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Induction of tetraploidy in zebrafish Danio rerio and Nile tilapia Oreochromis niloticus

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INDUCTION OF TETRAPLOIDY IN ZEBRAFISH *DANIO RERIO* AND NILE TILAPIA *OREOCHROMIS NILOTICUS*

A Thesis

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by
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To my family

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ABSTRACT

The goals of this thesis were to: 1) induce tetraploidy in zebrafish; 2) characterize single-female spawning in Nile tilapia for egg collection; 3) design and construct hatching systems for tilapia eggs; 4) induce tetraploidy in Nile tilapia.

Tetraploidy was induced in zebrafish *Danio rerio*, by applying a 41 C heat shock to eggs for 2 min at 11, 13, 15, 17, or 20 min after fertilization (AF). A trend of increasing percent tetraploid yield occurred the later the shocks were applied. Shocks applied at 11 min AF yielded 1% tetraploidy and shocks applied at 20 min AF yielded 10%. There were no significant differences ($\underline{P} = 0.20$) in induction among treatments. This is the first report on induction of tetraploidy in zebrafish.

Stocking of single-female Nile tilapia *Oreochromis niloticus* in ten 80-L aquaria yielded a reliable supply of eggs for polyploidy research conducted in this study. In one year, 72 spawns were recorded, yielding ~90,000 eggs. Peak spawning occurred in summer.

The two egg hatching systems for artificial incubation of tilapia eggs proved to be effective, with 75% of control fish surviving to hatch. Methylene blue and ultraviolet sterilization reduced losses of eggs to disease and use of glass test tubes as hatching vessels allowed for constant rolling of the eggs and easy viewing and access. The systems occupied little space (< 1 m^2) and could accommodate up to 30 treatments of eggs.

The use of egg baskets allowed for rapid and uniform heat shocking of multiple treatments of Nile tilapia eggs for polyploidy induction experiments. Tetraploidy and triploidy were induced by application of 42.8 ± 0.2 C heat shocks to eggs for 3 min at 22, 24, 26 and 28 min AF. Shocks applied at 24 min AF resulted in the highest tetraploid yield (10%) while shocks applied at 22 min AF resulted in the highest triploid yield (7%). This is the first study to report the

simultaneous induction of tetraploidy and triploidy in Nile tilapia within treatments from a single spawn and raises questions about technical, maternal and genetic effects on synchrony of zygotic development in this species.

CHAPTER 1
FOREWORD

Aquaculture, the husbandry of aquatic organisms, has undergone a progression directly linked to that of human history. Ancient cultures of the Far East and pre-Columbian societies of the Amazon were known to have harvested fish stranded in pools by receding floodwaters. Observation of fish reproducing in these pools may have led to the collection and transport of fry for stocking in ponds and eventually the artificial hatching of fish, the first account of which dates back over 2000 years in China (Avault 1996).

Today, aquaculture has a different face than it did 2000 years ago, with more than 32.9 million metric tons produced worldwide at the end of the 20th century (FAO 2001), and the expansion of aquaculture from foodfish production to include ornamentals and research animals for biotechnology. This increase in aquaculture production, especially at the end of last century has been made possible through the accumulation of knowledge and advances in technology.

Some of the advances made in fish culture include the closing of life cycles through artificial reproduction, and the hatchery rearing and domestication of fish species. These advances have facilitated genetic improvement through a number of methods including selective breeding, hybridization, creation of monosex populations, gynogenesis, androgenesis, polyploidy and gene transfer (the incorporation of foreign DNA into a recipient genome).

Although these techniques can improve and expand production, they also raise ecological and human health questions. For example, concerns have been raised about the possibility of contamination of wild gene pools and the introduction of exotic species if hybrids and farmed strains escape. The human health risks and ecological effects associated with the production of transgenic animals are poorly understood. The production of monosex populations by

administration of hormones to fish before they undergo gonadal differentiation also raises issues about consumer acceptance.

One way to address some of these issues that can also provide commercial benefits is the production of sterile fish. Sterility has been proposed as a possible safeguard against introductions of transgenic animals (Thorgaard 1992). Indeed, some researchers have used sterility to guard against the perpetuation of transgenic genomes (Marian 1997). Other researchers have suggested the use of sterility to protect against hybrids backcrossing with and adulterating the genome of native fishes (Scheerer et al. 1987).

This is not a theoretical issue. For example, only the stocking of functionally sterile grass carp is legally permitted in certain states including Louisiana, as a means of protecting against the establishment of this exotic species. The production of sterile fish can also help to protect investment and intellectual property when selling live fish. Furthermore and perhaps the most beneficial aspect of the use of sterility for aquaculture production is that it can be used as an effective management tool when growing fish that reach sexual maturity before they attain market size. Polyploidy, the possession of more than the normal two sets of chromosomes per cell, can be employed as a method for the production of sterile fish and research on this area of genetics began over 30 years ago (reviewed by Lutz 2001).

Polyploidy is a naturally occurring phenomenon in plants and non-mammalian vertebrates including fishes. Chapter 2 of this thesis introduces polyploidy in fish. Triploidy, a form of polyploidy where each cell contains three sets of chromosomes, causes functional sterility. Triploidy has been induced in a number of fish species including zebrafish (Kavumpurath and Pandian 1990) and tilapia (Don and Avtalion 1986). The process involves the application of a shock to eggs shortly after fertilization causing the retention of the second polar body (an extra

set of chromosomes normally lost when meiosis II is resumed in the egg) following fertilization. The induction of triploidy is not always 100% effective, however, and can result in decreased viability and other side effects. Another form of polyploidy, tetraploidy, has been used to facilitate the production of triploids.

Through the disruption of processes associated with first cleavage in newly fertilized eggs, it is possible to produce fishes with four sets of chromosomes (tetraploidy). Tetraploid rainbow trout *Oncorhynchus mykiss* have been crossed with diploids to produce triploids (Chourrout et al. 1986, Myers and Hershberger 1996). This method, referred to as production of interploid triploids, is reliable and avoids detrimental side effects associated with conventional methods of triploidy induction and shows promise for the commercial production of triploid stocks.

The studies in this thesis address the induction of tetraploidy by use of heat shock in two species of fish: zebrafish *Danio rerio*, and Nile tilapia *Oreochromis niloticus*. The main goal of this thesis research was to standardize methods to produce tetraploid zebrafish and Nile tilapia. The specific objectives were to: 1) evaluate the use of heat shocks for the induction of tetraploidy in zebrafish and to evaluate the effects of timing of application of shocks on percent tetraploidy, percent survival and percent tetraploid yield; 2) characterize single-female spawning in Nile tilapia for the collection of ripe eggs for ploidy induction experiments; 3) design and construct a hatching system for tilapia eggs; 4) develop protocols for the rapid and reproducible heat shocking of Nile tilapia eggs, and evaluate the effects of timing of application of shock on percent tetraploidy, percent survival and percent tetraploid yield.

Zebrafish are a common aquarium fish (Figure 1.1), which is likely responsible for their introduction to areas outside of their native range including the southeastern United States (USGS 2001). Over the last several decades, zebrafish have become a research model for the

study of vertebrate genetics, developmental biology and biotechnology (Kahn 1994, Kimmel 1989, Barinaga 1990). Studies on gene transfer, gynogenesis, androgenesis, and the

Figure 1.1. Zebrafish *Danio rerio* is a popular aquarium fish and an important research model for the study of genetics, developmental biology and biotechnology.

induction of triploidy have been conducted on this species. However, there are no published reports on tetraploidy in zebrafish. Chapter 3 of this thesis addresses the induction of tetraploidy in zebrafish by use of heat shocks and is the first report on the induction of tetraploidy in this species.

Nile tilapia *Oreochromis niloticus* are one of the most widely cultured foodfishes in the world (Young and Muir 2000) (Figure 1.2). Studies on gene transfer, gynogenesis, androgenesis, and polyploidy have also been conducted in this species. The artificial spawning of Nile tilapia has facilitated much of this research, but the collection and artificial incubation of eggs can pose some challenges in this species. Chapter 4 of this thesis addresses single-female spawning of Nile tilapia for egg collection and Chapter 5 describes the design and construction of a hatching system for tilapia eggs.

The widespread culture of Nile tilapia has led to their introduction to areas outside of their native range and they are listed as a non-indigenous species occurring in the southeastern United States (USGS 2001). The state of Louisiana only permits the culture of Nile tilapia indoors to safeguard against their introduction into natural habitats.

Figure 1.2. Nile tilapia *Oreochromis niloticus*, a foodfish cultured throughout the world.

Nile tilapia reach sexual maturity well before they attain market size and reproduction in culture systems often results in decreased production. The production of functionally sterile tilapia has been achieved by the induction of triploidy through conventional methods, but these methods have proved to be unviable on a commercial scale. A handful of studies have been aimed at the production of tetraploid Nile tilapia. However, none of these resulted in tetraploids surviving to sexual maturity and much variation in the reported effective timing of application of shocks to induce tetraploidy exists between studies. Chapter 6 of this thesis addresses the induction of polyploidy and synchrony of development in Nile tilapia.

The results of this thesis project have yielded four published abstracts in conference proceedings (Table 1.1). All chapters of this thesis have been prepared in the format of the *Journal of The World Aquaculture Society*. It is anticipated that Chapters 3, 4, 5, and 6 will be submitted for publication in peer-reviewed journals.

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Table 1.1. Conference presentations and abstracts of research presented in this thesis.

Year	Title	Conference	Location
2002	Four sets of chromosomes (tetraploidy) would kill a zebra. What about a zebrafish? Nile tilapia?	Annual Meeting of the Louisiana Academy of Sciences	Baton Rouge, Louisiana
2002	Induction of tetraploidy in zebrafish *Danio rerio*	Aquaculture America 2002	San Diego, California
2002	Is variable induction of polyploidy in Nile tilapia caused by asynchrony of zygotic development?	Joint Meeting of the Mississippi and Louisiana Chapters of the American Fisheries Society	Biloxi, Mississippi
2001	Production of tetraploid zebrafish *Danio rerio*	Annual Meeting of the Louisiana Chapter of the American Fisheries Society [1]	Baton Rouge, Louisiana

[1] Received Best Student Paper Honorable Mention.

CHAPTER 2
INTRODUCTION

This chapter is intended to provide an overview of polyploidy in fishes and the processes involved in the induction of triploidy and tetraploidy. A brief introduction to zebrafish *Danio rerio* and Nile tilapia *Oreochromis niloticus* and their status with respect to polyploidy is also provided. A more detailed account of these species, the subjects of this thesis, will be provided in subsequent chapters.

Polyploidy

Overview of Polyploidy

Diploidy, the possession of two chromosome sets per cell, is the most common condition in animals. Polyploidy, the possession of more than the normal two sets of chromosomes per cell occurs naturally in some plants and can result in the phenomenon of gigantism (Ehrlich et al. 1963). Gigantism results from an increase in cell size that is proportional to the increased DNA content of polyploid cells. The possibility of gigantism occurring in animals stimulated interest in the production of polyploid agricultural animals. In mammals, the condition of polyploidy will result in embryonic death. However, other vertebrates are able to tolerate polyploidy and it is a rare naturally occurring phenomenon in birds (Tiersch et al. 1991a), reptiles (Tiersch 1991b), amphibians (Frankhuaser 1945) and fishes (Thorgaard and Gall 1979). Unlike in plants, polyploidy in animals does not result in gigantism. Although there is an increase in the size of cell nuclei and cells of polyploid animals compared to those of diploids, this increase is compensated for by a decrease in the total number of cells in the organism. Thus, animal size is regulated by factors other than cell size and DNA content (Frankhauser 1945). The natural

occurrence of polyploidy can result from disruptions in the cellular events that occur between fertilization and the first mitotic division.

Polyploidy in Fishes

In fishes, a mature egg completes Meiosis I before fertilization, is arrested in Meiosis II, and contains two sets of chromosomes. Upon activation (usually by fertilization, changes in osmotic pressure, or contact with sperm from another species), Meiosis II is resumed and the separation of the two sets of chromosomes occurs. One set forms the second polar body, which is normally extruded from the egg, while the other set forms the egg pronucleus. The egg pronucleus fuses with the sperm pronucleus (also containing one set of chromosomes) restoring the number of chromosome sets to two and resulting in a diploid zygote. After the DNA has been replicated, first mitosis results in two identical diploid daughter cells (Figure 2.1).

If the resumption of Meiosis II is disrupted (either naturally or induced by application of a shock), the second polar body and the egg pronucleus fail to separate (non-disjunction) or the second polar body fails to be extruded and the egg will retain two sets of chromosomes. If fertilization has occurred, the set of chromosomes from the sperm can combine with the two sets from the egg and result in a triploid zygote (Figure 2.2). Triploidy is a form of polyploidy where each cell contains three sets of chromosomes. If activation occurs without fertilization or by fertilization by sperm that has been irradiated to inactivate the DNA, and the resumption of Meiosis II is disrupted, a diploid zygote receiving both sets of chromosomes from the mother can result. This is a form of gynogenesis (all-maternal inheritance) and is termed meiotic gynogenesis because it results from the disruption of Meiosis II (Figure 2.2).

Disruption of the first mitotic division (natural, or induced by application of a shock) can result in another form of gynogenesis, termed mitotic gynogenesis, androgenesis (all-paternal

inheritance), or tetraploidy, a form of polyploidy where each cell contains four sets of chromosomes (Figure 2.3).

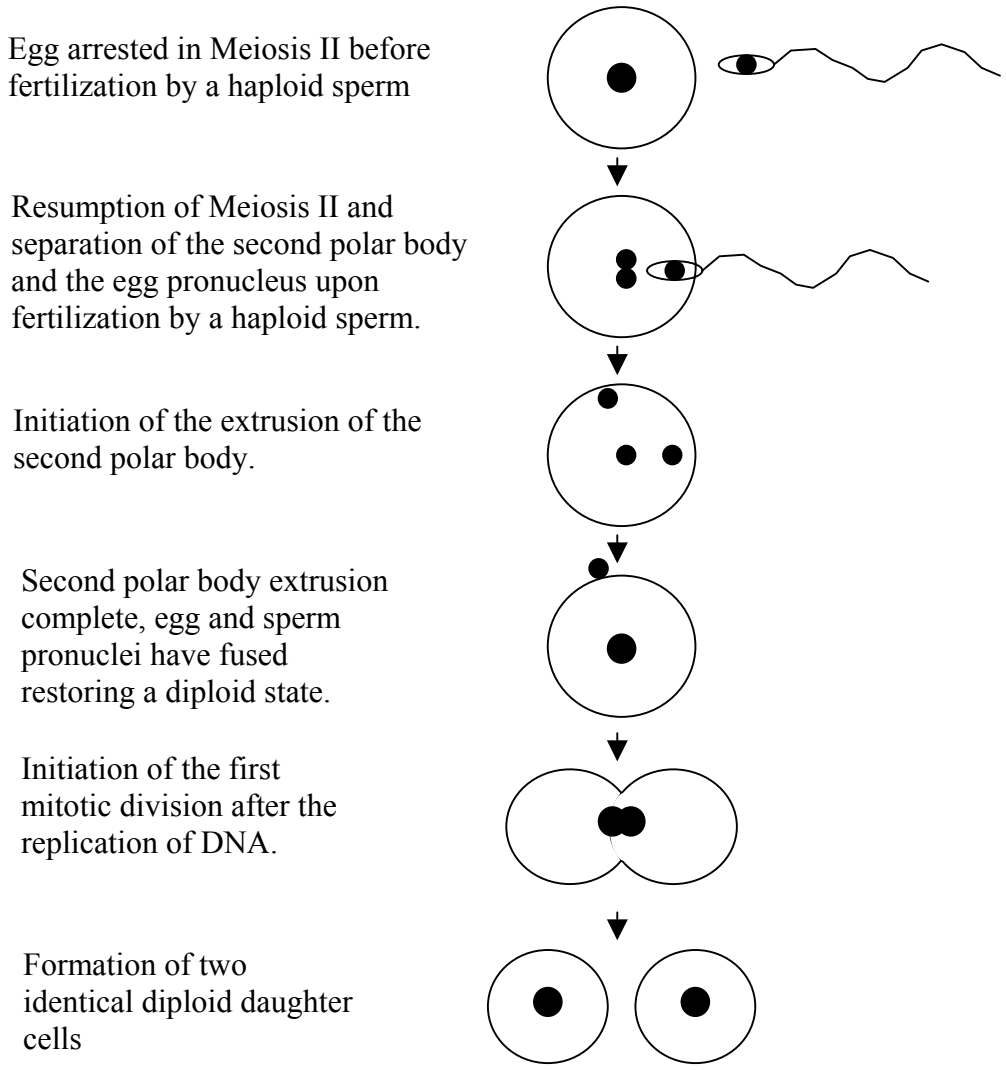

Figure 2.1. Diagram representing the normal progression of cellular events following fertilization of an egg from a normal diploid female by a sperm from a normal diploid male. Diagram modified from Tave (1993).

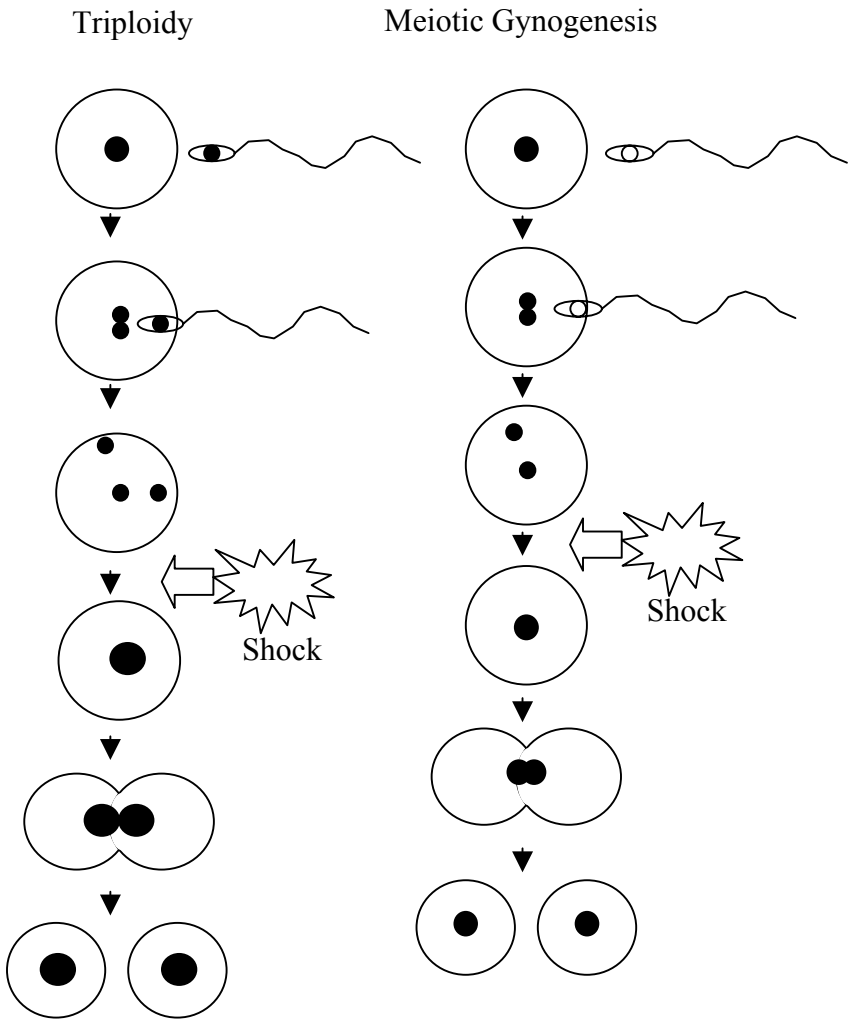

Figure 2.2. Disruption of cellular processes associated with Meiosis II causing the retention of the second polar body. For triploidy, the retention of the second polar body results in the formation of a nucleus with three sets of chromosomes. For meiotic gynogenesis, the sperm has been irradiated and the DNA inactivated. The retention of the second polar body reestablishes the diploid state. Diagram modified from Tave (1993).

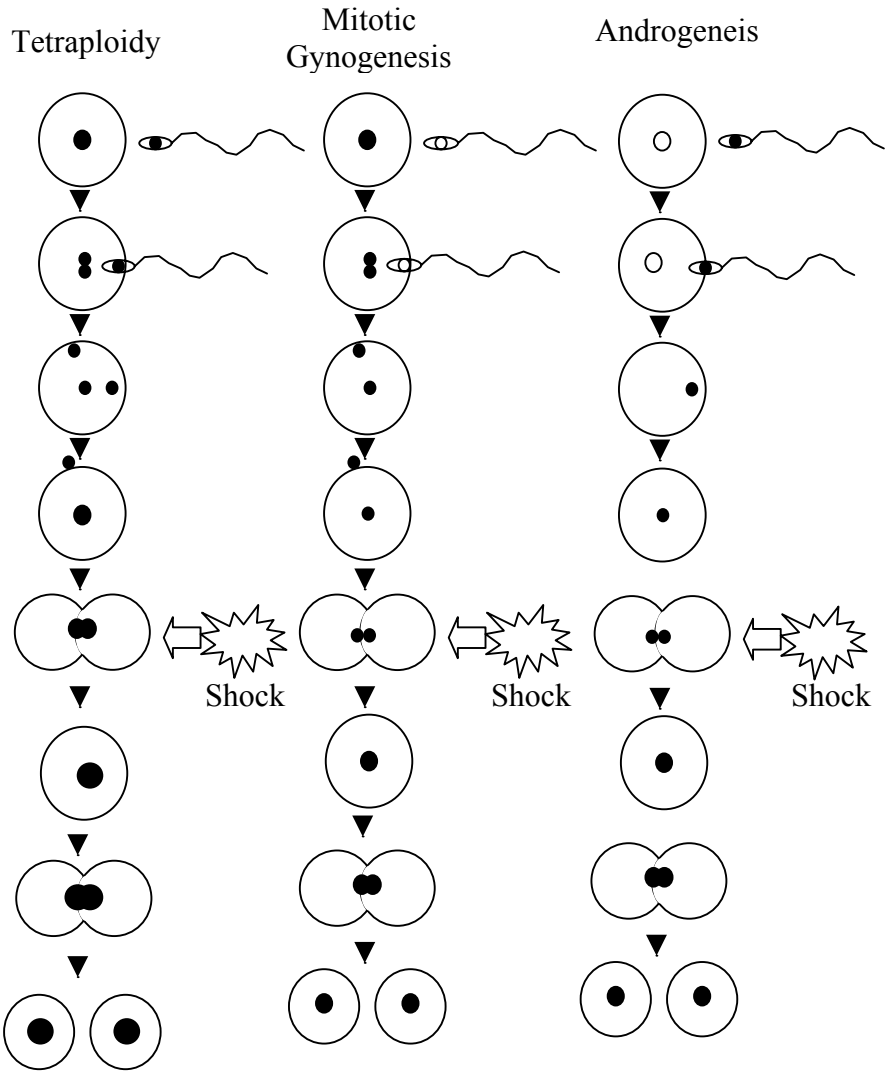

Figure 2.3. Diagram of the inhibition of the first mitotic division by the application of a shock resulting in tetraploidy, mitotic gynogenesis, and androgenesis. For tetraploidy, the inhibition of the first mitotic division occurs after the DNA has been replicated resulting in a nucleus with four sets of chromosomes. For mitotic gynogenesis, the sperm has been irradiated and the DNA inactivated. For androgenesis, the egg has been irradiated and the DNA inactivated. In both mitotic gynogenesis and androgensis, the diploid state is restored by preventing the first mitotic division after the DNA from the haploid egg pronucleus or sperm pronucleus has been replicated. Diagram modified from Tave (1993).

Gynogenesis and androgenesis are naturally occurring phenomena in fish. In fact, a species of parthenogenic fish, the Amazon molly *Poecilia formosa* has evolved using gynogenesis as a reproductive strategy. The females spawn with males of another species whose sperm activate the eggs, but do not fertilize them (Schlupp et al. 1994). Spontaneous androgenetic grass carp have resulted from the hybridization of common carp females with grass carp males. Evidently incompatibility of the of the two genomes results in the extrusion of the egg pronucleus as well as the second polar body leaving only the sperm (Ihssen et al. 1990). Gynogenesis and androgenesis are of interest to researchers and aquaculturists because they can facilitate the production of homozygous lines of fish and clones. Initial research on the artificial initiation of gynogenesis through disruption of cellular events led the production of polyploids.

Triploidy and tetraploidy are naturally occurring phenomena in fishes as well. Spontaneous triploids (naturally occurring, where a polyploid individual is found within a normal diploid population) have been reported in several species including the brown bullhead *Ameiurus nebulosus* (Tiersch et al. 1993) and rainbow trout *Oncorhynchus mykiss* (Thorgaard and Gall 1979). Spontaneously occurring tetraploids have also been reported in the loach *Misgurnus anguillicaudatus* (Arai et al. 1993). Whole families of fish (Catostomidae and Salmonidae) are thought to have evolved by tetraploid events (Ihssen et al. 1990). Tetraploidy can also result from hybridization as is reported with carps (Liu et al. 2001). Triploidy and tetraploidy have several practical applications for aquaculture.

Triploidy has been induced in a variety of fish species (Table 2.1) and is of interest to aquaculturists because it results in functional sterility (gonads may be able to produce gametes resulting in fertilization, but the chances of normal development and survival of progeny are minuscule). The production of sterile fish can reduce the risks of exotic introductions and

Table 2.1. Survey of studies during past 30 years on triploidy induction in fish.

Common name	Scientific name	Shock type	Highest reported induction	Reference
Plaice	*Pleuronectes platessa*	cold	*	Purdom 1973
Common carp	*Cyprinus carpio*	cold	*	Gervai et al. 1980
Channel catfish	*Ictalurus punctatus*	cold	100%	Wolters et al. 1981
Atlantic salmon	*Salmo salar*	anesthetics	80%	Johnstone et al. 1989
Nile tilapia	*Oreochromis niloticus*	heat, pressure, cold	100%	Hussain et al. 1991
Tench	*Tinca tinca*	cold, pressure	100%	Flajshans et al. 1993
Yellow perch	*Perca flavescens*	heat, pressure	30-70%	Malison et al. 1993a
Coho salmon	*Oncorhynchus kisutch*	heat, electric	100%	Teskeredzic et al. 1993
Hybrid sunfish	*Leopomis machrochirus* x *L. cyanellus*	pressure	100%	Wills et al. 1994
Zebrafish	*Danio rerio*	heat	100%	Marian 1997
Turbot	*Scophthalmus maximus*	cold	~80%	Piferrer et al. 2000

* Not reported.

contamination of wild gene pools. In addition, it can safeguard against unauthorized perpetuation of transgenic genomes and protect intellectual property. The development of sterile stocks for foodfish production can be used to control precocious spawning in fishes that reach sexual maturity before they reach market size and may improve growth.

The functional sterility of triploid fish has been established for several species. The probability of a competent sperm from a triploid grass carp *Ctenopharyngodon idella* fertilizing a normal egg and resulting in a reproductively viable offspring was estimated to be 4×10^{-11} for every reduction of hexaploid spermatagonia (Allen et al. 1986). Sperm from triploid rainbow trout *Oncorynchus mykiss* were reported to be anueploid (Benfey et al. 1986), meaning that the cells contained an abnormal amount of DNA, not equal to the haploid value and would result in non-viable offspring. Sperm from triploid zebrafish were not able to fertilize eggs from diploid females (Kavumpurath and Pandian 1990) while triploid tilapia sperm were able to fertilize eggs from a diploid female, but all resultant embryos were abnormal and died (Penman et al. 1987).

As mentioned earlier, the process of triploidy induction involves the application of a shock to eggs shortly after fertilization, which results in the retention of the second polar body (a set of chromosomes normally lost when meiosis II is resumed) (Figure 2.2). Hypotheses addressing the mechanism involved in retention of the second polar body include the breakdown of the spindle apparatus or actin filaments that pull the egg pronucleus and second polar body apart during Meiosis II (Mair 1993).

Although a high percentage of triploids can be produced, induction through the application of shocks is not always 100% effective and can result in lowered viability and other detrimental effects (Lutz 2001). Reports on the growth of triploids relative to normal diploids have varied between studies and among species and life stages. While no differences in growth between

triploid and diploid Nile tilapia were observed prior to sexual maturation, the growth of triploids surpassed that of diploids after the onset of sexual maturation (Bramick et al. 1995, Focken et al. 2000). However, faster growth of triploids when compared to diploids has been reported in Blue tilapia prior to sexual maturation (Valenti 1975). In contrast, growth of triploid rainbow trout has been reported to be inferior to that of diploid controls, especially prior to sexual maturity (Myers and Hershberger 1991b).

Another form of polyploidy, tetraploidy (the possession of four sets of chromosomes per cell), has been induced in a number of fish species (Table 2.2) and has been used to facilitate the production of triploids. As mentioned above, techniques used to induce tetraploidy are similar those used to produce triploids. However, the shocks are applied later, after the extrusion of the second polar body, but before the first cell division (Figure 2.3). Tetraploidy can be induced by application of shocks during different phases of the cell cycle leading to first mitosis. Application of shocks just prior to the first mitotic division (cytokinesis) is hypothesized to cause the breakdown of actin filaments and prevent the formation of the cleavage furrow (Chourrout 1982). Application of shocks just prior to or during metaphase is thought to prevent the separation of sister chromosomes (karyokinesis), possibly by disrupting the formation of the asters following the fusion of the pronuclei (Myers et al. 1995).

Although tetraploidy has been induced in more than 10 species, reports of reproductively viable tetraploids are limited to loaches and salmonids. Spontaneously occurring tetraploid loaches *Misgurnus anguillicaudatus* of both sexes have been crossed with each other to produce tetraploid offspring, and with diploids to produce triploids (Arai et al. 1993). Tetraploid rainbow trout have also been crossed with diploids to produce triploids (Chourrout et al. 1986, Myers and Hershberger 1996) (Figure 2.4).

Table 2.2. Survey of studies during past 30 years on tetraploidy induction in fish.

Common name	Scientific name	Shock type	Highest reported induction	Reference
Plaice	*Pleuronectes platessa*	cold	0%	Purdom 1973
Rainbow trout	*Oncorhynchus mykiss*	heat	*	Chourrout 1982
Channel catfish	*Ictalurus punctatus*	heat	62%	Bidwell et al. 1985
Blue tilapia	*Oreochromis aureus*	cold	25%	Don and Avtalion 1988
Tench	*Tinca tinca*	heat	79%	Flajshans et al. 1993
Yellow perch	*Perca flavescens*	heat, pressure	100%	Malison et al. 1993b
Bluegill sunfish	*Lepomis cyanellus*	cold , pressure	40%	Miller 1995
Nile tilapia	*Oreochromis niloticus*	heat	80%	El Gamal et al. 1999
African catfish	*Clarias gariepinus*	heat	9%	Varadi et al. 1999

* Not reported.

These progeny are referred to as interploid triploids. Because no shocks are applied, this method appears to be more reliable and avoids detrimental side effects associated with conventional methods of triploidy induction (Myers and Hershberger 1991b, reviewed by Lutz 2001).

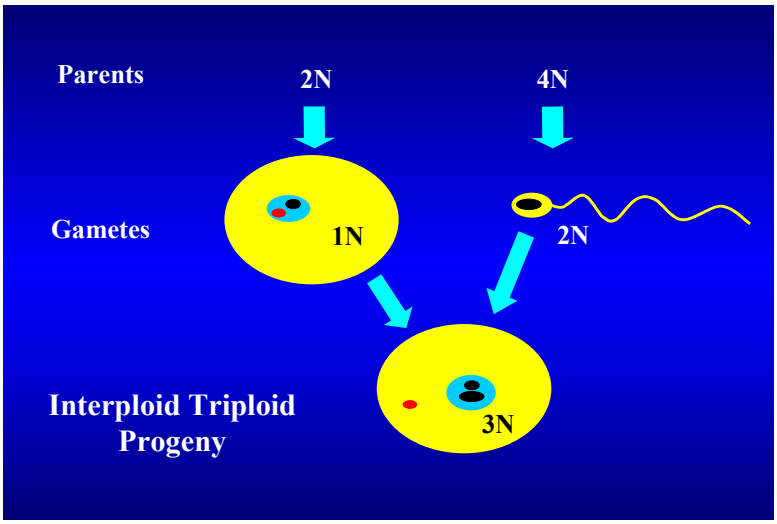

Figure 2.4. The production of interploid triploids is achieved by fertilization of a haploid egg from a diploid female with a diploid sperm from a tetraploid male. Haploidy, (the presence of one set of chromosomes) is designated as "1N" and is the normal condition of germ cells produced by diploid parents after completion of meiosis II. Diploidy is designated as "2N," triploidy is designated by "3N," and tetraploidy as "4N." No shock is required for the production of interploid triploids.

The production of tetraploid lines of fish is possible by crossing tetraploids with tetraploids (Fig 2.5). The availability of tetraploid lines could be a valuable tool for the commercial production of functionally sterile interploid triploids.

Challenges of Ploidy Induction

The disruption of cellular events that result in polyploidy induction requires control over the timing, intensity, and duration of shocks to newly fertilized eggs. In nature, activation of eggs and resumption of Meiosis II differ among fish species. In zebrafish, activation is triggered by changes that occur in osmotic pressure when eggs are released into the external environment

(Westerfield 1995). In Nile tilapia, activation occurs upon fertilization by the sperm (Myers and Hershberger 1991). Eggs of most freshwater fishes go through a hydration process upon

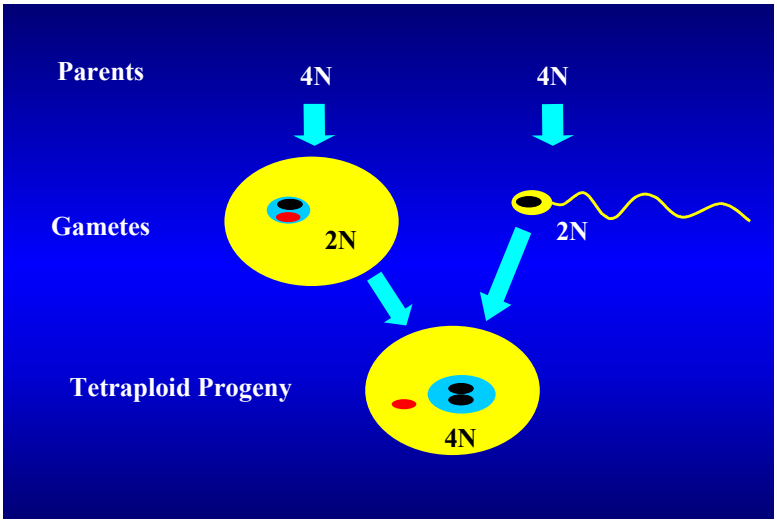

Figure 2.5. Maintaining tetraploid lines by fertilizing diploid eggs from a tetraploid female with diploid sperm from a tetraploid male. Diploid gametes are designated as "2N" and tetraploid parents and progeny as "4N." No shock is required.

exposure to reductions in osmotic pressure. This leads to swelling, the hardening of the chorion (outer egg membrane) and the closing of the micropyle (the site of sperm entry) after which natural fertilization is not possible. Fish sperm can also be activated by changes in osmotic pressure. The collection of gametes in physiological solutions during artificial spawning can help avoid unwanted activation of eggs and sperm, extend the working time, and allow the precise timing of artificial fertilization for polyploidy induction experiments.

The application of chemical or pressure shocks to induce polyploidy in fishes involves some inherent risks to researchers and can require specialized apparatus such as a pressure chambers. Heat shocks are effective at inducing polyploidy in fishes including zebrafish and tilapia, and are relatively simple.

Because polyploid organisms have an increased DNA content, nuclear size, number of chromosomes, and cellular size compared to diploids, ploidy can be determined by several methods that allow for the comparison of these characteristics between organisms in question and control organisms (usually diploids). One of the more definitive methods is the preparation of chromosome spreads and counting of individual chromosomes. To do this, cells are exposed to chemicals such as colchicine that arrest the cell division in metaphase followed by swelling of cells in hypotonic solutions. Chromosome spreads are obtained by dropping the swollen cells onto microscope slides and the number of chromosomes are counted using microscopy (Thorgaard and Disney 1990). Triploids will have 1.5 times the number of chromosomes of diploids and tetraploids will have twice the number. This method is labor intensive (Utter et al. 1983) and is not always successful (Thorgaard et al. 1982).

Other methods involve the collection of blood and measurement of erythrocyte nuclear diameters by microscopy or measurement of optical density of stained nuclei by image analysis (Cormier et al. 1993). Comparisons of erythrocyte nuclear volume can be made using a Coulter counter. Coulter counters are electronic devices that measure change in resistance caused by a cell or nucleus passing through a small orifice through which an electrical current is maintained. The change in resistance is proportional to the size of the particle (Wattendorf 1986). This method allows for the analyses of 1,000 or more samples per day, but may not always yield conclusive results unless cells are lysed for measurement of nuclei (Johnson et al. 1984). Another method for ploidy evaluation involves comparisons of DNA content by flow cytometry.

Flow cytometry allows for the simultaneous measurement of multiple cell parameters by incorporating fluidics, optics, and electronics. Measurements are made while the cells pass single file in a fluid stream through a laser beam. Upwards of ten thousand cells can be measured

in less than a minute allowing for quick and statistically powerful analyses. For ploidy evaluation, a fluorescent dye such as propidium iodide (PI) that binds to DNA can be used. The number of PI molecules that bind to the DNA is directly proportional to the amount of DNA. Passage of stained nuclei through the laser beam causes the dye to fluoresce. The fluorescence intensity is directly proportional to the amount of DNA present and is detected by photomultiplier tubes and converted to an analog electrical signal. The signals from individual cells are plotted to generate a histogram representing fluorescence intensity (Wachtel and Tiersch 1993). Cells from an organism of known DNA content are often combined with sample cells and used as an internal reference (Tiersch and Chandler 1989). When this is done, two populations can be seen, one population corresponding to the internal reference cells and the second population corresponding to cells from the organism in question. The ratio of fluorescence intensity between the two populations is directly proportional to the ratio of DNA content between the two organisms. If the DNA content of the internal reference is known, the DNA content of the cells from the organism in question can be computed. A triploid animal would have ~1.5 times the DNA content of a diploid and a tetraploid animal would have ~2 times the DNA content of a diploid.

Although flow cytometry allows for the precise and statistically powerful determination of ploidy and up to several hundred samples can be analyzed in a day, it is expensive, requires specialized equipment and trained personnel, and it is not always readily available (Thorgaard et al. 1982).

Zebrafish and Polyploidy

Zebrafish are members of the family Cyprinidae and are native to the Ganges River basin in India, Nepal and Bangladesh. They reach a maximum size of ~6 cm in length and although they

have no commercial value as a foodfish, they are a common ornamental fish and are found in home aquaria throughout the world. The production of zebrafish for the pet trade is thought to be behind the expansion of their range to include the waters of South America (www.fishbase.org) and their listing as a non-native fish in the Southeastern United States (USGS 2001).

In the past several decades, zebrafish have been identified as a valuable research animal in the areas of vertebrate genetics, developmental biology, and biotechnology (Kahn 1994, Kimmel 1989, Barinaga 1990) and are found in over 270 laboratories throughout the world (http://zfin.org/index.html). In fact, a book on laboratory techniques and rearing of zebrafish has been published (Westerfield 1995). Androgenetic (all-paternal inheritance) (Corely-Smith et al. 1996), gynogenetic (all-maternal inheritance) (Streisinger et al. 1981) and transgenic zebrafish (Marian 1997) have been produced. The only reports on polyploidy in zebrafish have been on the induction of triploidy (Kavumparath and Pandian 1990, Gestl et al. 1997). Given their value as a research animal, it is surprising that a literature search revealed no publications on tetraploidy in this species, especially with the potential tetraploidy offers for the production of interploid triploids. Chapter 3 of this thesis addresses tetraploidy induction in zebrafish.

Nile Tilapia and Polyploidy

Nile tilapia are members of the family Cichlidae and are native to Africa and the Middle East. They are one of the most widely cultured fish in the world. One of the challenges of tilapia culture is the prevention of pre-harvest spawning that leads to overpopulation and stunting. Decades of research in this species have produced several methods that address this problem. One of the most common methods is the production of monosex populations through administration of hormones prior to gonadal differentiation. However, this is not always 100%

effective and can raise concerns about consumption by humans. One of the more promising methods is the production of sterile stocks through ploidy manipulation. Research on the induction of polyploidy in Nile tilapia has yielded 100% triploidy, with survival comparable to controls (Hussain et al. 1991, Hussain et al. 1995, Focken et al. 2000). However, the logistics involved in the induction of triploidy on a commercial scale (including the need to control the exact timing of fertilization and shocks and reliable production of ripe eggs) have precluded the commercial implementation of these techniques. Research on the production of tetraploid tilapias has resulted in limited success. A handful of studies have reported on induction of tetraploidy in Nile tilapia (Table 6.2) and there are no reports of survival to adulthood. Furthermore, reports on the effective timing of shock application to induce tetraploidy in Nile tilapia ranged from 30 to 90 min after fertilization. There is one report of tetraploid blue tilapia *Oreochromis aureus* surviving to adulthood, although these fish exhibited retarded growth and were not able to reproduce (Don and Avtalion 1988). Chapter 6 of this thesis addresses polyploidy induction and synchrony of zygotic development (the amount of variation in the time to the first cell division among individuals from a single spawn) in Nile tilapia.

One of the challenges of inducing polyploidy in fish is the need to collect ripe eggs. A promising method for Nile tilapia is the stocking of single females in aquaria and monitoring the extension of the urogenital papilla on a daily basis. Although few studies have reported using this technique alone (El Gamal et al. 1990). Chapter 4 of this thesis characterizes the single-female spawning of Nile tilapia for egg collection. Another challenge associated with polyploidy induction is minimization of losses of eggs to bacteria and fungus after shock treatments. Nile tilapia females are mouth brooders (after spawning, eggs are collected and guarded in the mouth) and artificial incubation of eggs requires simulation of the natural rolling

experienced in the mother's mouth. Chapter 5 of this thesis describes the design and construction of a hatching system for Nile tilapia eggs.

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CHAPTER 3
INDUCTION OF TETRAPLOIDY IN ZEBRAFISH

Zebrafish *Danio rerio* are members of the family cyprinidae and reach a maximum size of about 6 cm. They are native to the Ganges river basin of India and Bangladesh, but their popularity as an aquarium fish has resulted in the expansion of their distribution to the waters of South America (www.fishbase.org), and the southeastern United States (USGS 2001). Traits including external fertilization, transparent embryos, rapid developmental rate and a short generation time (3 months) make zebrafish an invaluable research animal in the areas of vertebrate genetics, developmental biology and biotechnology (Kahn 1994, Kimmel 1989, Barinaga 1990).

Gene transfer is possible in fish and has been identified as a potential expression system for pharmaceutical products (Maclean and Penman 1990). If this were to occur, intellectual property could be safeguarded though sterilization of production fish. Transgenic zebrafish have been produced and unwanted transmission of transgenic genomes has been addressed by producing sterile animals though chromosome set manipulation (Marian 1997).

Polyploidy, the possession of more than the normal two sets of chromosomes per cell is a naturally occurring phenomenon in birds, reptiles, amphibians and fish (Tiersch and Watchel 1993). Triploidy, a form of polyploidy where each cell contains thee sets of chromosomes, causes functional sterility and has been induced in a variety of fish including rainbow trout *Oncorhynchus mykiss* (Purdom 1973), grass carp *Ctenopharyngodon idella* (Cassani and Caton 1985), Nile tilapia *Oreochomis niloticus* (Don and Avtalion 1988), and zebrafish *Danio rerio* (Kavumpurath and Pandian 1990). Polyploidy induction involves the application of a shock (pressure, temperature, chemical or electrical) to eggs shortly after fertilization to cause the retention of the second polar body, a set of chromosomes normally lost when meiosis II is

resumed. The application of shocks to induce triploidy is not always 100% effective and can cause detrimental side effects and decreased viability. Another form of polyploidy, tetraploidy, has been used to facilitate the production of triploids.

Through the disruption of processes associated with first cleavage in newly fertilized eggs, it is possible to produce tetraploid fishes with four sets of chromosomes. Tetraploid rainbow trout have been crossed with diploids to produce triploids (Chourrout et al. 1986, Myers and Hershberger 1996). This method, referred to as production of interploid triploids, is reliable and avoids detrimental side effects associated with conventional methods of triploidy induction. A literature search yielded no reports on the induction of tetraploidy in zebrafish. If a population of tetraploid zebrafish were to be established, it could provide a reliable means of producing triploids on a commercial scale.

Many areas of genetics and developmental biology research are facilitated by increased homozygosity or reduction in heterozygosity. Techniques to increase homozygosity include the production of fish containing DNA only from the mother (gynogens) or the father (androgens) (Lutz 2001). Mitotic gynogens and androgens are produced by applying a shock to inhibit the first mitotic division. The timing of application of shocks to produce tetraploids, gynogens and androgens in other species is similar (Hussain et al. 1993, Mair 1988, Chourrout et al. 1984).

Mitotic gynogenetic and androgenetic zebrafish have each been produced. Effective methods for the production of mitotic gynogens include pressure shocks, pressure shocks combined with ether, and heat shocks, of which heat has been reported as the most successful and simplest method (Streisinger et al. 1981). Androgenetic zebrafish have also been produced by application of heat shock (Corely-Smith et al. 1996).

In light of the successful production of gynogenetic and androgenetic zebrafish and the potential applications of tetraploidy in this species, the goal of this chapter was to induce tetraploidy in zebrafish by use of heat shocks. The objectives were to: 1) apply heat shocks ranging in time of application and duration to estimate parameters for the induction of tetraploidy; 2) evaluate the effects of timing of application of heat shocks on tetraploidy induction, survival, and tetraploid yield, and 3) evaluate the effects of heat shock on ploidy and survival to adulthood.

Materials And Methods

Broodstock

Broodstock zebrafish were obtained from a commercial supplier (Scientific Hatcheries, Huntington Beach, California) and housed in a recirculating aquaculture system comprised of ten 80-L aquaria, a 400-L sump, a 5-L upwelling bead filter, an 18-W ultraviolet sterilizer (Emperor Aquatics, Pottstown, Pennsylvania) and a 0.08 kW (1/8 hp) pump (Little Giant Pump Company, Oklahoma City, Oklahoma). A photoperiod of 14 h light :10 h dark was maintained and water temperature was kept at 26 C. Water quality parameters were maintained at 1 ppt salinity, 100 mg/L hardness ($CaCO_3$) and 100 mg/L alkalinity ($NaHCO_3$) (Poleo 2002). Broodstock were fed a variety of commercial diets including TetraFin Goldfish Flakes (Tetra, Blacksburg, Virginia), 42% protein and 8% crude fat, and catfish fry starter (Clover Brand, Farmland Industries, Kansas City, Missouri), 45% protein and 10% crude fat, and live *Artemia* nauplii (at least twice a week) hatched from cysts (INVE, Gruntsville, Utah).

Gamete Collection

In the afternoons on days prior to gamete collection, six 6-L breeding tanks were stocked with three females and two males each (Westerfield 1995). On the mornings of gamete collection,

males and females were separated shortly after the lights came on. Fish were anesthetized in water containing a concentration of 0.17mg/mL of tricaine methanesulfonate (methyl-m-aminobenzoate, MS-222, Argent Laboratories, Inc., Redmond, Washington) and gently blotted dry with paper towels and Kimwipes ® (Kimberly-Clark, Augusta, Georgia). Sperm were collected from males by holding them upside down for observation with a dissecting microscope and applying gentle pressure to the abdomen (Appendix A, SOP-6). Sperm were drawn into a 10μL pipette tip attached to a 10 μL pipette, transferred to a 1.5-mL microcentrifuge tube in an ~3:1 dilution with 300 mOsm/kg Hanks' balanced salt solution (HBSS) (Appendix A, SOP-1), and placed on crushed ice. Eggs were collected by placing females on their sides in petri dishes and gently applying pressure along the sides of the fish, starting at the pectoral fins and working posteriorly (Appendix A, SOP-6). The eggs were covered with HBSS and viewed with a dissecting microscope. Eggs that appeared to be activated (chorion of increased size) or of poor quality (irregular shape or color) were discarded.

Induction of Tetraploidy

After 300 to 600 eggs had been collected (usually from 3 - 4 females), they were mixed by drawing into a plastic eyedropper and were gently expelled into the petri dish 3 to 4 times. The HBSS was poured off and the eggs concentrated on one side of the petri dish. Sperm were distributed over the eggs with a disposable plastic eyedropper. Five mL of 27 C embryo medium buffer (EMB) (Appendix A, SOP-2) (Westerfield 1995) were added to activate eggs and sperm, which were swirled to ensure mixing. One min after fertilization (AF), ~5 more mL of EMB were added. Two min AF, eggs were poured into a 300-mL watch glass held in a 27 C water bath (VWR Scientific model # 1235, Shel Lab, Sheldon Manufacturing Inc., Cornelius, Oregon) and filled to 1.5 cm with EMB. At 8 minutes AF, eggs were drawn into plastic eyedroppers and

immediately placed in egg baskets located in the same watch glass. Baskets were made from 1.5-cm sections of 25-mL plastic cryopreservation goblets (Southland Cryogenics, Carrollton, Texas) with 30-µm nylon mesh melted to one end (Appendix A, SOP-3). Approximately 50 to 100 eggs were placed in each basket. At the time the shock was to be applied, the basket was taken out of the incubation watch glass, blotted on a paper towel and immediately placed in a 300-mL watch glass held in a 41 C water bath (VWR Scientific model # 1141, Shel Lab, Sheldon Manufacturing Inc., Cornelius, Oregon) filled to 1.5 cm with EMB and maintained at 41 C. At the end of the heat shock, baskets were removed from the heat-shock watch glass, blotted on a paper towel and immediately returned to the incubation watch glass (Appendix A, SOP-8). Temperatures in the incubation and heat-shock watch glasses were measured with a thermocouple and data logger (model HH21, Omega Engineering Inc., Stamford, Connecticut). In initial range-finding experiments, shocks were applied to eggs at 13, 15, 16 and 20 min AF for durations of 2 and 5 min. In later experiments, heat shocks were applied at 11, 13, 15, 17 and 20 min AF for a duration of 2 min.

Embryo and Larval Rearing

After the heat shocks, eggs from each basket were taken from the incubation watch glass and divided into two 100-mL watch glasses each containing ~50 mL of 27 C EMB. Watch glasses were transferred to an incubator (VWR Scientific, Cornelius, Oregon) set at 28 C. Twice each day, embryos were examined with a dissecting microscope; dead embryos were removed, and 50% of the EMB was replaced.

Over the course of the study, thee trials were used to evaluate tetraploid viability to further stages. The tetraploidy induction protocols described above were used except eggs were shocked at either 17 or 20 min AF. Embryo rearing protocols were as described above. However, ~96 h

AF, larvae were transferred from the incubator to a 40-L aquarium containing 8 L of "green" water. Before transfer, fish were acclimated for 1 h by the gradual addition of aquarium water to the watch glasses. "Green" water was collected from an outdoor pool. Algae and zooplankton were concentrated by passing 40 L of pool water though 112 and 40-µm screens. Eight liters of the filtered water were poured into the aquarium and the algae and zooplankton remaining on the 40-µm screen were washed into the aquarium with a squirt bottle containing distilled water. Gentle aeration was provided by a small airstone and aquarium air pump. Fish were fed live *Artemia* nauplii and catfish fry starter, from 9 days to 20 days AF and were fed as broodstock thereafter. Approximately 2 L of dechlorinated municipal water increased to ~1 ppt salinity with artificial marine salts (Marinemix, Wiegandt Gmblt, Krefeld, Germany) was added each day starting 7 days after fertilization until the aquarium was full. A standard home aquarium filter with an activated carbon cartridge was added and 30% of the water was replaced twice weekly.

Ploidy Evaluation

Ploidy of larvae was evaluated 170 hours (7 days) AF. Larvae were removed from the watch glasses and placed individually into 1.5-mL microcentrifuge tubes with 150 µl of lysis buffer, 0.1% sodium citrate and 0.1% Triton X-100 (Scintillar®, Mallinckrodt, Paris, Kentucky) in Dulbecco's phosphate-buffered saline (PBS) (0.8% NaCl, 0.02% KCl, 0.144% NaHPO4, 0.024% KH2PO4) (Life Technologies, Rockville, Maryland). Microcentrifuge tubes were placed on crushed ice and transferred to a –15 C freezer for 2 to 3 days. Tubes containing larvae were removed from the freezer, thawed at room temperature for 2 min and placed on crushed ice. A cell suspension was obtained by slowly passing the larvae though a 25-gauge needle fitted to a 1-mL syringe (four times). The cell suspension was filtered though 30-µm nylon mesh (Small Parts, Miami Lakes, Florida) into a 4-mL test tube. Cell nuclei were stained by the addition of

150 µl of lysis-staining buffer which was 0.5 mg/mL solution of propidium iodide (PI) (# P1304, Molecular Probes, Eugene, Oregon) containing 1 µg/mL RNase (Ribonuclease A, Sigma, St. Louis, Missouri), 0.1% sodium citrate, and 1% Triton X –100 (Tiersch and Chandler 1989, Tiersch et al. 1990). After 20 min of incubation at room temperature in the dark, DNA content was analyzed with a FACSCalibur® flow cytometer (Becton Dickinson, San Jose, California) equipped with a 480-nm air-cooled argon laser. The FACSComp® software (Becton Dickinson) was used to calibrate instrument settings. A sample of stained channel catfish blood (2.0 ± 0.01 pg DNA / cell) (Tiersch et al. 1989) was added as an internal reference. Measurements of DNA content of control (no shock) zebrafish larvae were compared to those of larvae that had received a heat shock. If the DNA content was two times that of controls, the fish were considered to be tetraploids.

Ploidy of adults was determined in the same way except fresh blood and sperm were used. Sperm were collected from males as described for artificial fertilization in ~50 µl of HBSS. Blood was collected with a 1-mL syringe fitted with a 27-gauge needle containing 0.1 mL acid-citrate-dextrose (ACD), a chelating anticoagulant. Fish were anesthetized with MS-222 and the needle was inserted in the ventral surface near the caudal fin. Blood was drawn from the caudal vessels ventral to the vertebral column. One to 3 µl of blood were collected and the needle was withdrawn as soon as blood could be seen in the translucent hub. Blood was expelled from the needle into a 1.5-mL microcentrifuge tube containing 20µl of ACD and placed on ice. Sperm and blood samples were added to 4-mL flow cytometry test tubes containing 200 µl of lysis-staining buffer, incubated in the dark for 20 min and analyzed with the flow cytometer. Channel catfish blood was used as an internal reference for zebrafish blood samples. The DNA content was compared to that of known diploid controls. Sperm were evaluated without an internal

reference by comparing the peak channel number of fluorescence intensity to that obtained from blood of known diploids.

Statistical Analysis

Statistical analysis was performed using the JMP IN® Software for Windows version 3 (SAS Institute, Cary, North Carolina). Differences in percent tetraploidy, percent survival, and percent tetraploid yield were evaluated among the treatments by a one-way analysis of variance (ANOVA). The level of significance was set at $P \leq 0.05$.

Results

Over the course of this study, 12 tetraploidy induction trials were conducted. Of these, 2 trials were lost with 100% mortality occurring before hatch. Flow cytometric analyses were inconclusive for two other trials due to initial problems in cell preparation that resulted in insufficient sample sizes. Data were collected and analyzed from two range-finding trials, three replicated experiments and three trials that were conducted to evaluate survival and percent tetraploidy of heat-shocked fish at adulthood.

Estimation of Parameters for the Induction of Tetraploidy

Tetraploidy induction in zebrafish was achieved in this study. Results from the range-finding trials indicated that tetraploidy induction was possible when 41 C heat shocks were applied for 2 min at 15, 16, and 20 min after fertilization AF. Shocks of 5-min duration proved to be ineffective, although survival between the two durations was similar.

Effects of Timing of Application of Heat Shocks

Results from thee replicated experiments showed that tetraploidy could be induced by shocks that were applied over a range of 11 to 20 min AF (Figure 3.1). Shocks applied at 13 min AF yielded 66% tetraploidy in larvae when evaluated 168 h AF. Shocks applied at 20 min AF,

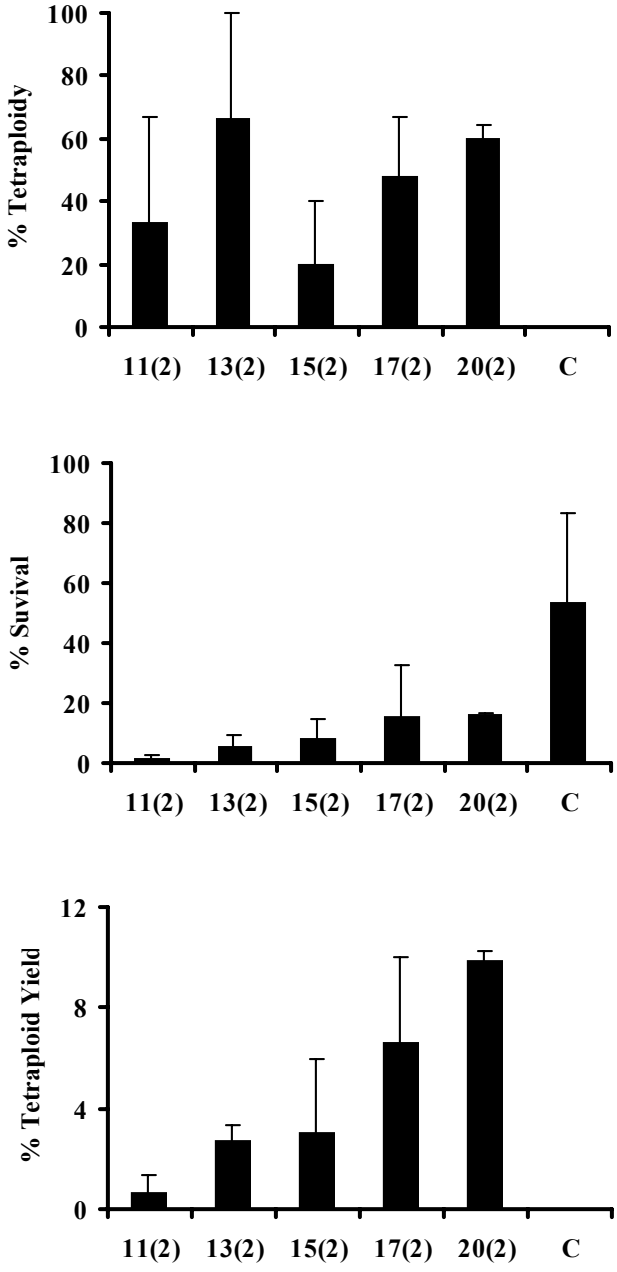

Figure 3.1. Percent tetraploidy, percent survival and percent tetraploid yield (mean ± SD). Data were combined from replicated experiments where 41 C heat shocks were applied to eggs at 11, 13, 15, 17 and 20 min after fertilization for a duration of 2 min. The letter C denotes control treatments where no shocks were applied. Data for percent tetraploidy and percent tetraploidy yield were lost from the third replicate due to technical failure and were not included in the analysis.

yielded 60% tetraploidy. Shocks applied at 15 min AF yielded the least percent tetraploidy (20%). There were no significant differences in percentage of tetraploidy induction among the treatments ($\underline{P} = 0.75$). There was a trend of increasing survival for later shocks. Shocks applied 11 min AF yielded 1% survival while those applied at 20 min AF yielded 16% survival. There were no significant differences in survival among the treatments ($\underline{P} = 0.07$). However, overall survival was significantly lower than that of controls (60%) for shocks applied at 11, 13, and 15 min AF ($\underline{P} = 0.02$). A trend of increasing percent tetraploid yield the later shocks were applied occurred as well. A tetraploid yield of 1% resulted from shocks applied at 11 min AF while a 10% yield resulted from shocks applied at 20 min AF. There were no significant differences among treatments ($\underline{P} = 0.20$).

Although about 10% of the fish that developed from heat-shocked eggs appeared normal, a range of physical abnormalities also occurred (Figure 3.2). Ploidy evaluation ~7 days AF revealed that some abnormal fish were diploids, while some normal-appearing fish were tetraploids.

Effects of Heat Shock on Survival and Ploidy at Adulthood

Forty-five fish, 11 males and 34 females, survived to adulthood from three trials where ~ 400 eggs were subjected to 2 min heat shocks (41 C) applied at 17 and 20 min AF. All sperm evaluated for ploidy were haploid, indicating that the males were all diploid. The collection and evaluation of blood from females showed that all were diploid with DNA content equal to that of controls, (3.15 ± 0.06 pg of DNA / cell). All females (n = 34) recovered after blood collection and no mortalities occurred.

Flow cytometric evaluation of ploidy yielded conclusive results when larvae were evaluated for ploidy at 7 days AF (Figure 3.3). Although, cell collection and preparation techniques needed

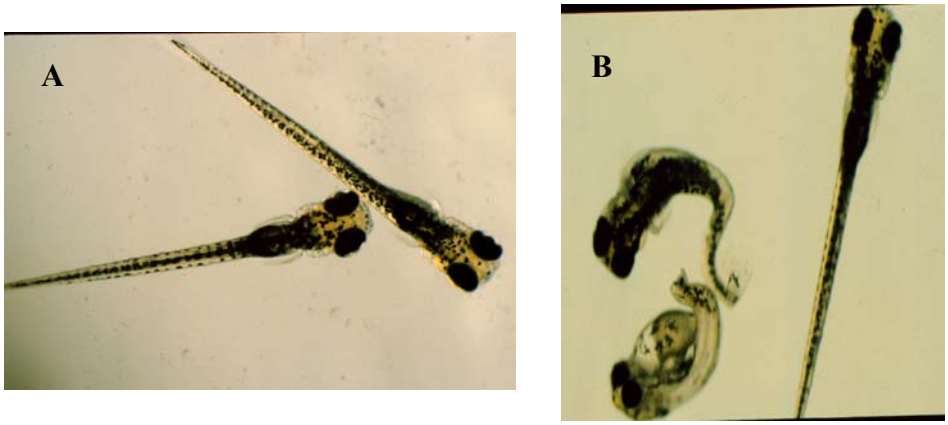

Figure 3.2. Photographs of 7-day-old zebrafish larvae. A) Zebrafish from the normal control group, which received no shock. B) Abnormal and normal appearing zebrafish resulting from eggs that received a 41 C heat shock for 2 min at 17 min AF.

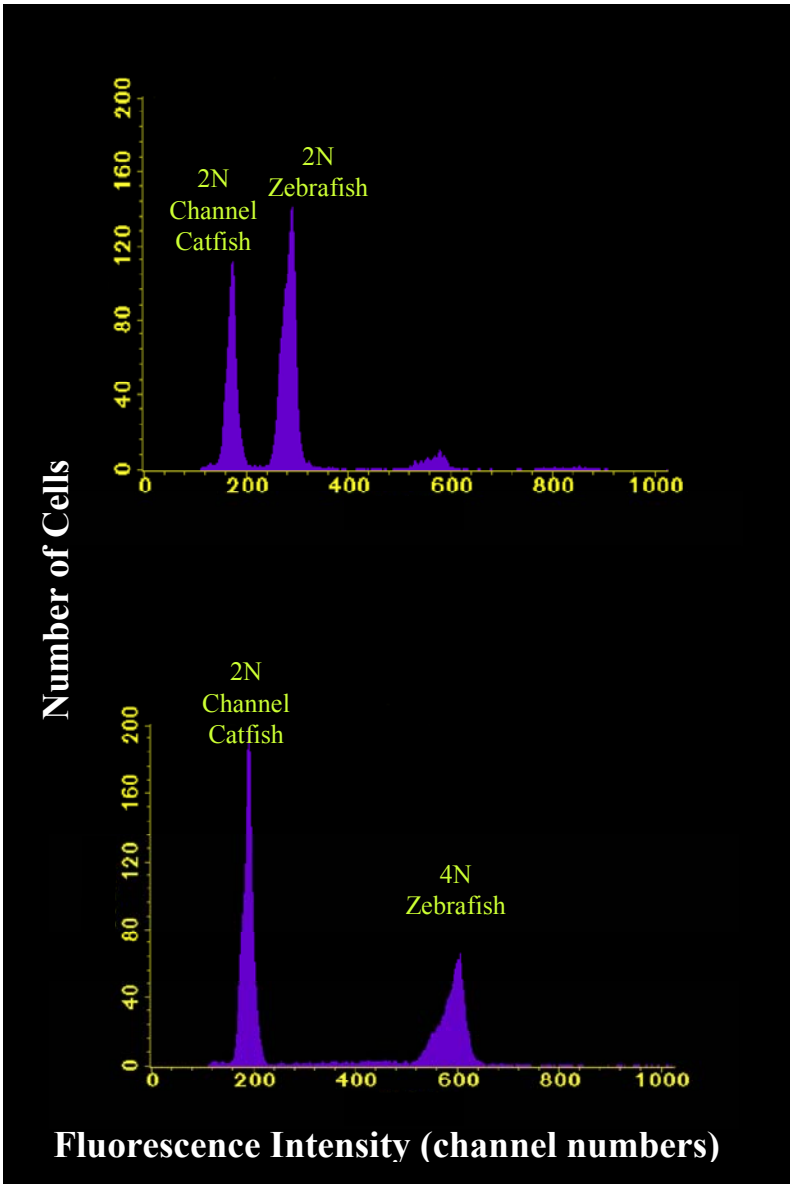

Figure 3.3. Flow cytometric histograms of fluorescence intensity showing diploid (2N) and tetraploid (4N) populations of zebrafish cells obtained from 7-day-old larvae in relation to diploid channel catfish blood cells (internal reference).

refinement in the beginning of the study. Conclusive results from larvae with large amounts of yolk stores, evaluated at 48 h AF, were difficult to obtain.

Discussion

Two min was the effective duration reported for heat shocks applied to inhibit the first mitotic division in the production of androgens and gynogens in zebrafish (Streisinger et al. 1981, Corely-Smith et al. 1996). This study showed that tetraploidy induction is also possible in zebrafish by application of heat shock. Tetraploidy was induced when shocks of 41 C were applied to eggs (incubated at 27 C) from 11 to 20 min AF for a duration of 2 min. Range-finding experiments in this study indicated that heat shocks of 5 min duration did not result in tetraploidy even though survival was comparable to that from shocks of 2 min duration.

The range of times that yielded tetraploidy in this study could be an indication of asynchronous development. In the trials, eggs were pooled from several females. It is possible that although eggs from individual spawns are synchronous (Streisinger et al. 1981), differences among spawns from individuals were the source of variation. Individual zebrafish females have been shown to produce eggs that are susceptible to heat shock for the production of gynogens and androgens at various times after activation (Streisinger et al. 1981, Horstgen-Schwark 1993) indicating that maternal effects play a role in the rate of zygotic development.

The significantly lower survival compared to controls for treatments where shocks were applied at 11 to 15 min AF, and the subsequently lower tetraploid yields may be indicative of heat-sensitive events occurring within that time frame. This is contrary to reports of 13 min AF as the effective timing of application for heat-shock production of androgens and gynogens. These differences in optimal time of application of heat shocks to prevent the first mitotic division may be a result of a lower incubation temperature in this study (27 C) compared to

previous studies (28.5 C). The developmental rates may also have been different between fertilized eggs used in this study and for gynogen production, and eggs that have been X-ray irradiated for production of androgens. However, if development of gynogenetic and androgenetic zygotes is slower than that of diploid controls as was reported for Nile tilapia, (Myers et al. 1995) and this is also true for zygotic development in zebrafish, heat shocks would have to be applied later to produce androgens and gynogens than for tetraploidy induction.

The developmental abnormalities observed in this study appeared to be caused by the heat shocks and not the presence of extra sets of chromosomes in the tetraploids. This assumption is based on the fact that some normal-appearing fish were tetraploid and some abnormal fish were diploid. Studies on chromosome set manipulation in other fishes have found abnormal appearing fish to be diploids (Chourrout 1982) adding support to the hypothesis that abnormalities result from the shocks and not from the extra chromosome sets. Furthermore, diploid androgenetic rainbow trout obtained through shock applications showed inferior survival compared to androgens derived from diploid sperm from tetraploids and were not shocked, indicating that the shocks are a source of detrimental effects (Thorgaard et al. 1990). Increased homozygosity of deleterious alleles in androgens and gynogens may also contribute to abnormalities.

Although a tetraploid yield of 10 percent was observed in fish evaluated at 7 days AF, no tetraploids were found among the 45 adults that survived from eggs that had been shocked at 17 and 20 min AF. This is not surprising considering that only 400 eggs were shocked and only 10% of these survived to adulthood. Further research with larger sample sizes is required to adequately assess the viability of tetraploid zebrafish to adulthood. Other types of shock should also be evaluated. Pressure shocks were found to be more effective than heat shocks for production of viable rainbow tetraploid trout (Chourrout 1984) and cold shocks (11 C) were the

only type of shock to produce tetraploid tilapia that survived to adulthood (Don and Avtalion 1988).

Although no tetraploid adults were produced, this is the first report of tetraploidy induction in zebrafish. Given that zebrafish are such a useful model for other areas of research, perhaps further studies into the induction of tetraploidy in this species will lead to a better understanding of polyploidy induction and the establishment of tetraploid lines of zebrafish and other species as well.

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CHAPTER 4
SINGLE-FEMALE SPAWNING OF NILE TILAPIA FOR EGG COLLECTION

Natural spawning of Nile tilapia *Oreochromis niloticus* occurs when a ripe female is attracted by a displaying male who has built a nest (an oval or circular clearing in the substrate). When the female is ready, she passes over the nest and releases eggs in small increments over the course of 45 min to 1 h. After the male fertilizes the eggs, the female collects them in her mouth and leaves the spawning area to incubate the eggs and fry in her buccal cavity for ~15 days. Hatching generally occurs within 4 to 5 days and sacfry are incubated until their yolk sac is absorbed, about 11 days after spawning. A 5-day nursing period may ensue during which the female continues to provide refuge for free-feeding fry. Because the female does not eat during the incubation period, as long as 4 weeks may be required before she can return to spawning condition (MacIntosh and Little 1995).

Nile tilapia can spawn repeatedly throughout the year in tropical regions. In areas where seasonal differences are more pronounced, spawning tends to occur during times of higher temperatures and longer photoperiods (Jalabert and Zohar 1982). The average reproductive cycle has been reported to be approximately 30 days (MacIntosh and Little 1995). The number of eggs produced from each spawn is variable and can be affected by the age and size of the female, diet, and environmental factors (Little and Hulata 2000).

For commercial production of tilapia seed, broodfish have been stocked at various densities and ratios of females to males in ponds, hapas or tanks. Fry are seined or netted, or females are captured and the fertilized eggs removed from their mouths for artificial incubation. Removal of eggs from mouth-brooding tilapia has been shown to decrease the spawning interval and increase fecundity (Lee 1979). These methods are effective for seed production and collection, but are inadequate for various types of genetic research such as induction of polyploidy, hybridization,

gynogenesis and implementation of complex mating designs that require precise timing of fertilization and specific matings of broodstock (Lutz 2001). Artificial spawning techniques are necessary for such genetic manipulations.

Collection of ripe gametes and correct timing are essential for artificial spawning and are especially important when collecting eggs. This has led to the stocking of broodfish in tanks or aquaria to facilitate observation and capture. One of the most common strategies for egg collection in controlled environments is the stocking of one or two males with three to eight females in aquaria. However, aggressive behavior of tilapia can lead to fighting, injury and death when stocked in confined environments (Guerrero 1975, Lee 1979, El Gamal et al.1999). This problem has been addressed by removing the premaxillae from males and dominant females (Lee 1979, Myers and Hershberger 1991) and the creation of broodfish "families" where fish are raised together in an aquarium from a small size (Rothbard and Pruginin 1975). Other methods include the separation of a spawning pair with a screen or plexiglass (Hussain et al. 1991, Mires 1982).

Initially it was thought that collection of ripe eggs from tilapia could only be achieved after the observation of courtship behavior, such as coloration changes and passage of the female over the nest of a displaying male (Valenti 1975, Chourrout and Itskovich 1983, Varadaraj 1990). Readiness of female tilapia to spawn has also been judged by combination of courtship behavior and the degree of extension of the urogenital papilla (Mires 1982, Rothbard and Pruginin 1975, Myers 1985, Myers and Hershberger 1991). Most notably, Nile tilapia females, stocked singly in aquaria, have been determined to be ready to spawn by degree of extension of the urogenital papilla alone and have been artificially spawned (El Gamal et al. 1999). This phenomenon is supported by observations of single-female Nile tilapia (Mires 1982) and *Oreochromis*

mossambicus (Silverman 1978a, 1978b) releasing eggs in aquaria, without interaction of males. Use of the extension of the urogenital papilla as an indicator of spawning readiness has also enabled the collection of ripe eggs from isolated blue tilapia *Oreochromis aureus* for use in experiments (Frimpong 2000). Nile tilapia have been observed to spawn naturally in tanks at 1400 to 1800 h, 8 to 10 h after the lights come on (Myers and Hershberger 1991).

Artificial spawning of eggs produced by females stocked singly in aquaria led to the evaluation of this method of egg collection for the research on tetraploidy induction in Nile tilapia for this thesis. The goal of this chapter was to characterize a Nile tilapia spawning system containing ten 80-L aquaria, each stocked with a single female. Objectives were to: 1) tabulate the number of spawns and spawning interval for each tank; 2) tabulate the overall number of spawns and spawning interval for the system and estimate the total number of eggs produced; 3) compare the number of eggs collected from strip-spawning of females to the number of eggs collected from the mouths of females after they had spawned in tanks, and 4) determine if tank position had an effect on the number of spawns occurring in each tank.

Materials and Methods

Broodstock and System Description

Female Nile tilapia *Oreochromis niloticus* were obtained from Til-Tech Aquafarm in Robert, Louisiana in October of 2000 and used in these investigations. These fish were from a line designated as the Swansea strain, which originated from Lake Manzala, Egypt. This strain was first established at the Institute of Aquaculture, University of Stirling, Scotland and subsequently obtained by researchers at the University of Wales, Swansea, UK in 1981 and 1982. Til-Tech Aquafarm in Robert, Louisiana, has obtained Swansea strain Nile tilapia from the University of Wales several times each year since 1997. Aquaria were stocked with single-females in a

recirculating system comprised of ten 80-L aquaria, a 200-L sump, an upwelling bead filter, a 25-watt ultraviolet sterilizer and a 1/4 hp pump (Figure 4.1).

Figure 4.1. Ten 80-L tanks each stocked with a single Nile tilapia female.

Ten females were also stocked as a group in an 800-L circular tank plumbed into a recirculating system adjacent to the spawning system, to be used as replacements if fish in the spawning system died. Water quality was monitored weekly, and salinity was maintained at ~1 ppt, alkalinity and hardness at ~200 mg/L as $CaCO_3$, pH at between 7 and 8.5, and unionized ammonia at < 0.05 mg/L. Fish were maintained on a natural photoperiod and were fed a commercial diet (28% crude protein and 4% crude fat, Cargill, Nutrena Feed Division, Minneapolis, Minnesota) twice daily, *ad libitum* (as much as they could consume in 10 min). Temperature ranged from 29 C in the summer to 26 C in the winter. Fish were weighed at the end of the study and weights ranged from 212 g to 530 g with a mean (± SD) of 399 ± 100 g. Although some fish started spawning within one month of stocking, the data presented in this study represent one calendar year (January 1, 2001 through December 31, 2001).

Egg Collection and Artificial Spawning

Spawning readiness was estimated by gauging the degree of extension of the urogenital papilla. Fish were checked daily every hour from 1300 to 1700 h. If the extension of a papilla

was ≥ 0.5cm, the fish was chosen for spawning and was anesthetized in ~150 mg/L tricaine methanesulfonate (methyl-m-aminobenzoate, MS-222, Argent Laboratories, Inc., Redmond, Washington). Upon the loss of equilibrium, the fish was blotted dry with paper towels and the eggs were stripped into a 300-mL watch glass containing ~ 75 mL of Hanks' balanced salt solution (HBSS) (Appendix A, SOP-1) by gentle squeezing of the abdomen between the thumb and forefinger and stroking in a head-to-tail direction (Appendix A, SOP-6).

Enumeration and Tabulation of Eggs and Spawning Interval

All spawning dates were recorded and the number of eggs collected from each spawn was estimated volumetrically. A total volume was obtained by pouring the eggs into a 15-mL graduated centrifuge tube. One mL of eggs was collected in a 1.5-mL microcentrifuge tube. The eggs were poured into a 100-mL watch glass and counted (repeated 3 times and averaged). Finally, the average number of eggs per mL was multiplied by the total volume of eggs to estimate the total number of eggs for that spawn. The total number of eggs produced by the system was estimated by taking the average number of eggs collected per strip spawn (the manual expression of eggs from a female) and multiplying it by the total number of spawns, including tank spawns. To compare the number of eggs obtained from strip spawns to the number of eggs collected from tank spawns (females that had spawned in tanks and collected the eggs in their mouths), the average number of eggs collected from ten strip spawns was compared to the average number of eggs collected from five tank spawns. The eggs from tank spawns were collected on the day following spawning by removing the fish from the tank, and flushing the eggs out of the mouth in water in a 20-L bucket. Female weight was measured to the nearest gram.

Effects of Tank Position on Number of Spawns

To determine the effect of tank position on the number of spawns, vertical positioning was evaluated by taking the total number of spawns produced from the top row of tanks and comparing it to the total of the bottom row. The number of spawns produced by each of the five two-tank columns were also compared to evaluate the effects of lateral positioning.

Statistical Analysis

Statistical analysis was performed using the JMP IN® Software for Windows version 3 (SAS Institute, Cary, North Carolina). A one-way analysis of variance with a significance level of $P \leq 0.05$ was used to detect differences between the number of eggs collected from strip spawning to the number of eggs collected from tank spawns. A Chi-Square test was performed to detect differences in the number of spawns produced by each row or column of tanks.

Results

Over the course of the study three females were replaced, two because of accidental deaths (one fish knocked over a standpipe, and another jumped from its tank. The third fish was replaced because flow cytometric evaluation indicated that it might have been a triploid. The number of spawns produced by each tank was highly variable. The most productive tank produced 12 spawns, and the least productive only 2 (Table 4.1). Spawning intervals were also highly variable. The longest average spawning interval for the year was 182 days and the shortest 30 days. The average spawning interval for all 10 tanks for the year was 73 days. However, individual fish only tended to spawn for a period of 5 to 7 months and the fewest number of days between spawns was 12. If spawning interval for individual fish was calculated for the time frame between the first and last spawn instead of the entire year, the average spawning interval was ~35 days. The spawning interval for the system as a single unit was 5

Table 4.1. Number of spawns per tank (X indicates a single spawn), overall number of spawns per month, total spawns for entire system and spawning intervals (average number of days between spawns) for each tank and the for the entire system of Nile tilapia females stocked singly in ten 80-L aquaria. Data were collected from January 01, 2001 through December 31, 2001.

Tank	Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.	Total (Year)	Spawning Interval
1	X	X			X	X	X	X	X				7	52
2	X					X	X	X	XX		X		7	52
3			X	X	X	X	XX	X	XX				9	41
4		X			XX	X		X					5	73
5			X	X	X	X	XX	XX	XXX	X			12	30
6			X	XXX	X		X	XX	X	X	X		11	33
7										XX			2	183
8							X	X					2	183
9			X		X		XX	X	XX			X	8	46
10		X			XX	X	X	X	XX	X			9	41
Total	2	3	4	5	9	6	11	11	13	5	2	1	72	5

days. A total of 72 spawns were recorded for the year producing ~90,000 eggs, an average of 9,000 eggs per tank. The average number of eggs per spawn used in the calculation was the average collected from strip spawns (1,276 ± 309).

More spawns occurred in the summer than in the winter. A gradual increase in spawning frequency (spawns per month) occurred from January to September, which had the highest spawning frequency (13 spawns). This was followed by a steady decline with December producing only one spawn (Figure 4.2).

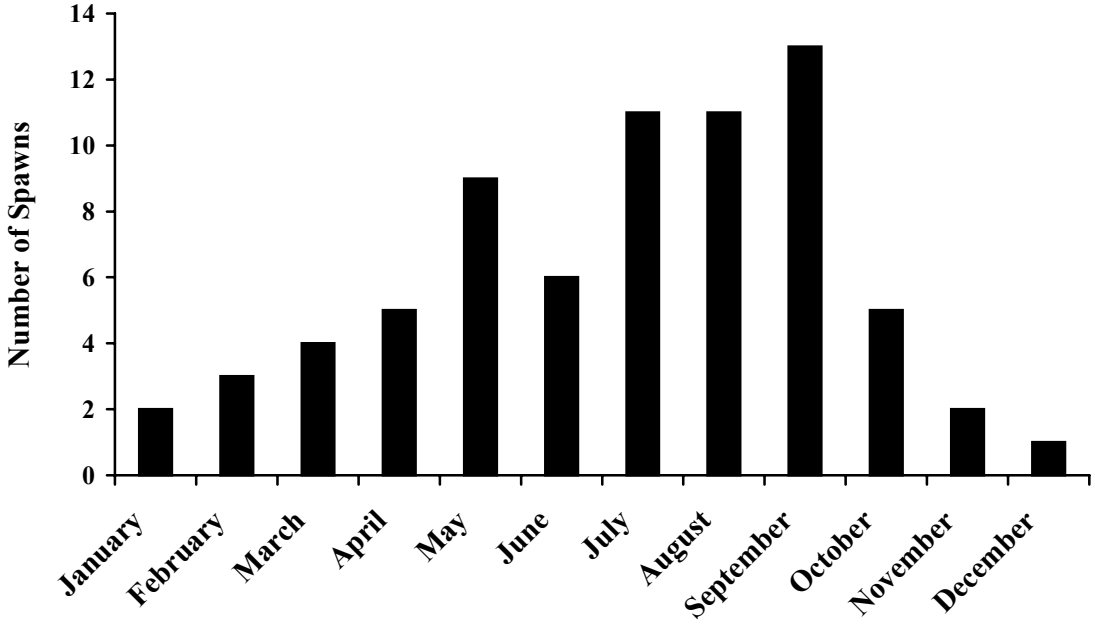

Figure 4.2. Histogram of the number spawns produced per month from January 01, 2001 through December 31, 2001, from ten 80-L tanks stocked with single Nile tilapia females. The total number of spawns was 72.

The number of eggs collected from tank spawns was significantly higher than the number collected from strip spawns ($\underline{P} < 0.05$) (Table 4.2).

Table 4.2. Comparison of several egg parameters (mean ± SD) between strip spawns (n = 10) and tank spawns (n = 5). Females were weighed at the end of the study for the strip spawns and at the time of egg collection for the tanks spawns. Eggs from strip spawns were placed in an extender solution and parameters were measured before fertilization.

Type of Spawn	Female Weight (g)	Volume of Eggs (mL)	Number of Eggs per mL	Number of Eggs per Spawn
Strip	399 ± 100	7.8 ± 1.6	166 ± 43	1,276 ± 309
Tank	405 ± 40	14.7 ± 0.5	119 ± 7.1	1,752 ± 71.8

Effects of Tank Position on Number of Spawns

The top row produced 40 spawns and the bottom row 32 (Table 4.3). There was no significant difference between the number of spawns due to vertical positioning ($\underline{P} = 0.35$). Columns 1 and 5, on either end of the spawning system, on average produced more spawns than did columns 2, 3 and 4. However, there were no significant differences among the number of spawns due to horizontal positioning ($\underline{P} = 0.14$).

Table 4.3. Number of spawns per tank in a 2 x 5 array for the single-female spawning system.

Row	Column 1	Column 2	Column 3	Column 4	Column 5	Total
Top	7	7	9	5	12	40
Bottom	11	2	2	8	9	32
Total	18	9	11	13	21	72

Discussion

The stocking of aquaria with single Nile tilapia females was an effective method for the collection of eggs for polyploidy experiments. The degree of extension of the urogenital papilla was an effective indicator of egg ripeness with less than 10% of all strip spawns being discarded

because of poor egg quality. Although the exact time of tank spawns was not recorded, all observed tank spawns occurred in the afternoon, which has been reported in Nile tilapia in other studies (Gautier et al. 2000).

A definite peak in spawning and shorter spawning intervals occurred during the summer in this study. This is consistent with results from other experiments that were conducted at latitudes outside the natural range for Nile tilapia (Lee 1979, Myers 1985). Although the production of 72 spawns from the 10 tanks stocked with single-females was adequate for this study, the number of spawns produced by this system could be increased and the spawning interval decreased in future studies.

Nile tilapia are known to spawn throughout the year in tropical areas and exhibit more seasonal spawning during months of higher temperatures and increased photoperiod in subtropical areas, a trend supported by this study. Seed production has been increased by increasing photoperiod and notable drops in seed production have been recorded when photoperiod was below 12 hr of light (Baroiller et al. 1997). Light intensity was also found to affect seed production with a photoperiod of 18 hr light and light intensity of 2500 lux producing the most seed (Ridah 2000). Higher temperatures in the present system during the summer (29 C) compared to the winter (26 C) may have been a factor responsible for more spawns occurring in the summer.

Diet is also reported to play a role in the spawning interval and number of spawns produced in tilapia. A diet with 20% protein has been found to be adequate for tilapia broodfish in green-water systems where additional nutrition is obtainable from the environment, while diets of 25 to 30% protein (as used in this study) were found to be optimal in clear water systems (cited by

Little and Hulatta 2000). However, other studies have shown improved spawning in fish fed diets of 40% protein compared to fish fed diets with 30% protein (El Naggar et al. 2000).

In this study aquaria were positioned side by side, allowing fish to have visual contact with at least one other female. Increased ovulation in tilapia has been reported when females in isolated aquaria were allowed visual contact with other tilapia regardless of sex (Silverman 1978a and 1978b).

When spawning families (1 to 2 males stocked with 3 to 8 females) are used for egg production, the majority of eggs produced can come from only a minority of the females. This has been attributed to hierarchies and the dominance of a few females over the others (Myers 1985, Baroiller et al.1997). In this study where the females were isolated (except for visual contact), three of the ten females had to be replaced, leaving only seven females that provided spawning data throughout the 1-year study. Of these, the number of spawns per fish ranged from 12 to 2. This could be indicative of genetic factors influencing individual spawning characteristics (Myers and Hershberger 1991, Lutz 2001) as much as social interaction.

A recent study found the interval of extension of the urogenital papilla to be several days less than spawning interval in Nile tilapia, indicating that monitoring spawns alone may underestimate the number of cycles in a given female. When males were added to aquaria with females possessing extended papillas, it led to an increase in the number of spawns following papillary distension (Gautier 2000). This seems to indicate that solitary females may not always deposit eggs and instead reabsorb them in preparation for the next spawning cycle. Perhaps more spawns could have been recorded in this study had all females been stripped of eggs following papillary distension.

Significantly fewer numbers of eggs were collected from strip spawns compared to the number of eggs collected from tank spawns, indicating that not all eggs are stripped from the ovaries. Naturally, one explanation would be that tilapia are more efficient at laying their eggs than researchers are at stripping eggs. Statistical analysis of data indicates that neither vertical positioning nor horizontal positioning of tanks had a significant effect on the number of spawns produced.

The production of ~9,000 eggs per tank per year in the single-female system compares favorably with intensive Nile tilapia seed production in net enclosures in ponds where individual females produced 20 seed per day or 7,300 seed per year (Wantanabe et al. 1997). However, further work needs to be done regarding the viability of this method to produce seed on a commercial scale. Benefits of the single-female spawning of Nile tilapia include prevention of losses of broodfish to fighting and the facilitation of genetic manipulations. Though this system may be more intensive than others, increased value from the production of hybrids, polyploids, monosex populations, and selectively bred fish may make it worthwhile.

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CHAPTER 5
A HATCHING SYSTEM FOR TILAPIA EGGS

Nile tilapia *Oreochromis niloticus* is a commercially important fish grown throughout the world and is one the most researched warmwater fish on the planet (Pullin et al. 1994). Genetics research on the induction of polyploidy, gynogenesis, androgenesis, sex reversal, hybridization, gene transfer, and selective breeding in this species are all facilitated by artificial spawning. One consideration for artificially spawning Nile tilapia is that they are mouth brooders and embryos and sacfry are incubated inside the mother's mouth (Trewavas 1982) (Figure 5.1). Constant churning within the buccal cavity cleans and aerates the developing eggs.

Figure 5.1. A female Nile tilapia incubating eggs in her mouth. Note the eggs on the bottom of the tank that have not yet been picked up.

Simulation of the gentle rolling that eggs experience during mouth brooding is essential for artificial incubation of Nile tilapia eggs (MacIntosh and Little 1995). Methods used to keep eggs in constant motion have included placement of stand-alone hatching jars on a constantly moving base (Rothbard and Pruginin 1975, Hulata et al. 1988, Varadaraj and Pandian 1990), aeration

(Nussbaum and Chervinsky 1968, Valenti 1975), and water flow (Woynarovich 1962, Rothbard and Hulata 1980, Myers and Hershberger 1991). Commercial egg hatching vessels can be expensive, often prompting researchers to make their own. Non-commercial egg-hatching vessels constructed for hatching of tilapia and other fish eggs have included inverted soda bottles (Bates and Tiersch 1995, MacIntosh and Little 1995, Myers 1985), upright soda bottles (Glenn and Tiersch 1997), inverted carboys (MacIntosh and Little 1995), Zuger bottles and jars (Mires 1974, Rothbard and Hulata 1980, Don and Avtalion 1986), Ehrlenmeyer flasks (Nussbaum and Chervinsky 1968), plastic funnels (Valenti 1975), polyvinyl chloride tubing (Dewey and Wagner 1993) and plastic centrifuge tubes (Frimpong 2000). However, some of these vessels may not be suitable for small aliquots of eggs and many do not have rounded bottoms, a characteristic of the most effective vessels for hatching of tilapia eggs (MacIntosh and Little 1995).

Bacterial and fungal infections can be a major cause of mortality in tilapia eggs (Subasinghe and Sommerville 1985) and preventative measures should be taken. In flow-through systems and recirculating systems that have biofiltration, eggs must removed from the system and disinfected with chemicals (El Gamal et al. 1999, Myers 1985, Myers and Hershberger 1991). Antibiotics have also been added to hatching systems to fight bacteria and fungi (Peruzzi et al. 1993, Valenti 1974). An effective method to combat bacteria and fungi that avoids chemical treatment is incorporation of an ultraviolet sterilizer in the system (Hussain et al. 1991, Peruzzi et al. 1993).

The most important characteristics of a Nile tilapia hatching system are its ability to maintain steady water flow through the incubator and good water quality (MacIntosh and Little 1995). A means to minimize egg loss to bacteria and fungi must also be incorporated. In addition to these design and operational requirements, further constraints existed on the hatching system design

for the research on the induction of polyploidy in Nile tilapia in this thesis. These included limited space and the need for the system to have a small footprint, the need to incorporate multiple hatching vessels to keep track of five or more treatment and control groups (~ 2 mL aliquots of eggs each) from a single spawn, and the need for easy access and viewing of developing embryos.

Two similar systems were designed and built to meet these constraints and incorporated many of the features of the hatching systems mentioned above. The first system, System 1, cost approximately US $125 and contained fifteen 75-mL hatching tubes. Methylene blue was used to combat bacteria and fungus (Blacklidge and Bidwell 1993). The system occupied 0.36 m^2 of space and was built in one day. The second system, System 2, cost approximately US $550 (excluding the sump), contained thirty 75-mL hatching tubes, and used biological, mechanical, and chemical filtration and UV sterilization to maintain water quality. This system occupied 0.90 m^2 and was also built in 1 day.

Materials and Methods

Construction of System 1

Frame

Two base supports (51-cm long 2 x 4s (lumber with a height of 5.1 cm and a width 10.1 cm)) were positioned on either side of a 40-L aquarium with the 10.1 cm side facing down (Figure 5.2). Uprights (a 69-cm 2 x 4 (left side) and an 81-cm 2 x 4 (right side)) were centered and screwed into each of the base supports, with the 5.1 cm side facing out and the 10.1 cm side facing the aquarium, using 7-cm sheetrock screws. Two cross members made of 59.7 cm 2 x 4s were attached to the posts with 7-cm sheetrock screws. The bottom cross member was

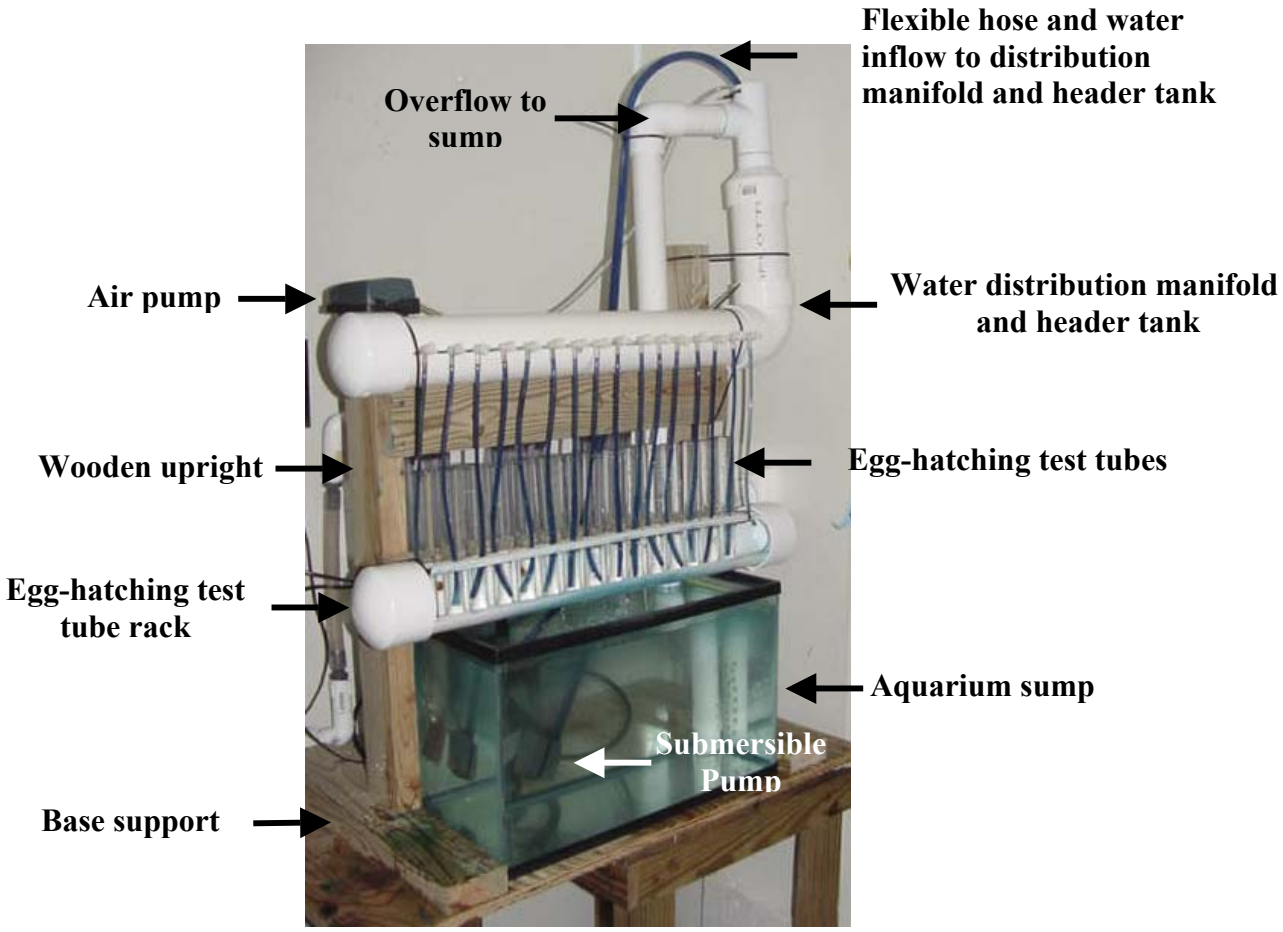

Figure 5.2. Components of System 1 containing fifteen 75-mL egg-hatching tubes. The system straddles a 40-L glass aquarium which contains a submersible pump.

positioned 1 cm above the height of the aquarium. The upper cross member was positioned with the lowest edge ~ 50 cm up the posts (measuring from the top of the base supports).

Egg-Hatching Tube Rack

A 20.3 cm section of 7.62 cm schedule 40 polyvinyl chloride (PVC) pipe was cut. A straight line was drawn from one end to the other. Fifteen 2.54-cm holes (spaced 3.81 cm apart on center) were made along the line starting 8.9 cm from the left side with a 2.54-cm hole saw. The holes were widened with a rattail file to accommodate the test tubes. Four more lines were

drawn and two sections were cut out with a jigsaw. End caps (7.62 cm) were fixed to both ends with PVC cement (Figure 5.3). The hatching tube rack was fastened to the middle of the bottom cross member of the frame with 6 stainless-steel screws. Four 0.87 cm holes were drilled along the bottom center to allow for drainage into the aquarium sump. A 75-mL test tube (25 mm x 200 mm borosilicate (Corning, Acton, Massachusetts)) obtainable at science supply stores for ~$0.95, was placed in each of the 2.54 cm holes (Figure 5.4).

Water Distribution Manifold and Header Tank

Fifteen 0.87-cm holes (spaced 3.81 apart cm on center) were drilled in a 70-cm section of 7.62-cm schedule 40 PVC pipe in a straight line starting 8.9 cm from the left side. The holes were threaded with a 0.32-cm x 10.63 threads / cm (1/8" x 27 threads / inch) national pipe thread (NPT) tap. A 7.62-cm end cap was fixed on the left side and a 7.62-cm elbow on the right side with PVC cement (making sure the open end of the elbow was facing straight up when the drilled holes were facing out). This piece was set on top of the top cross member of the frame and held in place with plastic cable ties (making sure the 0.87-cm holes were facing out). A 20.3-cm section of 7.62-cm PVC pipe was cut and fit into the open end of the elbow. A 7.62 cm to 3.81 cm reducer was glued on the other end of the 20.3-cm section. A 7.62-cm section of 3.81-cm PVC pipe was cut to allow for the addition of a 3.81-cm tee on top of the reducer. A 20-cm section of 3.81-cm PVC pipe was fit to the tee parallel to the ground and over the aquarium. A 3.81-cm elbow was attached facing directly down and a 96.5-cm section of 3.81-cm PVC pipe fit to the elbow. Twenty 0.87-cm holes were drilled in the bottom 25 cm of this section. Teflon tape was wrapped around the threaded ends of the plastic valves. The valves were screwed into the tapped holes with the open end of the valve facing downward (Figure 5.5).

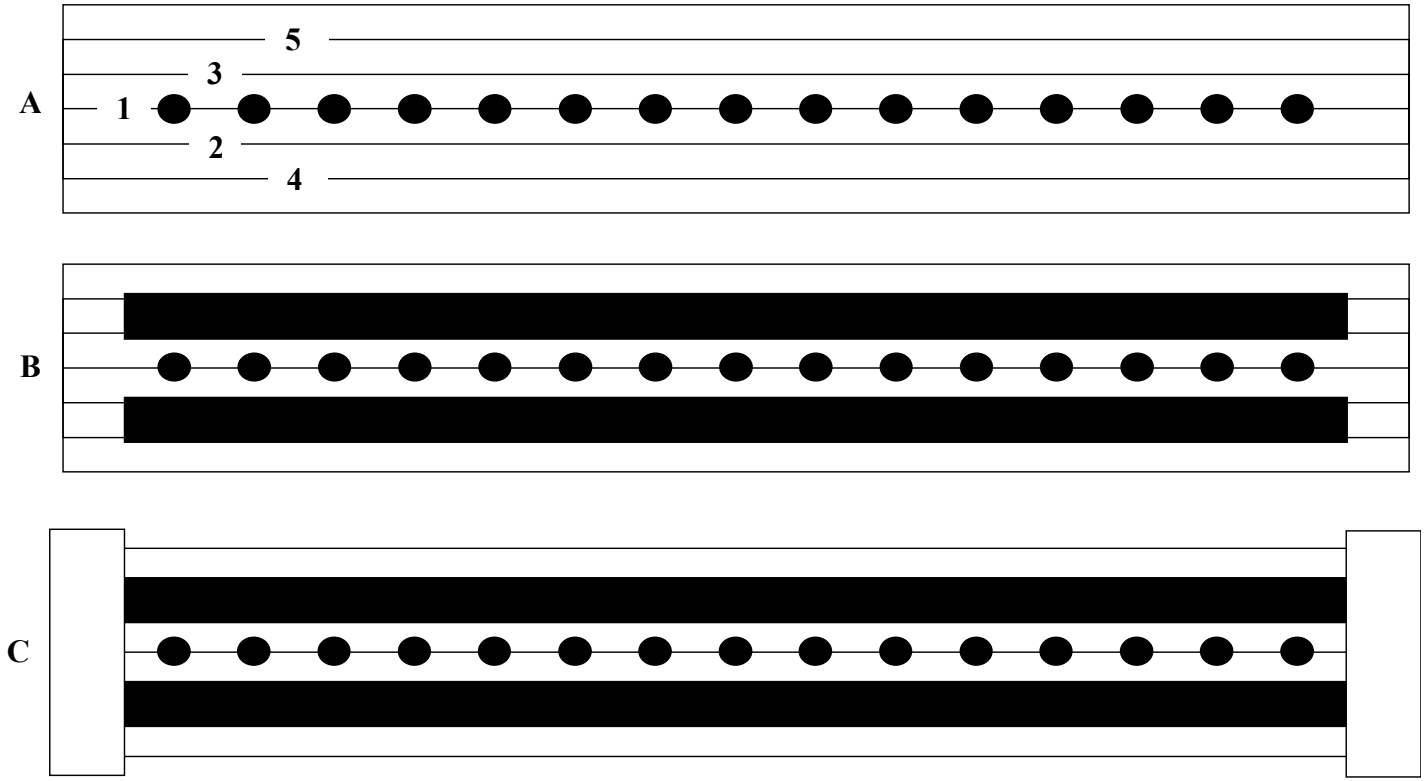

Figure 5.3. Construction and assembly of the hatching tube rack. A) Fifteen 2.54-cm holes (spaced 3.81 apart cm on center) were made along line 1. Lines 2 to 5 were drawn with: 3.81 cm between lines 1and 2 and 1and 3; 6.98 cm between lines 2 and 4, and 5.72 cm between lines 3 and 5. B) Sections between lines 2 and 4 and 3 and 5 were cut out leaving 5.08 cm on either end. C) End caps were fixed to the ends with PVC cement.

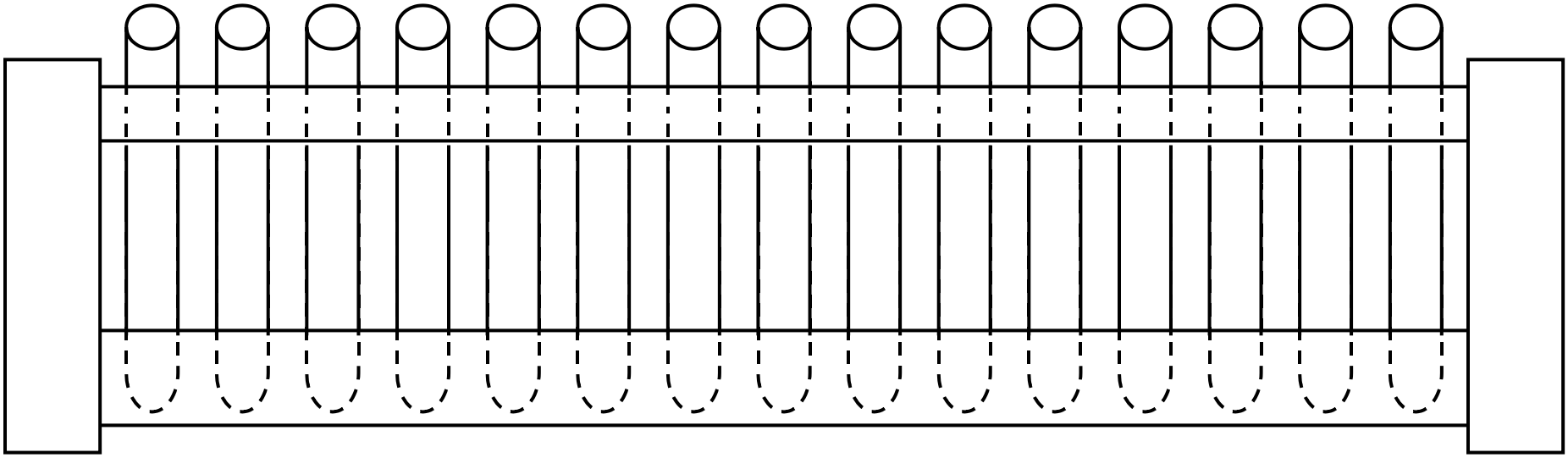

Figure 5.4. Front view of egg-hatching tube rack with fifteen 75-mL test tubes.

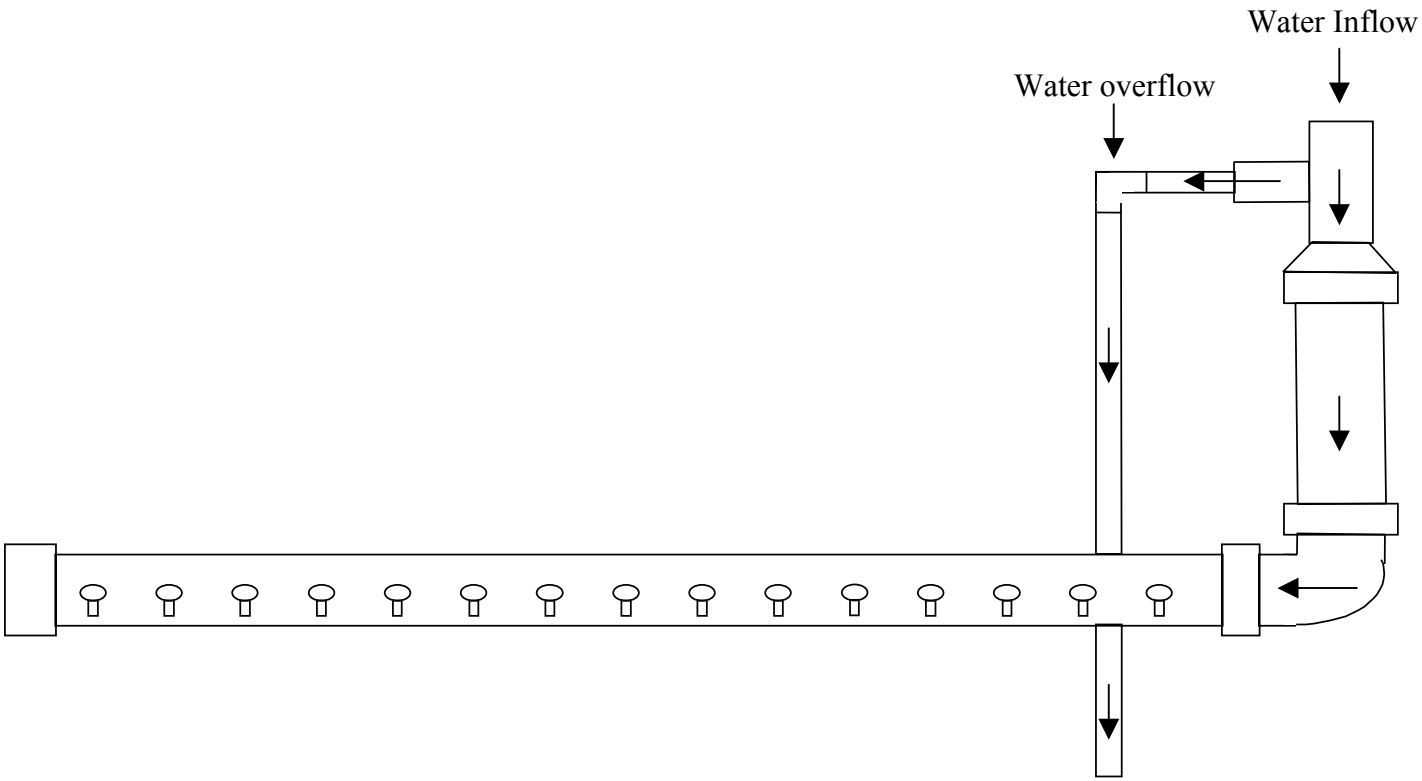

Figure 5.5. Water distribution manifold and head tank with 15 valves. Water overflow back to sump provided constant head pressure.

Fifteen 30.5-cm sections of 0.396-cm inside diameter vinyl tubing were cut and attached to each valve. The free ends were placed inside the egg-hatching test tubes below.

Water Pump and Aerator

A 122-cm section of 1.27-cm inside diameter vinyl tubing was attached to the aquarium pump with a cable tie. The pump was set in the aquarium and the tubing run up to the open end of the 3.81-cm tee on top of the water distribution manifold. Two holes were drilled in the tee near the open end and a cable tie was used to secure the vinyl tubing. An airstone connected to the air pump was placed inside the distribution manifold.

Operation of System 1

Water was pumped from the aquarium sump to the water distribution manifold and head tank. The inflow of water to the water distribution manifold tank was greater than the water exiting the valves to the hatching tubes. Thus, water was always overflowing from the water distribution manifold tank back to the aquarium and the height of the water remained constant. This resulted in constant head pressure and allowed for continual and consistent water flow through the manifold valves into the egg-hatching test tubes. Once the desired flow was achieved by adjusting the valve, further adjustments were not necessary. Overflowing water from the egg hatching tubes was collected in the gutter of the tube rack and went back into the sump.

Methylene blue was added to the system daily to reduce losses of eggs to bacteria and fungus. When the system was heavily loaded with eggs, and high mortalities from polyploid experiments were expected, 50% to 100% of the water was exchanged daily to maintain water quality.

The airstone in the water distribution manifold maximized the oxygen available to the eggs. Dead eggs were removed from the tubes with a siphon, or the tubes were removed, the eggs poured into a 300-mL watch glass and the dead eggs removed with a pipette.

Construction of System 2

This system was larger and held thirty 75-mL hatching test tubes, but construction was similar to that of system 1. The main differences were: 1) a larger, external pump (Little Giant Pump Company, Oklahoma City, Oklahoma) was used; 2) a Living Stream® (Frigid Units Inc., Toledo, Ohio) with biofiltration and a larger capacity (445-L) was used for a sump instead of the 40-L aquarium; 3) two cartridge filters (5-µm and 2-µm with activated carbon) and an 8-watt ultraviolet sterilizer (Aqua, Temecula, California) were plumbed in line from the pump to the water distribution manifold (Figures 5.6 and 5.7); 4) a shunt (equipped with valves) back to the sump was plumbed in between the pump and the water filters and UV sterilizer to allow for controlled flow rates through the UV sterilizer, and 5) the upright 7.62-cm section on the water distribution manifold tank was cut to 38 cm instead of 20.3 cm to allow for greater head pressure. A centrifugal air blower that supplied air for the entire laboratory also provided aeration to this system. Eggs were disinfected with methylene blue prior to their addition to the system, but no methylene blue was added to the system. Little water exchange was necessary because 2 baffles filled with clamshells provided biofiltration in the sump.

Results

A flow rate of 0.17 L per min per test tube was sufficient for 2 to 3 mL of eggs while 4 mL of eggs required 0.18 L per min. Approximately 75% of fertilized eggs from the control groups of the polyploidy induction experiments survived to hatch. Eggs from

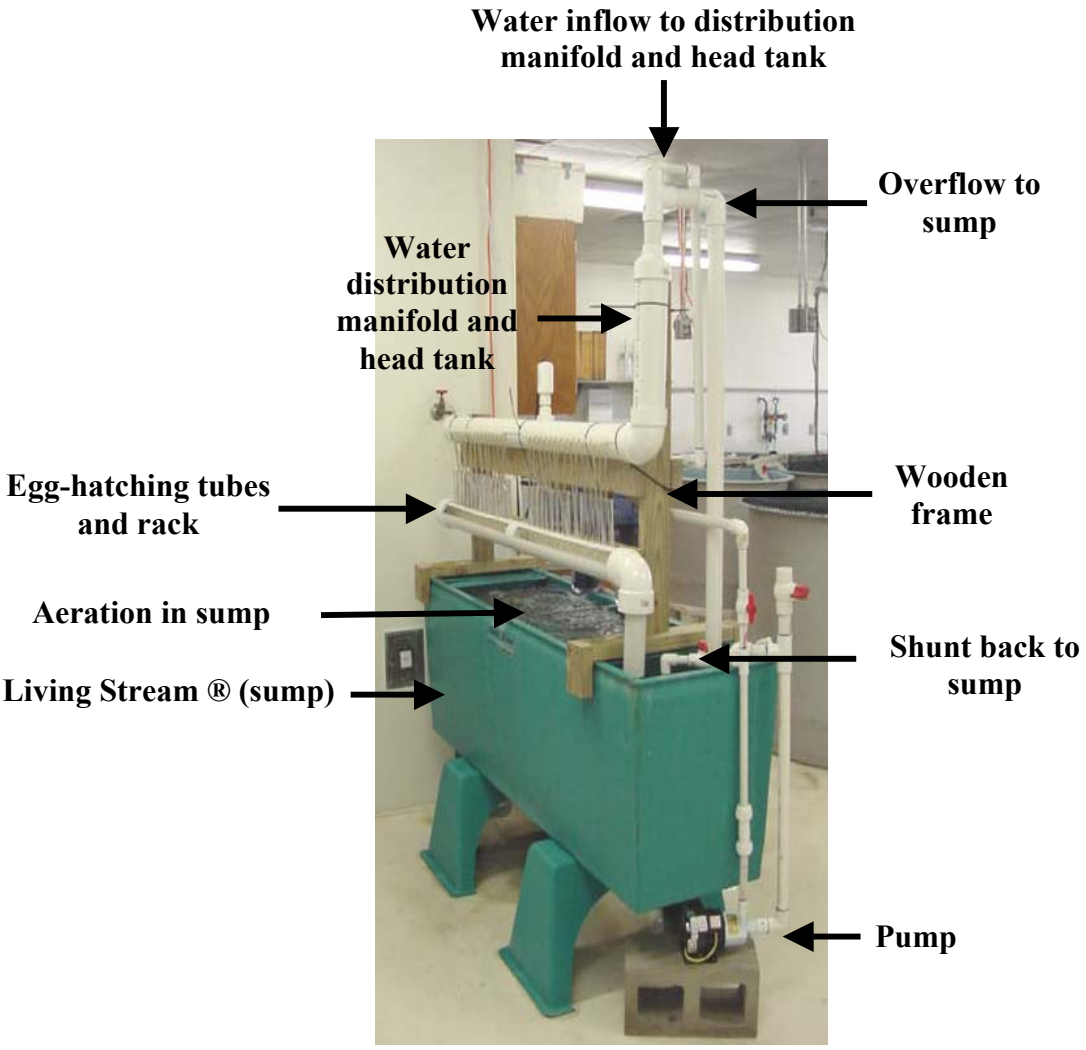

Figure 5.6. Labeled components of the front side of System 2 containing thirty 75-mL egg-hatching test tubes.

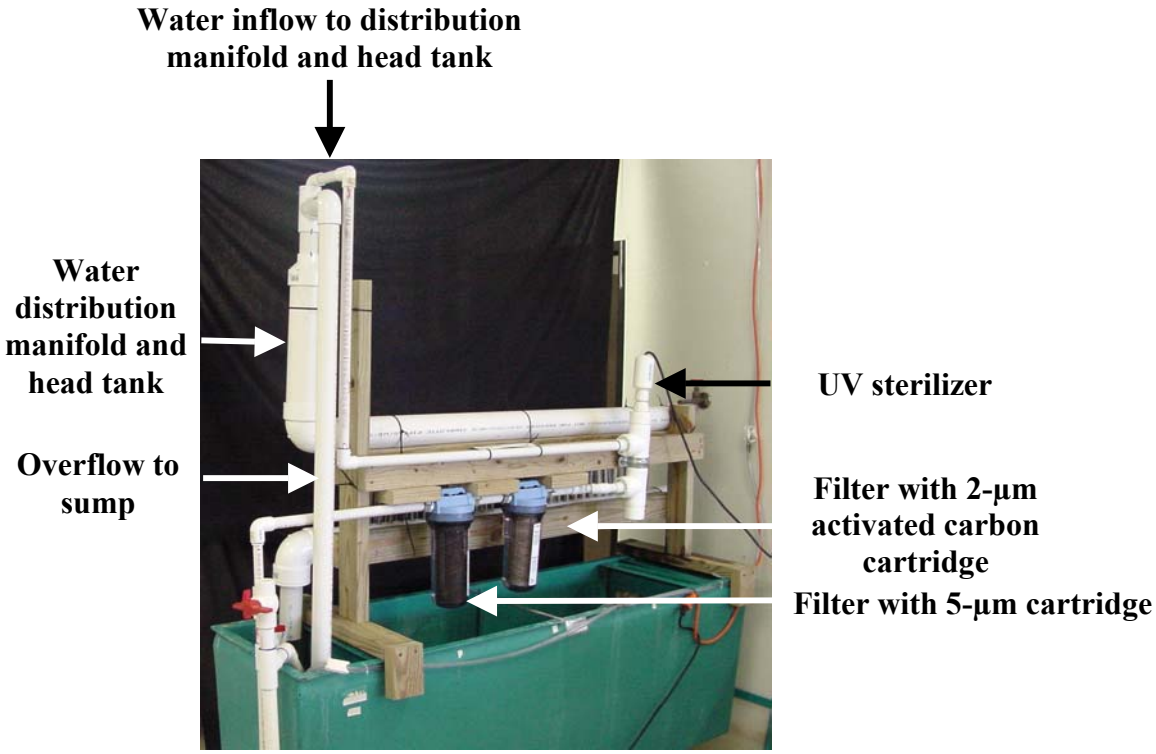

Figure 5.7. Photograph with labeled components of the back side of System 2 containing thirty 75-mL egg-hatching test tubes.

hybrid crosses of Nile tilapia x blue tilapia *Oreochromis aureus* were also incubated in the systems with similar survival to hatch. Over 90% survival to hatch was obtained from spawns that were not used in polyploidy experiments and fertilized immediately after stripping. No differences in survival were noted between the two systems.

Discussion

System 1 was economical to build and easy to operate. The addition of methylene blue was effective at preventing egg loss due to disease. However, the lack of biofiltration required frequent water changes when the system was heavily loaded, especially if high mortalities were anticipated due to experimental design. This was addressed with an extra aquarium that was filled with replacement water daily and allowed to come to the same temperature as the hatching system over a 24-hour period.

Another option that could reduce water changes would be to use a larger sump such as a 208-L plastic drum instead of the 40-L aquarium. In addition, for less than US $20 an extra pump could be added to the system and run concurrently in case of pump failure.

System 2, with mechanical and chemical filtration as well as UV sterilization, was also effective at preventing losses of eggs due to bacteria and fungus without the need to remove the hatching tubes daily for disinfection. However, these features raised the construction costs from US $125 to US $550. They also created a potential risk of inconsistent water flow to the eggs if the inflow to the water distribution manifold was reduced from clogged filters. However, the filter cartridges were changed every two weeks, and clogging never became a problem. Use of an additional cartridge filter could prevent the 5-µm and 2-µm filters from clogging as quickly.

Both systems were appropriate for the incubation of 2 mL of Nile tilapia eggs (~300) per hatching tube when conducting experiments with eggs from a single spawn and three or more treatment groups and a control were needed. Larger test tubes with increased egg-hatching capacity could be incorporated into the design of this system. The major advantage of these systems was their small footprint, which permitted more space to be allocated to rearing systems for other life stages. The isolation of these systems from rearing tanks allowed for easier maintenance of water quality and lowered the risk of transmission of disease to the eggs. The system design with the header tank and manifold allowed for constant water flow and the 75-mL hatching tubes with rounded bottoms provided for a gentle rolling of the eggs as well as uninhibited observation and easy access and handling.

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CHAPTER 6
POLYPLOIDY AND SYNCHRONY OF DEVELOPMENT IN NILE TILAPIA

Tilapia are members of the family Cichlidae and are native to Africa and the Jordan Valley and coastal rivers of Palestine (Philippart and Ruwet 1982). Today they are one of the most widely cultured fish in the world. Worldwide production has increased dramatically over the past decade with imports to the United States increasing from 15 million pounds in 1995 to over 127 million pounds in 2001 (http://www.ers.usda.gov/publications/so/view.asp?f=livestock/ldp-aqs/). Tilapia often reach sexual maturity in 6 months or less at a weight of ~40 g, well before they reach market size of ~500 g. Precocious spawning often leads to overpopulation and stunting. The allocation of energy to gamete production and spawning behavior may also increase feed costs, the highest variable cost in production.

Consequently, much research has been directed at preventing pre-harvest spawning in tilapias. One approach has been the alteration of environmental conditions. Growing tilapias at high densities has been shown to inhibit reproductive activity (cited by Guerrero 1982). Tilapia have also been grown in floating cages such that eggs fall through the mesh before they are fertilized (Goche 1982).

Another approach is the production and stocking of monosex populations. The hand sorting of males and females can be effective, but is too costly for large-scale production and is subject to human error. Monosex populations can be produced by administering hormones to fish prior to gonadal differentiation (Guerrero 1975). However, this is normally not 100% effective and can raise issues regarding consumer acceptance. Hybridization of certain tilapia species has produced 100% monosex populations (Pruginin et al. 1975, Lee 1979), although this has not been shown to occur in all pair matings (Hulata et al. 1981), perhaps due to difficulties in the

maintenance of pure lines of broodstock (Lovshin 1982). The production of sterile stocks of fish through chromosome set manipulation and the induction of polyploidy, a naturally occurring phenomenon in fish, has received much attention over the last several decades.

Triploidy, a form of polyploidy where organisms posses three complements of chromosomes, causes functional sterility. One of the most promising methods for the production of all-triploid stocks is the mating of tetraploid fish (possessing four sets of chromosomes) with normal diploid fish (Lutz 2001). This method has proven to be reliable in salmonids (Chourrout et al. 1986, Myers and Hershberger 1990) and avoids problems associated with application of pressure, temperature change, chemicals or electric shocks to fertilized eggs, which are often used to produce triploids.

Much of the work done on the induction of polyploidy in tilapia has centered on the search for the optimal timing, intensity, and duration of shocks to fertilized eggs. Specific timings for application of shocks that produce triploidy or tetraploidy have been identified, indicating that the mechanisms responsible for the prevention of the extrusion of the second polar body (triploidy) or the inhibition of first mitosis (tetraploidy) occur at specific stages of zygotic development. These windows are variable, however. Most windows of opportunity for the induction of triploidy or the production of meiotic gynogens (fish possessing only maternal DNA) have been reported to occur between 3 and 9 min after fertilization (AF). However, triploidy induction has been reported as early as 0.5 min AF and as late as 15 min AF (Table 6.1). Reported timing of shock application to prevent first mitosis in Nile tilapia for the production of tetraploids, mitotic gynogens (fish possessing only maternal DNA) and androgens (completely homozygous fish possessing only paternal DNA) (see Chapter 2 for details) have

Table 6.1. Survey of research during the past 15 years on the production of triploid and meiotic gynogenetic tilapias. These conditions involve the disruption of the second meiotic division and retention of the second polar body. Triploids (3N) possess three chromosome sets, meiotic gynogens (Mei Gyn) have two chromosome sets, each inherited from the mother.

Oreochromis Species	Ploidy	Incubation. Temperature C	Shock Type	Intensity	Duration (min)	Shock applied (min AF)	Optimal (min AF)	Induction at optimal	Source
niloticus	3N	28	Cold	7 C	20 - 50	7	7	100%	Hussain et al. 1991
niloticus	3N	28	Cold	9 C	30 - 50	7	7	100%	Hussain et al.1991
niloticus	3N	28	Cold	11 C	40 - 50	7 - 9	7	97%	Hussain et al. 1991
niloticus	3N	28	Cold	15 C	30 - 50	7	7	58%	Hussain et al. 1991
niloticus	3N	28	Cold	11 C	60	0.5 - 15	0.5, 5	100%	Don and Avtalion 1988a
niloticus	3N	*	Heat	39.5 - 41C	3.5 - 4	3.5 - 4	4	100%	Don and Avtalion 1988a
niloticus	3N	28	Heat	41, 42 C	3.5	2 - 7	5	100%	Hussain et al., 1991
niloticus	3N	*	Heat	41 C	4.5	4	*	*	Puckhaber and Horstgen-Schwark 1993
niloticus	3N	28	Pressure	8000 psi	2	8 - 12	8, 9	100%	Hussain et al. 1991
niloticus	3N	24 - 31	Heat	40 - 41C	4 - 5	4 - 10	4 - 6	100%	El Gamal et al. 1999
niloticus	3N	30 - 31	Cold	12.8 C	45 - 50	5	5	100%	El Gamal et al., 1999
niloticus	2N Mei Gyn	*	Heat	41 C	3.5	5	5	16%	Mair et al. 1987, Mair 1988
aureus	3N	25 - 26	Cold	11 C	60	0.5-15	0.5, 5	100%	Don and Avtalion 1988a
aureus	3N	25 - 26	Heat	39.5 - 41.5 C	3.5 - 4	*	4	100%	Don and Avtalion 1986
aureus x *niloticus*	3N	25 - 26	Heat	39.5 C	3	3	3	100%	Don and Avtalion 1988a
aureus	2N Mei Gyn	25 - 26	Heat	39.5 C	3.5 - 4	3	3	13%	Don and Avtalion 1988b
aureus x *niloticus*	3N	25 - 26	Heat	39.5 C	3	3	3	100%	Don and Avtalion 1988a
niloticus x *aureus*	3N	25 - 26	Heat	40.5 C	4	3.5	3.5	96%	Don and Avtalion 1988a
mossambicus	3N	*	Heat	42 C	3	2.5	2.5	100%	Varadaraj and Pandian 1990
mossambicus	2N Mei Gyn	27	Heat	42 C	3	2.5	2.5	27%**	Varadaraj 1990

* Not reported.

** Sperm from common carp *Cyprinus carpio.*

varied even more, with the successful timing reported at 27-30, 40-50, and 60-90 min AF (Table 6.2). Hypotheses addressing variation within and among studies regarding the timing of application of shock to produce polyploid, gynogenetic and androgenetic tilapia include: species and strain differences (Myers et al. 1995); interruption of different cellular processes that result in polyploidy (Mair 1993); methodological differences and asynchronous development (Don and Avtalion 1988).

Table 6.2. Survey of research during the past 15 years on the production of tetraploid, mitotic gynogenetic and androgenetic tilapias. These conditions involve the inhibition of the first mitotic division after the replication of DNA. Tetraploids (4N) possess four chromosome sets, mitotic gynogens (Mit Gyn) have two chromosome sets (2N), both inherited from the mother. Androgens also have two chromosome sets, both inherited from the father.

Oreochromis Species	Ploidy	Incubation temperature C	Shock type	Intensity	Duration (min)	Shock applied (min AF)	Optimal (min AF)	Induction at optimal	Source
niloticus	4N	*	Heat	41 C	5	30 - 33	30 - 33	4 - 12	Mair 1988
niloticus	4N	30	Heat	41 C	5 - 6	65 - 90	(double) 65+80	80	El Gamal et al. 1999
niloticus	4N	28 - 32	Cold, Pressure	7500 psi at 7.5 C	7	70	70	5	Myers 1986
niloticus	2N Mit Gyn	*	Heat	41 C	3.5	5	30	3	Mair 1988
niloticus	2N Mit Gyn	28	Heat	41C	3.5	25 - 48	30	2	Huassain et al. 1993
niloticus	2N Mit Gyn	28	Pressure	9000 psi	2	35 - 50	47.5	2	Huassain et al. 1993
niloticus	2N Mei + Mit Gyn**	28	Pressure	8000 psi	2	9 - 35	9, 17	14	Peruzzi et al. 1993
niloticus	2N Androgens	28	Heat	41-42.5 C	4	25 - 30	25	5 (42.5 C)	Myers et al. 1995
niloticus	2N Mit Gyn	28	Heat	41-42.6 C	4	20 - 30	27	10 (42.5 C)	Myers et al. 1996
aureus	4N	25 - 26	Cold	11 C	60	80 -104	92	25	Don and Avtalion 1988c
mossambicus x *niloticus*	4N	28 - 32	Cold, Pressure	7500 psi at 7.5 C	7	70	70	21	Myers 1986
mossambicus	2N Mit Gyn	*	Heat	41 C	3.5	33	33	1	Mair 1988

* Not reported.
** No test done to differentiate between mitotic and meiotic gynogens.

The goal of this chapter was to create tetraploid Nile tilapia by use of heat shocks. The objectives were to: 1) develop protocols that allow for rapid and uniform temperature change for

shocking of Nile tilapia eggs; 2) apply heat shocks ranging in of time of application, duration and intensity to estimate parameters for the induction of tetraploidy; 3) evaluate the effects of timing of heat shocks on survival, tetraploidy induction, and tetraploid yield.

Materials and Methods

Broodstock

Two strains of Nile tilapia, provided by Tiltech Aquafarm, Robert, Louisiana in October of 2000, were used in these investigations. Females were from a line designated as the Swansea strain, which originated from Lake Manzala, Egypt. This strain was first established at the Institute of Aquaculture, University of Stirling, Scotland and subsequently obtained by researchers at the University of Wales, Swansea, U.K. in 1981 and 1982. Til-Tech Aquafarm has obtained Swansea strain Nile tilapia from the University of Wales several times each year since 1997. Male Nile tilapia used in the study were from the Swansea strain, or from breeding stock at Til-Tech Aquafarm descended from the original Auburn-Egypt strain. This strain was established with fish collected from the Ishmalia Canal in the Nile delta of Egypt. Til-Tech obtained its original Nile tilapia breeding stock from Mississippi State University in 1992, which had obtained Auburn-Egypt strain broodstock from Auburn University.

Aquaria were stocked with single females in a recirculating system containing ten 80-L aquaria, a 200-L sump, an upwelling bead filter, a 25-watt ultraviolet sterilizer (Model QL-25, Rainbow Plastics, El Monte, California) and a 0.19 kW (1/4 hp pump) (Chapter 4). Ten females were also stocked as a group in an 800-L circular tank, plumbed into a recirculating system adjacent to the spawning system, and were used to replace fish in the aquaria system when the need arose. Another 800-L tank was stocked with three males for access to sperm. The following target values for water quality parameters were maintained throughout the study:

salinity, 1-2 ppt; alkalinity and hardness, 200 mg/L as $CaCO_3$; pH, 7 to 8.5; and unionized ammonia, < 0.05 mg/L. Fish were maintained on a natural photoperiod and fed a commercial diet (28% crude protein and 4% crude fat, Cargill, Nutrena Feed Division, Minneapolis, Minnesota), as much as they would ingest in 10 min, twice daily. Temperature ranged from 29 C in the summer to 26 C in the winter. Fish were weighed at the end of the study and weights ranged from 212 g to 530 g with a mean (± SD) of 399 ± 100 g.

Gamete Collection

Spawning readiness was estimated by gauging the degree of extension of the urogenital papilla as described in Chapter 4. Fish were checked every hour from 13:00 to 17:00 hours. If the extension of the papilla was > 0.5 cm, the fish was chosen for spawning and anesthetized in ~150 mg/L tricaine methanesulfonate (methyl-m-aminobenzoate, MS-222, Argent Laboratories, Inc., Redmond, Washington). Upon the loss of equilibrium, the fish was blotted with paper towels and the eggs were stripped into a 300-mL watch glass, containing ~80 mL of Hanks' balanced salt solution (HBSS) (Appendix A, SOP-1), by gentle squeezing of the abdomen between the thumb and forefinger and stroking in a head-to-tail direction (Appendix A, SOP-7). The eggs were judged to be of good quality and were used for experiments if they were easily stripped, uniform in shape and size, and connective tissue and blood were absent.

Sperm were collected from one or two males for each experiment. Males were not anesthetized but were held upside down with their heads wrapped in moist paper towels. The rest of the fish was blotted dry with paper towels and HBSS was squirted on the area around the papilla. Gentle squeezing of the abdomen caused sperm and sometimes urine to be released. If urine was expelled, the papilla was wiped dry with paper towels. When it appeared that milt only was released, it was collected with a 200 µL pipetter, transferred to 1.5-mL microcentrifuge

tubes, diluted with HBSS and placed on crushed ice until used for fertilization (Appendix A, SOP-7).

Protocols for Rapid Temperature Change

To ensure that eggs were subjected to rapid and uniform heat shocks, protocols were developed as follows. An incubation waterbath (29 C) (VWR Scientific model # 1235, Shel Lab, Sheldon Manufacturing Inc., Cornelius, Oregon) and a heat-shock water bath (42 C) (VWR Scientific model # 1141) were turned on 1 hr before the collection of gametes. A 1-L graduated beaker was placed in each of the baths and filled with freshly prepared egg hatching water (Appendix A, SOP-5) to the same level as the bath water on the outside. Airstones attached to an aquarium aerator were placed in each of the beakers to provide aeration and continuously mix the water. Five egg hatching baskets made from 50-mL centrifuge tubes (Appendix A, SOP-4) were stocked with 2 mL of eggs and placed in the incubation beaker.

To evaluate the standardization of the heat shocks, a thermocouple and data logger (model RD6112, Omega Engineering Inc., Stamford, Connecticut) was used to measure the water temperature of the incubation and heat-shock beakers to the nearest 0.1 C. The accuracy of the thermocouple was determined to be within 0.6 C. It yielded values of 0.6 ± 0.1 C when submersed in an ice slurry (mean ± SD, N = 5) and 100.3 ± 0.1 C when submersed in boiling water (N= 5). After 28 min, four of the egg baskets were removed from the incubation beaker, quickly blotted on a paper towel and placed in the heat-shock beaker. The thermocouple was placed inside one of the hatching baskets and temperatures were recorded every 10 sec for 1 min, and at 90, 120, and 180 sec. After 3 min the egg baskets were transferred back to the incubation beaker. This process was repeated five times with 45 min allowed to pass between each trial.

Artificial Fertilization and Heat Shocks

Eggs from single females were used in each experiment. Sperm were collected before the experiment to minimize the time between egg collection and fertilization (less than 15 min). Depending on the spawn, 1 to 2 mL of eggs were added to each of five 100-mL watch glasses and covered with HBSS. Fertilization of the five portions was carried out in a specific sequence. A timer was started upon fertilization of the first batch of eggs. The HBSS was poured off and one end of the watch glass raised to concentrate the eggs at the other end. Five drops of sperm were dispersed over the eggs with a plastic eyedropper and 5 mL of egg hatching water were taken from the incubation beaker and added to the eggs. Eggs and sperm were swirled several times and left to sit for ~1 min, after which time another 5 mL of water were added. Two min after the addition of sperm, the eggs were transferred to egg baskets and placed in the incubation bath (Appendix A, SOP-9). The process was repeated at the scheduled time interval for each treatment. The control group was the last to be fertilized (10 min after the first experimental group). At the desired time of heat shock, the four baskets containing the treatment groups of eggs were removed simultaneously from the incubation bath, quickly blotted on a paper towel and placed into the hot-water bath. The temperature was measured with a standard laboratory 76-mm immersion glass thermometer, –20 to110 C (manufacturer not specified). The accuracy of this thermometer was determined to be within 0.3 C. It yielded values of 0.0 ± 0.0 C when submersed in an ice slurry (N = 5) and 99.7 ± 0.1 C when submersed in boiling water (N = 5). At the end of the heat shock, the baskets were removed simultaneously, quickly blotted on a paper towel and placed back into the incubation bath. In preliminary experiments, shocks ranging from 41.0 to 43.0 C were applied from 22 to 80 min AF for durations of 2, 3 or 5 min

(Appendix E). In later experiments, 42.8 ± 0.2 C (mean ± SD, N = 7) heat shocks were applied at 22, 24, 26, and 28 min AF for a duration of 3 min.

Egg Incubation

At the end of the heat shocks, the beaker was removed from the incubation bath and taken to an egg hatching system (described in Chapter 5) where the eggs were acclimated for 15 min by the gradual addition of system water. Eggs were transferred from the baskets to hatching tubes and water flow was adjusted to provide a gentle rolling action. Dead eggs were removed twice a day, until hatch. At least 50 to 90% of the water was replaced daily depending on the number of spawns in the egg hatching system.

Ploidy Evaluation

Approximately 120 hours AF, newly hatched sacfry were counted and percent survival was calculated. A sample of twenty fish from each treatment group and at least five fish from the control group were used for ploidy analyses. If 20 fish did not survive in a treatment group, all fish were used for ploidy analysis. If more than 20 fish survived, those not evaluated were returned to the egg hatching system. The yolksac was removed from each fish by use of 25-gauge needles attached to 1-mL syringes. The remaining body was placed in a 1.5-mL microcentrifuge tube with 150 µl of lysis buffer which contained, 0.1% sodium citrate and 0.1% Triton X-100 in Dubecco's phosphate-buffered saline (PBS) (0.8% NaCl, 0.02% KCl, 0.144% $NaHPO_4$, 0.024% KH_2PO_4) (Life Technologies, Rockville, Maryland). Microcentrifuge tubes were placed on crushed ice and transferred to a –15 C freezer and stored for 2 to 3 days. Samples were removed from the freezer and allowed to thaw at room temperature. A cell suspension was obtained by slowly passing the fish through a 25-gauge needle fitted to a 1-mL syringe (four times). The cell suspension was drawn up with a 200-µl pipeter and filtered

through 20-µm mesh into a glass test tube. Cell nuclei were stained by the addition of 150 µl of lysis-staining buffer which was a solution of 0.5 mg/mL propidium iodide (PI) (#P1304, Molecular Probes, Eugene, Oregon) containing 1 µg/mL RNase (Ribonuclease A, Sigma, St. Louis, Missouri), 0.1% sodium citrate (SX0445-1, EM Science, Gibbstown, New Jersey) and 1% Triton X –100 (Scintillar ®, Mallinckrodt, Paris, Kentucky) (Tiersch and Chandler 1989, Tiersch et al. 1990). After incubation of 20 min at room temperature and in the dark, DNA content was analyzed with a FACSCalibur® flow cytometer (Becton Dickinson, San Jose, California) equipped with a 480-nm air-cooled argon laser. The FACSComp® software (Becton Dickinson) was used to calibrate the instrument settings. A sample of stained channel catfish blood cells (2.0 ± 0.01 pg DNA / cell) (Tiersch et al. 1989) was added as an internal reference. Measurements of DNA content of control tilapia (no heat shock) were compared to those of sacfry that had received heat shocks. The DNA content was estimated by dividing the modal channel number for fluorescence intensity obtained for tilapia by the modal channel number for channel catfish and multiplying the result by 2.0 (the picogram value for DNA content of diploid channel catfish).

Ploidy of juveniles (age ~ 2 months) and adults (age ~ 8 months) was evaluated using fresh blood. A 1-mL syringe was fitted with a 27 or 25-gauge needle that had been rinsed with acid citrate dextrose (ACD) (Vacutainer®, Becton Dickinson, New Jersey) a chelating anticoagulant. Fish were anesthetized with MS-222 and the needle inserted just below the lateral line, straight up from the posterior of the anal fin. Blood was collected from the caudal vessels on the ventral side of the vertebral column. Only 1 to 3 µl of blood were collected and the needle was withdrawn as soon as blood was visible in the translucent hub. Blood was expelled from the needle into a 1.5-mL microcentrifuge tube containing 20 µl of ACD and placed on ice. The

mixture of blood and ACD was added to a glass test tube containing 200 µl of lysis-staining buffer, incubated in the dark for 20 min and analyzed with the flow cytometer. Channel catfish blood was used as an internal reference.

Rearing of Fry and Juveniles

Sacfry that were not used for ploidy evaluation were kept in the hatching system until they reached the swim-up stage, 7 to 8 days AF. Fry were transferred to 40-L aquaria where they were placed in mesh nursery baskets. One or two days later, fry were fed newly hatched *Artemia* (INVE, Gruntsville, Utah) and catfish fry starter, 45% protein and 10% crude fat, (Clover Brand, Farmland Industries, Kansas City, Missouri), 3 to 5 times daily. Fish were later transferred to recirculating systems.

Over the course of the study, an additional 5,000 eggs from four spawns were shocked at 26 min or 28 min AF to evaluate production of adult tetraploids. Fish were reared and their ploidy analyzed 2 to 4 months AF.

Statistical Analysis

Statistical analyses were performed using the JMP IN® Software for Windows version 3 (SAS Institute, Cary, North Carolina). Differences in percent polyploidy, percent survival, and percent polyploid yield were evaluated among the treatments by use of a one-way analysis of variance (ANOVA). The level of significance was set at $P \leq 0.05$.

Results

Protocols for Rapid Temperature Change

The egg baskets and protocols provided rapid and uniform heat shocks with little variation occurring between the five trials (Figure 6.1). The water temperature in the hot water beaker dropped from 43.4 ± 0.1 C to 42.9 ± 0.2 C (mean ± SD) immediately upon the immersion of the

four egg baskets. The temperature inside the egg baskets increased gradually over the 3-min heat shock from 42.9 ± 0.2 C to 43.2 ± 0.3 C.

Flow cytometry proved effective for ploidy analysis of Nile tilapia fry. Data points from 10,000 cells were obtained from each sample and well-defined peaks (populations of cells) were obtained in flow cytometric histograms of fluorescence intensity. Diploid, triploid and tetraploid populations of Nile tilapia cells were visually distinguishable by comparing the proximity to the population of channel catfish blood cells (internal reference) (Figure 6.2). The DNA content calculated from whole diploid fry ranged from 2.5 to 3.4 pg with a mean of 2.8 ± 0.2 pg (N = 23).

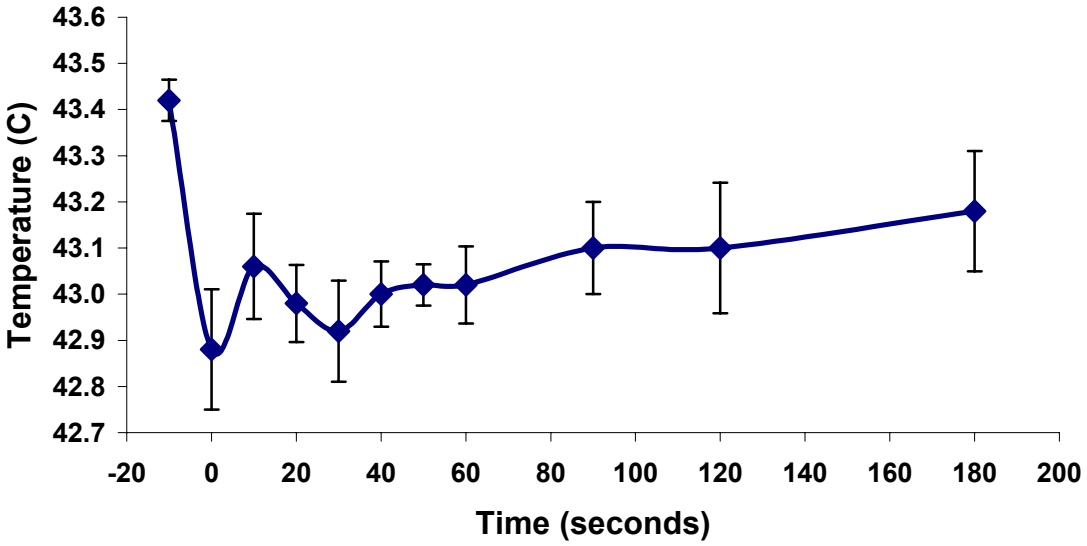

Figure 6.1. Temperature change profile for 3-min heat shocks applied to Nile tilapia eggs. The data point at –10 sec represents the temperature in the heat-shock beaker prior to the immersion of four heat-shock baskets with 1.5 mL of eggs each at 0 sec. Each point represents the mean (± SD) for 5 measurements.

The use of 27and 25-gauge needles for blood collection from the caudal vessels of juveniles and adults ranging in weight from ~8 to 100 g yielded 100% survival. The DNA content

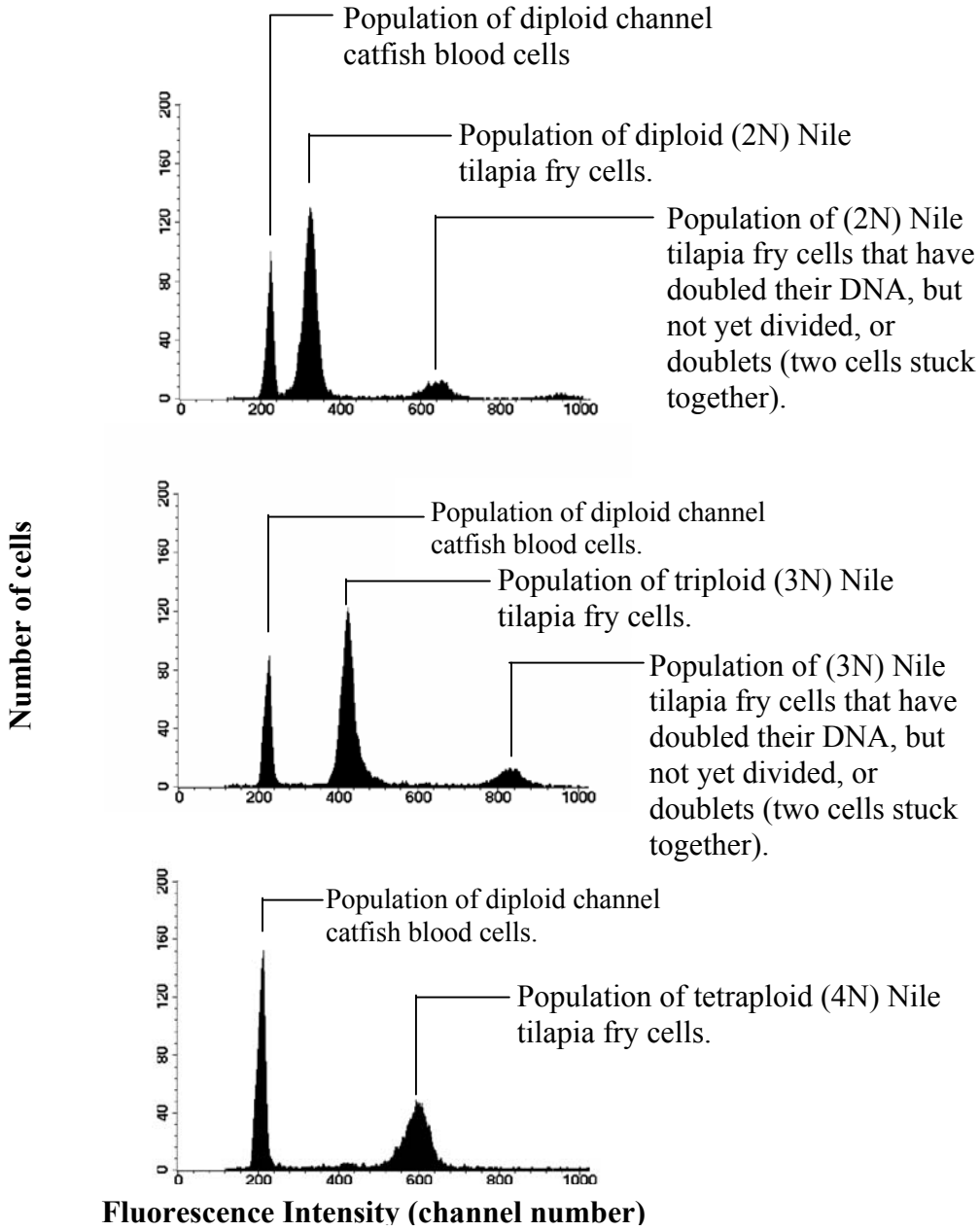

Figure 6.2. Flow cytometric histograms of fluorescence intensity of diploid (2N), triploid (3N), and tetraploid (4N) populations of Nile tilapia cells obtained from individual 5-day-old dissociated fry and diploid channel catfish blood cells, used as an internal reference.

calculated from blood of diploid juveniles and adults ranged from 2.3 to 2.4 pg with a mean of 2.3 ± 0.03 pg (N = 7).

Twelve spawns were used to estimate effective parameters for the induction of tetraploidy. Single and double heat shocks ranging from 42.0 C to 45.0 C were applied to eggs at 22 to 80 min AF for durations of 2 to 5 min. Tetraploids resulted only from treatments where shocks of 42.0 to 42.8 C were applied at 22 to 28 min AF for 3 min (Appendix E).

Seven spawns were used in replicated experiments where 42.8 ± 0.2 C heat shocks were applied at 22, 24, 26, or 28 min AF for a duration of 3 min. An unexpected result was the induction of triploidy in treatments that were expected to only yield tetraploidy. Percent triploidy and tetraploidy, percent survival, and percent triploid and tetraploid yield for treatments to eggs from all seven spawns were combined (Figure 6.3). Shocks applied at 28 min AF produced the highest percentage of tetraploidy (34%), while shocks applied at 22 min AF produced the lowest (14%). However, analysis of variance revealed no significant differences in percentage of tetraploidy among the treatments ($\underline{P} = 0.69$). Shocks applied at 22 min AF produced the highest percent triploidy (27%), while shocks applied at 28 min AF produced the lowest (1%). However, no significant differences in the percentage of triploidy among the treatments were found ($\underline{P} = 0.19$). Percent survival was similar in treatments where shocks were applied at 22, 24 or 26 min AF with 24 min being the highest (26%). Survival of eggs shocked at 28 min AF was lowest (9%). However, no significant differences in survival were found among the treatment groups ($\underline{P} = 0.55$). Survival in the control group, 74%, was significantly higher than in the treatment groups ($\underline{P} < 0.0001$). The highest percentage of tetraploid yield (9%) was obtained from shocks applied at 24 min AF while the highest percentage of triploid yield (7%) was obtained from shocks applied at 22 min AF. No significant differences were

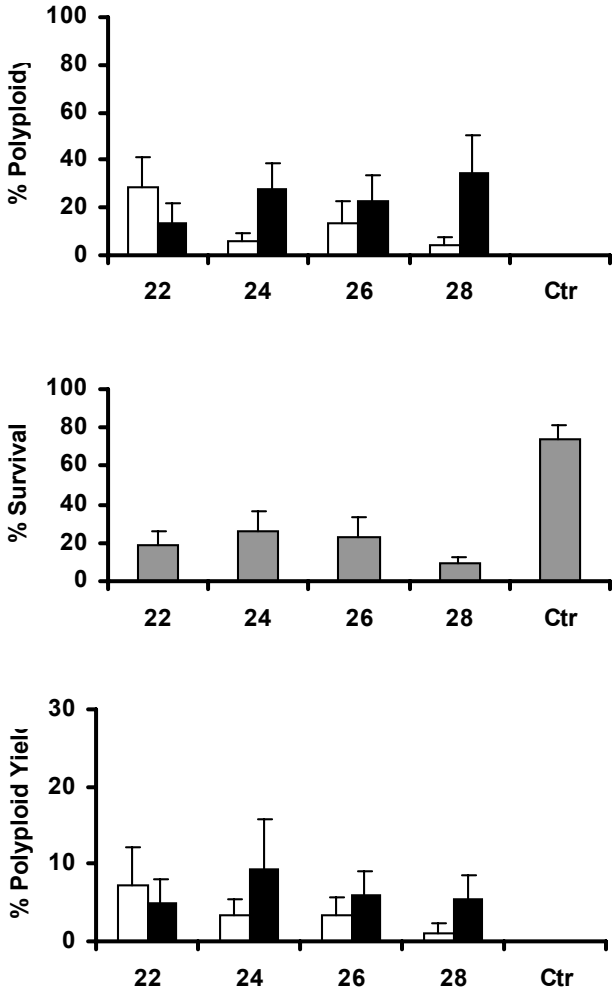

Figure 6.3. Mean percentage of triploidy (white bars) and tetraploidy (black bars), percent survival (gray bars), and percent triploid and tetraploid yield. Data were combined from seven replicated experiments where 42.8 C heat shocks were applied to eggs from individual spawns at 22, 24, 26, and 28 min AF for a duration of 3 min. The letters "Ctr" denote control where no shock was applied.

observed among the treatments for percentage of tetraploid yield ($\underline{P} = 0.89$) or percentage of triploid yield ($\underline{P} = 0.52$).

Variation in production of polyploid progeny occurred among females. One female produced > 95% polyploids in all treatments (Figure 6.4), while another failed to produce any polyploids in any of the treatments.

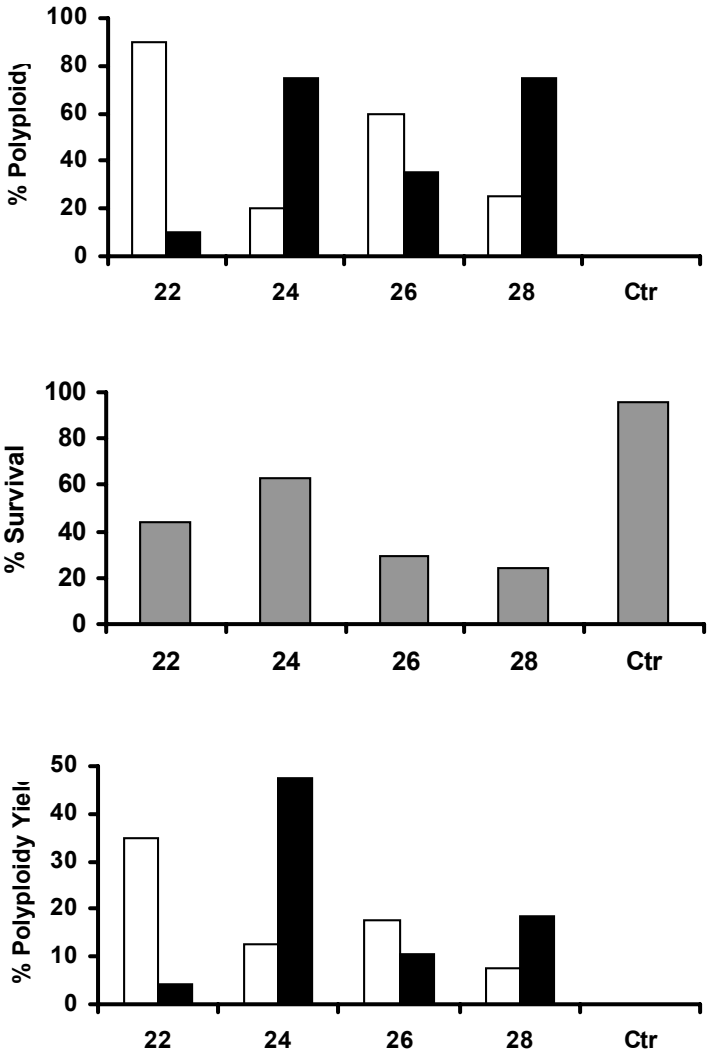

Figure 6.4. Percentage of triploidy (white bars) and tetraploidy (black bars), percent survival (gray bars), and percent triploid and tetraploid yield where 42.8 C heat shocks were applied to eggs from an individual spawn at 22, 24, 26, and 28 min AF for a duration of 3 min. The letters "Ctr" denote control where no shock was applied.

Of the four spawns that were used to produce tetraploids for evaluation at adulthood, only four fish survived. These fish were evaluated for ploidy at ~2 months of age. None of these were tetraploid: one was triploid and the other three were diploids. Of the seven replicated tetraploidy induction experiments where shocks were applied from 22 to 28 min AF to ~6,400

eggs, ~245 triploids and ~411 tetraploids were produced. Of these, 42 fish that were not evaluated for ploidy at hatch survived. However, when evaluated at the age of four months all were diploid. Of the three experiments where shocks were applied at 60 to 80 min AF, 104 fish from the ~3,600 shocked eggs survived for five or more months. All of these fish were diploid.

Discussion

The single-female spawning system was effective in providing spawns for the ploidy manipulation experiments in this study. More detailed information on the system is provided in Chapter 4 of this thesis.

The use of baskets was a suitable method for the heat shocking of eggs and facilitated the simultaneous shocking of multiple treatments. The direct immersion of eggs into heat shock baths of the desired temperature assured that duration and intensity of shocks were consistent among treatments. The use of netting baskets (Hussain et al. 1991) should provide similar advantages, while collection and straining of eggs through fine mesh and direct addition to heat shock baths (Valenti 1975) may increase variability in duration of shocks among treatments due to the time required for handling. The heat-shock temperature profiles showed little variation among the five trials with the temperature inside the egg baskets increasing gradually over the 3-min heat shock from 42.9 ± 0.2 C to 43.2 ± 0.3 C. This would indicate that heat shocks among spawns were consistent over the course of this study and it is unlikely that the observed variation in polyploidy induction was due to differences in heat shock profiles.

The egg incubation system was effective, with a mean of 75% survival to hatch for control groups. The range in survival from 55% to 98% in controls may be due to egg quality differences among spawns. More information on the egg hatching system is provided in Chapter 5 of this thesis.

Flow cytometric evaluation of ploidy in fish is a common practice and can be more reliable than traditional methods such as chromosome spreads and erythrocyte measurements (Allen and Stanley 1983, Don and Avtalion 1988b, Lamatsch et al. 2000). In this study, flow cytometry allowed for fast and statistically powerful evaluation of ploidy, where 10,000 cells per sample could be evaluated in less than 1 min. The DNA content obtained when using blood from juvenile and adult fish in this study showed little variation, 2.3 ± 0.03 pg (mean ± SD). Flow cytometric evaluation of blood from chickens also shows little variation in DNA content and chicken blood is often used as an internal reference (Tiersch and Chandler 1989). The evaluation of DNA content of channel catfish (2.0 ± 0.1 pg) is also consistent when blood is used (Tiersch et al. 1990).

Greater variation in diploid DNA content, 2.8 ± 0.2 pg (mean ± SD) occurred when whole fry were analyzed in this study. With newly hatched tilapia fry, collection of sufficient numbers of blood cells for analysis by flow cytometry was not possible. To provide sufficient numbers of cells, whole fry were passed through 25-gauge needles resulting in the collection of cells from multiple tissue types. This was most likely the source of the variation in the calculated DNA content when analyzing fry in this study. When DNA content was calculated using cells collected from different tissues from mosquitofish *Gambusia affinis*, as much as an 18% difference occurred when compared to the values obtained using blood alone (Tiersch and Wachtel 1992).

This study showed that the induction of tetraploidy in Nile tilapia can be accomplished by application of 42.8 C heat shocks to eggs over a range of times (22 to 28 min AF). This range is similar to other studies reporting the induction of tetraploidy (Mair 1988), mitotic gynogenesis (Peruzzi et al. 1993) and androgenesis (Hussain et al. 1993). No tetraploids were found when

shocks were applied at 65 and 80 min after fertilization as reported elsewhere (El Gamal et al. 1999). However, the fish that survived shocks applied at 65 and 80 min AF were not evaluated for ploidy at the time of hatch. Therefore, tetraploids may have been produced by these treatments and died prior to evaluation. The incubation temperature of 29.0 C used in this study was higher than that used in other studies (~28.0 C). Developmental rate in fish is correlated with temperature and this may have contributed to the highest tetraploid yield occurring when shocks were applied at 24 min AF in this study, 6 min earlier than the earliest previous report on optimum timing of shock application for tetraploidy induction (Mair 1988).

The unexpected induction of triploidy in this study occurred as late as 28 min AF, 13 min beyond the latest reported time of shock application to induce triploidy (Don and Avtallion 1988a). If the window of opportunity for triploidy induction is limited to the first 10 min after fertilization as previously suggested (Don and Avtalion 1986), no triploids should have occurred when shocks were applied from 22 to 28 min AF. The simultaneous induction of triploidy along with tetraploidy in this study raises questions about the synchrony of zygotic development. If different mechanisms are responsible for the induction of triploidy (prevention of the extrusion of the second polar body) and tetraploidy (prevention of the first mitotic division), and the window of opportunity to disrupt these mechanisms occurs at distinct stages of zygotic development, the results of this study indicate that asynchrony of development plays an important role in the variation in reported windows of opportunity to produce triploids and tetraploids.

Asynchrony of development was hypothesized to be the reason for the long window of opportunity (80 to 104 min AF) for tetraploidy induction in *O. aureus* (Don and Avtalion 1988b). Asynchrony may be an artifact of technical variations in the artificial spawning and

shocking processes such as the non-uniform shocking of eggs or the inadvertent activation of eggs upon stripping. These two possibilities do not seem relevant in this study given that all treatments were submersed and removed from the heat shock beakers at the same times. The small volume of eggs ($\leq$ 2 mL) in the baskets also ensured that all eggs were thoroughly mixed and exposed to identical temperatures upon immersion and throughout the duration of the shock. The heat shock profile in this study also indicated that little variation (with a maximum increase of 0.5 C) occurred over the duration of the 3-min heat shocks. The target temperature for this study was 42.5 C: temperatures of 42.8 ± 0.2 C were recorded at the end of the 3 min shocks. Therefore it can be inferred that eggs were immediately subjected to temperatures of 42.3 ± 0.2 C and the temperature increased to 42.8 ± 0.2 C over the 3 min duration.

This study is one of the few to report the standardization of the thermometer used to measure the heat shock temperature. The thermometer was found to be accurate to within ± 0.3 C. So, the actual temperatures of the shocks in this study should be within ± 0.3 C of those reported. Activation upon stripping is unlikely because eggs were stripped into HBSS, a physiological solution, and tilapia eggs are not normally activated until fertilization (Myers and Hershberger 1991, Poleo 2002). In this study, all treatment groups of eggs were fertilized within 20 min of stripping. In other studies, tilapia eggs that were stored in HBSS as long as 80 min showed no significant difference in percent fertilization when compared to eggs that were fertilized immediately after stripping (Poleo 2002).

Tilapia are known to lay eggs in a fractional manner where small increments are deposited over a period of 45 min to an hour (Lee 1979). If there is a lag time in development between increments of eggs released by females, it is possible that simultaneous stripping of multiple increments results in asynchrony. The use of the single-female spawning system and the

gauging of the ripeness of eggs solely on the extension of the urogenital papilla may not be the most accurate method of assessing spawning readiness. In some studies, extension of the urogenital papilla has been observed up to 24 hr prior to natural spawning (Myers and Hershberger 1991) although other reports indicate that extension of the urogential papilla occurs just prior to spawning in Nile tilapia (Myers 1985). The strip spawning of eggs after the deposition of the first increment of eggs (Valenti 1975) may help improve synchronization of eggs if technical variation in strip spawning protocols is a major factor. The possibility also exists that genetic effects contribute to asynchronous zygotic development. Individual genetic differences may be the cause for eggs from one female producing > 95% polyploids from a single spawn while eggs from other females produced less than 3% or none. Variation between females and percent polyploidy induction has been reported in other studies with Nile tilapia (El Gamal et al. 1999). Possible effects that could cause asynchrony in zygotic development are summarized in Table 6.3.

There are no reports on the viability of tetraploid Nile tilapia to adulthood. This study found no tetraploids surviving beyond 5 days AF. However, more than the 5,000 eggs exposed to 42.8 C heat shocks at 26 and 28 min AF for 3 min in this study may be necessary for the production of adult tetraploids in this species.

Table 6.3. Overview of the possible effects that may cause asynchronous zygotic development in Nile tilapia.

Effect	Example
Technical	Variation in shocks, activation upon stripping of eggs, method for evaluating spawning readiness affects developmental rate in individual eggs.
Maternal	Variation from incremental deposition of eggs, egg quality affects rate of development.
Genetic	Developmental rate of eggs is influenced by inheritance.

The only report of tetraploid *Oreochromis* sp. surviving to adulthood was in blue tilapia where cold shocks were used (Don and Avtalion 1988b). Although blue tilapia may be more amenable to tetraploidy induction, further research on the use of cold shocks to induce tetraploidy in Nile tilapia may improve the chances of tetraploids surviving to adulthood.

This is the first study to report the induction of both triploidy and tetraploidy in the same treatment from a single spawn. The questions this raises about the synchrony of zygotic development warrant further research. A better understanding of the synchrony of zygotic development and methods to improve synchrony may improve the chances of producing adult tetraploid Nile tilapia.

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CHAPTER 7
SUMMARY AND CONCLUSIONS

Chromosome set manipulation has several practical applications for aquaculture. The production of functionally sterile triploid (three sets of chromosomes per cell) fish can help reduce the risk of the establishment of populations of exotic species and the contamination of wild gene pools by domesticated strains. It can also safeguard against the perpetuation of transgenic genomes and serve as a management tool for producers of species that reach sexually maturity before they attain harvest size. Furthermore, sterile fish can help protect years of investment and intellectual property if novel fish or fish from selected lines are sold live. Tetraploid (four sets of chromosomes per cell) fish can be crossed with normal diploid (two sets of chromosomes per cell) fish to provide a reliable means for the production of all-triploid progenies. The studies in this thesis addressed the induction of tetraploidy in zebrafish *Danio rerio* and Nile tilapia *Oreochromis niloticus*. A general overview of the potential applications of this research is represented in Figure 7.1.

Zebrafish are a popular aquarium fish as well as an important research model. Although triploid, gynogenetic, and androgenetic zebrafish have been reported, this study is the first to report the induction of tetraploidy in this species (Chapter 3). A heat shock of 41 C was effective at inducing tetraploidy when applied at 11, 13, 15, 17, or 20 min after fertilization (AF) for a duration of 2 min. Low survival (< 10%) resulted when shocks were applied from 11 to 15 min AF indicating that heat-sensitive events may be occurring during these times. The most effective treatment was a 2-min heat shock of 41 C applied 20 min AF that resulted in ~10 percent tetraploid yield. Flow cytometry revealed the occurrence of diploidy and tetraploidy in

fish of normal and abnormal appearance, indicating that heat shocks and not extra sets of chromosomes were responsible for abnormalities (Thorgaard et al. 1990). Although no

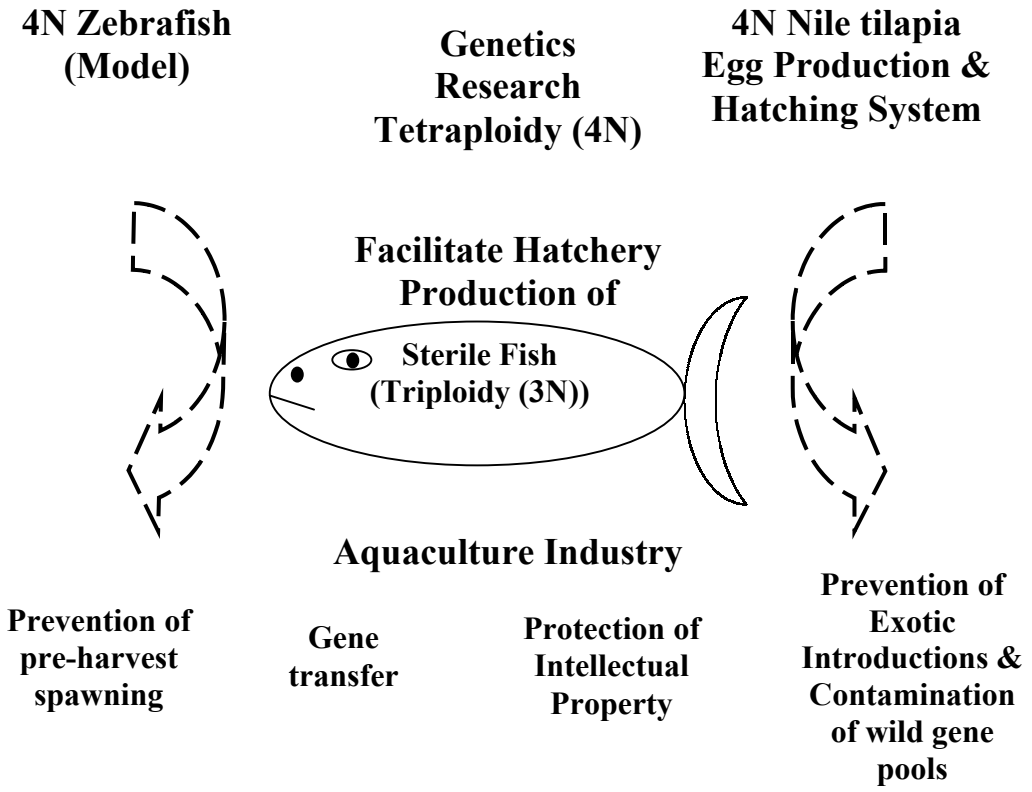

Figure 7.1. Overview of how research presented in this thesis could lead to a better understanding of tetraploidy induction in fishes, which may result in a means to produce sterile fish (triploids) on a commercial scale.

tetraploids were found among adults surviving heat shocks, it may have been possible to obtain adult tetraploids if larger numbers of eggs were shocked (beyond the 400 in this study). Other types of shocks may be more effective at inducing tetraploidy in zebrafish or in increasing survival to adulthood. Combined pressure and ether shocks were reported to be more effective than heat shocks at producing mitotic gynogenetic zebrafish (Horstgen-Schwark 1993). Zebrafish are an important research model yielding information in biotechnology, developmental biology and genetics. Further research on the induction of tetraploidy in zebrafish may provide a better understanding of tetraploidy induction in general and serve as a model for the study of

tetraploidy in other fishes. Furthermore, tetraploids could provide a reliable means of producing large numbers of sterile triploids. Triploids could be used for biomedical and gene transfer research and protect intellectual property and decrease the risk of the perpetuation of the transgenic genomes.

Nile tilapia are an important aquaculture species grown throughout the world. However, overpopulation resulting from pre-harvest spawning can result in decreased production. The production of triploids by shocking newly fertilized eggs is possible, but is not commercially viable. Although several studies have reported the induction of tetraploidy in Nile tilapia fry, there are no reports of tetraploids surviving to adulthood. Research on polyploidy is dependent upon access to ripe eggs and involves the application of multiple treatments to eggs. Furthermore, the shocking of eggs can increase the risk of early mortality. The single-female spawning of Nile tilapia and the egg hatching systems described in this thesis addressed these issues.

The stocking of ten 80-L tanks with single Nile tilapia females (Chapter 4) resulted in a reliable supply of eggs for research. The degree of extension of the urogenital papilla was a reliable indicator of spawning readiness with few strip spawns (< 10%) being discarded due to poor quality. There was a gradual increase in the number of spawns per month from January through September. This was followed by a decline in the number of spawns per month with only one occurring in December. Individual fish tended to spawn for periods of 5 to 7 months. One fish yielded a total of 12 spawns and others only 2. The longest spawning interval (calculated for the year) was 182 days and the shortest 30. On average, seven spawns were produced by each tank and the spawning interval was 73 days. The entire system produced 72 spawns for a total of ~90,000 eggs. The total number of eggs collected from fish that had

spawned in tanks (1,752 ± 72) was significantly higher than the number of eggs collected from strip spawns (1,276 ± 309) indicating that all eggs were not removed from the fish during stripping. Tank position had no significant effect on the number of spawns produced.

The hatching systems described in Chapter 5 were effective for the artificial incubation of tilapia eggs with ~75% of the controls surviving to hatch. The use of methylene blue in System 1 and ultraviolet sterilization in System 2 reduced losses of eggs to disease. The system designs, and use of test tubes as hatching vessels allowed for easy viewing and access. Possibly the most beneficial characteristic of the systems was their capacity to accommodate multiple treatments of eggs for genetics research while occupying little space.

The induction of polyploidy in Nile tilapia by use of heat shock is possible by applying 42.8 ± 0.2 C heat shocks (temperatures measured using a glass thermometer) to eggs for 3 min at 22 to 28 min AF (Chapter 6). Results from the generation of a heat-shock profile (temperatures measured using a thermocouple) indicated that shocks to eggs among the five trials were uniform, increasing gradually from 42.9 ± 0.2 C to 43.2 ± 0.3 C over the 3 min duration. This was made possible by the use of egg baskets that also facilitated the handling of multiple treatments of eggs.

Shocks applied at 28 min AF resulted in the highest percentage tetraploidy (34%). However, low survival in that treatment (< 10%) resulted in poor tetraploid yield (~5%). Shocks applied at 24 min AF accounted for the highest tetraploid yield (10%). The highest percentage of triploidy (27%) and triploid yield (7%) resulted from shocks applied at 22 min AF. The induction of tetraploidy with heat shocks at 22 min AF is the earliest reported time of tetraploidy induction in this species and the induction of triploidy at 28 min AF is the latest.

This is the first study to report the simultaneous induction of tetraploidy and triploidy within treatments from a single spawn. This raises questions about the synchrony of zygotic development in Nile tilapia and has implications for the reported variation in timing for polyploidy induction in this species. Asynchrony may be a technical artifact in the induction process, although the consistent protocols used in this study reduce the chances of that being the cause. Also, biological asynchrony may result from artificial spawning. The stripping of eggs and the gauging of spawning readiness solely on the degree of extension of the urogenital papilla may be key factors. Genetic effects may contribute to asynchronous zygotic development as well. One female produced eggs that resulted in almost 100% polyploidy in all treatments while another female produced eggs that did not result in a single polyploid in any of the treatments.

Although no tetraploid Nile tilapia survived to adulthood in this study, further studies using larger numbers of eggs (more than 5,000 eggs used in this study) may result in tetraploid adults. Other types of shocks initiated at similar times AF to the heat shocks in this study may also result in tetraploid adults. Cold shocks have been used to produce tetraploid blue tilapia, *O. aureus,* that survived to adulthood (Don and Avtalion 1988). Research on asynchrony of zygotic development may lead to a better understanding of the causes behind it, allow for increased synchrony and improve the chances of producing adult tetraploids. If successful, the development of tetraploid lines of Nile tilapia may provide a reliable means of producing triploids on a commercial scale.

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APPENDIX A
STANDARD OPERATING PROCEDURES (SOPS)

SOP-1. Preparation Of Hanks' Balanced Salt Solution (HBSS)

To make 1 L of HBSS combine the following in order:

8.00g NaCl

0.40g KCl

0.16g $CaCl_2 \cdot 2H_2O$

0.20g $MgSO_4 \cdot 7H_2O$

0.06g Na_2HPO_4

0.06g KH_2PO_4

0.35g $NaHCO_3$

1.00g $C_6H_{12}O_6$ (glucose)

~980 mL distilled water (Osmolality should be ~300 mOsmol/Kg)

Store at 4 C.

SOP-2. Preparation Of Embryo Medium Buffer (EMB)*

Make the following stock solutions and store at 4 C.

Solution 1: 8.0g NaCl, 0.4g KCl, 100 mL distilled water

Solution 2: 0.358g Na_2HPO_4 (anhydrous), 0.60g KH_2PO_4, 100 mL distilled water

Solution 3: 0.72g $CaCl_2$, 50 mL distilled water

Solution 4: 1.23g $MgSO_4 \cdot 7H_2O$, 50 mL distilled water

Solution 5: (Note, make fresh) 0.35g $NaHCO_3$, 10 mL distilled water

To make 1 L of EMB combine the following in order:

10 mL of solution 1

1 mL of solution 2

10 mL of solution 3

959 mL of distilled water

10 mL of solution 4

10 mL of solution 5

Adjust pH to ~7.2

Store at 4 C.

*Modified from Westerfield 1995.

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SOP-3. Egg Baskets For Heat Shocking Of Zebrafish Eggs

Materials needed:

25-mL plastic cryopreservation goblets
Scissors
30-μm nylon mesh
Aluminum foil
Hot plate

Procedure:

1. Cover the hot plate with aluminum foil.
2. Turn the hot plate up to ~8.
3. Cut plastic cryopreservation goblets into 1.5-cm sections.
4. Cut pieces of 30-μm nylon mesh to a size that covers the ends of the 1.5-cm sections.
5. Place a piece of nylon screen on the hot plate.
6. Place the end of a 1.5-cm section on top of the screen and apply pressure until the plastic melts into the screen. (Be careful not to touch the hot plate).
7. Remove, let cool, and trim the excess screen with scissors.

SOP-4. Egg Baskets For Heat Shocking Of Nile tilapia Eggs

Materials needed:

50-mL plastic centrifuge tubes
Scissors
Nylon window screening
Aluminum foil
Hot plate
Bunsen burner
Pliers
Small finish nail

Procedure:

1. Cover the hot plate with aluminum foil.
2. Turn the hot plate up to ~8.
3. Cut the conical bottom off of the 50-mL centrifuge tubes with scissors or a sharp knife.
4. Cut pieces of nylon window screening to a size that covers the cut ends of the 50-mL plastic centrifuge tubes.
5. Place a piece of screen on the hot plate.
6. Place the cut end of the 50-mL plastic centrifuge tube on top of the screen and apply pressure until the plastic melts into the screen. (Be careful not to touch the hot plate).
7. Remove, let cool, and trim the excess screen with scissors.
8. Hold the small finish nail with pliers and heat the tip over the Bunsen burner.
9. Make ~ 15 holes in the cap of the 50-mL centrifuge tube with the nail.

SOP-5. Preparation Of Egg Hatching Water

To make 1 L of Egg Hatching Water, combine the following:

1.1 g artificial sea salt (Marinemix, Wiegandt Gmblt, Krefeld, Germany)

1 L distilled water

SOP-6. Collection Of Zebrafish Sperm And Eggs And Artificial Fertilization

Sperm Collection*

Materials Needed:

Tricaine methanesulfonate (methyl-m-aminobenzoate, MS-222, Argent Laboratories, Inc., Redmond, Washington)
Latex gloves
Tank water
300-mL watch glass
Spoon
Kimwipes ®
100-mm plastic lid from a cell culture dish
Dissecting microscope
10-µL pipette and tip
1.5-mL microcentrifuge tube
Hanks' balanced salt solution (HBSS) (SOP-1)
Container with crushed ice

Procedure:

1. Put 10 µL of HBSS in the 1.5-mL microcentrifuge tube and place on ice.
2. Make 200-mL of a 0.17mg/mL MS-222 solution in the 300-mL watch glass using tank water. (Wear latex gloves when working with MS-222)
3. Place a male in the MS-222 solution.
4. When the fish loses equilibrium, remove with a spoon and gently blot dry with a Kimwipe, especially the area around the urogenital pore and pelvic fins.
5. Place the fish upside down on the plastic lid and view with the dissecting microscope.
6. Spread the pelvic fins away from the urogenital pore with the pipette tip.
7. Gently squeeze the abdomen with your fingers just anterior to the anal fin and draw the sperm into the pipette tip.
8. Put the male in a recovery container.
9. Expel the sperm into the HBSS in the microcentrifuge tube and place on crushed ice.
10. Repeat until you have collected the desired amount of sperm.
11. Maintain the sperm and HBSS on ice until artificial insemination.

Egg Collection*

Materials Needed:

Tricaine methanesulfonate (methyl-m-aminobenzoate, MS-222, Argent Laboratories, Inc., Redmond, Washington)
Tank water
300-mL watch glass
Spoon

Kimwipes ®
Paper towels
100-mm petri dishes
Plastic eyedroppers
Hanks' balanced salt solution (HBSS) (SOP-1)
Embryo medium (EMB) (SOP-2)
Procedure:

1. Make 200-mL of a 0.17mg/mL MS-222 solution in the 300-mL watch glass using tank water. (Wear latex gloves when working with MS-222)
2. Place a female in the MS-222 solution.
3. When the fish loses equilibrium, remove with a spoon, quickly immerse and remove fish in a dish containing EMB, lay on a paper towel and gently blot dry with a Kimwipe.
4. Place fish on side in a petri dish and express the eggs by applying gentle pressure with the finger along the side of the fish, starting near the pectoral fins and working toward the tail. If eggs are not produced, do not press harder as this may injure the fish.
5. If eggs are obtained, draw HBSS into a plastic eyedropper and gently wash the eggs away from the female.
6. Place the female in a recovery tank.
7. Make sure the eggs are covered with HBSS
8. Repeat until enough eggs are collected.

In Vitro* Fertilization

Materials Needed:
Embryo medium buffer (EMB) (SOP-2)

Procedure:

1. Tilt the petri dish to concentrate the eggs on one side and pour off the HBSS until eggs are just covered.
2. Distribute the sperm over the eggs with the pipette.
3. Gently mix the sperm and eggs with the pipette tip.
4. Add 1 mL of EMB to activate the sperm and eggs and gently swirl back and forth a few times and let sit for 1 min. (The time the of fertilization is considered to be when the EMB is added to the eggs and sperm, not when the sperm is added to the eggs.)
5. Add another mL of EMB, swirl and let sit for another min.

* Modified from Westerfield 1995.

References

Westerfield, M. 1995. *The Zebrafish Book. Guide for the Laboratory Use of Zebrafish (Danio rerio). Third edition.* University of Oregon Press, Eugene, Oregon, USA.

SOP-7. Collection Of Nile Tilapia Sperm And Eggs And Artificial Fertilization

Sperm Collection

Materials Needed:

Paper towels
200-µL pipette and tip
1.5-mL microcentrifuge tube
Hanks' balanced salt solution (HBSS) (SOP-1)
Container with crushed ice

Procedure:

1. Put 500 µL of HBSS in the 1.5-mL microcentrifuge tube and place on ice.
2. Remove a male from a tank, place a paper towel over its head so its eyes are covered and hold upside down.
3. Blot the fish dry with a paper towel, especially the area around the urogenital papilla.
4. Wash the urogenital papilla with a little bit of HBSS.
5. Gently squeeze the abdomen between your thumb and fingers just anterior to the urogenital papilla.
6. Draw the sperm into the pipette tip. (Try to collect only sperm. If urine is present, do not collect. Wipe away the urine with a dry paper towel and try again.)
7. Expel the sperm into the HBSS in the microcentrifuge tube and place on crushed ice.
8. Put the male back in its tank.
9. Maintain the sperm and HBSS on ice until artificial insemination.

Egg Collection

Materials Needed:

Tricaine methanesulfonate (methyl-m-aminobenzoate, MS-222, Argent Laboratories, Inc., Redmond, Washington)
40-L bucket
Tank water
300-mL watch glass
Paper towels
Hanks' balanced salt solution (HBSS) (SOP-1)

Procedure:

1. Add ~ 8 L of tank water to a 20-L bucket and add MS-222 to make a 0.2g/L solution.
2. Place a female in the MS-222 solution.
3. When the fish loses equilibrium, remove and blot dry with paper towels, especially the area around the urogenital papilla.
4. Place a paper towel over the fish's head covering the eyes and hold upright.

5. Express the eggs into the 300-mL watch glass (containing ~100mL HBSS) by gently squeezing the abdomen between the thumb and fingers and stroking in a head-to-tail direction (start near the pectoral fins and work toward the tail). Ripe eggs will be uniform in shape, size and color and are easy to strip. If eggs do not come out easily, do not press harder as this may injure the fish. Return the fish to its tank. If eggs are white, non-uniform in size and shape or if a good amount of connective tissue or blood is present, do not use in experiments.
6. Return the female to its tank and revive.

In Vitro Fertilization

Materials Needed:

Egg hatching water (SOP-5)

Procedure:

1. Tilt the 300-mL watch glass to concentrate the eggs on one side and pour off the HBSS until eggs are just covered.
2. Distribute sperm over the eggs using the pipette.
3. Gently mix the sperm and eggs with the pipette tip.
4. Depending on the amount of eggs, add 10 to 20 mL of egg hatching water to activate the sperm and eggs (allowing for fertilization), gently swirl back and forth a few times and let sit for 1 min. (The time of fertilization is considered to be when the egg hatching water is added to the eggs and sperm, not when the sperm is added to the eggs.)
5. Add another 10 to 20 mL of egg hatching water, swirl and let sit for another min.

SOP-8. Heat Shocking Of Zebrafish Eggs

Materials Needed:

Two warm-water baths
Four 300-mL watch glasses
EMB (SOP-2)
Thermocouple
Zebrafish egg baskets (SOP-3)
Disposable plastic pipette
Tweezers
Paper towels

Procedure:

1. Set up two warm-water baths, one at 28 C (incubation) and the other at 42 C (heat shock), at least 1 h prior to sperm and egg collection.
2. Place a 300-mL watch glass upside down in both baths.
3. Place another 300-mL watch glass right side up on top of the other watch glasses.
4. Add EMB to each of the watch glasses to a depth of ~1.5 cm (the height of the egg baskets).
5. Make sure the level of the bath water on the outside of the watch glasses is equal to or slightly above the level of the EMB on the inside of the watch glasses.
6. After 30 min, check the temperature of the EMB with a thermocouple.
7. Adjust the water bath temperature settings to get the EMB to the desired temperature.
8. Place the necessary amount of egg baskets in the incubation watch glass off to one side.
9. Collect sperm and eggs and fertilize (SOP-6). (When activating sperm and eggs for *in vitro* fertilization, be sure to use EMB from the incubation bath.)
10. Two min after fertilization (AF), transfer fertilized eggs to the incubation watch glass using a disposable plastic pipette.
11. Eight min AF, place the desired number of eggs into each basket using the plastic pipette.
12. At the desired time of shock application, remove a basket with tweezers, quickly blot on a paper towel and transfer to the heat shock watch glass.
13. At the end of the desired duration of the shock, remove the basket, blot on a paper towel and place back in the incubation watch glass.
14. Repeat the procedure with other egg baskets at the desired times and for the desired durations.

SOP-9. Heat Shocking OF Nile tilapia Eggs

Materials Needed:

Two warm-water baths
Two 1-L beakers
Egg hatching water (SOP-5)
Aquarium aerator with 2 airlines and airstones
Thermocouple
Five 100-mL watch glasses
Nile tilapia egg baskets (SOP-4)
Disposable plastic pipette
Paper towels
Hanks' balanced salt solution (HBSS) (SOP-1)

Procedure:

1. Set up two warm-water baths, one at 28 C (incubation) and the other at 42 C (heat shock), at least 1 h prior to sperm and egg collection.
2. Set a 1-L beaker filled with 800 mL of egg hatching water in each of the baths.
3. Make sure the level of the bath water on the outside of the beakers is equal to or slightly above the level of the egg hatching water on the inside of the beakers.
4. Place an airstone in each of the beakers.
5. After 30 min, check the temperature of the egg hatching water with a thermocouple.
6. Adjust the water bath temperature settings to get the egg hatching water to the desired temperature.
7. Rinse the required number (number of shock treatments plus control group) of 100-mL watch glasses with HBSS.
8. Collect the sperm and eggs (SOP-7).
9. Add the desired volume of eggs (1 to 2 mL usually) to each 100-mL watch glass (using a disposable plastic pipette with the tip cut off to accommodate tilapia eggs) making sure they are covered with HBSS.
10. Fertilize the eggs (SOP-7) of the first treatment group. (Be sure to use egg-hatching water from the incubation beaker).
11. After 1.5 min, set a Nile tilapia egg-hatching basket (SOP-4) off to the side in the watch glass.
12. Transfer the eggs into the egg basket with the disposable plastic pipette.
13. Place the egg basket to the incubation beaker.
14. After the elapse of the desired time interval between treatments, repeat the procedure for the next treatment group.
15. At the desired time of shock application, simultaneously remove all treatment egg baskets from the incubation bath, quickly blot on a paper towel and simultaneously immerse in the heat shock beaker. (This ensures that all treatment groups are subjected to an identical shock).
16. After the elapse of the desired duration of the shock, simultaneously remove all treatment egg baskets, quickly blot on a paper towel and transfer back to the incubation bath.

Figure A.1. A) The separation of Nile tilapia eggs into treatment groups for heat shocking experiments. B) Incubation of Nile tilapia eggs in egg baskets. C) Heat shocking of Nile tilapia eggs.

APPENDIX B
UNANALYZED DATA CHAPTER 3

Table B.1. Unanalyzed data for range finding trials to induce tetraploidy in zebrafish by application of 41 C heat shocks to newly fertilized eggs. The treatment terms indicate the time of shock application (min after fertilization) followed by shock duration (min) in parentheses. The letter C denotes control (no shock).

Trial	Treatment	Number of eggs	Number of fish (day 7)	% S	Number 4N	% 4N	% 4N yield
1	13(2)	41	16	39.0	0	0.0	0.0
1	13(5)	56	36	64.3	0	0.0	0.0
1	15(2)	66	50	75.8	few	.	.
1	15(5)	70	38	54.3	0	0.0	0.0
1	20(2)	51	37	72.5	few	.	.
1	20(5)	53	34	64.2	0	0.0	0.0
1	C	45	25	55.6	0	0.0	0.0
2	13(2)	56	18	32.0	0	0.0	0.0
2	13(5)	162	0	0.0	0	0.0	0.0
2	15(2)	48	3	6.3	0	0.0	0.0
2	15(5)	18	0	0.0	0	0.0	0.0
2	16(2)	65	7	10.8	6	85.7	9.0
2	C	35	28	80.0	0	0.0	0.0

Table B.2. Unanalyzed data for three replicated experiments to induce tetraploidy in zebrafish by application of 41 C heat shocks to newly fertilized eggs. The treatment terms indicate the time of shock application (min after fertilization), followed by shock duration (2 min) in parentheses.

Trial	Treatment	Number of eggs	Number of fish (day 7)	% S	Number 4N	% 4N	% 4N yield
1	11(2)	148	3	2.03	2	66.7	1.4
1	13(2)	139	3	2.16	3	100.0	2.2
1	15(2)	128	10	7.81	0	0.0	0.0
1	17(2)	127	6	4.72	4	66.7	3.2
1	20(2)	88	14	15.91	9	64.3	10.2
1	C	120	22	18.33	0	0.0	0.0
2	11(2)	37	0	0	0	0.0	0.0
2	13(2)	29	3	10	1	33.0	3.3
2	15(2)	33	5	15	2	40.0	6.0
2	17(2)	49	17	35	5	29.0	10.0
2	20(2)	52	9	17	5	56.0	9.5
2	C	11	8	73	0	0.0	0.0
3	11(2)	58	1	1.72	.	.	.
3	13(2)	63	3	4.76	.	.	.
3	15(2)	60	1	1.67	.	.	.
3	17(2)	67	4	5.97	.	.	.
3	20(2)	59	9	15.25	.	.	.
3	C	98	67	68.37	.	.	.

APPENDIX C
UNANALYZED DATA CHAPTER 4

Table C.1. Unanalyzed data of egg parameters for 10 Nile tilapia strip spawns.

Date	Female	V Eggs (ml)	Eggs/ml	Total # Eggs
12/03/01	T-9	6.5	188	1222
09/18/01	T-3	6.2	173	1066
09/17/01	T-4	6.0	128	768
09/10/01	T-9	7.6	238	1808
09/01/01	T-7	8.3	121	998
09/01/01	T-3	8.9	173	1534
08/26/01	T-9	6.9	222	1533
08/09/01	T-6	7.5	169	1265
08/08/01	T-1	10.0	111	1110
07/17/01	T-7	10.7	136	1455

Table C.2. Unanalyzed data of egg parameters for 5 Nile tilapia tank spawns.

Date	Female	Weight (g)	V Eggs (ml)	Eggs/ml	Total # Eggs	# Eggs/ g Female
02/21/02	P-A-4	387	14.8	113	1672	4.3
03/02/02	P-B-6	475	14.7	127	1867	3.9
03/03/02	T-10	372	14.6	120	1752	4.7
03/18/02	P-B-1	395	15.5	111	1721	4.4
04/05/02	P-B-1	398	14	125	1750	4.4

APPENDIX D
LIST OF MATERIALS CHPATER 5

List of Materials:

System 1

Two 2.44-m pressure treated 2x4s,
Three m of 7.62-cm schedule 40 polyvinyl chloride (PVC) pipe,
Three 7.62-cm PVC end cap,
One 7.62-cm PVC 90º elbow,
One 7.62-cm to 1.5" PVC reducer,
One 3.81-cm PVC Tee,
Three m of 3.81-cm schedule 40 PVC,
Fifteen plastic valves (Part # VPL1, Aquatic Eco-Systems Inc., Apopka, Florida),
One 1/8 X 27 NPT tap,
One 0.873-cm drill bit,
One roll Teflon tape,
Five m of 0.397-cm inside diameter vinyl tubing,
One 2.54-cm hole saw,
One rat's tail file,
Fifteen 75-mm test tubes (Pyrex®, Corning, Acton, Massachusetts),
One 40-L aquarium,
One submersible aquarium pump (MINI JET MN606, Aquarium Systems, Italy),
Six feet of 1.27-cm inside diameter vinyl tubing,
One bag of long cable ties,
Six 1.91-cm stainless-steel wood screws,
Fourteen 6.99-cm sheetrock screws,
An electric drill with screwdriver bits,
A hand-held jig saw,
A saw for cutting PVC and wood,
Methylene blue (Aquatrol Inc., Anaheim, California),
Aquarium aerator and airstone.
Total cost of materials: ~$125 (not including tools).

System 2

Six mof pressure treated 2X4s,
Five m of 7.62-cm schedule 40 polyvinyl chloride (PVC) pipe,
Three 7.62-cm PVC end cap,
Two 7.62-cm PVC 90º elbow,
One 7.62-cm to 1.5" PVC reducer,
One 3.81-cm PVC Tee,
Three m of 3.81-cm schedule 40 PVC pipe,
One 0.873-cm drill bit,
One 1/8 X 27 NPT tap,
Thirty plastic valves (Part # VPL1, Aquatic Eco-Systems Inc., Apopka, Florida),
One roll Teflon tape,

Nine m of 0.397-cm inside diameter vinyl tubing,
One 2.54-cm hole saw,
One rat's tail file,
Thirty 75-mm test tubes (Corning Pyrex®, Acton, Massachusetts),
One Living Stream (Frigid Units Inc., Toledo, Ohio),
Three m 2.54-cm schedule 40 PVC pipe,
Two 2.54-cm PVC 45º fittings,
One 2.54-cm PVC tee,
One 2.54-cm ball valve,
One 2.54-cm elbow,
One 2.54-cm threaded male adapter,
One 1/8 HP pump (Little Giant Pump Company, Oklahoma City, Oklahoma),
Three m of 1.27-cm schedule 40 PVC pipe,
One 1.27-cm female threaded adapter,
One 1.27-cm compression coupling,
One 1.27-cm PVC tee,
Two 1.27-cm ball valves,
One 1.27-cm elbow,
One 1.91-cm to 1.27-cm reducer,
Three m of 1.91-cm schedule 40 PVC pipe,
Four 1.91-cm elbows,
Six 1.91-cm male threaded adapters,
Two 1.91-cm 45º fittings,
Two water filters (Culligan model # HF-360, Sheboygan, Wisconsin),
One 5-µm filter cartridge,
One 2-µm activated carbon filter cartridge,
One 8-W ultraviolet sterilizer (Aqua, Temecula, California),
One bag of long cable ties,
Twelve 1.91-cm stainless-steel wood screws,
Twenty four 6.99-cm sheetrock screws,
An electric drill with screw driver bits,
A hand-held jig saw,
A saw for cutting PVC and wood.
Total cost of materials: ~$550 (not including: Living Stream and tools).

APPENDIX E
UNANALYZED DATA CHAPTER 6

Table E.1. Unanalyzed data from range finding trials to induce tetraploidy in Nile tilapia. Two heat shocks were applied to newly fertilized eggs.

Trial	Number of Eggs	Incubation Temp C	Shock 1 Application time (min AF)	Shock 1 Temp C	Shock 1 Duration (min)	Shock 2 Application time (min AF)	Shock 2 Temp C	Shock 2 Duration (min)	Age of Fish at Ploidy Evaluation (days)	Survival to Ploidy Evaluation (# of fish)	Tetraploidy (# of fish)	Tissue Evaluated
1	~1000	28.5	65	40.5	3	80	40.5	3	315	56	0	blood
2	~1000	28.8	66	41	5	80	43	2	300	13	0	blood
3	~1000	29	65	41.5	5	80	41.5	5	270	35	0	blood
4	~1000	29	36	43	3	50	43	3	3	24	0	sacfry

Table E.2. Unanalyzed data from range finding trials to induce tetraploidy in Nile tilapia. A single heat shock was applied to newly fertilized eggs.

Trial	Number of Eggs	Incubation Temp C	Shock Application time (min AF)	Shock Temp C	Shock Duration (min)	Age of Fish at Ploidy Evaluation (days)	Survival to Ploidy Evaluation (# of fish)	Tetraploidy (# of fish)	Tissue Evaluated
1	250	29	20	41.6	5	3	1	0	sacfry
1	250	29	25	41.6	5	3	2	0	sacfry
1	250	29	30	41.6	5	3	12	0	sacfry
1	250	29	35	41.6	5	3	1	0	sacfry
1	250	29	40	41.6	5	3	11	0	sacfry
1	250	29	45	41.6	5	3	1	0	sacfry
2	250	29	35	42	5	3	8	0	sacfry
2	250	29	40	42	5	3	11	0	sacfry
2	250	29	45	42	5	3	0	0	sacfry
2	250	29	50	42	5	3	0	0	sacfry
2	250	29	55	42	5	3	1	0	sacfry
3	300	29	30	41.8	5	3	43	0	sacfry
3	300	29	35	41.8	5	3	0	0	sacfry
3	300	29	40	41.8	5	3	0	0	sacfry
3	300	29	45	41.8	5	3	23	0	sacfry
3	300	29	50	41.8	5	3	0	0	sacfry
4	220	29	22	42.5	3	4	1	1	sacfry
4	220	29	24	42.5	3	4	0	0	sacfry
4	220	29	26	42.5	3	4	2	2	sacfry
4	220	29	28	42.5	3	4	1	1	sacfry
5	250	29	26	42	3	3	6	0	sacfry
5	250	29	28	42	3	3	12	0	sacfry
5	250	29	30	42	3	3	13	0	sacfry
5	250	29	32	42	3	3	2	0	sacfry
6	144	29	24	42	3	4	0	0	sacfry
6	144	29	26	42	3	4	0	0	sacfry
6	144	29	28	42	3	4	31	1	sacfry
7	130	29	22	42.2	3	4	0	0	sacfry
7	130	29	24	42.2	3	4	2	0	sacfry
7	130	29	26	42.2	3	4	2	0	sacfry
7	130	29	28	42.2	3	4	0	0	sacfry
8	124	29	26	42.8	3	4	44	1	sacfry
8	124	29	28	42.8	3	4	83	0	sacfry
8	124	29	30	42.8	3	4	96	0	sacfry
8	124	29	32	42.8	3	4	19	0	sacfry
8	163	29	26 to 32	42.8	3	210	41	0	blood

Table E.3. Unanalyzed data for percent survival (% S), percent triploidy (% 3N), percent triploid yield (% 3N yield), percent tetraploidy (% 4N) and percent tetraploidy yield (% 4N yield) for 7 replicated trials to induce tetraploidy in Nile tilapia (Triploids were an unexpected result). Heat shocks of 42.8 C were applied to newly fertilized eggs for a duration of 3 min. Treatment indicates the time of shock application (min after fertilization (AF)). The letter C denotes control (no shock). Ploidy was evaluated using dissociated 5-day-old fry.

	Trial 1	**Trial 2**	**Trial 3**	**Trial 4**	**Trial 5**	**Trial 6**	**Trial 7**
Treatment	% S	% S	% S	% S	% S	% S	% S
28	0.5	0.8	15.3	18.5	0.0	24.2	5.0
26	0.9	5.1	77.4	33.0	0.0	29.7	12.7
24	0.0	4.7	66.9	31.9	0.0	63.3	14.6
22	0.5	2.4	35.5	35.0	10.9	43.8	4.6
C	54.5	55.7	97.6	69.6	61.3	96.1	86.2

	Trial 1	**Trial 2**	**Trial 3**	**Trial 4**	**Trial 5**	**Trial 6**	**Trial 7**
Treatment	% 3N	% 3N	% 3N	% 3N	% 3N	% 3N	% 3N
28	0	0	0	0	0	25	6.05
26	0	0	0	5	0	60	30
24	0	0	0	10	0	20	12.66
22	0	0	0	35	15	90	58.33
C	0	0	0	0	0	0	0

	Trial 1	**Trial 2**	**Trial 3**	**Trial 4**	**Trial 5**	**Trial 6**	**Trial 7**
Treatment	% 3N Yield	% 3N Yield	% 3N Yield	% 3N Yield	% 3N Yield	% 3N Yield	% 3N Yield
28	0	0	0	0	0	7.69	0.30
26	0	0	0	1.65	0	17.82	3.81
24	0	0	0	3.19	0	12.66	8.03
22	0	0	0	12.25	1.635	35	2.66
C	0	0	0	0	0	0	0

	Trial 1	**Trial 2**	**Trial 3**	**Trial 4**	**Trial 5**	**Trial 6**	**Trial 7**
Treatment	% 4N	% 4N	% 4N	% 4N	% 4N	% 4N	% 4N
28	1	0	0	80	0	75	84.6
26	2	0	0	70	0	35	50
24	0	0	0	35	0	75	45
22	1	0	5	65	5.45	10	8.33
C	0	0	0	0	0	0	0

	Trial 1	**Trial 2**	**Trial 3**	**Trial 4**	**Trial 5**	**Trial 6**	**Trial 7**
Treatment	% 4N Yield	% 4N Yield	% 4N Yield	% 4N Yield	% 4N Yield	% 4N Yield	% 4N Yield
28	0.45	0	0	14.8	0	18.5	4.23
26	0.9	0	0	23.1	0	10.4	6.35
24	0	0	0	11.1	0	47.48	6.57
22	0.45	0	1.8	22.8	5.45	4.38	0.38
C	0	0	0	0	0	0	0

VITA

Eric Christopher Herbst was born January 31, 1970. In 1988 he enrolled in the University of North Carolina at Charlotte where he met Lorena Llano. In 1992 he received his bachelor of arts degree with a major in Spanish and a certificate of translation -Spanish to English. In 1993 he moved to Paraguay, South America, where he taught English at the language institute Stael Ruffinelli de Ortiz English and Campo Alto, a private high school. In 1994 he returned to the States and taught Spanish and coached soccer at E. Rowan Middle School, E. Spencer, North Carolina. In 1995, Eric returned to Latin America to teach English at Pro Ingles, a private language institute in San Jose, Costa Rica. In 1996 he moved to Isla Bastimentos, Bocas del Toro, Panama, where he worked as a tour operator. In 1997 he returned to the U.S. to change careers and work at North State Fisheries, a hybrid striped bass farm in Pintetown, North Carolina. Working there was enough to convince him to go back to school and pursue a graduate degree in aquaculture. In 1988, Eric reenrolled at the University of North Carolina at Charlotte as a post baccalaureate student. After a year of preparatory courses, he was accepted at Virginia Tech (VPI & SU) and started a master's degree in fisheries science (aquaculture). In 2000, Eric transferred to Louisiana State University where he is currently a candidate for the degree of Master of Science in fisheries science (aquaculture). Eric married Maria Lorena Pascual Llano on June 9, 2001.