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.

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 second program year to enable attainment of those objectives. The 2018 - 2019 and 2019 - 2020 workplans (Appendix A) list tasks to be undertaken during the first two project years for attainment of program objectives. The pages below summarize results of efforts in regard to the 2019 - 2020 plan plus work conducted to address tasks ongoing from the prior year.

Overview and Methods

Sex Reversal Trials

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 the review of histological samples and summarization of results from sex reversal trials initiated in the preceding several years. This wrap-up work included trials on Lake Trout and Northern Pike begun during 2016 and 2019, respectively. In addition, a new sex reversal trial for a new species (Brown Trout) was initiated at two state hatchery facilities during Fall 2019.

Lake Trout

Very limited prior work on sex differentiation and estrogenic sex reversal existed for Lake Trout with generally poor results in both studies (Wenstrom 1975; Herman and Kincaid 1991). Despite severe mortality problems with fish fed E2 treated feed at first feeding due to unrelated rearing conditions, Wenstrom (1975) reported best feminization (80 %) with a truncated treatment at 12 mg/kg but with a sample size of only 10 fish. The author also reported beginning and ending sex differentiation times for control fish reared at 4 °C as observed by histological examinations, and suggested that the process of differentiation seems more a function of cumulative temperature units than size or age of fish.

In Winter 2016 a study design that maximized the number of possible treatment or "recipe" evaluations was undertaken at IDFG's Grace Fish Hatchery (GFH). As GFH rearing temps were much higher at 12.2 °C, we adjusted planned dates for trial fish to receive treatment feed on an accelerated CTU basis. While the above authors orally administered E2 in food, other salmonids have proven remarkably susceptible to feminization by immersion of alevins in a solution of E2 for two brief periods, one week apart (Hunter et al. 1986). Haffray et al. (2009) suggested that use of both immersion and feed treatment combined would produce superior feminization results in the closely related charr, the Brook Trout. Accordingly, 12 possible recipes and a control were devised for evaluation that included several possible egg immersion treatments alone, a range of feed-alone treatments, and several treatments that combined both options (Table 1). The majority of the treatments tested (10) involved the use of E2, while two of the combination treatments were conducted with Estrone (E1), the first metabolic derivative of E2 that is roughly 20 % as powerful an estrogenic substance. Two replicates were conducted for each treatment and control group.

The immersions, whether for standalone or combination treatments, were conducted for two hours on incubating eggs (N = 120) in Heath trays at half hatch as recommended for salmonids (Feist et

al. 1996). Immersions were conducted either once or three times at a weekly interval. Dose concentrations for immersions were either 200 or 400 µ/l for E2 and were fixed at 200 µg/L for E1. Fish from all treatment and control groups were spawned at Story Fish Hatchery in Wyoming on 11 October 2016. Eggs were transferred to GFH and hatched in Heath trays. Developing fry were ponded to 14 L round tanks for rearing on 9 January 2017.

At first feeding, 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. Treatments involving feed were all ran from first feeding for 97 d and ranged in hormone dose from 12 to 100 mg/kg for four E2 recipes and were either 100 or 200 mg/kg for immersion/feed combination E1 treatments. Treatment feed was topcoated using a hand-held sprayer (Schill et al. 2016a). On June 2017 all surviving fish from the treatment and control groups were PIT-tagged using 8 mm tags and moved to an outdoor raceway to rear communally.

Table 1. Sex reversal trial framework for Lake Trout conducted at the Grace Fish Hatchery (IDFG), treatment feeding conducted 20 January to 27 April 2017.

Trial Code	Treatment Type	Immersion Level	Dose µg/l	Period	Tx Feed Level	Dose mg/kg	Period (day)
A	E2, Immersion only	Low	200	2hr, 1x	-		
B	E2, Imm only	High	400	2hr, 1x	-		
C	E2, Imm only	Periodic low	200	2hrs/1x weekly till 1st feed (3x)	-		
D	E2, Imm only	Periodic High	400	2hrs/1x weekly till 1st feed (3x)	-		
E	E2, Feed only	-	-	-	Low	30	97
E.2	E2, Feed only	-	-	-	Lowest	12	97
F	E2, Feed only	-	-	-	Moderate	60	97
G	E2, Feed only	-	-	-	High	100	97
H	E2, Combo	High	400	2hr, 1x	Low	30	97
I	E2, Combo	Low	200	2hr, 1x	Moderate	60	97
J	E1, Combo	Low	200	2hr, 1x	Low	100	97
K	E1, Combo	Low	200	2hr, 1x	High	200	97
Z	Control	-	-	-	-	-	-

Several samples of excess untreated fish were sampled to ascertain if study fish had sexually developed adequately to ascertain phenotype readily *via* necropsy. On 24 September 2018, 661 days post hatch (DPH) 10 excess control group fish from the holding raceway were euthanized and necropsied.

Only two females and one male had clearly discernable gonadal development and data collection for the remaining fish was delayed. On April 16 2019 an additional control fish was sampled, and its gonads still appeared indistinct based on visual examination. On 20 April 2019, 10 additional surplus control surrogates were collected for histological preparation and were interpreted in July 2019. Based on these results, on 29 August 2019, 1000 DPH, the main sampling consisted of fish sacrifice via anesthetic overdose and was where phenotypic designation during necropsy occurred. Lengths, weights, and phenotype were recorded for each individual fish and a fin clip was retained for possible genetic evaluation based on summarized sex ratio results. Based on the observed phenotypic sex ratios, tissues from 20 control fish from this event were preserved and subsequently histologically examined.

Northern Pike

No prior attempts to feminize a male Northern Pike have been conducted but two published studies reported successful masculinization of female pike (Demska-Zakes et al. 2000; Luczynski et al. 2003). Based on the above study results, a pike sex reversal trial using E2 was begun in Iowa, assisted by Alan Johnson at the Rathbun Fish Culture Research Facility (RFCRF), Moravia, IA.

Approximately 5000 Northern Pike fry (5 DPH) were truck transported on 28 April 2020 from the Spirit Lake Hatchery, Spirit Lake, IA, to RFCRF and reared communally to allow for acclimatization to station and account for transport mortality. Three test groups consisting of 1000 control fish and two 1000 fish treatment groups were subsequently stocked and reared in indoor 830 L circular flow-through tanks.

On 8 May 2019, a day prior to treatment initiation at 14 DPH, fry were counted directly into the circular tanks. Beginning the following day study fish were fed feed ranging from 3 - 8 % body weight per day over the course of the study. Fish were initially fed Otohime and were switched to BioVita. E2 treatment group feed was topcoated with a 15 mg/kg feed Estradiol solution suspended in non-denatured ethanol (EtOH) using a hand-held sprayer (Schill et al. 2016a). Control feed was topcoated with pure EtOH. The treatment groups were fed E2 coated feed for 14 or 21 days, beginning at 24 mm (approx. 15 DPH) for the longer duration and at 28 mm (approx. 19 DPH) for the shorter duration treatment (Table 2).

At 57 DPH, 60 fish from each treatment group were measured for length, weight, with genetic clips preserved on Whatman sheets, and preserved as whole fish for later histological analysis. At this time (19 June 2020), it was realized that, due to a communication miscue, the fish had been exposed to 50 % less E2 in the trials than originally planned based on the above-mentioned masculinization studies. They had been treated at 15 mg/kg (Table 2) rather than 30 mg/kg as originally intended, for the respective durations based on prior MT studies. While not ideal, it was hoped the lower dose at the longer duration would still be effective, so we proceeded with sampling and PIT tagging. A total of 200 fish from each group were then PIT-tagged on 19 June 2019 to rear communally until final sampling.

On 3 October 2019, all remaining trial fish were killed via anesthetic overdose, length and weight collected, genetically sampled and gonad tissue collected for later histological analysis. At this time, gonadal tissue was weighed, visually examined, and phenotype recorded for each fish. Individual fish from each treatment group were identified as either male, female or intersex (presence of both oocytes and sperm cells).

Table 2. Northern Pike sex reversal trial undertaken at the Rathbun Fish Culture Research Center, April 2019.

Treatment Type	Dose	Size at initiation	Duration
E2	15mg/kg	24mm	21 days
	15mg/kg	28mm	14 days

Brown Trout

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

During November and December 2019 sex reversal trials using Estradiol (hereafter E2) on treatment groups of swim-up Brown Trout were initiated in hatcheries in Colorado and South Dakota. The first trial was conducted with George Schisler, State Fish Research Supervisor, Colorado Parks and Wildlife, and Brad Neuschwanger, Hatchery Manager, at the Colorado Fish Research Hatchery (COFRH), Bellvue, Colorado. In this trial, three test groups consisting of 1200 control fish (four replicates of 300) and two 1200 treatment groups (four replicates of 300 for each group) were stocked and reared indoors in 75 L flow-through aquaria. Study fish were fed dry pelleted feed (Bio-Oregon) for the course of the study. Treatment group feed was topcoated with 20 mg/kg feed E2 solution diluted with non-denatured ethanol (EtOH), using a hand-held sprayer (Schill et al. 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). A sister study of similar design (four replicates per treatment group) was started 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 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 3). At COFRH, eggs were hatched out in heath trays and fry counted into aquaria prior to swim-up. In South Dakota, eyed eggs were counted out and placed into 400 L flow-through circular tanks, where they then hatched out. However, on 27 February 2020 at 85 DPH, due to MFH facility operational needs, fish were relocated to 100 L flow-through circular tanks where, on 13 April 2020 at 129 DPH, they were culled to 200 fish per replicate. A sample for histological analysis of five random fish from each replicate was taken at this time.

Table 3. Sex reversal trial framework for Brown Trout receiving Estradiol (E2) via treated dry feed at two different facilities, initiated Winter 2019.

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

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

Common Carp

Based on the positive preliminary results of prior carp studies as reported by Schill and Mamer (2019), outside funding was sought to enlist additional Common Carp reproductive and aquaculture expertise for a 2020 study. See "Additional Funding" section below for a description of this successful grant solicitation effort. Funds were obtained and extensive planning was undertaken in February and March 2020 with Bill Shelton, Common Carp reproduction expert at the University of Oklahoma (Emeritus) as well as Kurt Kuklinski, Fisheries Research Supervisor, Oklahoma Dept. of Wildlife Conservation, stationed at the Oklahoma Fishery Research Laboratory, Oklahoma City, OK, and Carl Kittel, Hatchery Supervisor, and Mike Matthews, Hatchery Manager, Texas Parks and Wildlife Department, at the A.E. Woods Hatchery, San Marcos, TX. A duration and concentration design similar to that described above for the two-state Brown Trout trial was developed with Texas actually spawning test fish. Unfortunately, the COVID-19 pandemic subsequently forced shutdown of the effort in both states. A request was submitted to the USFWS to postpone the work as permitted under the contract and the work on this important invasive species will hopefully be re-initiated in 2021 with a completion date of 20 June 2022.

Sex Markers

Methods

To develop genetic sex tests or sex markers for the five species of initial interest to the WAFWA Consortium, the EFGL used existing Y-chromosome (sdY) DNA sequences available for salmonids or generated new DNA sequence data using Restriction site associated DNA sequencing (RADseq). For the RADseq work, mature adult fish of wild origin were collected by various personnel, killed via anesthetic overdose, necropsied and visually sexed. Fin tissues were only taken from fish with clearly identifiable gonads and were placed on numbered Whatman filter paper sheets for storage. DNA was subsequently extracted from the fin tissue of Brown Trout, Lake Trout, Common Carp and Walleye by IDFG's Eagle Fish Genetics Lab (EFGL) personnel and cut into fragments using specific restriction enzymes. Selected DNA fragments were then sequenced so that the exact order of nucleotides (i.e. A,C,T,G) was determined. These sequences were then compared between phenotypic males and phenotypic females to find specific single nucleotide polymorphisms (SNPs) specific to each sex. For more detailed example descriptions of the procedures used by EFGL personnel in conducting species-specific analyses see Appendix B1 - B4.

Background and Sample Collections

The objective of genetics work in FY2019 was initially to develop Y chromosome-linked markers that would permit the differentiation of XX and XY individuals and secondarily, to initiate

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

Walleye

During FY2019 IDFG's Eagle Fish Genetics Laboratory staff made several concerted efforts to develop a Walleye sex marker but the work did not yield useful markers (Schill and Mamer 2019; EFGL - Appendix B1). Additional efforts in FY2020 are described below and in Appendix (B1). Large samples from two additional wild populations in Colorado and Wyoming were recently secured (Table 4) to buttress a new marker development approach.

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 (Mathew Campbell, EFGL, personal communication). 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). An effort to fund this follow-up effort was secured via the MSCGP Grant program discussed above but this work was subsequently cancelled due to COVID-19. This work will be resumed and reported on fully in spring 2022 assuming pandemic issues resolve.

Lake Trout

In 2019, 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). This marker (CushSdy) should assist in the future development of an YY Broodstock In addition, a candidate bi-allelic sex marker was identified (Sna_433923_27) through RADseq that successfully differentiated XX and XY individuals. In FY2020, our work objective was to continue optimization of this marker and test its accuracy on samples collected from five Lake Trout populations (Table 4).

Brown Trout

In 2019, the EFGL developed an sdY presence/absence marker from sdY sequences on Genbank and demonstrated its accuracy by screening 53 phenotypic male and 42 phenotypic female Brown Trout from the South Fork Snake River (Mamer and Schill 2019, EFGL- Appendix B4). This marker (BrownT_Sex) should assist in the future development of an YY Broodstock. In addition, a candidate

bi-allelic sex marker was identified (Stru9767_37_15) through RADseq that successfully differentiated XY and YY individuals. In FY2020, our objective was to continue optimization of this marker and test its' accuracy on samples from 5 Brown Trout populations (Table 4).

Northern Pike

As noted in the workplan (Appendix A) the Alaska Department of Fish and Game (AKFG) volunteered to take the lead on sex marker development for Northern Pike. AKFG secured the requisite FY2020 funding for development of RAD sequencing technology at their Genetics Lab. Sequencing results to date will be reported below.

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

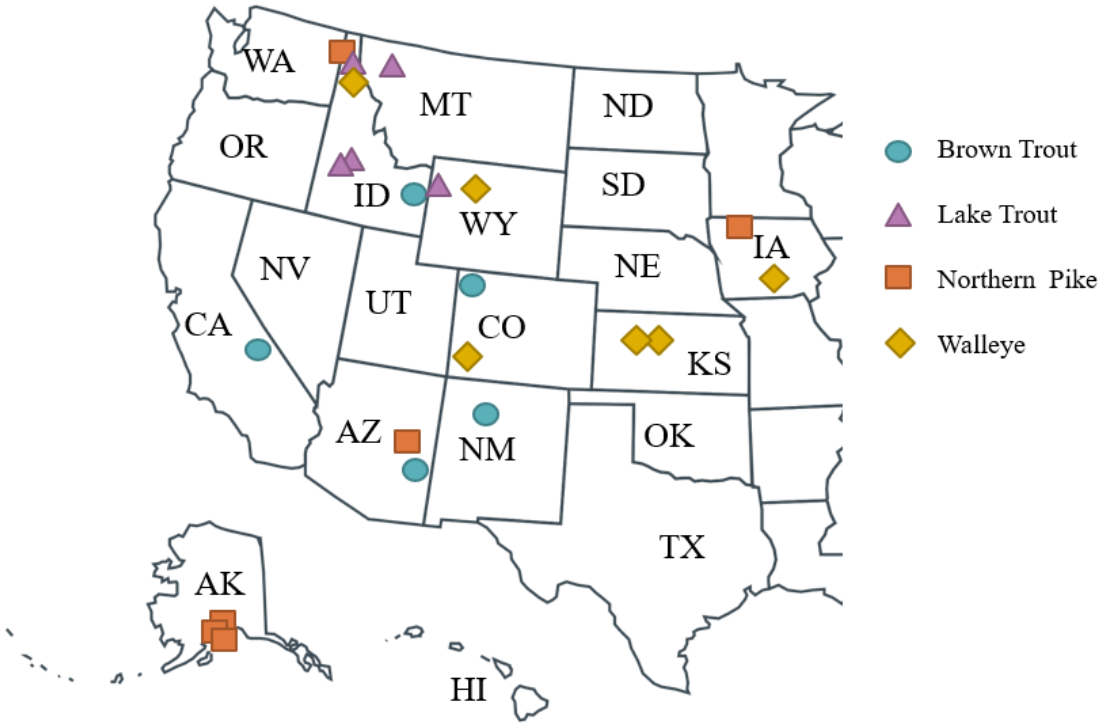


Table 4. Waters, state, sampling date and N's for individual populations of four fish species involved in sex marker development, 2018-2020.

Species	Water	ST	Collection Date	n
Brown Trout	WF Black River	AZ	Apr-18	198
Brown Trout	Owens River	CA	Nov-19	193
Brown Trout	Yampa River	CO	Sep-19	106
Brown Trout	SF Snake River	ID	Oct-18	200
Brown Trout	SF Snake River	ID	Oct-18	106
Brown Trout	Rio Cebolla	NM	Sep-19	146
Lake Trout	Payette Lake	ID	Spring 2020	21
Lake Trout	Stanley Lake	ID	Spring 2020	149
Lake Trout	Lake Pend Oreille	ID	Spring 2008 & 2017	167
Lake Trout	Flathead Lake	MT	Dec-19	218
Lake Trout	Yellowstone Lake	WY	Oct-19	97
Northern Pike	Alexander Lake	AK	May-18	96
Northern Pike	Threemile Lake	AK	Jun-18	96
Northern Pike	Tote Road Lake	AK	Summer/Fall 2018	96
Northern Pike	Rainbow Lake	AZ	May-19	87
Northern Pike	Big Spirit Lake	IA	Apr-19	186
Northern Pike	Roosevelt Lake	WA	Jan-20	107
Walleye	Narraguinnep Reservoir	CO	Mar-19	147
Walleye	Narraguinnep Reservoir	CO	Mar-20	89
Walleye	Rathbun Lake	IA	Apr-17	100
Walleye	Lake Pend Oreille	ID	Spring 2018	281
Walleye	Wilson Reservoir	KS	Oct-17	27
Walleye	Cedar Bluff Reservoir	KS	Oct-17	20
Walleye	Buffalo Bill Reservoir	WY	Jun-20	150

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) although 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 should be thought of as a possible density-related change in *phenotypic* sex. In this vein, Lake Superior Lake Herring (Bowen et al. 1991) have been suggested as a candidate regarding DDSC capability although this modeling study provided little empirical or genetic evidence for their assertion. 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 especially 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 5). 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 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, 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.

There was initially high mortality, particularly with the higher density groups and fish in the highest groups appeared to approach the upper limit of pot carrying capacity in late September. 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. Fish sampling was delayed by COVID-19 but occurred on 22 June 2020. Data collected included total lengths, weights and observed phenotypic sex. Gonads of a subsample 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 (Schill and Mamer 2019).

Table 5. Environmental Sex Determination Trial framework for Lake Trout initiated January 2018at the Grace Fish Hatchery, Grace, Idaho.

Pot #s	Initial Starting Density
1-9	5
10-18	10
19-20	20
21-22	50
23-24	100
Total	475

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 three years. Based on preliminary data analysis, roughly 75 % of the population has beenremoved 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. The remaining results in this report are presented under separate topical headings.

Walleye

Sex Reversal Trials

Schill and Mamer (2019) previously reported two successful E2 treatment protocols for feminizing walleye with 100 % of fish in both the 84 and 100 d treatment groups histologically identified as female. Thus, assuming the treatment groups contained males and females, both treatment regimes appear effective at generating sex reversed neofemales though the exact proportion is unknown due to difficulties in obtaining a working sex marker (see below).

Sex Marker Development

During the past year IDFG's Eagle Fish Genetics Laboratory staff made new efforts to develop a Walleye sex marker but, like the prior year, the work has not yielded useful results. Analyses to find additional candidate markers to test are currently underway. For additional explanation on 2020 results

see Appendix B1. An alternative approach for developing a YY Male broodstock without using a sex marker would involve a breeding program that tracks adult crosses and offspring sex ratios *via* progeny testing. These types of approaches have been used to produce monosex fish populations since the late 1970s (reviewed in Dunham 2011). Mair et al (1997) describes the process for producing YY Tilapia in this manner but their approach took five generations. Reducing the generations required without a sex marker would require a Parentage Based Tagging (PBT) approach where adult broodstock would be genetically sampled at the time of spawning and offspring would be tracked using genetic parentage methodologies (Steele et al. 2019). A SNP panel has already been developed for Walleye and its accuracy for parentage analyses has been demonstrated (Bootsma et al. 2020). Given the urgent need for a YY Male broodstock in Idaho and elsewhere due to increasing illegal angler spread of invasive Walleye, we recommend that a YY breeding approach, utilizing PBT, be pursued in the upcoming year concurrently with ongoing work on the sex marker. Genetic sub-samples of current Walleye broodstock being spawned at those hatchery facilities being considered for possible development of a YY Male broodstock should be sampled next spring.

Lake Trout

Sex Reversal Trial 2016

Overall LKT 2016 results – sampled at 1000 DPH in August 2019

On 29 August 2019 at 1000 DPH 618 Lake Trout were examined visually *via* necropsy for phenotype. Unfortunately none of the 12 treatment protocols tested skewed the sex ratio towards females. The proportion female in treatment groups ranged from 40.7 to 63.0 % but all confidence intervals contained 50 % and were thus not significantly different from a 50:50 sex ratio. Particularly disappointing was a lack of better result for the combination treatments, an approach which had sought to combine the occasionally strong feminization results with early immersion for some salmonids with the more typical feed method of delivering sex hormones. Paradoxically, the only skewed sex ratio was for the control group where 69.8 % of 53 fish were females, a statistically significant difference (Table 6). Because of this anomalous finding, we histologically examined 20 controls. All of these fish were either male or female with normal appearing gonads and no incidence of intersex observed.

Table 6. Percent phenotype ascertained by visual gonad observation of necropsied fish (1000 DPH) of Lake Trout for 12 E2 treatment regimens, Grace Fish Hatchery, Idaho, 28 August 2019.

Name	n	% F	95% Confidence Interval
Immersion Low	49	42.9 %	28.0 - 58.0
Immersion High	51	49.0 %	34.3 - 63.7
Periodic Low	54	40.7 %	26.7 - 54.8
Periodic High	56	50.0 %	36.0 -6 4.0
Feed only v Low	27	59.3 %	39.0 -80.0
Feed only Low	27	63.0 %	43.0 - 83.0
Feed only Mod	56	50.0 %	36.0 - 64.0
Feed only High	45	42.2 %	27.0 - 58.0
Imm High, Feed Low	54	50.0 %	36.0 - 64.3
Imm Low, Feed Mod	54	46.3 %	32.1 - 61.0
Imm Low, Feed Low	53	54.7 %	40.3 - 69.1
Imm, Low, Feed High	39	48.7 %	31.8 - 66.0
Control	53	69.8 %	56.5 - 83.1
Grand Total	618	50.6 %	

Results of this sex reversal trial were perplexing with several possibilities for the lack of success despite the considerable number of treatments examined. To begin, water temperature at the GFH (mean = 12.2 °C) was much higher than those of the prior two E2 sex reversal trials that had been conducted on Lake Trout at 7 °C (Herman and Kincaid 1991) and 4 °C (Wenstrom 1975). We converted Wenstrom's treatment periods into CTU treatment times, converted the exposure dates based on the same CTU's and ran tests during that period accordingly. That author had obtained reasonable results for 12 mg/kg E2 (80 % F) but an N of only 10 trial fish. Given these feeble but positive results, we tried this feed treatment regimen plus a number at higher E2 feed concentrations (Table 1). However, we may have erred by attempting to directly convert his treatment periods to GFH based on the thermal regime differences. Given the general lack of overall success by the prior two Lake Trout studies, both conducted at much lower temperature regimes, we ran a large variety of treatment groups including feed, immersion, and combinations. However, we likely erred in not testing later exposure periods. Based on our inability to even nudge sex ratios, in the future, a smaller number of treatment concentration options with a far stronger temporal component is recommended including later initiation of drug exposure (perhaps at 120 days after first feeding) and a series of later treatment ending dates.

Sex Marker Development

As noted above, with the successful FY2019 development of a sex marker for a single population in Idaho (Lake Pend Oreille) project focus in FY2020 was geographic expansion of population samples to broaden sex marker utility. Samples were collected from four additional populations with a total N of 652 (Table 4). However, additional samples will be added to the existing Yellowstone Lake and Payette Lake samples during the upcoming field season. After acquisition of these samples, a complete analysis will be done and sex marker work for this species will be finalized in the FY2021 report. In addition, the EFGL will continue efforts on development of a bi-allelic sex marker for Lake Trout that will be able to differentiate XY males from YY individuals to aid in future broodstock development and field evaluations.

Northern Pike

Sex Reversal Trial

Results of the sex reversal trial conducted at the Rathbun Hatchery in Iowa indicated that we failed to sex reverse Northern Pike in either of the two treatment groups. The sex ratios of the 14 and 21 d treatments were both virtually 50:50 with 50.4 and 51.4 % females, respectively (Table 7). Given the above-noted miscommunication, whereby only half the planned concentration of drug was administered, this result was not surprising. However, it had been hoped that the lower treatment would still be effective as the previous studies on which the planned dosage had been based involved Methyltestosterone, and it was thought possible that a different sensitivity threshold to the female sex hormone might have existed for Northern Pike. A small proportion of fish in both E2 treatment groups (0.9 - 2.4 %) exhibited intersex gonads as opposed to none in control fish so there was some indication of very light treatment effect. In addition, mean length comparison by sex across the study groups showed minimal growth differences (Table 7). However, a comparison of mean weights for males suggested the 21 d treatment dose might have slightly suppressed growth. Such an occurrence is not unexpected although even for fully sex reversed individuals, growth of feminized fish typically catches up to untreated fish as maturity approaches (Schill et al. 2016a). We observed an anomalous sex ratio biased towards females in the control group. Given the large control group sample size (160 individuals) the observed ratio of females was strongly skewed and confidence limits did not overlap 50 % (Table 7). Like the Lake Trout control result reported above, there is no ready explanation for this observation. Taken collectively our results suggest a repeat of this trial at the E2 dose concentrations originally planned (30 mg/kg). If a facility can be found that would permit either replication of the treatment and control groups and/or examination of at least one higher dose would be desirable. Such was not feasible

at the Rathbun Hatchery but given the fact that this state is not even a part of WAFWA, we were particularly grateful for their willingness to participate.

Table 7. Growth measurements and percent phenotype of Northern Pike via visual observation of necropsied fish (DPH) at Rathbun Fish Health Research Center in Iowa, sampled 3 October 2019.

Phenotypic Sex by Trial	n	Average Length (mm)	Average Weight (g)	% Phenotype (95% CI)
Control				
F	120	243.8	93.8	75.0 (67.9 - 82.0)
M	40	247.0	96.1	25.0 (18.0 - 32.0)
E2-14				
F	63	243.5	91.2	50.4 (41.2 - 60.0)
IS	3	255.3	107.9	2.4 (0 - 5.4)
M	59	249.1	97.4	47.2 (38.0 - 56.3)
E2-21				
F	55	241.6	86.0	51.4 (41.5 - 61.3)
IS	1	222.0	61.0	0.9 (-1.3 - 3.2)
M	51	246.7	92.9	47.7 (37.7 - 58.0)
Grand Total	392			

Sex Marker Development

AKFG genetics staff reported building a genome scaffold, and identifying regions with high sex associations (Chris Habicht, AKFG, personal communication). They also noted that the highest signal of sex association is located at the end of one linkage group (chromosome), but there were also other locations with elevated associated scattered over a handful of chromosomes. Their staff suggested this may be an indication that the sex-determining gene is jumping across chromosomes which may explain why other researchers have had difficulty in identifying markers that consistently correlate with sex across their circumpolar range (Dan Prince, AKFG, personal communication). Unfortunately, severe budget cuts across all of AK state government has since precluded additional work and project personal are now searching for additional outside funding opportunities to complete sex marker work. In the meantime, additional samples were collected across additional U.S. states for eventual incorporation into a sex marker (Table 4). If AKFG funding shortfalls continue, some of the pike sex marker work may need to eventually be picked up by the EFGL.

Brown Trout

Sex Reversal Trial

Based on the Walleye and Northern Pike sex reversal work in IA and KS, undertaking trials at two facilities allows for more recipe testing without overly burdening a given facility and also provides a measure of safety in regard to unforeseen aquaculture hazards or miscues. The basic trial design involves a test of E2 treatment duration in CO (30 vs 60 days) and a test of dosage (20 vs 30 mg/kg) in SD. There is an overlapping treatment at both facilities (20 mg/kg for 60 days starting at first feeding), the best guess option based on prior salmonid studies including the successful Brook Trout recipe of Schill et al. (2016a). Preliminary CO trial results indicate that both mean weight and length of the 30 d duration trials closely matched those of controls (Table 8) while growth of the 60 d treatment fish was slightly depressed. The 60 d result is not necessarily cause for concern and may in fact be a sign of proper or optimal treatment levels as E2 sex reversed fish, or even exposed genetic females, often experience reduce initial growth. Preliminary SD results show a slightly different response with length and weight of both treatment groups experiencing slightly less growth than the control group (Table 9). All fish measured in these preliminary samples were subsequently preserved in formalin for subsequent histology. Sampling of study fish to evaluate the efficacy of sex reversal recipes (sex ratios of treatment groups relative to controls) will be conducted at both hatcheries later in 2020 while smaller subsamples of trial fish from each study group will be retained for growout to full maturity.

Table 8. Preliminary length and weight results 138 DPH for a subsample of Brown Trout following 30- or 60-day E2 exposure versus controls, Colorado Fish Research Hatchery, CO, 24 March 2020.

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

Table 9. Preliminary length and weight results 128 DPH for a subsample of Brown Trout following 20- or 30 mg/kg E2 exposure for 60 d versus controls, McNenny State Fish Hatchery, SD, 12 April 2020.

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

Sex Marker Development

This year EFGL's work on Brown Trout was chiefly an effort to test the accuracy of the prior year's sex marker assay developed from a single population across a broader geographic group of WAFWA states (Figure 1). Details of the work including methods and more specific results are presented by EFGL staff in Appendix B4. For all five populations combined, the presence/absence marker proved quite accurate with a 96% concordance rate between predicted sex and observed phenotype. Concordance ranged from 95 to 100% for four populations in Idaho, New Mexico, California and Arizona. On the Yampa River in Colorado, concordance was estimated to be 80% though the veracity of this estimate is limited by sample size (see details in Appendix B4). Additional Yampa River samples would be desirable before concluding that the existing sex marker is measurably less accurate there than for the other populations. The sex marker easily appears accurate enough across a broad sweep of WAFWA states to permit straight-forward development of YY Male broodstocks for this species in most localities. Even in populations where the sex marker-phenotype is not 100 % concordant, we would only spawn concordant fish and subsequently would expect that the marker would correctly identify genetic sex for progeny from these crosses (M. Campbell, EFGL, personal communication).

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

The EFGL staff evaluated two potential biallelic SNP candidates for Brown Trout over the past year (see Appendix B-4 for details). In short, neither candidate was successful. However, the lab identified four additional candidate biallelic markers which will be evaluated during FY2021.

Density Dependent Sex Change

Lake Trout

This summary reports on progress made on the 2017 ESD trial initiated in Fall 2017. Table 10 below depicts numbers of Lake Trout rearing in individual pots at the start of the trial on 17 January 2018 until 2 October 2018 when they were PIT-tagged and transferred to a common garden raceway. As noted above, these fish were recently sampled on 22 - 23 June 2020 and resulting data yet to be analyzed,

therefore the results documenting genotype-phenotype comparisons will be reported in the 2021 annual report.

Table 10. Number of fish per pot trend for individual pots containing Lake Trout reared in controlled density environments.

	Date Inventoried				
Pot #	1/17/2018	3/20/2018	7/11/2018	8/27/2018	10/2/2018
1	5	3	2	2	1
2	5	3	2	2	2
3	5	2	1	1	1
4	5	2	2	2	2
5	5	3	3	3	2
6	5	3	3	2	2
7	5	2	2	2	2
8	5	5	4	4	5
9	5	1	1	1	1
10	10	5	4	4	4
11	10	6	6	6	6
12	10	3	3	3	3
13	10	4	4	3	2
14	10	3	3	3	3
15	10	4	4	4	4
16	10	4	3	3	3
17	10	4	4	4	3
18	10	5	4	3	3
19	20	12	11	11	10
20	20	5	5	5	5
21	50	6	6	6	5
22	50	25	20	19	17
23	100	49	32	31	29
24	100	42	27	26	25
Total n	475	201	156	150	140

Brook Trout

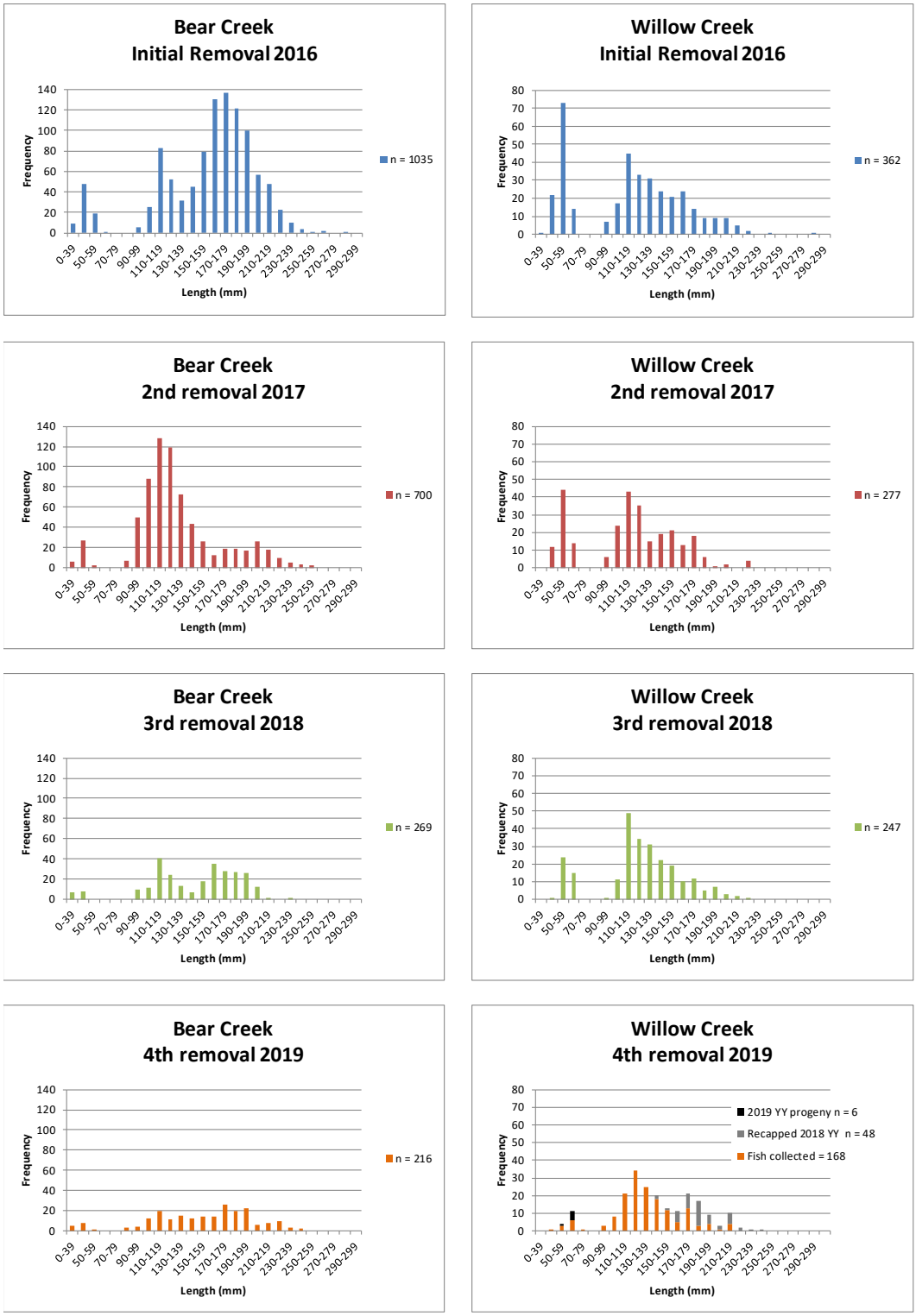
To date a total of 2673 wild Brook Trout in the two study streams have been visually sexed for phenotype and genetically sexed using a sex marker. Of the 1180 and 381 fish examined in 2016 and 2018, respectively, no discordance between genotype and phenotype was detected. However, nine mis-matches originally 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 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 though we doubt it to be a case of phenotypic sex reversal given all results before and since as no phenotype-genotype mismatches were observed in 2018 or 2019. Results from these latter two years when Brook Trout abundance in both streams was markedly lower than previously 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.

The intent of this study was and is to keep a watchful eye out for potential sex change as two isolated Brook Trout populations are being strongly reduced by electrofishing suppression and now YY Male introduction. A summary of length-frequencies for all four years of the study suggest that considerable population suppression, particularly for YOY fish has indeed been attained (Figure 2). Further, the declining sample sizes by year reported in Table 11 indicate that fish of adult size are also declining at a rapid rate. A total of 173 YY Male fish were stocked for the first time into Willow Creek in 2018. The length frequency for Willow Creek in 2019 (Figure 2) depicts the 47 stocked YY Males collected in the stream a year after that stocking (comprising 21.3 % 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. Based on predictive modeling (Schill et al. 2017), both populations are expected to continue marked decline. The primary aim of this study is to continue examining individual fish for genotype-phenotype mismatches, (and hence Density-Dependent Sex Change) as both populations continue to approach total collapse and hopefully become fully eradicated.

Table 11. Phenotype and Genotype for 2673 Brook Trout collected from two Idaho isolated streams, Bear Creek and Willow Creek, during the ESD trial, 2016-2019.

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				193
		F	110	0	
		M	0	83	
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	
Grand Total					2673

Figure 2. Frequency of size of Brook Trout removed over four years from two Idaho streams, 2016-2019. Willow Creek was stocked with YY fish in July 2018. Remaining YY Males and their progeny observed in July 2019 are depicted.



Coordination of INAD Coverage

Much time was spent during the report period coordinating various aspects of the YY Male Brook Trout program. This included working with staff from the Aquatic Animal Drug Approval Partnership (ADAAP) on various coordination aspects of INAD coverage with the Food and Drug Administration. Two update meetings (one in person and one via phone conference) were held with FDA and AADAP personnel. Preparatory phone conference strategy sessions with AADAP staff were planned and held before both of the formal FDA interactions. Much dialog was made in soliciting a private company (Novaeel) to serve as either the Estradiol drug sponsor (in the case of the Brook Trout INAD) or as an index holder. While no formal commitments from Novaeel have been garnered to date, considerable energy was expended enlisting their assistance in this regard. They are extremely interested in partnering with WAFWA, AADAP and others on a nationwide scale in moving the YY Male ball forward, both from a treated fish food sales perspective and possibly a research program. A key accomplishment in the drug approval arena was this project's Coordinator (Schill) raising the issue of drug Indexing with the FDA at the AADAP annual meeting in Bozeman in July 2019. An intense discussion among many parties ensued, resulting in one FL participant penning a two-page essay in the September issue of World Aquaculture. This essay, along with the above mentioned AADAP meeting dialog with FDA supervisory personnel has resulted in renewed interest in the FDA regarding Indexing as a pathway for drug approval. A subsequent meeting was held at the 2020 Aquaculture America meeting with that individual and AADAP personnel. Lastly, there was continued involvement of staff regarding distribution of YY Brook Trout eggs to the other receiving states and the USFWS in Washington.

YY Brook Trout Technical Team

The intent of team formation was to assist the other YY Brook Trout egg receiving entities in collectively planning their own research and monitoring activities. A total of 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. Two GoToMeeting conference calls were held during FY2020 on 2 July and 5 November 2019. General results of both sessions were quite productive and personnel from a total of 5 states participated. Bill Baker gave an in-depth presentation on WA YY stocking approach in two streams in July while NMSU student, Ben Armstrong presented summary results from his 2019 fieldwork in November. Other participants provided updates on their ongoing YY BK work in both sessions. During 2019, in concert with other program activities, the YY Male project Coordinator (Schill) was able to travel to NM, OR, and WA during the

year to meet and field tour the stocking locations or rearing hatcheries for YY Males that are being shared with states as well staff as the USFWS at Abernathy. The expectation is that the next Tech Team meeting will be held late this fall or early winter so the five involved research entities can compare notes on the field season results.

Investigate Additional Funding Opportunities

This project sought and obtained a Multi-State Conservation Grant administered by the U.S. Fish and Wildlife Service (USFWS) for FY 2020. Entitled "Development of YY Male Broodstocks for Eradication of Invasive Common Carp Populations", a proposal was submitted that featured two main objectives: 1) to perfect a sex reversal protocol for feminizing male Common Carp and 2) expand the geographic utility of the Common Carp sex marker developed and reported upon by this project in the prior year. The proposal was developed by the present authors and submitted to MSCGP by WAFWA in late July 2019 and $75,704 in funds were awarded with a performance period of Jan 1, 2020 to June 30, 2021.

As a valuable partner on the YY BK work being done by WDFW within the Pend Oreille River watershed, a second-year of funding was sought and secured from the Kalispell Tribe for FY 2020 financial support. Additional inquiries from several tribes in regard to YY Brook Trout involvement were fielded, though neither of these entities were working in direct partnership with a WAFWA member and decisions on how to handle such interest needs discussed among the participating Fish Chiefs.

Identify YY Male Broodstock Partners

Extensive communication efforts at multiple meeting venues and via conference calls were made during the contract period with upper level administrators of the USFWS regarding possible involvement with YY broodstock development. Project presentations regarding YY Male Brook Trout were made to USFWS staff in Boise ID and Portland OR. The YY Male project Coordinator (Schill) also traveled to the Abernathy Fish Technology Center in Washington to meet with staff currently raising YY Brook Trout for a partnership project with WDFW and subsequently presented a webinar in Vancouver virtually attended by a variety of Service offices nationwide. Ongoing dialog with USFWS Fisheries and Aquatic Conservation staff, along with a June 2020 online meeting held in concert with Iowa Department of Natural Resources staff on Walleye larvaculture techniques resulted in the Service committing to development of a YY Male broodstock for Walleye at Garrison National Fish Hatchery in North Dakota. In addition, in Spring 2020, the USFWS committed to participation in a Brown Trout sex reversal trial by

growing out FY2019 study fish initially treated and reared at the SDGFP McNenny Fish Hatchery. A virtual meeting was held with staff at the nearby D.C. Booth Hatchery on 26 June 2020 regarding growout of the study fish and additional discussion focused on subsequent development of a YY Brown Trout broodstock at the Booth facility. No decisions on that have been made as yet but we are hopeful regarding their eventual participation if the facility can be modified for broodstock development. A focused effort will be made to identify a willing partner to expand the Brook Trout program to an additional state facility during the upcoming year as we currently have (literally) all our YY Males of that species in the same basket. We also continue to look for additional funding sources and partners to expand fiscal support for the program.

Project Communication

An annual progress report for FY2020 was completed on schedule and YY Male Consortium project results were presented at the Kansas WAFWA Chief's meeting during July 2019. To develop interest in YY Males, numerous communication presentations on YY Male fish were made, including four virtual and one in person presentation to USFWS staff, platform presentations at the East Coast Wild Trout Conference, the annual SCCS meeting in southwest OR, the annual AADAP drug approval workshop, the Aquaculture America Meeting and the Idaho AFS meeting. A virtual webinar on YY Males was presented to the Northwest Power Council-funded ISAB group. The obvious benefit of attendance at such regional, national, and virtual meetings is the opportunity to meet and open dialogs with administrators from various agencies and entities that might be willing to assist fiscally or with manpower or hatchery facilities in regard to future YY Male program development. A meeting of the DAWG, the Drug Approval Working Group, was also attended at the Hawaii Aquaculture America Conference. This annual gathering is attended by key ADAAP staff and regulatory members of the FDA involved in the current YY Brook Trout INAD and is thus an important annual event for this project. Lastly, a paper documenting simulated results of stocking YY Male Common Carp was submitted to the North American Journal of Fisheries Management in March. The paper is just back from review and all three reviewers and the associate editor were quite positive with minor changes and responses requested. We are thus confident that the paper will be in press by the end of July and it should garner considerable interest in YY Male technology for C. carp, considered one of the 10 most destructive of any invasive species (plant or animal) worldwide.

Acknowledgements

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

For the Sex Reversal & ESD Trials:

- We thank the staff at the Grace Hatchery led by M. Gallagher, E. Pankau and currently W. Fowler, for their efforts in conducting the Lake Trout Sex Reversal and ESD trials.
- Northern Pike eggs and culturing advice were provided by K. Hawkins at Spirit Lake Hatchery, (IDNR) and cultured by A. Johnson and staff at Rathbun FCRC.
- Brook Trout ESD field work required massive manpower contributions and enthusiasm by B. Gamett (USFS) and staff , Matt Campbell and EFGL staff, and able assistance in both the field and laboratory from B. Schill.
- Common Carp expertise was generously and copiously provided by B. Gomelsky (Kentucky State University), Bill Shelton (University of Oklahoma) , Kurt Kuklinski (ODWC); Carl Kittle and Mike Matthews (TXPW), T. Delomas (IDFG).

Sex Marker Sample Collections:

For a sex marker to be robustly investigated requires physical samples to be collected across the geographical range occupied by the species in question. That being said, we thank those that submitted samples to the IDFG EFGL for processing and realize many remain anonymous beyond an agency name. Their contributions are no less appreciated, however.

- AZGF: B. Giordano, V. Corbett, M. Lopez
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- NMGF: Joanna Hatt; Bran Bakevich
- IDNR staff
- KDWPT staff
- USGS: J. Reid
- WADFW: B. Baker and staff
- Yellowstone National Park: P. Bigelow and staff
- A.E. Woods Hatchery, TX
- WG&F: J. Burkhardt

Sex Marker Development:

Without the incredible 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. Several lab geneticists (in chronological hire order) had the front-line responsibility of tackling the four sex marker efforts chronicled in this report including K. Coykendall, and T. Delomas. They were assisted by a long list of lab technicians, particularly D. Eardley who does nearly all the bench work on the sex markers. 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.

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Appendix A

WAFWA YY Consortium

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

Entity abbreviations

- 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 and are shown here for clarity

WAFWA YY Consortium Exhibit A, Continued

July 1, 2019 to June 30, 2020

I.	**Ongoing species work**		
	a.	Complete sex marker investigations- BRN and NP	EFGL & AKFG
	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.

Appendix B – Results of sex marker development efforts by the EFGL

B1 –Walleye (K.Coykendall, T. Delomas, M. Campbell)

Over the performance period we completed six RADseq libraries; three using the restriction enzyme PstI and three using the restriction enzyme BamHI. Sequence data produced from these libraries was initially analyzed "de novo" (lacking a reference genome). We later had access to an unpublished, draft walleye genome from colleagues at the University of Wisconsin, Madison. This allowed us to reanalyze our data using this reference genome. Having a reference genome allows for building loci with aligned reads, which can lead to better SNP calls. Using this data we identified 9 candidate SNPs and ordered assays for testing. Of these 9, we have tested 6 and none were diagnostic between the sexes when run on a larger sample size. Testing is underway on the remaining five candidate SNP markers. We are also doing a second round of SNP identification and assay design using different bioinformatics parameters and criteria. If all of this additional testing, fails to yield a diagnostic sex marker, we plan to utilize a newer methodology, Pool-seq, that involves sequencing pools of individuals (Scholotterer et al 2014). Pool-seq requires a reference genome, which as mentioned previously is now available for walleye. The tremendous advantage of this technology is that if can achieve remarkably high genome coverage (~50-100%) at lower costs than sequencing single individuals.

B2 – Common Carp

No work was done on this species during FY20 due to COVID-19 restrictions but a placeholder in this FY2020 Appendix was thought useful for report consistency.

B3 - Lake Trout

No work was done on this species by the EFGL during the report period. As noted in the methods section above, a large number of additional population samples were collected in FY20 and will be completely analyzed during the coming year.

B4 - Brown Trout (K. Coykendall, T. Delomas)

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

1.) We designed an assay within the sex determination gene on the Y chromosome (sdY), previously identified in salmonids (Yano et al. 2013). In its most complete form, this assay will quantitatively discriminate between females (XX; no amplification) and males (XY, YY; amplification). Details are below.

2.) We completed a restriction site-associated DNA sequencing (RADseq) study to identify candidate biallelic markers to discriminate between XX, XY, and YY individuals future YY male experiments. We discuss these two approaches in more detail below.

Sex marker on the Y chromosome

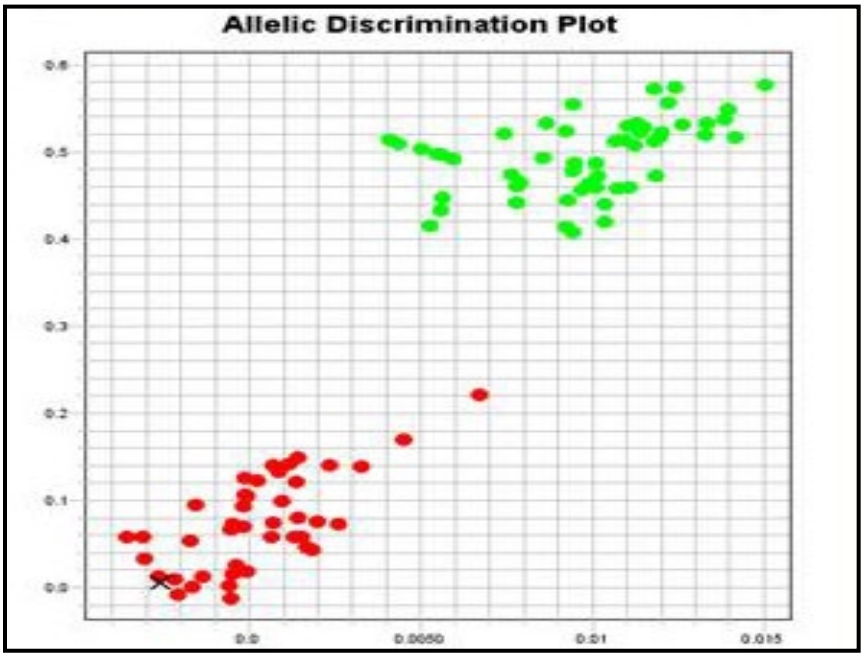
The sex determination gene, sexually dimorphic on the Y chromosome (sdY) has been found in a number of salmonids, including Brown Trout, *Salmo trutta* (Yano et al. 2013). To develop a genetic marker that interrogates the sdY gene in brown trout, we aligned sequences of sdY from Genbank and designed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

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

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

The graph below shows the assay run on 42 phenotypic females (red) and 53 phenotypic males (green) from the South Fork Snake River, Idaho.

Appendix B4 – Figure 1



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

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

We used values of ΔRn, defined as the fluorescent signal from the FAM probe divided by the signal from the passive, reporter probe. Since there was no reporter probe present in our assay, the ΔRn value is not truly normalized. We used the supervised machine learning algorithm, knn (k nearest

neighbors), to build a model that predicts sex from ΔRn values. We performed the analysis using the programming language R version 3.6.3 (R Core Team 2020) and R packages class (Venables et al. 2002), gmodels (Warnes et al. 2018), and caret (Kuhn, 2020). We split the dataset into a training set and a testing set. The algorithm uses the data from the training set to predict the sex within the testing set. When the model is predicting which group the unknown sample belongs to, it considers the closest points of known sex surrounding the unknown sample. The parameter, k, is the number of nearest neighbors the algorithm should take into account when predicting the value of the unknown. We typically chose the square root of the sample size rounded either up or down to the nearest odd number to avoid ties. In some cases, we tested different k-values to ascertain whether it had an effect on the accuracy of the model.

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

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

Future Directions

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

RADseq for Bi-allelic sex marker identification

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

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

The resultant VCF files were used as input into custom Python scripts that looked for patterns of SNP calls that were heterozygous in one sex, homozygous in the other sex, and lacking the third genotype class expected in normal autosomal chromosomal segregation. SNPs that were present in more than three of the 15 run results were ranked higher than those that did not. Candidates were further screened based on the number of individuals that were successfully genotyped at that locus, where the SNP occurred in the locus, and the number of SNPs nearby. Optimal assays of this type require 18 - 25 nucleotide primers that flank a stretch of DNA that should be 80 - 120 nucleotides long. The probes should be 20 - 25

nucleotide long and the SNP should fall as close to the center of the probe as possible. Initial genotyping assays were designed for the top two candidates using Primer3 v 0.4.0 (http://bioinfo.ut.ee/primer3-0.4.0/), but neither were successful.

Future Directions

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

Appendix B4 - Table 1. Results of Presence/Absence sex marker panel results versus observed phenotype for five western USA Brown Trout populations. Sampled collected in 2018-2019.

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

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

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

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

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