers et al. (1991) reported some success using 50mg/kg E2 from 10 - 15 weeks. Unfortunately, details in this conference proceedings are sparse. A more recent study yielded a Common Carp feminization rate of 58% for a single sample of 48 male fish, though the treatment protocol used involved a high dose of Estradiol (200 mg/kg) along with a second drug, the mammalian antiandrogen, Flutamide. Fish in that study were treated from 8 - 21 weeks DPH. Despite the lack of success in the present study, we remain skeptical that Common Carp feminization requires the use of Flutamide. Focus on a future sex reversal trial by the WAFWA Consortium should revolve around the identification of a partner highly experienced in indoor larvaculture of Common Carp, and treatments that begin and end later in the differentiation process.

Sex Marker Development

As in a prior FY2019 effort, the FY22 carp genetics work provided useful results with an overall concordance rate between known phenotype and genetic sex averaging 89.5% across five new U.S. populations. For a more detailed explanation of FY22 results see EFGL descriptions in Appendix C1. An additional RAD sequencing effort is currently underway to elucidate unexplained but apparent unidirectional discordance between phenotype and genotype in several of the populations sampled. A Final analysis of this effort will be documented in a final MSCGP report in spring 2023. However, existing results from both the FY19 and FY22 efforts indicate that an adequate sex marker is in hand to enable the development and field evaluation of a YY Male broodstock of Common Carp across a sizeable proportion of the continental U.S.

Walleye

Sex Reversal Trial

Main Trial Sampling - 279 DPH

While Control sex ratio closely approximated the expected value of 48.9%, all E2 treatment regimes evaluated resulted in exceptionally strong female biased sex-ratios of 100%, with the exception of the lowest dose regime tested. That treatment (5mg/kg for 84d) resulted in 98.7% females and also resulted in the lone

intersex fish observed in the study (Table 18). The stark differences between the control group replicates and the 15 mg, 85 d replicates, along with robust samples sizes of 75 fish per rep make the need for a confirmatory statistical test superfluous.

Growth of Control fish exceeded that of all treatment groups at the time of sampling which occurred at about 9 months of age. In terms of length, Control lengths averaged 199.3 mm and dropped slightly as treatment dose/duration increased, bottoming out in the 15mg 84d group at 166 mm before rebounding to 170.2 mm for the 75 mg/84d group. The same pattern was observed for group weights with a decline with increasing dose with the exception being an increase in weight for the highest intensity treatment. Growth typically lags in sex reversed fish but catchup growth is often observed in such fish relative to controls as the fish approach their second year of life (Schill et al. 2016a).

Combining the two growth parameters using Fulton's condition factor (W/L^3 x 1000), a different pattern was apparent with fish receiving E2 treatment having poorer condition values than the Control fish with the exception of those receiving the highest E2 dose of 75 mg (Figure 3). One possible explanation for this seemingly spurious result is that the highest treatment regime may have resulted in increased mortality of male fish that could be more negatively affected by greater estrogen treatment, leaving more females whose growth might not be as negatively affected. The lack of a sex marker for this species to date (Schill and Mamer 2021; also see 2022 marker results below) makes it difficult to evaluate this possibility, but such a retrospective analysis will be done if the EFGL successfully develops such a marker in the future as we have fin clips for all individual study fish catalogued.

Table 18. Percent phenotype ascertained by visual observation of gonads from necropsied Walleye, at 279 DPH, following exposure to various durations and concentrations of E2 treated dry feed beginning at first feeding versus Controls, Garrison National Fish Hatchery, hatched Jun 2021 – sampled Mar 2022.

				Phenotype								
				Female			Male			Intersex		
Treatment	Tank #	Total n	% F	n	Ave L (mm)	Ave W (g)	n	Ave L (mm)	Ave W (g)	n	Ave L (mm)	Ave W (g)
Control	ND 01	85	50.6%	43	205.9	64.6	42	202.0	61.0			
	ND 02	85	44.7%	38	195.1	53.0	47	196.7	54.8			
	ND 03	85	51.8%	44	198.2	56.6	41	200.9	59.4			
Control Total		255	49.0%	125	199.9	58.3	130	199.7	58.3			
5mg 84d	ND 8	84	98.8%	84	186.2	46.7				1	165.0	34.0
15mg 60d	ND 9	85	100%	85	172.5	36.0						
15mg 84d	ND 4	85	100%	85	166.8	32.1						
	ND 5	85	100%	85	166.6	32.2						
	ND 6	85	100%	85	165.5	31.7						
15mg 84d Total		255	100%	255	166.3	32.0						
75mg 84d	ND 7	85	100.0%	85	170.9	40.5						

Fish Health Sampling - 279 DPH

Liver size as measured by HSI increased in all treatment groups relative to Controls, though not severely. The increases were relatively small with the exception of fish in the highest treatment regime of 75mg 85d, which experienced an increase in liver HSI of roughly 25% compared to Controls (Figure 4). These results are similar to those reported by Haux and Norberg (1985) in an injection study of Rainbow Trout where low level injections of E2 resulted in only slight hypertrophy of the liver, while greater dosages increased liver size more. However, in the cited study, liver weights increased 2-fold following weekly "high" E2 injections, gains well above those we observed in the present study, even at the highest treatment regimen evaluated.

The overall Health Index values for Walleye in the study declined with increasing treatment dose. However, the two lowest treatment levels (5mg 84d and 15mg 60d) had average scores virtually identical to Control groups while the two higher treatment fish had lower scores (Figure 5).

Not surprisingly, the heaviest dose administered to trial fish (75mg 84d) had considerably lower overall average scores than all other treatment groups. In general, there was less decrease in both the Pelvic

and Dorsal/Caudal fin scores than that observed for the Head/Gills/Eyes and Body score categories. Once again, the high dose regimen had a noticeably greater negative effect than the other regimens. The Health Index values in the treatment groups will be evaluated again by project personnel at two years of age to examine longer term trends as was reported above for Brown Trout.

Summary- BY2021 WAE Sex Reversal Trial

Our BY 2020 trial results demonstrate that male Walleye are relatively easy to sex reverse, results consistent with our earlier efforts where the 15mg dose for either 84 or 100 days resulted in 100% female phenotypes in trials in two different state hatcheries (Schill and Mamer 2019). In the present study all but one treatment regimen resulted in 100% females with the remaining regimen producing 98.7% females. However, the 2022 results are far stronger, with robust sample sizes, compared to the earlier efforts. A large n is a useful feature considering that a sex marker is not yet available to quantify the actual feminization rate of genotypic males as was possible in the Brown Trout work above. The relatively large sample sizes of 75 fish per treatment groups thus provides a solid measure of comfort that at least some phenotypic females in the trial groups are actually feminized males.

Deciding upon the best of the BY20 treatment regimens will ultimately depend on several factors, one being the use of the least amount of E2 as possible. Given the 100% phenotypic female sex ratio and lack of intersex fish, the 15 mg 60 day regimen would appear to be the most desirable. The lowest dose tested (5 mg) had lower % females and an intersex fish, both undesirable for construction of a YY Male broodstock. However, given the nearly 99% ratio of females at this dose, the 5mg regimen maybe prove the most desirable. Longer exposure duration such as the 84 or 100 day regiments at the same exposure level (15 mg) tested in BY19 do not seem necessary. Coming into the BY 2021 trial we assumed the 15mg 84d regimen would be a highly efficacious treatment given our 2019 results and the fact that the regimen was the same used to effectively sex reverse another closely related percid, the Yellow Perch *Perca flavascens* (Malison et al. 1986). It was because of both earlier study regimens that a five fold exposure 75 mg for 84 days was selected to emulate the "worst case scenario" in a typical INAD Target Animal Safety protocol study testing 5X the effective dose.

A total of 358 fish (ideally 40 from each replicate, range 37 - 41) from the various treatment tanks remained alive at the cessation of the sex reversal trial. These tagged fish, split into two replicates (one as a backup) are currently being reared for growout communally in two tanks at GNFHand will be examined in Spring 2023 to ascertain long-term survival, growth, and time to maturity. The best treatment protocol may ultimately depend upon survival, growth and time to maturity of fish from the three lowest-dose test groups.

Figure 3. Condition factor (K) of Walleye (279 DPH) following exposure to various durations and concentrations of E2 treated dry feed starting at first feeding. Data include all fish from both the Health and Main sampling events, n = 623, with 95% CI's and n's above bars. Garrison Nat'l Fish Hatchery, Mar 2022.

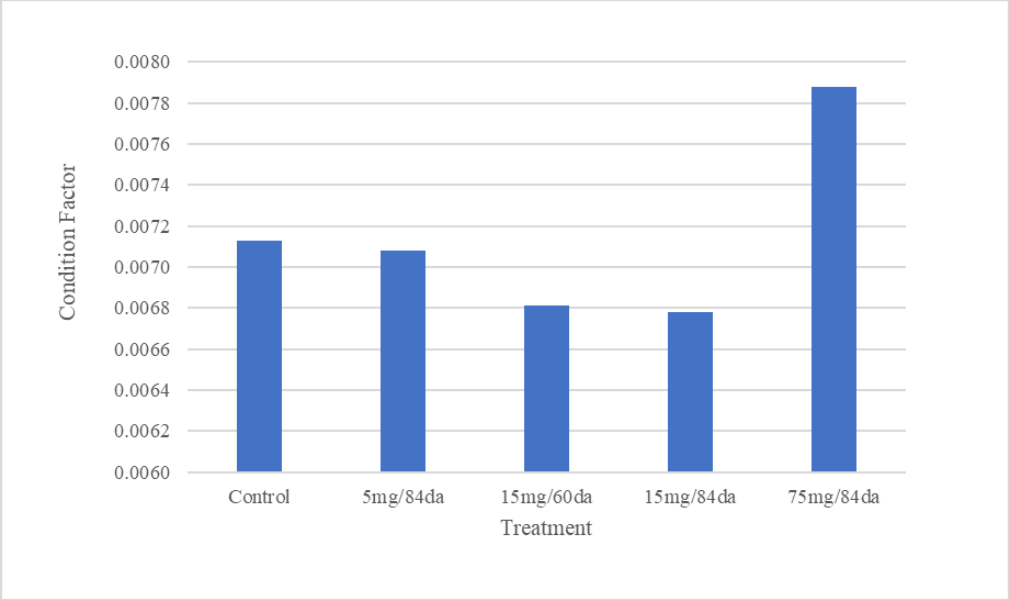


Figure 4. Hepatosomatic index of livers from necropsied Walleye, aged 279 DPH, following exposure to various durations and concentrations of E2 treated dry feed starting at first feeding versus Controls, Garrison National Fish Hatchery, hatched Jun 2021 – sampled Mar 2022. Dashed lines indicate average across replicates for a treatment group.

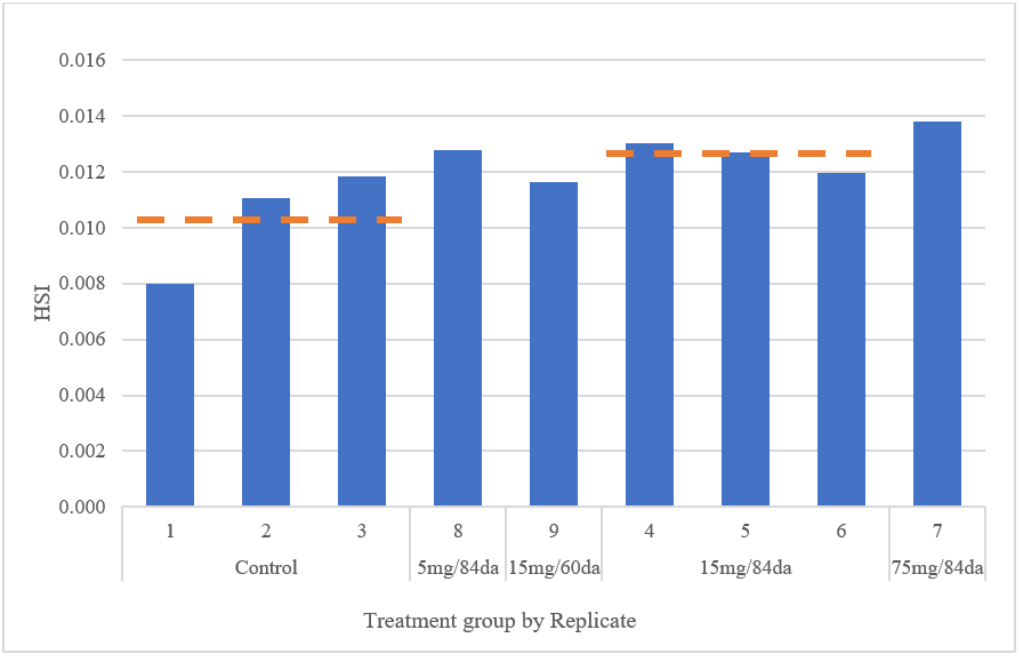
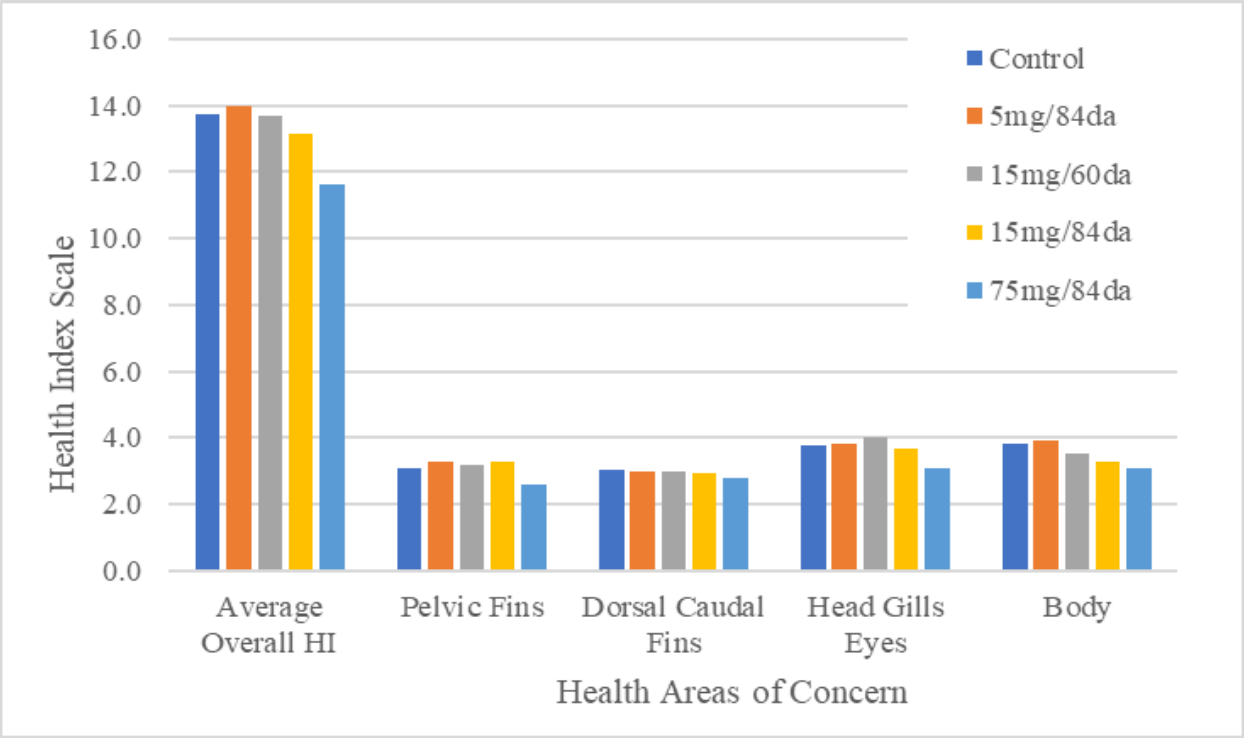


Figure 5. Health Index values by treatment group from a subsample of Walleye having been exposed to various doses and durations of E2, when compared to Controls, 279 DPH, Garrison National Fish Hatchery, Mar 2022. See Appendix B - Fig 1 for Health Index description.



Sex Marker Development

During the past year IDFG's Eagle Fish Genetics Laboratory staff made new efforts to develop a Walleye sex marker. Compared to prior year's challenges, considerable progress was made with a putative marker predicting sex with about 90% accuracy though additional work is needed. However, if this marker is subsequently verified and accepted, it would mean walleye females are the heterogametic sex (ZW), which is contradictory to previous published studies. For a detailed description of findings see Appendix C2. Additional work is planned with both US and European collaborators to solidify these FY22 results. This work will be completed and reported on by EFGL staff in FY23.

Lake Trout

Differentiation Study

Data was collected from 23 bi-weekly sampling events from Dec 2020 to Dec 2021, resulting in a clear differentiation profile for Lake Trout. Of the four sampling events that were analyzed histologically

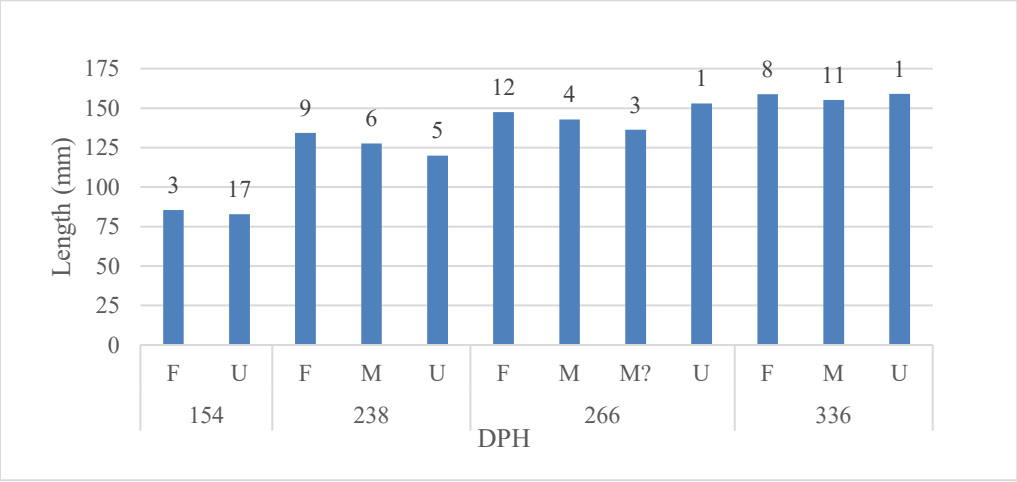
(Table 19), we were able to follow the progress of differentiation and note that male development lagged behind that of females.

At 154 DPH (2727 CTU), the only identifiable sex cells found were in very few females (n = 3), with the majority of the 20 samples examined still being in a sexually undifferentiated state of primordial germ cells (PGCs; Table 19). An intermediate sample, taken at 266 DPH (3922 CTU), still had ~25% of the samples being completely undifferentiated, and those identified as possible males ("M?") exhibiting spermatogenic cytology, but in an early state and not definitively committed to being male yet at that age. By 336 DPH (4776 CTU) only one of the twenty samples examined was not differentiated, the remainder being clearly developed into either sex. Being differentiated does not imply maturity. None of the fish examined were mature by 1 YO.

Table 19. Visual phenotype of Lake Trout, ascertained from histological samples of gonads, having been reared through 1 year of age at Grace Fish Hatchery, ID, Dec 2020-Dec 2021.

		Visual Phenotype			
DPH	Sample Date	F	M	U	M?
154	5/10/2021	3		17	
238	8/2/2021	9	6	5	
266	8/30/2021	12	4	1	3
336	11/6/2021	8	11	1	
Grand Total		32	21	24	3

Figure 6. Average length by age (days post hatch; DPH) and phenotype showing trend of gonadal differentiation of Lake Trout reared at Grace Fish Hatchery, ID, Dec 2020 – Dec 2021. N's above bars.



At this juncture, a follow-up LKT sex reversal trial should have greater potential to yield improved results. With this information, a future Lake Trout feminization trial (given similar CTUs as that of IDFG Grace FH) should consider the differentiation window of males starting much later than previously thought, suggesting that exposure to E2 should begin as late as 150 DPH, which would be approximately the age of differentiating females, and continue through 1 YO, perhaps longer, given that the final sample at 336 DPH still had males in early stages of differentiation.

Density Dependent Sex Change

Brook Trout ESD

To date a total of 3300 wild Brook Trout in the two study streams have been visually sexed for phenotype and successfully sexed genetically using a sex marker (Table 20). Of the 1180 and 381 fish examined in 2016 and 2018, respectively, no discordance between genotype and phenotype was detected. However, nine mis-matches originally occurred in 2017 out of 772 fish (Schill and Mamer 2019). 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). 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 or a genotyping error. However, we doubt it to be a case of phenotypic sex reversal given that no phenotype-genotype mismatches have been observed 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 mis-match reported for 2017 was likely a visual phenotyping error. Based on results to date on such a large sample, we conclude that ESD in wild Brook Trout via DDSC has not occurred in either study stream. We will continue examining individual fish for genotype-phenotype mismatches, as both populations continue to approach total collapse and wild fish hopefully become fully eradicated (see below).

Population Response to Suppression and Stocking

As noted above, 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 3300 Age 1 wild Brook Trout, only three weren't successfully genotyped in the entire study. These fish were disregarded in the population analyses reported below.

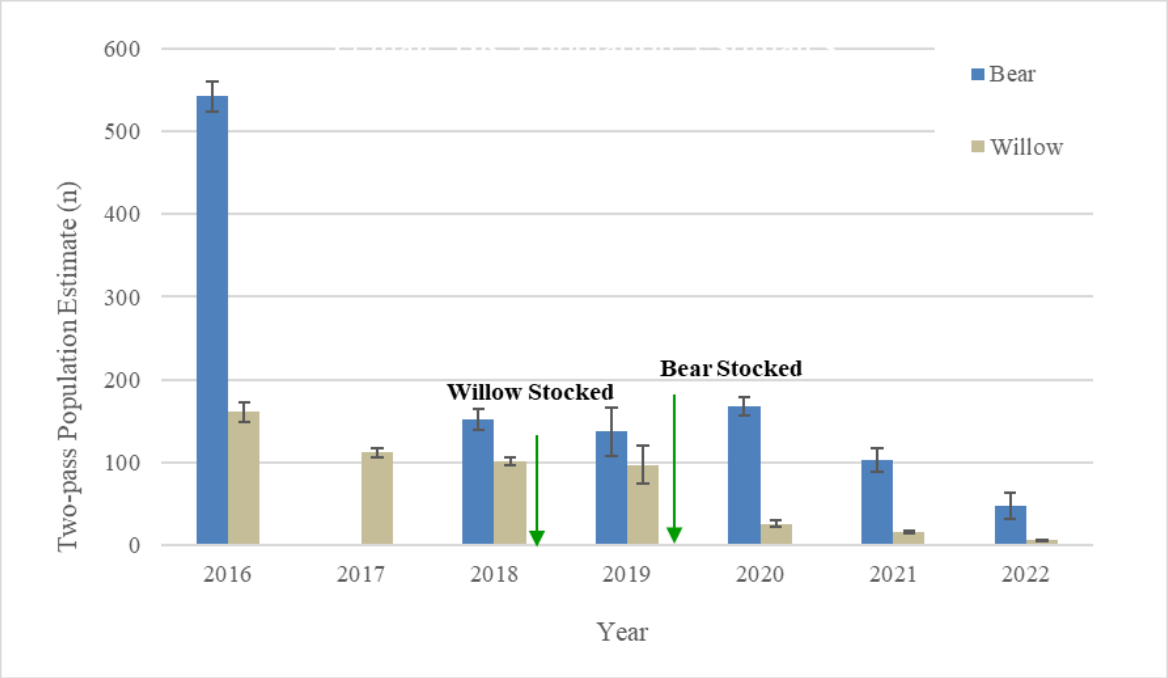
Population estimates derived for wild females in both study streams has declined precipitously since the first year of suppression. The initial female population in Bear Creek has been reduced from 542 fish in 2016 to 48 in 2022, a 91% decrease (Table 20). Female abundance has declined even more in Willow Creek at 96% with only an estimated 6 females remaining in the entire stream prior to the 2022 removal effort in July, after which we estimate that the entire Age1+ population was removed (Table 20). The decrease in abundance of females in both streams declined in the initial suppression years but dropped to very low levels in the years immediately following stocking (Figure 7). The rapid decrease in female abundance is not surprising given that the estimated exploitation rate 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 20).

Table 20. 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 2016-2022. 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.

	Removals						
	Day 1	Day 2	Total	Capture Probability	Population Estimate	95% Confidence Interval	Pop % Removed
Bear Ck							
2016	411	100	511	0.759	542	524-560	94.3%
2017	180	127	307	0.304	-	-	-
2018	110	31	152	0.727	152	140-164	92.8%
2019*	76	35	111	0.561	137	108-166	81.0%
2020	126	32	158	0.752	168	157-179	94.0%
2021	68	24	92	0.667	103	89-117	89.3%
2022	27	13	40	0.58	48	32-64	83.3%
Willow Ck							
2016	117	33	150	0.732	161	149-173	93.2%
2017	91	18	109	0.82	112	106-118	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	2	16	0.889	16	15-17	100.0%
2022	5	1	6	0.857	6	5-7	100.0%

* stocking year

Figure 7. Two-pass electrofishing removal estimates of population size (95% CL) for wild female Brook Trout in Bear and Willow Creeks, near Mackay Idaho, 2016-2022. Willow and Bear Creeks first stocked in 2018 and 2019, respectively.



Population estimates derived for Age 1+ XY males in both study streams has also declined precipitously since the first year of suppression. Population estimates in Bear Creek declined from 496 Age 1+ XY males in 2016 to 82 in 2022, a 93% decrease (Table 21). The decrease in XY males in Willow Creek was smaller at 68% across the same time period. High rates of population exploitation during the two pass removals were observed (85.6 to 100%) similar to the rates reported above for wild females. The 2022 abundance estimates of XY males following the management activities of suppression and stocking were 1.7 and 6-fold greater for males than females in Bear and Willow Creeks, respectively. However, it is important to recall that many of these males are likely the progeny of YY Males from prior matings with wild XX females (see below).

Table 21. Results, by year, of annual removal efforts, population estimate, and proportion removed of Age 1+ XY male Brook Trout from two study streams involved in the YY Brook Trout evaluation in eastern Idaho, July 2016-2022. Estimates are from 2-pass removals and stocking of YY BKT 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	77	24	101	0.706	110	98-122	91.8%
2022	57	18	75	0.701	82	72-92	91.5%
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	19	4	23	0.852	23	21-25	100.0%
2021	15	6	21	0.677	23	16-30	91.3%
2022	31	5	36	0.878	36	34-38	100.0%

* stocking year

Along with the number of females remaining in a water following a concerted Integrated Pest Management, or IPM, program such as the current study, 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). Such a phenomenon is apparent in the current study where consecutive removal day data sets within a year for both streams do not allow for multiple pass removal estimation (Table 22). Despite this observation, large reductions in Age 0 abundance is apparent when combining Day 1 and Day 2 YOY or fry catch. On Bear Creek, total fry collected along the entire length of the stream decreased from 77 to 8 fish from 2016 to 2022. On Willow Creek, the decline in fry collected was even sharper, where 110 fry were initially collected along the entire 2.9 km study reach in 2016 and where none were found in 2022 (Table 22).

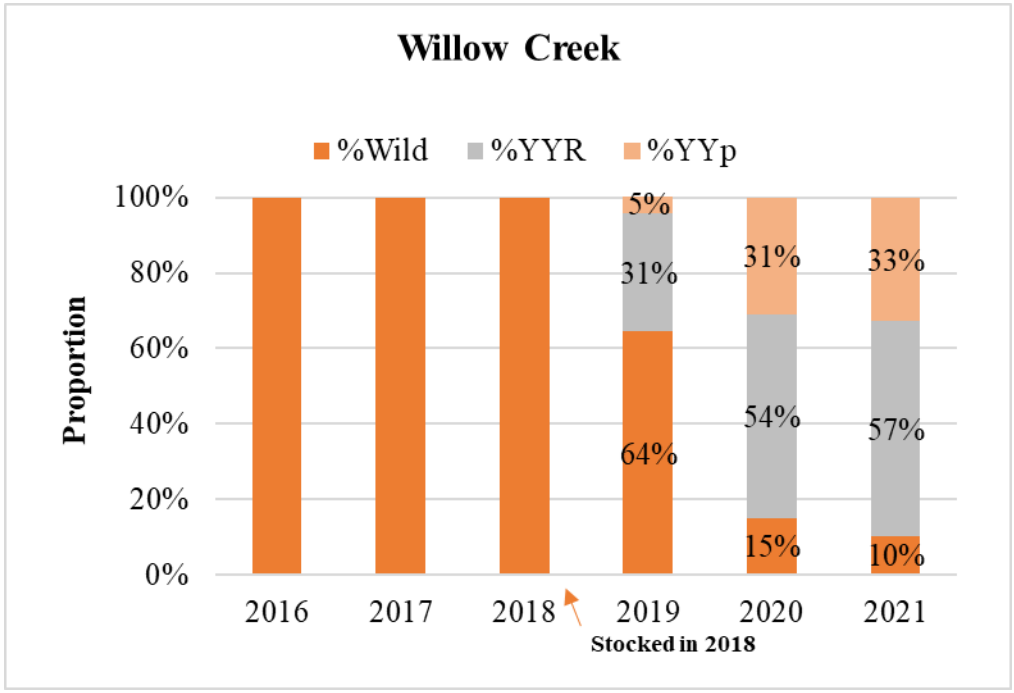
Table 22. Number of YOY Brook Trout collected during back-to-back electrofishing removal runs on consecutive days in Bear and Willow Creeks near Mackay Idaho, July 2016-2022.

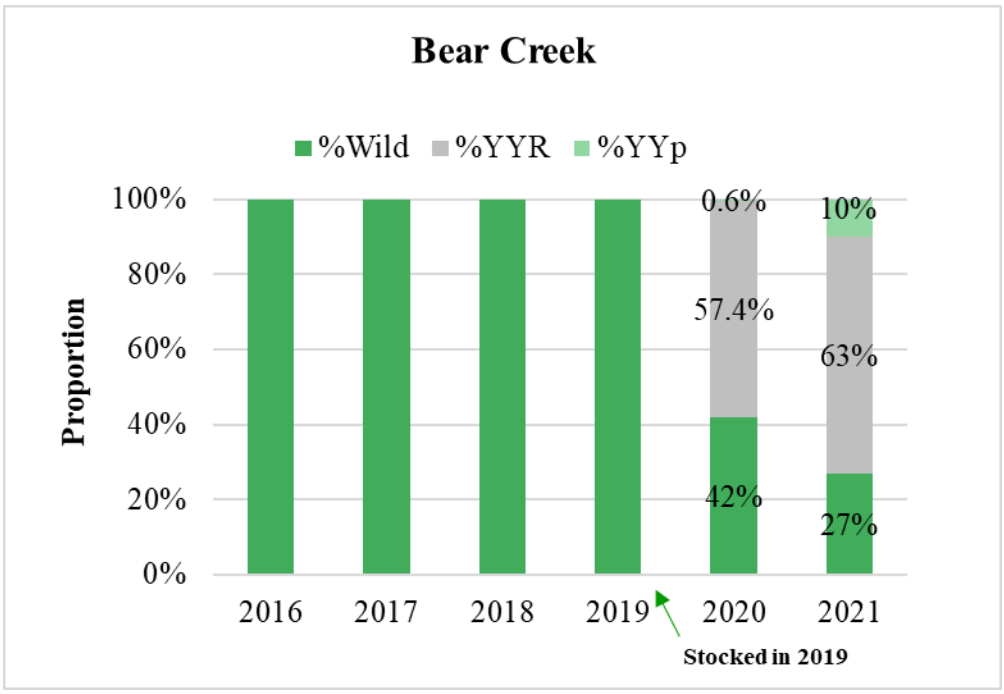
Stream Name	Sample Date	2016	2017	2018	2019	2020	2021	2022
Bear Ck	7/5/16	22						
	7/6/16	55						
	7/5/17		13					
	7/6/17		22					
	7/5/18			3				
	7/6/18			12				
	7/9/19				6			
	7/10/19				8			
	7/7/20					10		
	7/8/20					8		
	7/6/21						32	
	7/7/21						32	
	7/6/22							6
	7/7/22							2
Bear Ck Total		77	35	15	14	18	64	8
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
Willow Ck Total		110	70	40	16	48	41	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. The progeny of YY Males (n = 6) comprised 38 % of all 16 fry collected in Willow Creek in 2019 (Schill and Mamer 2021). Based on fin clip observations and GSI screening of fish collected annually, after the first three years of YY male stocking, the Willow Creek population was composed of 10, 57, and 33% wild fish, stocked YY Males (YYR) and YY progeny (YYp) respectively (Figure 8). A greater proportion of YY Males were sampled in Bear Creek two years after stocking (63%) while a smaller proportion of progeny were detected (10%) relative to second year

Willow Creek results. The proportion of wild fish in Willow Creek has declined by over six-fold in the three years since the stocking of YY Males began in 2018 (Figure 8).

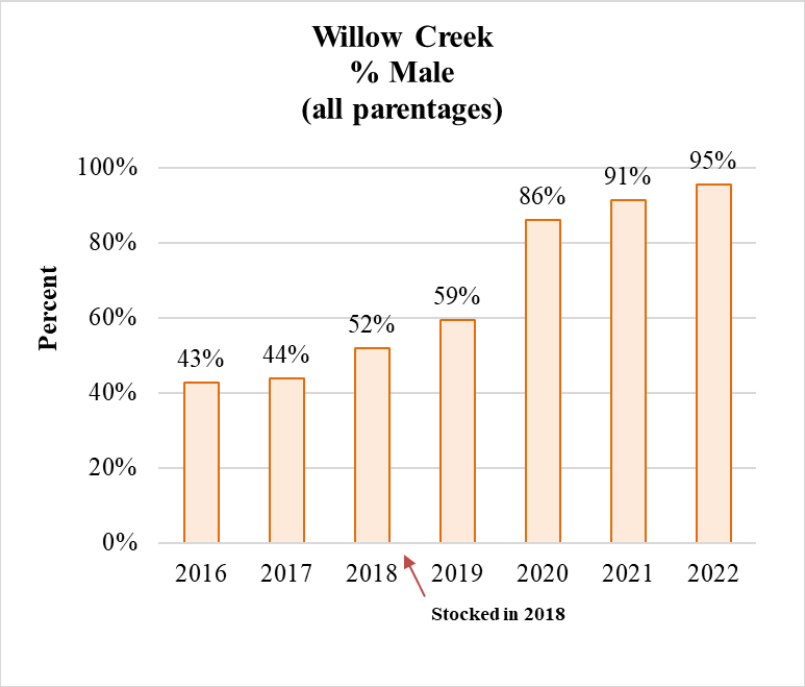
Figure 8. Proportion of Brook Trout by origin collected during 2-pass electrofishing in Willow Creek and Bear Creek Idaho, 2021-2021. YY males enumerated by observation of adipose fin clips on YY Males released (YYR) while YY progeny (YYp) ascertained *via* Genetic Stock Identification or GSI. Wild origin fish numbers observed also verified by GSI.

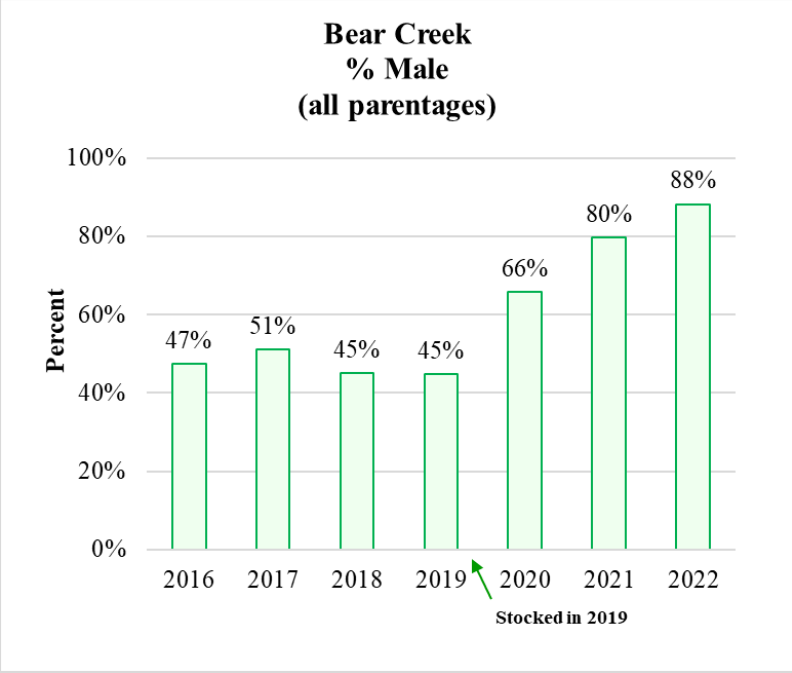




A final population metric available to evaluate the Bear-Willow IPM effort is the overall population sex ratio. Prior to the initiation of YY Male stocking, the population sex ratio averaged 46 and 47 percent male for Willow Creek and Bear Creek across suppression-only study years, respectively. In the three years since stocking in Bear Creek, the sex ratio for all fish sampled in the stream in mid-July has surged to 88%. Four years post-stocking in Willow Creek, the male sex ratio has reached 95% (Figure 9).

Figure 9. Sex ratio of male Brook Trout (all parentages) over five years from two Idaho streams, 2016-2022. Willow Creek was first stocked with YY fish in July 2018; Bear Creek first stocked in July 2019.





While additional years of data are clearly needed for Willow Creek, we suspect the lack of any observed fry recruitment and the 95% male sex ratio observed in 2022, plus the near complete lack of wild females remaining in the population portend imminent population collapse and hopefully complete wild female eradication. It is important to note that the observed 95% sex ratio reported above for Willow Creek was further buttressed on Day 2 of the 2022 removal effort with the subsequent stocking of additional YY Males at 50% of 2016 Age 0+ wild fish abundance. Thus the sex ratio skew and number of resident YY Males will be even greater going into the Fall 2022 spawning season. Few wild Brook Trout in Willow Creek reach 4 years of age (DJ Schill, unpublished data) and the few remaining adult females surviving in face of the IPM program are not likely to live long. Bear Creek was first stocked one year later than Willow and a similar population collapse appears close at hand. The seeming rapid collapse of both study populations has begat a new question, that being how long both streams should be stocked once fry production ceases? The logical answer to that question seems no longer than the typical lifespan of Brook Trout in each stream.

Coordination of INAD Coverage

Much time was spent during the reporting period coordinating various drug approval aspects of the YY Male Brook Trout program. This included working with staff from the Aquatic Animal Drug Approval Partnership (ADAAP). Two zoom meetings were attended. The first one was sponsored by AADAP and focused on several aspects of drug approval *via* both the INAD and little used "Indexing" route. The second session was the annual INAD check-in call with CVM. Several preparatory phone conference strategy sessions with AADAP staff were held before the formal annual FDA interaction on the Brook Trout INAD. Considerably more time was spent during the reporting period on the drug approval aspect than anticipated. This was largely due to the FDA seeking public comment on possible changes to their longstanding policies regarding the use of Indexing as a route for easier use of drugs in Aquaculture, particularly broodstocks where the fish are not released from the hatchery. Considerable time was spent with Drug Approval Working Group Co-Chair Alan Johnson, Iowa DNR, working on their comments to the FDA on Indexing and assistance in developing similar comments by the FAS. Schill, via FMS, Inc. submitted a separate written set of comments on the positive nature of a revised policy on Indexing to the FDA.

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 around about 30 individuals across states and federal agencies who are copied on team emails, with a core group of

roughly 10 individuals regularly involved, including the EFGL Manager, Matt Campbell, who provides guidance on field genetics sampling. Substantial interactions occurred between individual tech team members and the coordinator (Schill) throughout the year.

A virtual meeting was held on 2 February, 2022, and 24 personnel from a total of nine agencies including the states of NM,NV,WA,OR, ID,CO, the Kalispell Tribe, USFS, and the USFWS, with an additional 8 guests sitting in. NMSU student, Mike Miller presented some very positive results from his 2021 fieldwork related to stocked MYY survival in his study streams. Other State/Federal participants (Baker, Meewig, Roth, Schill, Poirier and Peterson) provided updates on their ongoing YY BK field work, and various genetic and hatchery studies were presented by Kaeli Davenport, Christian Smith and Doug Peterson.

Group consensus was to continue with the annual meeting concept, especially with the Fall 2022 YY Symposium on the horizon. The next Tech Team meeting will likely be winter 2023.

Identify Additional YY Partners and Funding Opportunities -

Bruce McIntosh came on board at the beginning of the fiscal year to assist in securing additional program funding. Bruce initially assisted with reminder memos in regard to "internal" YY funding by participating State Chiefs and contacted a number of prospective Federal partner employees in the USFS and USFWS. Three zoom presentations/discussions were held by McIntosh and Schill with upper level USFS staff, two with Pacific Northwest program managers and one at the Nationwide management level. Three Zoom presentations regarding YY funding were made to USFWS management level staff across the western US and zoom sessions and/or personal meetings were held with the Branch Chiefs of the USFWS Invasive Species and Aquaculture Programs in Washington DC. A dialog was continued with the Trinchera Ranch in southeastern CO regarding future possible funding of work being done on YY Brook Trout there that would include additional financial support for YY in general by the Moore Charitable Foundation. Although to date none of these contacts has produced in-hand funding, considerable progress has been made and funding from several of these sources seems likely in the 2023 FY.

Project Communication

The annual WAFWA progress report for FY2021 was completed and submitted to WAFWA on schedule along with an interim MSCGP report to the USFWS on the Common Carp work. YY Male Consortium project results were presented at the virtual WAFWA Chief's meeting during July 2021 and, by request, to the 2022 Idaho AFS meeting as part of a special aquaculture technology session. A zoom session was held with new UI researcher Matt Falcy to hopefully move modeling of YY Lake Trout forward in partnership with IDFG staff. A zoom session was held with George Schisler and CO hatchery staff to discuss

possible holding facilities for BY19 and BY20 Brown Trout study fish. Subsequent dialog with CDOW staff on YY fish culminated in a decision by CO to develop a backup YY Brook Trout broodstock, a long-sought positive move forward for the entire YY Consortium program. Both feminized and sperm-producing fish were transported by IDFG Hayspur hatchery staff and project personnel to the CO Bellvue research hatchery in June 2022 as a result. A paper documenting the results of the BY19 sex reversal trial on Brown Trout at two South Dakota facilities was prepared by McNenny, WAFWA contractors Schill and Mamer, and DC Booth Hatchery staff, and submitted to the Journal Aquaculture and Fisheries. Publication of sex reversal research studies are an important part of eventually obtaining drug regulatory approval by the FDA.

YY Symposium update

At the National American Fisheries Society meeting held in Spokane, Aug 2022, a 1.5 day symposium was held focusing on the development and implementation of YY Male technology. Seventeen presenters shared their projects with an attentive audience, ranging from modeling the technology, the challenge of developing genetic sex markers, feminization attempts for different species, Brook Trout field implementation results, FDA regulatory oversight of E2 use, and finally wrapping up with a panel discussion on where we should go from here. The list of speakers is below (Table 23).

Table 23. Participants and topics covered in the first YY Male Symposium held in Spokane, WA, Aug 2022.

Overview	Dan Schill	719	Dan	Schill	The Trojan Y Chromosome or YY Male Approach to Invasive Fish Eradication: Session Overview
Modeling		643	Casey	Day	Investigating factors affecting the success of YY-male programs using spatial simulation modeling
Modeling		462	Josh	McCormick	Simulation of YY Male stocking and suppression for eradicating Common Carp Populations
Modeling		191	Matthew	Ziegler	Modelling the effect of Trojan sex chromosomes on a Channel Catfish population
Modeling	Jeff Heindel	644	Jon	Amberg	The importance of life history on the successful use of YY-males
Genetics		244	Katharine	Coykendall	Sex Marker Discovery for Use in Trojan YY Programs
Genetics		146	Matt	Campbell	Methodologies for evaluating the reproductive success of MYY Brook Trout following release
Genetics		717	Chad	Teal	Attempts at the development of Trojan sex chromosome carrying Green Sunfish (*Lepomis cyanellus*)
Genetics		694	Kaeli	Davenport	Lab-based Evaluation of the RRS of MYY Brook Trout
Modeling	Matt Campbell	138	Mike	Miller	Using data from MYY suppression to simulate potential eradication of Brook Trout
Sex Reversal		234	Chad	Teal	The development of YY Red Shiner (*Cyprinella lutrensis*) for invasive population control
Sex Reversal		506	Liz	Mamer	Sex Reversal of Brown Trout Exposed to Differing Estradiol Treatments
Sex Reversal		189	Jared	Reimenschneider	Creation of Sexually Reversed Brook Trout Broodstock in a Hatchery Setting
YY Field Work	Mike Ruhl	402	Dan	Schill	The use of MYY fish to eradicate non-native Brook Trout populations in Idaho
YY Field Work		165	Bill	Baker	Male YY Chromosome Brook Trout – Encouraging results from the real world
YY Field Work		236	Ben	Armstrong	Stream-wide Evaluation of Survival and Reproduction of MYY and Wild Brook Trout
Regulations	Dan Schill	470	Paige	Maskill	INADs and Indexing: Potential Options For Use Of Unapproved Aquatic Animal Drugs
Regulations		766	Paul	Smith	ESTRAQUA® Eel INAD Human Safety Technical Section: Implications for Invasive Species Eradication
Panel	Mike Ruhl	Paul Smith, Dan Schill, Matt Campbell, Paige Maskill, Julie Carter			

Acknowledgements 2022

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, especially given the challenges and effects of the past pandemic year. 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.

For the Sex Reversal & ESD Trials:

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 National FH) for providing vigilant oversight of the product of this multi-facility venture.

The Common Carp Feminization trials wouldn't be possible without the generous expertise and willing time commitment of both facilities: OK team – W. Shelton (University of Oklahoma), and TX team – C. Kittle and M. Matthews (TXPW).

Many thanks to the USFWS and Garrison Dam National FH, working at the time R. Holm (now retired) and B. Oldenburg (now at Governor Tommy G. Thompson Hatchery, Spooner, WI), for heading up the Walleye feminization trial. Ben managed this work effectively on his own and his willingness to undertake the involved study design speaks volumes and we thank you. We continue to appreciate GFH support for maintaining our study growout population which we will be examining next Spring. Much appreciation to A. Johnson of Iowa DNR's Rathbun FHRC for his continued advice and support of this effort.

We thank the staff at the Grace Hatchery for hard work on the long-running YY Lake Trout work initiated by M. Gallagher and E. Pankau , and now led by W. Fowler with able assistance from S. Stowell.

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 , C. Roth, J. Vincent, M. Campbell and many others on the EFGL staff including C. Koykendall, Jesse McCaine, John Hargrove and others.

Sex Marker Development:

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 the bench work on these markers. Lastly, we thank the entire staff at the EFGL involved in DNA extraction etc., led by L. Schrader, 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.

For the YY Symposium

This was definitely a work of passion for all involved, including the Nat'l AFS coordinators that accommodated the constantly shifting target of an effective, informational meeting despite a rash of airline cancellations. We wish to specifically thank L. Earley (Pres-Elect, Western AFS), T. Fry (AFS) and B. Missildine (WA DFW) for their patience and assistance in herding cats (presenters) while we curse airlines. An additional heartfelt thanks to P. Smith of Novaeel for traveling across our continent to participate as both a speaker and on the panel, adeptly moderated by M. Ruhl. M. Campbell, and J. Heindel ably moderated other Symposium modules.

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:

Ten Fish Chiefs from the States of AZ, CO, ID, KS, NM, NV, OR, TX, 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 FY22 work. The list of names supporting financial state contributions is too long to mention all here and several of the original Chiefs have already moved on. We thank both the original and current Chiefs and program managers for their support. In addition, we thank the Kalispell Tribe for an additional contribution to the Consortium in relation to their ongoing Brook Trout removal work with WDFW. We would be remiss in not specifically thanking IDFG Deputy Director J. Fredericks for helping to conceptualize the Consortium as IDFG's Fish Chief and he remains a source of ideas on Federal and NGO funding.

Literature Cited

Ashby, K. R. 1957. The effect of steroid hormones on the brown trout (*Salmo trutta L.*) during the period of gonadal differentiation. Development, 5:225-249.

Beardmore, J. A., G. C. Mair, and R. I. Lewis. 2001. Monosex male production in finfish as exemplified by tilapia: applications, problems, and prospects. Aquaculture 197:283-301.

Bongers, A.B.J., M. C. H. Holland, R. H. M. Leenen, J. Komen, J. and C. J. J. Richter. 1991. Effect of 17ß-estradiol on sex differentiation in inbred (XX; MAS-1/MAS-1) males of common carp, *Cyprinus carpio L.* In Abstract 4th Int. Symp. *Reproductive Physiology of Fish*. Univ. East Anglia, Norwich, UK (p. 268).

Bowen, S. H., D. J. D'angelo, S. H. Arnold, M. J. Keniry, and R. J. Albrecht. 1991. Density-dependent maturation, growth, and female dominance in Lake Superior lake herring (*Coregonus artedii*). Canadian Journal of Fisheries and Aquatic Sciences, 48:569-576.

Chevassus, B., and F. Krieg. 1992. Effect of the concentration and duration of methyltestosterone treatment on masculinization rate in the Brown Trout (*Salmo trutta*). Aquatic Living Resources, 5:325-328.

Cotton, S., and C. Wedekind. 2007. Control of introduced species using Trojan sex chromosomes. Trends in Ecology and Evolution 22:441-443.

Degani, G. and D. Kushnirov. 1992. Effects of 17β-estradiol and grouping on sex determination of European eels. The Progressive Fish-Culturist, 54:88-91.

Docker, M. 1992. Labile sex determination in lampreys: The effect of larval density and steroids on gonadal differentiation. Ph.D. Dissertation, 283pp.

Feist, G., C. B. Schreck, and A. J. Gharrett. 1996. Controlling the sex of salmonids. Oregon Sea Grant, Ploidy/Sex Manipulation Work Group, ORESU-H-96-001: 26pp.

Gutierrez, J. B., and J. L. Teem. 2006. A model describing the effect of sex-reversed YY fish in an established wild population: the use of a Trojan Y chromosome to cause extinction of an introduced exotic species. Journal of Theoretical Biology 241:333-341.

Haux, C. and B. Norberg. 1985. The influence of estradiol-17B on the liver content of protein, lipids, glycogen and nucleic acids in juvenile rainbow trout, *Salmo gairdneri*. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry 81:275-279.

Herman, R. L., and H. L. Kincaid. 1991. Communications: Effects of orally administered steroids on Lake Trout and Atlantic Salmon. The Progressive Fish-Culturist, 53:3, 157-161,. http://dx.doi.org/10.1577/1548-8640(1991)053<0157:CEOOAS>2.3.CO;2

Hamilton, W. D. 1967. Extraordinary sex ratios: A sex-ratio theory for sex linkage and inbreeding has new implications in cytogenetics and entomology. Science 156:477-488.

Jiang, M., X. Wu, K. Chen, H. Luo, W. Yu, S. Jia, Y. Li, Y. Wang, P. Yang, Z. Zhu and Hu, W. 2018. Production of YY supermale and XY physiological female common carp for potential eradication of this invasive species. Journal of the World Aquaculture Society, 49:315-327

Johnstone, R., T. H. Simpson, A. F. Youngson, and C. Whitehead. 1979. Sex reversal in salmonid culture: Part II. The progeny of sex-reversed rainbow trout. Aquaculture 18:13-19.

Kennedy, P. A., K. A. Meyer, D. J. Schill, M. R. Campbell and N. V. Vu. 2018. Survival and reproductive success of hatchery YY male Brook Trout stocked in Idaho Streams. Transactions of the American Fisheries Society 147:419-430.

Komen, J., P. A. J. Lodder, F. Huskens, C. J. J. Richter, and E. A. Huisman. 1989. Effects of oral administration of 17α-methyltestosterone and 17β-estradiol on gonadal development in common carp, *Cyprinus carpio L.* Aquaculture 78:349-363.

Krueger, William H., and K. Oliveira. 1999. Evidence for environmental sex determination in the American eel, *Anguilla rostrata*. Environmental Biology of Fishes 55: 381-389.

Lowe, S., M. Browne, S. Boudjelas, and M. De Poorter. 2000. 100 of the world's worst invasive alien species. A selection from the Global Invasive Species Database. Auckland, New Zealand: The Invasive Species Specialist Group.

Luckenbach, J.A., R. J. Borski, H. V. Daniels, and J. Godwin, J., 2009.. Sex determination in flatfishes: mechanisms and environmental influences. In *Seminars in Cell & Developmental Biology*. 20:256-263. Academic Press.

Mair, G. C., J. S. Abucay, D. O. F. Skibinski, T. A. Abella, and J. A. Beardmore. 1997. Genetic manipulation of sex ratio for the large scale production of all-male tilapia *Oreochromis niloticus* L. Canadian Journal of Fisheries and Aquatic Sciences, 54:396-404.

Malison, J. A., T. B. Kayes, C. D. Best, C. H. Amundson, and B. C. Wentworth. 1986. Sexual differentiation and the use of hormones to control sex in Yellow Perch, *Perca flavescens*. Can. J. Fish. Aquati. Sci 43: 26-35.

McFadden, J. T., G. R. Alexander, and D. S. Shetter. 1967. Numerical changes and population regulation in Brook Trout *Salvelinus fontinalis.* Journal of the Fisheries Research Board of Canada 24:1425–1459.

Mills, C. 2009. Operation sex change. Conservation Magazine. 10:21-26.

Ospina-Álvarez, N., and F. Piferrer. 2008. Temperature-dependent sex determination in fish revisited: prevalence, a single sex ratio response pattern, and possible effects of climate change. PloS ONE 3 (7), e2837. http://dx.doi.org/10.1371/journal.pone.0002837.

Reinboth, R. 1980. Can sex inversion be environmentally induced? Biology of Reproduction 22.1:49-59.

Saunders, W. C. P. Budy, and G. P. Thiede. 2015. Demographic changes following mechanical removal of exotic brown trout in an Intermountain West (USA), high-elevation stream. Ecology of Freshwater Fish 24:252-263.

Schill, D. J. and E. R. J. M. Mamer. 2019. Progress Report to the Western Association of Fish and Wildlife Agencies on WAFWA YY Male Consortium Activities. Contract Period: July 1, 2018 to June 30, 2019. WAFWA, Boise, ID.

Schill, D. J. and E. R. J. M. Mamer. 2020. Progress Report to the Western Association of Fish and Wildlife Agencies on WAFWA YY Male Consortium Activities. Contract Period: July 1, 2019 to June 30, 2020. WAFWA, Boise, ID.

Schill, D. J. and E. R. J. M. Mamer. 2021. Progress Report to the Western Association of Fish and Wildlife Agencies on WAFWA YY Male Consortium Activities. Contract Period: July 1, 2020 to June 30, 2021. WAFWA, Boise, ID.

Schill, D. J., J. A. Heindel, M. R. Campbell, K. A. Meyer, and E. R. J. M. Mamer. 2016a. Production of a YY male Brook Trout broodstock for potential eradication of undesired Brook Trout populations. North American Journal of Aquaculture 78:72-83.

Schill, D. J., J. A. Heindel, M. R. Campbell, K. A. Meyer, and E. R. J. M. Mamer. 2016b. Supplement: Development of Y-chromosome-specific assays and rules for differentiating XY and YY Genotypes. DOI: 10.1080/15222055.2015.1100149.

Schill, D. J., K. A. Meyer, and M. J. Hansen. 2017. Simulated Effects of YY-Male Stocking and Manual Suppression for Eradicating Nonnative Brook Trout Populations. North American Journal of Fisheries Management 37:1054-1066.

Shelton, W.L., V. Wanniasingham, and A. E. Hiott. 1995. Ovarian differentiation in common carp (Cyprinus carpio) in relation to growth rate. Aquaculture 137:203-211.

Simpson, T. H. 1976. Endocrine aspects of salmonid culture. Proceedings of the Royal Society of Edinburgh, Section B: Biological Sciences, 75:241-252.

Teem, J. L., and J. B. Gutierrez. 2010. A theoretical strategy for eradication of Asian carps using a Trojan Y Chromosome to shift the sex ratio of the population. American Fisheries Society Symposium 74:1–12.

Wenstrom, J. C. 1975. Sex differentiation and hormone directed sex determination in the Lake Trout (*Salvelinus namaycush*). Masters thesis, Northern Michigan University. Marquette.

Zerrenner, A. and E. Marsden. 2005. Influence of larval sea lamprey density on transformer life history characteristics in Lewis Creek, Vermont. Transactions of the American Fisheries Society. 134:687-696.

Appendix A

WAFWA YY Consortium

Exhibit A2 - Workplan 2021-2024

Year Four: July 1, 2021 – June 30, 2022

		Entity involved
1. Ongoing Species Work		
a.	Continue ongoing feminization trials for optimal sex reversal recipes Brown Trout, Walleye, Common Carp	FMS,USFWS, CO/SD/OK/TX
b.	Growout of sex reversed fish – Normal gonads/reproduction? Brown Trout, Walleye, Common Carp	FMS,USFWS, CO/SD/OK/TX
c.	Conduct sex marker investigations Walleye, Common Carp *Northern Pike*	 EFGL *ADG&F*
d.	Evaluate Density-Dependent Sex Change/Extirpation in 2 Idaho streams (Brook Trout)	FMS/EFGL
e.	*Conduct modeling studies on YY use:* *Lake Trout* *Northern Pike*	 *IDFG* *ADG&F*
f.	Provide technical guidance on YY BK field evaluations to other agencies	FMS
g.	Initiate the start of a backup YY BK broodstock	FMS, IDFG and partners
h.	Pursue formal Indexing of E2 for salmonids	FMS, Novaeel Inc. and AADAP
i.	Pursue additional funding for the YY male program	FMS
2. Project communication		
a.	Annual Progress Report (2021-2022) – due 30 Sep 2022	FMS
b.	Annual WAFWA mtg update	FMS
c.	AFS or Aquaculture presentations (n = 2)	FMS

- EFGL = Eagle Fish Genetics Laboratory- Idaho Fish and Game
- ADG&F = Alaska Game and Fish
- IDFG = Idaho Fish and Game

- FMS = Fishery Management Solutions Inc. (Dan Schill and Liz Mamer)
- AADAP = Aquatic Animal Drug Approval Partnership-
- USFWS = US Fish Wildlife Service

Note – Work in *italics* outside of WAFWA/FMS duties

Workplan 2021-2024, Continued

Year Five: July 1, 2022 – June 30, 2023

		Entity involved
1. Ongoing Species Work		
a.	Growout of sex reversed fish – Normal gonads/reproduction? Brown Trout, Walleye, Common Carp	FMS, CO/SD, USFWS, TX
b.	Initiate Northern Pike sex reversal trial (Spring 2023)	FMS and WAFWA partners
c.	Finalize sex markers for Walleye and Northern Pike	EFGL
d.	Obtain Index coverage for salmonids from the FDA (Brook Trout and Brown Trout)	FMS/Novaeel Inc./AADAP
e.	Continue development of backup YY BK broodstock	FMS, IDFG and partners
f.	Evaluate Density-Dependent Sex Change/Extirpation in 2 Idaho streams (Brook Trout)	FMS/EFGL
g.	Provide technical guidance on BK field evaluations	FMS/IDFG
h.	Pursue additional funding for the YY male program	FMS
2. Project communication		
a	Annual Progress Report (2022 - 2023) – due 30 Sep 2023	FMS
b.	Annual WAFWA mtg update	FMS
c.	AFS or Aquaculture presentations (n = 2)	FMS
d.	Publication of prior study results	FMS/EFGL/WAFWA partners

Workplan 2021-2024, Continued

Year Six: July 1, 2023 – June 30, 2024

		Entity involved
1. Ongoing Species Work		
a.	Growout of sex reversed fish – Normal gonads/reproduction? Brown Trout, Walleye, Common Carp	FMS, CO/SD, USFWS, TX
b.	Initiate sex reversal trials for Lake Trout (Fall 2024)	FMS
c.	Initiate Development (Phase One) of a YY BRN Broodstock	FMS/WAFWA partners
d.	Evaluate Density-Dependent Sex Change/Extirpation in 2 Idaho streams (Brook Trout)	FMS/EFGL
e.	Continue technical guidance on BK field evaluations	FMS/IDFG
f.	Pursue additional funding for the YY male program	FMS
g.	Begin transition to new Consortium staff	FMS and ???
2. Project communication		
a.	Annual Progress Report (2023 - 2024) – due 30 Sep 2024	FMS
b.	Annual WAFWA mtg update	FMS
c.	AFS or Aquaculture presentations (n = 2)	FMS
d.	Publication of prior study results	FMS/EFGL/WAFWA partners

Note: Initiation of new YY Brown Trout broodstock will be dependent upon successful approval by the FDA.

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				

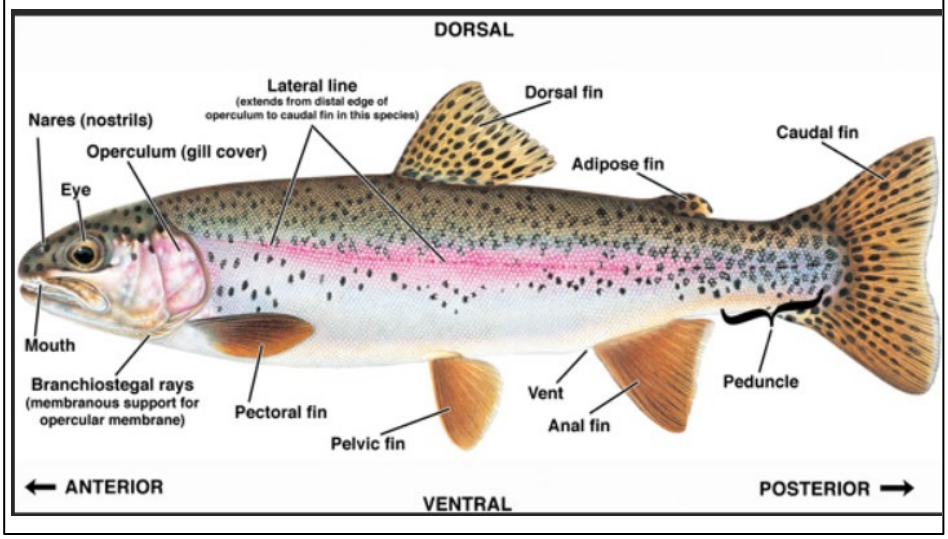
SCORING:
(*cumulative score could provide an early indication of arising health issues*):

15-16 – Very healthy

11-14 – Healthy

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

< 8 - Significant health concerns - requires action



Appendix C

Results of sex marker development efforts by the EFGL

(K. Coykendall and M. Campbell)

C1 - Common Carp

During the last performance period, the EFGL screened 5 additional sample collections using the genetic sex marker previously developed for carp (Cca744444_87). The population samples were collected during FY21 in Midwest and Eastern states by project staff under a MSCGP Grant (Schill and Mamer 2021). DNA extraction, analysis and data workup was conducted by EFGL staff in FY22. Overall concordance rates between phenotypic and genetic sex high and equaled or exceeded 96% in three of the waters (Appendix C, Table 1). Concordance rates for the Kentucky Lake population was slightly lower at 89% and one population exhibited significantly lower concordance (Guthrie City Lake, CcaGUTH21C = 69%). Overall concordance between phenotype and genotype for all five populations was 89.9%, a slightly lower rate than the 93% overall concordance level reported by EFGL staff 10 carp samples including seven from Idaho and three from the Midwest (Schill and Mamer 2019).

Table 1. Population and corresponding pedigree of study collections and the number of samples that were not concordant or concordant between phenotypic and genetic sex (Assay Cca744444_87). The number of failed samples, total samples genotyped, and overall concordance is also shown.

Population	State	Date Collected	Pedigree	Not Concordant	Concordant	Failed	Total	Concordance
Black Hawk L.	IA	May '21	CcaBLHL21C	1	195	4	200	0.99
Foster Joseph Sayers Res.	PA	May '21	CcaFJSR21C	6	132	1	139	0.96
Kentucky L.	TN	May '21	CcaKYLK21C	22	175	7	204	0.89
Milford Reservoir.	KS	Nov '17	CcaMILF17C	8	189	3	200	0.96
Guthrie City L.	OK	Apr '21	CcaGUTH21C	62	138	0	200	0.69

In the Guthrie City population, intriguingly all discordance errors were due to some phenotypic males screening out genetically as females. In contrast, all phenotypic females in CcaGUTH21C were identified as genetic females. We saw a similar pattern in the discordant samples in the other populations. We theorized that this pattern of discordance could be due to an unaccounted for SNP within the probe region of the assay. To investigate this, we complete directed Sanger sequencing of the Cca744444 locus to assess if unaccounted snps might be present that would impact probe annealing. While we were successful in

sequencing the Cca744444 locus on a subset of samples, we did not detect any SNPs that would explain the discordant results.

To address the issue of the discordance between Cca744444_87 and phenotypic sex in the Guthrie City Lake population, we constructed two Radseq libraries with a total of 13 females and 13 males. We aligned the resultant DNA sequences to a *Cyprinus carpio* genome published in 2021. (This genome was not available to us when we first developed Cca744444_87.) The following are putative sex markers we found, based on the rate of genotyping success in each sex:

Locus	F	M	Total
CM031287.1_902425	13	13	26
JAEOAB010006541.1_803032	13	13	26
JAEOAB010006541.1_802967	13	9	22
CM031272.1_4338895	12	13	25
CM031272.1_4338985	12	13	25
CM031261.1_9378126	10	9	19
CM031267.1_35740248	9	8	17

Looking at carp chromosomes where there were a high number of SNPs found to conform to sex-linked patterns, we have:

Chromosome	# SNPs
CM031267.1	20
CM031288.1	17
CM031245.1	15
CM031250.1	12
CM031277.1	10

The next steps will be to find the location of each SNP in the top table and design an assay if feasible. Also, we will look at the SNPs on each chromosome listed in the bottom table and see if they cluster together. Any assays we design will be tested on the remaining Guthrie City Lake samples we have and other populations of carp to determine if this marker works on a broader geographic scale. Final analysis of this follow-up RAD sequencing effort will be reported on in the final MSCGP report this spring.

C2 - Walleye

Walleye

This year we have not generated new data, but have focused on re-analyzing the data we do have. Since last year, two different labs have sequenced a walleye genome or improved a draft genome. Heiner Kuhl from the Leibniz Institute of Freshwater Ecology and Inland Fisheries (IGB) and Yan Guigen from the

National Research Institute for Agriculture, Food and Environment in France (INRAE) are collaborators that have sequenced and assembled a draft genome to the chromosome level. They shared their genome with us and we shared our genomic DNA sequences from them. Based on our data and data from other walleye from the US provided by Peter Euclide (Purdue University), Heiner found a putative genomic location associated with phenotypic sex where one allele is a string of eight T nucleotides (T8) and the other allele is a string of nine T nucleotides (T9). Females had both alleles (T8/T9) and males had two of the T9 alleles, which would make the sex determination system ZZ/ZW. This type of variation (number of repeated, single nucleotides) is difficult to develop into a genotyping assay. Therefore, Matt Campbell designed primers that flanked the marker and we spiked the primers into our existing GTseq panel and ran it on 95 walleye samples, 51 males and 44 females from Lake Pend Oreille in Idaho. If there was 100% concordance between the marker and phenotypic sex, we would expect the outcomes in the left table. What we actually observed in the data is in the right table. The "alt" row refers to an alternative allele that was amplified by these primers that looks very different from the sex marker. Heiner determined that it was an artifact from the GTseq library.

Nucleotides	M	F	Total
T8/T8	0	0	0
T8/T9	0	44	44
T9/T9	51	0	51

T8/T8	1	1	2
T8/T9	6	34	40
T9/T9	37	2	39
alt	7	7	14
Total	51	44	

This marker predicted sex with about 90% accuracy. The mismatches could be due to genotyping error, an environmental component to sex determination, additional genes influencing phenotypic sex, or less than 100% linkage between this marker and the sex determining gene. If this marker does hold up, it would mean walleye females are the heterogametic sex (ZW), which is contradictory to previous studies by Malison and Garcia-Abiado (1996) and Malison et al (1998).

Meanwhile, our colleagues at the University of Wisconsin at Milwaukee (Angela Schmoldt, Rebecca Klaper) and The Great Lakes Genomics Center (Olaf Mueller) have improved upon the original draft genome they shared with us two years ago. This new genome has been assembled to the chromosome level (24 chromosomes, 8,445 un-scaffolded sequences). Moving forward, we plan to apply additional bioinformatics techniques that look at read coverage, k-mer analysis, etc. along with these new genome resources to see if additional sex determining regions can be uncovered. Also, we plan to continue collaboration with Heiner Kuhl and his colleagues to see if the putative ZZ/ZW marker can be optimized.