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Factors affecting the first cleavage interval and effects of parental generation on tetraploid production in rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

Tetraploidy is induced in rainbow trout by applying a pressure shock at a specific time point between insemination and first cleavage, or the first cleavage interval (FCI). Previous studies suggested that variation in the FCI among individuals and populations of fish prevents the identification of a single time point that can be used for all trout. In this study we confirmed the optimal time to apply pressure is 65 ± 5% of the FCI. In addition, we found that variation in FCI of fish from a common environment can be within limits that allow a single time point to be established for that group of fish, if ova post ovulatory aging is taken into account. Aging of ova, either in vivo or in vitro, increased FCI to a degree that is a concern for tetraploid induction. The FCI was about 12 min longer at 7 days post ovulation, and 30 min at 10–14 days, than at 1 day. The FCI for a group of fish was consistent throughout the spawning season. Survival to hatching and frequency of spinal abnormalities were similar for progeny of first and second generation tetraploid males, but survival was doubled and abnormalities reduced by approximately 90% in second generation tetraploid females compared with first generation females. All progeny of tetraploid by tetraploid crosses were determined to be tetraploids based on flow cytometry of embryonic cells. In summary, attention to ova aging and use of second generation female tetraploids allows efficient production of a tetraploid rainbow trout broodstock.

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1. Introduction

Chromosome set manipulation is used in aquaculture to induce sterility (see review; Nichols, 1990; Piferrer et al., 2009). Fish with odd sets of chromosomes are generally sterile whereas those with even sets of chromosomes are generally fertile. The most common chromosome manipulation is to use a physical shock, usually a pressure or heat shock, to induce retention of the second polar body during meiosis, resulting in triploid (3N) fish. This approach takes advantage of the fact that fish ovulate a non-reduced 'ovum' from which the second polar body is expelled in response to fertilization. In rainbow trout, triploidy induces sterility in both sexes, but only inhibits gonadal development in females. Therefore, triploidy induction of both sexes can be used if the purpose is genetic containment, but all-female lines are used if the purpose is to mitigate the effects of sexual maturation on performance traits such as growth and fillet quality.

An alternative method for producing triploids is to first make tetraploids (4N) by suppression of early cell division in the zygote (Chourrout, 1984; Zhang and Onozato, 2004) and then mate the fertile tetraploids with normal diploids (2N) to generate triploid offspring. Generation of tetraploid-derived triploids has been achieved in rainbow trout (Blanc et al., 1987, 1993; Chourrout and Nakayama, 1987; Chourrout et al., 1986; Myers and Hershberger, 1991). In theory, tetraploid-derived triploids should have multiple advantages over triploids produced by using a mechanical shock. Progeny of tetraploid by diploid crosses should all be triploids whereas the induction rate of shock-induced triploidy is less than 100%, although often exceeding 99% (Devlin et al., 2010). Furthermore, tetraploid-derived triploids should have a higher degree of heterozygosity than mechanical triploids (Diter et al., 1988) and the tetraploid-derived triploids are not exposed to the trauma of the induction shock as are mechanical triploids.

In practice, induction of tetraploidy and production of tetraploid-derived triploids have been problematic. Induction of tetraploidy requires the precise timing of the application of the shock treatment, usually a pressure shock. The timing is based on a percentage of the first cleavage interval (FCI), which is the time span between insemination and first cleavage of the zygote (Chourrout, 1984; Myers et al., 1986). Unfortunately, determination of the FCI is labor intensive and there is considerable variation in FCI among individual animals (Hershberger and Hostuttler, 2005, 2007). Viability of first generation tetraploids has also been found to be very low. Improvement, leading to eyeing rates of up to 30%, is seen in what we will refer to as induced second generation tetraploids, which are tetraploids derived by insemination of ova from diploid females with sperm from tetraploid males, then heat-shocking the zygote to induce polar body retention (Blanc et al., 1987, 1993; Chourrout et al., 1986). In addition, tetraploid by

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tetraploid crosses and tetraploid by diploid crosses, involving either first generation tetraploids or induced second generation tetraploids, have resulted in small percentages of the progeny displaying unexpected chromosome numbers (Blanc et al., 1993; Chourrout and Nakayama, 1987; Chourrout et al., 1986). Possible explanations for unexpected euploidy and aneuploidy of the progeny include failures of chromosome reduction in tetraploid females (Chourrout and Nakayama, 1987), spontaneous androgenesis with tetraploid males (Blanc et al., 1993), or mosaicism of first generation tetraploids (Zhang and Onozato, 2004). The objectives of the current study were to identify factors that contribute to variation in FCI among individual fish and to compare survival and ploidy of progeny from crosses of first and true second generation tetraploids.

2. Methods and materials

2.1. Experimental animals and rearing conditions

Gametes from diploid rainbow trout were obtained from multiple sources dependent on the specific study. All tetraploids were produced and reared at the National Center for Cool and Cold Water Aquaculture (NCCCWA; U.S. Department of Agriculture, Agricultural Research Service), in Leetown, West Virginia. Fish were fed commercial trout feed and maintained on treated spring water that was at times partially recirculated and ranging in temperature at the tanks between 11 and 14 °C. Additional details are provided with the experimental designs for each study.

2.2. Polyploid induction, flow cytometry, first cleavage analysis, fertilization rate

Tetraploid and triploid induction, flow cytometry, and first cleavage analysis were conducted following procedures described by Hershberger and Hostuttler (2005, 2007). Unless otherwise specified, tetraploid induction was conducted by applying 633 kg/cm^2 (9000 psi) for 8 min starting at 62–65% of FCI at a constant temperature of 10.0 ± 0.3 °C. Triploid induction was conducted by applying the same pressure shock but at 30 min post insemination. Ploidy levels in red blood cells or dispersed cells from eyed-stage embryonic tissues were determined based on DNA content by use of a Becton Dickinson Flow Scan flow cytometer and the Beckton Cycle TEST PLUS reagent kit. At least 20,000 nuclei were analyzed per run and diploid rainbow trout red blood cells or embryonic tissues were used as control materials. Blood samples were analyzed for every animal used as broodstock. Ten zygotes were analyzed individually for each cross. First cleavage analysis to determine FCI was conducted at 10.0 ± 0.3 °C 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 to 10 h post insemination and immediately placed in modified Davidsons fixative (Hershberger and Hostuttler, 2005), then later examined microscopically to determine viability and the number of zygotes that had reached first cleavage at each collection time. 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). Fertilization rate was based on percent of zygotes that reached first cleavage during the last five time points collected, which are well after all zygotes should have reached first cleavage.

2.3. Experimental designs

Data on variation in the FCI among groups of fish were accumulated over 8 years and includes data from 2003 to 2005 that have been published previously (Hershberger and Hostuttler, 2005, 2007). Populations include a commercial population from Troutlodge Inc. (TL; Sumner, Washington); a second was from the Donaldson strain, (UW; University of Washington, Seattle); and a third population from the Shasta strain (SH; Ennis [Montana] National Fish Hatchery). Some lots designated as imported were shipped to NCCCWA as gametes whereas others were derived from fish raised at NCCCWA. Means were derived from FCI determinations for multiple clutches, each made up of ova from a single female exposed to pooled milt from two or more males.

We investigated the effect of applying pressure at different times after insemination based on percent of FCI, on ploidy induction and survival at eyeing. The females used in the study were checked for ovulation on a weekly basis. Six crosses, each consisting of ova from a single female and milt from a pool of three males were used in the study. Ova were stripped from each female and placed in individual plastic bags with the coelomic fluid and added O_2, and refrigerated at 4 °C. The FCI for each batch of ova from a single female was determined the next day, day 1, by fertilizing a subset of ova. The remainder of the ova from each batch were fertilized on day 2 and an aliquot of fertilized eggs were either not pressure treated (control) or pressure treated at 55, 65, 67.5, 70, or 75% of the FCI using the FCI calculated for each batch of ova the previous day. When the embryos reached the eyed stage, samples were collected for ploidy determination and survival was determined.

Three studies were conducted to determine factors affecting FCI. Fish used in the studies were derived from gametes obtained from Troutlodge Inc., but were bred and reared at NCCCWA for a minimum of two generations. The first study investigated the effect on FCI of when during the spawning season the fish ovulated. The study was repeated over 2 years. In this study a tank of 100 fish were checked for ovulation every 7 days. Early spawning fish were the 4th–9th fish found to have ovulated, mid-season fish were the 51st through 56th fish found to have ovulated, and late season fish were the 88th through 93 rd fish found to have ovulated each year. When a fish of interest was found to have ovulated, a sample of the ova was fertilized using a pool of milt from at least three males. Samples were subsequently collected for determination of FCI and fertilization rate.

The second and third studies investigated the effects of in vivo and in vitro ovum aging, respectively, on FCI. For these studies, a set of fish which had not yet ovulated were placed in a tank. The fish were checked daily for ovulation as indicated by the release of ova in response to the application of pressure to the abdomen. Fish that were found to have ovulated were considered to be at day 0. In the study of in vivo aging, fish that were identified as having just ovulated (day 0) were tagged and placed in a separate tank. In the first experiment of the study on in vivo aging, samples of about 300 ova were collected from each of 30 fish on day 1 for FCI determination. Ova were also collected multiple times from ten of these fish including on days 3, 7, and 14 post ovulation, for determination of FCI and fertilization rate. To control for the possible effects of handling stress associated with stripping of the fish multiple times, ova from another ten of these fish were collected on day 7 and another ten on day 14 for FCI and fertilization rate determination. In the second experiment conducted 1 year later, ova were collected as in the previous experiment, however ova were collected on days 1, 3, 7, 10, and 14 from the ten fish repeatedly sampled, and from ten fish each on days 1 and 7, or 1 and 10, or 1 and 14 for fish only sampled twice, serving as controls for handling stress. Water temperatures ranged from 12 to 12.5 °C in both runs of the study.

In the study of in vitro aging, all of the ova along with coelomic fluid were stripped from each of the ovulated females on the day they first ovulated (day 0). The ova from each individual female were placed in a separate 1 gal plastic bag filled with O_2 and placed in a refrigerator at 4 °C. Ova from day 0 and ova removed from the bags at days 1, 3, 7, and 14 were analyzed for determination of FCI and fertilization rate using milt from a pool of males. Bags were refilled with O_2 each time they were opened.