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

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Introduction

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

The development of a Trojan Y Chromosome broodstock for actual use in 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.

YY Male Consortium Program Objectives

1. Work with the Aquatic Animal Drug Approval Project ("AADAP"), the Food and Drug Administration ("FDA") and WAFWA partners on continued annual distribution of YY Male Brook Trout eggs.
2. Provide technical guidance on field evaluations of YY Brook Trout to WAFWA partners receiving eggs, including formation of a technical team.
3. Undertake sex marker development for a total of five candidate YY Male species including Common Carp, Walleye, Lake Trout, Northern Pike and Brown Trout.
4. Evaluate potential sex reversal recipes for the same five species.
5. Evaluate the likelihood of density-dependent sex change in lab studies for Common Carp, Brook Trout and Lake Trout.
6. Identify WAFWA partners or other collaborators willing to undertake creation of YY Male broodstocks for the above species.
7. Work with AADAP and WAFWA partners to provide Investigational New Animal Drugs ("INAD") coverage for development of new YY Male broodstocks developed under this agreement.
8. Assuming positive results are obtained via the above objectives; begin development of YY Male broodstocks for a minimum of three candidate species by 2021.
9. Investigate additional funding opportunities from interested collaborators.

This report documents results of the activities conducted during the third program year to enable attainment of those objectives. Appendix A lists tasks to be undertaken during the first three project years 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 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. In addition, new sex reversal trials were initiated in Spring 2021 in North Dakota for Walleye and for Common Carp at hatcheries in Texas and Oklahoma.

Brown Trout Trials – BY2019 & BY2020

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 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 BY2019 at or near this concentration and also examined treatment duration.

BY2019 Trials

Colorado Trial

During November and December 2019 sex reversal trials using Estradiol (hereafter E2) on treatment groups of swim-up Brown Trout were initiated in hatcheries in Colorado and South Dakota. The first trial was conducted 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). Trial eggs were hatched out in heath trays and fry counted into aquaria prior to swim-up. Mortality rate was documented daily. Treatment group feed was topcoated with a 20 mg/kg feed E2 solution diluted with non-denatured ethanol (EtOH), using a hand-held sprayer (Schill et al. 2016a). Control feed was topcoated with pure EtOH at the same concentration as treatment groups. The treatment groups were fed E2 coated feed for either 30 or 60 days, beginning at first feeding (approximately 30 DPH).

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

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

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)

South Dakota Trial

A sister study of similar design (four replicates per treatment group) 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 dose of E2 to be applied to the feed varied at either 20 or 30 mg/kg feed for a fixed period of 60 days, beginning at first feeding (Table 1). Eyed eggs were counted out and placed into 400 L flow-through circular tanks, where the fish hatched out and were subsequently treated as above. On 29 February 2020 at 85 DPH, due to facility operational needs, fish were relocated to 100 L flow-through circular tanks where, on 13 April 2020 at 129 DPH, they were culled to 200 fish per replicate. Mortality rate was monitored daily.

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

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

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

BY2020 Trial - Colorado

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

The 2020 trial consisted of 10 treatment groups experiencing various concentrations of E2 and exposure length, plus a Control group and a Control Interval group with 10 fish sampled every

30 days for histological preservation to document timing of gonadal differentiation (Table 2). To produce study fish, brood fish were genetically sampled then spawned on 6 Oct 2020 by Glenwood Springs Hatchery. Eggs were shipped to COFRH on 16 Nov 2020 where they were mixed and placed in heath trays for hatching. Prior to swim-up, hatched-out fry were counted into 75 L flow-through aquaria. The study fish were fed dry pelleted feed (Bio-Oregon, transitioning to Rangen and Skretting) for the course of the study. Treatment group feed was top-coated with E2 and EtOH as above. All treatment groups and the Control group feed were top-coated with the same volume of pure EtOH as that received by the highest dosage treatment group. Control Interval feed was not top coated with EtOH. The treatment groups (n = 100 fish) were fed E2 coated feed for varying durations of 60 to 120 days, beginning at first feeding (26 DPH) and mortality rate documented daily.

Table 2. Sex reversal trial framework for Brown Trout receiving Estradiol via treated dry feed at Colorado Fish Research Hatchery, Bellvue CO, initiated 29 Nov 2020. Numbers in matrix are replicates n's by treatment type (dosage and duration) for each treatment. Fry (n = 100) received either treated or control feed beginning at swim-up (26 DPH).

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

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

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

The first trial was started with 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 and a Control Interval group with 10 fish sampled every 21 days for histological preservation to document differentiation (Table 3). 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 3). 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. A Control Interval sample was taken when the first tanks went on treated feed (25 DPH) and will continue every 3 weeks until all groups are off treated feed in Sep 2021.

Table 3. 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)	None (No EtOH)
Short	60	90	2			
Mid	25	125		2		
Long	25	125		2		
Control					1	
Control Interval						1

Texas

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 4). An important feature of this trial was the inclusion of lower treatment levels for longer durations than that recently reported (200 mg/kg) to feminize roughly half of male Common Carp (Jiang 2020). Using AE Woods Koi maintained on station as feeder fish, broodstock were hand spawned and eggs hatched and reared to scalation in outside ponds on natural feed. Fry were counted into indoor 14' x 3' raceways (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 is documented daily. A Control Interval sample was taken at first day on treated feed (25 DPH) and will continue every 3 weeks until all groups are off treated feed in Nov 2021.

Table 4. 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

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. Last May 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 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 5). 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 42mm in size.

Table 5. 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 will receive 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. Ultimately once established in the terminal tanks, fry will be reared at 1.7 fish/l to continue the course of the trial. Study fish will be 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 will be top-coated with the same volume of pure EtOH as that received by the highest dosage treatment group. Control Interval feed will not be top-coated with any EtOH. The treatment groups will be fed E2 coated feed for varying durations of 60 to 84 days, beginning at first feeding (DPH not yet known at time of writing). Genetic fin clip samples were collected from broodstock at spawning, and 100 fry genetic samples will be taken at the time of post-scalation tank splitting to document early sex ratio.

At time of writing, fry have yet to go on treated feed or through the tank splitting/density balancing process.

Lake Trout Trial – BY2020

BY2020 Differentiation Study

Given both 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 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 are being sampled at two-week intervals, eventually advancing to monthly intervals once cytological differentiation has been observed. Fertilized eggs were received 16 Nov 2020 from Story Fish Hatchery, WY, hatched and bi-weekly sampling begun on 7 Dec at 14 DPH (677ctu's). Ten samples collected on 10 May 2021 have been sent for histological examination to confirm whether or not cytological differentiation has begun. At this point sampling will move to a monthly interval until approximately 18 - 24 mo (Jul - Nov 2022) and will finish once a complete (terminal) differentiation DPH date is established. At that juncture a follow-up LKT sex reversal trial should yield improved results.

Sex Markers

General Approach

To develop genetic sex tests or sex markers for species of initial interest to the WAFWA Consortium, the 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) personnel 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. In FY2021, the primary fieldwork objective was to collect additional samples for several species to hopefully help improve marker accuracy over a broader range of populations. Additional Northern Pike samples were collected from Lake Roosevelt and samples from a new Lake Trout water (Odell Lake OR) were added to the LKT dataset. In addition, new Common Carp populations were sampled (see below).

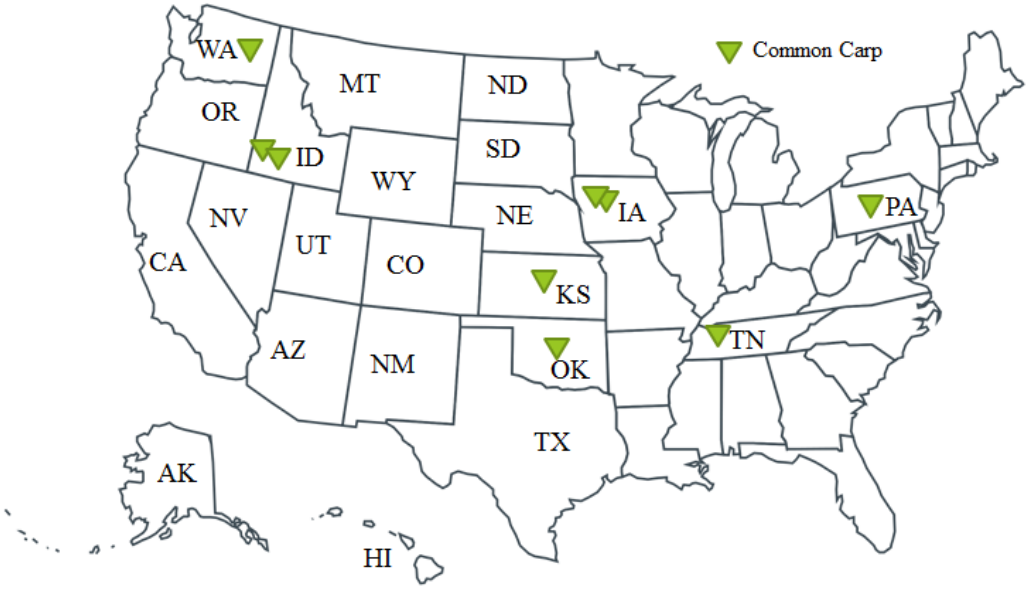
Walleye

During FY2019 and FY2020 IDFG's Eagle Fish Genetics Laboratory staff made several concerted efforts to develop a Walleye sex marker but unfortunately that work did not yield useful markers (Schill and Mamer 2019; Schill and Mamer 2020). Additional efforts were made by the lab in FY2021 and the approaches attempted in FY2021 are briefly described by EFGL staff in Appendix B1.

Common Carp

During FY 2019 EFGL staff identified two candidate bi-allelic loci and screened one (Cca744444_87) on 800 samples from 11 populations, 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, EFGL - Appendix B2). No lab work was conducted on Common Carp during FY2021. In FY2021, samples from five new waters in PA, TN, IA, WA and OK were collected and added to several large existing carp population samples involved in FY19 work to enhance the geographic utility of the marker. The combined samples (Figure 1) will collectively be analyzed and reported on in FY2022.

Figure 1. Location of Common Carp populations sampled for sex marker development. Samples and data to be analyzed in FY2022.



Lake Trout

In FY2019, the EFGL developed an sdY presence/absence marker from sdY sequences on Genbank and demonstrated its accuracy by screening 30 phenotypic male and 26 phenotypic female Lake Trout from Lake Pend Oreille. (Mamer and Schill 2019, EFGL - Appendix B3). At that time, the lab also discovered a candidate biallelic sex marker locus (Sna_433923_27) also potentially useful in building a YY Male broodstock. In FY2021, project staff added an additional population sample to the work (Odell Lake OR) and the EFGL continued optimization of the Bi-allelic marker and tested both of the above markers for accuracy on samples collected from six Lake Trout populations See Appendix B-3 for a detailed description of their methods.

Northern Pike

As noted in the workplan (Appendix A) 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). Unfortunately, due to Covid-related fiscal issues AKFG was fiscally unable to conduct any work to solidify markers during FY2021. However, new funding was secured work and a target for Northern Pike sex marker completion by their lab is now the summer of 2022.

Density-dependent Sex Change

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.

ESD Evaluations

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 initiated at Opaline Aquafarm and the null finding (no ESD observed) was reported previously in Schill and Mamer (2019).

Lake Trout

Based on our experience with the above-mentioned Common Carp pilot trial, a larger ESD trial was developed with managers of the Grace Fish Hatchery (GFH) including Malia Gallagher, Eric Pankau, and Wayne Fowler. Five initial rearing densities were selected for evaluation (Table

6). More pots were devoted to low density trials, nine and eight pots for the two lowest density levels respectively, because few fish would be available to examine at low densities and presumably about 50 % would already be genetic females, further limiting the possibility to observe male to female phenotype shifts.

Three female Lake Trout were spawned with three males and their eggs were mixed thoroughly and placed in Heath trays at the Story Fish Hatchery (Wyoming Game and Fish, Banner, WY). These were then shipped as eyed eggs to GFH, where they hatched on 6 December 2017. Fry were reared in heath trays to button-up and then transferred to 24 14 L circular pots for growout, on 17 January 2018 (42 DPH). Based on histological observations of developing Lake Trout (Wenstrom 1975), adjusted for GFU CTU, development of the bipotential genital ridge of our study fish placed in the pots should have been occurring from approximately 39-59 days. Thus the fish should not have begun differentiating before being placed in the pots. At first feeding and after, fish were fed dry pelleted feed (Rangen) over the course of the study. A rough guideline of 4.6 % of body weight per day was used though fish were typically fed to satiation.

Wenstrom (1976) reported that untreated control Lake Trout reared at 4 °C had all differentiated with respect to sex after 227 days. All fish in the present study were PIT-tagged on 2 October 2018 at 300 DPH and placed in a common raceway assuming sex differentiation had already occurred but that phenotype would not always be readily discernable without a considerable growout period. Final sampling of 118 surviving Lake Trout occurred on 22 June 2020 at 930 DPH. Data collected included total lengths, weights and observed phenotypic sex. Gonads of a subsample (20%) of study fish were preserved for histological examination to verify visual phenotype calls on fish from various pots. A fin clip was taken for genetic analysis and genetic sex will subsequently be compared to observed phenotype to ascertain if any phenotypic shifts occurred.

Table 6. Environmental Sex Determination Trial framework for Lake Trout initiated January 2018at the Grace Fish Hatchery, Grace, Idaho, demonstrating range of densities (fish per pot).

Density Type	Replicates	Initial Starting Density
Low	9	5
Low-Mod	9	10
Mod	2	20
Mod-High	2	50
High	2	100

Brook Trout

A far larger field study of ESD, initiated by IDFG and Bart Gamett of the United States Forest Service, has been ongoing on two Idaho Brook Trout streams since 2016. Bear Creek and

Willow Creek are two short, 1 - 2 km, isolated streams containing only invasive Brook Trout. The entire lengths of both streams have been subjected to Pulsed DC electrofishing removal on two consecutive days in early July for the past five years. Based on preliminary data analysis, roughly 75 % of the population has been removed annually. All fish collected were killed and a fin clip taken and stored on numbered Whatman sheets. 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 made only on fish with clearly identifiable gonads and the remainder were classified as unknown. Genetic sex was independently determined by personnel at the Eagle Fish Genetics Lab (EFGL) for all phenotypically sexed fish using a sex marker (Schill et al. 2016b) although more advanced SNP panels are presently available to identify Brook Trout sex (Mathew Campbell, IDFG, EFGL, personal communication). Phenotype and genetic sex data were subsequently compared for each individual fish and any discrepancies noted.

The aim of this ongoing evaluation is to 1) search for genotype:phenotype mismatches and hence potential Density-Dependent sex change as the total wild populations of the two streams are reduced *via* ongoing suppression efforts including concomitant stocking of YY Male Brook Trout and 2) document time to extirpation of these Brook Trout populations.

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.

Walleye

Sex Reversal Trials

Results of the ongoing Garrison National Hatchery trial will be reported on in the FY22 report.

Sex Marker Development

During the past year IDFG's Eagle Fish Genetics Laboratory staff made new efforts to develop a Walleye sex marker. Unfortunately, as in the prior two years, the FY2021 work did not yielded useful results. Analyses to find additional candidate markers are underway and will be reported on in FY2022. For a more detailed explanation see Appendix B1.

Lake Trout

Differentiation Study

No actual sex reversal trial was undertaken during FY21. However, as noted in the methods, the differentiation study initiated at the Grace Hatchery should aid in a future sex reversal trial by highlighting the exact period that juveniles need to be exposed to E2 to be successfully sex reversed.

Sex Marker Development

During FY2021 the EFGL ran both the presence-absence and biallelic markers on a total of 995 Lake Trout outside Lake Pend Oreille where they were initially developed (Schill and Mamer 2019). Concordance between observed phenotype and genotype was good at 93% overall and there was only 1 disagreement between the presence-absence and bi-allelic genotype prediction (Appendix B-3). There were sizeable differences in concordance between the 2019 and 2020 samples from Yellowstone Lake and both markers performed poorest relative to known genotype at Stanley Lake. The reason for reduced concordance in these two groups is unknown. Nonetheless, the overall rate reported above (93%) should be more than necessary to build a YY Lake Trout broodstock assuming an effective sex reversal protocol can be found That is because the potential parents and progeny of those Lake Trout involved in gamete stripping during spawning in Phase 1 of YY broodstock production (see Schill et al. 2016a) whose phenotype at the time of gamete stripping does not match their genotype from prior genetic analysis can be culled from the population (Mathew Campbell, EFGL personal communication).

Brown Trout

BY2019 Sex Reversal Trial

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

Colorado

Based on visual examination of gonads nearly 11 month after the trial start, sex ratios in the control group (n = 239) approximated the expected 50:50 sex ratio at 48.5% female (Table 7). In the 30 day treatment exposure group (n = 238) the female ratio was exactly 50% while the 60d exposure group was comprised of 74.3% females. Confidence bounds around the difference

between the mean control versus 30d treatment ratios (n = 4) were not statistically different (Figure 2). However, the difference between the mean control and 60d female ratios (n = 4) did not overlap zero indicating a statistically significant difference for the longer treatment group. Not surprisingly, there was also a statistical difference between the unsuccessful 30 day treatment and the 60 treatment group with the latter clearly being preferable (Figure 2). As expected there were no intersex fish visually observed in the control group and only two in the 30 treatment while 7 were observed in the 60 day group leading us to suspect that a longer treatment interval might improve on the feminization rate. In addition, our histological examination of 240 fish in the two E2 treatment groups yielded only 10 intersex fish that were not detected visually (4%). As examination of all fish histologically would have minimal effect on "true" feminization rates, we concluded that expensive histological examination of treated fish 11 months or older was unnecessary and thus did not pay for histological sampling of the 13 month old fish at time of sampling in the South Dakota trial below.

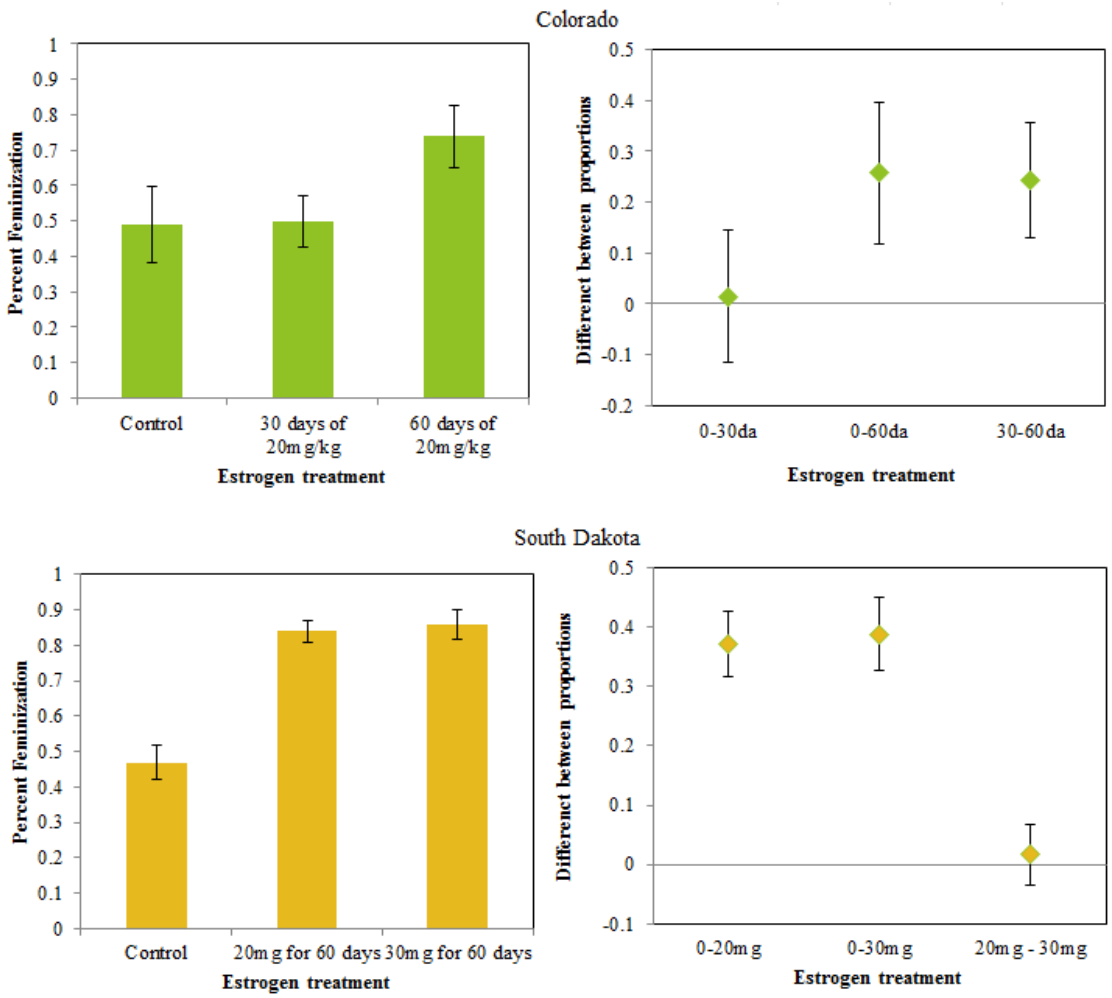
With one exception, mean lengths and weights by sex within study groups declined as the duration of E2 exposure increased from none (Control) to 60 days (Table 7). This result is not necessarily cause for concern and may in fact be a sign of successful treatment levels because male fish sex reversed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

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

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

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

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



South Dakota

Based on visual examination of gonads nearly 15 months after the trial start, sex ratios in the control group (n = 238) approximated the expected 50:50 sex ratio at 47.1% female (Table 8). In the 20 mg/kg, (n = 224) the female ratio was 84.2% while the 30 mg/kg group was only slightly higher at 85.8% female. Mean difference in this proportion female (n = 4) between the control versus 30d treatment and between the Control and 60d treatment groups were both statistically

significant as their differences did not overlap zero (Figure 2). Sixteen intersex fish were visually observed in the 60 day group, again leading us to suspect that a longer treatment interval might improve on the feminization rate. Not surprisingly, less intersex fish (8) were observed in the higher 60 day, 30mg/kg E2 feed concentration group.

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

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

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

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

Summary of BY2019 Trials

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

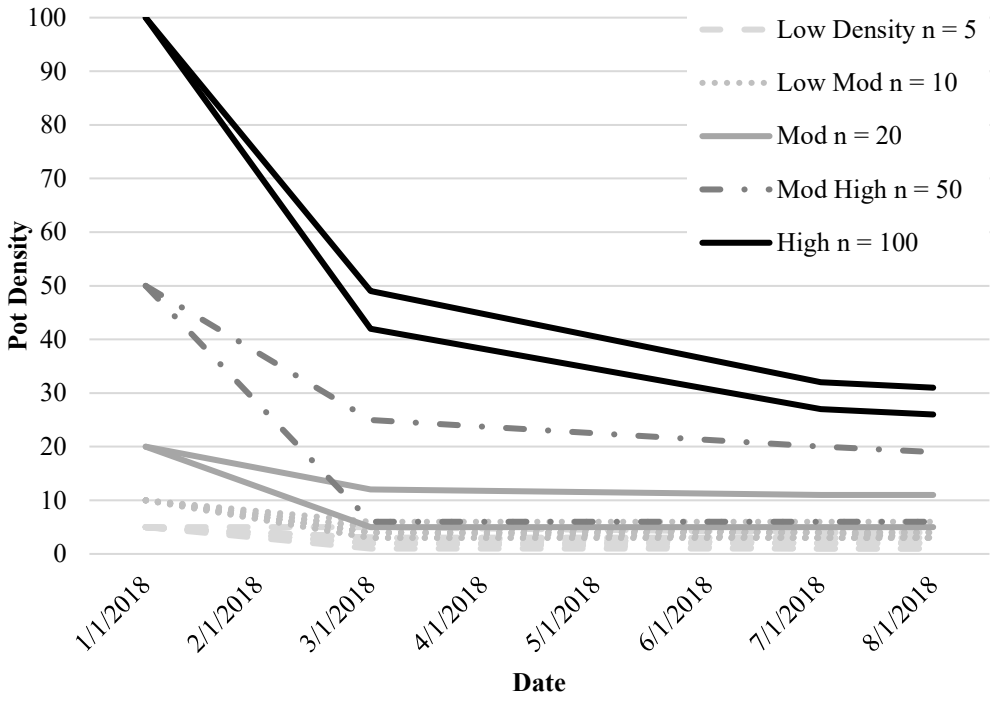
Given the above results and the possibility that the BY2020 work could improve upon the already positive 2019 feminization results in this report, Brown Trout have become the best candidate for starting the next YY Male broodstock. Given this reality, additional focus will be paid in FY2022, working in concert with Novaeel Inc., to obtaining FDA authorization to proceed on a YY Male Brown Trout. The most likely route for authorization to start a broodstock will be working in concert with Novaeel Inc. *via* the Indexing process currently undergoing review by FDA's Center for Veterinary Medicine.

Density Dependent Sex Change

Lake Trout

Mortality was tracked throughout the length of this 2.5 year study and not surprisingly, heavy mortality occurred during the first 90 DPH time period (Figure 3). The mortality range during this period averaged 47% in the Low density pots to 69% in the Moderate High pots with the remaining treatments falling in between. Of the five different initial density environments in which these fish were reared (5, 10, 20, 50 & 100 fish per 14 L pot up to 300 DPH), 100% were found to have matching pheno-geno types and there were zero instances of intersex observed in 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-based study.

Figure 3. Mortality trends of Lake Trout reared to 300 DPH at five different densities within twenty-two 14 L pots from Jan 2018 through Oct 2018 and subsequently PIT-tagged and reared in a common garden raceway until 22 Jun 2021, Grace Fish Hatchery,



Populations were maintained successfully in all pots for the duration of the trial and there were a total of 16 fish examined for phenotype-genotype mismatch or shift in the low density pots which, in terms of the YY Male approach, were the primary fish of interest. Similar observations were observed in the Common Carp pilot study where carp held in low densities tanks showed no phenotypic shift (Schill and Mamer 2019). While these two laboratory studies provide some indication that these two gonochoristic species cannot to shift phenotype at low abundance, the

ongoing work on Brook Trout in the wild discussed below comprise a much more rigorous evaluation of the question.

Brook Trout

To date a total of 3014 wild Brook Trout in the two study streams have been visually sexed for phenotype and successfully sexed genetically using a sex marker (Table 9). 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 (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 sex marker panels. 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 all results before and since, where no phenotype-genotype mismatches were observed from 2018 to 2020. 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. Continued annual searching for future mismatches will provide additional support for this interpretation.

While the initial and primary intent of this study was and remains keeping a watchful eye out for potential sex change, a secondary goal is to document results of suppression and YY Male stocking on the status of the two populations. An examination of length-frequencies for all five years of the study suggest that considerable wild Brook Trout population suppression, particularly for YOY fish has been attained in both streams (Figure 4). Further, relative to the first sampling year, wild fish of adult size in Willow Creek are also declining at a rapid rate with the larger sizes in 2020 being dominated by stocked YY Males that had overwintered from prior stocking. Larger fish (>130mm) are were also predominantly overwintering YY Males in Bear Creek in 2020 but wild males in the same sizes did not decline since the prior sampling year, remaining somewhat stable. However, an uptick in wild Age 0 wild fish appeared to have occurred in Bear Creek in 2020, perhaps due to either compensatory survival in remaining wild fry or optimal overwinter rearing conditions. An alternative possibility is that the weir on lower Bear Creek has been breached but no large uptick in larger wild fish appeared to have occurred so this would seem unlikely.

Table 9. Phenotype and Genotype for 3014 Brook Trout collected from two Idaho isolated streams, Bear Creek and Willow Creek, during the ESD trial, 2016-2020.

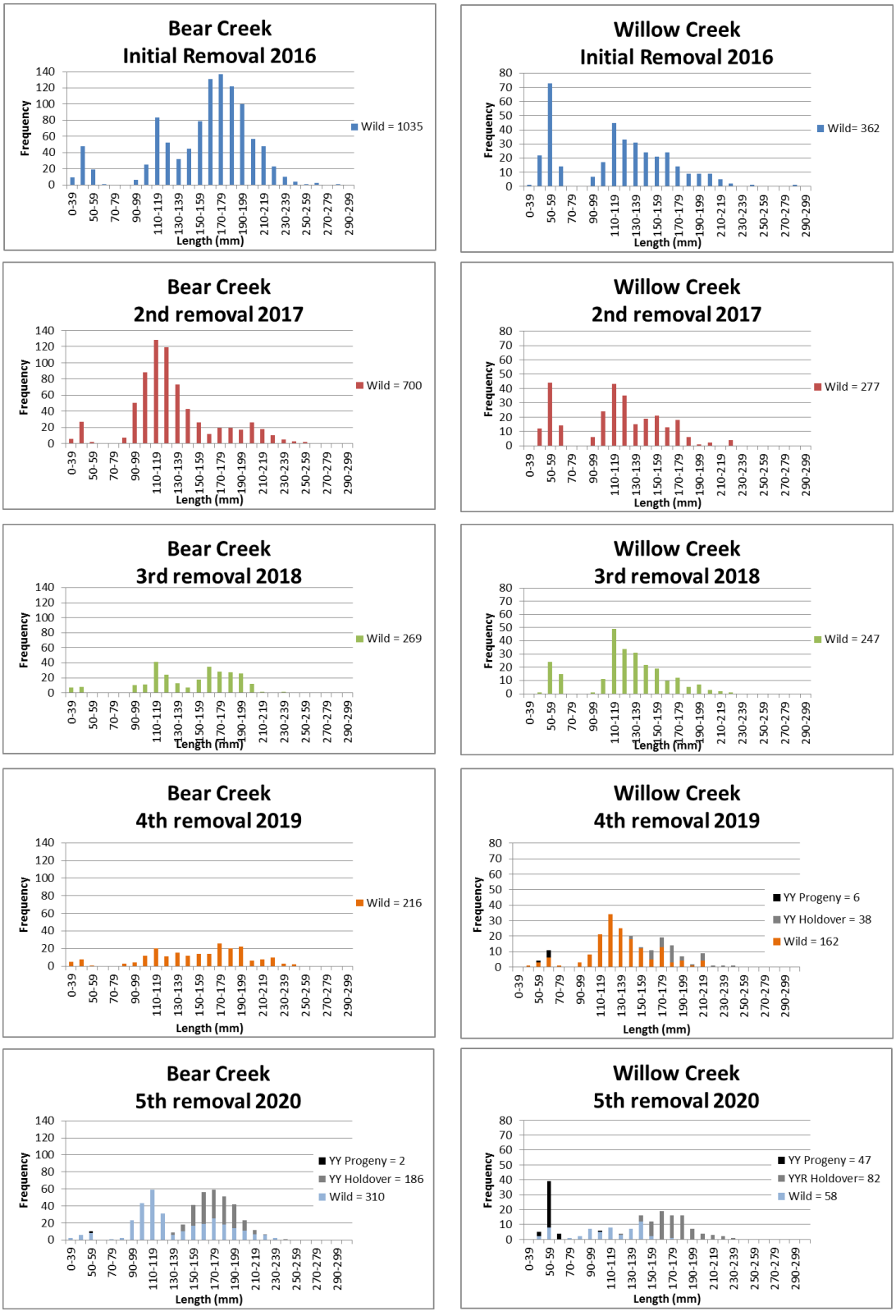
Year	Stream	Phenotype	Genotype		Grand Total
			F	M	
2016	Bear Ck				929
		F	495	0	
		M	0	434	
	Willow Ck				251
		F	149	0	
		M	0	102	
2017	Bear Ck				594
		F	283	0	
		M	1	310	
	Willow Ck				178
		F	102	0	
		M	0	76	
2018	Bear Ck				182
		F	102	0	
		M	0	80	
	Willow Ck				199
		F	90	0	
		M	0	109	
2019	Bear Ck				189
		F	101	0	
		M	0	88	
	Willow Ck				147
		F	77	0	
		M	0	70	
2020	Bear Ck				288
		F	162	0	
		M	0	126	
	Willow Ck				57
		F	25	0	
		M	0	32	
Grand Total					3014

YY Male fish (n = 173) were stocked for the first time into Willow Creek in 2018. The length frequency for Willow Creek in 2019 (Figure 4) depicts the 38 stocked YY Males collected in the stream a year after that stocking (comprising 18.4% of the sample) and also identifies their progeny based on the use of Genetic Stock Index techniques (Matthew Campbell, EFGL unpublished data). The progeny of YY Males (n = 6) comprised 35.3 % of all fry collected in Willow Creek in 2019. After two years of YY stocking, Age 0 progeny of YY Males comprised a large majority (79%) of Willow Creek fry in 2020 (Figure 4).

YY Male fish were stocked for the first time into Bear Creek in 2019 and comprised a strong majority of Brook Trout >130mm one year later (Figure 4). However, only two progeny of that first YY Male year class were observed. The exact reasons for the disparity in first year progeny production between Willow and Bear Creek is unknown but the latter was mistakenly understocked by 40% the first year which may explain the difference.

Based on predictive modeling (Schill et al. 2017), both populations are expected to continue declining markedly. We will continue examining individual fish for genotype-phenotype mismatches, (and hence Density-Dependent Sex Change) as both populations continue to approach total collapse and wild fish hopefully become fully eradicated.

Figure 4. Frequency of size of Brook Trout removed over five years from two Idaho streams, 2016-2020. Willow Creek was first stocked with YY fish in July 2018; Bear Creek first stocked in July 2019.



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 with FDA and AADAP personnel. The first one was sponsored by CVM 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. The Brook Trout INAD is now held by AADAP with technical assistance from us. 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 Novaeel Inc's formal stated recent interest in helping to support the use of E2 in the YY Male arena, though their interest lies largely in the Indexing route for use of the drug. This mid-winter work involved a detailed summary and review of existing Hayspur Hatchery data considered potentially helpful by Novaeel Inc. in moving drug approval forward, initially for salmonids *via* the Indexing route.

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. A total of about 20 individuals are copied on team email but a core group of roughly 10 individuals were regularly involved including the EFGL supervisor, 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 GoToMeeting conference call was held on 3 December and personnel from a total of 4 states and the USFWS participated. NMSU student, Ben Armstrong presented some very positive results from his 2020 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 work and Ron Twibell from the Abernathy Fish Technology Center presented results from a laboratory feed study on growth and maturity rates observed in fingerling YY Brook Trout at their facility. There was discussion regarding the need for a Spring 2021 meeting. Group consensus was to not hold two semi-annual meetings and move to a single, mid-winter meeting in FY2022.

Identify Additional YY Partners and Funding Opportunities

In FY2019, this project sought and obtained a grant administered by the U.S. Fish and Wildlife Service (USFWS) for FY 2020. A Multi-State Conservation Grant proposal entitled "Development of YY Male Broodstocks for Eradication of Invasive Common Carp Populations" was submitted and awarded by the USFWS for 75K with a performance period of Jan 1 2020 to June 30, 2021. The work had to be cancelled due to Covid in 2020 but an extension was granted and the work is currently underway and will straddle FY21 and FY22. A follow-up Preliminary Proposal was recently submitted by project staff and accepted by the MSCGP Grants Committee for a second year of work. A full proposal will be submitted for consideration this summer by program staff.

FY 2021 financial support ($15K) for the Consortium was also sought and secured from the Kalispell Tribe for funding Brook Trout YY work being done in partnership with WDFW within the Pend Oreille River watershed.

A dialog was initiated with the Trinchera Ranch in southeastern CO regarding future possible funding of work being done to build a second (backup) YY Brook Trout broodstock. Although no funds were committed at that time, a recent request by a ranch representative for more dialog on possible support was recently expressed and this dialog is expected to continue into FY2022.

In perhaps the most substantial progress to date on pursuit of new funding opportunities, project staff sought and obtained support from WAFWA fish chiefs to add an additional person to the Consortium staff whose expressed purpose is to pursue new program funding. Although initially funded for a modest amount of time annually, it is hoped this new addition will be able to grow the program as work hopefully begins on construction of additional YY broodstocks.

Project Communication

Detailed fiscal and narrative reports were delivered to AZ to wrap up Brown Trout sex marker work funded under their Heritage Grant Program. The annual WAFWA progress report for FY2021 was completed and submitted to WAFWA on schedule. YY Male Consortium project results were presented at the virtual WAFWA Chief's meeting during July 2020. As part of a YY Consortium planning effort, an Executive Summary was prepared that detailed work undertaken during the first three years of the program. Many of these results and additional budget info was presented via Zoom during an April 2021 WAFWA Chiefs planning meeting where a proposal for the next three years of YY Male work was presented and subsequently approved by the funding Chiefs. To develop interest in possibly receiving/using YY Male Brook Trout in research studies in

three new states, Zoom presentations on YY Male Brook Trout were made to State and Federal staff in IA, CO, and NV. To broaden understanding of work being done by the Consortium, Zoom presentations were given to the 2021 virtual Idaho AFS meeting and the National AFS FAS Section meeting at the North American. Lastly, a paper documenting simulated results of stocking YY Male Common Carp was finalized and published in North American Journal of Fisheries Management in March.

Acknowledgements 2021

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 Nat'l 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 R. Snow (ODWC), and TX team – C. Kittle and M. Matthews (TXPW). We again thank B. Gomelsky (Kentucky State University) for his continued advice on all aspects of C. Carp rearing.

Under the direction of the now retired R. Holm, the USFWS at Garrison Dam National FH has dedicated a staff member, B. Oldenburg, heading up the Walleye feminization trial. Their willingness to undertake the involved study design speaks volumes and we thank you. 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 Lake Trout ESD study 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, S. Unsworth, M. Campbell and many on the EFGL staff.

FY2021 Sex Marker Sample Collections:

We thank those that helped collect samples in the past year for eventual processing by the IDFG EFGL and realize many involved may remain anonymous beyond an agency name. Their contributions are no less appreciated, however.

Common Carp

ODWC: R. Snow and staff

PAF&B: D. Nihart, J. Detar and staff

TNWRA: J. Habera, E. Ganus and staff, Hart's Fish Market staff

A.E. Woods Hatchery, TX: C. Kittel and M. Matthews

WADFW: B. Baker and staff

Iowa DNR: M. Hawkins and B. Wallace

Lake Trout

IDFG: G. Schoby, K. Estep and Region 2 staff; J. Messner and Region 3M staff

ODFW: S. Clements and staff

Yellowstone National Park: P. Bigelow and staff

Northern Pike

WADFW: B. Baker and staff

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 chronicled in this report assisted on Walleye by T. Delomas. 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.

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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.

Bootsma, M. L., K. M. Gruenthal, and G. J. McKinney. 2020. A GT-seq panel for walleye (Sander vitreus) provides a generalized workflow for efficient development and implementation of amplicon panels in non-model organisms. bioRxiv. DOI:10.1101/2020.02.13.948331.

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.

Demska-Zakes, K., M, Luczynski, K. Dabrowski, M. Luczynski, and J. Krol. 2000. Masculinization of Northern Pike fry using the Steroid 11 β-Hydroxyandrostenedione. North American Journal of Aquaculture 62:294-599.

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

Dunham, R. A. 2011. Aquaculture and Fisheries Biotechnology Genetic Approaches. Chapter 11: Sex Reversal and Breeding. Cambridge: CABI Publishing. P. 128-135.

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.

Haffray, P., V. Petit, Y. Guiguen, E Quillett, P. Rault, and A. Fostier. 2009. Successful production of monosex female brook trout *Salvelinus fontinalis* using gynogenetic sex reversed males by a combination of methyltestosterone immersion and oral treatments. Aquaculture 290:47-52.

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

Hunter, G. A., I. I. Solar, I. J. Baker, and E. M. Donaldson. 1986. Feminization of Coho Salmon *Oncorhynchus kisutch* and Chinook Salmon *Oncorhynchus tshawytscha* by Immersion of alevins in a solution of Estradiol-17β.

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.

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.

Luczynski, M., K. Demska-Zakes, K. Dabrowski and M. Luczynski. 2003. Masculinization of gynogenetic Northern Pike juveniles using 17 α-Methyltestosterone. North American Journal of Aquaculture 65:255–259.

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.

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 iIntermountain 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., 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.

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

Steele, C., M. Hess, S. Narum and M. R. Campbell. 2019. Parentage-based tagging: Reviewing the implementation of a new tool for an old problem. Fisheries. DOI:10.1002/fsh.10260

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

Yano A., B. Nicol, E. Jouanno, E. Quillet, A. Fostier, R. Guyomard, and Y. Guiguen. 2013. The sexually dimorphic on the Y-chromosome gene (sdY) is a conserved male-specific Y-chromosome sequence in many salmonids. Evolutionary Applications. 6:486-96.

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

Workplan 2018-2021

Year One: July 1, 2018 to June 30, 2019

		Entity Involved
I.	**Ongoing species work**	
	a. Complete sex marker investigation(SM) (Lake Trout, Walleye, Common Carp)	EFGL
	b. Analyze fem/SM for a successful recipe (Lake Trout, Walleye, Common Carp)	FMS
	c. Evaluate Density-Dep Sex Change (Lake Trout, Brook Trout, Common Carp)	FMS/IDFG
	d. Finalize modeling study on LT	*IDFG-Region 1*
	e. Initiate INAD dialog with FDA if above work is successful for CC, WAE, LT	FMS/AADAP
	f. Growout of sex reversed fish-normal gonads? (Lake Trout, Walleye, Common Carp)	FMS/KS/IDFG
	g. Coordinate AADAP BK Trout INAD coverage for other states receiving YY Male eggs	FMS/AADAP/AZ, NM, WA, OR
	h. Provide technical guidance on field evaluations to WAFWA partners receiving Brook Trout eggs.	FMS/IDFG
II.	**New Species work**	
	a. Sex Marker Development NP & BRN	
	1. Field maturity data and clips (n = 3-5 populations)	WAFWA partners and FMS
	2. Sex Marker investigations	EFGL and *ADG&F for NP*
	b. Modeling study NP	*ADG&F*
	c. Identify NP and BRN "recipe" trial facilities	FMS
	d. Initiate NP sex reversal trial Spring 2019	FMS/WAFWA partners
III.	**Project communication**	
	a. Annual Progress Report (2018-2019)- June 30, 2019	FMS
	b. Annual WAFWA mtg update	FMS
	c. AFS or Aquaculture presentations (n = 2)	FMS

- EFGL = Eagle Fish Genetics Laboratory- Idaho Fish and Game
- IDFG = Idaho Fish and Game
- FMS = Fishery Management Solutions Inc. (Dan Schill and Liz Mamer)
- AADAP = Aquatic Animal Drug Approval Partnership- USFWS
- Note - Entities in *italics* are doing associated work outside of WAFWA funding proposal

WAFWA YY Consortium Exhibit A, Continued

Year Two: July 1, 2019 to June 30, 2020

I. Ongoing species work

a.	Complete sex marker investigations- BRN and NP	EFGL & AKF&G
b.	Evaluate results of NP sex reversal trials	FMS/EFGL
c.	Evaluate 2018-19 results- if successful, Identify WAFWA hatchery facility for housing YY Broodstocks for potential species (Lake Trout, Walleye, Brook Trout, Common Carp)	FMS/IDFG/WAFWA partners
d.	Solicit INAD Coverage for 2 new species (LT, WAE, NP or CC?)	FMS/IDFG/AADAP/WAFWA
e.	Begin Phase I of YY Male Broodstock (n = 2 species)	FMS/IDFG/WAFWA partners
f.	Initiate BRN sex reversal trials Fall 2019	WAFWA partners and FMS
g.	Maintain YY BK egg distribution network	FMS/AADAP
h.	Provide technical guidance on BK field evaluations	FMS/IDFG

II. New Species Work- None planned for this period

III. Project communication

a.	Annual Progress Report (2019-2020) - June 30, 2020	FMS
b.	Annual WAFWA mtg update	FMS
c.	AFS or Aquaculture presentations (n = 2)	FMS
d.	Publication of 2018 - 2019 results	FMS/EFGL/WAFWA partners

Note: Initiation of YY Broodstock Development (and selection of species) will be dependent on the success of key aspects of the prior year's work between July 1, 2018 and June 30, 2019.

WAFWA YY Consortium Exhibit A, Continued

Year Three: July 1, 2020 to June 30, 2021

I. Ongoing species work

a.	Maintain BK egg distribution network	FMS/AADAP/WAFWA partners
b.	Evaluate results of BRN sex reversal trials	FMS/EFGL
c.	Initiate Development of BRN YY Broodstock	FMS/WAFWA partners
d.	Ongoing oversight of YY Male Broodstocks n=3	FMS/IDFG/WAFWA partners
e.	Continue technical guidance on BK field evaluations	FMS/IDFG

II. New Species Work- None planned for this period

III. Project communication

a.	Annual Progress Report (2020-2021) - June 30, 2021	FMS
b.	Annual WAFWA mtg update	FMS
c.	AFS or Aquaculture presentations (n=2)	FMS
d.	Publication of 2018-2021 results	FMS/EFGL/WAFWA partners

Note: Initiation of new YY Broodstock Development and maintenance of existing ones will be dependent on the success of key aspects of the prior two year's work between July 1, 2018 and June 30, 2020.

Appendix B

Results of sex marker development efforts by the EFGL

(K. Coykendall, T. Delomas, M. Campbell)

B1 - Walleye

Over the performance period (FY21) we utilized a newer methodology, Pool-seq, which involves sequencing pools of individuals (Scholotterer et al 2014) and requires a reference genome. The tremendous advantage of this technology is that if can achieve remarkably high genome coverage (~50-100%) at lower costs than sequencing single individuals. The drawback is lower sequencing coverage per locus and so lower confidence in the SNP calls. We used a rough draft of a walleye genome provided to us by colleagues at University of Wisconsin Madison. The sample pools were 100 females and 100 males from Lake Pend Oreille in Idaho. From these results, we identified genomic positions that had SNPs where the allele proportion in males was near 0.5 and the proportion of the same allele in females was near 0.0 or 1.0, which indicates an XY, sex-linked inheritance pattern. Our data yielded approximately 65 genomic positions with this pattern. To date, we have designed primers and probes for 11 loci that fit the criteria. After optimization, nine of the assays were not concordant with phenotypic sex calls in assayed fish. Testing is still underway on the remaining two assays of this set. If they do not work, we can design more assays from the remaining 54 loci with the XY inheritance pattern. This work will be undertaken during FY22.

B2 - Common Carp

No laboratory work was done on samples for this species during FY21 but a placeholder in this FY2020 Appendix was thought useful for report consistency.

B3 – Lake Trout

I. At EFGL, we developed two different assays to determine genetic sex in lake trout. Previous work in several salmonid species have identified the sex determination on the Y gene (sdY). This small genomic region is present in males and absent in females. We developed a presence/absence assay using sequences of sdY from other salmonid species published by Yano et al 2013 as well as publically available DNA sequences from the sdY region of lake trout. We designed a forward and reverse primer that flank the sdY region and a probe that should anneal to the region between the primers in male fish. In female fish, the probe should not anneal because the sdY region is absent in females. The names of the forward and reverse primers and probe, and the DNA sequences for each are below.

Forward primer, CushSdyF: 5’-CCCTCATGGAGGGTGGAGT-3’

Reverse primer, CushSdyR: 5'-GCTTGGCTATGCCGTTCAG-3'

Probe, CushSdyP: 5'-GCTCTAGGGAGGAAGGCATC-3'

This assay was optimized in lake trout from Lake Pend Oreille in northern Idaho and has very high 100% accuracy in that population.

II. Additionally, we discovered a biallelic sex marker from a radseq library constructed with 10 females and ten males from Lake Pend Oreille, Idaho. This marker is a SNP that is homozygous for the same allele in all females and heterozygous in all males. We developed a two-probe assay where one probe anneals to the X-linked allele and the other probe anneals to the Y-linked probe. As with the sdY assay, this assay has very high 100% accuracy in the Lake Pend Oreille population.

III. During 2020-21, we ran our assays on 995 lake trout outside of Lake Pend Oreille. The seven sampling locations from lakes in Oregon, Idaho, Montana, and Wyoming as well as sample sizes are listed in Table B1. Initially, we ran our biallelic, probe-based assay on all fish. If there was a discrepancy between phenotypic sex calls and genotypic sex calls, we also ran our sdY presence/absence assay. For further confirmation of genotypes of the Yellowstone Lake samples, we ran our lake trout GTseq panel assay these samples. Both sex markers mentioned above have been incorporated into our panel. However, instead of relying on probe annealing, the assay results in sequence data to discriminate between alleles in the case of the biallelic marker or the presence/absence of sdY. Therefore, each sample was genotyped at 1) the biallelic locus or 2) both the biallelic and sdY locus or 3) both using the probe-based assays and the sequence-based GTseq assay. The results are listed in Table B1. Concordance between phenotypic and genotypic sex across all sample groups was 93%. Concordance within groups ranged from 83% to 100%. Higher discrepancies between phenotypic and genotypic calls were observed in 2020 in Stanley Lake and Yellowstone Lake. Of the 29 discrepancies in the Stanley Lake population, 18 were phenotypic females, but genotypic males, seven were phenotypic males, but genotypic females, and four were phenotypic males but genotyped as homozygous for the male allele. In all 26 instances of discordance in the 2020 Yellowstone Lake population, the phenotypic sex was male and the genotypic sex was female. In a single case, the two genotypic sex markers did not agree. This was not counted as a mismatch.

Table B1. Waters, state, sample date, N and percent accuracy for populations of Lake Trout involved in sex marker development, sampled 2017 - 2020.

Water	ST	Year	Genetic Label	N	Number	Correct	%
Flathead Lake	MT	2019	SnaFLHD19C	218	8	210	96.33%
Grace Fish	ID	2017	SnaGRAC17B	119		119	100.00%
Odell Lake	OR	2020	SnaODLL20C	26		26	100.00%
Payette Lake	ID	2020	SnaPAYL20C	189	7	182	96.30%
Stanley Lake	ID	2020	SnaSTLK20C	167	29	138	82.63%
Yellowstone Lake	WY	2019	SnaYELL19C	97		97	100.00%
Yellowstone Lake	WY	2020	SnaYELL20C	179	26	153	85.47%
Grand Total				995	69	926	93.06%