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ARTICLE

Production of a YY Male Brook Trout Broodstock for Potential Eradication of Undesired Brook Trout Populations

Daniel J. Schill* and Jeff A. Heindel
Idaho Department of Fish and Game, 600 South Walnut Street, Post Office Box 25, Boise, Idaho 83707, USA

Matthew R. Campbell
Idaho Department of Fish and Game, 1800 Trout Road, Eagle, Idaho 83616, USA

Kevin A. Meyer and Elizabeth R. J. M. Mamer
Idaho Department of Fish and Game, 1414 East Locust Lane, Nampa, Idaho 83686, USA

Abstract

Brook Trout *Salvelinus fontinalis* introduced outside of their native range often negatively impact native aquatic fauna or provide marginal fisheries and are frequently targeted for manual or piscicide removal in lakes and streams. Unfortunately, complete eradication of exotic Brook Trout populations via these methods is rarely achieved; new approaches are needed. A potential alternative is a Trojan Y Chromosome (TYC) program in which hatchery-produced genetically YY male fish would be regularly released into an undesired population over time, skewing the population towards 100% males, theoretically resulting in wild population extirpation. We developed two genetic sex markers for Brook Trout and employed juvenile sex reversal methods commonly used in commercial aquaculture to develop a YY broodstock that can produce offspring for possible future use as biological control agents. Our search for genetic sex markers proved successful, with genotypic sex determination for two assays matching the observed phenotype for 90 out of 90 individuals. In the first phase of the program, estradiol-infused feed readily feminized genetic XY males into neofemales (F_{XY} fish) at a high rate (99.6%; $n = 224$). Survival of progeny from such egg-laying F_{XY} fish averaged 88% to eye-up and 91% from eye-up to ponding, values similar to untreated Brook Trout reared at the same facility. In the second program phase, we cultured both sperm- and egg-producing supermales (YY fish), a vital step towards development of TYC technology on a large aquaculture scale. Results showed that, in the hatchery, estradiol treatment does not reduce Brook Trout growth. This study demonstrates that hatchery production of a YY Brook Trout broodstock is feasible, modest in cost (less than US$10,000), and can be completed in 4 years. Although several hurdles remain before a full-scale stocking program could occur, we believe that future work on the TYC strategy for Brook Trout is warranted.

Brook Trout *Salvelinus fontinalis* introduced outside of their native range often provide marginal fisheries (Rabe 1970; Donald and Alger 1989) or negatively impact native aquatic fauna (reviewed in Dunham et al. 2002) and are thus increasingly targeted for manual removal in lakes and streams across western North America (e.g., Shepard et al. 2014). Unfortunately, complete eradication of established Brook Trout populations is often not achieved, and empirical studies suggest that, in most instances, manual or chemical eradication of such undesirable nonnative populations is not practical for a variety of reasons (Britton et al. 2010). The development of alternative approaches for eliminating select nonnative Brook Trout populations and other exotics is needed. One such alternative is a sex-skewing approach in which the anthropogenic shifting of the population

*Corresponding author: dan.schill@idfg.idaho.gov
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sex ratio toward males would reduce the long-term viability of an undesired population, eventually resulting in extirpation of the population.

Perhaps the most promising of such sex-skewing methods being considered, the Trojan Y Chromosome approach (hereafter TYC) relies on the development and release of males that are genetically YY rather than the typical XY arrangement (Gutierrez and Teem 2006; Teem and Gutierrez 2010). The TYC method is so named because it involves introducing stocked YY individuals capable of incorporating "hidden" Y chromosomal material into the undesirable population. Assuming that enough YY males are released into the undesirable population over time and that they successfully survive and reproduce, the population will strongly skew toward males, theoretically eradicating the wild population once the sex ratio reaches 100% males (Teem and Gutierrez 2010). The general TYC approach suggested by these authors was to (1) use exogenous estrogen exposure methods well established in commercial aquaculture to produce YY males, typically known as supermales (Varadaraj and Pandian 1989; Liu et al. 2013), (2) sex reverse half of the supermales, or M_{YY} fish, at the fry stage, again using estrogenic hormones, to produce fish hereafter referred to as feminized supermales (F_{YY}), and (3) maintain groups of both M_{YY} and F_{YY} fish as a broodstock capable of producing large numbers of YY fish for release into the wild. Along with a heterogametic XY male and homogametic XX female reproductive system, the development of a YY broodstock ideally requires the development of genetic tools to identify sex and the above-noted ability to feminize young male fish (Gutierrez and Teem 2006; Cotton and Wedekind 2007).

Techniques for feminizing standard XY male fish are readily available for salmonids (Feist et al. 1996), including Brook Trout (Johnstone et al. 1978). However, it is believed that YY males in other species may be more resistant to feminization than XY males (Mair et al. 1997; Liu et al. 2013), although viable feminized YY fish were subsequently produced by the same authors. We are unaware of any previous attempt to feminize a YY salmonid and this must be successfully accomplished if large-scale production of supermales is to occur (Mair et al. 1997; Liu et al. 2013).

The goal of this study was to investigate the feasibility of producing viable supermale Brook Trout (M_{YY} and F_{YY}) in a hatchery environment for use as a possible future TYC control agent in undesired Idaho Brook Trout populations. Specific study objectives were to (1) develop genetic sex markers for Brook Trout, including a quantitative PCR assay to differentiate XX from XY and YY fish, (2) produce a viable YY Brook Trout broodstock comprised of both phenotypic male (sperm-producing) and feminized (egg-laying) fish, (3) evaluate fecundity and growth of feminized XY and YY fish in the hatchery relative to untreated fish, and (4) use progeny tests to confirm the accuracy of the genetic male YY assay and empirically confirm the production of feminized supermales in a salmonid.

METHODS

Development of Brook Trout Sex Markers

The availability of a genetic sex marker greatly simplifies the production of monosex broodstocks. The Y-chromosome-specific assays used in this study to differentiate sex in Brook Trout were developed from amplified fragment length polymorphism (AFLP) sequence data of the Y chromosome region provided by Joseph Brunelli, Washington State University (unpublished data). An AFLP-based approach for the identification of sex-linked markers has been successfully used for a variety of species (e.g., Brunelli et al. 2008). The assays we developed were run in two different configurations: as a presence–absence assay in a multiplex microsatellite panel screened on Brook Trout and as a TaqMan-based allelic discrimination assay. The later assay was used specifically to discriminate between XY and YY males. For a detailed summary of marker development and results, see the Supplement provided in the online version of this article.

Trojan Y Chromosome Brook Trout

Phase 1: Neofemale (F_{XY}) development.—The first phase of TYC broodstock development involved the creation of feminized, genetically male fish known as neofemales or F_{XY} fish. On November 24, 2008, 3,026 eyed Brook Trout eggs from four 1 female : 1 male spawn pairings were transferred from the Wyoming Game and Fish Department Story Fish Hatchery to the Idaho Department of Fish and Game Ashton Fish Hatchery. Eggs were incubated in vertical-stack incubators on a constant 10°C spring-fed water source. Hatching began on December 8, 2008, and on January 2, 2009, 250 swim-up fry from each family were split into two equal groups (Figure 1). One group of 125 fish from each family was fed for 60 d beginning at first feeding on January 7, 2009, with commercially produced salmonid starter diets (Rangen; #0 starter) treated via spraying with 17β-estradiol (hereafter referred to simply as estradiol) at a concentration of 20 mg of steroid per kilogram of diet. This food was prepared as in Johnstone et al. (1978), except that diets were not defatted prior to steroid treatment. Fish in the four other paired lots were fed the same food, but it was not treated with estradiol. A subsample of male fish from these untreated groups was subsequently used as standard M_{XY} breeders at the beginning of Phase 2 (Figure 1). Fish in all eight lots were reared separately in isolated rearing vessels until they were large enough to be individually tagged. This ensured that no siblings were bred together in subsequent generations when deriving the TYC broodstock. All fish were fed hourly from 0800 to 1700 hours until feed trained and thereafter fed 3–4 times per day to apparent satiation throughout their remaining culture.

On October 14, 2009, all fish were measured for total length (nearest mm), weighed (nearest g), tagged in the body cavity with a passive integrated transponder (PIT) tag (Prentice et al. 1990), and fin-clipped for genetic sex identification (see below). After tagging, all fish were subsequently pooled and reared

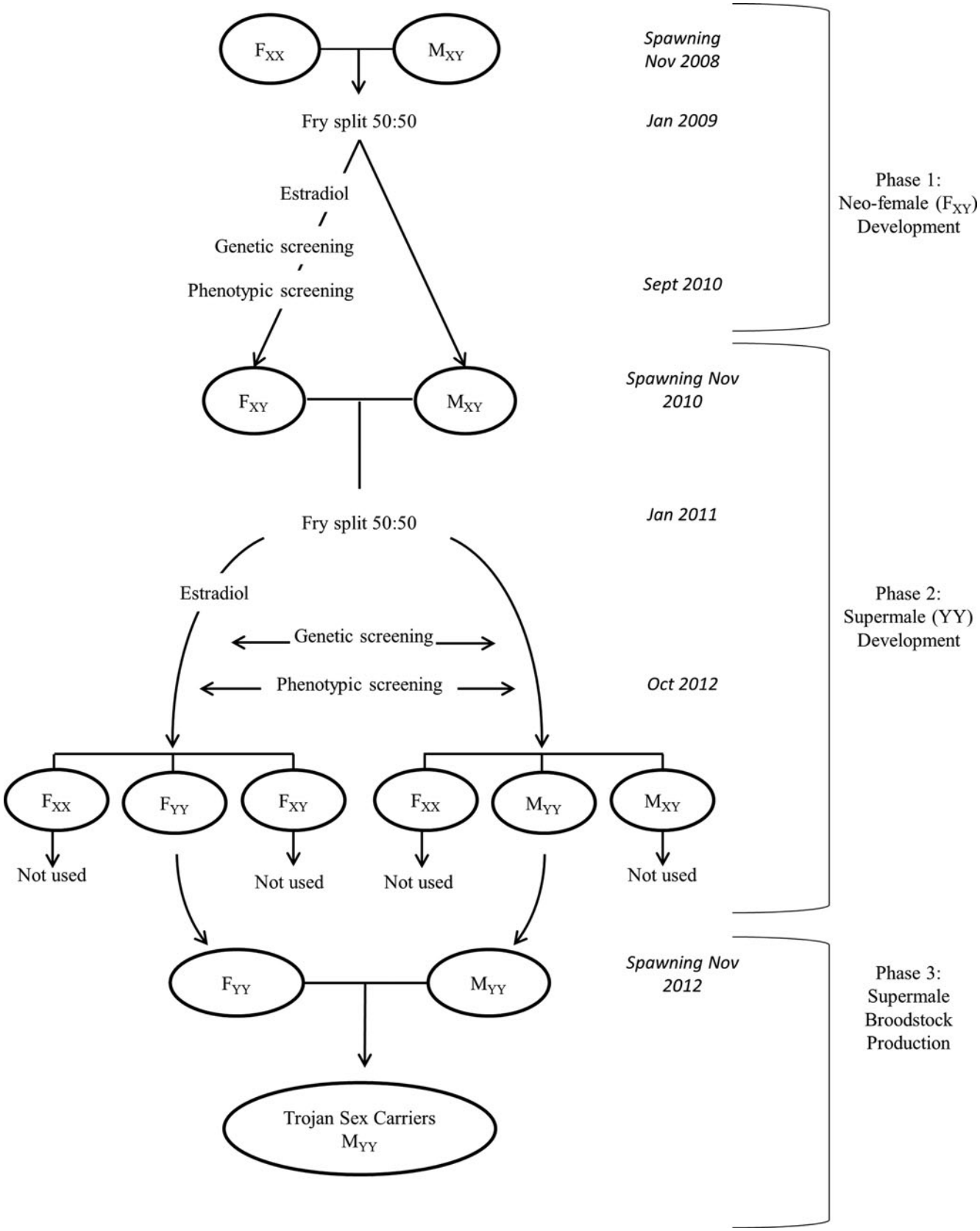

FIGURE 1. Schematic outlining the general method of Trojan YY Brook Trout production, 2008–2012.

together until maturation. During this rearing period, genetic sex markers were developed using a sample of unrelated known-sex Brook Trout to enable identification of putative neofemales in the estradiol treatment groups. This effort proved successful (see online Supplement for details) and by late October 2010, a suite of informative microsatellite markers were run on fin clips from all fish in the treatment lots to individually identify potential egg-laying neofemales (F_{XY}) and standard XX females. All genotypic XX females from the treatment lots were culled leaving only putative neofemales.

The remaining fish in the treated and untreated groups were then examined physically to determine maturational status (Figure 1). Although phenotypic sex could be determined in most maturing fish via secondary sex characteristics, all genetic XY fish in the treatment groups were examined via a hand-held ultrasound system (SonoSite Vet180Plus) to identify egg producing neofemales, which were held separately until mature. All identified neofemales were either spawned in Phase 2 or were subsequently necropsied to evaluate the proportion successfully feminized.

Once both genotypes and phenotypes for individually tagged study fish were known, we retrospectively compared the size of treated F_{XY} neofemales with that of untreated M_{XY} males. A first comparison was made for data collected on October 14, 2009, 309 d posthatch (221 d after termination of estradiol treatment). Mean total lengths (mm) and weights (g) of fish were compared from both groups via confidence interval (CI) examination; if overlap existed, we calculated the 95% CI around the difference between two estimates and considered these estimates statistically different if the CI did not include zero (Zar 1996; Johnson 1999). We also compared mean lengths (weights were not taken) for the same individually PIT-tagged F_{XY} neofemales and M_{XY} males later in the rearing cycle at 797 d posthatch (709 d after estradiol treatment) and constructed and evaluated CIs as above.

Phase 2: Supermale development (F_{YY} and M_{YY} fish).—Phase 2 of this study involved the development of supermale fish, defined in this study as either egg- or sperm-producing fish with a YY genotype. During the week of November 10, 2010, eight F_{XY} neofemales were crossed with eight standard M_{XY} males produced from corresponding untreated egg lots in Phase 1 to produce eight unique family groups. Developing eggs from the eight crosses were separated by spawn pairings and reared as in Phase 1. Standard genotypic crosses were expected to yield 50% XY males, 25% XX females, and 25% YY males, with the latter group (supermales) being sought for the next generation TYC broodstock (Cotton and Wedekind 2007).

In January 2011, prior to first feeding, $F_{XY} \times M_{XY}$ progeny were split into equal-sized treated and untreated family groups of approximately 400 fish/group. The treatment groups were again fed estradiol-treated starter feed (described above) for a period of 60 d and then returned to standard commercial (Rangen) fry diets and reared as above. Untreated groups from the same eight families were fed an identical untreated diet. Fish were reared by individual spawn pairings in 39-L isolated tanks until they were large enough for tagging.

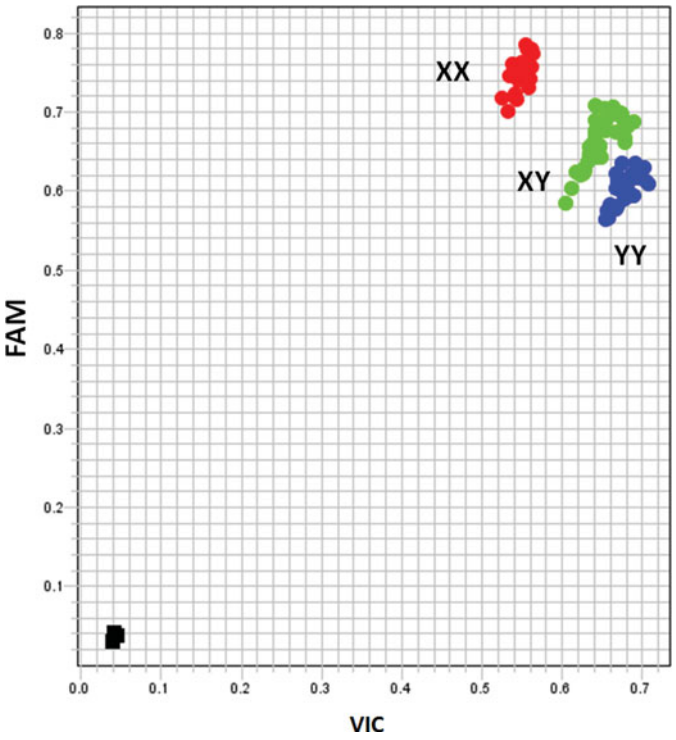

FIGURE 2. An allelic discrimination plot showing diagnostic clustering of XX females, XY males, and presumed YY supermales from a subsample (93 out of 416) of individually PIT-tagged Brook Trout reared in an Ashton Fish Hatchery raceway in October 2012. The VIC fluorophore (*x*-axis) is associated with the probe for the Y-specific product (males), while the FAM fluorophore (*y*-axis) labels the autosomal product. The black squares on the bottom left of the plot are no-template controls, which omit DNA from the PCR reaction to verify the absence of contamination across samples and reagents.

On August 4, 2011, 26 fish from each treated and untreated family group (416 fish total) were PIT-tagged, fin-clipped for genetic sexing, and measured for length (mm) and weight (g). After tagging, fish were subsequently combined in a common concrete nursery raceway at the Ashton Fish Hatchery and reared to maturity. Culture techniques followed standard hatchery protocols for salmonids (density, flow indices), and rearing temperature remained a constant 10°C from a spring-fed water source. Genetic markers as described above were used to individually identify XX, XY, and YY fish in the raceway (Figure 2). The observed yields of XX, XY, and YY fish were compared to expected yields obtained via standard Punnett square calculations. In October 2012, maturing feminized supermales (F_{YY}) and supermales (M_{YY}) in the raceway were identified by combining both genetic marker information and observed phenotypic secondary sex characteristics (Figure 1).

Once genotypes and phenotypes were available, mean lengths and weights of all PIT-tagged F_{YY} and M_{YY} fish as measured on August 4, 2011, at 213 d posthatch (125 d after estradiol treatment termination) were compared by examining 95% CIs, as above. On November 20, 2012 (474 d later), with spawning imminent, weights from a subsample of fish measured in August 2011 were taken from the five groups of fish present

in the common raceway (F_{XX}, F_{XY}, F_{YY}, M_{YY}, and M_{XY} fish). Mean weight and 95% CIs were constructed and evaluated as above. After these measurements, all F_{XX}, F_{XY}, and M_{XY} fish present in the raceway were culled, leaving only F_{YY} and M_{YY} fish (i.e., the supermale broodstock).

Phase 3: Supermale broodstock production.—Phase 3 of this study involved the production of YY supermales in large enough numbers to both renew the supermale broodstock and support future research-scale stocking of M_{YY} fish if deemed warranted. In November 2012, all putative M_{YY} supermales identified previously via genetic markers and phenotypic characters were checked for milt expression. Sperm motility checks were performed on a subsample ($n = 21$) of the M_{YY} supermales prior to use to ensure the presence of motile sperm once activated (Mounib 1978). Of the 21 M_{YY} fish examined, milt from 9 individuals was cryopreserved for use in future years using techniques described by Cloud et al. (1990) and Wheeler and Thorgaard (1991). After cryopreservation, milt from one supermale (one 0.5 mL straw) was thawed and activated to confirm the presence of live, motile sperm cells.

A total of 51 putative F_{YY} fish were available for spawning, but many were deemed unsuitable for use (see Results below). Throughout November 2012, 19 spawning crosses of $M_{YY} \times F_{YY}$ fish were performed by bisecting the body ventrally, manually removing ovulated eggs, and fertilizing them with stripped milt. Of 51 potential F_{YY} broodfish, 48 were necropsied postspawn or postmortem to determine their degree of feminization. These fish were classified as supermales, feminized supermales, or intersex. All eggs produced were incubated by family type in vertical-stack incubators until swim-up to confirm live production of M_{YY} fry.

Progeny tests.—To confirm the production of YY broodstock, we completed confirmatory crosses with known genetic sex individuals (e.g., Mair et al. 1997) for a number of spawning crosses in Phase 3 above. Crosses were made between identified YY individuals and known XX and XY individuals. For each successful confirmatory cross, we lethally sampled up to 22 fry offspring, extracted their DNA, and screened them with the allelic discrimination assay (see online Supplement). The identification of any fry with an XX genotype from these crosses would be of concern, because it would suggest that the YY broodstock had been incorrectly sexed genetically. All screening included a known XX female to ensure that we could, in fact, detect a female chromosome in each cross if one was present.

RESULTS

Development of Brook Trout Sex Markers

Our search for genetic sex markers proved successful. For the presence–absence assay, the genotypic sex determination matched the observed phenotypic sex for all individuals (90 out of 90). For the TaqMan-based allelic discrimination assay, the genotypic sex determination also matched the observed phenotypic sex for all individuals (90 out of 90). Using these markers, a total of 189 XX females (out of the fish being reared to initiate Phase 2) were identified from genetic clips and culled from the F_{XY} spawning pool. Based on the TaqMan-based allelic discrimination assay and on progeny test results (see below) we were able to successfully discriminate between XY and YY males (Figure 2).

Trojan Y Chromosome Brook Trout

Phase 1: Neofemale (F_{XY}) development.—The F_{XY} Brook Trout fry treated with estradiol were initially smaller than the untreated males produced by the same parents, but the difference narrowed as rearing progressed. At 309 d posthatch (221 d after estradiol treatment termination), the relative mean weights and lengths of F_{XY} neofemales were 30% and 11% less, respectively, than that of standard M_{XY} males produced from the same parents; both size comparisons were statistically significant

TABLE 1. Comparison of mean weight (g) and length (mm) for estradiol-treated and untreated Brook Trout during two phases of TYC production. The 95% confidence intervals are given in parentheses and blank cells indicate values that were not measured.

Production phase	Year spawned	Putative sex	Group	Sample date	Days posthatch	Days after treatment	Sample size	Mean weight	Mean length
1	2008	F_{XY}	Treated	Oct 2009	309	221	143	21.1 (20.0–22.2)	126.3 (124.3–128.3)
	2008	M_{XY}	Untreated	Oct 2009	309	221	31	30.6 (26.8–34.4)	141.7 (136.2–147.2)
	2008	F_{XY}	Treated	Feb 2011	797	709	143		337.1 (333.5–340.7)
	2008	M_{XY}	Untreated	Feb 2011	797	709	30		329.3 (322.2–336.4)
2	2010	F_{YY}	Treated	Aug 2011	213	125	208	9.3 (8.6–10.0)	92.9 (91.8–94.0)
	2010	M_{YY}	Untreated	Aug 2011	213	125	208	13.5 (12.9–14.1)	106.4 (105.0–107.8)
	2010	F_{YY}	Treated	Nov 2012	687	599	15	422.8 (372.7–472.9)	
	2010	M_{YY}	Untreated	Nov 2012	687	599	7	469.0 (342.1–595.9)	
	2010	Fxx	Untreated	Nov 2012	687	599	6	326.1 (265.8–386.4)	
	2010	F_{XY}	Treated	Nov 2012	687	599	29	373.5 (340.5–406.5)	
	2010	M_{XY}	Untreated	Nov 2012	687	599	20	417.5 (382.5–452.5)	

TABLE 2. Fecundity of neofemale (F_{XY}) fish (i.e., green eggs) and survival to eyed and ponded stages for eight individual Brook Trout crosses ($M_{XY} \times F_{XY}$) in Phase 2 of supermale (M_{YY}) production.

	Number of eggs by stage			Egg survival by stage (%)	
Cross number and mean	Green	Eyed	Ponded	Green to eyed	Eyed to ponded
1	1,393	985	840	70.7	85.3
2	1,050	995	920	94.8	92.5
3	1,713	1,580	1,507	92.2	95.4
4	842	800	757	95.0	94.6
5	1,242	1,227	1,177	98.8	95.9
6	1,011	902	838	89.2	92.9
7	1,472	1,211	1,022	82.3	84.4
8	1,526	1,238	1,076	81.1	86.9
Mean	1,281	1,117	1,017	88.0	91.0

(Table 1). However, by 709 d posttreatment and within a few days of spawning, the neofemales were similar in length to standard males (difference = 8 mm; 95% CI = −0.2 to + 15.7 mm).

The sex reversal of genetically male (M_{XY}) Brook Trout to F_{XY} neofemales did not prove difficult. As noted above, eight neofemales were spawned with normal male fish. Necropsies performed on the remaining 216 putative neofemales genetically identified from the estradiol treatment group (but not used in supermale production) indicated a near 100% success in full feminization to neofemales, as only one individual intersex fish was observed. Thus for all treated XY Brook Trout reared to maturity, 223 of 224 fish (99.6%) had fully formed, functional ovaries.

Phase 2: Supermale development (F_{YY} and M_{YY} fish).—The fecundity of F_{XY} neofemale Brook Trout at age 2 ranged from 842 to 1,713 eggs/fish and averaged 1,281 eggs (Table 2). The 10,249 green eggs produced, and our subsequent rearing efforts, yielded 8,938 eyed eggs or 1,117 eyed eggs/female. Survival for the progeny of egg-laying neofemales averaged 88% to eye-up and 91% from eye-up to ponding.

The estradiol treatment regimen appeared to negatively affect the early growth of feminized supermales relative to untreated fish, but by the time spawning occurred, F_{YY} growth was either the same as or superior to several untreated groups. The mean weights and lengths of F_{YY} fish 213 d posthatch (125 d after estradiol treatment termination) were 31% and 13% less, respectively, than that of M_{YY} fish; both size comparisons were statistically different (Table 1). At spawning (599 d after estradiol treatment) M_{YY} and F_{YY} weights were not statistically different (difference = 46 g; 95% CI = −90 to +183 g), though this finding was likely due to small sample sizes (Table 1). Though similar in weight to M_{YY} and F_{XY} fish at spawning, F_{YY} fish were 30% heavier on average than standard untreated F_{XX} fish (Table 1). This size discrepancy was statistically different (difference = 97 g; 95% CI = 18–175 g).

Using genetic sex markers and a phenotypic screening of prespawners, 51 maturing F_{YY} fish and 49 maturing M_{YY} fish were preliminarily identified in October 2012. Regardless of phenotypic sex determinations, a total of 100 YY fish were confirmed using genetic markers. Genotypic expectations for the 416 fish reared to maturity in Phase 2 predicted 104 homozygous YY individuals. In general, there was little difference between expected and observed production in the three genotypic groups (Table 3).

Phase 3: Supermale broodstock production.—The phenotypic sex of maturing M_{YY} supermales was apparent with spawning coloration and pronounced secondary-sex characteristics (e.g., development of a kype, laterally compressed body, spermiation) typical of normal (M_{XY}) maturing Brook Trout males (Figure 3A–C). Live, motile sperm were expressed from all M_{YY} supermales examined microscopically (21 of 49 were examined). The presence of live, motile sperm cells in a cryopreserved specimen was also confirmed; thus, sperm from the 2012 supermales will be available in the future.

Of the 51 putative F_{YY} fish identified from prespawning checks and genetics, a total of 48 were necropsied postspawn to determine the degree of feminization. Of these, 45 (94%) were classified as intersex, with the presence of mature testes and ovaries in various states of maturity (Figure 3D). Two fish (4%) were identified as true males (mature testes, no maturing oocytes present), and only one fish (2%) was classified as a complete phenotypic female (only paired ovaries present, no evidence of intersex).

There were 14 of the 48 (29%) putative F_{YY} fish that failed to produce external phenotypes typical of "standard" female

TABLE 3. Expected and observed genotypes of 416 progeny from eight $F_{XY} \times M_{XY}$ parent crosses in November 2010 based on standard Punnett square calculations.

Genotype	Expected	Observed	Percent agreement
XX	104	98	94.2
XY	208	218	104.8
YY	104	100	96.2

FIGURE 3. Photographs of YY supermale Brook Trout at Ashton Fish Hatchery, November 2012, showing **(A)** an untreated M_{YY} supermale (laterally compressed body, kype presence, coloration), **(B)** a feminized supermale (F_{YY}) on the left versus an untreated supermale (M_{YY}) on the right (note the phenotypic differences), **(C)** the same fish, with the M_{YY} fish on top and the F_{YY} fish on the bottom, and **(D)** an estradiol-treated (intersex) supermale (F_{YY}) that is an external phenotype female and produces eggs but shows the presence of testes and vas deferens.

Brook Trout, and they were culled. The external appearance of these fish varied but was generally more characteristic of the male phenotype (in coloration, kype, and lateral compression), suggesting incomplete feminization. Several of these fish produced viable (motile) milt when checked for the presence of ovulated oocytes, and necropsies later confirmed the presence of fully developed vas deferens in these instances.

Of the remaining 34 putative feminized supermales (F_{YY}) produced, 25 fish demonstrated predominantly female-like external phenotypes (pronounced ovipositor, color, head and body shape) and the remaining 9 displayed varying degrees of both male (coloration, kype, lateral compression, milt production) and female (ovipositor, body shape, mature oocytes) phenotypes (Figure 3D). Of the 19 actual $F_{YY} \times M_{YY}$ crosses made, 6 failed to produce viable progeny (eyed eggs) while 13 yielded approximately 5,500 viable YY green eggs. All surviving eggs were incubated by family type in vertical-stack incubators until hatch, at which time approximately 500 supermales were isolated for future Trojan supermale broodstock and the balance of approximately 4,500 were combined and reared for potential future field experiments. Although the majority of successful egg-producing parents in Phase 3 were intersex, we subsequently refer to them as feminized supermales (F_{YY} fish) in this paper.

Progeny tests.—All progeny test crosses were not successful, but results of genetic screening indicated that we did, in fact, feminize YY supermales to produce egg-yielding fish. As noted above, the identification of fry with an XX genotype would be concerning as it would indicate that the YY broodstock had been incorrectly genetically sexed. We obtained fry samples from 16 separate crosses, with a total of 231 successfully genotyped (93%) out of the 249 sampled (Table 4). No samples from confirmatory crosses exhibited XX genotypes; thus, we are confident that we successfully produced egg-laying supermale Brook Trout (Table 4).

TABLE 4. Progeny test of Brook Trout fry screenings from 16 confirmatory crosses via the diagnostic TaqMan-based allelic discrimination assay.

Trojan parent genetic sex and total	Confirmatory parent genetic sex	Number of fry successfully genotyped	Number of XX genotypes observed
YY	XY	15	0
YY	XX	16	0
YY	XX	16	0
YY	XY	16	0
YY	XX	15	0
YY	XX	13	0
YY	XY	5	0
YY	XX	20	0
YY	XY	11	0
YY	XX	9	0
YY	XY	22	0
YY	XY	21	0
YY	XY	5	0
YY	XY	6	0
YY	XY	20	0
YY	XY	21	0
Total		231	0

DISCUSSION

Although the process we used to produce a TYC broodstock for Brook Trout is complex (Figure 1), the monosex production of numerous species has been accomplished for decades by commercial aquaculturists because it is often economically advantageous. For example, in Nile Tilapia *Oreochromis niloticus*, producing monosex YY progeny at the production scale solves the twin problems of early maturation and uncontrolled pond reproduction, thereby increasing yields by up to 58% compared with mixed Nile Tilapia of the same strain (Mair et al. 1995). In salmonids, the monosex culture of all-female lines of Rainbow Trout *Oncorhynchus mykiss* approached commercial feasibility by 1975 (Simpson 1976) and has been the norm for over two decades, largely to eliminate early maturation and lower product quality in males (Bye and Lincoln 1986; Piferrer 2001; Razmi et al. 2011). More recently, Wang et al. (2008) sought to produce a monosex male stock of supermale Bluegill *Lepomis macrochirus* due to the superior growth of males in commercial aquaculture settings. In all of these cases, monosex culture required the sex reversal of parental broodstock via early exogenous exposure of fry to sex hormones in a manner similar to that reported in the present study. Such methods have become ubiquitous, and estrogen has been administered to over 56 different fish species worldwide using at least 12 different estrogenic substances (Piferrer 2001).

The results of our Brook Trout work have established that broodstock-scale production of M_{YY} fish for possible use as TYC control agents is feasible in a salmonid and can be done at modest cost. The work took 4 years, but broodstock development was completed with minimal manpower needs, which were primarily focused in the 2–3-d spawning period at the end of each production phase and during a single day of PIT-tagging and fin-clipping. Nearly all of the elapsed time from project initiation in fall 2008 to successful F_{YY} and M_{YY} broodstock spawning in fall 2012 was needed simply to allow fish to mature between production phases. Feed costs for rearing TYC fish in all phases was minimal, roughly US$700. Overall, the total costs for the development of this broodstock, including the genetic testing, feed, and labor, were less than $10,000, in large part because a presumably sex-linked AFLP sequence had already been identified and the costs of developing a working sex marker were minimal. Once confirmatory testing was completed, the consumables cost per fish to screen the sex marker was only about $5.

This study developed sex-specific assays for Brook Trout from DNA sequences identified using AFLP technologies. The

accuracy of our sex-specific assays has not been thoroughly tested in Brook Trout across their range. Previous genetic studies indicate that Brook Trout across their native range are subdivided into two major lineages (Northern and Southern; Danzmann et al. 1998). Further testing of these sex-specific assays should include additional populations from both lineages. The development of TYC technology in other salmonid species will greatly benefit from the recent discovery of *sdY* (sexually dimorphic on the Y chromosome), the master sex-determining gene in Rainbow Trout (Yano et al. 2012a). The confirmation that this gene is conserved in other salmonid species (Yano et al. 2012b) suggests that it should be straightforward to develop sex-specific assays for species within this family. Development of TYC technology in other species besides salmonids will also require the identification of sex-specific markers. Several recent studies have demonstrated the utility and substantial cost reduction of restriction-site associated DNA sequencing (RAD-seq) technology for resolving sex-determining systems and identifying sex-specific markers in a variety of organisms that lack visually heteromorphic sex chromosomes and that do not have reference genomes (e.g., Palaiokostas et al. 2013; Gamble and Zarkower 2014).

The availability of such sex markers has been identified as one of the crucial steps in the development of a TYC program (Cotton and Wedekind 2007), and the lack of such markers has been one of the major obstacles to male monosex culture in general (Beardmore et al. 2001). When developing a monosex stock using sex reversal, such markers eliminate the need for time-consuming progeny tests that, for species like Rainbow Trout, can often take 3 years (Kirankumar et al. 2003). Devlin et al. (1994) developed a rapid PCR-based sex marker for Chinook Salmon *O. tshawytscha* for a similar reason. The development of Brook Trout sex markers in the present study, including a method to differentiate YY from XY males, reduced TYC broodstock production time markedly. For example, it took Mair et al. (1997) 5.5 generations to develop YY supermale broodstock in Nile Tilapia, largely due to the lack of genetic tools and the need to grow fish to older ages to verify the sex of specific individuals before spawning them. In contrast, our development of genetic markers for Brook Trout and use of PIT-tagging and the other production methods employed enabled a relatively short development time of only three generations and 4 years for the development of a supermale broodstock.

Our work on Brook Trout also met the second major challenge regarding the potential utility of a TYC approach for eliminating a given exotic species: the ability to feminize male fish for egg production (Cotton and Wedekind 2007). The estradiol-infused food approach of Johnstone et al. (1978) readily feminized XY fish into neofemales at a high rate (99.6%) in the first production phase of the program. We also cultured egg-producing supermales, a vital step towards the development of YY technology on a large aquaculture scale (Mair et al. 1997; Liu et al. 2013). We are aware of five prior attempts to feminize YY supermales (Yamamoto 1967; Vera Cruz et al. 1996; Mair et al. 1997; Scholz et al. 2003; Liu et al. 2013), and in all instances many YY fish of several species proved capable of egg production. Furthermore, large-scale production of supermale fish was accomplished in several of these studies, specifically for the Nile Tilapia, Japanese Medaka *Oryzias latipes*, and Yellow Catfish *Pelteobagrus fulvidraco (*Mair et al. 1997; Scholz et al. 2003; Liu et al. 2013). Despite our success in producing egg-laying supermale Brook Trout, we clearly experienced difficulties achieving complete feminization. Instead, nearly all fish in the estradiol-treated YY broodstock (93.8%) displayed intersex characteristics, a sign of suboptimal steroid treatment (Pandian and Kirankumar 2008). We circumvented the problem of self-fertilization of these broodfish during artificial spawning by bisecting the body cavity ventrally and manually removing ovulated eggs.

Additional experimentation will almost certainly yield better supermale feminization rates. For example, immersion in estradiol-treated rearing water for 2-hr periods when about 50% of the eggs have hatched often provided nearly 100% rates of sex reversal in salmonids (Piferrer and Donaldson 1989, 1994; Feist et al. 1996). Alternatively, a combination of immersion and feed treatment often produces similarly high rates of feminization (Goetz et al. 1979; Feist et al. 1996). Perhaps the most likely recipe for optimal feminization of supermale Brook Trout are two such immersion and dietary estradiol treatments, as suggested by Parks and Parks (1991). It has been suggested that Brook Trout have an earlier and longer sex lability period than most other salmonids, resulting in the need to initiate immersion treatments in fry well before the start of gonadal differentiation (Haffrey et al. 2009). Although our use of the Johnstone et al. (1978) recipe produced nearly 100% feminization of genetic XY males to F_{XY} neofemales, rates approaching that for feminizing genetic YY supermales to F_{YY} fish will simply require more refinement. This observation should not be surprising, since it is generally believed that supermales are more difficult to feminize than are standard males (Vera Cruz et al. 1996; Liu et al. 2013). However, the attainment of feminization rates superior to those observed in this study may not be necessary. For example, in the existing program we currently have on hand a TYC broodstock capable of producing about 15,000 supermale Brook Trout fingerlings in the upcoming year with rapid expansion being quite feasible (J. A. Heindel, unpublished data).

A recent review (Senior et al. 2012) concluded that fish that have undergone hormonal sex reversal often have a reduced gonadal somatic index, leading to questions regarding whether F_{XY} and F_{YY} fish have reduced fecundities in the hatchery. Further, individual-based modeling (Senior et al. 2013) has found that, of several life history variables examined, changes in fecundity hold the most influence over how effective a TYC program would be at eradicating fish populations. We did not collect fecundity data from our F_{YY} fish during the current work due to their relative scarcity and the high ratio of intersex fish in this early juncture of the program. However, the available data for our neofemale fish would suggest that estradiol treatment

did not result in lower fecundity and, in fact, had the opposite effect. The average fecundity of the four standard F_{XX} Brook Trout we obtained from Story Hatchery in Wyoming to start the program averaged only 757 eyed eggs/female, similar to their hatchery average for a standard 2-year-old at 716 eggs/female (S. Diekema, Wyoming Game and Fish Department, unpublished data). However, both of these values are well below the average fecundity observed (1,281 eggs/female) for 2-year-old F_{XY} fish in the present study (Table 2). This estimate also exceeded that of Story Hatchery 3-year-olds at 1,180 eggs/female (Diekema, unpublished data). Thus despite the finding of Senior et al. (2012) regarding the reduced gonadal somatic index of sex-reversed fish and the subsequent suggestion that their fecundity may be reduced (Senior et al. 2013), we did not observe this for neofemale (F_{XY}) Brook Trout. For future broodstock planning purposes, additional observations are needed to evaluate this possibility for feminized supermales.

In their summary, Senior et al. (2012) also concluded that the growth of sex-reversed fish appeared to slow during hormone exposure, although such fish subsequently caught up in size relative to untreated fish. These observations are consistent with the findings in the present study. While we did not directly compare the survival of treated and untreated groups, during early rearing both eggs and fry produced from neofemales experienced survival rates comparable to normal Brook Trout reared at the Ashton facility (J. Heindel, personal observation). During both the neofemale and supermale development phases of the current study, the exposure of fish to estradiol initially reduced growth, but in both instances treated fish weights or lengths subsequently equaled those of untreated fish. Such a growth response to hormonal sex reversal has been observed in numerous studies, but it is unclear if the effect is due to compensatory growth that occurs in response to an earlier growth decline (Ali et al. 2003) or a positive effect of the hormone. Further, in regard to supermale growth at later rearing periods, small sample sizes limited the statistical rigor of the comparisons, F_{YY} and M_{YY} fish were the two heaviest of the five Phase 2 study groups measured at the end of two rearing years. Thus in the hatchery at least, our results show that estradiol treatment does not reduce Brook Trout growth. This concurs with Johnstone et al. (1978) who reported that the growth of an all-female stock treated with estradiol was identical to that of control females. Estradiol treatment in some species actually improves growth performance, such as in Yellow Perch *Perca flavescens* for which exogenous estrogen exposure stimulated food consumption (Malison et al. 1988).

The present work demonstrating the successful production of a supermale broodstock in Brook Trout is the first step in a possible TYC program, but significant hurdles remain. The next logical step forward would be to conduct stochastic population simulations to evaluate the probabilities of success for variants of a TYC Brook Trout program and to guide experimental field research. A major issue not mentioned by prior TYC modeling studies is the need for regulatory approval to release feminized fish or their progeny into the environment. Feist et al. (1996) cautioned on the need to check the rules and regulations of the U.S. Food and Drug Administration (FDA) before using hormonal sex control agents. The acquisition of estradiol for research in fish must be done by a licensed veterinarian from a licensed compounding pharmacy. Further, its use requires stringent storage and record keeping, even if treated fish are not anticipated to be released into the environment (USOFR 2015). If the use of hatchery-reared supermale Brook Trout as a biological control agent is undertaken as a field experiment, the use of estradiol for this purpose in the USA may involve oversight by the FDA.

Another important issue will be the public acceptance of a TYC program, although for several reasons we may believe this should not pose a major challenge. First, the sex reversal methods used to produce the TYC Brook Trout broodstock in this study are virtually identical to routine monosex aquaculture techniques currently in use across most of the developed world for freshwater fish production (e.g., Rainbow Trout, Common Carp *Cyprinus carpio*, and multiple species of tilapia *Oreochromis* spp.). From a food safety standpoint, assuming untreated M_{YY} fish prove efficacious by themselves, monosex aquaculture and supermale production via the indirect approach we used should not be objectionable since no fish destined for human consumption have been treated with hormones (Bye and Lincoln 1986). Worldwide, a large percentage of commercially grown food fish are currently the progeny of sex-reversed fish, and hundreds of millions of all-female salmonids, including Rainbow Trout and Brook Trout, have been produced by commercial growers and also stocked for sportfishing (e.g., Bye and Lincoln 1986). Second, the TYC method is specific to the target exotic species, not native species, so there is little to no possibility of direct ecological collateral damage (Stelkins and Wedekind 2010).

Perhaps most importantly in regard to public acceptance, a TYC fish is not a genetically modified food organism, or GMO, since no new genetic material is infused into the released fish. Instead, the sex-reversal manipulations comprise a simple reassortment of preexisting sex chromosomes among individuals, and therefore, a TYC stocking program is reversible via cessation of stocking supermales (Cotton and Wedekind 2007; Stelkins and Wedekind 2010). Largely for this reason, a TYC program is the least likely of various "genetic" approaches for exotic fish suppression to generate public controversy (Thresher et al. 2014).

Finally, there may be concerns by biologists or regulatory entities over the release of estradiol into the environment from the production of supermale fish at scales similar to that which we have described. The total amount of estradiol fed to fish over 5 years in this study was 15 mg or less and its release to the environment was further reduced via charcoal filtration (Contreras-Sánchez 2001). In contrast, a single pregnant human releases approximately 6.9 mg of total estrogens in urine daily (Wise et al. 2011). Assuming a 70–90% removal rate of estrogen via primary and secondary sewage treatment (Shore and Shemesh 2003), in 7–21 d that single person would ex-

crete more estrogen directly into the aquatic environment than the worst-case amount required for the production of our entire TYC broodstock over 5 years. Indeed, the amount of estrogen required to produce supermale fish in this study is inconsequential compared with the amount of natural estrogen discharged into the environment by humans and livestock (Lange et al. 2002), the latter being about 49 metric tons annually.

Conclusion

To our knowledge, this study represents the first successful attempt to obtain viable eggs from a supermale YY salmonid. In 4 years, with minimal cost, we were able to produce a viable YY broodstock comprised of both egg-laying F_{YY} supermales and sperm-producing M_{YY} supermales. To date, this broodstock has produced large numbers of M_{YY} Brook Trout progeny, including over 5,000 in 2012 and over 15,000 in 2014. Despite these positive results, much work remains before TYC methods can be broadly field tested. The highest priorities would be to (1) conduct a stochastic population simulation study to ascertain whether a stocking strategy could likely drive exotic populations toward extirpation, (2) refine feminization methods for supermale Brook Trout, and (3) obtain FDA authorization to conduct field experiments evaluating the results of releasing TYC fish. Despite the successful creation of a YY male Brook Trout broodstock, some skepticism seems advisable in regard to the TYC concept as there are many obstacles yet to be overcome. Nonetheless, there are few viable nonpiscicide options available for the complete removal of exotic fish populations, and future work on the topic is thus clearly warranted.

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