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

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June 26, 2019

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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 = 250mm) 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 BK 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 first program year to enable attainment of those objectives. The 2018-2019 workplan (Appendix A) lists tasks to be undertaken during the first project year for timely attainment of program objectives. The pages below summarize results of those efforts.

Methods

Sex Reversal Trials

Overview

The ability to feminize male fish for subsequent egg production is one of two requirements 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 by IDFG staff for Common Carp, Walleye and Lake Trout during the prior two years. In addition, in the case of Walleye and Common Carp, we grew out study fish during the current workplan year to determine if sex reversed males or neofemales could spawn at two years of age. Finally, a new sex reversal trial was initiated during spring 2019 for a new species (Northern Pike).

Walleye (Sander vitreus)

During May 2017 sex reversal trials using Estradiol (hereafter E2) on treatment groups of recently hatched walleye were initiated in two Midwestern walleye hatcheries. The initial trial was conducted with Alan Johnson, Fisheries Resource Biologist at the Rathbun Fish Culture Research Facility (RFCRF), operated by the Iowa Department of Natural Resources. In this trial, three test groups consisting of 1200 control fish and two 1200 fish treatment groups were stocked and reared in 284 L circular tanks. For a detailed description of intensive walleye larvaculture rearing methods used at this facility see Summerfelt and Johnson (2015). Study fish were fed dry pelleted feed (Otohime) ranging from 3-8% body weight per day over the course of the study. Treatment group feed was topcoated with a 15mg/kg feed Estradiol solution suspended in 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 84 or 100 days, beginning at 20mm (17DPH). A similar design was used working with Aaron Andrews, Fish and Wildlife Biologist 1 at the Meade Fish Hatchery operated by the Kansas Department of Wildlife, Parks and Tourism. However at Meade there was only one treatment group and the E2 exposure period was earlier, starting at 7 DPH and running for 84 days. Because of space limitations, study fish from both hatcheries were PIT-tagged and transferred to Daric Schneidewind. at the Milford Fish Hatchery in Kansas on16 November 2017 to be reared communally. On 21 March 2018, the majority of surviving trial fish from both the Rathbun and Meade trials were killed via anesthetic overdose and preserved for subsequent gonad histological analysis. Gonadal tissue was examined and phenotype was recorded for each fish.

A small number of both treatment and control fish from both the Iowa and Kansas trials were retained for further overwinter growout. Three days prior to our return to the Milford facility on 3 April 2019 both these fish were injected with hCG with the intent being to possibly spawn these fish at the same time as other Milford hatchery walleye. After this effort, all 2017 study fish were sacrificed and gonads removed and sent off for histological analysis to determine if oocytes from fish in the treatment groups were developing normally.

Common Carp (Cyprinus carpio)

On 2 July 2017, sex reversal trials using E2 and Estrone (hereafter E1) were initiated by IDFG staff at Opaline Aquafarm, a private warmwater facility near Nampa, Idaho. Trial groups of approximately 500 recently hatched Common Carp were stocked and reared in 7 108 L rectangular tanks in a thermo-modulated flow through system. With one exception trial groups were initially fed artemia to improve survival. Study fish were subsequently fed dry pelleted feed (Otohime) 4% body weight per day over the course of the study. Treatment group feed was topcoated with 100 mg/kg E1 or 40 mg/kg E2 using a hand-held sprayer (Schill et al. 2016). The treatment groups were fed E1 or E2 coated feed for different time periods based on the design (Table 1). Control feed was topcoated with pure EtOH. On 21 May 2018, a sample of fish from each study group was killed via anesthetic overdose and preserved for subsequent histological analysis. A fin clip was also taken for genetic analysis.

Table 1. Sex reversal trial framework for Common Carp undertaken by IDFG staff at a private warmwater facility (Opaline Aquafarms) in southern Idaho, 2017.

Treatment Type	Dosage	Artemia (da)	Dry Feed (da)	Total Tx Duration (da)
E1	100 mg/kg	0	140	140
		10	130	140
		20	90	110
		20 (unTx'd)	90	110
E2	40 mg/kg	20	40	60
		20	70	90
		20	90	110
Control	0 mg/kg	20 (unTx'd)	Un Tx'd till completion	0

Gonadal tissue was prepared histologically and subsequently examined in July 2018. Individual fish from each trial group were identified as either male, female or intersex (presence of both oocytes and sperm cells). To determine if individual treatment fish were sex reversed we compared results of these phenotype designations with the fish's genetic sex as determined by a sex marker developed by the IDFG

Eagle Fish Genetics Lab (see Sex Marker methods section below). The percent of fish in each study group comprised of sex reversed neofemales was then calculated and is reported.

A small number of fish from both treatment and control groups were retained for subsequent growout over the 2019 winter period to determine if potentially sex reversed neofemales could be spawned in their second year. Use of the above-mentioned sex marker enabled identification of true genetic males and females that were subsequently PIT tagged. The reproductive status of genetic males that had been treated with an estrogen (potential neofemales) were the primary fish of interest in this effort. On 23 May, 2019, we injected one known genetic control group female, and four surviving potential neofemales with carp pituitary hormone in an attempt to initiate spawning at two years of age.

Lake Trout (Salvelinus namaycush)

Very limited prior work on sex differentiation and estrogenic sex reversal existed for Lake Trout with notably poor results (Wenstrom 1975; Herman and Kincaid 1991). Consequently, staff at IDFG's participating Grace Hatchery requested a study design that maximized the number of possible treatment or "recipe" evaluations. In addition, Haffrey 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 2). The majority of the treatments tested (10) involved use of E2 while two of the combination treatments were conducted with E1. 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. 1995). 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 transferred from Heath Trays and ponded to 14L 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 ranged in hormone dose from 30 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. 2016). On June 2017 all surviving fish from the treatment and control groups were PIT tagged using 8mm tags and moved to an outdoor raceway to rear communally.

Table 2. Sex reversal trial framework for Lake Trout currently ongoing at the Grace Fish Hatchery (IDFG).

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 fish have been sampled to ascertain if study fish have sexually differentiated visually. On 24 September, 2018, ten 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 2018 an additional control fish was sampled and its gonads still appeared indistinct based on visual examination. On 20 April 2019, ten additional surplus control surrogates were collected for histological preparation and will be interpreted in July 2019. It is hoped that the gonads of this late maturing species will finally be discernible histologically in the summer of 2019 and allow for an evaluation of the various recipes.

Northern Pike (Esox Lucius)

No prior attempts to feminize a male northern pike have occurred but two published studies reported successful masculinization of female pike (Demska-Zakes et al, 2000; Luczynski et al 2003). Using these two studies as a guide, a pike sex reversal trial using E2 was recently begun in Iowa again assisted by Alan Johnson at the Rathbun Fish Culture Research Facility (RFCRF). This trial, which began in April 2019 includes a control and two treatments groups (Table 3).

Table 3. Sex reversal trial framework for Northern Pike 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

Sex Markers

The development of sex markers for each species of interest involves genetic sampling and DNA sequencing. First, mature adult fish of wild origin were collected by 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 by IDFG's Eagle Fish Genetics Lab (EFGL) personnel and cut into fragments using specific enzymes. Selected DNA fragments were then sequenced so that the exact order of nucleotides (i.e. A,C,T,G) was determined. These sequences were then compared between phenotypic males and females to find specific single nucleotide polymorphisms (SNPs) specific to each sex.

For more detailed example descriptions of the procedures used by EFGL personnel in conducting these analyses see Appendix B.

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-Alvarez and Piferrer 2008). Such a form of phenotype change (female to male) is not a threat to the YY Male technique. However, Density-Dependent changes in the sex ratio of recruits has been suggested for Lake Superior Lake Herring (Bowen et al. 1990) although this study is not without limitations and provided no direct genetic evidence. 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. In both cases, perhaps the best way to look for phenotypic shift at low abundance is to examine gonads of fish rearing at low abundance and compare resultant observed phenotype to genotypes 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 trial for Common Carp was initiated at the above noted Opaline Aquafarm. Three 108 L tanks were stocked with two densities of Common Carp fry. As the fish grew, culling was required in the high density tank. After the initial cull, we sought to maintain a 10:1 ratio between high and low density tanks during subsequent culling events (Table 4). All study fish were killed via anesthetic overdose on 10 January 2018. Fish were necropsied and sexed as above and fin clips were taken. Individual fish phenotype designations were then compared to their genetic sex which was derived using the fin clips and sex marker techniques for Common Carp as described above. A similar study design was implemented for Lake Trout at the Grace Hatchery but because results are also unavailable due to the late maturity of this species, further detail will not be presented until next year.

Table 4. Rearing tank density levels of Common Carp (fish per 108 L tank) in ESD trial conducted at Opaline Aqua-Farms 2017-2018

Tank number	Treatment	Initial Density 9 Jul	1st cull 16 Aug	2nd cull 30 Aug	3rd cull 6 Sep
1	Low 1	50	25	15	10
2	Low 2	50	25	15	10
3	High	1500	250	150	100

A far larger ESD study 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 is removed annually. All fish collected are killed and a fin clip taken and stored on coded Whatman sheets. Those fish deemed large enough to sex phenotypically based on prior sub-sampling efforts were placed in individual bags, frozen on dry ice, and returned to the laboratory. Bagged fish were subsequently defrosted, necropsied and their phenotypic sex was determined visually with the aid of microscopy when required. Only fish with clearly identifiable gonads were sexed and the remainder were classified as unknown. Genetic sex was independently determined for all phenotypically sexed fish using a published Brook Trout Sex marker (Schill et al. 2016b). The phenotype and genetic sex data were subsequently examined for each individual fish and any discrepancies noted. The intent of this ongoing evaluation is to continue searching for genotype:phenotype mismatches as the total wild populations of the two streams are reduced via ongoing suppression efforts and eventually, concomitant stocking of YY Male Brook Trout.

Results

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 initially 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

The trials at both facilities produced positive sex reversal results. Based on histological sampling on March 21 2018, nearly a year after the trial started, sex ratios in the control groups at both the Meade and Rathbun facilities approximated the expected 50:50 sex ratio (Table 5). In contrast, 100% of fish in both the 84 and 100 day treatment groups were histologically identified as female. Thus both treatment regimes were effective at generating sex reversed neofemales though the exact number is unknown due to difficulties in obtaining a working sex marker (see below).

Nearly two years after the initiation of the trial, all surviving fish were netted, identified by PIT tag, and handled by Milford Hatchery personnel on 3 April 2019 to ascertain spawning potential. A total of 8 control males from both hatchery trials (67%) were flowing sperm and four control females (2 MFH, 2 RFCRF; 57%) had ripened to the point of flowing eggs upon pressure. None of the treatment fish that had been retained had ripened. Due to hatchery space considerations and a desire to obtain insight on oocyte development in females from the hormone exposed groups, a decision was made to sacrifice all remaining trial fish for histological examination.

Table 5. Results of histological samples from control and hormonally exposed Walleye at two mid-west hatcheries (Kansas, Iowa) sampled 21 March 2018.

Treatment Type	Phenotypic Sex (Visual)	
	F	M
Meade Fish Hatchery		
Control	19	21
E2 84	35	0
Rathbun Fish Culture Research Facility		
Control	23	16
E2 84	32	0
E2 100	29	0

Table 6. Phenotype of Walleye via visual observation of necropsied fish (709DPH) at two mid-west hatcheries (Kansas, Iowa) sampled 3 April 2019.

Treatment Type	Phenotypic Sex (Visual)	
	F	M
Meade Fish Hatchery		
Control	4	1
E2 84	4	0
Rathbun Fish Culture Research Facility		
Control	3	11
E2 84	8	0
E2 100	6	0

Visual gonadal examination results indicated that all retained fish in the three E2 treatment groups were phenotypic females (Table 6) though histological slides have yet to examined. Not surprisingly, based on the visual necropsies, ovarian tissues in the majority of these unripe treatment group fish were not fully developed along the entire longitudinal plane of the body cavity at the time of sampling. The lack of full reproductive development in treatment fish is not surprising given that only four of seven of the control females had ripened and full gonadal development in sex reversed fish often lags slightly behind untreated females. Further, a strong majority of feral wild Walleye females spawned in the hatchery mature at age 3 (Alan Johnson, personal communication). Overall, results of the sex reversal trials in both states are quite positive and Walleye appear to be readily sex reversible. A dialog with possible WAFWA and Federal collaborators willing to consider initiating the first phase of a YY

Male Walleye broodstock construction have begun though a decision to actually begin such an effort is dependent on the EFGL attaining a functioning walleye sex marker (see below).

Sex Marker Development

During the past year IDFG's Eagle Fish Genetics Laboratory staff made a concerted effort to develop a Walleye sex marker but to date the work has not yielded useful markers. Analyses to find more suitable candidate markers are currently underway.

For a more detailed explanation see Appendix B1.

Common Carp

Sex Reversal Trials

Our 2018 review of the histology samples for phenotype, and integration of these results with 2019 true genetic sex designations derived using a sex marker for Idaho populations (see section immediately below) permits an unusually informed assessment of the 2017 sex reversal trial results. All previous sex reversal studies on a wide range of species we have reviewed simply report the overall sex ratio of treatment and control groups and test for statistical differences. Our ability to integrate known genetic sex into the study results allows us to note actual sex-reversal related phenotype shifts for individual fish well before sexual maturity.

An examination of the results suggests that all three E2 treatments tested were not of sufficient duration to effectively feminize Common Carp (Table 7). A strong majority (77.8%) of the 60 day dry food group remained true males. However, the number of true males in both the 90 and 110 day groups declined while the number of intersex (IS) fish increased markedly in them. The only true sex reversed neofemales obtained in the E2 trials were for the 110 dry feed groups again suggesting the need for longer duration hormone delivery.

In contrast to the E2 trials, feminized neofemales were obtained in all four of the E1 trials, all of which were ran for 110 or 140 days. The proportion of desirable neofemales in the two 110 day E1 trials remained low at 8.2-17.4% but rose to 50-55.6% for both 140 d treatment groups. This suggests that additional exposure times to either E1 or E2 above the 140 days we tested may produce better feminization results.

Table 7. Results of the Common Carp sex reversal trial undertaken by IDFG staff at a private warmwater facility (Opaline Aquafarms) in southern Idaho, sampled 21 May 2018. The table summarizes the integration of known genetic sex for individual carp with their histologically derived phenotype. Those fish of either True or Sterile condition have concordant geno-phenotypes. Neofemales are fish that have a phenotypic sex that has shifted to the opposite sex when compared to genotype. An asterisk indicates a shift in phenotype from known genetic sex.

Treatment Type	Exposure (Days artemia, total days)	Geno-Pheno Shift	Count	% Genetic Male by Phenotype (excluding F)
E1	0, 140	True F	6	-
		Sterile F	1	-
		Neofemale*	2	50.0
		Sterile M	2	50.0
	10, 140	True F	25	-
		Sterile F	2	-
		Neofemale*	5	55.6
		M to IS*	4	44.4
	20, 110	True F	21	-
		Sterile F	2	-
		Neofemale*	4	17.4
		M to IS*	16	69.6
		Sterile M	3	13.0
	Control 20, 110	True F	27	-
		Neofemale*	2	8.2
		M to IS*	21	87.5
		True M	1	4.2
E2	20, 60	True F	22	-
		M to IS*	5	18.5
		True M	21	77.8
		Sterile M	1	3.7
	20, 90	True F	17	-
		M to IS*	7	87.5
		Sterile M	1	12.5
	20, 110	True F	21	-
		Neofemale*	3	13.6
		M to IS*	15	68.2
		True M	2	9.1
		Sterile M	2	9.1
Control	0	True F	30	
		True M	13	100.0

Overall, despite the small sample sizes, it would appear that we are close to an effective feminization recipe for Common Carp. Indeed, the feminization results (males shifting to neofemales) obtained for the two 140d treatment groups (50-55.6%) might be sufficient to initiate building a YY Male

broodstock if based on a larger sample size. However, because of private hatchery water quality issues in our trial, space limitations, and the resultant small sample sizes, a final sex reversal trial seems necessary. To avoid the challenges we experienced, it is paramount that this effort be conducted at a facility familiar with the rearing of Common Carp (or at least Koi) and a dialog on possible states or facilities willing to do this is ongoing. In addition, to ensure adequate resources for a strong final effort, we have submitted a Multi-state DJ grant proposal on behalf of WAFWA specific to conducting a sex reversal trial for Common Carp. It is hoped that a successful proposal may provide enough extra resources to offset any extra infrastructure costs for a willing partner and to also allow for the involvement of additional Common Carp reproductive expertise to ensure success.

Results of the 2019 growout effort yielded similar results as the walleye trial noted above in that the four potential neofemales did not mature and spawn at two years. However, the single control genetic female did flow eggs after pituitary hormone injection. These results suggest that, like walleye, feminization likely delays maturity in this species.

Sex Marker Development

EFGL staff identified two candidate sex markers for Common Carp based on samples of 31 phenotypic females and 30 males collected from a single population in Idaho. For a more detailed description of procedures used see Appendix B2. These two candidate markers were screened on 728 Common carp genetic samples of known phenotypic sex collected from four states. The concordance rate for all samples screened was 93%. There was some variation in genotyping completeness and concordance across Idaho populations, but the weighted average for Idaho populations was higher at 96%, a level deemed acceptable for YY Male Broodstock construction by EFGL staff. Marker-phenotype concordance rates were generally lower for Midwest samples with rates ranging from 50 to 77% in Iowa and Texas, respectively (Appendix B2). Two major Common Carp strains (Eurasian and European) have been distributed worldwide and the differences observed in concordance rates are most likely the result of strain differences (Matt Campbell, EFGL supervisor). Future genetic work would involve sequencing more samples from both strains, more populations and screening more of the genome. It is possible that separate genetic markers may have to be developed to achieve high accuracy for each strain.

Lake Trout

Sex Reversal Trial

As noted above in methods, we are currently awaiting results of a 10 fish histology sample in regard to sacrificing the majority of trial fish to evaluate recipe options. However, because of the strong Grace Hatchery staff commitment to the project and the resultant large number of treatment options

tested, it is hoped that a successful one will be found from the current trial. Lake Trout are well known for their late maturity which presents considerable challenges for YY Male broodstock development using the three generation approach of Schill et al. (2016a). In anticipation that another, shorter route to creation of YY Male Lake Trout may need to be found, IDFG research staff in northern Idaho conducted successful preliminary spawning experiments during the past fall designed to pave the way for possible androgenesis experiments in the near future. That work was conducted outside of the WAFWA Consortium funding stream and thus will not be reported upon here.

Sex Marker Development

The sex determining gene known as the sexually dimorphic on the Y chromosome (sdY) was identified by Yano et al. (2013) and is conserved in most salmonids. The original sex marker for Lake Trout required running samples using gel electrophoresis. To decrease per sample costs and the time required to run samples, the EFGL designed a new genetic sex marker based on the sdY gene. This new genetic sex marker can accurately differentiate XY males from XX females, with its accuracy demonstrated by running samples of known phenotypic sex (See Appendix B3 Figures). The EFGL is currently working on developing another genetic sex marker for Lake Trout that will be able to differentiate XY males from YY individuals in the future.

Northern Pike

Sex Reversal Trial

This work has just begun in Iowa and other than noting its start, there is little to report.

Sex Marker Development

As noted in the workplan (Appendix A) sex marker development for Northern Pike is slated for completion by both the EFGL and the Alaska Department of Game and Fish (AKDG&F) genetics lab. Based on both an in-person site visit and a phone conference call, ADG& F opted to take the lead on this work and secured additional funding for marker development using RAD sequencing technology. Since the last conference call, permanent staff changes at the Alaska lab have resulted in delays and slowed initiation of this work. However, since the above sex reversal trial has just started, the delay over the last several months should not be problematic.

Brown Trout

Sex Reversal Trial

Identification of appropriate WAFWA member hatcheries for conducting a Fall 2019 sex reversal trial for Brown Trout was an identified work task for the reporting period. We surveyed Fish Chiefs of all

contributing members of the YY Consortium to ascertain which states might be willing to aid in this effort. The states of Colorado and South Dakota both volunteered and initial contacts and plans are underway for research-oriented facilities in both states to conduct trials. Based on the walleye work in IA and KS, undertaking trials at two facilities allows for more recipe testing without burdening a given facility and also provides a measure of safety in regard to unforeseen aquaculture hazards.

Sex Marker Development

The sex determining gene known as the sexually dimorphic on the Y chromosome (sdY) was identified by Yano et al. (2013) and is conserved in most salmonids. The original sex marker for Brown Trout required running samples using gel electrophoresis. To decrease per sample costs and the time required to run samples, the EFGL designed a new genetic sex marker based on the sdY gene. This new genetic sex marker can differentiate XY males from XX females, with its accuracy demonstrated by running samples of known phenotypic sex (See Appendix B4 Figure). The EFGL is currently working on developing another genetic sex marker for Brown Trout that will be able to differentiate XY males from YY individuals in the future.

Density Dependent Sex Change

Common Carp

Results of the density-dependence trial did not find evidence of sex change at relatively low fish densities. In both of the low density replicates, the sex ratio was 60% female and no phenotype shifts were observed (Table 8). However, 7 of 85 fish (8%) in the high rearing density tank showed evidence of phenotype shift. These anomalies included one shift from genetic female to phenotypic male, four shifts from genetic male to phenotypic female, and two shifts from genetic male to phenotypic intersex. Not surprisingly, the fish in the high-density tank were considerably smaller (Mean = 183 mm) than those from the low density tanks (Mean = 248 mm). While the shifts are of concern, it is unknown if the extreme high rearing densities and associated biomass in the high group that precipitated the several culling events (Table 4) are remotely reflective of nature. In regard to the initial goal of examining shifts at low densities, a more solid test would have been to rear even less fish in the tanks, perhaps even one or two per tank. Such a design was not feasible in the private hatchery at our disposal. Assuming an additional Common Carp sex reversal trial will be done in a more favorable environment in the future, additional work on rearing density effects on common carp phenotype should also be conducted if facility size permits.

Table 8. Results of the Common Carp Environmental Sex Determination Trial undertaken at a private warmwater facility (Opaline Aquafarms) in southern Idaho, sampled 10 Jan, 2018. Those fish of either True or Sterile condition have concordant geno-phenotypes (histologically derived). An asterisk indicates a shift in phenotype from known genetic sex.

Tank Density	Geno-Pheno Shift	Count	% by Sex
High	True F	35	41.2
	F to M*	1	1.2
	Sterile F	1	1.2
	M to F*	4	4.7
	M to IS*	2	2.4
	True M	41	48.2
	Sterile M	1	1.2
Low Rep 1	True F	6	60.0
	True M	4	40.0
Low Rep 2	True F	6	60.0
	True M	4	40.0

Brook Trout

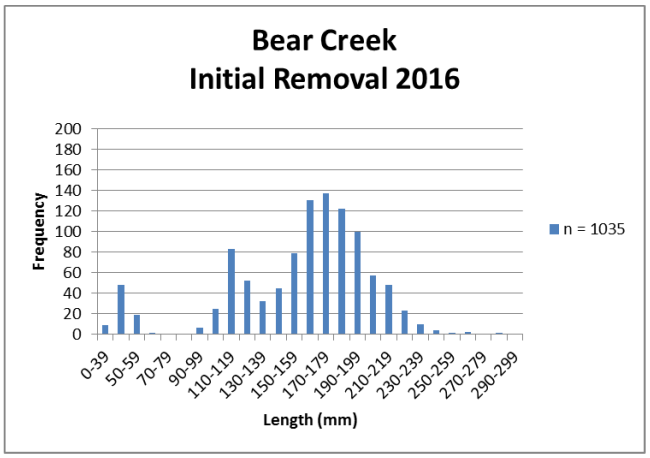
To date a total of 2316 wild Brook Trout in the two study streams have been visually sexed for phenotype and genetically sexed using a sex marker. Of the 1180 and 336 fish examined in 2016 and 2018, respectively, no discordance between genotype and phenotype was detected. However, nine mis-matches occurred in 2017 (Table 9). At present, it is unknown if these discrepancies are due to actual phenotype shift or represent errors in either the genetic sex characterization, phenotype designation, or human error in record-keeping. Stored DNA from the fish in question are currently being re-analyzed.

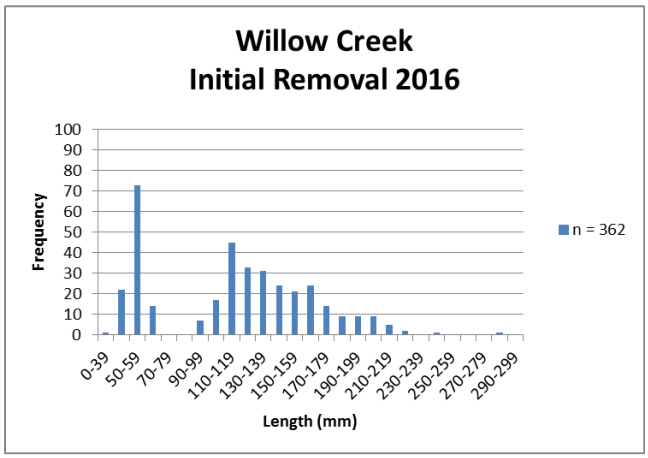
The intent of this study was and is to keep a watchful eye out for potential sex change as two isolated Brook Trout populations are being strongly reduced by electrofishing suppression. A summary of length-frequencies for all three years of the study suggest that considerable population suppression, particularly for YOY fish has indeed been attained (Figure 1). Further, the declining sample sizes by year reported in Table 9, indicate that fish of adult size are also declining at a rapid rate. In addition, YY Male fish were introduced into Willow Creek in 2018 for the first time and will be released into both streams in 2019. Based on predictive modeling (Schill et al. 2017), both populations are expected to decline markedly in the near future. The intent of this study is to continue to examine individual fish for genotype-phenotype mis-matches as both populations further decline.

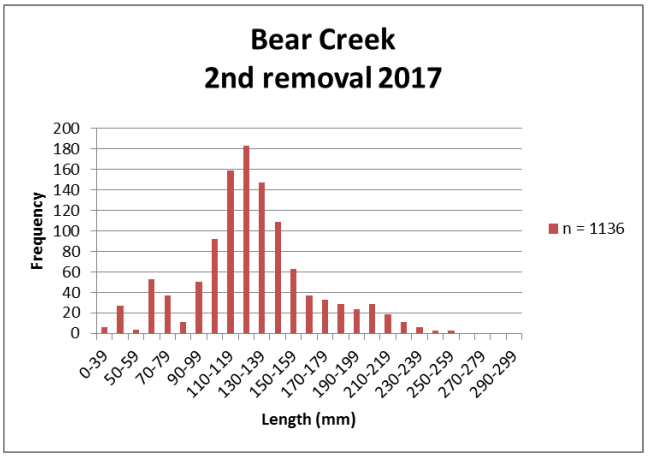
Table 9. Results of the Brook Trout field ESD trial collected from two isolated Idaho streams from 2016-2018.

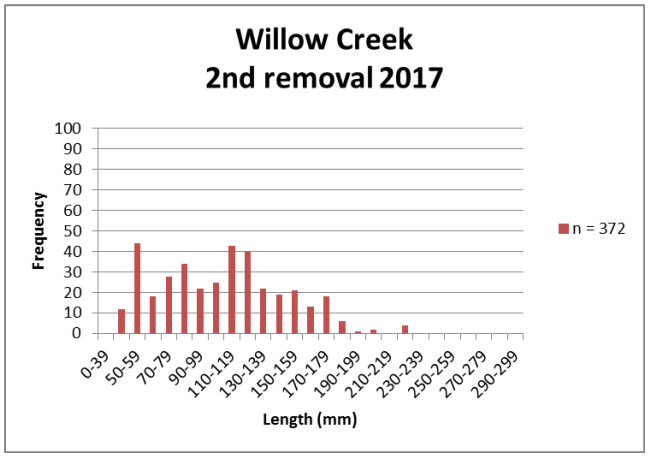
Year	Stream	Phenotype	Genotype F	Genotype M	Grand Total
2016	Bear Ck				929
		F	495	0	
		M	0	434	
	Willow Ck				251
		F	149	0	
		M	0	102	
2017	Bear Ck				602
		F	283	4	
		M	1	314	
	Willow Ck				198
		F	109	4	
		M	0	85	
2018	Bear Ck				182
		F	102	0	
		M	0	80	
	Willow Ck				154
		F	72	0	
		M	0	82	
Grand Total					2316

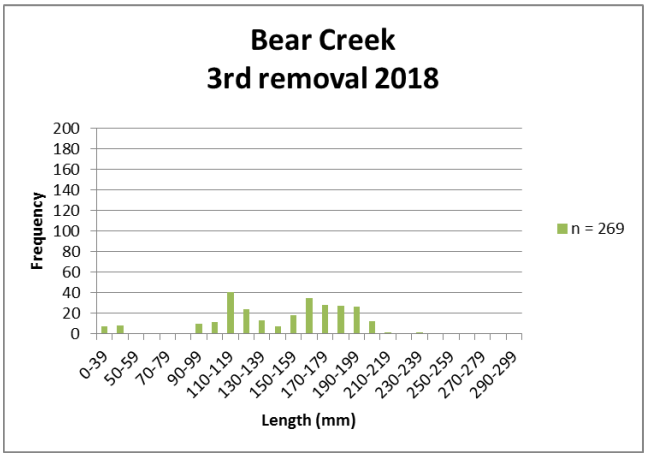
Figure 1. Frequency of size of Brook Trout removed over three years from two Idaho streams, 2016-2018.

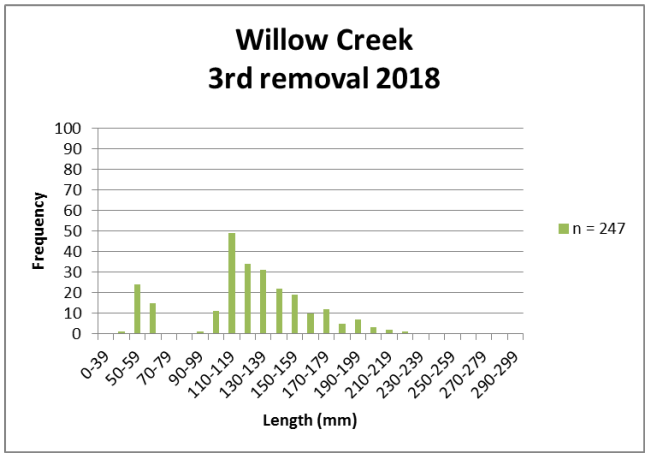












Coordination of INAD Coverage

Considerable 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. A key accomplishment, initiated by Bonnie Blair of ADAAP was a revision of the YY Brook Trout INAD Food Use Authorization or Slaughter Permit. Prior to 2019, the four states currently releasing YY Male Brook Trout (ID, WA, NM, and OR) had been subjected to a stocking cap. This cap has now been removed and the Food Use Authorization is now more appropriately related to the number of Brook Trout being treated to maintain the Hayspur Hatchery broodstock. Additional time was also spent coordinating egg distribution in general, helping the first Federal agency stocking YY Brook Trout obtain appropriate state stocking authority, and working with all five stocking entities and ADAAP staff on a Categorical Exclusion request required by the FDA.

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. Three conference calls were held during 2018 along with a single one to date in 2019. General results of the calls were quite productive and group interest centers around not duplicating past or ongoing research efforts and collectively deciding remaining research priorities. Based on the success of this meeting, a second YY Brook Trout working group chaired by Marc Garst, IDFG Hatchery Supervisor was formed to troubleshoot and coordinate YY Brook Trout rearing for the three egg receiving states plus the USFWS at the Abernathy facility, Longview, WA.

Project Communication

An annual progress report was completed on schedule and YY Male Consortium project results were presented at the Oregon WAFWA Chief's meeting during 2018. As identified in the workplan, two communication presentations on YY Male fish were made, one at the World Aquaculture Society

Meeting in New Orleans and a second at the National American Fisheries Society meeting in Atlantic City. An obvious benefit of attendance at at such national scale meetings is the opportunity to meet and open dialogs with high level administrators from various agencies 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 New Orleans meeting. This annual gathering is attended by key ADAAP staff and regulatory members of the FDA involved in the current YY Brook Trout INAD. Involvement with the DAWG group is key as many of the attendees will also be directly involved in any future INAD coverage for new YY Male species. The 2019 Midwest Warm and Coolwater Fish Culture Workshop in Iowa was also attended with the primary purpose of learning more about Northern Pike Aquaculture techniques and solidifying plans with the Iowa DNR staff to undertake the now ongoing pike sex reversal trial.

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:

- These would not be possible without the culturing prowess of A. Johnson and J. Rudacille and staff at Rathbun FCRC, IDNR, IA, and, A. Andrews and J. Vajnar and staff at Meade FH, KDWPT, KS for Walleye. We thank Doug Nygren and KDWPT for navigating travel challenges for us.

- Common Carp expertise was generously and copiously provided by B. Gomelsky (Kentucky State University), T. Delomas (IDFG), R. Cunningham (Opaline Aquafarms) and culture assistance by K. Stevenson (IDFG). Collection of broodstock assistance provided by M. Peterson (IDFG).

- We thank the extensive staff at the Grace Hatchery (M. Gallagher, W. Fowler, K. Kincaid, E. Pankau, IDFG) for their long-term commitment to our Lake Trout trials, given that we didn't appreciate just how long it would take for this late maturing fish to provide us with phenotype results.

- 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 , and able assistance in both the field and laboratory from B. Schill.

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
- IDFG: B. High and Region 6 staff, J. Thiessen and Region 4 staff, N. Vu, D. Burton, L. Schrader, K. Stevenson and the entire staff at the EGL, led by M. Campbell, supported by T. Delomas, J. McCane, A. Boone and many others
- IDNR staff
- KDWPT staff
- NMDGF: B. Bakevich
- University of Michigan
- University of Minnesota
- A.E. Woods Hatchery, TX

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 N. Vu, K. Coykendall, and T. Delomas. They were assisted by a long list of lab technicians, particularly A. Boone and D. Eardley who specialize in much of the frontline sex marker work.

Administrative support and assistance:

Thank you to C. Campbell, D. VonDeBur and C. Boyd from WAFWA for providing guidance throughout the Consortium building process as well as deftly stewarding the administration of the program.

Financial Support:

Thirteen Fish Chiefs from the States of AK, AZ, CA, CO, ID, KS, NE, NM, OR, SD, UT, WA, WY and many of their program managers were enthusiastic enough about the potential of YY Male fish that they contributed closely guarded funds from their budgets to support this work. The list of names supporting financial state contributions is too long to mention all here and several of the original Chiefs have already moved on. We have not forgotten who they are and thank both the original and current Chiefs and program managers for their support. In addition, we thank the Kalispell Tribe for a donation to the Consortium in relation to their ongoing Brook Trout removal work. We would be remiss in not

specifically thanking IDFG Chief Jim Fredericks for helping to conceptualize the Consortium and providing an exceptional share of funding and manpower to initiate it.

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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

Appendix B – Results of sex marker development efforts by the IDFG Eagle Fish Genetics Lab

B1 – Walleye (M. Campbell)

We completed RADseq on 11 phenotypic females and 11 phenotypic males from Lake Pend Oreille using the SbfI restriction. We identified 3 candidates SNPs that exhibited differences between males and females. However, these were unsuccessful as diagnostic biallelic or presence/absence markers upon further testing. We recently completed RADseq on the same set of samples as used previously using a second restriction enzyme (BAMHI). Analyses to find candidate markers are currently underway. We hope to have a new candidate list by the end of the month.

B2 – Carp (M. Campbell)

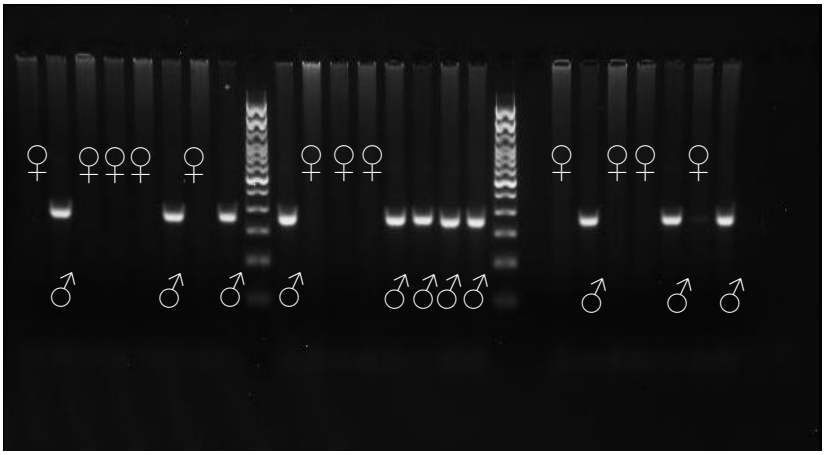
We completed Restriction site-associated DNA sequencing (RADseq) on 31 phenotypic females and 30 phenotypic males using the SbfI restriction enzyme (cuts every 8 b.p.). We identified 2 candidate loci (Cca_49666 and Cca_1852). Cca_49666 was found in 27 males and 1 female. Cca_1852 was found in 27 males and No females. We subsequently screened Cca_1852 on approximately 800 samples and observed an overall concordance rate of ~93% between genetic and phenotypic sex.

Future proposed work would use a second restriction enzyme (PstI) that cuts the genome more frequently (cuts every 6 b.p.).

	Concordance?		Unknown	Unsuccessful		Geno	Concordance	Location/ year	
Collection	no	yes	Gender	Geno	Total	Rate	Rate	inventory	State
CcaLOWL14C		31		7	38	81.6%	100%	Lake Lowell '14	ID
CcaLOWL15C		45		1	46	97.8%	100%	Lake Lowell '15	ID
CcaLOWL15S	1	10			11	100.0%	91%	Lake Lowell '15	ID
CcaLOWL16C	3	147		1	151	99.3%	98%	Lake Lowell '16	ID
CcaPURG15C	5	53		2	60	96.7%	91%	Purgatory Cr. '15	MN
CcaRLYP15C		17	1	3	21	85.7%	100%	Riley Pond '15	ID
CcaSNAR17C	18	196	2	4	220	98.2%	92%	Snake R. '17	ID
CcaAEWH15S	20	66		14	100	86.0%	77%	A.E. Woods FH '15	TX
CcaANDP15C		23			23	100.0%	100%	Anderson Pond '15	ID
CcaMSSP16C	2	2		1	5	80.0%	50%	Mississippi R. '16	IA
CcaSTML16C	2	50		1	53	98.1%	96%	Storm Lake '16	IA
Total	51	640	3	34	728	95.3%	93%		

B3 - Lake Trout (K. Coykendell, T. Delomas)

The sex determination gene, sexually dimorphic on the Y chromosome (*sdY*) has been found in a number of salmonids, including lake trout, *Salvelinus namaycush* (Yano et al 2013). The primer combination of Sdy_E2S1 and SdY E2AS1 from a previous study successfully amplified a product in males (Figure 1). However, this product is too large for real-time PCR assay development.

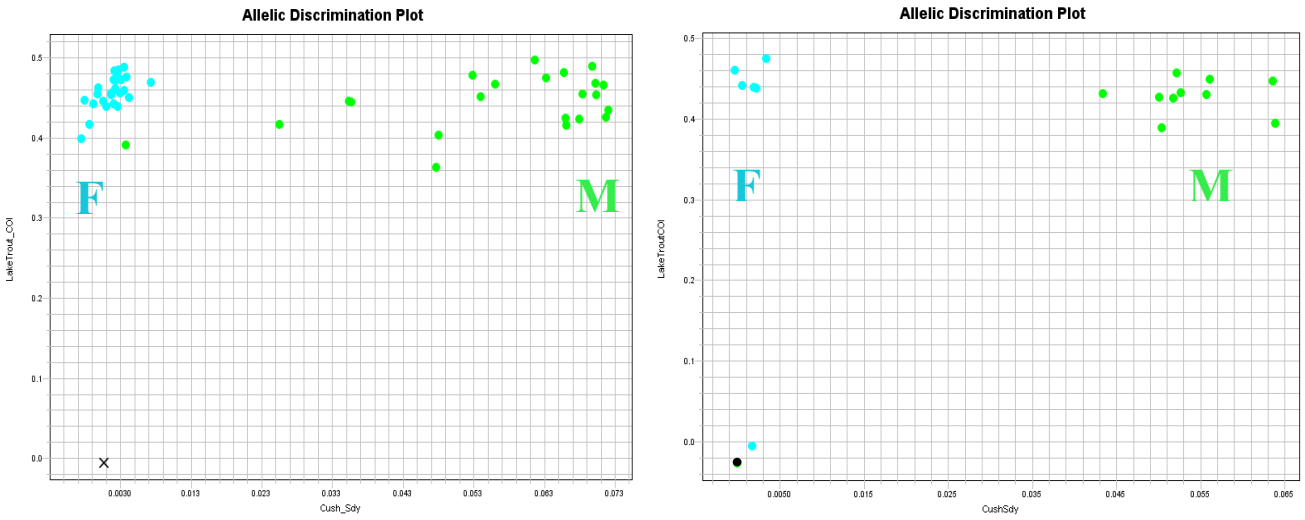


To develop a sex-specific assay that would be more efficiently screened with a real-time PCR assay, we aligned 10 sequences of the *sdY* from Genbank and designed primer and probe sequences from this aligned sequence. The primer and probe sequences are as follows:

CushSdyF: 5'-CCCTCATGGAGGGTGGAGT-3'
CushSdyR: 5'-GCTTGGCTATGCCGTTCAG-3'
CushSdyP: 5'-GCTCTAGGGAGGAAGGCATC-3' (labeled with Cy3)

For an internal positive control, which should amplify in both males and females, we included a second primer pair from Lacoursiere-Roussel et al (2015) that amplifies a 66 bp segment of the mitochondrial *COI* gene:
LakeTrout_COI_F: 5'- GGGCCTCCGTTGATTTAACTATC -3'
LakeTrout_COI_R: 5'- TGGCCCCTAAAATTGAGGAA -3'
LakeTrout_COI_Probe (5'-CTCTCTTCATTTAGCTGGC -3' (labeled with FAM)

In the assays primers and probes for both genes are present. The genes were co-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 55°C for 1 minute (annealing), followed by a 4°C hold for 10 minutes.



Bi-allelic sex marker identification

A presence/absence marker such as the *sdY* gene can discern between XX and XY samples. However, it can be difficult if not impossible to discriminate between XY and YY individuals as the marker would amplify in both. Therefore, a biallelic marker (in this case, a single nucleotide polymorphism (SNP)) that discriminates between XX, XY, and YY individuals would be useful for future Trojan male experiments. The fish used in the RADseq runs were captured from Lake Pend Oreille in 2017. RADseq was performed mostly following the Rapture protocol (Ali et al. 2016), using the restriction enzyme *PstI* (recognition site: CTGCAG). Each sample was tagged with a unique barcode so that they could be combined into libraries, then separated bioinformatically. A total of 10 females and 10 males were split into four separate libraries that were run on an Illumina NextSeq using mid- or high-300 v2 output sequencing kits, with an expected output of 32-120 Gigabases. We took the resulting sequences through the Stacks pipeline (Catchen et al. 2013) with a minimum depth of coverage (*m*) of 3, and a maximum distance allowed between stacks (*M*) within samples varying from 3 to 6 in ustacks, and the number of mismatches allowed between sample loci (*n*) among samples varied from 2 to 7 in cstacks, depending on the M-value. The average number of paired end reads used in the stacks analysis was 359,426,147, average number of genotyped loci recovered was 1,381,257 with a mean coverage of reads per locus of 32 and a mean coverage of reads per sample of 269. The average number of loci that contained at least one SNP was 821,775.

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. Both XX/XY and the less common ZZ/WZ sex chromosome patterns were considered. A separate script was run that looked for the expected

presence/absence pattern expected in a classical sex-linked marker (e.g. *sdY*). 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. Genotyping assays were designed for the top candidates of each species using Primer3 v 0.4.0 (http://bioinfo.ut.ee/primer3-0.4.0/). Probe sequences were designed so that the SNP fell within the first few bases on the 5' end.

Our preliminary results indicate that a candidate locus (Sna_433923_27) was successfully genotyped in all 20 individuals and is closely related to Chinook interferon alpha 1-like gene (related to *sdY*). The standard PCR recipe is identical to the one for *sdY* amplification above as are the cycling conditions. Optimization is currently underway that includes doubling the concentration of DNA, varying the concentrations of primers and probes from 0.25X to 2X and varying the annealing temperature from 55-62°C to achieve two distinct clusters representing males and females on the amplification plots as above.

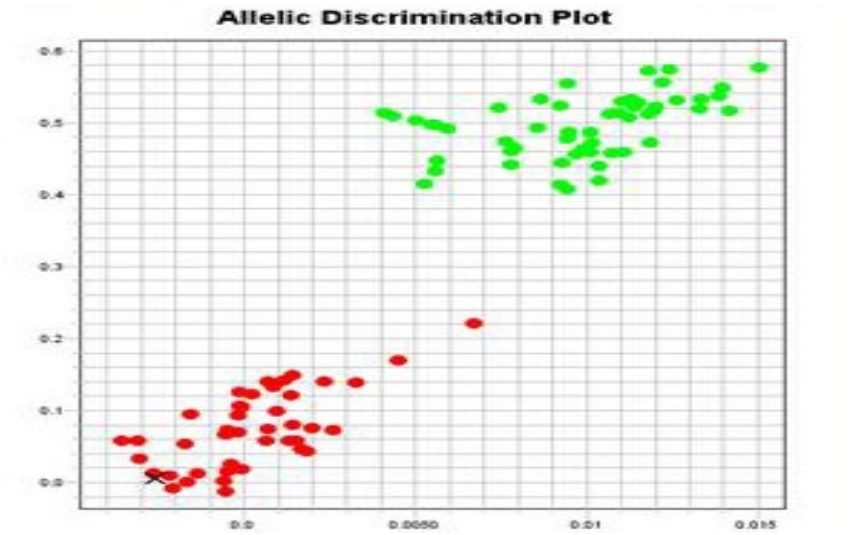
B4 - Brown Trout (K. Coykendell, T. Delomas)

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). EFGL has developed assays using the sdY gene and a control gene that discriminates between males and females. To achieve this, 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_Sex_F: 5'-TACTGCGAAGAGGAGGTGCT-3'
BrownT_Sex_R: 5'-GGTTGAACGGTCAGAGGAGA-3'
BrownT_Sex_P: 5'-AAGCCCTTC/ZEN/TCCCTGATGAT-3' (labeled with FAM)

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 females (red) and 53 males (green) from the South Fork Snake River, Idaho.



Bi-allelic sex marker identification

A presence/absence marker such as the *sdY* gene can discern between XX and XY samples. However, it can be difficult if not impossible to discriminate between XY and YY individuals as the marker would amplify in both. Therefore, a biallelic marker in this case, a single nucleotide polymorphism (SNP)) that discriminates between XX, XY, and YY individuals would be useful for future Trojan male experiments. The fish used in the RADseq runs were captured from the South Fork Snake River, Idaho. RADseq was performed mostly following the Rapture protocol (Ali et al. 2016), using the restriction enzyme *PstI* (recognition site: CTGCAG). Each sample was tagged with a unique barcode so that they could be combined into libraries, then separated bioinformatically. 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 sequences through the Stacks pipeline (Catchen et al. 2013) with a minimum depth of coverage (*m*) of 3, and a maximum distance 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 varied from 1 to 9 in cstacks, depending on the M-value. The average number of paired end reads used in the stacks analysis 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. Both XX/XY and the less common ZZ/WZ sex chromosome patterns were considered. 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. Genotyping assays were designed for the top candidates of each species using Primer3 v 0.4.0 (http://bioinfo.ut.ee/primer3-0.4.0/). Probe sequences were designed so that the SNP fell within the first few bases on the 5' end.

Our preliminary results indicate that two candidate loci was successfully genotyped in 15 individuals. An assay has been developed for one of the putative sex markers, Stru9767_37_15. The standard PCR recipe is identical to the one for *sdY* amplification above as are the cycling conditions. Optimization is currently underway that includes doubling the concentration of DNA, varying the concentrations of primers and probes from 0.25X to 2X and varying the annealing temperature from 55-62°C to achieve two distinct clusters representing males and females on the amplification plots as above.