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Induction and viability of tetraploids in brook trout (*Salvelinus fontinalis*).

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Short Version of Title: Tetraploidy in Brook Trout

Abstract

Brook trout (*Salvelinus fontinalis*) populations are threatened by introduction of invasive species, habitat loss, and habitat degradation in their native range; and are a problem invasive species in western Unites States and Canada, and in Europe. Stocking sterile triploids has been promoted as an approach to reduce negative effects of stocking of brook trout for recreational fishing on native fish populations. Crossing a tetraploid with a diploid is a method of triploid

production that may help hatcheries meet demand. We induced tetraploidy in brook trout by application of $633 \text{ kg}\cdot\text{cm}^{-2}$ of hydrostatic pressure for 8 minutes at 70-72.5% of the first cleavage interval. Yields of above 50% tetraploid progeny at hatching were readily achieved although few animals reached one year of age. We crossed a male tetraploid with female diploid fish and produced interploid-triploids with eyeing rates in excess of 50%, demonstrating male tetraploids are fertile and capable of siring triploid progeny. Female tetraploid fish were reared to 16 months post-hatching and possessed follicles in secondary vitellogenesis, suggesting tetraploid females are also fertile. Tetraploid induction rates in excess of 96% were achieved applying the same hydrostatic pressure treatment to zygotes of tetraploid x diploid crosses at 30 minutes post-fertilization.

Key words: Aquaculture, fishery management, polyploidy, trout, reproduction

Introduction

Triploidy has been promoted as a tool to induce sterility and thereby reduce negative effects of stocking of brook trout (*Salvelinus fontinalis*) for recreational fishing on native fish populations including maintaining genetic diversity and integrity of local brook trout populations (Ihssen et al. 1990; Habera and Moore 2005; Budy et al. 2012). Maintaining small populations of genetically diverse species of native fish is of increasing importance as the species adapts to changing environmental conditions including climate change (Flebbe et al. 2006; Hudy et al. 2008). Conventional means of producing triploid brook trout by shocking embryos by means of a temperature or pressure shock to induce retention of the second polar body during meiosis, induced-triploids, are established and in practice (e.g. Dubé et al. 1991; Galbreath et al. 2000;

Budy et al. 2012). Meeting increasing demand for triploids can be difficult for existing hatchery infrastructure and resources. Triploid production is more labor intensive and often results in lower survival and increased deformity rates compared with diploid production; it requires additional equipment to deliver the shock treatment; and certification of triploid induction rate is sometimes mandated. Production of interploid-triploids generated by crossing a tetraploid ($4n$) with a diploid ($2n$) is an alternative approach first developed for rainbow trout (*Oncorhynchus mykiss*) that may benefit brook trout fisheries management and aquaculture (e.g. Chourrout et al. 1986; Myers and Hershberger 1991; Weber et al. 2013, 2014). Development of a tetraploid broodstock allows production of triploid fish without needing to shock all production fish to induce triploidy. Furthermore, milt from tetraploids produced from a centralized source can be easily shipped to increase the amount of hatcheries with capabilities to produce triploids. Studies in rainbow trout also suggest interploid-triploids exhibit superior growth performance and possibly disease resistance compared with induced-triploids (Weber et al. 2013, 2014).

Tetraploidy can be induced by suppression of early cell division in the zygote (Chourrout 1984; Zhang et al. 2007). Tetraploid induction has been accomplished by pressure, temperature, or chemical shocking of zygotes in many species including tilapias (*Oreochromis* spp.; Myers 1986; El Gamal et al. 1999), channel catfish (*Ictalurus punctatus*; Bidwell et al. 1985; Goudie et al. 1995), yellow perch (*Perca flavescens*; Malison et al. 1993; Malison and Garcia-Abiado 1996), and Masu salmon (*Oncorhynchus masou*; Sakao et al. 2006). However, production of viable tetraploid broodstock animals and production of interploid-triploids using this approach has only been accomplished in a few species including rainbow trout (Chourrout et al. 1986; Myers and Hershberger 1991; Weber and Hostuttler 2012) and two Cypriniformes, the blunt snout bream (*Megalobrama amblycephala*; Zou et al. 2004; Li et al. 2006) and the mud loach

(*Misgurnus mizolepis*; Nam and Kim 2001, 2004). Tetraploid broodstocks have also been developed through interspecies distant hybridization and used for mass production of triploids, however, this approach appears limited to the unique genetics of some Cypriniformes (Luo et al. 2011; Zhang et al. 2014). Major impediments to establishment of tetraploid broodstocks has been the low survival of first generation tetraploids, reduced fertility of tetraploids, and unexpected ploidy level in progeny from tetraploid broodfish (see review, Piferrer et al. 2009). The objective of the present study was to develop a protocol for inducing tetraploidy in brook trout and determine if the tetraploid brook trout is viable and fertile.

Materials and Methods

Experimental animals and rearing conditions

Broodstock were obtained from Paint Bank Fish Culture Station (PBFCS; Virginia Department of Game and Inland Fisheries), Paint Bank Virginia, or from West Virginia University Reymann Memorial Farm (WVU), Wardensville West Virginia, or reared from these initial stocks at the National Center for Cool and Cold Water Aquaculture (NCCCWA; U.S. Department of Agriculture, Agricultural Research Service), Leetown West Virginia. The WVU fish were obtained as eyed eggs from Story Fish Hatchery, Story Wyoming. Tetraploids were produced and reared at NCCCWA or PBFCS. Fish were maintained at ambient photoperiod at each location and on flow-through spring water although sometimes on treated spring water at NCCCWA. Fish were fed commercial trout feed. Eggs were fertilized with milt pooled from a minimum of three males unless otherwise indicated. Fish were anesthetized for stripping of gametes and blood sampling using 100 mg·L⁻¹ tricaine methanesulfonate (MS-222, Western

Chemicals, Ferndale, CA). Eggs were incubated in Heath tray incubators at both locations. Additional details are provided with the experimental designs for each study. Animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals (1996) and use of animals was reviewed and approved by the NCCCWA Institutional Animal Care and Use Committee (Kearneysville, WV).

First cleavage interval analysis, polyploid induction, flow cytometry

First cleavage interval (FCI) analysis, tetraploid induction, and flow cytometry, were conducted following procedures described by Hershberger and Hostuttler (2005; 2007). First cleavage analysis to determine FCI was conducted at 10.0 ± 0.3 °C unless otherwise indicated and ‘time zero’ for determination of the time to first cleavage was the addition of activating fluid to the mixture of eggs and milt. Ten zygotes were collected at 10 min intervals from 7-10 h post insemination and immediately placed in modified Davidson’s fixative (Hershberger and Hostuttler 2005) for FCI determination. We added 150 mg of methylene blue stain to 1 L of Davidson’s fixative to enhance visualization of the cleavage furrow. The FCI was defined as the time at which 50% of the zygotes had reached first cleavage as calculated according to Hershberger and Hostuttler (2005).

Tetraploid induction in zygotes from diploid x diploid crosses was conducted by applying $633 \text{ kg}\cdot\text{cm}^{-2}$ (9000 psi) for 8 min starting between 60-75% of FCI, depending on the study. All FCI determinations were conducted at NCCCWA. Tetraploid induction was conducted at 10.0 ± 0.3 °C when conducted at NCCCWA, and ambient water temperature at PBFCS, ~ 12.5 °C. Temperature at NCCCWA was recorded using a Traceable® Fisher Scientific Digital Thermometer Model # 15-077-8 (Thermo Fisher Scientific, Waltham, MA) calibrated and

certified by the manufacture to within 0.05 °C. The FCI value was adjusted based on thermal minutes (FCI in minutes x temperature in degrees Celsius) when the temperature at which FCI was determined was different from the temperature at which tetraploidy was induced. Tetraploid induction in zygotes from male tetraploid x female diploid crosses was conducted at NCCCWA using the same hydrostatic pressure treatment applied at 30 minutes post-fertilization. Ploidy levels were determined based on DNA content of cells by use of a Becton Dickinson Flow Scan flow cytometer (Franklin Lakes, NJ) and the Becton Dickinson Cycle TEST PLUS reagent kit, staining with propidium iodide. At least 20,000 nuclei of red blood cells, or cells from embryonic or larval tissues were analyzed per run, with the same tissue type from diploid brook trout used as control materials. Ten progeny were analyzed individually for each treatment group unless otherwise noted. Blood from up to 20 fish was pooled for ploidy determination for presumptive triploid batches by pooling the same volume of heparinized whole blood from each individual before preparation for flow cytometry. Embryonic tissues were from eyed stage embryos removed from the chorion and with yolk sac removed. Tissues from larval stage fish were post-hatch with the head and abdomen, including yolk sac, removed.

Eyeing and hatching rate determination

Survival at eyeing was determined by sorting dead from live embryos by hand based on color and eye development at approximately three days before hatching, expressed as a percentage of all eggs at fertilization. Hatching rate was determined by counting all surviving fry by hand at three days post-hatching, expressed as a percentage of all eggs at fertilization. Day of hatch was considered the day at which approximately 50% of eggs had hatched. The

number of eggs at fertilization was determined for each batch of eggs based on the Von Bayer method (Von Bayer 1950) by counting the number of eggs in a six-inch trough.

Histology and oocyte diameter measurement

Histological analysis was performed in paraffin embedded, 4 µm, hematoxylin and eosin stained sections of testis fixed in Z-fix™ (Anatech, Ltd., Battle Creek, MI) and ovary fixed in Prefer™ (Anatech, Ltd., Battle Creek, MI). Slides were viewed using a Nikon Eclipse E600 compound microscope. Images were captured with a Penguin 150CL cooled CCD camera (Pixera) via Image-Pro Plus 6.2 (Media Cybernetics, Silver Spring, Maryland) image analysis software. Follicle measurements were conducted in ovarian samples stored in 70% EtOH for two days by measuring the diameters of 19-22 of the largest cohort of follicle-enclosed oocytes to the nearest 100 µm using a dissecting stereomicroscope fitted with an ocular micrometer.

Experimental design

We conducted two studies investigating the effect of applying pressure at different times after insemination based on percent of FCI, on ploidy induction and survival at eyeing and hatching. In Study 1 green eggs (unfertilized ova) and milt were obtained from 2-yr-old fish from WVU. Three crosses, each consisting of ova from a single female and milt from a pool of three males were used in the study. An aliquot of fertilized eggs from each batch were pressure treated at 60, 62.5, 65, 67.5, or 70% of FCI using the FCI calculated for each batch of ova the previous day. In Study 2 we extended the time frame up to 75% of FCI and compared using ova from 2-yr-old and 3-yr-old fish. Milt and green eggs were obtained from 2-yr-old and 3-yr-old fish from PBFCS. Six crosses, each consisting of ova from a single female and milt from a pool

of three males were used in the study. Three of the crosses included ova from 2-yr-old females and three from 3-yr-old females. An aliquot of fertilized eggs from each batch were pressure treated at 60, 65, 67.5, 70, 72.5, or 75% of FCI using the FCI calculated for each batch of ova the previous day. In both studies samples were collected for ploidy determination at approximately three days after 50% of the eggs had hatched. Ten fish were analyzed individually for each treatment group. Survival at eyeing and hatching were recorded for each treatment group in each study. Fish were retained after both studies to follow survival of the tetraploids and obtain broodstock.

A third study was conducted to determine the effect of incubation temperature on FCI. In Study 3 milt and green eggs were obtained from 2-yr-old fish from PBFCS. Three crosses, each consisting of ova from a single female and milt from a pool of three males were used in the study. An aliquot of fertilized eggs from each batch were incubated at 10 ± 0.2 , 11 ± 0.2 , or 12 ± 0.2 °C, for collection of zygotes for FCI determination. The time to FCI expressed in thermal minutes was compared among temperature treatments.

Fertility of a single tetraploid male was investigated. A single male from Study 2, pressured at 72.5% of FCI, survived to two years of age. The male was small and produced little expressible milt so it was euthanized by an overdose of tricaine methanesulfonate ($1.5 \text{ g} \cdot \text{L}^{-1}$) and testes were excised to obtain more sperm while eggs were available. Testes were placed in a plastic bag with 3 mL of Cortland's solution per gram of tissue, slit open, and then squeezed by hand to release milt into the buffer. The testes were then removed and placed in Z-fix™ for histology. The diluted milt was stored at 4°C with added oxygen. Milt from testes excised from this male was used to fertilize eggs on three separate days, up to five days after the testes were removed. Each day a separate pool of eggs from 2 or 3 females that were 22 months of age were

used to make one batch of progeny on day 1 and 2, and two batches on day 5. Sperm motility was estimated at greater than 80% by casual observation on each of these days. Ploidy was determined in ten embryos at eyeing for each of the four batches of progeny produced. Survival at eyeing was determined. Ploidy was also determined for over 40 offspring at 8 months of age for each of these batches.

A second generation of tetraploids was produced from the zygotes obtained from tetraploid x diploid crosses by applying the same hydrostatic pressure treatment to the zygotes at 30 minutes post-fertilization. Ploidy was determined in ten embryos at eyeing for each of the six batches of progeny produced. Survival at eyeing was determined. Ploidy was also determined for 11-49 offspring at 8 months of age for five of these batches.

Tetraploid fish from diploid parents were produced at PBFCS on several occasions that were not part of experiments, and fish leftover from our various studies were reared to monitor growth and survival of tetraploids, and to serve as broodstock. Since survival of tetraploids was limited, the data collected were primarily observational in nature. The methods for rearing and evaluating these fish were diverse and therefore we present the methods as part of presenting the results of these efforts.

Statistical analyses

Statistical analyses were performed using SigmaPlot 12 (Systat Software Inc., San Jose, CA) software. The means for tetraploid yield, eyeing rate, or hatch rate were compared among treatments by the two-way ANOVA model including female donor as a factor, followed by a Holm-Sidak multiple comparisons test. In no analysis was the effect of female significant but the power for this factor was always very low. In some comparisons, age of the female was also

a factor and found to be significant. When there was no interaction between time of pressure application (percent of FCI) and age, age was not included in the analysis. When there was an interaction, the data for the fish of the two different ages were analyzed separately as described above. A t-test was used to compare the FCI of two groups. All data met normality and equal variance assumptions. All data were considered significant at the $P = 0.05$ level. Data are shown as mean $\pm$ SEM.

Results

Induction of tetraploidy in diploid x diploid crosses

Timing of the start of the application of pressure treatment affected the rate of tetraploid induction as determined at hatching. In Study 1, pressure was applied at time points centering on the optimal time determined for rainbow trout, 65% of FCI (e.g. Myers et al. 1986; Weber and Hostuttler 2012). Tetraploid induction rate increased the later pressure was applied ($P = 0.007$; Fig. 1A). The range was extended in Study 2, centering on 70% of FCI. In addition, tetraploid induction rates using eggs from 2-yr-old and 3-yr-old females were compared. There was a significant time x age interaction ($P < 0.001$) so effect of time was analyzed separately for 2-yr-old and 3-yr-old fish. The highest percent of tetraploid induction was observed at 72.5% of FCI for 2-yr-old fish ($P < 0.001$) and 70% of FCI for 3-yr-old fish ($P < 0.001$; Fig. 2A).

Survival following treatment for tetraploid induction in diploid x diploid crosses

Survival to eyeing was low in Study 1, below 20% for all time points, but mortality from eyeing to hatching was minimal (Fig. 1B). Power of the analysis with $\alpha = 0.050$ was low

due in part to the high mortality (eyeing = 0.206; hatching = 0.372). There were no effects of age (eyeing, $P = 0.427$; hatching, $P = 0.746$) or time x age interactions (eyeing, $P = 0.648$; hatching $P = 0.310$) for eyeing or hatching in Study 2 so age was not included in the models. Survival to eyeing was higher in Study 2, above 40% in all treatments (Fig. 2B). Survival to hatching was low, less than 25% for all treatments (Fig. 2B). In all, survival to hatching was low in both studies with survival to hatching generally decreasing the later pressure was applied (Study 2 eyeing, $P < 0.001$; hatching, $P < 0.001$).

Survival of tetraploids to fingerling stage was extremely low for all our studies at NCCCWA and efforts at PBFCS. Often no tetraploids were found by one year of age. As an example, there were no tetraploids present from Study 1 when 300 fish were tested at nine months post-hatching. In Study 2, only the 67.5-75% of FCI treatment fish were retained past hatching. Fish were tested at swim-up for polyploidy. A total of 75 out of 87, or 86.2%, of deformed fish at swim-up were tetraploid. In contrast, only 17 out of 180, or 9.4%, of normal appearing fry were tetraploid, with the 72.5% of FCI treatment from the 2-yr-old fish having the highest percent of tetraploids, 30%. For Study 2, when the fish were nine months post-hatching, nine very stunted and deformed fish were removed from the 72.5% of FCI group for separate rearing to avoid competition with healthier animals. Five of the nine were identified as tetraploid and retained, of which only one was able to swim. At the same time, all 60 non-deformed fish from these groups that were tested were diploid. At 14 months post-hatch the remaining fish in the study were analyzed. None of the surviving normal-appearing 145 fish, 31-42 per time treatment, were tetraploid. At that time the fish averaged 214 ± 6.2 g in body weight and five had ovulated and eight were releasing milt. Best survival of tetraploids was obtained at PBFCS when 13 of 57 fish at 14 months of age were identified as tetraploid and were swimming well. However, the

tetraploids averaged 127.2 ± 13.9 g whereas the diploids weighed 340.1 ± 14.7 g. Unfortunately the ten tetraploid fish from this group that survived until 16 months post-hatching died because of a hatchery mishap. The tetraploid fish averaged 191.4 ± 21.0 g at that time.

Factors affecting FCI

The FCI did not differ significantly with incubation temperature when quantified in thermal minutes (time in minutes x temperature in degrees Celsius). The FCI were 4789 ± 25 ; 4829 ± 18 ; and 4767 ± 53 thermal minutes for 10, 11, and 12 °C respectively. The greatest difference among temperature treatments in observed mean FCI based on thermal minutes was between 11 °C and 12 °C in Study 3 and was only about 1.3% of FCI. The FCI varied among different groups of fish we observed throughout our studies. The FCIs were close among females from the same population on a given day but could differ over a 2-day span and from year-to-year from the same population. The FCI for eight females from WVU in 2010 was 481.8 ± 1.5 min on one day and 488.7 ± 1.2 min for three females two days later ($P = 0.018$), determined at 10 °C. The FCI from PBFCS females determined at 10 °C was 516.1 ± 2.9 min for four females in 2010 and 480.4 ± 0.8 min for six females in 2012 ($P < 0.001$). Although sample number was too low to allow meaningful statistical analysis, age of the female did not appear to have much of an effect on FCI. The FCI of three 2-yr-old females was 481.0 ± 1.0 min and 479.8 ± 1.3 min for three 3-yr-old females from PBFCS in 2012, determined at 10 °C. In all situations, the fish were checked for ovulation weekly so eggs were no more than nine days post-ovulation in age at the time of FCI determination.

Fertility of tetraploids

In all, only four fish were able to provide information on potential fertility of tetraploid brook trout. Ovaries were obtained from three of the ten tetraploid fish that died at 16 months post-hatching. The fish were examined after they had been dead in the tank for 1 to 2 days followed by 2 days of refrigeration. Ovarian samples were collected for histology and follicle diameter measurement. All three fish had reached secondary vitellogenesis (Fig. 3A) and mean follicle diameters of 1.04 ± 0.05 , 1.25 ± 0.06 , and 0.88 ± 0.05 mm. These data support that the female tetraploid fish would have reached sexual maturity. One of the five deformed fish from Study 2 survived to maturation. It was only 13 cm fork length despite being 16 months post-hatching when sacrificed for milt. The testes were mature (Fig. 3B insert). Shown is a testis after milt was collected (Fig. 3B). The milt was used on three occasions at 1, 2 and 5 days post-collection to make a total of four batches of triploid progeny and six batches of second generation tetraploid progeny. At eyeing, ten embryos were tested for ploidy for each batch and all 40 embryos tested from the presumptive triploid batches were determined to be triploid and all but two of the 60 embryos from the presumptive tetraploid batches were determined to be tetraploid with two triploids observed in one of the batches. Furthermore, at 8 months post-hatching a total of 336 fish from the presumptive triploid batches were tested and all identified as triploid; and 152 out of 158 fish tested from the presumptive tetraploid batches were found to be tetraploid with the remaining six from the same batch being triploid. The batch of presumptive tetraploids identified as having some triploid embryos was different from the batch with triploids at 8 months of age. Eyeing rates ranged from 49-60% for the presumptive triploid batches and 4-45% ($21.6 \pm 6.4\%$) for the presumptive tetraploid batches.

Discussion

299

300 We have developed procedures to produce limited numbers of tetraploid brook trout from
301 diploid parents and provide evidence that tetraploid brook trout are fertile. The yield of
302 tetraploids at hatching, often over 50% of all fish being tetraploid, should be sufficient for
303 development of a tetraploid broodstock. However, both pre- and post-hatching survival is poor.
304 Many fish that were identified as tetraploid were severely deformed. Even those that were
305 among the most normal looking tetraploid fish were smaller than diploid fish of the same cohort
306 by about 14 months post-hatch, most likely due to difficulty competing with the diploid fish.
307 Thus, sorting the fish early based on size and extent of deformity will likely increase chances of
308 obtaining tetraploid adults. Rearing deformed fish is not necessary as long as survival rates
309 exceed tetraploid induction rates. Increased deformity is common in shock induced polyploids
310 including triploids and results in losses at harvest (Fraser et al. 2013; Weber et al. 2014).
311 Although the occurrence of deformed fish is high in de novo tetraploids, the rate is reduced in
312 second generation tetraploids (Weber and Hostuttler 2010). Most important, the deformity rate in
313 triploids derived from crossing a tetraploid with a diploid is much lower than in shock-induced
314 triploids (Weber et al. 2014), and therefore a primary benefit of establishment of a tetraploid
315 broodstock is an overall reduction in deformity rates in triploid production.

316 The optimal time to apply pressure was 70% of FCI for eggs obtained from 2-yr-old females
317 and 72.5% of FCI for 3-yr-old females. We did not observe a clear effect of the age of the
318 brooder on tetraploid induction rate or survival to hatching, but only three fish of each age were
319 compared. Although timing of when pressure is applied is a critical determinant of tetraploid
320 induction success and survival, the pressure and duration of the applied pressure are also critical
321 factors that we did not attempt to optimize (Myers et al. 1986; Foisil and Chourrout 1992). In

rainbow trout, tetraploid induction rates of nearly 100% can be achieved with optimized protocols, although overall survival is still relatively low and yield high proportions of deformed fish (Hershberger and Hostuttler 2007; Weber and Hostuttler 2012; Weber et al. 2014). Nevertheless, with rainbow trout, some tetraploids can be obtained from each attempt at induction with some degree of regularity (Weber et al. 2013, 2014).

Correctly identifying the FCI for a particular batch of eggs has been shown to be a critical factor in successful tetraploid induction in rainbow trout (Hershberger and Hostuttler 2005, 2007) and likely is equally important for brook trout. We observed considerable year-year variation in FCI even from the same population of brook trout at PBFCS, yet there appeared to be little variation within the population in a given year, or with age even though the 2-yr- and 3-yr-old fish at PBFCS were maintained in separate raceways. Among factors shown to affect FCI in rainbow trout is the age of the ova post-ovulation (Weber and Hostuttler 2012). It is therefore likely important to keep the post-ovulatory age of the brook trout eggs similar for when FCI is determined and tetraploidy is induced. Incubation temperature has a large effect on embryonic development rate in poikilotherms. It is not always possible to determine FCI and induce tetraploidy at the same temperature. The difference in FCI in thermal minutes when FCI was conducted at 10, 11, and 12 °C were not significantly different supporting the use of the thermal minute conversion.

As mentioned, although tetraploidy has been successfully induced by shocking of embryos in many species of fish, to our knowledge fertile adults have only been obtained in three, two of which are Cypriniformes (Chourrout et al. 1986; Nam and Kim 2004; Li et al. 2006). It was therefore important to show a tetraploid brook trout can reach adult stage and be fertile. We have shown a tetraploid male brook trout can mature and produce milt capable of fertilization.

The observation that three female tetraploid brook trout reached well into secondary vitellogenic growth at 16 months post-hatching supports female tetraploid brook trout are also capable of producing viable gametes. It is important to note the females died from a hatchery accident and not due to health issues. Triploid brook trout at much reduced proportions have also been found to be able to produce mature oocytes, but not until they are about four years of age and with drastically reduced numbers of oocytes (Schafhauser-Smith and Benfey 2001). In contrast, follicle development appeared similar in all three females in our study in that they had follicles of similar size both among and within females, and many follicles had reached this advanced stage in each of the three females suggesting ovarian development was progressing normally.

Studies have shown tetraploids to have reduced reproductive potential. In rainbow trout first generation male and female tetraploids have low reproductive potential (Chourrout 1984; Chourrout et al. 1986; Blanc et al. 1987, 1993; Myers et al. 1986; Hershberger and Hostuttler 2005, 2007). Egg quality is improved in second generation tetraploids compared with first generation tetraploids in terms of both hatching and abnormality rates (Weber and Hostuttler 2012). Nevertheless, a continuing problem in rainbow trout is the increased diameter of tetraploid sperm impedes the sperm's ability to traverse the micropyle canal of the egg (Chourrout et al. 1986). Sperm diameter was determined to be moderately heritable in rainbow trout suggesting this impediment can be alleviated through selective breeding (Chourrout et al. 1986; Blanc et al. 1993). Sexual maturation was found to be delayed in tetraploid mud loach and blunt snout bream but fertilization and hatching rate was only reduced for progeny from female tetraploid blunt snout bream (Nam and Kim 2004; Zou et al. 2004; Li et al. 2006). Eyeing rate in the present study was consistently between 50-60% for each batch of eggs fertilized suggesting

sperm diameter might not be a critical problem for brook trout, however, sperm from only one male was used so it remains possible this individual possessed unusually small diameter sperm.

Production of even a limited number of fertile tetraploids provides potential for developing significant numbers of second generation tetraploids by crossing gametes from a limited number of tetraploids of one sex with gametes from many diploid individuals of the other sex, then shocking the embryos to cause retention of the second polar body, as in triploidy induction (see Chourrout et al. 1986). We have shown this procedure works well for brook trout. Six batches of second generation tetraploids were produced with greater than 96% of offspring being tetraploid. Survival to eyeing was low, less than 21%, but again this is more than sufficient to expand a tetraploid broodstock or to allow a hatchery with the capability to make triploids using hydrostatic pressure to establish a tetraploid broodstock by obtaining milt from tetraploids from another stock. Furthermore, few deformed tetraploids were observed at 8 months post-hatching.

Progeny from tetraploids having unexpected chromosome numbers has been reported and suggested to be an impediment to using interploidy for triploid production (see Piferrer et al. 2009). This concern derives from work with a single population of tetraploid rainbow trout. Chourrout and Nakayama (1987) reported a high incidence and diversity of unexpected chromosome numbers in progeny of tetraploid female rainbow trout which they attributed to both androgenesis and failure in chromosome reduction in the ova. Blanc et al. (1993) working with the same population of tetraploids as Chourrout and Nakayama (1987) also found some males presumed to be tetraploid, to generate progeny with a lower ploidy level than expected. On the other hand, all progeny of tetraploids were found to be of the expected ploidy level in studies by Weber and colleagues regardless if the tetraploid was male or female (Weber and Hostuttler 2012; Weber et al. 2013, 2014). Studies in mud loach have shown sires identified as

tetraploid by chromosome analysis of erythrocytes do not always produce all $2n$ sperm (Nam and Kim 2004). A low percentage of tetraploid sires were found to produce all $2n$ sperm whereas others produced all $1n$ sperm or a mix of $2n$ and $1n$ sperm. The male tetraploid in our study was identified as tetraploid by analysis of erythrocytes. We identified all 376 tested progeny of the male tetraploid brook trout crossed with diploid females as being triploids supporting the tetraploid sire produced all $2n$ sperm. Our results do not address if using tetraploid females might be problematic but they do support high percentages of triploid progeny can be expected if a tetraploid sire is used. Our findings thus far are in agreement with the recommendation that the ploidy of the sperm produced by tetraploids of any species should be confirmed before being used for triploid production, or the progeny should be tested to confirm triploidy (Benfey 1999; Nam and Kim 2004). Nevertheless, assuming these problems with ploidy are due to the incomplete action of the shock treatment on chromosome set doubling (see Zhang et al. 2007), it would be expected that true second generation tetraploid males, those derived from tetraploid parents, should always produce $2n$ gametes.

In summary, we have developed a protocol capable of generating very limited numbers of fertile male tetraploid brook trout and likely fertile female broodstock. We have shown milt from tetraploid brook trout can contain at least a high percentage of $2n$ gametes and yield good eyeing rates. Together, these findings demonstrate production of interploid-triploid brook trout is possible. Increased yield can likely be obtained by optimizing pressure and duration of pressure used in the induction process and concentrating on husbandry of deformed fish. Nevertheless, we have also demonstrated milt from a single tetraploid male can easily be used to develop a viable tetraploid broodstock by fertilizing many eggs from diploid females and then using hydrostatic pressure, as currently performed by many hatcheries to produce triploids, to

make second generation tetraploids. Thus, only a few de novo tetraploid males are needed to generate and perpetuate many tetraploid males for triploid production even if tetraploid females turn out to be sterile. Availability of tetraploids for interploid-triploid production should increase availability and decrease cost of sterile triploid brook trout for stocking. Further research is still required to confirm the requisite proportion of progeny from interploid crosses are indeed sterile.

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Figure legends

Fig. 1. Effect of applying pressure at different times after insemination based on percent of the first cleavage interval (FCI), on tetraploid induction (A) and on survival from eggs at fertilization to eyeing and three days post-hatching (B). Bars represent mean $\pm$ SEM for the percent tetraploid induction (A) or percent survival (B) for three crosses. Bars with the same letters are not significantly different at the $P \leq 0.5$ level (A). Low statistical power prohibited statistical comparisons in B.

Fig. 2. Effect of applying pressure at different times after insemination based on percent of the first cleavage interval (FCI), and age of the egg donor, on tetraploid induction (A) and on survival at eyeing and three days post-hatching (B). There was no significant effect of age on survival so the data were combined (B). Bars represent mean $\pm$ SEM for the percent tetraploid induction (A) or percent survival (B) for three crosses. Bars with the same letters are not significantly different at the $P < 0.5$ level among time points for females of the same age (A) or survival at eyeing and hatching (B).

Fig. 3. Images of ovarian tissue from a tetraploid female brook trout at 16 month post-hatching (A); and testis tissue from the male tetraploid brook trout after milt was collected for spawning (B). The insert in panel B shows the intact testes. The bars in A and B represent 100 mm.

Fig. 1

Fig. 2