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Variation in Time to First Cleavage in Rainbow Trout *Oncorhynchus mykiss* Embryos: A Major Factor in Induction of Tetraploids.

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Abstract

This study was undertaken to analyze the sources of variation in timing of first cleavage in rainbow trout *Oncorhynchus mykiss* embryos in order to adjust treatment protocols to maximize tetraploid induction. The first cleavage interval (FCI), or the time from fertilization to the mid-point of the appearance of first cleavage, was determined at several temperatures for eggs from individual females from each of four different strains. Statistical analyses of the data did not reveal any significant differences among samples taken on different dates or among females within populations, but there was a significant difference shown among the populations. Further analyses revealed this difference was due to a significantly shorter FCI in one population. Data on two of the populations incubated at elevated temperatures showed a decreased FCI within the populations, but the differences between the populations remained. The results suggested that modification of the treatment protocols for induction of tetraploidy (and, perhaps, gynogenesis and androgenesis) are needed to compensate for variation noted among populations if maximum induction and minimum mortality are to be achieved.

Results reported in the literature on manipulation of rainbow trout *Oncorhynchus mykiss* chromosome sets by interrupting the first mitotic division in developing embryos to produce tetraploid, gynogenetic, or androgenetic individuals have been very variable (Ihssen et al. 1990). Survival among different investigations has ranged from 8% to 40% and the percent of successful induction has extended from 8% to 100%. With the severity of the treatments utilized for these manipulation processes (brief exposure to temperatures ≥ 26 C or pressures ≥ 422 kg/cm^2 (6,000 psi)) during a sensitive developmental period, low survival values are anticipated. However, optimization of the treatment protocols in terms of: 1) treatment intensity, 2) treatment duration, and 3) timing of treatment initiation for the embryos should result in less variation in induction of the desired chromosome configuration. Such increased predictability for induction is particularly important in a program designed to utilize the individuals resulting from the chromosome manipulation efforts as founding material for further genetic work.

We have undertaken an investigation into the production of tetraploid strains of rainbow trout as part of a program to develop improved broodstock for the aquaculture industry. Development of tetraploid rainbow trout strains has been recognized as a potentially viable approach for the direct production of triploid offspring by mating with diploids (Chourrout et al. 1986; Horstgen-Schwark 1993). However, the low levels of post-treatment survival and of tetraploid induction success limit the genetic variation among fish, which presents problems for the formation of strains with improved traits that can be used for production of superior triploids. Consequently, studies were conducted to identify factors in the treatment procedures or traits in the development of rainbow trout embryos that could yield more reliable/consistent results.

One of the assumptions on which the induction of tetraploidy (as well as gynogenesis and androgenesis) is based is the consistent timing of the first mitotic division. Pressure or heat shocks of sufficient intensity are applied at a time after fertilization and for a duration that effectively suppresses the first cleavage in developing embryos. Foisil and Chourroutt (1992) demonstrated that the use of pressure is more effective than high temperature (≥ 26 C) for interrupting the first mitotic division to produce tetraploids, gynogenetics, and androgenetics. The timing of most treatments is

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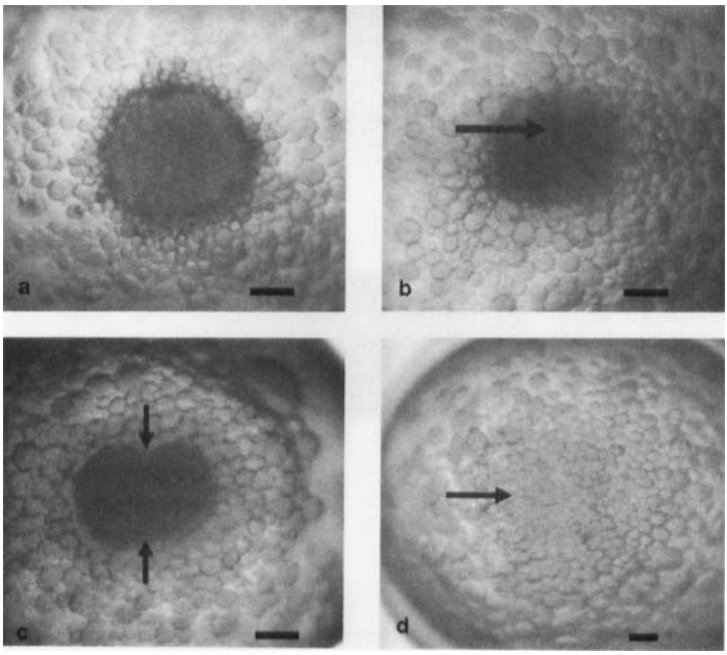

FIGURE 1. *Four microscopic images of rainbow trout eggs during very early development. (a) Germinal disc prior to first cleavage initiation. (b) Germinal disc at first cleavage — arrow points to the cleavage plate formed prior to division into two cells. (c) Germinal disc after first cleavage is completed — arrows point to the cleavage furrow between cells at the two-cell stage. (d) A nonviable egg with no germinal disc structure present — arrow points to location where germinal disc should be located. Scale bar = 0.03 mm*

apparently based on the published developmental rate of rainbow trout at a given temperature (e.g., Knight 1963). However, the variability in the results between treatments using the same parameters within the same laboratory and between laboratories suggests that there are additional sources of variation that are not being accounted for in the procedures being utilized. Consequently, this investigation was initiated to determine if variation exists in the time to first cleavage for rainbow trout and whether these differences are characteristic of the populations being tested.

Methods and Materials

Gametes were obtained at several times from four different populations of rainbow trout. One of the populations is a commercially used strain from the west coast of the U.S. (Troutlodge – TL), one is a strain employed primarily for academic research from the west coast of the U.S. (Donaldson – UW), and two strains are utilized mostly for natural resource management in the U.S. (Shasta – SH and Erwin – ER). Eggs were collected as individual lots from five or six 2-yr-old females while the milt was pooled from several males.

Samples of about 1,000 eggs were fertilized by addition of 2.5–3.0 mL milt and approximately 15 mL of activating fluid (Scheerer and Thorgaard 1989). For determination of the time to first cleavage, the addition of the activating fluid was considered "time zero." The activator and egg containers were placed in a controlled temperature water bath at the same temperature as the incubator. After 2 min the gametes from each female were rinsed and placed in a separate container in a recirculating controlled temperature incubator. Egg lots from all populations were incubated at 10.0 C maintained within a range of ± 0.3 C; temperature control units were electronically regulated, and digital thermometers calibrated to ± 0.05 C were used for temperature measurement. In addition, lots from some of the populations were incubated at 12.0 C or at 14.0 C, also controlled within a range of ± 0.3 C. At the 10.0 C incubation temperature, ten eggs were carefully collected at 10 min intervals from 7–10 h post-fertilization (PF) and immediately placed in 20 mL of fixative consisting of equal volumes of methanol, acetic acid, and water. After 10 min, this fixative was removed and replaced with a modified Davidson's fixative consisting of 200-mL formalin, 100-mL glycerol, 300-mL ethanol, 100-mL acetic acid, and 300-mL water. At the 12.0 C and the 14.0 C incubation temperatures, ten eggs were collected and fixed at the same interval

from 6–9 h PF and from 5–7 h PF, respectively, to compensate for more rapid division at the elevated temperatures.

Approximately 200 eggs from each of 63 females (total of >12,000 eggs) were examined microscopically to determine the number of eggs that had reached first cleavage at each collection time. Each egg/embryo was classified into one of three categories: 1) pre-first cleavage; 2) at first cleavage; and 3) post-first cleavage (Figs. 1a, 1b, 1c). Additionally, apparently non-viable eggs/embryos, as noted by the lack of a defined germinal disc (Fig. 1d), were recorded. Total non-viability in egg lots led to the elimination of data from several females. In addition, any ten-egg collection with ≥ 5 non-viable eggs was eliminated from further analysis. First cleavage was considered to have begun when the germinal disc in the egg showed a distinct cleavage plate, or the beginning of a cleavage furrow dividing it into two equal-sized cells (Fig. 1b). The frequency of this occurrence in each collection of eggs for each time period was recorded and used to calculate the first cleavage interval (FCI) for each female sampled.

The FCI for each female was defined as the time PF at which 50% of the eggs/embryos had reached first cleavage. It was calculated as the sum of the products of the number of eggs at first cleavage (corrected for the number of nonviable eggs noted) and the time (min) at which the sample was taken, divided by the total corrected number of eggs at first cleavage over the entire sampling period. The FCI value (min) for each female was then utilized to calculate mean FCI estimates for each sampling date in each population.

FCI estimates for each sample date were analyzed by *t*-test or single factor ANOVA to test for differences among females within populations. No significant differences were found among the females sampled or the different dates of sampling, so the individual values were combined for subsequent analyses. The combined data were analyzed with Bartlett's test to assess homogeneity of variances among the populations and a significant ($P < 0.05$) amount of heterogeneity was discovered. Consequently, the data were analyzed by a nonparametric Kruskal-Wallis single-factor analysis of variance (Zar 1999) to determine whether significant differences existed among the populations studied. A Tukey-type multiple comparison test was conducted to identify the populations that were significantly different.

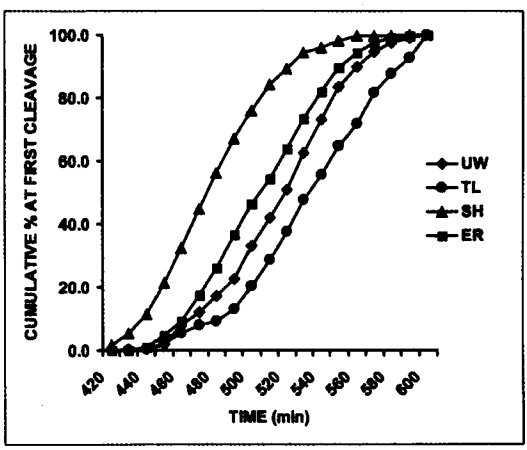

FIGURE 2. *Plot of cumulative mean percent at first cleavage over time PF in four populations of rainbow trout.*

Results

At the 10.0 C incubation temperature, the apparent time to completion of the first cleavage division was about 560 min (9.0 h) to 600 min (10.0 h) PF (Fig. 2). The lapse of time from the initial to the final observation of first cleavage initiation within a population sample of eggs averaged 2 h 40 min. This value was fairly consistent across populations, suggesting that differences among populations may reside in the time PF that first cleavage is initiated.

The variation in mean FCI among the populations tested in this study was fairly large (maximum difference > 40 min) (Table 1). On the other hand, the coefficient of variation within the populations was rather small (2–4%), suggesting homogeneity among the egg lots within each population. In fact, statistical tests (*t*-test and ANOVA) revealed no significant differences ($P > 0.05$) among the egg lots collected on different dates. Therefore, the data were pooled across dates within each population.

Analysis of the pooled data with a nonparametric Kruskal-Wallis ANOVA revealed significant differences ($P < 0.05$) among FCI values for the different populations. A subsequent multiple comparison test among these values indicated

TABLE 1. *Averages and ranges of first cleavage interval (FCI) values in developing embryos of four different populations incubated at 10 + 0.3 C.*

Population	Sample Date	No. Females	Average FCI ± SD (min)[1]	Range
ER	6/10/03	5	515.1 ± 18.3	
	6/18/03	6	510.8 ± 10.0	
	All	11	512.7 ± 13.8[a]	497.5 – 539.8
UW	1/24/03	5	530.0 ± 2.8	
	9/5/03	5	522.3 ± 19.6	
	All	10	526.1 ± 13.8[a]	487.4 – 533.2
SH	1/24/03	6	482.7 ± 8.7	
	3/19/03	6	482.2 ± 13.3	
	All	12	482.5 ± 10.7[b]	464.2 – 502.2
TL	1/24/03	4	520.9 ± 9.4	
	3/19/03	6	537.5 ± 17.5	
	5/2/03	5	513.0 ± 16.9	
	All	15	524.9 ± 18.3[a]	491.4 – 557.4

[1]*Values with the same superscript are not significantly different.*

that in one population (SH) the FCI was significantly shorter ($P < 0.05$) than in the other three populations, among which no significant differences were found. It is worth noting from the data in Table 1 that the span of time in mean FCI values among the populations that did not differ significantly was only 13.4 min, while the eggs from the significantly different SH population had a mean FCI value at least 30 min shorter than eggs from the other populations.

Fertilized eggs from the TL and ER populations were also incubated at 12 C and 14 C and analyzed for FCI by the same protocol. As is shown in Table 2, the FCI values in the egg lots from each population decreased with higher incubation temperatures. In addition to the decrease in time to first cleavage, the standard deviation decreased with an increase in incubation temperature. However, the CV for each population remained relatively similar to that shown at 10 C (2–4%). Further, the difference in FCI value between the populations remained fairly constant irrespective of the incubation temperature.

TABLE 2. *Average FCI values (± SD) in embryos from two populations of rainbow trout incubated at different temperatures.*

Population	No. Females	Incubation Temp. (C)	Average FCI ± SD (min)
TL	15	10	524.9 ± 18.3
TL	4	12	432.7 ± 9.0
TL	5	14	357.1 ± 7.2
ER	11	10	512.7 ± 13.8
ER	6	12	420.6 ± 10.1

Discussion

The primary goal of this study was to analyze the time to first cleavage in rainbow trout embryos to determine whether any variability was present and whether this would indicate that adjustment of treatment protocols is needed to more consistently yield tetraploids. There were suggestions from the results of a number of studies that a reasonable amount of variability in time to first cleavage existed, both between populations and between and within egg lots (Knight 1963; Myers et al 1987; Horstgen-Schwark 1993). Further, research on development rate in rainbow trout (Ferguson et al 1985; Robison and Thorgaard 2004) and other salmonids (Tallman 1986; Beacham 1988; Hebert et al. 1998) has demonstrated genetic variation among populations. However, most of these results deal with traits that are much later in embryological development (e.g., hatching) which may or may not be correlated to the time to first cleavage. Since time to first cleavage is very critical to the

TABLE 3. *Percent tetraploidy and survival in individual female lots of eggs from the TL strain subjected to hydrostatic pressure at 633 kg/cm2 (9,000 psi) at different initiation times and for two different durations (6 and 8 min).*

No. Treated	% FCI	Initiation Time (min)	Treatment Duration (min)	% 4N	% Survival
1,000	N/A	320	6	25.0	0.5
1,000	N/A	320	8	50.0	1.0
1,000	N/A	320	8	10.0	0.2
700	61.6	325	6	69.2	1.9
700	61.6	325	8	100.0	5.0
700	61.6	325	8	100.9	2.0
700	62.0	328	6	2.3	18.7
700	62.0	328	8	88.1	16.9

definition of successful treatment protocols to induce tetraploids, this study was undertaken to better define the variability in this trait in several rainbow trout populations.

The method chosen to measure the time to first cleavage was microscopic examination of the developing germinal disc to determine the time PF when the initial cleavage plate/furrow appeared. The translucent nature of the chorion in rainbow trout did not present major obstacles to microscopic observation and was satisfactorily addressed by the combination of fixatives applied when the developing eggs were sampled. Further, a single observer conducted all of the analyses to minimize potential interpretation error.

Analyses of FCI data revealed no significant differences between collection dates or individual females; however, there was a significant difference noted among the four populations sampled. Further analyses demonstrated this was apparently due to the more rapid development in one of the populations (SH) that yielded an FCI value about 20 min shorter than the next closest group (Table 1). Whether this difference in average time to first cleavage is related to the variation in rate of development to hatch noted in populations of rainbow trout (Robison et al. 1999; Robison and Thorgaard 2004) has yet to be determined. However, the differences among the populations studied suggest this variation has a genetic basis.

The presence of differences of such magnitude in timing to first cleavage could also explain some of the variation in the level of tetraploid induction and, perhaps, post-treatment mortality in rainbow trout. Closer examination of the results from two reports on production of tetraploid breeding stock in this species revealed that the highest level of success with a specified treatment protocol was achieved with crosses of individual females and males or with crosses derived from a particular familial background (Foisil and Chourrout 1992; Horstgen-Schwark 1993). One interpretation of these results is that due to genetic variability in the time to first cleavage, the treatment conditions were optimum only for the groups with specific genetic constitutions.

Most of the reported treatment protocols for the induction of chromosome set duplication are based on a defined time post-fertilization (Chourrout et al. 1986; Foisil and Chourrout 1992; Horstgen-Schwark 1993) which assumes a constant time to first cleavage for rainbow trout eggs. On the other hand, Myers et al (1987) based their treatment on a percentage of the time to first cleavage, in this case 65%. With the appropriate analyses prior to treatment, such an approach would enable adjustment of the treatment protocol to compensate for differences among groups of eggs.

Initial results from work in our lab to define the most effective set of treatment conditions for induction of tetraploidy in rainbow trout strains suggest that adjustment of the time of pressure treatment initiation based on the population FCI values yields major improvement in tetraploid induction and post-treatment mortality (Table 3). Comparison of percent tetraploid and percent sur-

vival values between groups treated at a published, standard time (320 min) (Foisil and Chourrout 1992) and those treated at a time based on population FCI values (325 and 328 min) shows the latter to be a more effective approach. Although these results are preliminary and require additional validation, they suggest that it would be prudent for tetraploid induction to determine at least a population FCI value, as a basis for defining time of treatment.

One technique that could possibly be employed to compensate for differences in FCI is the use of incubation temperatures to change the rate of the first cleavage division. Results from this study demonstrated that increased incubation temperatures decreased FCI, as anticipated in a poikilothermic animal. However, this approach should be cautiously considered since the data suggest more than one treatment parameter would need to be modified. At higher temperatures first cleavage not only occurs sooner but it appears to be more homogeneous (Table 2) and, even with the use of a modified time of treatment initiation, a standard duration time may have negative impacts on induction and embryo survival. It would seem prudent to simply evaluate FCI for the genetic groups being treated and adjust only one parameter to optimize induction results.

In conclusion, significant variation in the time to first cleavage among four populations of rainbow trout was identified. This variation was due to a shorter FCI (30–40 min) in one population while no significant differences were found among the other three populations studied. The data suggest genetic differences among the populations that may involve the activation of the egg to initiate development. A better understanding of the mechanisms of egg activation (Coward et al. 2002) may yield definitive information on the explanation and the genetic interpretation of the variation found. However, differences of the magnitude shown in this study, even the differences among the populations that were not statistically significant could have a major impact on the success of tetraploid induction. Successful hydrostatic treatment lasts only 6–8 min (Foisil and Chourrout 1992) and initiation of the treatment at an inappropriate time would lead to a low percentage of tetraploid induction and, perhaps, higher mortality. Thus, to achieve the maximum levels of tetraploidy in rainbow trout with the minimum level of mortality it is recommended that at least population-based FCI values should be used to guide definition of the time of treatment initiation.

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Literature Cited

Beacham, T. D. 1988. A genetic analysis of early development in pink and chum salmon at three different temperatures. Genome 30:89–96.

Chourrout, D., B. Chevassus, F. Krieg, A. Happe, G. Burger, and P. Renard. 1986. Production of second generation triploid and tetraploid rainbow trout by mating tetraploid males and diploid females — potential of tetraploid fish. Theoretical and Applied Genetics 72:193–206

Coward, K., N. R. Bromage, O. Hibbitt, and J. Parrington. 2002. Gamete physiology, fertilization and egg activation in teleost fish. Review of Fish Biology and Fisheries 12:33–58.

Ferguson, M. M., R. G. Danzman, and F. W. Allendorf. 1985. Developmental divergence among hatchery strains of rainbow trout (*Salmo gairdneri*): pure strains. Canadian Journal of Genetics and Cytology 27:289–297.

Foisil, L., and D. Chourrout. 1992. Chromosome doubling by pressure treatments for tetraploidy and mitotic gynogenesis in rainbow trout, *Oncorhynchus mykiss* (Walbaum): re-examination and improvements. Aquaculture and Fisheries Management 23:567–575.

Hebert, K. P., P. L. Goddard, W. W. Smoker, and A. J. Gharrett. 1998. Quantitative genetic variation and genotype by environment interaction of embryo development rate in pink salmon. Canadian Journal

of Fisheries and Aquatic Science 55:2048–2057.

Horstgen-Schwark, G. 1993. Initiation of tetraploid breeding line development in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Aquaculture and Fisheries Management 24:641–652.

Ihssen, P. E., L. R. McKay, I. McMillan, and R. B. Phillips. 1990. Ploidy manipulation and gynogenesis in fishes: cytogenetic and fisheries applications. Transactions of the American Fisheries Society 119:698–717.

Knight, A. E. 1963. The embryonic and larval development of the rainbow trout. Transactions of the American Fisheries Society 92:344–355.

Myers, J. M., R. N. Iwamoto, and W. K. Hershberger. 1987. Tetraploidy induction in salmonids. Journal of the World Aquaculture Society 17:1–7.

Robison, B.D. and G. H. Thorgaard. 2004. The phenotypic relationship of a clonal line to its population of origin; rapid embryonic development in an Alaskan population of rainbow trout. Transactions of the American Fisheries Society 133:455–561.

Robison, B. D., P. A. Wheeler, and G. H. Thorgaard. 1999. Variation in developmental rate among clonal lines of rainbow trout (*Oncorhynchus mykiss*). Aquaculture 173:131–141.

Scheerer, P. D. and G. H. Thorgaard. 1989. Improved fertilization by cryopreserved rainbow trout semen with theophylline. Progressive Fish-Culturist 51:179–182.

Tallman, R. F. 1986. Genetic differentiation among seasonally distinct spawning populations of chum salmon, *Oncorhynchus keta*. Aquaculture 57:211–217.