ANDROGENETIC ZEBRAFISH (*DANIO RERIO*): GENERATION, CONFIRMATION AND GENETIC UTILITY.

by

Graham Corley-Smith

B.Sc., University of Victoria, 1980

M.Sc. University of Victoria, 1989

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APPROVAL

Name: Graham Edmund Corley-Smith

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Examining Committee:

Chair: Dr. C. Boone, Professor

Dr. B. Brandhorst, Professor, Senior Supervisor
Department of Biological Sciences, SFU

Dr. J. Price, Assistant Professor
Department of Biological Sciences, SFU

Dr. R. Devlin, Research Scientist
Canada Department of Fisheries and Oceans

Dr. D. Baillie, Professor
Department of Biological Sciences, SFU
Public Examiner

Dr. D. Grunwald, Associate Professor
Department of Human Genetics, Univ. of Utah
External Examiner

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ABSTRACT

To help investigate the evolutionary origin of the imprinting (parent of origin mono-allelic expression) of paternal genes observed in mammals, I developed methods to produce haploid and diploid androgenetic zebrafish (*Danio rerio*). Androgenotes receive their genomic DNA solely from their male parent, just as gynogenotes receive their genomic DNA from their female parent. Haploid androgenotes were produced by fertilizing eggs that had been x-ray irradiated to eliminate the maternal genome. Diploid androgenotes were produced by inhibition of the first mitotic division by heat shock. Analysis of parentally polymorphic DNA markers (Random Amplified Polymorphic DNA and Simple Sequence Repeats) confirmed the lack of significant maternal transmission to the androgenotes. Haploid androgenotes completed embryonic development but arrested as larvae, showing defects typical of the "haploid syndrome". Diploid androgenotes developed normally and have been bred. The survival of androgenetic zebrafish suggests that if paternal imprinting occurs in zebrafish, it does not result in essential genes being inactivated when their expression is required for development. Production of haploid androgenotes is being used to determine the meiotic recombination rate in male zebrafish. Androgenesis has other useful genetic applications which are discussed.

To produce androgenotes and to provide genetic evidence that androgenotes had been produced, two techniques were developed. Zebrafish eggs must normally be inseminated within a few minutes of being expressed from the female, allowing insufficient time to irradiate eggs for production of androgenotes. I developed the use of ovarian fluid from coho salmon (*Oncorhynchus kisutch*) for delayed *in vitro* fertilization, allowing manipulations of the eggs prior to fertilization. I also developed the Fluorescent Random Amplified Polymorphic DNA (FRAPD) technique for use on an automated DNA sequencer. This technique allowed the efficient production of strong genetic evidence to confirm the paternal origin of the genomes of the androgenotes produced. None of the 157 maternal-specific DNA markers analyzed using FRAPD, some of which were apparently homozygous, were passed on to any of the 18 putative androgenotes analyzed.

DEDICATION

This thesis is dedicated to a pioneer of zebrafish research and friend, Charline Walker. Charline Walker's willingness to competently help new through experienced zebrafish researchers with fish rearing and research techniques has contributed greatly to zebrafish becoming an important model organism of vertebrate development. In her own research and by helping others, she has made, and continues to make, a great contribution to the field.

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community spirit of cooperative research that has been fostered by the many zebrafish researchers at the University of Oregon has benefited me greatly, for which I am very grateful. I would also like to extend my thanks to the many graduate students in IMBB that helped me find answers to the myriad of questions I asked. I am particularly indebted to the late great Mr. Grumpy (John Boom) for his assistance. The assistance of Chinten James Lim in the research we collaboratively performed is appreciated. George Streisinger, Charline Walker and their colleagues at the University of Oregon developed many of the techniques crucial to the research presented in my thesis. For assistance in caring for the zebrafish, I would like to thank Joy Schembri, Scott Stuart, Sue Gillespie, Amy Chow, Shawn Dunbar, Mandy Poitras and Corrie Layher.

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GENERAL INTRODUCTION

Organization of thesis:

This thesis is composed of three chapters: 1) Delayed *in vitro* Fertilization of Zebrafish Eggs. 2) Efficient Detection of DNA Polymorphisms by Fluorescent RAPD Analysis (FRAPD) (Corley-Smith et al. 1997) and 3) Production of Androgenetic Zebrafish (*Danio rerio*) (Corley-Smith et al. 1996). The first chapter was written for this thesis, whereas chapters 2 and 3 are published manuscripts. This General Introduction explains the connections between the three chapters. The significance of this research in the context of vertebrate development and genetics is described in the Concluding Discussion (p. 124). References for the General Introduction and Concluding Discussion are located at the end of the thesis in the General References section (p. 131).

Overview of research performed:

This thesis describes the production of androgenetic zebrafish. I developed methods to produce haploid and diploid androgenetic zebrafish and confirmed the androgenetic nature of their genomes. Androgenotes receive their genomic DNA solely from their male parent, just as gynogenotes receive their genomic DNA from their female parent. Parthenogenesis is a form of reproduction in which an unfertilized egg develops into a new individual. Gynogenesis is a specialized form of parthenogenesis in which the egg, or oocyte, is activated by sperm, but without fusion of the egg and sperm nuclei. I produced haploid androgenotes by fertilizing eggs (secondary oocytes) that had been x-ray irradiated to eliminate the maternal genome. Diploid androgenotes were produced by

inhibition of the first mitotic division by heat shock. Analysis of polymorphic DNA markers (Random Amplified Polymorphic DNA and Simple Sequence Repeats) confirmed the lack of significant maternal transmission to the androgenotes. Haploid androgenotes completed embryonic development but arrested as larvae, showing defects typical of the "haploid syndrome". Diploid androgenotes developed normally and have been bred. The survival of androgenetic zebrafish suggests that if paternal imprinting (parent of origin mono-allelic expression) occurs in zebrafish, it does not result in essential genes being inactivated when their expression is required for development. Production of haploid androgenotes is being used to determine the meiotic recombination rate in male zebrafish. Androgenesis has other useful genetic applications that are discussed.

To produce androgenotes and to provide genetic evidence that androgenotes had been produced, two techniques were developed. Zebrafish eggs must normally be inseminated within a few minutes of being expressed from the female, allowing insufficient time to irradiate eggs for production of androgenotes. I developed the use of ovarian fluid from coho (*Oncorhynchus kisutch*) and chinook (*Oncorhynchus tshawytscha*) salmon for delayed *in vitro* fertilization, allowing manipulations of the eggs prior to fertilization. I also developed the Fluorescent Random Amplified Polymorphic DNA (FRAPD) technique for use on an automated DNA sequencer. This technique allowed the efficient production of strong genetic evidence to confirm the paternal origin of the genomes of the androgenotes produced. None of the 157 maternal-specific DNA markers analyzed using FRAPD, some of which were apparently homozygous, were passed on to any of the 18

putative androgenotes analyzed. Although, as discussed below, production of haploid and diploid androgenetic teleosts had been reported prior to my research, strong genetic evidence confirming the lack of transmission of maternal DNA was lacking. Thus, my major contribution has been in not only producing androgenetic zebrafish but also in providing the first case for a vertebrate in which the androgenetic nature of the genome is well documented.

Chromosome Set Manipulations:

Various chromosome set manipulations may be possible. Fertilization with normal sperm and inhibition of the second meiotic division of the oocyte could produce triploid (3n = 3 sets of chromosomes, 2 maternal sets and 1 paternal set). By inhibition of the first mitotic division, the chromosome number should be doubled. This could either be used to restore diploidy to a haploid embryo or to induce tetraploidy in a diploid embryo. If sperm or eggs (secondary oocytes) are irradiated to destroy nuclear DNA, haploid embryos could result from fertilization. If the sperm is irradiated and then used to activate the oocyte, only the maternal genomic DNA is transmitted to the progeny, and they would be called gynogenotes. Irradiation of eggs to eliminate the maternal genome and insemination with normal sperm could result in production of androgenotes.

Several methods to prevent transmission of nuclear DNA from one of the gametes have been attempted. To date, chemical methods have not been very successful. Physical methods, including UV, X-ray and gamma-ray irradiation (e.g. ^{60}Co and ^{137}Cs sources) have been more successful than chemical methods. UV irradiation has the advantage that

it cross-links the DNA. If the DNA is sufficiently cross-linked, the DNA is not properly distributed to daughter cells. Unfortunately the attenuation of UV irradiation in aqueous media is so great that it is only practical for irradiating cells of normal size cells (e.g. sperm). Giant cells, like teleost oocytes are too large to allow sufficient penetration of UV light to adequately cross link DNA. Higher energy irradiation (X-rays and gamma-rays) penetrate aqueous media with less attenuation. These high-energy irradiation sources fragment DNA by breaking covalent bonds. The degree of fragmentation is dependent mainly on the total dose delivered. The rate of irradiation may also be important when the rate of DNA repair is appreciable in relation to rate of fragmentation. In practice, UV light is used to irradiate sperm for gynogenesis and X-rays or γ-rays are used to irradiate oocytes for androgenesis.

Several methods have also been used to inhibit the second meiotic or first mitotic division. Chemical treatments are usually not successful, probably as the effect of the treatments persist and alters cellular events including subsequent mitotic events. Thus, transient physical treatments have been used more extensively for treatments to block the second meiotic or first mitotic division. Heat shock (increased temperature), cold shock (decreased temperature) and pressure shock (increased pressure) have all been used with variable success. To my knowledge, details of the mechanisms by which heat treatments inhibit the first mitotic or second meiotic division of teleosts have not been established. Two possible events that could be targeted are karyokinesis or cytokinesis. Heat and pressure shocks probably act to depolymerize microtubules during the period of shocking.

Factors that influence the success of shocking include: the time after fertilization to initiate the shock, the intensity of the shock (Pa or °C), and the duration of the shock. Success is often measured in percent survival and the fraction which have undergone the desired increase in ploidy. The conditions used to inhibit the first mitotic division in zebrafish are presented in Chapter 3.

Background on Androgenesis in Teleosts:

To assess androgenesis, the following are required: 1) scoring of transmission of parentally polymorphic DNA markers to progeny, 2) demonstration that the markers are transmitted in Mendelian fashion to progeny of a normal bi-parental mating and 3) demonstration that markers are not closely linked (i.e. are not length variants of same locus). The analysis of DNA polymorphisms allows for direct assessment of parental alleles, irrespective of their state of expression. Thus, it can provide more compelling genetic evidence for lack of maternal inheritance to androgenetic progeny than can assays that rely on gene expression. The above three requirements to assess androgenesis have not been achieved in previous reports for the successful production of androgenetic teleosts (discussed below).

Attempts to produce haploid and diploid androgenetic fishes have been reported by several groups (reviewed by Ihssen *et al.* 1990). Putative haploid androgenetic embryos did not survive to the active feeding larval stage (Romashov and Belyaeva 1964; Arai et al. 1979; Parsons and Thorgaard 1984). Diploid loach *(Misgurnus anguillicaudatus)* have been reported (Masaoka et al. 1995) but up to 10% of treated eggs expressed maternal

derived pigmentation. For the 0.2% that survived to the feeding stage and lacked expression of the maternal pigmentation gene (putative diploid androgenotes), it is not clear to what extent they lacked other maternal genes.

Due to the commercial value of salmon, there have been several attempts to produce diploid androgenetic salmon. Successful production has been reported (Parsons and Thorgaard 1985; May et al. 1988; Scheerer et al. 1986, 1991). These fishes were reported to be androgenetic based on their being homozygous at several loci, as determined from enzyme expression assays. Androgenesis has also been reported based on isogenicity of what the authors believe to be androgenetically derived lines of rainbow trout (Young et al. 1996). Although DNA was accessed directly in this study and many markers were shown to be in all progeny of a line, the number of markers that were polymorphic between parents was not reported. The number of presumed homozygous and heterozygous maternal specific markers was not reported nor was the number of androgenotes in which these markers were assessed. Although most markers occurred in all individuals of a line, the fact that the majority of assessed markers were not shown to be polymorphic between parents weakens the evidence for isogenicity and androgenesis.

In previous reports of successful production of androgenetic teleosts (Romashov and Belyaeva 1964; Arai et al. 1979; Parsons and Thorgaard 1984, 1985; May et al. 1988; Scheerer et al. 1986, 1991; Masaoka et al. 1995; Young et al. 1996), I believe that strong genetic evidence confirming the androgenetic nature of the genomes was lacking. I

present a more thorough assessment of the androgenetic nature of putative androgenetic genomes than has previously been reported.

Ploidy Manipulations using Salmon and Zebrafish:

I will designate the set of chromosomes normally found in one gamete as the haploid number of chromosomes. It has been speculated that pacific salmon have four sets of chromosomes (Ohno 1993; Bailey et al. 1969; Klose et al. 1968). Thus in my usage, haploid is not necessarily equivalent to one set of chromosomes. Zebrafish have 25 pairs of chromosomes that are distinguishable by replication banding (Daga et al. 1996). Zebrafish act as typical diploid organisms in the extensive mutational analyses performed on them (Grunwald 1996; Eisen 1996).

My present interest in zebrafish is as a model system of vertebrate development. However, my original research in the area of chromosome set manipulations was, at least in part, influenced by the desires of the B.C. salmon aquaculture industry. My initial investigations on producing teleost androgenotes, tetraploids and triploids were performed on salmonids (Eastern Brook Trout, *Salvelinus fontinalis*; coho salmon *Oncorhynchus kisutch*; chinook salmon, *Oncorhynchus tshawytscha*; and rainbow trout *Oncorhynchus mykiss*). Funding was available to study these species because they are important to the West Coast recreational and commercial fisheries, and some are of interest from an aquaculture perspective.

A sentiment I have heard expressed many times in the B.C. aquaculture industry is reflected in the words of Hackett (1996): "There is a chronic need to develop growth-enhanced fish for aquaculture." Virtually any form of genetic engineering that permits fish to grow larger and faster and still be marketable would be welcomed by the aquaculture industry. For salmon and some other fish species, the price a fish farmer (a.k.a. fish rancher) receives for his fish increases dramatically as the size of his fish increases; not only does the fish farmer receive money for more pounds of fish, but he/she also receives more money per pound for larger fish. To optimize profits, several factors have been investigated including: use of alternate species (including exotic species like Atlantic Salmon (*Salmo salar*), introduction of transgenic growth hormone genes (Devlin et al. 1994; Devlin et al. 1995), production of mono-sex fish (Benfey 1996; Solar and Donaldson 1991), and production of triploid fish (Benfey 1989). Various species have been used. During the mid 1980's, coho salmon were the main farmed fish in B.C., followed by chinook salmon which grew larger and for which techniques for production of all female stocks were developed. For a few years, over 80% of all salmon reared in aquaculture operations in B.C. were mono-sex (all female) chinook salmon. For chinook salmon, rearing only females was financially preferable as approximately 20% of males developed as jacks (precocious maturation) and thus did not develop to a large marketable size. All salmon undergo fundamental changes which lower the quality of the meat when they become sexually mature and most Pacific salmon raised in seapens are terminal spawners; they die shortly after spawning. Presently the predominant farmed salmon in

B.C is Atlantic salmon. The impact on native salmonid stocks and on the environment from Atlantic salmon that escape from netpens is presently not known.

Some aquaculture managers were interested in developing triploids because of the following hypotheses: 1) triploids should be sterile, 2) triploid cells should be larger, thus if the number of cells is conserved, organs including muscle would be larger, 3) triploid salmon should grow larger as little energy is directed to egg development, and 4) precocious maturation should be avoided. When I started my Ph.D. program, triploids had been produced for all salmonids commonly used in aquaculture and were found to be sterile, but the percentage of triploids produced was often undesirably low, (Benfey et al., 1986; Benfey, 1989). In triploids, mitosis occurs normally but meiosis does not. Presumably the meiotic machinery has difficulty coping with homologous pairing and segregation of three sets of chromosomes. Although most triploids are sterile, this may not always apply. Anecdotal evidence of a successful mating of a triploid male to a diploid female salmon in Japan has been reported (personal communication: Edward Donaldson, Fisheries and Oceans Canada, West Vancouver, B.C.). Small and Benfey (1987) discovered that triploid cells of coho and Atlantic salmon are larger than comparable diploid cells, but organ size is approximately similar due to a decrease in number of cells per organ. Although triploids are usually sterile, the utility of using triploidy to prevent breeding of escaped farm fish is hampered by the difficult of producing exclusively triploid progeny (usually a mixture of diploid and triploid progeny result). Testing all progeny for ploidy is not practical. Thus, one of my early goals was to

produce tetraploid salmon, which in theory, if bred to diploids, should result in production of all triploid progeny. Although I did succeed in optimizing conditions to block the second meiotic division using heat shock (100% triploidy in three families, with 20 progeny sampled per family), I did not succeed in producing any tetraploids. I attempted to use the shock conditions (temperature and duration) successfully used to inhibit the second meiotic division, to prevent the first mitotic division. My lack of success could have been attributed to number of factors including 1) tetraploids are not viable or, 2) I had found the appropriate conditions to inhibit the first mitotic division. Unfortunately I did not try restoring diploidy of gynogenetic haploids, which in retrospect may have distinguished whether the difficulty lay in timing of shocking and conditions of shocking used, or simply that tetraploids have low viability. Ploidy was determined by fluorescent analysis of individual red blood cells. Some salmon I analyzed were mosaic (triploid and tetraploid cells) which might indicate that tetraploidy is unstable in salmonids. In humans, tetraploid embryos can undergo early cleavages (Wojcik et al. 1995) but tetraploid embryos may form hydatidiform moles (Fukunaga et al. 1996) and evidence exists that postconceptional nondisjunction leading to tetraploidy results in demise during pregnancy (Rudnicki et al 1991). Tetraploidy is also associated with certain types of cancer in humans. For example, of 42 Wilms' tumors on which flow cytometry was performed, 12 were tetraploid and 17 aneuploid (Chen et al. 1994). In one study on ductal carcinomas (breast cancer), near-triploid and near-tetraploid karyotypes were a common finding, especially in grade-III tumors and in tumors showing high mitotic activity *in vivo* (Pandis et al. 1996).

Research on chromosome set manipulations, including triploidy, tetraploidy and androgenesis are difficult using salmonids: obtaining eggs for experimentation is difficult, salmonid eggs are nearly opaque which interferes with cytological analysis, and the time from fertilization to hatch is approximately 2 months. Furthermore, most salmon require at least 2 years before they produce eggs. Raising them for this period is expensive, and most species die shortly after breeding. Although funding was available for the research, salmonids are a difficult system for assessing methods for manipulations of chromosome sets.

Thus, I turned to the zebrafish (*Danio rerio*) for my experiments. Zebrafish are a small aquarium fish. The females produce eggs starting at approximately 3 months of age and for over a year produce a few hundred eggs every few days. The eggs are transparent, offering the possibility of following cytological events with vital stains and a microscope. The work by George Streisinger and colleagues at the University of Oregon had demonstrated that *in vitro* fertilization was possible, as was the production of gynogenotes. Haploid gynogenotes were produced but their development arrested near the time of hatching (Streisinger et al. 1981). Diploid gynogenotes produced either by inhibition of the second meiotic division or by inhibition of the first mitotic division survived to adulthood (Streisinger et al. 1981). This latter observation was important as it indicated that lines of homozygous zebrafish could be produced, indicating that recessive lethals should not be an insurmountable problem for production of androgenotes. Furthermore, the careful work of Streisinger and colleagues had established timing of the

second meiotic division and the first mitotic division under controlled conditions (Streisinger et al.1981). This defined the timing of treatments used to inhibit these events.

Oocytes of zebrafish arrest at prophase I of meiosis as they develop and grow within the ovarian follicle (Selman et al. 1993). In response to a hormone signal they undergo oocyte maturation (Selman et al. 1994), and arrest in prophase II of meiosis (Streisinger et al. 1981). During mating, eggs are extruded from the female as secondary oocytes with completion of meiosis II taking place within a few minutes of fertilization. A single sperm fertilizes the egg via the micropyle (Hart and Donovan 1983). Although studies on zebrafish oocyte maturation (Selman et al. 1993; Selman et al. 1994) and insemination (Hart and Donovan 1983) have been performed, I am not aware of any detailed studies of events immediately following insemination which include: extrusion of the second polar body and fusion of the maternal and paternal pronuclei. In zebrafish, the sperm enters the micropyle, and the head of the sperm fuses with a specialized portion of the plasma membrane, and the paternal nucleus enters the ooplasm (Hart and Donovan 1983). The rising chorion then moves the sperm body and tail away from the plasma membrane (Hart and Donovan 1983). I am unaware of any studies that have investigated if other constituents enter the ooplasm along with the paternal nucleus (e.g. mitochondria, activation factors).

Imprinting:

Androgenesis allows the investigation of a poorly understood epigenetic phenomenon; that of paternal imprinting. Paternal imprinting results in the parental

specific mono-allelic expression of certain genes. Although imprinting is known to occur in humans and mice (McGrath and Solter 1984; Surani et al. 1984; Surani 1986; Barra and Renard 1988; Sapienza 1990; Renard et al. 1991; Varzuma and Mann, 1994; Gold and Pederson 1994; Ohlsson et al. 1994; Chaillet et al. 1995), whether it was characteristic of all vertebrates was previously not known. An important experimental observation which led to the discovery of parental imprinting in mice was arrest of gynogenotes and androgenotes during early development (McGrath and Solter 1984). The production of gynogenotes zebrafish (Streisinger et al. 1981) has demonstrated that if genes are inactivated by maternal imprinting they are not required for development in zebrafish. There are several reports of the production of viable fish and amphibian androgenotes, though the documentation of lack of maternal genes is not compelling (see above). The successful production of a genetically confirmed androgenetic vertebrate would help determine if imprinting has an essential function required during vertebrate development. If paternal imprinting of essential genes required during embryogenesis occurs in divergent vertebrate taxa (e.g. eutherians and teleosts), it would support the hypothesis of an essential function required during vertebrate development. If it only occurs in some vertebrate taxa (e.g. in eutherians but not in teleosts), alternate or modified hypotheses need to be considered. Paternal imprinting has been documented in mice (McGrath and Solter 1984). Thus determining if paternal imprinting of essential genes required for development occurs in zebrafish, provides an opportunity to consider function and adaptive significance of paternal imprinting in those taxa where it occurs.

Several adaptive functions for parental imprinting have been proposed in speculative discussions (reviewed in Haig and Trivers 1995), including prevention of parthenogenetic development (Solter 1988), an expression of genetic conflicts between maternal and paternal genomes (Haig and Westoby 1989), an outcome of dominance modification (Sapienza 1989), a means to restrain the growth of the placenta (Hall, 1990), a mechanism of growth factor regulation (Cattanach, 1991), a consequence of host defense mechanisms (Barlow 1993), and a mechanism to protect females against malignant germ-cell tumors (Varzuma and Mann 1994). The genetic-conflict hypothesis is the only model which explains parental imprinting as a function (as distinguished from a side-effect; see Haig and Trivers 1995) and is fully consistent with the current information about parental imprinting (Haig and Trivers 1995). It provides function for imprinting and also explains how and why imprinting could have evolved.

Imprinting may function as an epigenetic mechanism that has evolved in some mammals because of the conflicting interests of maternal and paternal genes in relation to the transfer of nutrients from the mother to her offspring (Moore and Haig 1991). This genetic-conflict model explains the existence of parental imprinting as an expression of genetic conflicts between maternal and paternal genomes (Haig and Trivers, 1995). In polygamous species, to maximize genes of particular parents being perpetuated in subsequent generations, the father can benefit by promoting the fitness of offspring resulting the mating he is involved in (i.e. large offspring to maximize fitness from single mating). Whereas the female can benefit by the maximizing the total number of progeny

she can produce with adequate fitness (i.e. maximize fitness for all matings). Over-investment by the mother of resources to progeny of a single mating may adversely affect the number and fitness of subsequent offspring. Thus, reducing perpetuation of the mothers genes in subsequent generations. The genetic-conflict hypothesis predicts that parental imprinting should occur when there is a large maternal investment during gestation and that parental imprinting will have an important role during embryonic development in viviparous taxa; but will be less important in oviparious taxa. Thus, according to the genetic-conflict hypothesis, parental imprinting should not prevent the production of androgenetic teleosts, even though completion of mouse embryogenesis requires both the maternal and paternal genomes because of imprinting of essential genes in male and female gametes (McGrath and Solter 1984; Surani et al. 1984; Surani 1986; Barra and Renard 1988; Sapienza 1990; Renard et al. 1991; Gold and Pederson 1994; Ohlsson et al. 1994; Chaillet et al. 1995). Thus, our successful production of diploid androgenetic zebrafish is consistent with the prediction of the genetic-conflict model of parental imprinting and thus adds evidence to support the model.

Technologies Required to Produce Androgenetic Zebrafish:

To produce androgenetic zebrafish, it was necessary to refine some technologies. A method to irradiate eggs to prevent transmission of maternal genome to progeny was required, as was a practical method to demonstrate the lack of the maternal transmission of DNA to progeny. To prevent transmission of the maternal genome, I used X-irradiation using a Torrex 150D X-ray inspection system (Faxitron X-Ray Corp., Buffalo

Grove, IL.). Based on my previous dose response curves on eggs of chinook salmon (Hertwig effect; please see chapter 3) done using irradiation from a ^{137}Cs source, I predicted irradiation doses up to 100,000 R would be required. With our x-ray machine, this would require holding zebrafish eggs for at least 30 minutes for irradiation. *In vitro* fertilization methodologies were already available when I started the research (Hart and Messina 1972; Streisinger et al. 1981). However, once zebrafish eggs are extruded from the female, they must be fertilized almost immediately, since the fertilization rate drops to zero within approximately 15 minutes. Thus, I needed to refine *in vitro* fertilization methods to allow holding zebrafish fish eggs for at least 30 minutes. Development of this delayed *in vitro* fertilization technique is described in Chapter 1. I also required methods to optimize collection of high quality zebrafish eggs for production of androgenotes. Our protocol for obtaining high quality eggs for *in vitro* fertilizations, which was honed by a colleague James Lim, is presented in Appendix 1.

Another requirement for my research was obtaining compelling genetic evidence that the putative androgenotes were receiving all or most of their genomic DNA from their father. This requires having markers (e.g. visible phenotypes, isozymes, allozymes, or DNA markers) that are polymorphic between parents and for which the transmission to progeny is scoreable. While a few visible phenotypic markers have been described for zebrafish, establishing that the maternal genome had been eliminated with a high degree of statistical certainty required use of many scoreable markers. We desired a direct method for assessing the DNA content of progeny and decided that Random Amplified

Polymorphic DNA (RAPD) analysis should be an efficient method to find DNA markers polymorphic between parents. RAPD markers are dominant markers. An important consideration was the ability to confidently score maternal markers as absent in progeny. Scoring for the presence of RAPD markers separated on an agarose gel with ethidium bromide staining is possible, but to score with high confidence the absence of a band (RAPD marker) is much more difficult due to the background staining within lanes of an agarose gel. Thus, we developed a sensitive and reproducible DNA marker technology that uses fluorescent RAPD primers to allow detection of amplified RAPD markers separated and detected on an ABI 373A DNA Sequencer. Although, this technique was supposedly already possible according to ABI literature, we were unable to effectively perform their protocol. ABI personnel in Foster City, CA were also unable to perform analysis on fluorescent RAPD (FRAPD) amplification products that we sent to them for analysis. The protocol we developed (Chapter 2) allows for reproducible amplification and detection of FRAPD products and allows for confident assessment of the absence of maternal FRAPD products in the progeny.

Successful Production of Haploid and Diploid Zebrafish:

The successful production of haploid and diploid androgenetic zebrafish is presented in Chapter 3. Delayed *in vitro* fertilization (Chapter 1) was used for producing the androgenotes and FRAPD analysis (Chapter 2) was used to assess the androgenetic nature of the genomes. The utility of the androgenetic zebrafish and lessons gained from their production are discussed at the conclusion of the thesis.

Contributions of Graham Corley-Smith to Co-Authored Publications.

For all papers mentioned in this thesis which I co-authored, both peer-reviewed and others, I planned and designed the experiments and I either conducted the experiments myself or supervised their implementation. I analyzed the data and I wrote the various papers in consultation with co-authors. I also constructed and managed the zebrafish facility at Simon Fraser University that was required to conduct the research presented in these publications. Acknowledgments of people other than co-authors who assisted in various aspects of the research are listed in each chapter.

James Chinten Lim competently performed experimental techniques and was a major contributor to both of the published papers included in this thesis. Dr. Kalmar was involved in developing the FRAPD technology. He supervised the making of fluorescent primers, and his expertise in molecular biology and in the use of the ABI 373A DNA sequencer was invaluable. Dr. Brandhorst supervised and supplied monetary support for the research and contributed extensively to the preparation of the manuscripts. The research in both papers was supported on operating grant from the National Science and Engineering Council of Canada to Dr. Brandhorst.

CHAPTER 1: DELAYED *IN VITRO* FERTILIZATION

This chapter, unlike chapters 2 and 3, is not a reproduction of peer-reviewed manuscripts. Much of it was written for this thesis, but some of the information in this chapter appears in previous publications (Corley-Smith et al. 1996; Corley-Smith 1996; Corley-Smith et al. 1995a; Corley-Smith et al. 1995b; Corley-Smith et al. 1995c; Corley-Smith et al. 1995d) and on my web page (http://darwin.mbb.sfu.ca/imbb/brandhorst/zfish.html).

Abstract

For successful *in vitro* fertilization, zebrafish eggs must normally be fertilized almost immediately after being collected from the female. However, I found that zebrafish eggs can be held in the ovarian fluid of coho salmon (*Oncorhynchus kisutch*) or chinook salmon (*Oncorhynchus tshawytscha*) for periods exceeding 1.5 hours with high subsequent fertilization rates; this fluid surrounds the mature eggs in the ovary. To delay fertilization of eggs after they are extruded from the female, zebrafish eggs are held at room temperature (18-22°C) in the salmon ovarian fluid. Eggs can be successfully held for periods exceeding 6 hours, although the fertilization rate is compromised.

Being able to delay fertilization of zebrafish eggs for periods up to a few hours after they are extruded from the female zebrafish enables a variety of experimental operations. It allows time for manipulation prior to fertilization (e.g. irradiation of eggs for androgenesis or microinjection for transgenic experimentation). Delayed fertilization also permits sequential fertilization of small groups of eggs, thus allowing time to perform operations on embryos at a particular post-fertilization developmental stage.

Introduction

Ordinarily the union of a haploid sperm cell and a haploid egg cell results in restoring a diploid set of heterozygous chromosomes and activation (initiation of cleavage divisions) of the zygote. Thus, restoring diploidy and activation are normally linked. However, these events can be unlinked in zebrafish (Streisinger et al. 1981). Insemination with irradiated sperm results in activation without restoring diploidy (Streisinger et al. 1981). Feulgen-stained squashes of whole eggs have revealed that at 28.5°C, metaphase of meiosis II occurs at 4 min post-activation and that metaphase of the first mitotic division occurs at approximately 23.5 min post-activation (Streisinger et al. 1981). These Feulgen-stained squashes also indicate at fertilization the oocyte appears to be in prophase. This is prophase II of meiosis (Selman et al. 1994). Thus, zebrafish eggs either when laid by the female during normal mating or collected by gently squeezing the abdomen of female zebrafish, are secondary oocytes (arrested in prophase of second meiotic division). Ordinarily, insemination results in completion of the second meiotic division, extrusion of the second polar body (shown for other teleosts and assumed for zebrafish), fusion of the male and female pro-nuclei and subsequent initiation of the mitotic cell divisions.

In vitro fertilization of zebrafish eggs (Hart and Messina 1972; Streisinger et al. 1981; Walker and Streisinger 1995) is a valuable research technique. It allows for carefully controlled genetic crosses. *In vitro* fertilization of zebrafish eggs was required for production of gynogenetic progeny. This has allowed performing haploid mutation screens which are very useful. Mutants are detected in the F1 generation, in contrast to

diploid screens used with vertebrate animals in which mutants are commonly detected in the F3 generation. A zebrafish linkage map based on female meiotic recombination rates determined from gynogenetic progeny has been developed (Postlethwait et al. 1994; Johnson et al. 1996). This is one of the few closed (i.e. the number of linkage groups equals the number of chromosomes) vertebrate linkage maps and presently has approximately 652 PCR-based markers, 100 cloned genes and 10 mutations (Personal Communication: Dr. J. Postlethwait, University of Oregon, Eugene, OR). The linkage map and haploid screens, both of which have both been facilitated by *in vitro* fertilization, enhanced the genetic utility of zebrafish as a vertebrate model system.

Delayed *in vitro* fertilization would be useful. In normal mating of zebrafish the males bump against the female and as soon as the demersal eggs are extruded into the water, the male ejects sperm on the eggs. The sperm swim vigorously upon contact with water for 30-60 seconds and fertilize the zebrafish egg via the micropyle. Only one sperm head can fit far enough down the micropyle to permit insemination (Hart and Donovan 1983), thus preventing polyspermy. A specialized region of the head of the sperm fuses with the egg and the male pro-nucleus enters the egg cytoplasm. The sperm body and tail do not enter the cytoplasm. The chorion then rises away from the underlying membrane, thus physically lifting the micropyle away from the underlying membrane. This is probably a secondary physical block to further fertilization. If zebrafish eggs are placed in water, the elevation of the chorion starts within a few minutes. Thus there is only a short period in which insemination is normally possible. If zebrafish eggs are gently squeezed from a female, the eggs can be fertilized successfully for 10-15 minutes. Afterwards, the chorion

has noticeably risen when viewed under a dissection microscope and attempts at fertilization are not successful.

Transgenic and knock-out genetic strategies as used for the mouse (Galli-Taliadoros et al. 1995; Brandon et al. 1995; Copp 1995) are not fully developed for zebrafish. The ability to hold eggs in a viable state, although not essential for production of transgenic embryos, would allow more working time to inject more eggs (secondary oocyte, zygote or early embryo) and thus is desirable. Delayed *in vitro* fertilization may also have some utility in developing knock-out strategies for zebrafish. Once embryonic stem cells lines are developed for zebrafish and methods of homologous recombination are developed, delayed fertilization may be helpful in allowing time to inject cells into more embryos at a particular developmental stage. This could be facilitated by sequential fertilization of small groups of eggs.

To produce androgenetic zebrafish, we decided to eliminate the maternal genome from eggs prior to fertilization. To accomplish this, we needed to develop a method to eliminate the maternal genomic DNA. Since irradiation of eggs within the ovary of live fish is not practical, it is necessary to irradiate eggs after they have been extruded. Thus a method for holding extruded zebrafish eggs was required. Considering the dose rate of our X-ray machine, it was calculated that to perform the dose response experiments (please refer to Chapter 3) eggs must remain fertilizable and viable for a period of at least 30 minutes after extrusion.

I have refined a method to allow holding zebrafish eggs for a period of time prior to delayed *in vitro* fertilization. This method can be used for a number of operations that

can benefit either from having more time to manipulate eggs prior to fertilization, or operations in which expanded time at a particular stage is advantageous. The latter objective can be achieved by sequential fertilization of small groups of eggs.

In Materials and Methods, I present the methods for performing delayed *in vitro* fertilization. In Results, I present experimental data which demonstrate the usefulness of using ovarian fluid for delayed *in vitro* fertilization. Three important criteria for delayed fertilization were considered: 1) the length of delay (at least 30 minutes is desirable), 2) the mortality and fertilizability of the eggs, 3) and the degree of normal development of embryos which underwent delayed *in vitro* fertilization.

Methods and Materials

Collection of Coho and Chinook Salmon Ovarian Fluid:

Coho and chinook salmon are terminal spawners; thus eggs are normally collected at hatcheries immediately following lethal cranial trauma. To collect ovarian fluid, the euthanized female salmon was immediately hung by its tail and all the gills on both sides of its head were slit to drain blood. A wad of paper towels was then pushed under the operculum to help soak up blood and impede coagulation. After 5 minutes, the fish was dried with a towel so that no water could drip into eggs and ovarian fluid when they were collected. One person held the fish by its head and tail, with its belly downward over a clean dry bowl, and a second person then slit the fish from anus to the front of the body cavity. Eggs and ovarian fluid then fell into the bowl. We then removed eggs from skeins (ovarian connective tissue) and removed pieces of skein from bowl containing free eggs and ovarian fluid. Some of the ovarian fluid (~ 75%) was collected into a 50 ml conical plastic tube and stored on ice. The suspension was poured through a non-abrasive stainless steel kitchen strainer to separate eggs from ovarian fluid. Eggs were handled gently at all times to prevent breakage. The hatchery staff then fertilized salmon eggs according to their normal practice. Back at the laboratory, each batch of ovarian fluid was centrifuged at 5500g for 5 min at 4°C to sediment cellular debris. The supernatant was decanted to a fresh 50 ml plastic tube and stored frozen at -80°C.

Collection of Zebrafish Eggs for Delayed *In Vitro* Fertilization

1. Approx. 100 µl of salmon ovarian fluid was pipetted into a 50mm diameter petri dish at room temperature. It formed a small dome near the centre of the dish.
2. A female zebrafish was then anaesthetized in 17 ppm (w/v) Tricaine (3-aminobenzoic acid ethyl ester, Sigma A-5040; pH adjusted to approximately 7 with sodium bicarbonate).
3. The female was then placed belly up under a stereo microscope, resting in a V-shaped slit of a damp sponge.
4. The belly and genital pore were then dried with Kleenex or Kimwipe.
5. Some ovarian fluid from the petri dish was then drawn up in a silanized glass capillary tube (Kimax-51, Kimble Products Art. No. 34502, ID 0.8-1.1mm, length 100mm) and expelled back into the dish. This was done to pre-wet the tube, reducing friction and lessening the chance of breaking eggs.
6. The fish was then squeezed gently and the eggs gently drawn up into the glass capillary tube.
7. The eggs were then observed under a dissecting microscope and assessed for quality by appearance.
8. The eggs were then gently expelled into the ovarian fluid on the petri dish. We tried to avoid placing eggs on a dry dish, which may reduce subsequent fertilization rates.
9. The fish was then returned to water.
10. A lid was then placed on the petri dish to reduce evaporation. Although perhaps not necessary, a black plastic sheets was placed over the dishes to shield out light.

11. Eggs were held at room temperature until fertilization.

Collection of Sperm for Delayed *In Vitro* Fertilization

1. 50µl of sperm extender (Table 1 below) was pipetted into 500µl microcentrifuge tubes on ice.
2. Steps 2 through 4 as described above for the female were then performed.
3. The fish was squeezed gently and milt was taken up into a 2 or 5µl glass capillary tube (Drummond Microcaps) by capillary action.
4. The milt from one male was then gently expelled into a microcentrifuge tube containing sperm extender on ice. Typically 1-2 µl of milt was added to 50 µl sperm extender.
5. The sperm and sperm extender were then gently swirled to mix them.
6. The mixture was stored on ice until needed.

Performing Delayed *In Vitro* Fertilization

1. Most of the ovarian fluid was removed from the eggs with a sterile pipette to a clean 0.5 ml microcentrifuge tube. Later when time permitted, this used ovarian fluid was centrifuged at 5500g for 5 min at 4°C, the supernatant decanted and frozen. Ovarian fluid can be reused several times.
2. 5-15 µl of sperm extender containing sperm was spread evenly over all the eggs in the petri dish.

3. Immediately, 0.5 ml fish water (described in Appendix 1) was added and swirled very gently to mix.
4. After 1 minute, 28.5°C water was very gently added to 3/4 fill the petri dish, and left at 28.5°C for 1 hour. To promote gas exchange, a space was left between the top of the water and petri dish cover.
5. After 1 hour, a stock solution of 0.02% methylene blue was added by pipette to a final concentration of 0.3 PPM. This was done to inhibit fungal growth. Care was taken to make sure eggs do not touch each other, to reduce the spread of fungus that may grow on dead eggs from attacking developing embryos.
6. The petri dishes were then placed in a 28.5°C incubator.
7. After 24 hours, dead embryos were removed and methylene blue was flushed out.

Testing Salmonid Ovarian Fluid for *In Vitro* Fertilization

Individual batches of coho or chinook ovarian fluid vary in their effectiveness in allowing for successful *in vitro* fertilization of eggs collected from zebrafish. Therefore each batch of salmon ovarian fluid collected was tested for its capacity to maintain viability of zebrafish eggs for approximately one hour. The desired scheme was to collect a large number of high quality zebrafish eggs from a single female, divide the eggs into a number of groups. One group was fertilized immediately (control group) the other groups of eggs were held in aliquots of ovarian fluids from different individual salmon for approximately one hour and then fertilized. If possible all groups were fertilized with milt from the same male zebrafish.

The number of developing embryos was scored under a stereo microscope approximately 4-6 or 24 hours after fertilization was attempted.

Lyophilization of Ovarian Fluid

To facilitate distribution to other institutions, aliquots of 1 or 1.5 mls salmon ovarian fluid were place in a microcentrifuge tube (2 ml screw cap type with sealing O-ring) and lyophilized. Following storage it was restored to its original volume using purified water (distilled or reverse-osmosis and de-ionized in a Barnstead Nanopure Ultrapure Water System). We then re-tested the ovarian fluid for its ability to maintain zebrafish eggs in a viable state.

Nomenclature for Ovarian Fluid Samples

We collected salmonid ovarian fluid on seven occasions. A single letter is used as shorthand to designate sequential samples dates, A-F inclusive. On each sample date, ovarian fluid was collected from only one salmon species: A-C were collected from rainbow trout, D-E were collected from coho salmon, and F was collected from chinook salmon. For each sample date, I sequentially numbered the females from which ovarian fluid was collected. As ovarian fluid has been supplied to other researchers and may be collected elsewhere in future, I have used the prefix of SFU. Thus SFU-D8, was collected by SFU researchers, collected on the 4th collection date (coho salmon collection date), and is the 8th female from which we collected ovarian fluid on that date. As ovarian fluid is aliquoted into small volume for ease of handling, I have used the phrase "batch" to indicate ovarian fluid collected from a single female. The batches of salmon ovarian fluid

for which I will present data below are SFU-D, SFU-E and SFU-F. The former two were collected from coho salmon and the latter from chinook salmon.

Reagents and Buffers

Table 1: Sperm Extender Recipe

Chemical Name	Chemical Formula	Concentration (mM)
Hepes	$C_8H_{18}N_20_4S$	10
Potassium Chloride	KCl	80
Sodium Chloride	NaCl	45
Sodium Acetate	$C_2H_3NaO_2$	45
Calcium Chloride	$CaCl_2$	0.4
Magnesium Chloride	$MgCl_2$	0.2

Bring up to volume with ddH_2O, and adjust to pH 7.7 with 1M NaOH. Filter through 0.22µm filter, and store at 4°C.

Results

Coho Salmon Ovarian Fluid

In many trials (data not shown), no fertilization resulted when fertilization was attempted on zebrafish eggs held for over 20 minutes either on ice or at room temperature (18-22°C). In these trials, eggs were either collected in capillary tubes and deposited onto a dry petri dish or squeezed directly into a dry petri dish. Microscopic observations indicate that when zebrafish secondary oocytes are extruded into water or water is subsequently added to eggs in a petri dish, cytoplasmic streaming and elevation of the chorion begins almost immediately. The chorion becomes fully raised within a few minutes of this "activation." I have never succeeded in fertilizing an egg (secondary oocyte) after the chorion is fully raised. Thus exposure to water initiates an activation process culminating in elevation of the chorion and loss of fertilizability. This takes substantially less than the 30 minutes required to irradiate eggs for the production of androgenotes. Elevation of the chorion was arrested or delayed when zebrafish eggs were stored at room temperature in salmon ovarian fluid.

When zebrafish eggs were stored for 60 minutes in various batches of coho salmon ovarian fluid (SFU-D8 & SFU-E1 through SFU-E10), fertilization success measured at ~24 hrs ranged from 56-84% (Table 2). Due to the small number of eggs available, no control groups were performed, but the observed fertilization rates were similar to those for other batches of eggs of similar quality fertilized immediately after extrusion (squeezing) into water.

Table 2: Percentage of eggs held in various batches of ovarian fluid for 60 minutes observed as normal developing embryos at 24 hours after fertilization.

Batch of Ovarian Fluid	Numerical designation of zebrafish from which eggs were collected	Number of Eggs Tested	Percent developing as normal embryos at 24 hours[2]
SFU-E1	1	70	66
SFU-E2	1	79	62
SFU-E3	1	65	68
SFU-E4	2	61	72
SFU-E5	2	66	65
SFU-E6	2	89	69
SFU-E7	3	69	67
SFU-E8	3	74	82
SFU-E9	3	76	84
SFU-E10	4	65	80
SFU-D8[1]	4	73	56

[1] D8 is a batch of ovarian fluid collected previously which we compared to the new batches of ovarian fluid.

[2] Percentages are not adjusted relative to a control. Percentages calculated as ((embryos at 24 hrs / eggs tested) x 100).

Charline Walker and I performed similar tests at the University of Oregon. Even after holding eggs for 90 minutes, more than 50% of the eggs were fertilized and showed normal development (See Table 3).

Table 3: Survival of eggs held in coho ovarian fluid scored 1 day after fertilization.

Female (#)	Eggs per Clutch (#)	Control			Delayed Fertilization				
		Eggs (#)	2n (#)	2n (abs %)	min.	Eggs (#)	2n (#)	2n (abs %)	2n (rel %)
4	137	30	20	67	15	107	72	67	100
1	50	14	12	86	27	36	32	89	103
2	39	15	6	40	29	24	14	58	145
3	26	---	---	---	45	26	6	23	---
5	127	35	13	37	60	92	8	9	24
6	71	22	16	73	90	49	26	53	72

Survival scored at ~ 1 day after fertilization as number of diploid (2n) embryos observed. Coho ovarian fluid E9 used. Females were of the S4302 (*AB) line; males were from various lines and pooled. Date eggs squeezed: Feb 22, 1996. Absolute (abs) % survival calculated based on number of eggs in group with relative (rel) % survival calculated relative to control group. Embryos were scored as surviving to ~24 hrs if they appeared to be reasonably normal diploids (i.e. AA-like; ref. page 2.20 of Walker and Streisinger 1995).

Clutch numbers correspond to females eggs collected from. Egg quality by appearance: Clutch 1 & 2: good; clutch 3: mediocre; clutch 4: some broken eggs; clutch 5: chorions did not rise properly; clutch 6: debris (i.e. broken and resorbing eggs) and chorions did not rise to full normal height.

Sperm quality: sperm was held in Hanks Medium (recipe in The Zebrafish Book, p. 10.8) on ice and an aliquot was tested for mobility in response to addition of water 3 hours after being collected. This was after all groups of eggs had been fertilized. Viewed under compound microscope, >80% of sperm were swimming vigorously.

To investigate if salmon ovarian fluid affects development of zebrafish embryos, several groups of embryos were raised for 24 hours. Charline Walker viewed all embryos surviving to 24 hours (278 embryos) for possible developmental abnormalities using a stereo microscope at 50X or lower magnification. Although she detected many abnormalities and a mutant phenotype (a known mutant carried in a line of zebrafish we used) in some of the progeny, she did not detect any defects that were either solely or mainly contained in treated groups. She cautiously concluded that she had not detected any consistent developmental differences between the control and treated groups. The percentage of fish without swim bladders six days after fertilization was not consistently different in treated versus control groups (Table 4).

Table 4: Percentage of surviving fish scored at day 6 with and without swim bladders.

Treatment	Clutch	Embryos (#)	With S.B. (%)	Without S.B. (%)
control	4	19	89	11
delay 15 min	4	69	83	17
control	1	12	100	0
delay 27 min	1	34	100	0
control	2	6	100	0
delay 29 min	2	12	75	25
control	3	--	--	--
delay 45 min	3	5	100	0
control	5	--	--	--
delay 60 min	5	5	80	20
control	6	16	94	6
delay 90 min	6		100	0

Clutch numbers correspond to the females that eggs were collected from as listed in Table 3. # of embryos is indication of sample size and not measure of fish surviving.

We concluded from these trials that:

1. Coho salmon ovarian fluid was useful for holding eggs for at least 90 minutes.
2. No developmental abnormalities associated with holding eggs in coho ovarian fluid were detected.

Lyophilization of Coho Salmon Ovarian Fluid

An aliquot of SFU-E9 coho salmon ovarian fluid was lyophilized, stored at room temperature for 2 weeks and then reconstituted with distilled water to its original volume. This ovarian fluid was then tested for its capacity to hold zebrafish eggs for ~0.7 and ~1.0 hours in comparison to controls which were placed in SFU-E9 that had not been reconstituted (Table 5). These eggs were irradiated with gamma rays from a ^{137}Cs source

which may have compromised survival and fertilization, but comparison with other groups of eggs irradiated at the same dose was possible. Approximately 10-20 minutes handling time must be added to the irradiation times to estimate total time elapsed between collecting and fertilizing the eggs. While the sample size was small, it is clear that lyophilization and reconstitution did not inhibit the function of coho salmon ovarian fluid for use in delayed *in vitro* fertilization of zebrafish eggs.

Table 5: Comparison of lyophilized and non-lyophilized coho ovarian fluid E-9. Eggs were irradiated with ^{137}Cs. Eggs pooled from 8 females.

Dose (R)	Irradiation time (hrs)	Lyophilized (Yes/No)	Number of Eggs (#)	Fertilized (%)
38,000	0.7	No	71	72
38,000	0.7	Yes	62	77
54,300	1.0	No	98	86
54,300	1.0	Yes	116	96

Chinook Salmon Ovarian Fluid

Chinook salmon ovarian fluid was found to be effective as a delayed *in vitro* fertilization medium for zebrafish eggs (Table 6). Zebrafish eggs were held for approximately 1 hr and survival measured at 24 hours ranged from 18 to 88%. Four batches of chinook ovarian fluid were compared against one of the best batches of coho

salmon ovarian fluid (SFU-E9; see Table 2). For these four batches, the survival relative to SFU-E9 was calculated as (percent survival of eggs held in chinook ovarian fluid / percent survival of eggs from same zebrafish cross held in SFU-E9) × 100%. The survival relative to SFU-E9 ranged from 88% to 372%, indicating that not only is chinook salmon ovarian fluid useful for delayed *in vitro* fertilization of zebrafish eggs, but that it is at least as good, or better on average than coho ovarian fluid for this purpose. Two to three times as much ovarian fluid can be collected from chinook as from coho salmon, which is another advantage of using chinook ovarian fluid. This reduces the amount of testing of ovarian fluid and allows for more consistency when a large number of delayed *in vitro* fertilizations are performed.

Table 6: Testing of chinook salmon ovarian fluid. Survival was measured at 24 hours after fertilization.

Ovarian Fluid Tested	Female	Male	Time Held (hrs:min)	Eggs (#)	Survival Abs. (%)	Survival Relative to SFU-E9 (%)
SFU-F3	1	2	1:10	70	64	88[a]
SFU-F4	1	2	1:10	61	75	103[a]
SFU-F6	2	2	1:02	17	88	N/A
SFU-F8	3	1,2	1:00	37	59	149[b]
SFU-F7	4	3	1:10	34	18	N/A
SFU-F9	5	3	1:05	46	70	N/A
SFU-F11	4	4	1:05	48	77	N/A
SFU-F12	6	5	0:45	22	82	N/A
SFU-F13	8	4	1:00	30	53	N/A
SFU-F14	9	6	0:55	35	66	372[c]

[a] For SFU-E9: number of eggs=35 and survival abs.=73%

[b] For SFU-E9: number of eggs=30 and survival abs.=40%

[c] For SFU-E9: number of eggs=17 and survival abs.=18%

Discussion

There are two important criteria that must be met for delayed fertilization 1) mortality should not be excessive, and 2) the development of surviving embryos should not be significantly different from control embryos. From the results obtained, I conclude that salmon ovarian fluid, from either coho or chinook, is useful for holding zebrafish eggs for at least one hour. Secondly, I conclude that no consistent developmental abnormalities associated with holding eggs in coho ovarian fluid were detected (discussed in more detail below).

Gibbs *et al.* (1994) reported that rainbow trout ovarian fluid can be used for holding zebrafish eggs for delayed fertilization. Our preliminary results suggest that coho or chinook salmon ovarian fluid is superior using our conditions for delayed *in vitro* fertilization.

The utility of various batches of salmon ovarian fluid varied considerably, perhaps due to ovarian fluid being diluted with water or ovarian fluid contaminated from rupturing of salmon eggs. Thus each group of ovarian fluid collected was individually tested. In general we found that chinook ovarian fluid was more effective than that of coho salmon, while ovarian fluid from rainbow trout was least effective. Chinook, coho and rainbow trout are closely related pacific salmon (McKay et al., 1996). We considered it possible that the most effective ovarian fluid will be from species that undergo prolonged upstream migrations to their spawning streams and consequently probably hold their eggs longer. However, it may be that ovarian fluid from any fish species might be useful if properly collected.

When coho ovarian fluid containing zebrafish eggs is diluted with water, the chorions of the eggs rise, and cytoplasmic streaming that leads to formation of the blastodisc is initiated. Although the blastodisc forms, few if any cell divisions take place. Thus, egg activation without insemination is initiated, and the egg can no longer be fertilized. Very little dilution (< 10 % v/v) of coho ovarian fluid is required for initiation of activation. Increasing the proportion of water added, up to a plateau, increases the rate of chorion elevation.

The fact that a slight dilution of ovarian fluid results in activation is not understood. The activation response is likely not simply a reaction to the osmolality of the fluid. Defined culture media having similar or even twice the osmolality of ovarian fluid (1X L-15 medium and 2X L-15 medium, Sigma) do not prevent egg activation. When such media are mixed with small amount of ovarian fluid the rate of activation is retarded. This suggests the presence of a factor with inhibits activation. We have performed a detailed chemical analysis of salmon ovarian fluid. By comparing its chemical constituents to a defined tissue culture medium that retards but does not prevent activation of zebrafish eggs, we hope to identify components that are important for inhibiting activation. Thus we not only hope to understand the mechanisms of egg activation, but also hope to develop a defined medium that can be used for delayed fertilization. The ability to uncouple fertilization and activation in zebrafish offers some new experimental opportunities.

For rainbow trout, coho salmon and chinook salmon ovarian fluids, certain batches of ovarian fluid are more effective for holding zebrafish eggs for the purpose of delayed

fertilizations. We have observed that dilution of the fluid with even a small amount of water resulted in activation of eggs: the chorions elevated and fertilization became impossible. Thus, the utility of salmonid ovarian fluid for delayed *in vitro* fertilization is compromised if diluted with water. Care must be taken to prevent introduction of water during collection of ovarian fluid. Water could be introduced into the ovarian fluid either while still in the salmon (if eggs are retained too long) or while collecting ovarian fluid.

Results presented at the Annual General Meeting of British Columbia Fish Culturalists, (Richmond, B.C., March 1991) indicate that the contents of ruptured salmon eggs compromises fertilizability. It was postulated that the protein from egg cytoplasm may interfere with insemination. Thus, it may follow that the utility for holding eggs of a batch of ovarian fluid may be compromised if some eggs were ruptured during collection.

A key measure of testing ovarian fluids was the fraction of embryos that developed to 4-6 hours (dome to shield stage which extends from late blastula to early gastrulation periods; Kimmel et al. 1995), or at 24 hours (primordia 5 in the pharyngula period; Kimmel et al. 1995). This is usually a good indicator of successful fertilization and normal development. Even in unfertilized eggs, I observed a few cell divisions (usually incomplete, unequal and in abnormal cleavage plane) may occur when water is added. Thus fertilization was assessed in embryos 4-6 hrs after fertilization, or at 24 hrs. Embryos developing normally at dome stage (4-6hrs), usually showed normal development at 24 hrs as well. Thus, survival data collected during these two periods is comparable for our purposes.

It should be noted that fertilization results are approximate and vary depending on the quality of zebrafish eggs used. Using survival data as a measure of fertilization success appears to be appropriate. Fertilization is composed of syngamy and karyogamy, although plasmogamy is minimal in zebrafish. Oogamous fertilization in zebrafish is the union of two gametes to produce a zygote usually followed by cell divisions leading to a multi-cellular organism. Thus by measuring survival to dome or later stage, an underlying assumption is that unless fertilization occurred, a multi-cellular organism would not normally form. Under the conditions used, this is a reasonable assumption for zebrafish since eggs extruded, but not fertilized, do not develop into embryos. The assumption that all eggs fertilized will develop to at least the dome stage is not certain. Thus the fertilization rates reported are minimal rates, as fertilization may have occurred without subsequent development to the dome stage.

Several batches of chinook salmon ovarian fluid were useful in holding zebrafish eggs for 1 hour with fertilization rates up to 88% observed. Lyophilization of ovarian fluid did not appear to decrease the effectiveness of coho salmon ovarian fluid. We now routinely lyophilize all ovarian fluid that we send to other research institutes or which we supply to a commercial distributor in the US (SeaTech Bioproducts), and to date we have not been notified of any problems arising from use of this fluid. Several labs have requested ovarian fluid. At least one use of the fluid has led to a publication (Lee et al. 1996).

Definitively determining if using salmonid ovarian fluid has any impact on subsequent development of zebrafish is not practical. However, Charline Walker and I carefully observed hundreds of embryos up to the swim-up stage (free swimming and feeding stage) by stereo microscopy and did not detect any abnormalities consistently associated with using salmon ovarian fluid. Charline Walker has had extensive experience in mutation screens and I believe she is likely to have noticed any significant and consistent developmental anomalies, but subtle defects might not have been noticed. Eggs held in salmon ovarian fluid prior to fertilization have been raised to adulthood and bred. We have held zebrafish eggs for over 6 hours after extrusion from the female; when subsequently fertilized, they developed into apparently normal zebrafish. Further evidence that ovarian fluid does not lead to developmental abnormalities comes from comparing the percentage of fish that develop swim bladders to those that do not develop swim bladders. Ordinarily, this is the last developmental stage at which is associated with high mortality. Once healthy fish develop swim bladders they begin active swimming and feeding. If juvenile zebrafish engage in both of these activities, they will normally survive to adulthood in our tanks. Thus, our observation that juveniles resulting from eggs held in salmon ovarian fluid progressed past this key developmental point with the same frequency as control juveniles suggests that holding zebrafish eggs in coho salmon ovarian fluid does not have adverse impacts on development or survival.

There are several events normally linked to fertilization of zebrafish eggs including, an initial transient rise in intracellular free calcium. This "Calcium Flash" (Lee et al. 1996) is probably followed by transient calcium waves (as observed in other protostomes and

deuterostomes; Stricker 1996), exocytosis of cortical granules (Donovan and Hart, 1982), membrane retrieval (Donovan and Hart 1986; Hart and Collins 1991), raising of the chorion and micropyle away from plasma membrane (Hart and Donovan, 1983), microtubule dependent ooplasmic segregation (observed in Medaka by Abraham et al., 1993), and completion of meiotic maturation (Streisinger et al. 1981; Selman et al. 1994).

The elevation of the chorion (activation) of unfertilized zebrafish secondary ooctyes has been linked to an accompanying wave of elevated Ca^{2+} traversing the zebrafish egg (Lee et al. 1996), as has been observed for a variety of deuterostomes and protostomes (Jaffe 1993; Eckberg and Miller 1995; Sticker 1996). Although an explosive rise in free calcium appears to provide most of the activating stimulus for activation in zebrafish eggs (Lee et al. 1996), the events that actually trigger the free rise in calcium are presently not fully understood in zebrafish. In *Xenopus*, a receptor of the RGD sequence on the egg plasma membrane is believed to cause egg activation through an intracellular signal transduction system (Iwao and Fujimura 1996). They postulate that a sperm agonist including the RGD sequence may play an important role in egg activation through an egg membrane receptor during the normal fertilization process. As activation of zebrafish eggs occurs on contact with water, with or without sperm present, it is clear that a sperm agonist is not a prerequisite for raising of the chorion, which is normally an indicator of activation of zebrafish eggs. In the protostome worms *Cerebratulus lacteus* (Stricker, 1996) and *Chaetopterus pergamentaceus* (Eckberg and Miller, 1995), treatment with K^+ to depolarize the oolemma results in a "cortical flash" of elevated calcium that spreads rapidly around unfertilized oocytes. If cobalt is used to block calcium channels,

the cortical flash is eliminated (Stricker 1996). In conjunction with other observations, this observation led Stricker (1996) to conclude that "fertilization initially triggers an influx of calcium ions through voltage-gated calcium channels in the oolemma." Following the cortical flash, oscillating waves of calcium were observed for approximately 60-100 minutes post fertilization as meiotic maturation is completed, and such waves continue to occur in cobalt-containing seawater or calcium-free seawater (Stricker, 1996). This later observation suggests that wavelike calcium oscillations after fertilization are dependent on internal calcium stores. The previous observation that a sperm epitope may trigger a signal transduction pathway leading to activation in *Xenopus* (Iwao and Fujimura, 1996), may also be true for the protostomous worm (*Cerebratulus lacteus*). If whole sperm are injected into unfertilized oocytes of these worms, repetitive calcium waves results, however no such oscillations result if the sperm is boiled first (Stricker 1996). This suggests the presence of a heat labile epitope in sperm that can trigger a signal transduction pathway leading to oscillations of free Ca^{2+} ions. The observation in the starfish *Asterina miniata*, that trypsin, chymotrypsin or pronase can induce fertilization-like responses (Carroll and Jaffe 1995), suggests that a protein receptor exists and that it may transduce a signal from the sperm. An alternate hypothesis is that the sperm may introduce into the ooplasm a soluble factor that can trigger calcium release without involving an oolemmal-related signaling step (Stricker 1996). Recent evidence indicates that the differences in fertilization-induced calcium fluxes between protostomes and deuterostomes are less clear cut than once believed (Stricker 1996). Cortical flashes

have been observed to be present and absent in both the protostome and deuterostome lineages (Stricker 1996).

The functional significance of the cortical Ca^{2+} flash remains unresolved (Stricker, 1996) and how the events of fertilization, cortical granule exocytosis, elevation of the fertilization membrane (or chorion), cytoplasmic streaming and meiotic maturation are linked is not fully understood. Transient calcium waves may be involved with these events (Speksnijder et al., 1990) and also with gene regulation (Stricker 1996), but further research remains to be done. In the past, a major obstacle to investigating these events in zebrafish was the inability to delay *in vitro* fertilization. Lee et al. (1996) have taken the first step by showing a positive correlation between increases in intracellular Ca^{2+} in unfertilized secondary oocytes of zebrafish and elevation of the chorion.

Research on egg activation will continue in Dr. Brandhorst's laboratory.

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Chapter 2: Efficient Detection of DNA Polymorphisms by Fluorescent RAPD Analysis (FRAPD)

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Graham E. Corley-Smith, Chinten James Lim, Gabriel B. Kalmar
and Bruce P. Brandhorst.

Institute of Molecular Biology and Biochemistry,
Simon Fraser University,
Burnaby, British Columbia, V5A 1S6,
Canada.

Address correspondence to:

Graham E. Corley-Smith, Institute of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, B.C., V5A 1S6, Canada

e-mail: corley@sfu.ca

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ABSTRACT

A method is presented for analysis of fluorescently labeled Random Amplified Polymorphic DNA (FRAPD) fragments. An Applied Biosystems (ABI) 373A DNA Sequencer and GeneScan software were used to estimate the sizes of DNA fragments based on their mobilities relative to inlane size markers. This allowed confident identification and comparison of FRAPD markers both within and between polyacrylamide gels. In comparison with analysis of RAPD products using ethidium bromide stained agarose gels, fluorescent analysis improved the sensitivity, resolution, and precision of sizing of RAPD products of about 50-2100 bps. FRAPD fragments produced from amplification of zebrafish DNA are informative as genetic markers which segregate with Mendelian inheritance. FRAPD analysis was found to be very efficient for identifying new DNA polymorphisms.

INTRODUCTION

Detection of sequence differences in DNA samples is useful for many applications including: positional cloning, generation of high resolution genetic maps, and inheritance analysis. Random Amplified Polymorphic DNA (RAPD) allows the generation of many potentially polymorphic fragments from a single short primer (usually a decamer) of arbitrary sequence in a polymerase chain reaction (PCR) and are usually detected using agarose gel electrophoresis and ethidium bromide staining (7,10). We present a technique using polyacrylamide (PA) gel electrophoresis, fluorescent detection and inlane size markers that increases the sensitivity and reliability of identification of RAPD products.

The ABI 373A DNA Sequencer and GeneScan 672 collection and analysis programs are routinely used for analyses of polymorphic microsatellites and other simple sequence repeats (SSR)(5). However, we are unaware of any reports of their application to analysis of RAPD products. Analysis of fluorescent RAPD (FRAPD) products introduces some technical difficulties in comparison with analysis of microsatellites, since FRAPD fragments are fluorescently labeled on both ends, and some are larger than fragments normally used for microsatellite analysis. We found it difficult to discern if two closely spaced bands on a denaturing PA gel represent two different FRAPD fragments or two strands of the same fragment resulting from single strand conformational polymorphisms. Thus we used native conditions for electrophoresis. We present methods, including an adjustment of the size standards, which result in the reproducible and sensitive detection and precise identification of FRAPD products.

METHODS AND MATERIALS

PCR and Gel Conditions

Tissue samples were collected from zebrafish (*Danio rerio*) and DNA prepared by phenol/chloroform extraction. Quantification of DNA was done by spectrofluorometry using Pico-Green (Molecular Probes Inc., Eugene, OR). We synthesized and fluorescently labeled RAPD decamer oligonucleotide primers; 6-FAM (Applied Biosystems Inc., Foster City, CA) was attached to the 5′ end (1). For simplicity we focus on results obtained using a single RAPD primer (sequence of RAPD primer number 210 produced by the Nucleic Acid-Protein Service Unit, University of British Columbia, Vancouver, B.C., Canada).

For RAPD amplifications, 4 ng of each template DNA was suspended in PCR cocktail containing: 3 mM $MgCl_2$, 50 mM Tris-HCl pH 8.3, 0.25 mg/ml crystalline bovine serum albumin, 100 μM of each dNTP (Pharmacia Biotech, Uppsala, Sweden), 0.06 U/μl of Taq DNA polymerase (in storage buffer B; Promega Corp., Madison, WI), and 1.6 μM of a fluorescently labeled (6-FAM) RAPD decamer primer. Thermocyling was performed in heat sealed glass capillary tubes containing a total volume of 10 μl using an Idaho 1605 Air Thermo-Cycler (Idaho Technologies, Idaho Falls, ID). Two cycles of 91° for 60 s, 42° for 7 s, and 72° for 70 s were followed by 38 cycles of 91° for 1 s, 42° for 7 s, and 72° for 70 s, which was followed by a 3 min hold at 72°.

For each FRAPD reaction, an 8.0 μl aliquot was loaded onto a 1.8% agarose gel (Bio-Rad Laboratories, Hercules, CA, Cat. #162-0126) in 0.5 × TBE buffer (TBE: 0.09

M Tris base, 0.09 M boric acid, 0.002 M EDTA) containing 0.5 μg/ml ethidium bromide. DNA products were visualized with a 300 nm transilluminator and the images digitized using a UVP Gel Documentation System (Ultraviolet Products Inc., San Gabriel, CA). Digitized images were analyzed using the GelReader program, Macintosh ver. 2.0.5 (National Center for Supercomputing Applications, University of Illinois at Urbana-Champaign). The GelReader program compensates for variability between lanes by using isomolecular weight lines connecting DNA size standards in multiple lanes.

An aliquot of each FRAPD reaction was diluted 5 times and 2 μl of this solution was mixed with 3 μl of ABI agarose loading buffer containing 4 fmol of fluorescent size standards (ABI GS-2500 ROX). This mixture (5μl/well) was loaded onto a 4% native polyacrylamide gel (Bio-Rad Cat. #161-0144) in TBE and electrophoresed on an ABI 373A DNA Sequencer. Data were collected using the ABI GeneScan Collection Software (version 1.1) and analyzed with the ABI GeneScan PCR Analysis Software (version 1.2.2-1). RAPD markers are dominant markers and were scored as either present or absent; this is facilitated by the low baseline observed. Markers were named according to the convention adopted for the zebrafish RAPD linkage map (4,8).

Reassignment of Size Standard Values

The GeneScan program identifies and sizes peaks based on internal algorithms and specifications entered by the user, including a list of sizes to be assigned to the inlane size standards. Initial separations of FRAPD fragments performed according to ABI specifications, and in collaboration with the ABI CORE lab in Foster City, CA, resulted in only some of the 24 lanes per gel being analyzable. Lanes could be analyzed separately by

changing user defined specifications, but this prevents comparisons of data from lane to lane within a gel, undermining confident peak identification. The problem was associated with the anomalous mobilities observed for some of the fragments of the size standards, as shown in Figure 1.

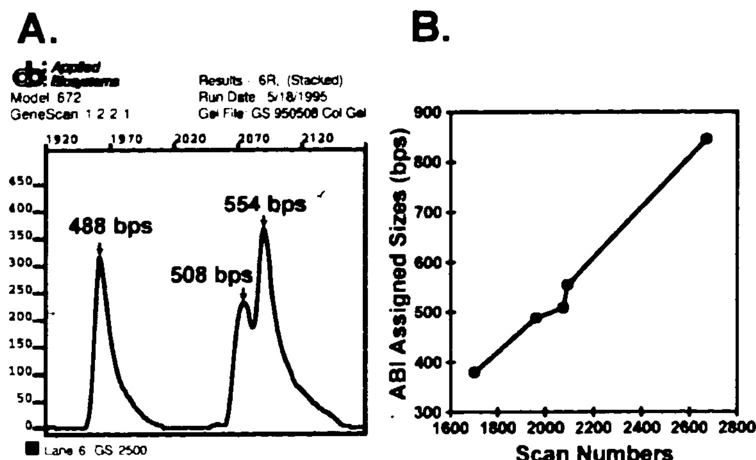


Figure 1. Anomalous mobility of the GeneScan-2500 ROX inlane size standards. A) GeneScan electropherogram of size markers separated on a 4% native polyacrylamide gel under the conditions described in Methods. The X-axis indicates mobility (scan number; scans every 6 seconds). The Y-axis indicates arbitrary units of fluorescence. Sizes indicated above peaks were assigned according to ABI (2). B) Plot of peak sizes assigned by ABI against scan number, which shows the anomalous mobility of the 508 bp marker.

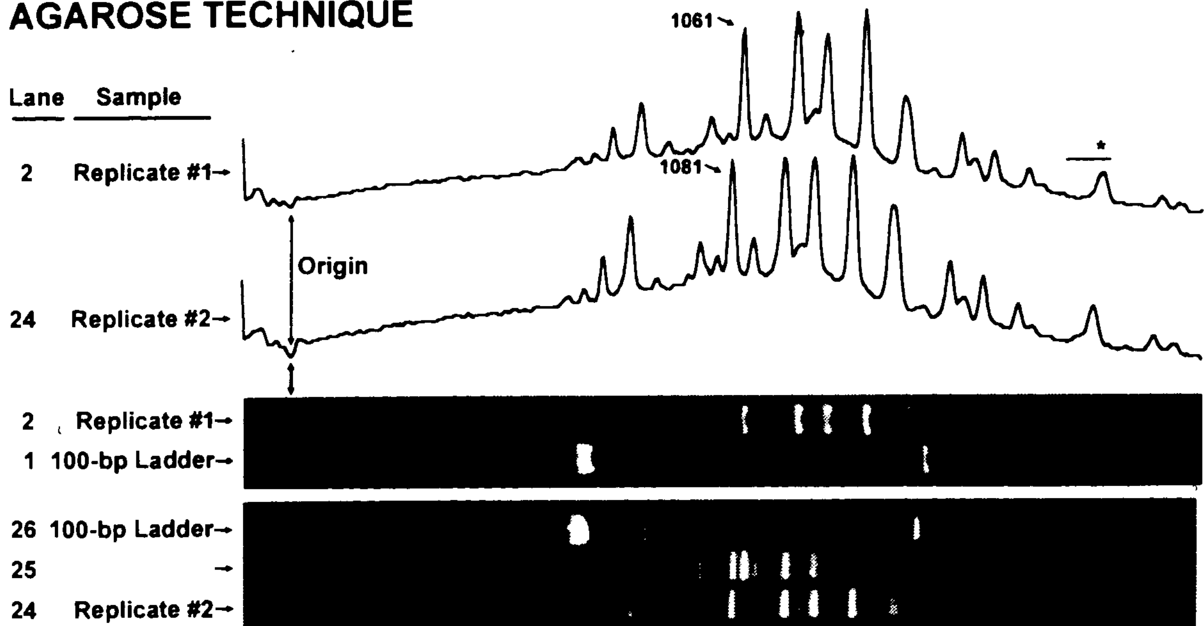
The peaks of the size standards, fragments of λ phage DNA fluorescently end-labeled with ROX, corresponded to ABI specifications (2, and insert supplied with the GeneScan-2500 ROX kit): 55, 112, 127, 134, 190, 204, 240, 251, 256, 287, 304, 379,

488, 508, 554, 845, 1133, 1199, 1740 and 2026 bps. Using the SIZE program (6), adapted to accept input of mobility as scan number, we reassigned size values to these peaks based on the best fit curve of peak size plotted against scan number: 58, 112, 125, 132, 187, 202, 237, 249, 254, 290, 308, 378, 494, 553, 856, 1128,.1194, 1741, 2100 bps. Note that, as shown in Figure 1, the 508 and 554 peaks are poorly resolved and have been re-assigned a single value of 553. These re-assigned size standard values were used in all subsequent analyses with the GeneScan software and allowed reproducible assignment of sizes for all 24 lanes in the range from approximately 50 bps to 2100 bps.

RESULTS AND DISCUSSION

In Figure 2, products from two separate PCR amplifications using the same primer-template combination were compared after separation on agarose and PA gels. The number of markers detected by each method is an indication of sensitivity. Agarose analysis detected 21 bands and FRAPD analysis detected at least 43 products. The sensitivity of detection of FRAPD fragments (2 fluorescent labels/fragment) is constant over the size range of fragments in comparison to agarose analysis where size of fragment influences intensity of fluorescent signal. Thus, the sensitivity of detecting small fluorescent DNA fragments using the fluorescent method is especially improved compared to ethidium staining. What appeared as a single RAPD product on the agarose gel (sized as 336 bp by the GelReader program in lane 2) was resolved as seven products using fluorescent analysis. Improper identification of two or more markers as a single marker can confuse interpretation of inheritance data.

Identification of RAPD markers is based on their sizes calculated from their mobilities relative to size standards. Replicates of the same primer-template combination were analyzed on different, widely separated lanes of the same gel (Figure 2). The coefficient of variation (12) was less for GeneScan calculated sizes (0.07%) than for GelReader calculated sizes (2.09%). For a given template-primer combination, even when FRAPD reactions were performed several days apart and analyzed on different PA gels, all products were assigned reproducible sizes that varied by less than 0.22% and showed similar peak heights relative to other peaks (data not shown). Thus, corresponding FRAPD peaks could be identified with confidence between gels.



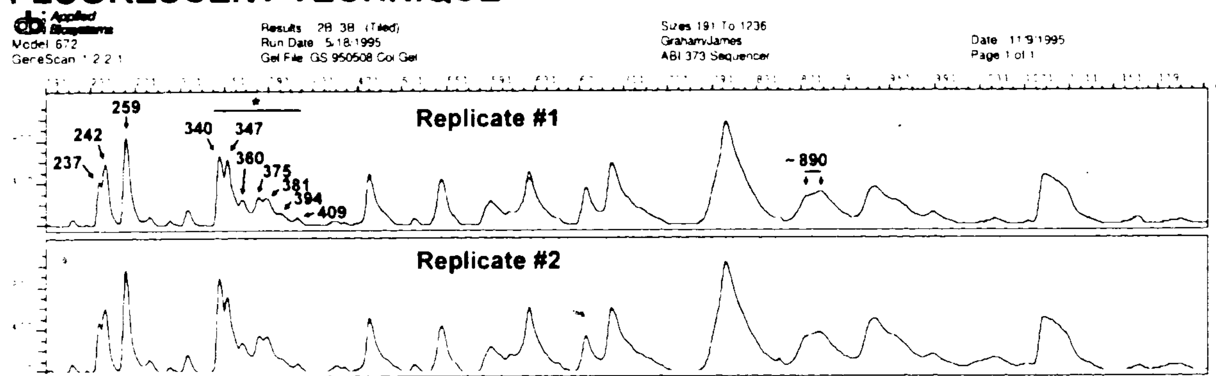


Figure 2: Comparison of methods for analysis of RAPD products. Two separate PCR reactions were prepared using the same template DNA and fluorescent primer. An aliquot of each reaction was analyzed on a 1.8% agarose gel with ethidium bromide staining (Agarose Technique Panel) and on a 4% native PA gel using fluorescent detection (Fluorescent Technique Panel). Agarose Technique Panel: Agarose gel and densitometric

comparisons of the two samples in lanes 2 and 24; the densitometric plots are shown as output from GelReader. The gel had 26 lanes, with 230 ng of 100 bp ladder (Cat. No. 15628; Life Technologies™, Gaithersburg, MD) loaded in lanes 1,12,17, and 26; the bright band is the 600 bp marker. Lane 25 had a different PCR sample to provide spacing. Fluorescent Technique Panel: Traces shown are output from the GeneScan Analysis program. The * indicates a corresponding region of the agarose and PA gels. The single peak on the agarose gel was resolved into 7 peaks by GeneScan.

Although the densitometric scans of replicates run on an agarose gel are similar, peaks did not precisely coincide nor were they precisely sized when compared with size markers in nearby lanes (e.g., the peak sized at 1061 and 1081 bp in different lanes in Figure 2). When the template is the same, it is easy to identify corresponding markers by counting peaks. However, this is less reliable when comparing RAPD products amplified from different DNA samples which do not share all markers. FRAPD analysis allows more confident identification of markers in comparing different DNA samples.

Inheritance analysis requires reproducible detection and confident identification of markers from different DNA samples, an example of which is shown in Figure 3. In extensive pedigree analyses on zebrafish (4; our unpublished observations), FRAPD markers were found to be informative for inheritance analysis for the following reasons: 1) they are highly reproducible; 2) all scorable markers observed in progeny to date have been observed in one or both parents; 3) segregation of markers is consistent with

Mendelian inheritance according to Chi-square analysis; and 4) of 15 polymorphic FRAPD markers analyzed for segregation, none were found to be closely linked, indicating that they are not length variants of the same locus and that these markers appear to be randomly distributed through the genome of zebrafish. Peaks for homozygous markers are consistently larger than for heterozygotes. For example, in Figure 3, two parental specific markers (210bcf.452 and 210bcf.799) were both apparently homozygous in that parent since they were present in all 12 progeny tested, but as smaller peaks.

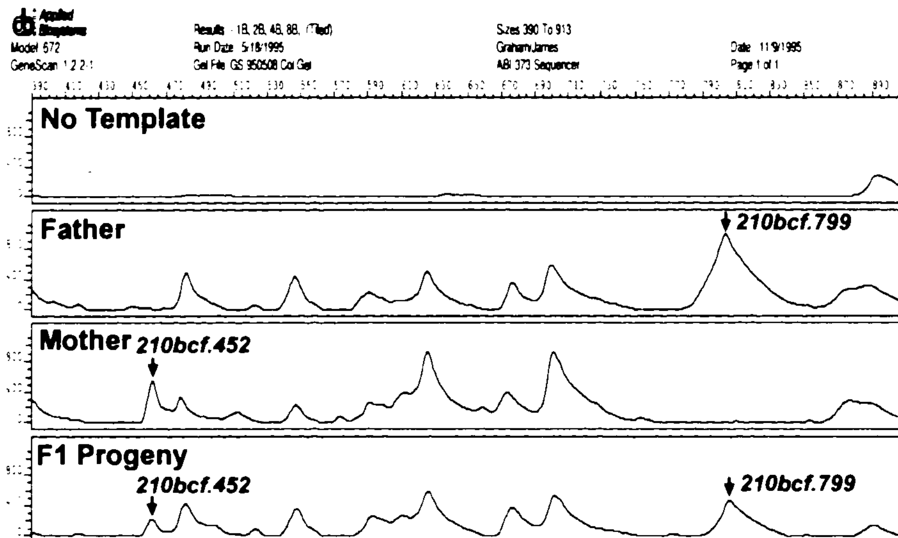


Figure 3: Demonstration of transmission of FRAPD markers to progeny. FRAPD products of zebrafish DNA are shown for a father, mother, and one of their progeny, as well as a reaction to which no DNA template was added. All FRAPD peaks in the progeny had a corresponding peak of very similar calculated size in one or both parents: e.g., the maternal specific marker 210bcf.452 was sized as 452.63 in the mother and 452.13 in the progeny. Modified from (4).

RAPD technology has sometimes been criticized for a lack of reproducibility and the generation of artifactual products. Inheritance studies of RAPD markers indicate that both of these reported problems have been mitigated (4,7,8). Reproducibility can be assured by careful quantification of all thermocycling reaction components, particularly template DNA concentration (10) (we recommend fluorescent rather than UV quantification, as impurities have less effect), combined with stringent thermocycling conditions. A slow ramp speed between denaturing and annealing temperatures may allow annealing of non-complementary template strands to each other, thus resulting in heteroduplex (non-parental) band formation (3,9). This can be mitigated by low concentration of the DNA template relative to primer, fast ramp speeds (as achieved in capillary tube electrophoresis), and sufficient stringency for primer annealing.

Using three FRAPD primers applied to 10 pairs of individual zebrafish from the SFU and *AB lines, from 5 to 9.5 polymorphisms per primer were detected. More frequent detection of FRAPD markers would be expected in a highly polymorphic cross. FRAPD markers were detected in an inbred line of fish. Other methods for detecting polymorphic DNA markers which can utilize fluorescent labels include SSR/microsatellite analysis and amplified fragment length polymorphisms (11). The former requires cloning and sequence analysis to create custom primers before being able to scan for new polymorphic markers, while the latter requires several operations besides PCR amplification. FRAPD technology is much simpler to apply in scanning for new polymorphic markers and is more random in sampling the genome than SSR analysis, which we have found to be considerably less efficient (4). While an automated DNA sequencer is expensive to

acquire and operate, the cost per analyzed sample can be decreased and throughput increased by multiplexing of three FRAPD samples per lane. The cost of producing a fluorescent primer is $75-150 more than for a standard RAPD primer (<$0.04/reaction). The efficiency and precision of detection of new FRAPD polymorphisms may justify the increased cost, especially if kits of fluorescent primers become commercially available.

ACKNOWLEDGMENTS

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Chapter 3: Production of Androgenetic Zebrafish (*Danio rerio*).

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Graham E. Corley-Smith, Chinten James Lim and Bruce P. Brandhorst

Institute of Molecular Biology and Biochemistry,

Simon Fraser University,

Burnaby, British Columbia,

V5A 1S6,

Canada.

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ABSTRACT

To help investigate the evolutionary origin of the imprinting (parent-of-origin mono-allelic expression) of paternal genes observed in mammals, we constructed haploid and diploid androgenetic zebrafish (*Danio rerio*). Haploid androgenotes were produced by fertilizing eggs which had been x-ray irradiated to eliminate the maternal genome. Subsequent inhibition of the first mitotic division of haploid androgenotes by heat shock, produced diploid androgenotes. The lack of inheritance of maternal specific DNA markers (RAPD and SSR) by putative diploid and haploid androgenotes confirmed the androgenetic origin of their genomes. Marker analysis was performed on 18 putative androgenotes (five diploids and 13 haploids) from six families. None of 157 maternal specific RAPD markers analyzed, some of which were apparently homozygous, were passed on to any of these putative androgenotes. A mean of 7.7 maternal specific markers were assessed per family. The survival of androgenetic zebrafish suggests that if paternal imprinting occurs in zebrafish, it does not result in essential genes being inactivated when their expression is required for development. Production of haploid androgenotes can be used to determine the meiotic recombination rate in male zebrafish. Androgenesis may also provide useful information about the mechanism of sex determination in zebrafish.

INTRODUCTION

Zebrafish, (*Danio rerio*, formerly known as *Brachydanio rerio;* MEYER *et al.* 1993) are an important model for studying vertebrate development, and are amenable to genetic analysis (STREISINGER *et al.* 1981; KIMMEL 1989; BARINAGA 1990; NÜSSLEIN-VOLHARD 1994; CONCORDET and INGHAM 1994; DRIEVER *et al.* 1994; KAHN 1994; BARINAGA 1994). Their use as a model organism for genetic analysis is facilitated by a linkage map of DNA markers (POSTLETHWAIT *et al.* 1994) and large scale screens for mutations are underway (MULLINS *et al.* 1994; DRIEVER *et al.* 1994; KAHN 1994).

To investigate imprinting in vertebrates and to help develop zebrafish as a genetic system, we constructed haploid and diploid androgenotes. Construction of individuals with uniparental inheritance can facilitate genetic analysis. Haploid and diploid gynogenotes have been produced by fertilizing zebrafish eggs with sperm irradiated to eliminate the paternal genome (STREISINGER *et al.* 1981; HÖRSTGEN-SCHWARK 1993). Haploid gynogenotes were used to produce a zebrafish linkage map based on rates of meiotic crossing over in oocytes (POSTLETHWAIT *et al.* 1994). Haploid gynogenotes complete embryogenesis and arrest as larvae. Thus, haploid embryos can be used for F1 mutant screening (KIMMEL 1989): mutations in the stem cells of the maternal germ line are introduced by fertilization with mutagenized sperm or by mutagenesis of early embryos. Such screens

require maintenance of considerably fewer progeny to recover an interesting recessive mutation from the maternal stock than conventional diploid screens which require production of an F3 generation (MULLINS *et al.* 1994; KAHN 1994). Diploid gynogenotes can be produced by inhibiting extrusion of the second polar body or by inhibiting the first mitotic division of the gynogenote (STREISINGER *et al.* 1981; HÖRSTGEN-SCHWARK 1993). In the latter instance, the progeny are homozygous and clonal lines of gynogenetic zebrafish have been produced (STREISINGER *et al.* 1981; KIMMEL 1989).

Androgenetic haploid progeny would result from fertilization of eggs that have been treated to eliminate the maternal genome. A method for production of androgenetic zebrafish has not been reported, but would facilitate determination of rates of meiotic recombination in males, mapping of male specific DNA markers and linkage groups (if any), and analyses of sex determination and genomic imprinting. Use of androgenetic haploids may have some usefulness for F1 mutant screens if interesting mutations can be reliably recovered from cryopreserved milt long after the screen was performed.

Like haploid gynogenotes, haploid androgenotes are expected to arrest following embryogenesis, but inhibition of the first mitotic division should produce homozygous diploid androgenotes. A major technical impediment to producing androgenetic zebrafish has been the short period of time following

egg collection during which successful fertilization can be accomplished. This restricts the opportunity for manipulations, such as irradiating to destroy the maternal genome. Other technical impediments include the possibility that irradiation of eggs might damage the egg cytoplasm, maternal RNA, or mitochondrial DNA. Genomic imprinting of essential genes that are irreversibly suppressed at a required developmental stage and derived from the paternal genome as in mammals (MCGRATH and SOLTER 1984; SURANI *et al.* 1984; SURANI 1986; BARRA and RENARD 1988; SAPIENZA 1990; RENARD *et al.* 1991; GOLD and PEDERSON 1994; CHAILLET *et al.* 1995) would make the survival of androgenotes impossible. Recessive lethal mutations could limit the successful production of androgenotes, but this is not expected to be an insurmountable problem since gynogenetic homozygous zebrafish have been produced (STREISINGER *et al.* 1981; HÖRSTGEN-SCHWARK 1993), and inbred lines are available. We present genetic evidence that haploid and diploid androgenetic zebrafish can be constructed. To confirm androgenesis, lack of inheritance of maternal markers is a crucial part of the analysis. Markers relying on gene expression (e.g., phenotypic traits, isozymes, and allozymes) can be affected by many factors including imprinting, tissue specific expression, and developmental specific expression. Failure to detect a maternal marker that results from gene expression in a putative androgenote can be attributed to lack of maternal DNA in the putative androgenote or to

lack of expression. Thus, we directly assayed the DNA of putative androgenotes for maternal specific markers using PCR (polymerase chain reaction) methods.

METHODS AND MATERIALS

Production of androgenetic fish: Androgenetic haploids are produced by irradiating eggs to destroy the maternal genome, followed by fertilization. By inhibition of the first mitotic division, diploid androgenotes can be produced.

A Torrex 150D x-ray inspection system (Faxitron X-Ray Corp., Buffalo Grove, IL.) was used to irradiate eggs. X-ray dosimetry was performed with a MDH1515 dosimeter using a MDH model 10X5-180 ion chamber (paddle chamber). This was calibrated with a known ^{137}Cs source (NBS source #47455). The appropriate dose to eliminate the maternal DNA without unduly decreasing subsequent survival rates was determined based on the Hertwig effect (HERTWIG 1911). Eggs were collected from fish anaesthetized in 17 ppm (wv) Tricaine (3-aminobenzoic acid ethyl ester, Sigma A-5040; pH adjusted to approximately 7 with sodium bicarbonate) by gently squeezing the abdomen. Eggs were collected into a silanized capillary tube and placed into approximately 100 µl of coho salmon (*Oncorhynchus kisutch*) ovarian fluid (the fluid surrounding mature eggs) in a petri dish. The milt was collected just prior to being used for fertilization and was held in sperm extender: 80 mM KCl, 45 mM NaCl, 0.4 mM $CaCl_2$, 0.2 mM $MgCl_2$, 45 mM sodium acetate, and 10 mM Hepes, pH 7.7 (Gibbs *et al.* 1994).

In the first experiment, eggs were collected from one female, and divided into 8 groups. Each group of approximately 100 (88 -112) eggs was held in coho salmon ovarian fluid and was exposed to a different total accumulated irradiation dose. All eggs were simultaneously inseminated, and survival was scored at 1 day post-fertilization (p.f.). In the second trial, 5 groups of eggs (69 -102 eggs) from a single female were irradiated with different total accumulated doses of x-rays. Living embryos were scored at 1 day p.f. and at 4 days p.f. according to appearance. Based on the results of these two dose response trials, a dose of 10,000 R (Roentgens) was used in the following experiments to produce androgenotes.

The first mitotic division was inhibited by heat shock treatment. After fertilization, eggs were held at 28.5±0.5° for 13 min, then heat shocked for 2 min at 41.4±0.05°, and returned to 28.5° (modified from STREISINGER *et al.* 1981). Temperatures were measured with a calibrated thermometer (Fisher Scientific, Cat. No. 15041A) having an uncertainty certified not to exceed 0.03°.

Families analyzed: Fish from two laboratory lines of fish were used: 1) *AB line (star AB line), which has been screened to reduce recessive lethals (C. WALKER, personal communication); 2) the SFU line, which originated from zebrafish bought from pet stores on Vancouver Island, B.C., Canada.

Production of haploid or diploid androgenotes was attempted in 14 families (two families used for Hertwig experiments plus families A - H; Tables 4-5, plus seven other families). In six of these families (Table 5) inheritance of parental DNA markers was assessed in a sample of the putative androgenetic progeny, with Family A being subjected to the most thorough genetic analysis.

Family A: Eggs were collected from one female of the SFU line, and the milt was from one male of the *AB line. The eggs were held in coho salmon ovarian fluid at room temperature for 50 min, the time required for irradiation of eggs. Of the 280 eggs collected, 76 eggs were not x-ray irradiated (NI) and 204 eggs were irradiated (I) with 10,000 R of x-rays. All eggs were then simultaneously inseminated. Of the irradiated eggs, 49 were not heat shocked (I/NHS; treatment to produce putative haploid androgenotes: PHA) and 155 were heat shocked (I/HS, treatment to produce putative diploid androgenotes: PDA). The NI/NHS (not irradiated and not heat shocked) treatment was used to produce normal biparental diploid progeny: BDP.

DNA extraction: The following tissue samples were collected to prepare DNA extracts: both parents (caudal fin clips); 12 biparental diploid progeny (whole fish collected at five days post-fertilization (p.f.); two putative haploid androgenotes (whole fish collected at 5 days p.f.); one putative diploid

androgenote (whole fish at nine days p.f.); and a second putative diploid androgenote (caudal fin clip at 1.5 months p.f.). Whole fish were collected before feeding to decrease DNA contamination. All tissue samples were thoroughly rinsed with pure water before DNA extraction. DNA was prepared by phenol/chloroform extraction as described by ASHBURNER (1989). The protocol was slightly modified by extracting twice with phenol, twice with 1:1 phenol/chloroform and once with chloroform; DNA precipitation was done by adding 1/10th volume 2.5 M sodium acetate (pH 5.5) and two volumes cold absolute ethanol. Quantification of DNA was done by spectrofluorometry using Pico-Green (Molecular Probes, Inc., Eugene, OR) on a SLM 4800™C Subnanosecond Spectrofluorometer (SLM Aminco®, SLM Instruments Inc., Urbana, IL). DNA quantification standards were prepared from calf thymus DNA (Sigma, D 1501).

Fluorescent DNA Primers: RAPD (Random Amplified Polymorphic DNA) (WILLIAMS *et al.* 1993) oligonucleotide primers were synthesized and fluorescently labeled with 6-FAM on an ABI 392 DNA/RNA synthesizer (Applied Biosystems Inc.). FAM amidite (6-FAM) is a carboxyfluorescein derivative and was attached to the 5' end of the primer by amino linker chemistry according to ABI user bulletin number 67. Quantification of fluorescent primers was based on absorbance at 260 nm using a Beckman DU640 Spectrophotometer. Primers were diluted to their working

concentration of 5.0 µM in pure water and stored frozen. RAPD primer 208BCF is ACGGCCGACC, RAPD primer 210BCF is GCACCGAGAG, and RAPD primer 269BCF is CCAGTTCGCC. These sequences correspond to RAPD primers numbers 208, 210 and 269, respectively, that were produced by Nucleic Acid - Protein Service (NAPS) Unit, University of British Columbia, Vancouver, B.C., Canada.

PCR conditions: For RAPD amplifications, 4 ng of each template DNA was added to an aliquot of PCR cocktail containing: 1X Idaho 3 mM Mg buffer (Idaho Technology Inc., Idaho Falls, ID), 100 µM of each dNTP (Pharmacia) and 0.06 U/µl of Taq DNA polymerase (Promega in storage buffer B) and 1.6 µM of one of the three fluorescently labeled 10-mer primers. Thermocyling was performed in heat sealed glass capillary tubes containing a total volume of 10 µl in an Idaho 1605 Air Thermo-Cycler. Two cycles of 91° for 60 s, 42° for 7 s, and 72° for 70 s was followed by 38 cycles of 91° for 1 s, 42° for 7 s, and 72° for 70 s, which was followed by a 3 min hold at 72°. SSR (simple sequence repeats) conditions were identical except that annealing was at 69° for 10 s.

Analysis of RAPD-PCR products: Aliquots of each PCR reaction were separated by electrophoresis on agarose and polyacrylamide gels. One aliquot of each PCR reaction was loaded onto 1.8% agarose gels in 0.5 X TBE (0.045 M Tris base, 0.045 M boric acid, 0.001 M ethylenediaminetetraacetic

acid) containing 0.5 µg/ml of ethidium bromide. Electrophoresis was performed at 3 V/cm. Ethidium bromide staining was visualized with a 300 nm transilluminator and the images were captured on a UVP Gel Documentation System (UVP San Gabriel, CA). These images were analyzed using the NCSA GelReader program, Macintosh version 2.0.5 (National Center for Supercomputing Applications, University of Illinois at Urbana-Champaign). The second aliquot of each RAPD-PCR reaction was loaded onto a 4% native polyacrylamide gel on an ABI 373 DNA Sequencer. ABI's fluorescent ROX-GS2500 inlane size standard was also loaded onto all lanes. Data were collected with the ABI GeneScan Collection Software (version 1.1) and analyzed with the ABI GeneScan Analysis Software (version 1.2.2-1). RAPD markers are dominant markers and were scored as either present or absent. By scoring for presence or absence of each marker in 12 normal biparental diploid progeny, it was determined if the marker was heterozygous or apparently (see below) homozygous in the parent. To check that the markers were segregating in a normal Mendelian fashion, for each of the heterozygous markers the number of BDP containing that marker were scored. The observed present:absent ratio in the F1 was then checked against the theoretical ratio of 1:1 using chi-square analysis, employing the Yates correction for continuity. To check that markers were assorting independently, each heterozygous marker was then tested for *cis* or *trans*

linkage with each other marker. Markers were named according to the convention adopted for the zebrafish RAPD linkage map (POSTLETHWAIT *et al.* 1994): markers are prefixed with the RAPD primer used and suffixed with the size designation obtained from the Genescan software. For example, *210bcf.453* is a marker amplified with the sequence of primer 210 from the UBC primer series, the F designates a FAM labeled primer, and 453 designates the size of the amplified fragment.

MHC and SSR pedigree analysis: To further investigate inheritance to putative androgenetic progeny, other PCR-based methods were used to screen for polymorphisms between parental DNA's. The MHC (Major Histocompatibility Complex class II genes) primers Tu360 and Tu385 (ONO *et al.* 1992) were used as they often show polymorphisms between the AB line of zebrafish and other zebrafish strains (POSTLETHWAIT *et al.* 1994). We also used a set of 16 forward and reverse simple sequence repeat (SSR) primers (GOFF *et al.* 1992). The fragments amplified by the MHC primer Tu360 and Tu385, and the fragments amplified by the SSR primers have been placed on the zebrafish linkage map (POSTLETHWAIT *et al.* 1994).

RESULTS

Irradiation dosage: In the first Hertwig dose response survival trial (Figure 1), a shoulder was observed at 10,000 R. Survival in the first trial included embryos that developed as masses of cells without any discernible body axis at 24 hrs p.f. (post-fertilization). In the second trial, only those surviving embryos which developed a body axis are represented in Figure 1. For 5,000 R dosage group at 24 hr, 5% had developed a body axis and 52% had developed as a mass of cells with no body axis. These were both scored as alive in experiment 1. When viewed at 4 days p.f., in the unirradiated group, all embryos alive at 24 hr were still alive and all appeared to be normal diploids (see below and Figure 2), the group irradiated with 500 R had four surviving embryos that appeared to be normal diploids and the rest of the group had moderate to severe abnormalities, none of which displayed the haploid syndrome (see below). All embryos from the 5,000 R group were dead and had arrested as grossly abnormal embryos. In the 10,000 R group, all embryos displayed the haploid syndrome (see below), 13 appeared to be normal haploids and 12 appeared to be anatomically abnormal haploids. In the 15,000 R group, 1 normal haploid and 13 abnormal haploids were seen. No embryos with a diploid appearance were seen in the 5,000, 10,000, nor 15,000 R groups. Based on these results, 10,000 R was used in later experiments to produce androgenotes.

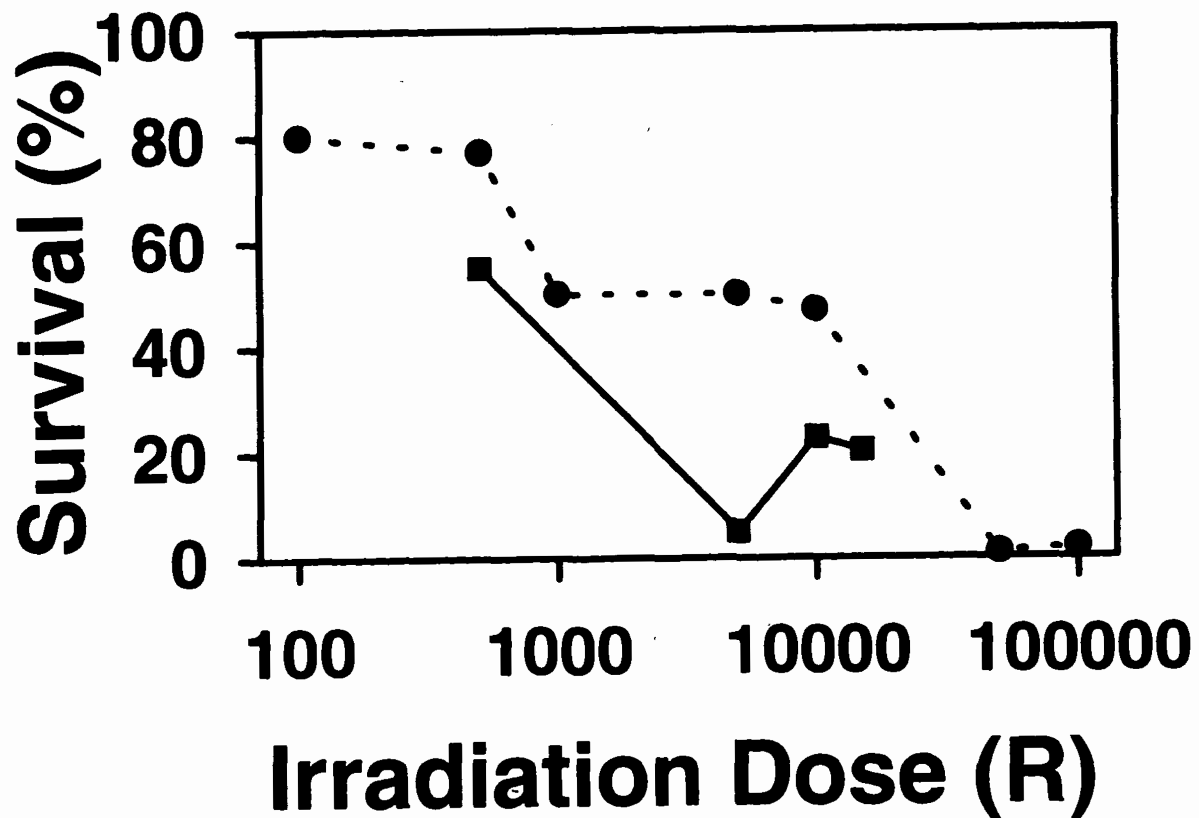

FIGURE 1. -- Irradiation dose response curve. The percentage of eggs irradiated at each dose surviving at 24 hrs p.f. is shown. ●, first experiment in which embryos were considered surviving if the chorions were transparent and some cleavage was observed; ■, a second experiment in which only those embryos having transparent chorions, some cleavage and a distinct body axis were scored as survivors.

Production of androgenotes: No embryos with a diploid phenotype were observed among 49 eggs for the I/NHS (irradiated and not heat shocked) group and two were observed among the 155 eggs for the I/HS (irradiated and heat shocked) group (Table 1).

Table 1

Progeny of Family A Surviving at 24 hrs

Treatment	Initial group size	Surviving at 24 hours	
		Haploids	Diploids
	(# eggs)	(# embryos)	(# embryos)
NI/NHS	76	0	55
I/NHS	49	5	0
I/HS	155	0	2

Symbols and abbreviations used: I, irradiated; NI, not irradiated; HS, heat shocked; NHS, not heat shocked. Data for family A.

A syndrome similar to the haploid syndrome of gynogenetic haploids (STREISINGER *et al.* 1981; HÖRSTGEN-SCHWARK 1993) was seen at 24 hours as a shortened body phenotype (Figure 2), which was obvious at 48 hours in I/NHS embryos. Melanocytes are characteristically smaller in haploid embryos. This became noticeable at 48 hours (Figure 2) and was pronounced by 96 hours (not shown). The development of putative androgenetic diploid embryos was initially slightly retarded (Figure 2). However, by the end of

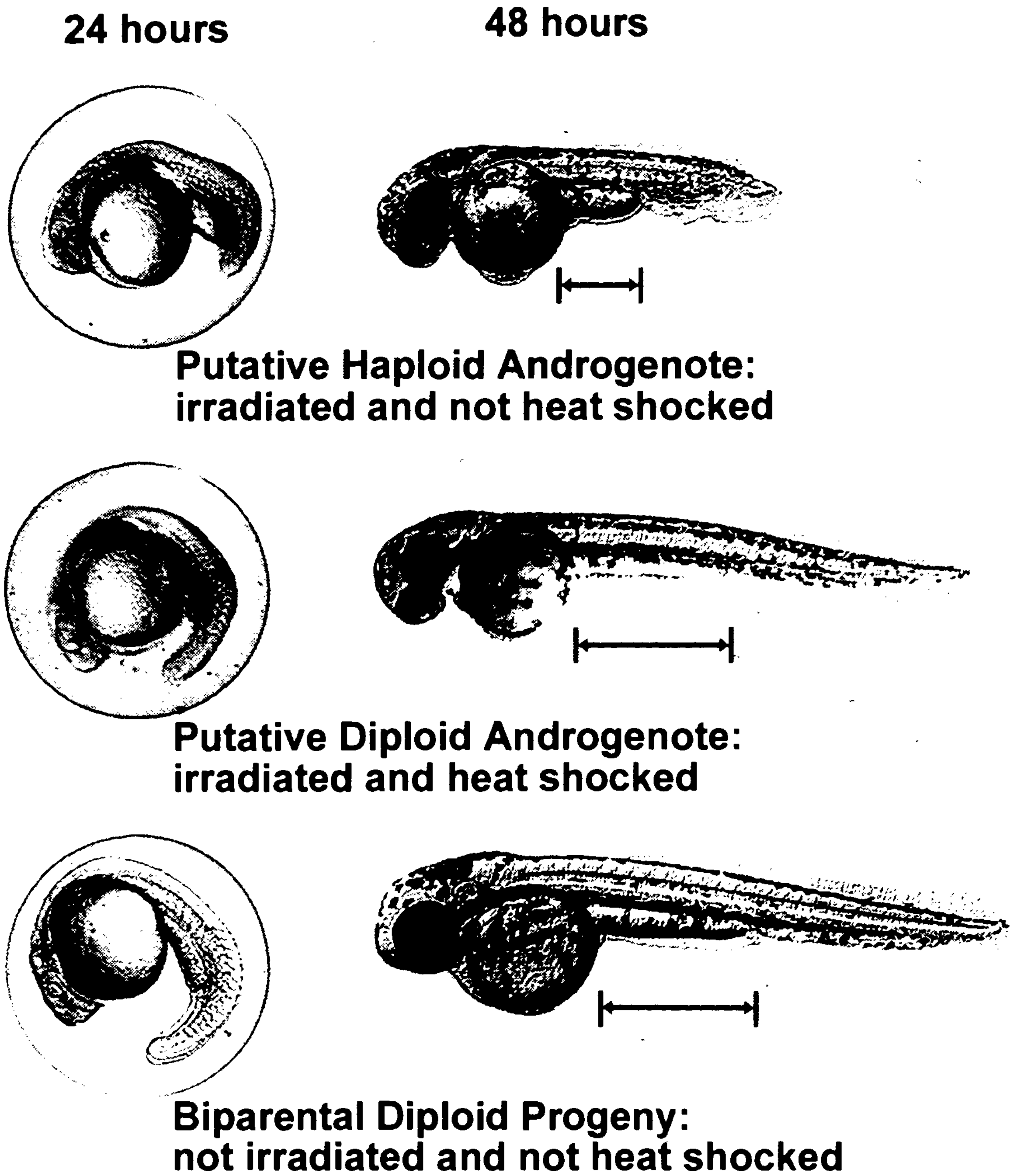

FIGURE 2. -- Putative haploid and diploid androgenetic embryos and biparental diploid embryos. Developing embryos of each type were

photographed at 24 hrs (left side of figure). The same embryos were photographed again at 48 hrs (right side of figure) after removing the chorion. The distance between the posterior yolk sac margin and the anal pore (as shown by horizontal bars) is greater for the diploid phenotype than for the haploid phenotype. The appearance of two eyes in the putative androgenotes and not in the biparental diploid progeny is the result of differences in the angle of photography and is not a phenotypic difference.

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the first month, the PDA fish in this experiment, and several in other experiments, were approximately the same size as the diploid control fish. In this experiment, the percentage of haploid and diploid androgenotes produced relative to our control group was 14% and 2%, respectively.

Evaluation of the pedigree analysis technique: Analysis of DNA polymorphisms was used to determine the inheritance of maternal and paternal DNA to putative androgenetic offspring. Using RAPD primer 208BCF, three maternal markers, but not paternal markers, were detected by agarose gel electrophoresis (Figure 3). None of the maternal markers was inherited by any of the four putative androgenetic progeny. The ABI 373 Automated DNA Sequencer allows for the separation of PCR products with greater resolution and sensitivity than agarose electrophoresis, and the use of inlane fluorescent size markers allows for more precise sizing of fragments,

facilitating identification of markers. Thus, most of our genetic analyses were based on fluorescent RAPD products separated on the ABI sequencer.

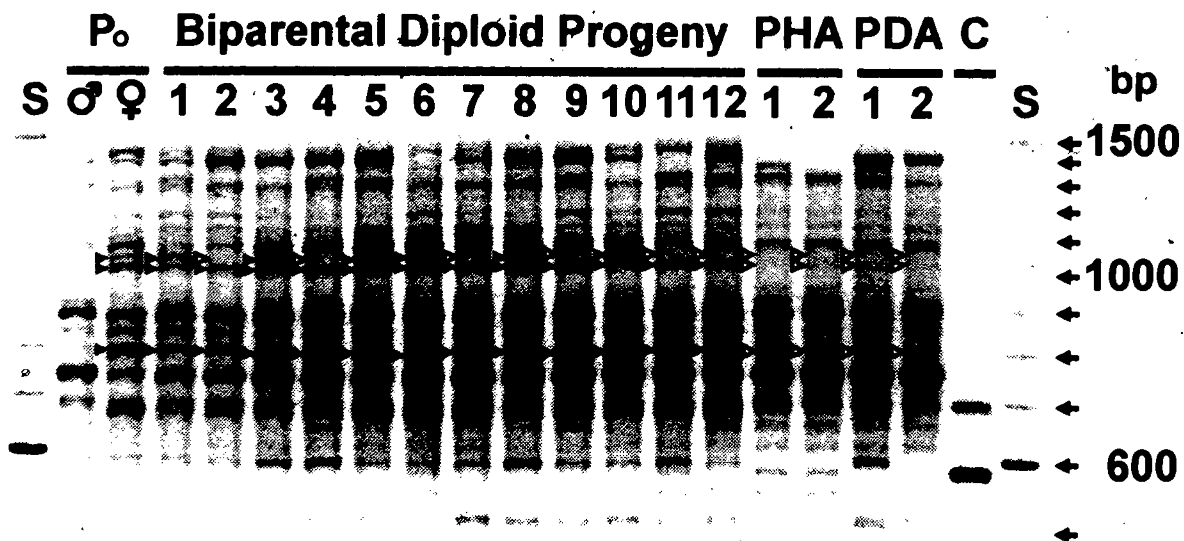

FIGURE 3. -- Inheritance of three maternal polymorphic markers for RAPD primer 208BCF. The inheritance of three maternal RAPD markers is shown for 12 biparental diploid progeny and four putative androgenetic progeny. The PCR products were separated by electrophoresis on a 1.8% agarose gel and stained with ethidium bromide. Abbreviations and symbols used: PHA, putative haploid androgenote (1 and 2); PDA, putative diploid androgenote (1 and 2); C, control (no template); S, sizing standard (Gibco BRL, 100 bp ladder); ►, presence of band; ▷, absence of band. Three bands (RAPD markers) in the maternal (Po♀) lane are marked with ►. These bands are absent from the paternal (Po♂) lane and were designated as maternal. One

of the maternal markers is seen in only some of the biparental diploid progeny and is thus considered heterozygous in the female parent. Two of these maternal bands are seen in all 12 biparental diploid progeny and thus are presumed homozygous in the female parent. None of these three maternal markers was detected in any of the four putative androgenotes tested.

Figure 4 shows output from the Genescan program. A comparison of two separate RAPD-PCR reactions replicated for each of two DNA templates (parents of putative androgenotes) using the same fluorescent primer are shown. Although some peak heights vary slightly, all major peaks can be seen in both PCR reactions that contained an aliquot of the same DNA template, demonstrating that fluorescent RAPD-PCR markers are amplified reproducibly and that they can be reproducibly detected. While the resolution is much better than on agarose gels, some peaks overlap. The zoom feature in the GeneScan software allows resolution of more peaks than can be seen in Figure 4. For our analysis, we used only those markers that were clearly distinguishable. In all the ABI GeneScan electropherograms we have viewed to date, we have never detected a RAPD-PCR product in a progeny which was not detected in one of the parents, which is consistent with our RAPD markers acting as Mendelian markers. A clearly

polymorphic peak specific to the father is seen in the top two panels at 799 bp's in Figure 4. A small amount of amplification product was observed when no template was included in the PCR reaction (Figure 5). Amplification products appearing in the absence of template DNA, which disappeared when template DNA was included in the PCR reaction, have been previously noted for RAPD reactions (e.g., WILLIAMS *et al.* 1990). PCR markers used in our analyses are clearly distinct in mobility from those amplified in the absence of DNA template.

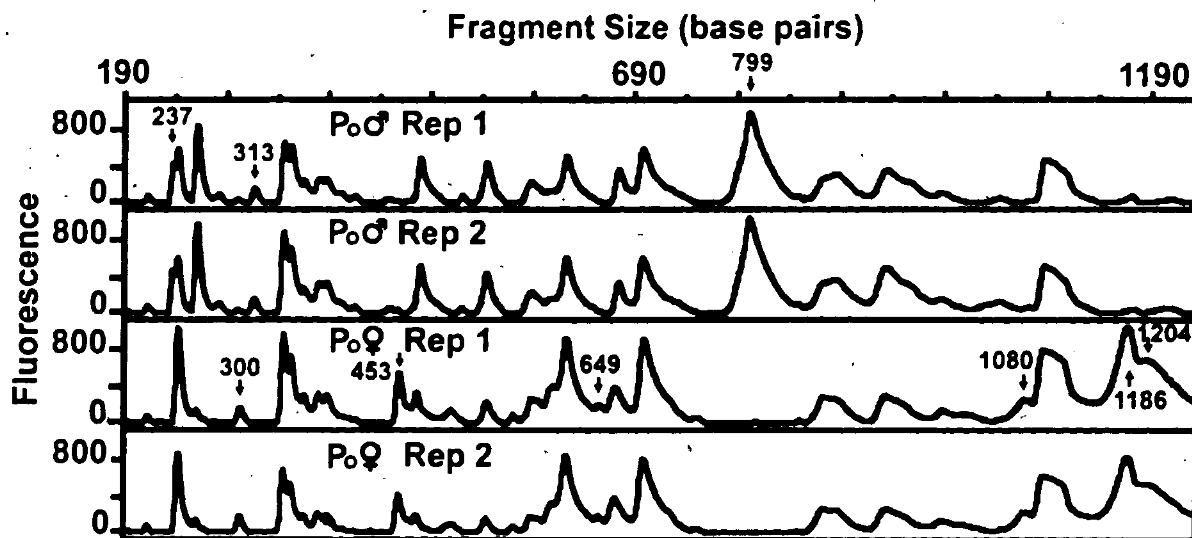

FIGURE 4. -- Detection of DNA polymorphisms by RAPD analysis using fluorescent primers. Replicate PCR reactions (Rep 1 and Rep 2) are shown for the paternal and maternal DNA templates. Each template was PCR amplified with the fluorescent (6-FAM) RAPD primer 210BCF. Fluorescent RAPD products were separated and detected during electrophoresis on an

ABI 373 DNA sequencer. Fragment sizing was performed by ABI Genescan software, using fluorescent inlane size standards. Each panel is an electropherogram output by the ABI Genescan program. Genescan electropherograms were captured as print files and imported into Photoshop version 3.0 to add labels and thicken lines to allow for photoreduction. Fluorescence is shown in arbitrary units. Arrows and associated numbers indicate sizes of parentally polymorphic peaks used in the single family analysis to assess the androgenetic nature of putative androgenetic progeny. Three paternal specific and six maternal specific markers are shown. Although maternal marker *210bcf.300* appears in the figure to be present in paternal electropherograms, the peak in the paternal lane near this location is of a different size, which is more evident using the zoom feature of the Genescan program. Likewise the valley surrounding *210bcf.649* is more pronounced when the x-axis is amplified using the zoom feature.

Examples of a maternal marker and a paternal marker and their inheritance to a normal diploid progeny and a putative diploid androgenetic progeny are shown in Figure 5. The maternal specific marker *210bcf.453*, and the paternal specific marker *210bcf.799* shown in Figure 5 were found in all 12 biparental diploid progeny tested (only one of which is shown in Figure 5) and are presumed to be homozygous in the parent (see Table 2).

Henceforth, any marker referred to as maternal or paternal, will refer to a marker of a particular size that was observed in only one of the parents. The apparently homozygous maternal marker (Figure 5) was not inherited by any of the four putative androgenotes (only one of which is shown in Figure 5), while the apparently homozygous paternal marker was inherited by all four putative androgenotes tested.

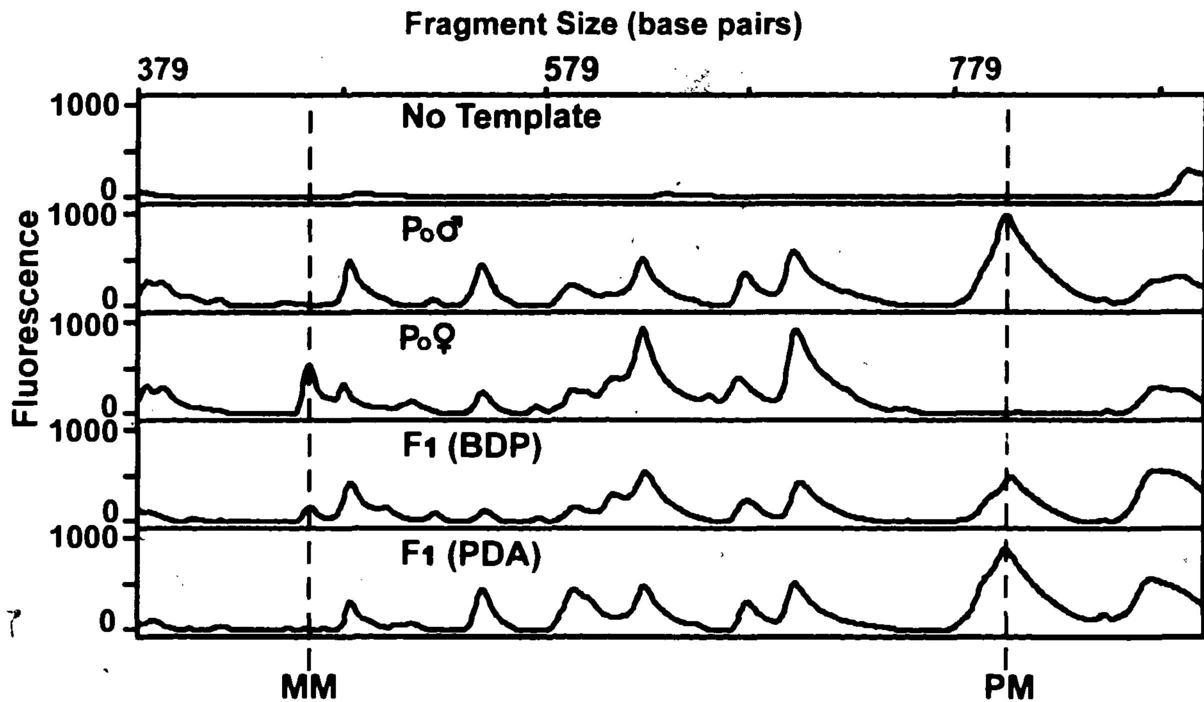

FIGURE 5. -- Inheritance of fluorescent RAPD markers by putative androgenotes for same primer (210BCF) as used in Figure 4. This example shows a maternal marker (MM) *210bcf.453* and a paternal marker (PM) *210bcf.799* and their inheritance to a biparental diploid progeny (BDP) and a

putative diploid androgenote (PDA). Both markers were found in all 12 biparental diploid progeny (not shown in this figure) and are presumed to be homozygous in the parents (see footnote to Table 2). The reduced peak height from the parent where the marker is homozygous (PM and MM) to the BDP is consistent with there being a single allele, as expected for a heterozygote, in the BDP. However, our results are based only on presence or absence of a marker and do not rely on quantitative PCR. The bottom panel shows that the homozygous maternal marker is not inherited by the putative diploid androgenote and that the homozygous paternal marker is inherited by the putative diploid androgenote.

Inheritance of RAPD-PCR markers by putative androgenotes:

Using three fluorescent primers, 16 maternal (11 heterozygous and 5 apparently homozygous) and seven paternal (4 heterozygous and 3 apparently homozygous) markers were identified (Table 2). Markers were considered heterozygous if only some of the progeny received the marker and apparently homozygous if all 12 test BDP received the marker (Table 2, footnote *a*).

To test whether these RAPD markers were segregating in a Mendelian fashion, the 11 heterozygous maternal markers were scored in the 12 BDP, and the 4 heterozygous paternal markers were scored in the 16 progeny (12

BDP + 2 PHA + 2 PDA). For each marker, the present:absent count in the F1 progeny was tested using chi-square analysis for goodness of fit (Zar, 1974) to the theoretical ratio of 1:1. The null hypothesis of no difference to a 1:1 ratio

TABLE 2

Number of parentally polymorphic RAPD markers in Family A

Primer	Maternal Markers		Paternal Markers	
	Homozygous[a]	Heterozygous	Homozygous	Heterozygous
208BCF	2	3	0	0
210BCF	1	5	1	2
269BCF	2	3	2	2
Total	5	11	3	4

[a] Markers were designated homozygous if they occurred in all 12 biparental diploid progeny tested. The probability (P) of making an error by calling a marker homozygous, based on it being found in 12 BDP, was calculated:

$P = 1/2^n$ with n = number of progeny tested.

$P = (1/2)^{12} = 0.00024414$

Thus, the chance that a marker designated as homozygous is in fact heterozygous, is 0.00024414 and the chance that it is homozygous is (1 - 0.00024414 = 0.99975586).

was not rejected, with $\alpha = 0.05$, for 14 of the 15 heterozygous markers. Thus, 14 of the 15 heterozygous markers appear to behave as Mendelian factors in our analysis. Marker *269bcf.793* was clearly present in both replicates for the maternal template and was clearly present in the one BDP progeny it was observed in. It was considered a statistical outlier, and was not excluded from the data. Its exclusion would have very little effect on our androgenetic analysis.

It is important for our analysis to show that each marker represents a different locus, rather than some of them being length variants of the same locus. The 11 maternal heterozygous markers were tested for independent assortment against all other maternal markers. A similar analysis was performed for the four heterozygous paternal markers. For this analysis it was assumed there was a recessive (un-amplified) allele for each of the dominant RAPD markers. As the markers are dominant and parent specific, the cross can be viewed as a test cross. By arbitrarily assigning the two markers being compared as A and B, we use the notation for the cross as AaBb X aabb. If unlinked, the four categories in the cross will have a ratio approximating 1:1:1:1. If the two dominant markers are closely linked on the same chromosome (*cis*-linked), the AaBb and aabb categories would strongly dominate. If the dominant form of A is on the same chromosome as the recessive form of B (*trans*-linked), the Aabb and aaBb categories would

strongly predominate. In the 55 comparisons done between maternal heterozygous markers, only one pair of markers appeared *cis*-linked and this was not complete. The degree of linkage was not calculated due to the small sample size. None of the paternal marker comparisons indicated linkage. In summary, our heterozygous RAPD markers appear to be segregating as Mendelian markers and (with perhaps one exception) appear to be assorting independently.

To verify the androgenetic nature of PHA and PDA progeny of family A, the inheritance of maternal and paternal markers by these progeny was analyzed. All three of the homozygous paternal markers were inherited by all four of the putative androgenetic progeny tested, whereas none of the 16 maternal markers, five of which are probably homozygous, were detected in any of the four putative androgenetic progeny. Heterozygous paternal markers were inherited by putative androgenotes 10 times out of a possible 16 (Table 3).

TABLE 3

Inheritance of heterozygous paternal markers by four putative androgenetic progeny tested in Family A

Marker	PHA		PDA	
	1	2	1	2
210bcf.237	+	+	-	-
210bcf.313	-	+	+	-
269bcf.615	+	+	+	-
269bcf.637	+	+	-	+

Symbols and abbreviations used: 210BCF, signifies the fluorescent RAPD primer used and the digits after the decimal indicate the size of the amplified fragment; PHA, putative haploid androgenotes (#1 and #2); PDA, putative diploid androgenotes (#1 and #2): +, marker present; -, marker absent.

Inheritance of MHC and SSR-PCR markers by putative androgenotes: The MHC primer pair did not produce an informative

marker as it was monomorphic between the parents. Likewise, 15 of the 16 SSR primers tested did not detect parental polymorphisms. SSR primer set 29 (GOFF *et al.* 1992), fluorescently labeled, detected two maternal specific and two paternal specific markers. The paternal markers are *ssr29f.153* and *ssr29f.189*, and the maternal markers are *ssr29f.164* and *ssr29f.179*. The two maternal markers appear to be different loci as both markers occur in some BDP. Likewise the paternal bands are assumed to be different loci as both markers occurred in some BDP. Maternal marker *ssr29f.164* was found in four out of five BDP tested and is assumed to be heterozygous. Maternal marker *ssr29f.179* was found in five out of five BDP tested, and is assumed to be homozygous. Neither of these maternal markers were found in any of the four putative androgenotes. Both paternal markers were found in all four putative androgenotes.

Confirmation of fertility of a diploid androgenote: We have produced several putative androgenotes that have survived to adulthood. The androgenetic nature of a male fish (progeny of family B) that has sired hundreds of offspring was analyzed using fluorescent RAPD markers (primer 208BCF was used). Two paternal markers, which were designated as homozygous based on their occurrence in all 7 BDP tested, were both found in the breeding putative male diploid androgenote. Two homozygous and one

heterozygous maternal markers were not transmitted to this breeding male diploid androgenote.

Efficiency of production of androgenotes: Five crosses were made to test the percentage of normal haploid appearing embryos resulting from irradiating eggs with 10,000 R of X-rays; the range varied from 8 to 28% (Table 4). If normalized relative to the control groups, the percentages range from 10 to 37%. The first four crosses were performed in one morning and the fifth the following morning.

TABLE 4

Production Rates of Androgenetic Haploids

Family	Po		Control		Irradiated			
	Male	Female	n (#)	AA (%)	n (#)	A (%)	B (%)	C/D (%)
D	SFU	SFU	67	76	101	28	27	14
E	*AB	SFU	85	66	62	15	18	23
F	*AB	SFU	39	69	131	21	14	11
G	*AB	SFU	27	78	89	8	20	12
H	*AB	SFU	57	72	87	24	7	22

Eggs in the control group were held at room temperature and fertilized at same time irradiated eggs were fertilized. Milt and eggs for the five families were collected from five separate males and females. The five groups of eggs

were irradiated separately at 10,000R. Embryos were scored two days after fertilization based on appearance. Percentages are not normalized relative to control groups. Symbols and abbreviations used: n, sample size; AA, diploid phenotype with no morphological abnormalities apparent; A, haploid phenotype as described in Figure 2; B, haploid phenotype with noticeable morphological imperfections such as bent tail or missing part of tail; C/D, grossly abnormal embryos (classification based on WALKER and STREISINGER 1994c). In the control groups, only dead eggs and embryos that were normal diploid in appearance were observed. In the irradiated group, no embryos having a diploid appearance were observed. Data from families A through C, inclusive, are not included in this table as the progeny were not double checked by a second observer for agreement of classification into above categories.

I/NHS (irradiated and not heat shocked) embryos, when viewed at 24 and 48 hr p.f., displayed a range of morphological phenotypes, ranging from haploid appearing with no noticeable morphological abnormalities (scored as "A" in Table 4, with example of one shown in Figure 2) to balls of cells that had arrested development before 24 hrs. Examples of phenotypes observed more than once in embryo scored as category "B" (Table 4) included: developed head with diminished body and no tail, developed body and head

with no tail, and body and tail with little or no head. The occurrence of certain morphological abnormalities was more common in some families than others.

When the milt for use in producing androgenotes was obtained from a fish of the SFU line, which has not been screened for recessive lethals as has the *AB line, the efficiency of production of putative haploid androgenotes (category "A" in Table 4) was similar to that when milt was obtained from a *AB fish.

We have scored over 1,200 embryos from 12 families to date that resulted from eggs irradiated with 10,000 R of x-rays and not heat shocked. We have never observed an embryo with a diploid appearance resulting from this I/NHS treatment. Data on seven of these 12 families are not presented in Table 4, as the morphological characterization of abnormal androgenotes was less thorough.

To date we have produced 44 putative diploid androgenotes; thirteen of them survived past 20 days. Production of large numbers of diploid androgenotes has not been attempted, as our rearing facility is not large.

Genetic Analysis in Multiple Families: Using RAPD primers 208BCF and 210BCF, we surveyed a sample of putative haploid androgenotes (category "A" in Table 4) from four additional families (D-F) for inheritance of maternal and paternal specific markers (Table 5). Thus, 18 putative

androgenotes in total were genetically analyzed. No maternal markers were found in any of the 18 embryos analyzed which had been irradiated with 10,000 R of x-rays.

Table 5

Summary of Genetic Marker Analysis for Six Families of Androgenotes

Family	Indiv.	Parental Specific Markers in Family		Parental Markers Observed in Putative Androgenotes		Probability of not Observing Maternal Markers
		Po ♂	Po ♀	Po ♂	Po ♀	
A	PHA1	7	16	5	0	1.3×10^{-23}
A	PHA2	7	16	7	0	1.3×10^{-23}
A	PDA1	7	16	7	0	1.3×10^{-23}
A	PDA2	7	16	4	0	1.3×10^{-23}
B	PDA1	2	3	2	0	7.6×10^{-6}
C	PDA1	6	9	6	0	$\leq 2.0 \times 10^{-3}$
C	PDA2	6	9	6	0	$\leq 2.0 \times 10^{-3}$
C	PHA1	6	9	6	0	$\leq 2.0 \times 10^{-3}$
C	PHA2	6	9	6	0	$\leq 2.0 \times 10^{-3}$
D	PHA1	1	2	1	0	$\leq 2.5 \times 10^{-1}$
D	PHA2	1	2	1	0	$\leq 2.5 \times 10^{-1}$
D	PHA3	1	2	1	0	$\leq 2.5 \times 10^{-1}$
E	PHA1	5	7	5	0	$\leq 7.8 \times 10^{-3}$

E	PHA2	5	7	4	0	$\leq 7.8 \times 10^{-3}$
E	PHA3	5	7	5	0	$\leq 7.8 \times 10^{-3}$
F	PHA1	10	9	8	0	$\leq 2.0 \times 10^{-3}$
F	PHA2	10	9	7	0	$\leq 2.0 \times 10^{-3}$
F	PHA3	10	9	8	0	$\leq 2.0 \times 10^{-3}$
All	All	102	157	89	0	$\leq 2.0 \times 10^{-124}$

Genescan RAPD marker analysis is summarized. Symbols and abbreviations used: Indiv., Individual androgenote analyzed; PHA, putative haploid androgenote; PDA, putative diploid androgenote. Individuals analyzed were assigned numbers within families (e.g. PHA1, indicates putative haploid androgenote number one in family indicated). Probabilities indicate the chance of not observing the maternal specific markers in a normal biparental diploid progeny. See Discussion section for method of calculating probabilities, and the associated assumptions. Family A is the family for which in-depth analysis was performed. Eight of the maternal markers for this family are apparently homozygous as discussed in text. PDA1 of family B, is the male diploid androgenote which we have bred. Two of the maternal markers in this family are apparently homozygous as discussed in text. It was not determined if the markers in families C through F, inclusive, were homozygous or heterozygous; thus probabilities were calculated for these families based on the conservative assumption that all markers are

heterozygous in the mother. The underlying assumptions of the markers being unlinked and acting as normally segregating Mendelian markers was not tested for markers in families C through F.

DISCUSSION

The genetic analysis presented here, and discussed below, demonstrates the successful production of diploid androgenetic zebrafish surviving to adulthood. The results are significant for the use of androgenotes in genetic research and the evolutionary origin of genetic imprinting.

Confirmation of androgenetic inheritance in Family A: The androgenetic nature of progeny in the one family experiment was confirmed by lack of inheritance of 12 (11 RAPD + 1 SSR) heterozygous maternal and six (5 RAPD + 1 SSR) apparently-homozygous maternal DNA markers to all four androgenetic progeny tested. Although it is possible that some DNA leakage from the mother occurred, none was detected and the results strongly indicate that genomic DNA inheritance to the progeny was mostly or entirely from the male parent.

A RAPD marker was presumed homozygous in the parent if it occurred in all 12 biparental diploid progeny tested (Table 2, footnote 1). The chance of a biparental diploid progeny inheriting a marker designated as homozygous, based on it being found in all 12 previously tested biparental diploid progeny, is[1]: $(1.0 \times \text{chance of marker being homozygous}) + (0.5 \times \text{chance of marker being heterozygous}) = (1 \times 0.99975586) + (0.5 \times 0.00024414) = 0.99987793$.

[1] *The large number of significant figures are included for calculation purposes only and do not indicate an exact probability as some markers, as discussed in text, may not be assorting independently to progeny.*

Thus, the chance of not finding one of these presumed homozygous markers in a biparental diploid progeny is 1 - 0.99987793 = 0.00012207, or approximately 1 in 10,000. The chance that none of the 11 heterozygous, and none of the five apparently homozygous maternal markers, being inherited by a single biparental diploid progeny can also be estimated: $(0.5)^{11} \times (0.00012207)^5 = 1.3235 \times 10^{-23}$. This calculation assumes all markers are segregating as Mendelian markers and are independently assorting. Although some of the maternal heterozygous markers may be weakly linked, our analysis of random assortment of markers showed no complete linkage between any two markers. The chance that four biparental progeny would receive none of these 16 maternal RAPD markers is: $(1.3235 \times 10^{-23})^4 = 3.068 \times 10^{-92}$.

The androgenetic nature of the putative androgenetic progeny is further supported by the lack of maternal SSR-PCR markers and presence of paternal SSR-PCR markers in these progeny. The chance that neither the maternal heterozygous nor the apparently homozygous SSR markers (see results) would be found in four biparental diploid progeny is 3.7×10^{-9}. Combining the RAPD and SSR maternal marker data, the chance that none of the markers would be found in four biparental diploid progeny is 1.1×10^{-100}. This strongly suggests androgenetic inheritance.

All of the apparently-homozygous paternal RAPD markers and a proportion of the heterozygous paternal markers were inherited by all four putative androgenotes analyzed. The proportion of paternal RAPD markers inherited by the progeny is consistent (P=0.45) with the Mendelian expectation that heterozygous markers will be inherited by half the progeny by Chi-square testing for goodness of fit (ZAR 1974). Thus, it appears that androgenotes are inheriting paternal markers in a Mendelian fashion and not inheriting maternal markers.

The androgenetic nature of these fish is further supported by the phenotype of the irradiated embryos. Only severely abnormal embryos or embryos exhibiting the haploid syndrome were observed when irradiated eggs were inseminated. Following insemination of eggs irradiated with 10,000 R, in over 1,200 embryos observed, we have never observed the diploid phenotype (Figure 2), unless the zygotes were subsequently treated to inhibit the first mitotic division. This evidence suggests that the irradiation dose is sufficient to eliminate most or all the maternal DNA, and also that the heat shock procedure is effective in restoring embryos to the normal diploid phenotype. That the irradiation dose used (10,000 R) is sufficient to prevent inheritance of maternal DNA is further supported by the coincidence of this dose with the secondary peak on a plot of survival, as a function of dosage (Figure 1), known as the Hertwig effect (HERTWIG 1911). The initial decline

in survival is thought to be due to partial destruction of the maternal genome leading to aneuploidy, while further irradiation leads to complete destruction of the irradiated genome (ARAI *et al.* 1979; DON and AVTALION 1988). Although the Hertwig effect was originally observed for irradiated sperm (HERTWIG 1911), we have noted similar survival curves for irradiated zebrafish, chinook salmon (*Oncorhynchus tshawytscha*), and rainbow trout (*Oncorhynchus mykiss*) eggs.

In combination, the RAPD marker evidence, the SSR marker evidence, the absence of the normal diploid phenotype in the irradiated and not heat shocked group of progeny, and the observation of the Hertwig effect, provide strong evidence to support androgenetic inheritance.

Confirmation of fertility of a diploid androgenote: Genetic analysis of a putative androgenetic breeding male zebrafish, indicated it has an androgenetic genome. The chance of a BDP not inheriting two homozygous maternal (found in all 7 BDP), nor one heterozygous maternal marker is 7.6 X 10^{-6}, indicating that this breeding fish has an androgenetic genome.

Morphological appearance of haploid androgenotes: Abnormalities, viewed at 24 and 48 hr p.f., including underdeveloped heads, bodies, or tails, could be attributed to mutations carried in some individuals of the paternal line of fish, or to damage resulting from irradiation. Similar abnormalities have been observed in haploid gynogenotes produced from the *AB line of

zebrafish (C. WALKER, personal communication). This suggests that some of the abnormalities result from background mutations in the *AB line.

Efficiency of production of androgenotes: The observed efficiency of production of haploid androgenotes (category "A", Table 4) in five families (D-H) ranged from 8 to 28%. If categories "A" and "B" (Table 4) are combined, the production efficiency of haploid androgenotes ranged from 28 to 55%. Although we initially used milt from *AB males because this line was screened to reduce recessive lethals, we have achieved good results with milt from the SFU line of fish which are believed to be relatively heterozygous as they originated from several pet stores and presently are not homogenous in appearance. This suggests that milt useful for producing androgenotes, does not need to be obtained from a line of fish screened for recessive lethals.

The efficiency of production of diploid androgenotes in family A was 1.3%. To date, we have achieved success rates up to 2.1% for production of diploid androgenotes. In our facility, if fish live past the first 20 days, they usually survive through adulthood. This applies both to diploid biparental and diploid androgenetic progeny. Thus, survival was measured at day 25. At 2% efficiency, six diploid androgenotes can be expected from a batch of 300 eggs.

Some abnormalities observed in haploid androgenotes are likely to have resulted from irradiation damage to cytoplasmic components of the oocyte.

Since cytoplasmic components are known to be damaged by soft (low energy) x-rays, the efficiency of production of androgenotes might be increased by: 1) filtering out soft (low energy) x-rays, 2) using an x-ray machine with a higher KeV output, or 3) using a gamma irradiation source (e.g. ^{60}Co or ^{137}Cs).

Androgenesis in other teleosts: Attempts to produce androgenetic fishes have been reported by several groups (reviewed by IHSSEN *et al.* 1990). Putative haploid[2] androgenetic embryos did not survive to the active feeding larval stage (ROMASHOV and BELYAEVA 1964; ARAI *et al.* 1979; PARSONS and THORGAARD 1984).

The production and survival of diploid androgenetic salmonids has been reported (PARSONS and THORGAARD 1985; MAY *et al.* 1988; SCHEERER *et al.* 1986, 1991). These fishes were reported to be androgenetic based on their being homozygous at several loci, as determined from enzyme expression assays. However, the use of DNA polymorphisms allows for direct assessment of parental alleles, irrespective of their state of expression. Thus, it provides more compelling genetic evidence for lack of maternal inheritance to androgenetic progeny.

[2] *Haploid is used here to designate the set of chromosomes found in one normal gamete. It has been speculated that pacific salmon may have four sets of chromosomes (*KLOSE *et al. 1968;* BAILEY *et al. 1969). Thus, in our usage, haploid is not necessarily equivalent to one set of chromosomes.*

Androgenesis as a genetic tool: The production of androgenetic zebrafish has significance for investigation of several biological phenomena and provides a useful genetic tool. The process of collecting eggs and milt and irradiating and fertilizing them can be accomplished by one person in less than one hour. If heat shocking is performed, an additional 20 minutes is required. Tens of thousands of eggs can be irradiated simultaneously in the x-ray machine we use.

Male specific meiotic recombination rates: Knowledge of the meiotic recombination rate in each sex during gametogenesis is important for genetic studies. In humans and mice the male meiotic crossover rate is approximately half that found in females and no crossing over occurs during meiosis in Drosophila males. Postlethwait *et al.* (1994) has determined the female specific cross over rate for numerous RAPD markers on all 25 zebrafish chromosomes by analyzing markers inherited by gynogenetic haploid zebrafish. The male specific cross over rates could be determined using a similar procedure, except that inheritance would be assessed in haploid androgenetic rather than haploid gynogenetic progeny. As the present RAPD map is based on female meioses, male specific markers (if any exist) would not have been observed. Thus any new linkage group that might show up during mapping with androgenetic haploids, might be male specific, and might include sex determining genes.

Only 94 gynogenetic haploid embryos were used to produce the zebrafish linkage map (POSTLETHWAIT *et al.* 1994). To produce 100 androgenotes for a linkage map based on male cross over rates would require irradiating 1,000 eggs, assuming a 10% production rate of androgenetic haploids. Assuming 100 eggs/female, eggs would need to be collected from 10 females. Since more than 800 eggs can on occasion be collected from one female, a 28% efficiency of production would produce 224 haploid androgenotes from a single cross.

Sex determination: The mechanism of sex determination in zebrafish is presently unknown (HÖRSTGEN-SCHWARK 1993; MARTIN and MCGOWAN 1995a), but androgenotes may provide some insight. If zebrafish have an XY-like sex determining system, those genes required on the X chromosome for survival and fecundity must also reside on the Y chromosome as male androgenotes both survive and breed. If zebrafish have an XY sex determining mechanism, then male androgenotes (YY) when bred to a normal female (XX), would result in only male (XY) progeny. STREISINGER (1981) and HÖRSTGEN-SCHWARK (1993) both found strongly skewed sex ratios in diploid homozygous gynogenetic progeny. STREISINGER (1981) observed mainly females and HÖRSTGEN-SCHWARK (1993) observed only males (two experiments: n=9 and n=8). If the probability of being male or female is equal, the chance of 17 progeny all being male is 1 in 130,000. Thus, HÖRSTGEN-SCHWARK'S result is unlikely to be due to small sample size alone.

There is likely to be an environmental influence that may override any genetic mechanism of sex determination in zebrafish, which must be considered in interpreting sex ratios of progeny from androgenotes.

Cryopreservation of allelic combinations: Androgenesis may be useful for storing and retrieving desirable combinations of certain alleles, clonal lines or wild (e.g., salmon) stocks. Zebrafish milt can be cryopreserved, (HARVEY *et al.* 1982; WALKER and STREISINGER 1994a, 1994b) but we are not aware of any reports of successful fertilization of any previously frozen teleost eggs. Frozen sperm is generally not as effective in fertilizing eggs as normal sperm (HARVEY *et al.* 1982 report that frozen sperm on average was 51% as effective as fresh sperm in fertilizing eggs). However, even very low rates of production of diploid androgenotes may be acceptable for some applications as the milt from one fish can be used to attempt fertilization of thousands of eggs. Although we have never attempted to produce diploid androgenotes from frozen sperm, we cannot foresee any reason why it should not be possible. We are hopeful that in the future the efficiency of both fertilization using frozen sperm and efficiency of production of diploid androgenotes will improve.

Mutation screening: Androgenetic F1 haploid screens in theory might have certain advantages over gynogenetic haploid screens in mutagenesis protocols. Mutations can be induced, as for gynogenetic screens, by

irradiation of sperm, eggs, or early embryos. Part of the milt obtained from the male containing the mutagenized germ-line could be used to produce androgenetic haploids for the F1 screen, and the rest frozen and used only if mutations of interest were detected. Thus, in principle, the mutagenized parent need not be retained as the mutation can be recovered from cryopreserved sperm following mutation screening.

If a haploid androgenesis screen is attempted, a background set of haploid abnormalities is expected, similar to those which are found during mutation screening using gynogenetic haploids (C. WALKER, personal communication). Induced mutations can be identified by the new appearance of specific haploid abnormalities that are particular to a certain family.

Genomic imprinting: Completion of mouse embryogenesis requires both the maternal and paternal genomes because of imprinting (parent-of-origin mono-allelic expression) of essential genes in male and female gametes (MCGRATH and SOLTER 1984; SURANI *et al.* 1984; SURANI 1986; BARRA and RENARD 1988; SAPIENZA 1990; RENARD *et al.* 1991; GOLD and PEDERSON 1994; OHLSSON *et al.* 1994; CHAILLET *et al.* 1995). This does not appear to be the case for zebrafish. Diploid homozygous gynogenotes not only complete embryogenesis, but survive to adulthood (STREISINGER *et al.* 1981). Our results show that this is also true of diploid homozygous androgenotes. These results suggest that imprinting, in either of the parental gametic

genomes, does not result in essential genes being irreversibly inactivated during a time when required for development.

While parent-of-origin (gametic) inactivation of essential genes has been ruled out in zebrafish, parent-of-origin effects on a transgene have been detected (MARTIN and MCGOWAN 1995b). A decrease in methylation with maternal passage and an increase in methylation with paternal passage of a transgene in zebrafish was consistently observed. Thus, it appears that epigenetic phenomena associated with genomic imprinting occur in zebrafish and that parent-of-origin imprinting may occur in zebrafish but not for genes essential for development.

Zebrafish androgenetic haploid embryos are morphologically slightly abnormal (Figure 2) and arrest around day four. Zebrafish gynogenetic haploid embryos exhibit a typical 'haploid syndrome': they have short, stocky bodies, their eyes are incompletely formed at the ventral furrow and the brain is poorly sculptured. Cell size is often smaller in gynogenetic haploids than in diploids, as observed for melanocytes. Eventually they become edematous and die after four-five days. (C. WALKER, personal communication). Haploid androgenotes are indistinguishable in appearance from haploid gynogenotes, suggesting that the abnormalities are not due to parent-of-origin, but may be dependent on gene dosage.

We believe that this is the first report of the production of a viable and fertile androgenetic diploid vertebrate in which the extent of elimination of the maternal genome has been assessed by the use of DNA markers. There have been other reports of production of fertile androgenetic and gynogenetic adult teleost fishes (reviewed by IHSSEN *et al.* 1990) and amphibians (e.g., GILLESPIE and ARMSTRONG 1981). Collectively, these reports indicate that the failure of androgenesis and gynogenesis reported for some mammals (McGRATH and SOLTER 1984; SURANI *et al.* 1984; SURANI 1986; GOLD and PEDERSON, 1994) is not characteristic of vertebrates in general. Thus, genomic imprinting of genes essential for development may be a specialized phenomenon which arose during mammalian evolution.

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CONCLUDING DISCUSSION

The research reported in this thesis is not meant to represent a finished program of research but an opening of doors to a number of interesting and important developmental and genetic issues. During the last fifteen years, zebrafish have gained attention as an experimental organism for understanding vertebrate embryonic development. Classical experiments using avian and amphibian organisms have helped elucidate basic aspects of vertebrate embryology. However, for decades the ability to understanding the underlying mechanisms of vertebrate development has been inhibited by the inability to carry out convenient genetic studies. During the 1970's, a seminal observation was made in Eugene, Oregon. Visible embryonic phenotypes could easily be viewed in the developing embryos of zebrafish (e.g. picture on cover of The Zebrafish Book, Westerfield 1995). External development and transparent embryos make it easy to identify and score mutant phenotypes for zebrafish. Merely 15 years after Streisinger and colleagues published their findings in Nature (Streisinger et al. 1981), zebrafish have become a major developmental model system. More extensive genetic screens have been carried out in zebrafish than for any other vertebrate (Driever, et al. 1996; Haffter et al. 1996; Eisen 1996; Grunwald 1996). Two F3 screens, one in Boston (Driever et al. 1996) and one in Tübingen (Haffter et al. 1996) resulted in the isolation and initial characterization of an impressive 1858 mutations affecting almost every aspect of embryonic development assessed (Eisen 1996).

Accurate staging of zebrafish embryos is possible (Kimmel et al. 1995) and techniques to label cells have been developed for zebrafish, enabling studies of cell lineage

and cell fate (Wilson et al. 1993; Helde et al. 1994). Transplanting cells between embryos and following labeled cells has enabled studies of autonomy of mutant genes (Ho and Kane 1990). The ability to grow cells in culture away from the clamor of biochemical signals in the intact embryo allows the induction of cell fate by cell signaling to be investigated (Rousch 1996b; Shih and Fraser 1996). A new arena of understanding of vertebrate development is unfolding as the level on which processes are understood progresses toward the molecular genetic level. It is this arena in which zebrafish will be able to significantly add to the knowledge gained from other notable vertebrate models (e.g. mouse, chicken, frogs). In the past, understanding development at the molecular genetic level, has been mainly studied in non-vertebrate model systems (e.g. *Caenorhabditis elegans*, Drosophila, yeast). Although some molecular genetics have been performed in vertebrates (e.g., the mouse and *Xenopus*), external fertilization and transparency of the embryos (Driever and Fishman 1996) has made the zebrafish a more tractable system in which the large scale mutation screens have been performed (Driever et al. 1996; Haffter et al. 1996; Grunwald 1996; Eisen 1996; Rousch 1996a).

It is in this quest for progressing our understanding toward development at a molecular genetic level, that I feel the refinement of delayed *in vitro* technology (Corley-Smith et al. 1995b and 1995d) and the production of androgenetic haploid and diploid zebrafish (Corley-Smith et al. 1996) have significance. I have supplied salmonid ovarian fluid upon request to researchers working on zebrafish at several institutions including: MIT, Harvard Medical School, Carnegie Institution of Washington, University of Oregon

and University of Utah and to a commercial distributor in the USA (Corley-Smith 1996). Experiments not previously possible have been enabled by using delayed *in vitro* fertilization (e.g., Lee et al. 1996).

To study molecular genetic events leading to normal development requires investigating dysfunctional genes. Many mutations have been identified in zebrafish and a number of genes have been cloned. A present difficulty lies in relating a specific observed mutant phenotype with a DNA sequence (the disrupted gene). Once mutant phenotypes are identified, the disrupted gene can be sought using the candidate gene approach or the positional cloning approach. These methods are still very difficult in zebrafish although occasionally successful (Talbot et al 1995). A preferable scheme would be to cause a mutation in such a way as to allow easy cloning, sequencing and mapping of the mutant gene. This could be enabled if insertional mutagenesis can be developed for zebrafish. Insertional mutagenesis requires transgenic technologies and germline transmission is essential to enable recovery of mutant phenotypes.

A very powerful tool, that has enabled a variety of experiments in Drosophila, *C. elegans*, yeast and the mouse, is transgenesis. It is not commonly used in zebrafish yet. Transgenesis is a necessary step for site directed gene inactivation (knockouts) (Galli-Taliadoros et al. 1995; Brandon et al. 1995; Copp 1995), and forward genetic screens (insertional mutagenesis) (Meisler, 1992). Transgenics can also be used to study regulatory elements, specific portions of gene products, and to drive ectopic gene expression. Transgenesis using GFP (Green Fluorescent Protein) reporter system, holds

considerable promise for use in the optically clear zebrafish embryo (Amsterdam et al. 1996; Moss et al. 1996). One technical difficulty in producing transgenic zebrafish has been the short period of time in which microinjections can be performed before fertilization. Thus, delayed *in vitro* fertilization which allows sequential fertilization of small groups of eggs may help overcome a hurdle to producing transgenic zebrafish.

Until recently, transgenic zebrafish were produced by the microinjection of plasmid DNA into the cytoplasm of the one-cell stage embryo (Stuart et al. 1988; Stuart et al. 1990; Culp et al. 1991), in a similar manner used in salmonids (e.g. Devlin et al. 1994; Devlin et al. 1995). Although this method is useful, efficiency is variable, and transgenes are frequently present in tandem arrays and can have complex and unpredictable structures (Stuart et al. 1988; Stuart et al. 1990; Culp et al. 1991; Devlin et al. 1995). Recent advances in the efficiency of introducing transgenes using a modification of a pseudotyped virus developed for human gene therapy (Lin et al 1994; Gaiano et al. 1996; Gaiano and Hopkins 1996) indicates a potential for transgenics and insertional mutagenesis in zebrafish. Methods of transgenesis and insertional mutagenesis have been developed for the mouse (Meisler 1992; von Melchner et al 1992) and transgenesis has been developed for *Xenopus* (Kroll and Amaya 1996; Smith 1996). The transgenic method (REMI or Restriction Enzyme Mediated Integration) via permeablized sperm developed recently by Kroll and Amaya (1996) for *Xenopus*, to my knowledge has not be tried on zebrafish. I can see no reason the method could not be adapted for zebrafish. A method for large-

scale transgenesis in zebrafish would be a major step forward for molecular genetic studies in zebrafish.

Transgenics in combination with embryonic stem cell lines (not presently developed for zebrafish) would enable gene replacement ("knockouts") studies, a very powerful molecular genetic tool. ES-like cell lines have been developed for zebrafish (Sun et al. 1995). Although these cell lines are pluripotent and ES-like in morphology and can be successfully transplanted into embryos, they have not been observed to contribute to the germline and thus do not meet the crucial criteria of an embryonic stem cell line.

Recent advances in zebrafish genetics include: the construction of zebrafish linkage maps (Postlethwait et al. 1994, Johnson et al. 1996; Knapik et al. 1996), half-tetrad analysis (Johnson et al. 1995; Johnson et al. 1996) and demonstration that a genomic clone containing the wild allele of a gene can locally rescue a mutation in that gene when injected into early cleavage stage homozygous embryos (Talbot et al. 1995).

The present RAPD linkage map was produced using haploid gynogenotes (Postlethwait et al. 1994; Johnson et al. 1996) and thus reflects the cross-over rates during female meioses. Haploid androgenotes (Corley-Smith et al. 1996; Corley-Smith et al. 1995a and 1995c) can be used to produce a linkage map based on the cross-over rate during male meioses. This research is underway in a collaborative effort between myself and John Postlethwait's and Chuck Kimmel's Labs (both at University of Oregon, Eugene OR). I helped generate the haploid androgenotes for the mapping cross and production of

a RAPD linkage map for three large chromosomes is presently underway in Dr. Postlethwait's lab.

The FRAPD technique which I developed, should find applications in molecular genetic methods. It is useful for studying inheritance of markers to a progeny, especially in cases where a high degree of sensitivity and reproducibility is desired. It also has applicability for paternity issues.

The similarity of conserved mechanisms across families, orders and phyla is becoming more evident as genetic pathways in the various systems are resolved. Some aspects of basic body patterning are conserved across phyla. For example the alternating expression of the Drosophila pair-rule genes were once considered a phenomenon particular to insect development, but recently a zebrafish homologue (*hairy*) has been discovered to have analogous pattern of expression that is coincident with body patterning into somites (Müller et al. 1996). The astute zebrafish biologist, I believe will continue to pay attention to not only what can be inferred about human development from zebrafish development but also to pay attention to lessons learned in a variety of model systems including: yeast, *C. elegans*, Drosophila, mice, frogs and *Fugu rubripes*. To an increasing degree, I believe that understanding developmental processes will rely on applying knowledge obtained in a variety of model systems and across phyla. The molecular genetic techniques discussed above will accelerate the molecular genetic analysis of zebrafish development, and hence speed the functional analysis of the vertebrate genome.

This will aid biologists in their search for understanding the underlying processes of vertebrate development.

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Appendix 1: Breeding Fish for *in vitro* Fertilization

Introduction

For delayed *in vitro* fertilization it is important to obtain high quality eggs. We consider eggs to be of high quality if over 80% of them complete embryonic development after fertilization. Keeping zebrafish alive is often easy but obtaining good quality eggs requires both good nutrition and good water quality. Although water quality is very important, a comprehensive discussion of production of good water quality for breeding zebrafish is outside the scope of this protocol.

In our facility, tap water is unsuitable for raising zebrafish, even with the chlorine removed. Thus we use reverse-osmosis and de-ionized water (from recirculation system in building; ~ 0.05 µmhos). This water is passed through a series of filters on its way to a mixing tank (one Hytrex 10 µm particle filter, one Omni charcoal filter, 2 Barnstead HN Ultrapure DI resin canisters). This water is then poured through a column in the mixing tank that contains enough oyster shell to raise the pH to ~ 7. The water in the mixing tank is continually mixed with air pumped through a large air stone. A conductivity probe monitors salinity in the mixing tank and calls for injection of water containing 30 PPT Coral Life Salt (an artificial sea salt) to maintain the salinity in mixing tank at approximately 0.06 PPT. The water then is gravity fed to aquarium tanks. The system is flow through, with a flushing rate of approximately once every three days maintained for each aquarium tank containing fish. Each aquarium tank contains a heater to maintain water at approximately 28.5°C.

Outbred zebrafish lines may be undesirable for various experiments requiring uniformity of material. However, in my experience they not only yield more eggs but the eggs are usually of better quality and the fish are more robust. Our best females have produced over 800 eggs per squeeze. Typically, when viewed under a stereo microscope at 24 hours after fertilization was attempted, over 90% of the eggs were observed developing as AA embryos (classified according to Westerfield 1995, Page 2.20).

Following is the protocol we used at SFU to obtain high quality eggs for *in vitro* fertilization. This protocol was honed by a colleague, James Lim. It is more labor intensive than the protocol presented in the Zebrafish Book (Westerfield 1995), but nearly always results in obtaining good eggs from squeezed fish. We try to avoid squeezing fish more than once per month.

Maintenance of breeding females (pre-conditioning):

- Females were always kept in tanks with breeding males.
- Fish were fed less than what they would eat if given food to satiation. On weekends we often feed once on Saturday and not at all on Sunday. I believe these periods of low food are important to allow egg development to become synchronized so that a mixture of good and bad eggs are not obtained on breeding days.
- We use full spectrum fluorescent lighting. We suspect this affects males more than females, but the interaction of males and females is believed highly desirable in obtaining good quality eggs.

Day 1: (follows pre-conditioning).

- Males and female still together
- Fish were fed to satiation (trout starter pellets and copious amounts of brine shrimp)
- Water flow was turned up slightly and residual food and fecal material removed twice daily to maintain good water quality.

Day 2:

- Males and females still together.
- Again fish were well fed and water quality maintained.
- We found that females usually did not breed much during these first 2 days on high food but did on 3rd and 4th day).
- Either in the afternoon of day 2 or in morning of day 3 before the light went on, we separated fish by sex. We then double checked to make sure that no males are in with females.

Day 3:

- Fish are now separated by sex.
- Fish were fed well and water quality maintained.

Day 4: Breeding day.

- Generally 3 males and 3 females were put together per 5 to 10 gal tank as soon as the light came on. We then watched for signs of breeding behaviour (see below

for description of breeding behaviour). Although this watching is labor intensive, we found it worth the effort. When breeding behavior was observed, immediately upon seeing eggs, the female extruding eggs was removed, using a net. If only that female was shedding eggs and the other two were not, the breeding behavior abruptly ended. If the wrong fish was netted out, or more than one was breeding, the breeding behavior persisted. When a female was obtained this way, nearly all the eggs obtained fertilized and developed. Without this selection scheme, eggs obtained by squeezing included a mixture of eggs of good and poor quality. Occasionally, the selected female fish produced only a few eggs (all of which were usually good quality), but usually females produced hundreds of eggs of good quality.

Squeezing to obtain eggs

- Please refer to Methods and Materials section in Chapter 1.

Breeding behavior

What we observed.

- Breeding behaviour was observed to consist of the males chasing the females, often bumping into the sides of females. Before eggs were expelled, this chasing usually became increasingly frenzied and often moved to a corner of the tank with all breeding males being involved. The

non-breeding females often joined the group, presumably to be present for the anticipated meal of zebrafish eggs.

- Breeding behavior alone did not guarantee eggs will be good quality eggs. We watched breeding behavior until we saw the first eggs being expelled by the females during their interaction with the males before netting out the female.

How we observed.

- We taped black plastic to back of tanks to increase contrast so that eggs could be seen more easily.
- We used overhead full spectrum fluorescent lighting during this breeding selection. We observed that with cool white fluorescent tubes males often did not become sexually active and failed to bump into the sides of the females. In this case, the female did not begin to shed eggs, and the selection scheme failed to identify females having eggs of good quality. Thus, a female could be full of good quality eggs but if we did not see any being expelled, we would not know to net out that female.
- We often used a floating breeding cage with a slatted bottom through which eggs could drop making them easier to see.
- We viewed fish from the front of tank. The observers eyes were roughly horizontal to the fish being observed.

How long we observed:

- Usually if breeding activity was observed it was observed during the first hour of observation. Fish not shedding eggs on the first day, often did shed eggs on the second day of breeding. Thus, if fish did not produce eggs on day 4, we fed them well for rest of that day and repeated observation for egg laying on day 5.

Literature Cited:

Westerfield, M. 1995. The zebrafish book -- A guide for the laboratory use of zebrafish (*Danio rerio*), edition 3, edited M. Westerfield, Institute of Neuroscience, University of Oregon, Eugene, OR.