New Conditions for Generation of Gynogenetic Half-Tetrad Embryos in the Zebrafish (*Danio rerio*)

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The generation of gynogenetic half-tetrads is an important tool for genetic mapping and mutant screens in zebrafish (*Danio rerio*). Half-tetrad gynogenesis can be accomplished using hydraulic pressure to disrupt microtubule-mediated segregation during meiosis II, which normally occurs after fertilization. However, the equipment used in the original studies is no longer available, and the reported pressures have not proven effective. Here, microsatellite markers were employed in a redetermination of the pressures required to generate zebrafish half-tetrads with currently available equipment. Two criteria were used to choose conditions for half-tetrad gynogenesis: expected ploidy as confirmed with microsatellite markers and maximal egg viability. Pressures between 6140 and 6840 psi generate half-tetrad embryos, with optimal viabilities attained at 6140 and 6240 psi. As predicted, early pressure treatment of eggs fertilized with nonirradiated sperm created triploid embryos.

The zebrafish (*Danio rerio*) is an attractive experimental system for a variety of reasons. The transparency of the embryos, easy collection of large numbers of embryos, and development ex vivo allow the observation of development in great detail (Concordet and Ingham 1994; Kahn 1994; Strähle and Ingham 1992). The fecundity of the zebrafish makes screening for mutants practical. Sexual maturity is attained in 3–4 months, and the fish live about 2 years (Streisinger et al. 1981). These and other experimental features of the zebrafish have lately made possible the production of hundreds of developmental mutants, which increases the urgency of genetic mapping (Mullins et al. 1994; Solnica-Krezel et al. 1994). Genetic mapping in turn is assisted in the zebrafish by the ability to produce half-tetrad embryos (Streisinger et al. 1981).

Half-tetrad embryos are produced by activation of oocyte cell division with UV-irradiated sperm, followed by disruption of microtubule-mediated meiosis II disjunction (meiosis II normally occurs post-fertilization) using hydraulic pressure (early pressure, or EP) (Streisinger et al. 1981, 1986) (Figure 1). Pigment phenotypes of half-tetrad embryos were used by Streisinger et al. (1981) and Johnson et al. (1995) to calculate marker-centromere distances for recessive pigment markers (Johnson et al. 1995; Streisinger et al. 1986). While attempting to generate half-tetrad embryos using EP, we found that the original equipment was no longer available and that the reported pressure of 8000 psi (Streisinger et al. 1981; Wester-

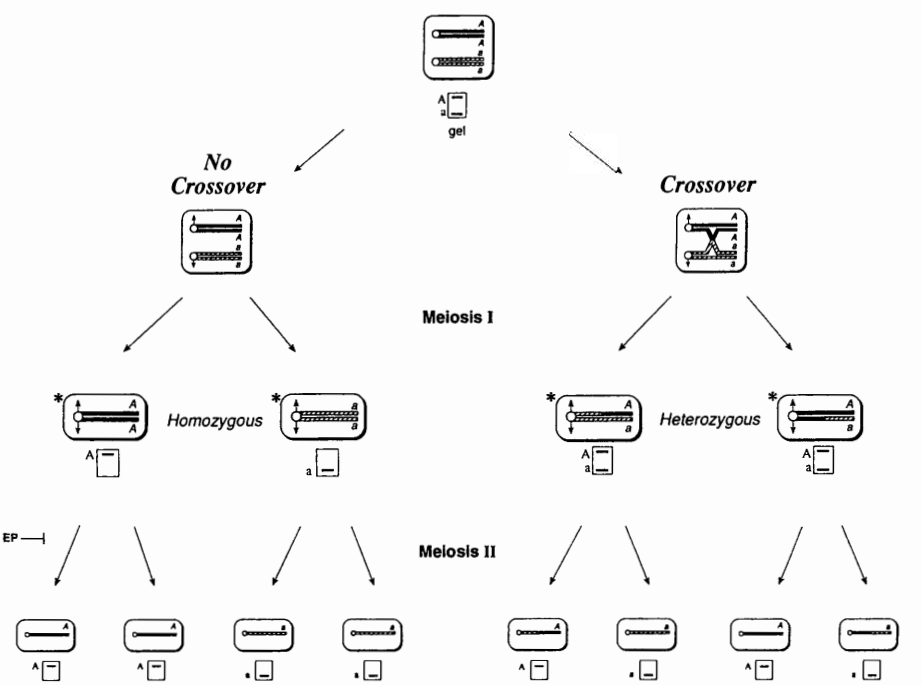

Figure 1. Early pressure parthenogenesis yields half-tetrad embryos. In each intermediate, the chromosome configuration at a hypothetical locus *A* is drawn. Alleles *A* and *a* represent microsatellite alleles which contain more (*A*), or fewer (*a*) repeat units. PCR amplification of this locus followed by electrophoretic fractionation of the products and autoradiography yields the schematicized gel bands shown below each corresponding intermediate. No crossovers or an even number of crossovers between a marker and its centromere yields homozygous half-tetrad genotypes (* under "No crossovers"); an odd number of crossovers between a marker and its centromere yields heterozygous half-tetrad genotypes. Early pressure parthenogenesis inhibits the segregation of sister chromatids that would normally occur during meiosis II, as indicated on the bottom left. The genotypes of the secondary oocytes (*) are identical to those of the half-tetrads generated by EP parthenogenesis. Reproduced with permission from Academic Press.

Table 1. Effect of pressure on embryo viability and ploidy during early pressure parthenogenesis

Pressure (psi)	No. of eggs immediately after EP	No. of viable embryos 3 h after EP (%)	No. of live embryos 6 days after EP (%)	Ploidy
8,000	0*	n/a	n/a	n/a
6,840	120	100 (83)	18 (15)	Diploid
6,740	~200	20 (10)	17 (9)	Diploid
6,590	98	9 (9)	7 (7)	Diploid
6,440	~70	27 (39)	27 (39)	Diploid
6,240	298	188 (63)	167 (56)	Diploid
6,140	259	190 (73)	160 (62)	Diploid
6,030	n.d.	33	25	Haploid

Data for each pressure is derived from one clutch of eggs.
Percentages are of the number of eggs immediately after EP.
* lysis; n/a, not applicable; n.d., not done.

field 1995) to produce gynogenetic diploid embryos resulted in egg lysis.

In order to find pressures that give a maximal yield of half-tetrad embryos on currently available equipment, we tested pressures ranging from 6000 to 8000 psi. $(CA)_n$ microsatellite markers (hereafter, SSRs for simple sequence repeat markers) (Goff et al. 1992) were used to prove three predictions of successful half-tetrad gynogenesis: 1) lack of paternal alleles, (2) generation of expected ploidy, and (3) demonstration that one embryo may be heterozygous for some markers and homozygous for others, depending upon recombination. Lastly, triploids were generated as expected using nonirradiated sperm and early pressure as an alternative to heat shock (Kavumpurath and Pandian 1990).

Materials and Methods

Fish

Wild-type zebrafish (*D. rerio*) were gifts from Mike Rust (University of Washington) and Lile's Tropical Ponds (Ruskin, Florida); *golden* zebrafish were from C. Kimmel (University of Oregon).

Preparation of Genomic DNA

DNA was extracted from dechorionated embryos or from approximately 2 × 2 mm clippings of caudal fins from anesthetized adults according to a protocol from C. Midson and J. Postlethwait (University of Oregon). In brief, samples were incubated in 50 μl of lysis buffer (10 mM Tris-HCL, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.3% Tween 20, 0.3% NP40) for 10 min at 98°C; proteinase K was then added to 1 mg/ml, and samples were incubated at 55°C for 30 min and 98°C for 10 min. After centrifugation at 16,000 g for 1–2 min, addition of 1.25 ml of TE (10 mM Tris, pH 7.6, 1 mM EDTA) to the supernatant gave DNA concentrations of 1–5 ng/ml.

Oligonucleotides

Sixteen pairs of oligonucleotides for scoring SSR polymorphisms in the zebrafish were a gift from C. Tabin (Harvard).

Polymerase Chain Reaction

Reactions were performed in volumes of 10 μl using a Perkin-Elmer 480 DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT) with 1.0× reaction buffer (Promega Corp., Madison, WI), 1.8 mM $MgCl_2$ (Promega Corp.), 200 μM each dATP, dCTP, dGTP, and dTTP, 0.05 μM each sense and antisense primers, 1 μg BSA (New England Biolabs, Beverly, MA), 0.50 U of Amplitaq polymerase (Perkin-Elmer Cetus), and 2 μl of genomic DNA. The "forward" primer was radiolabeled using T4 polynucleotide kinase and 0.5 μCi γ-^{33}P-ATP (DuPont Company, Boston, MA). The Taq DNA polymerase was added during an initial 10 min incubation at 93°C; this was followed by 27 cycles of 15–30 s at 93°C, 45 s at 55°C, and 30–90 s at 72°C; a 5–10 min incubation at 72°C followed the completion of the cycles. PCR products were examined using 6% denaturing acrylamide gel electrophoresis and autoradiography.

Early Pressure (EP) Parthenogenesis

Diploid gynogenetic embryos were produced by in vitro fertilization using timings described by Streisinger et al. (1981). Hydraulic pressure was applied using a 12 ton Carver model C hydraulic laboratory press and a Carver model 3343 pressure cell. Since the cross-sectional area of this

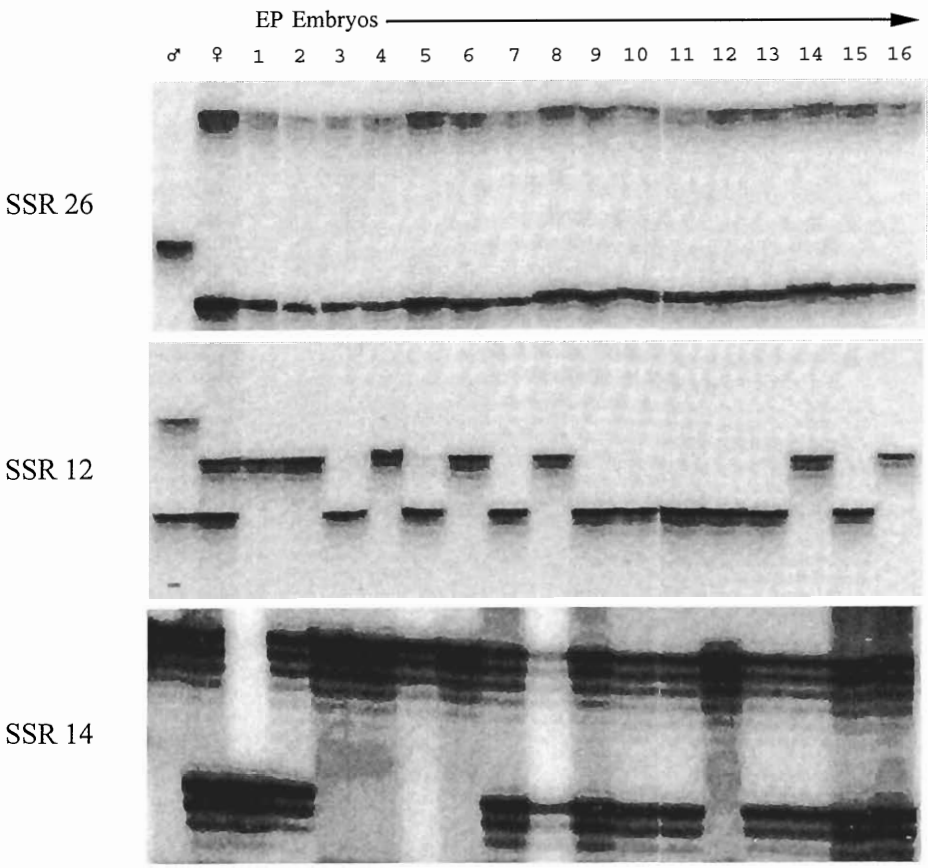

Figure 2. Half-tetrad zebrafish embryos show no paternal genetic contribution, and are either homozygous or heterozygous for different SSR markers. DNA was prepared from paternal (♂) and maternal (♀) fins and from each of 16 gynogenetic half-tetrad offspring produced during the early pressure process as described in Materials and Methods. SSR alleles amplified by PCR were used to determine the parental contribution and genotype of the offspring. Note that these half-tetrad genotypes are represented by those of the secondary oocytes (*) in Figure 1.

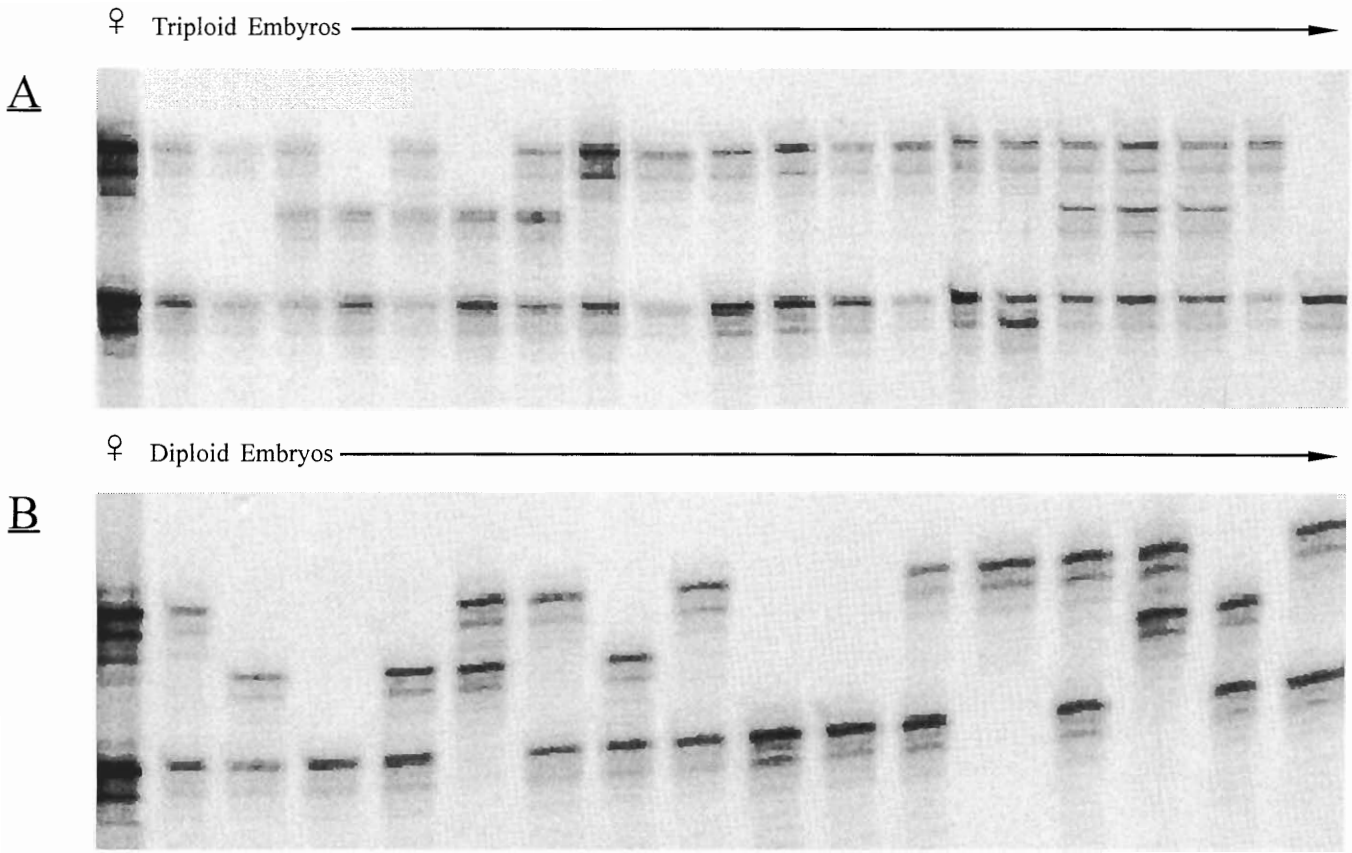

Figure 3. **(A)** Triploids were created by fertilizing with nonirradiated, pooled sperm and then applying early pressure. **(B)** Diploid embryos were created using eggs and nonirradiated, pooled sperm without early pressure. PCR amplification and gel analysis were performed as described in Materials and Methods and in Figure 2.

pressure cell is 1 in.^{2}, the pounds "applied load" on the dial directly translates to psi. A pressure of 6140–6840 psi was applied 84–97 s after egg activation, and continued until 6 min postactivation. Pressure was decreased slowly over the next minute, eggs were left in 60 mg/ml Instant Ocean® solution at 25°C for 2–4 h, and the number of viable embryos were then counted.

Results and Discussion

Egg viability and the ability to produce half-tetrad embryos were evaluated after EP treatments ranging from 6000 to 8000 psi (Table 1). Our three predictions of successful production of gynogenetic half-tetrads are demonstrated from the genotypic analysis of one set of 16 half-tetrad embryos generated at 6840 psi using SSRs 26, 12, and 14 (Figure 2). Embryos were collected 6 days after EP since they begin eating after that age; this maximizes DNA yield while minimizing food-derived DNA contamination. As is typical for $(CA)_n$ repeats, each allele is represented by a primary band accompanied by a ladder of smaller artifact bands differing from the primary band by steps of two nucleotides. The corresponding lane in each of three gels of Figure 2 represents PCR amplified products from a single parent or embryo.

The first two predictions—lack of contribution of paternal alleles to the embryos, and diploidy—are shown using SSR 26 (Figure 2). First, the paternal allele shown in the first lane for SSR 26 was not present in any of the EP embryos. This was also true for the upper paternal allele for SSR 12; the lower paternal allele is shared with the mother. Second, all of the embryos were heterozygous for SSR 26, consistent with diploidy. This frequency of heterozygosity is consistent with the high recombination frequency characteristic of this marker (Kauffman et al. 1995). The third prediction, the possibility of heterozygosity for one marker and homozygosity for another marker in the same embryo, is caused by the varying positions of the markers relative to crossovers during meiosis I (Figure 1). For SSR 12, all of the embryos were homozygous, having inherited only one of two maternal alleles. This is consistent with tight linkage of this marker to its centromere (Kauffman et al. 1995). SSR 14 shows a mixture of heterozygous and homozygous genotypes, again, for the same embryos. In support of the third prediction, for SSRs 26, 12, and 14, the first embryo was heterozygous, homozygous, and homozygous, respectively, and the second embryo was heterozygous, homozygous, and heterozygous, respectively. All three predictions were fulfilled for pressures between 6140 and 6840 psi.

Viability was assessed 3 h and 6 days following EP. At 6030 psi, a majority of the eggs survived EP intact, but were haploid. This was shown by an arrested developmental phenotype typical of haploids and by haploidy at three maternally heterozygous SSR loci. For example, 0 of 21 embryos were heterozygous for SSR 18 (data not shown). This differs from an expected frequency of 0.64 (13 heterozygotes expected out of 21 embryos) for successful gynogenesis (Kauffman et al. 1995). Embryos surviving the higher pressures tested were diploid. However, 6440, 6590, and 6740 psi appeared to cause greater damage to the eggs (Table 1). While the immediate survival of eggs at 6840 psi was high, the proportion surviving to 6 days was only 15%. At 6140 and 6240 psi, a majority of eggs viable after 3 h survived to 6 days and the

overall survival of embryos was maximal (56% and 62%, respectively). Thus, any pressure from 6140–6840 psi may be used to generate half-tetrad embryos, with maximal survival at the two lowest pressures, 6140 and 6240 psi.

An additional prediction of successful suppression of the second meiotic disjunction by EP is the production of triploid zygotes when eggs are fertilized with nonirradiated sperm and subjected to EP (Figure 3). Eggs from a single clutch were fertilized using nonirradiated sperm and divided into two groups: one was subjected to EP and the other group was allowed to complete the second meiotic division and develop normally. Lane 1 in each group shows the maternal genotype, and the subsequent lanes show embryo genotypes. SSR genotypes show that embryos exposed to EP were triploid (Figure 3A), while the normal embryos were diploid (Figure 3B). The lower panel also indirectly indicates that the pooled sperm DNA contains all three alleles. The evidence consistent with triploidy consists of the presence of three alleles for SSR 15 in 6 out of the 18 triploids (third, fifth, seventh, and last three embryo lanes). Furthermore, when only two alleles were present, one was darker than the other, consistent with the presence of two copies of the darker allele. In contrast, untreated diploid embryos allowed to complete normal meiosis and fertilization contained only one or two of the parental alleles; when two alleles were present, they were of equivalent intensity.

Generation of triploids in zebrafish may be used to study the effects of gene dosage on recessive and dominant mutations (Ashburner 1989). Application of this pressure-based generation of triploids offers an alternative to heat shock for generation of triploids in other organisms in which sterile animals may be desired to control overpopulation, and in which triploids may be associated with increased growth rates, final size, or survival in mature fish (Thorgaard 1983).

In order to determine whether the derived pressure of 6240 psi produced results consistent with previously published data (Streisinger et al. 1986), we determined the frequency of *golden* half-tetrad embryos produced by six heterozygous *golden* mothers in a wild-type background. Of 506 half-tetrad embryos, 27 (0.053 of total) were phenotypically *golden*. Assuming reciprocal genetic exchange and the absence of recessive lethal mutations in the region, the frequency of half-tetrads that are homozygous wild type at the *golden* locus is equal to the frequency of *golden* homozygotes. Therefore, the frequency of *golden* heterozygotes is 0.89, or $1–2m$, where m is the frequency of *golden* homozygotes. This is identical to the value derived by Streisinger et al. (1986) from the examination of 1,151 half-tetrad progeny of *golden* heterozygotes in a wild-type background.

In conclusion, SSR genotypes of embryos were determined to confirm the generation of gynogenic half-tetrads using specific pressures. Similar microsatellite analysis may be used to confirm the generation of candidate half-tetrad or triploid embryos in any organism for which microsatellite markers are available. Utilizing our improvements in the EP technique, linkage data deriving from these experiments have been successfully used to confirm strong chiasma interference in the zebrafish, map centromeres, and confirm linkage of nearby markers (Kauffman et al. 1995). Half-tetrad analysis was used to map the remaining centromeres to the current linkage map (Johnson et al. 1996) and may increase our understanding of chiasma interference. Finally, half-tetrad gynogenesis has been used to screen for recessive mutations in carrier females at the University of Oregon (Johnson and Weston 1995; Henion et al. 1996). The methods described herein are successfully being used to find mutants in other mutant screens in progress (Moore JL, Gestl E, Tsao-Wu G, Cheng KC, unpublished data).

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