

2002

Intracytoplasmic sperm injection (ICSI) in fishes

German Antonio Poleo

Louisiana State University and Agricultural and Mechanical College

Follow this and additional works at: https://digitalcommons.lsu.edu/gradschool_dissertations

Recommended Citation

Poleo, German Antonio, "Intracytoplasmic sperm injection (ICSI) in fishes" (2002). *LSU Doctoral Dissertations*. 3615.

https://digitalcommons.lsu.edu/gradschool_dissertations/3615

This Dissertation is brought to you for free and open access by the Graduate School at LSU Digital Commons. It has been accepted for inclusion in LSU Doctoral Dissertations by an authorized graduate school editor of LSU Digital Commons. For more information, please contact gradetd@lsu.edu.

INTRACYTOPLASMIC SPERM INJECTION (ICSI) IN FISHES

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the degree of
Doctor of Philosophy

in

The School of Forestry, Wildlife, and Fisheries

by
Germán A. Poleo
B.S., Universidad Central de Venezuela, 1992
M.S., University of Ottawa, 1998
May 2002

To my mom

ACKNOWLEDGMENTS

I thank Dr. Terrence Tiersch, Professor of Aquaculture, for acting as my major professor and providing guidance throughout my Ph.D. program and also for tolerating and sometimes understanding my sincerity. I also thank Dr. Robert Romaine, Professor of Aquaculture, Dr. Charles Lutz, Aquaculture Specialist with the Louisiana Cooperative Extension Service, Dr. Michael Vernon, Scientific Director of the Reproductive Endocrine Laboratories at Woman's Hospital, Dr. Robert Godke, Professor of Animal Science, and Dr. Kevin Carman, Associate Professor of Biological Sciences for serving on my Graduate Committee.

I also extend special thanks to all that I have encountered in my time at the Aquaculture Research Station who helped to reduce the stress of being a graduate student. I would like to acknowledge Dr. Masao Murakami, Dr. Arief Boediono, Richard Denniston and Brett Reggio of the LSU Embryo Biotechnology Laboratory and Research Associates Gang Yu and Yanli Li for help and useful tips during the realization of this work.

I specially thank Gina Cheuk for her intellectual and technical assistance during this dissertation, Mr. Stephen Abernathy from Til-Tech Aquafarm for providing the Nile tilapia used in this work and Mr. Ronald Becker from the Louisiana Sea Grant College Program for providing administrative support. Funding for this project was provided by Consejo Nacional de Investigaciones Científicas y Tecnológicas de Venezuela (CONICIT) and the Louisiana Sea Grant College Program.

And last but most importantly, I thank my family Lucia, Emilia and Andres who gave me complete support during this journey.

TABLE OF CONTENTS

DEDICATION	ii
ACKNOWLEDGMENTS	iii
LIST OF TABLES	vi
LIST OF FIGURES	vii
ABSTRACT	xiii
CHAPTER 1: FOREWORD	1
REFERENCES.....	6
CHAPTER 2: INTRODUCTION	8
FERTILIZATION AMONG VERTEBRATES	9
FERTILIZATION IN ZEBRAFISH.....	12
TILAPIA	19
OVERCOMING THE FERTILIZATION BARRIERS IN FISH	21
REFERENCES.....	22
CHAPTER 3: FERTILIZATION OF ZEBRAFISH EGGS BY INTRACYTOPLASMIC SPERM INJECTION (ICSI)	26
MATERIAL AND METHODS	27
RESULTS	34
DISCUSSION	42
REFERENCES.....	45
CHAPTER 4: INTRACYTOPLASMIC SPERM INJECTION IN SITES OTHER THAN THE MICROPYLE IN ZEBRAFISH EGGS	50
MATERIAL AND METHODS	52
RESULTS	57
DISCUSSION	60
REFERENCES.....	64
CHAPTER 5: FERTILIZATION OF NILE TILAPIA EGGS BY INTRACYTOPLASMIC SPERM INJECTION (ICSI)	67
MATERIAL AND METHODS	69
RESULTS	77
DISCUSSION	87
REFERENCES.....	91
CHAPTER 6: INTRACYTOPLASMIC SPERM INJECTION WITH CRYOPRESERVED, FIXED AND FREEZE DRIED SPERM IN NILE TILAPIA	95
MATERIAL AND METHODS	96

RESULTS	102
DISCUSSION	106
REFERENCES.....	111
CHAPTER 7: EVALUATION OF INTRACYTOPLASMIC SPERM INJECTION IN EGGS OF THREE TELEOST FISHES.....	115
MATERIAL AND METHODS	115
RESULTS AND DISCUSSION.....	116
REFERENCES.....	127
CHAPTER 8: SUMMARY AND CONCLUSIONS.....	129
REFERENCES.....	134
APPENDIX A. STANDARD OPERATING PROCEDURES	137
APPENDIX B. FERTILIZATION OF ZEBRAFISH EGGS USING SPERM FROM OTHER SPECIES	149
APPENDIX C. LETTER OF PERMISSION	155
VITA	156

LIST OF TABLES

3-1. Effect of sperm injections from wild-type, pigmented males into eggs from wild-type, pigmented females with activated and non-activated gametes. All embryos were pigmented	35
3-2. Effect of injections of sperm from wild-type, pigmented males into eggs from gold, long-fin females with activated and non-activated. All embryos that developed beyond 42 h were pigmented indicating genetic contribution from injected sperm	36
3-3. Probability of fertilization by ICSI using gametes in different conditions	39
4-1. Development of zebrafish embryos produced by injecting sperm cells through the micropyle or 100 μ m from the micropyle. Control injections were made without sperm	60
5-1. Intracytoplasmic sperm injection in Nile tilapia.....	84
6-1. Effect of cryopreservation and methanol fixation on the ability of Nile tilapia sperm to fertilize after ICSI. Control injections were made without sperm	104
7-1. Characteristics of three teleost species for performance of intracytoplasmic sperm injection (ICSI)	122
7-2. Results of ICSI in zebrafish, Nile tilapia and channel catfish	126
8-1. Potential applications of intracytoplasmic sperm injection (ISCI) in fish	133

LIST OF FIGURES

2-1.	The zebrafish <i>Danio rerio</i> is native to India, Pakistan, and Nepal. Currently the zebrafish is distributed around the world due to its value as a research model and aquarium fish. This picture was downloaded from FishBase a global information system on fishes (www.fishbase.org). Bar is 0.6 cm.	13
2-2.	Early development in zebrafish. Development can be followed easily due to the transparency of the eggs. It is interesting to note the velocity of embryonic development. A) unactivated and unfertilized egg. B) 30 seconds after insemination, the animal pole (AP) and vegetal pole (VP) are clearly defined: arrow shows the micropyle. C) 45 min after fertilization, blastodisc formation (arrow). D) 3 h after fertilization, blastula stage. E) 5 h after fertilization, gastrula stage. F) 20 h after fertilization, embryo shows many of the adult structures: eyes (arrow), all parts of the brain, and movement begins. Hatching occurs ~48 h after fertilization at 28°C.	14
2-3.	Events before, during and after fertilization in zebrafish, <i>Danio rerio</i>	16
2-4.	Calcium wave and cortical reaction in zebrafish eggs.	17
2-5.	Fertilization and activation of zebrafish eggs. Gametes were tracked during fertilization and activation by staining with the DNA-specific dye Hoechst 33342. Panels A, B and C show female and male gametes during the first 10 min after insemination. A) 30 sec after insemination the sperm head (left arrow) is on the surface of the egg membrane. The female chromosomes (right arrow) are positioned close to the sperm entry site at the moment of insemination. B) 5 min after insemination female chromosomes start to split, half will form the female pronucleus and the other half will be extruded as the second polar body. C) 10 min after insemination the second polar body has been extruded from the egg. It is shown surrounded by actin filaments (green). Double staining was performed using Hoechst 33342 to stain DNA and Alexa Fluor® 488 phalloidin to stain filaments of actin. Panels D, E and F show eggs during the first 10 min after activation by placement in fresh water. These panels correspond with the uppers panels and demonstrate that the cell cycle is resumed despite the absence of sperm (i.e. polar body formation and extrusion)	18
2-6.	One of the female Nile tilapia <i>Oreochromis niloticus</i> used for egg production in this study. Scale bar is 4 cm	20
2-7.	Political world map showing some of the countries where Nile tilapia <i>Oreochromis niloticus</i> are cultured (black dots). The information is from FishBase a global information system on fishes (www.fishbase.org).	20

3-1.	A) Zebrafish embryos at 48 h after fertilization: yolk sac (ys), wild-type, pigmented (p), and gold, long-fin (g). B) Activated zebrafish egg: the micropyle is indicated by the arrow. C) Non-activated zebrafish egg during microinjection with sperm: animal pole (a), vegetal pole (v). D and E Injection procedure (note pipette to right of egg): arrow in E shows the location of the sperm. F) Retraction of pipette (note adherence by egg). Scale bars in A and B, 200 μ m; C, 1 mm.....	28
3-2.	Microinjection equipment used during the course of this dissertation. Included two mechanical micromanipulators that moved the holding and injecting pipettes in three dimensions. The eggs were held in a 100-mm tissue culture dish during injections	31
3-3.	Comparison of embryonic stages. A, B and C) Normal zebrafish embryos obtained by artificial insemination. D-H) Embryos produced by ICSI. D) ICSI embryos obtained by injecting sperm from wild-type, pigmented male into eggs from wild-type, pigmented females. A, D and G) Fully developed embryos inside the chorion (48 h after fertilization). B, E and H) Hatched embryo (56 h). C, F) 5-day-old larvae. G-H) Zebrafish embryo produced by injecting sperm from wild-type, pigmented males into eggs from gold, long-fin females. The resulting fish shows the dominant pigmented phenotype.....	38
3-4.	Effect of time after egg collection on fertilization rate after ICSI (n = 153). Eggs were placed in HBSS containing 0.5% BSA immediately after collection. Closed circles indicate unfertilized eggs after ICSI. Open circles indicate fertilized eggs. Squares indicate fish with developmental abnormalities. Pictures show the developmental stages that each embryo passed through	40
3-5.	Development of zebrafish embryos after ICSI. A) Normal (control) embryo (top) at 48 h after artificial insemination and abnormal ICSI embryos at the same time. B-E) The anterior-posterior axis is affected, and the eyes are reduced in size. B) 24 h after ICSI the head is not present. C, D and E) Abnormal larvae (72 h after ICSI) showing pigmentation produced by injection of sperm from pigmented males into eggs from gold females.....	41
4-1.	Injection procedure. A) Sperm injection through the micropyle. B) Sperm injection ~100 μ m from the micropyle	55
4-2.	Fertilization in zebrafish. A) 30 sec after insemination, the sperm (arrow) is still present at the sperm entry site and the maternal chromosomes are about 40 μ m from it; bar 6.8 μ m. B) 5 min after insemination, DNA was labeled with Hoechst 33342 and actin filaments with Alexa fluor® 488 phalloidin, sperm head (blue) is surrounded by a meshwork of actin filaments (green); bar 2 μ m. C) 5 min after insemination, the maternal chromosomes begin to split; bar 6 μ m. D) At 15 min after insemination, female and male pronuclei	

appear decondensed and united. E) At 25 min after insemination, zygote pronuclei can be seen. F) Second polar body labeled with Hoechst and phalloidin	58
4-3. Sperm injected into zebrafish eggs. A) 10 min after injection, eggs were fixed in 4% paraformaldehyde. In 5 of 7 fixed eggs, the sperm was observed (arrow) in proximity to the maternal chromosomes (circle). Two pictures of the same frame but at different levels were taken and aligned for this image. B) DNA of the polar body surrounded by actin filaments. The entire egg surface was labeled with fluorescence. Extrusion of the polar body was observed in 7 of 7 injected eggs.....	59
4-4. Zebrafish embryos 20 h after fertilization. A) Normal embryo after artificial insemination; B) Abnormal embryo after ICSI	61
5-1. System for holding of female Nile tilapia composed of nine 80-L aquaria linked to a biofilter and an ultraviolet sterilizer (UV). This system is located in room 142 at the LSU Aquaculture Research Station	71
5-2. Nile tilapia egg shown in position for sperm injection. The tip of the injecting pipette is pointing to the micropyle of the egg. Scale bar ~100 μ m. The vegetal pole (v) represents more than 70% of the egg volume. The animal pole (a) is present at the narrow end of the egg.....	75
5-3. Fertilization of Nile tilapia eggs stored in Hanks' balanced salt solution (HBSS) from 0 to 80 min.....	78
5-4. Fertilization of Nile tilapia eggs that were stored in Hanks' balanced salt solution (HBSS) for 5 min or 3 hours	80
5-5. A) 30 seconds after insemination, the sperm head (arrow) was present at the micropyle canal (circle dot); bar 2 μ m. B) 30 seconds after insemination, lower magnification than in A: the distance between the sperm head (left arrow) and the maternal chromosomes (right arrow) was ~40 μ m; bar 3.6 μ m. C) 10 min after insemination, the maternal DNA (arrow) has begun to split; bar 3.6 μ m. D) 25 min after insemination, male and female pronuclei were in contact.....	81
5-6. Activation in Nile tilapia eggs. A) 30 min after placement in embryo medium buffer (EMB). There are no signs of activation (water hardening and blastodisc formation). B) 30 min after insertion of the injecting pipette, the chorion is raised and the egg membrane is more rigid. The blastodisc is developed at the animal pole (arrow); scale bar is ~ 750 μ m.....	82
5-7. Activation in Nile tilapia eggs. Eggs were injected with sperm (ICSI), injected only with injecting buffer (Buffer), placed in embryo medium buffer	

without sperm (EMB), or inseminated with goldfish sperm. Shared letters above error bars (\pm SD) represent no significant difference.	82
5-8. Activation in Nile tilapia eggs after DNA was labeled with Hoechst 33342. A) 10 min after insemination with Nile tilapia sperm, meiosis II is resumed as indicated by the division of the female chromosomes (arrow). The incorporated sperm head is shown in the circle. B) 10 min after the eggs were placed in embryo medium without injection, meiosis II was not resumed.....	83
5-9. A) Normal Nile tilapia embryo produced by ICSI, 52 h after injection. B) Abnormal Nile tilapia produced by ICSI, 52 h after injection. Pigment cells looked larger than normal. A large space between the yolk and the epithelium is seen (arrow). This embryo did not survive beyond 72 h.....	85
5-10. ICSI in Nile tilapia. A) 3 days after artificial insemination with fresh Nile tilapia sperm, control. B) 3 days after sperm injection C) same fish (in B) 3 months after hatching. It showed male phenotype	86
6-1. A) Pearl Nile tilapia. This line of fish presents a white coloration in the skin, all other phenotypic characteristics remain the same as wild-type fish B) Wild-type Nile tilapia; bar 12 cm	98
6-2. Intracytoplasmic sperm injection using fresh Nile tilapia sperm. A) 3 days after fertilization, Nile tilapia sac fry produced from artificial insemination (Control). B) 3 days after fertilization, Nile tilapia sac fry produced by ICSI. The pericardial cavity (arrow) was larger than normal, and fish died 7 days after hatching. C) 3 days after fertilization, Nile tilapia sac fry produced by ICSI showing characteristics of normal sac fry. D) The same fish (shown in C) 3 months later.	103
6-3. Intracytoplasmic sperm injection using cryopreserved Nile tilapia sperm. A) Morula stage, 6 h after artificial insemination (Control). B) Morula stage, 6 h after ICSI. The embryos stopped development at this stage. Cells are at the animal pole (arrows).....	105
6-4. A) Fresh and B) Freeze-dried Nile tilapia sperm stained with Hoechst 33342. Note the diffuse appearance of the freeze-dried nuclei.....	107
7-1. Embryonic development of zebrafish <i>Danio rerio</i> . A) Unfertilized egg. B) Activated egg showing the animal pole (a), micropyle (arrow) and the yolk (y) which occupies most of the egg. C) One-cell embryo, 1 h after fertilization. D) Blastula stage, 2.5 h after fertilization. E) Gastrula stage, 5 h after fertilization. F) Embryo 20 h after fertilization, at this time the head (bottom arrow), somites (top arrow) and the tail bud (t) are distinguishable	118
7-2. Embryonic development of Nile tilapia <i>Oreochromis niloticus</i> . A) Activated egg showing yolk (y) in the vegetal pole, and blastodisc (b). B) 2-cell	

embryo, 2 h after fertilization. C) 8-cell embryo, 4 h after fertilization. D) Embryo 48 h after fertilization. The head is forming, the optic bud is evident (arrow) and chromatophores have begun to appear over the dorsal part of the yolk. E) Embryo 52 h after fertilization. Chromatophores are larger. F) Hatched sacfry, 3 days after fertilization	119
7-3. Embryonic development of channel catfish <i>Ictalurus punctatus</i> . A) Unfertilized egg. Animal pole (a), vegetal pole (v), micropyle (arrow) and the chorion covering the egg are easy to differentiate. B) Early blastula, 5 h after fertilization. Blastomeres are visible at the animal pole. C) Blastula stage, 9 h after fertilization. D) Embryo 30 h after fertilization, the antero-posterior axis has developed forming the embryonic keel. Somites (arrow) can also be seen at this stage. E) Sacfry during hatching, 5-days after fertilization. F) Sacfry 6 days after fertilization.....	120
7-4. Size and morphology of unfertilized eggs from zebrafish, Nile tilapia and channel catfish	121
7-5. Micropyle localization for eggs of zebrafish, Nile tilapia and channel catfish. A) Non-activated zebrafish eggs. The animal and vegetal pole as well as the micropyle were difficult to localize. B) Non-activated Nile tilapia egg. The micropyle (arrow) is located at the narrow end of the egg. C) Non-activated channel catfish egg. The animal pole (a) the vegetal pole (v) and the micropyle (arrow) were easily distinguished.....	125
8-1. With the techniques described in this dissertation, ICSI could be use to create small broodstock populations to be used to produce offspring for research or commercial production.....	135
A-1. Diagram of chambers used for the observation of the animal pole of zebrafish and Nile tilapia eggs	145
A-2. Egg collection from Nile tilapia. A) An extended genital pore or papilla (arrow) indicates that the fish is ready to spawn. B) The anesthetized female is striped by gently squeezed the abdomen from the head to the anal fin. C) Ripe egg present a uniform shape and reduce blood and connective tissue in the ovarian fluid.....	148
B-1. Fertilization of zebrafish eggs using sperm from three different families of fish (Ictaluridae, Cyprinidae and Cichlidae). A) Zebrafish embryo 3 h after fertilization (Control). B) Embryo 3 h after fertilization produced using Nile tilapia (<i>Oreochromis niloticus</i>) sperm. C) Embryo 3 h after fertilization produced using goldfish (<i>Carassius auratus</i>) sperm, D) Undeveloped egg inseminated with channel catfish (<i>Ictalurus punctatus</i>) sperm. E) Zebrafish embryo 48 h after fertilization (Control). F) Embryo 48 h after fertilization produced by inseminating zebrafish eggs with goldfish sperm. Yolk sac is	

elongated compared with controls as well as chromatophores. G) Normal zebrafish embryo 48 h after fertilization. H) Embryo produced by inseminating zebrafish eggs with goldfish sperm.	151
B-2. DNA content measured by flow cytometry. DNA values are reported in arbitrary units expressed as fluorescence channel number. A) Wild-type zebrafish (M2, green peak) showing genome size of 3.15 pg/cell. B) Haploid zebrafish (M1, blue peak) showing DNA content of ~1.58 pg/cell were produced by using sperm from Nile tilapia. C) Goldfish (M2, blue) showing genome size of 3.5 ± 0.06 . D) Embryos produced by inseminating zebrafish eggs with goldfish sperm (M2, purple) showing a genome size of ~3.37 pg/cell that was the combination of zebrafish (1N) and goldfish (1N) genomes. Chicken blood was used as internal references (M1) 2.5. pg of DNA/cell	152

ABSTRACT

Sperm from zebrafish, *Danio rerio*, and Nile tilapia, *Oreochromis niloticus*, were microinjected directly into egg cytoplasm to evaluate the potential for developing a novel method of fertilization. In zebrafish, the sperm of two lines (wild-type and gold, long-fin) were injected with or without activation into activated and non-activated eggs. No significant difference ($P = 0.997$) in fertilization by intracytoplasmic sperm injection (ICSI) was observed between the two lines or when the sperm were activated or not ($P = 0.057$). There was significance difference in fertilization between activated and non-activated eggs ($P = 0.010$). The highest fertilization rate was achieved by injection of activated sperm into non-activated eggs (35%). From a total of 188 zebrafish eggs injected, 31 (16%) were fertilized, 10 (5%) developed as abnormal larvae and 3 (2%) developed normally and hatched.

Damage of maternal chromosomes by the injection procedure could have caused the developmental abnormalities observed after ICSI. This was investigated by fluorescence microscopy using a DNA-specific stain (Hoechst 33324). Fixed and stained animal poles of zebrafish 30 sec after artificial insemination revealed that female chromosomes were located ~40 μm from the sperm injection site (micropyle). Staining of the animal pole after sperm injection showed no disruption of the formation of the second polar body or its extrusion. Evaluation of two sperm injection sites in zebrafish showed no difference in fertilization rate ($P = 0.8264$) or reduction of abnormal development.

Nile tilapia eggs placed in Hanks' balanced salt solution retained their viability for at least 3 hours after collection. Of a total of 160 Nile tilapia eggs injected with fresh sperm, 16 (10%) were fertilized, 10 (6%) developed abnormally to neurula and 5 (3%) developed normally and hatched, two of which reached adulthood. From 45 eggs injected with cryopreserved sperm, 9 (20%) were fertilized but none developed beyond blastula stage. Injections of sperm fixed in methanol did not yield fertilization. These results demonstrate for the first time that injection of single sperm cells into the cytoplasm of a fish egg allows fertilization and subsequent development of normal larvae to hatching and beyond.

CHAPTER 1

FOREWORD

“Grown-ups never understand
anything by themselves,
and it is exhausting for children to
have to provide explanations
over and over again”

Antoine de Saint-Exupéry, *The Little Prince*

The words from Antonie de Saint-Exupéry could not be truer. Perceptions vary with perspectives and the amount of knowledge that has been gathered and internalized. As biologists, we often face the problem of communicating experiences to the rest of the world. Although it is tiring to explain the jargon and ideas that comprise our everyday vocabulary we need to find the words and the mechanism by which our work can reach different audiences and avoid misunderstandings. I tried to write this dissertation in the easiest and clearest way I could. This foreword explains the rationale behind this work by beginning with a bit of history of aquaculture and the evolving technology that has accompanied it through two millennia.

The culture of fish is thought to have begun more than 2,000 years ago in China with the culture of common carp, *Cyprinus carpio*. It spread to the Indo-pacific region where other species began to be cultured (reviewed by Avault 1996). Fish culture has become a common practice in Asian countries where seafood is the major source of animal protein. Records of fish culture before European colonization have been found in the New World, specifically in the Bolivian Amazon (Erickson 2000). The indigenous peoples of this region transformed the savanna landscape into fish weirs, causeways and artificial ponds to control and harvest fish. It was only in the late 1850s that fish began to be cultured commercially in North America (Parker 1989).

As with other human activities, aquaculture improved with the use of technologies developed to satisfy individual needs. These developments accompanied increased biological knowledge of the cultured animal such as breeding time, spawning behavior, interaction of animal and environment, food requirements, fertilization mechanisms and genetics. Increased knowledge of spawning behavior allowed culturists to reproduce different fishes in artificial ponds and tanks. Knowledge of the mechanisms of fertilization permitted the artificial spawning of fish that did not spawn readily in captivity, and the spawning of fish outside of normal spawning seasons. Another technique, already in use for centuries, is the genetic improvement of cultured species by selection of desired traits (selective breeding). In the past century, other more invasive genetic methods have been used to improve aquatic species. Some of these methods are gynogenesis, androgenesis, polyploidy, and most recently transgenesis. Gynogenesis and androgenesis refer to the production of fish that carry genetic material from only one parent. These methods can be employed to develop broodstocks for the production of genetically identical clones, monosex populations, and populations with high levels of homozygosity (Lutz 2001). Polyploidy refers to the production of animals that possess more than the normal two sets of chromosomes. Although polyploidy in mammals typically yields abnormalities or death, viable offspring have been produced in many fish and invertebrates (reviewed in Lutz 2001). Transgenesis or the incorporation of foreign genes into a new host was accomplished in mammals 30 years ago (Brackett et al. 1971), and 14 years later the first report in fish was published (Zhu et al. 1985) signaling the beginning of genetic engineering in aquatic animals.

A technique that has been used for almost a century (Lillie 1914) for basic studies of fertilization, and is a potential tool for the genetic improvement of fishes, is the direct injection of sperm cells into the egg cytoplasm. This is called intracytoplasmic sperm injection (ICSI). The first experiments reported using ICSI were accomplished in invertebrates such as starfish (Lillie 1914) and sea urchin (Hiramoto 1962, Dale et al. 1985). Eighty years later, this technique has been incorporated in the assisted reproduction of livestock and humans (Goto et al. 1990, Palermo et al. 1992, Catt et al. 1996, Cochran et al. 1998, Kolbe and Holtz 2000). However, despite its early development in aquatic invertebrates and its regular use in humans and farm animals, ICSI has not been studied in fish. The goal of this dissertation project was to evaluate the potential for performing ICSI in fishes and to develop basic protocols for two species, zebrafish *Danio rerio* and Nile tilapia *Oreochromis niloticus*. These species have characteristics that make them good candidates for the development of ICSI. Both spawn year-round without the need for hormone injections, are easy to maintain, and do not produce adhesive eggs. The zebrafish is intensively studied as a vertebrate research model for biomedical and developmental biology, and the global economic and social importance of Nile tilapia makes it a good model for aquaculture. These points are addressed in Chapter 2.

The specific objectives of the research reported within this dissertation were: 1) to evaluate conditions for injection of sperm into zebrafish eggs (e.g., media, egg quality, site of injection, effect of injection timing; 2) to evaluate the rate of fertilization by injecting sperm in places other than the micropyle in zebrafish eggs; 3) to identify conditions for injection of sperm into Nile tilapia eggs, and 4) to evaluate in Nile tilapia

the success of ICSI when using cryopreserved sperm, freeze-dried sperm and sperm preserved in methanol.

Accordingly, the first question was: Is it possible to overcome the fertilization barriers in fish by injecting sperm directly into the egg cytoplasm? This question is answered in Chapter 3 where different conditions such as injecting solution, gamete activation and the effect of elapsed time after egg collection were investigated. The knowledge obtained from this Chapter provided the basic methods for sperm injections that were used throughout the subsequent Chapters.

Intracytoplasmic sperm injection can mechanically damage some of the structures involved in fertilization and development such as chromosomes, spindle apparatus, cell membrane and cytoskeleton. These could result in a lack of fertilization or compromised development. In mammalian species, some of these problems can be avoided by injecting in specific places where the damage is minimized. In Chapter 4 improvement of ICSI was sought in fish by localization of maternal chromosomes and injection of sperm cells in sites that reduced the possibility of cell injury.

Among the most widely cultured fish in the world are two genera of the family Cichlidae, *Tilapia* and *Oreochromis*. They are native to Africa and the Middle East and are presently produced worldwide due to their popularity as food fish. Their economic value and spawning characteristics also make them good models for aquaculture research. Hybridization, androgenesis and gynogenesis have been employed for the improvement of tilapia. However, the use of ICSI has not been studied. Chapter 5 addresses the potential of using ICSI in Nile tilapia.

The need for germplasm repositories has encouraged researchers to develop techniques to preserve gametes and embryos for extended periods of time. The most common and effective method is cryopreservation. However this method is costly because it requires a constant supply of liquid nitrogen, and risky because it can easily fail. Other methods have been proposed such as the preservation of sperm in different solvents (Katayose et al. 1992); freeze-drying of sperm (Hoshi et al. 1992, Wakayama and Yanagimachi 1998) and anhydrobiosis or suspended animation (Holt 1997). Unlike cryopreservation, sperm that have been chemically stored or freeze-dried are conventionally “dead” because they lose motility and membrane integrity, although the sperm nucleus can maintain genetic integrity when injected (Wakayama and Yanagimachi 1998). Chapter 6 evaluates the potential of using ICSI with Nile tilapia sperm that has been cryopreserved, fixed in methanol or freeze-dried.

Although gametes of most of the fishes that spawn externally have similar characteristics, each species presents unique traits such as fecundity, egg size and morphology, localization of the sperm entry site and mechanisms of activation. These characteristics dictate the approach that must be taken during ICSI. Chapter 7 compares representatives of three genera of fish with research and commercial value, zebrafish, Nile tilapia and channel catfish *Ictalurus punctatus*, and describes the considerations required to perform ICSI in each species.

A manuscript based on Chapter 3 has been published in the journal *Biology of Reproduction* (Poleo et al. 2001). This chapter has been modified to conform to dissertation format. The dissertation was written following format put forth by 6th

Edition of The Council of Biology Editors Manual for Authors, Editors and Publishers
(Council of Biology Editors 1994).

REFERENCES

- Avault, J. W. 1996. Chapter 1 Getting started. Page 4-19 in *Fundamentals of Aquaculture*. AVA Publishing Company, Inc. Baton Rouge, Louisiana.
- Brackett, B., G. W. Baranska, W. Sawicki, and H. Koprowski. 1971. Uptake of a heterologous genome by mammalian spermatozoa and its transfer to ova through fertilization. *Proceedings of the National Academy of Sciences of the United States of America* 68:353-357.
- Catt, S. L., J. W. Catt, G. Evans, and W. M. C. Maxwell. 1996. Birth of male lamb after intracytoplasmic sperm injection of a presumptive male sperm. *Veterinary Research* 139:494-495.
- Cochran, R., M. Meintjes, B. Reggio, D. Hylan, J. Carter, C. Pinto, D. Paccamonti, and R. A. Godke. 1998. Live foals produced from sperm-injected oocytes derived from pregnant mares. *Journal of Equine Veterinary Science* 18:736-740.
- Council of Biology Editors. 1994. *Scientific Style and Format: CBE Manual for Authors, Editors, and Publishers*. 6th edition. Press Syndicate of the University of Cambridge, Cambridge. 825 pp.
- Dale, B., L. J. DeFelice, and G. Ehrenstein. 1985. Injection of a soluble sperm fraction into sea-urchin eggs triggers the cortical reaction. *Experientia* 41:1068-1070.
- Erickson, C. L. 2000. An artificial landscape-scale fishery in the Bolivian Amazon. *Nature* 408:145-6.
- Goto, K., A. Kinoshita, Y. Takuma, and K. Ogawa. 1990. Fertilization of bovine oocyte by the injection of immobilized, killed spermatozoa. *Veterinary Record* 24:517-520.
- Hiramoto, Y. 1962. Microinjection of the live spermatozoa into sea urchin eggs. *Experimental Cell Research* 27:416-426.
- Holt, W. V. 1997. Alternative strategies for the long-term preservation of spermatozoa. *Reproduction, Fertility and Development* 9:309-319.
- Hoshi, K., K. Yanagida, H. Katayose, and H. Yazawa. 1994. Pronuclear formation and cleavage of mammalian eggs after microsurgical injection of freeze-dried sperm nuclei. *Zygote* 2:237-242.

- Katayose, H., M. Junichiro, and R. Yanagimachi. 1992. The ability of dehydrated hamster and human sperm nuclei to develop into pronuclei. *Biology of Reproduction* 47:277-284.
- Kolbe, T. and W. Holtz. 2000. Birth of a piglet derived from oocyte fertilized by intracytoplasmic sperm injection (ICSI). *Animal Reproduction Science* 64:97-101.
- Lillie, F. R. 1914. Studies of fertilization. *The Journal of Experimental Zoology* 16:523-590.
- Lutz, C. G. 2001. Chromosomal genetics. Page 120-161 in *Practical Genetics for Aquaculture*. Fishing News Books, Oxford, United Kingdom.
- Palermo, G., H. Joris, P. Devroey, and A. C. Van Steirteghem. 1992. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet* 340:17-18.
- Parker, N. C. 1989. History, status, and future of aquaculture in the United States. *Reviews in Aquatic Science* 1:97-109.
- Poleo G. A., R. S. Denniston, R. C. Reggio, R. A. Godke, and T. R. Tiersch. 2001. Fertilization of eggs of zebrafish, *Danio rerio*, by intracytoplasmic sperm injection. *Biology of Reproduction* 65:961-966.
- Wakayama, T. and R. Yanagimachi. 1998. Development of normal mice from oocytes injected with freeze-dried spermatozoa. *Nature Biotechnology* 16:639-641.
- Zhu, Z., H. Xu, G. Li, L. He, and S. Chen. 1985. Novel gene transfer into the fertilized eggs of goldfish. *Zeitschrift für angewandte Ichthyologie* 1:31-34 (in German).

CHAPTER 2

INTRODUCTION

This chapter is intended to provide an overview of the general features of fertilization and early development in fishes, which provide the basis for the work in this dissertation. In addition, the fundamental aspects of intracytoplasmic sperm injection and the characteristics of the species studied in this project are discussed.

Microinjection is a useful technique for the study of cellular phenomena, allowing direct injection of substances in specific areas of the cell. It allows measurement of internal and external parameters such as pH and electric potential. This technique has helped to uncover physiological events such as membrane depolarization and structure and function of ion channels. The tools required for microinjection have also been used for nuclear transfer in mammals (Wilmut et al. 1997) and in fish (Shaoyi et al. 1980) and for the production of transgenic animals (reviewed by Chan 1999). It has also yielded crucial information for the study of mechanisms of fertilization by injection of sperm directly into the egg cytoplasm, a procedure called intracytoplasmic sperm injection (ICSI).

The first studies of ICSI were performed with aquatic organisms including echinoderms (Lillie 1914, Hiramoto 1962, Dale et al. 1985) and amphibians (Brun 1974). The first work in mammals appeared 25 years ago with reports that injections of sperm cells into hamster eggs progressed to pronuclei and early cleavage (Uehara and Yanagimachi 1976). Since then, ICSI has gained considerable attention and is now used in assisted reproduction in different mammalian species (Hosoi et al. 1988, Mann 1988, Goto et al. 1990, Catt et al. 1996, Cochran et al. 1998, Pope et al. 1998, Gomez et al. 2000, Kolbe and Holtz 2000) including humans (Palermo et al. 1992), as well as for

the production of transgenic animals (Kroll and Amaya 1996, Perry et al. 1999, Sparrow et al. 2000)

FERTILIZATION AMONG VERTEBRATES

The development of protocols for *in vitro* fertilization (IVF) or ICSI must take into consideration the physiology of normal fertilization. Therefore a brief discussion follows comparing fertilization among vertebrates such as amphibians, mammals and fishes to lay groundwork for the development of suitable protocols for ICSI in fishes.

The physiological status of sperm and eggs at the moment of fertilization are fundamental for the subsequent development of the embryo. For example, during normal fertilization in fishes, amphibians and mammals, the cytoplasm of the egg is arrested in meiotic metaphase II prior to contact with sperm (reviewed by Stricker 1999). Sperm cells must undergo structural membrane changes to allow penetration and fertilization of the egg. This process is called sperm capacitation. In amphibians, the process of sperm capacitation occurs when the sperm comes in contact with the jelly coat that covers the egg (Shiver and James 1970, Brun 1974).

In mammals, sperm capacitation occurs in the female reproductive tract (reviewed in Markert 1983, Topfer-Petersen 1999). Some of the changes that occur during capacitation are modifications to the lipid, and protein composition of the cell membrane and motility of the sperm. Changes in the chemical composition of the sperm membrane make it able to react with the extracellular matrix of the egg (zona pellucida in mammals, jelly coat in amphibians) by releasing enzymes from a structure called the acrosome that digest matrix material (acromosomal reaction). During capacitation, sperm motility is apparently regulated by changes in the intracellular

concentration of calcium ions through a calcium channel (named “CatSper”) that is only expressed in the tail of mature sperm (Ren et al. 2001). It is thought that the capacitation phenomenon is necessary for sperm to fuse with the ooplasmic membrane, but not for subsequent steps of fertilization such as DNA decondensation and pronucleus formation. This is supported by experiments in *Xenopus laevis* (Brun 1974), mice (Kimura and Yanagimachi 1995), and humans (Palermo et al. 1992) in which injection of intact spermatozoa into the egg cytoplasm resulted in development of normal offspring.

However, discrepancies exist. For example, the capacitation steps appear to be critical in bovine fertilization (Goto et al. 1990, Sutovsky 1997) and in other farm animals where ICSI is problematic (Catt 1995). The failure of ICSI in some farm animals may be due to the failure of removal of the perinuclear theca, a cytoskeletal capsule present between the sperm membrane and the nuclear envelope (Sutovsky et al. 1997). This structure could block the access of cytoplasmic factors involved in decondensation of the sperm nucleus. This is supported by results where artificially capacitated bull sperm resulted in pronucleus formation and production of a normal calf after ICSI (Goto et al. 1990).

In fish with external fertilization, the term “sperm capacitation” is usually not mentioned. If capacitation does occur, it probably happens when the sperm comes in contact with water. Fish spermatozoa, in contrast to mammalian spermatozoa, are not motile when in the seminal plasma or in isotonic or isoionic solutions. The sperm become motile when they contact the external medium (fresh water or salt water). For example, change in osmolality regulates sperm motility in the saltwater pufferfish,

Takifugu niphobles, and in the freshwater zebrafish, *Danio rerio* (Takai and Morisawa 1995). On the other hand, in salmonid fishes such as the rainbow trout, *Oncorhynchus mykiss*, sperm activation is triggered by changes in ion concentration. A decrease in extracellular potassium results in a potassium efflux and increases in intracellular calcium, which trigger sperm motility (Tanimoto et al. 1994). Although sperm activation and the catabolism of ATP in fishes have been studied, little has been published on structural changes associated with sperm activation.

It is important to point out that fusion of sperm and eggs in teleost fishes occurs in a fashion different from that of mammals. Teleost sperm typically do not possess an acrosome (Otha and Iwamatsu 1987, Koch and Lambert 1990), the specialized structure involved in the digestion of the extracellular egg coat. In zebrafish, the sperm plasma membrane binds directly to the egg membrane and a direct fusion occurs. Minutes later, the sperm head and the tail are present in the cytoplasm (Hart 1992). Fertilization is a partnership where the sperm and the egg are each part of the machinery of inheritance. In teleost fishes, eggs are composed mostly of the yolk in the vegetal pole and unlike other vertebrates, fertilization occurs only at a single location or sperm entry site (a microvillar cluster) (Wolenski and Hart, 1987) beneath an inner aperture called the micropyle.

A necessary condition for fertilization to occur is the activation of the egg. Activation in vertebrates begins with a rapid rise in intracellular calcium that propagates across the egg (Steinhardt et al. 1974, Iwamatsu 1992, Lee et al. 1999) that is usually triggered by the sperm. However, the activation mechanism of fish eggs seems to be species-specific. In zebrafish, activation does not occur when the sperm reacts with the

egg surface, but rather when the eggs come in contact with the spawning medium (Hart and Yu 1980, Hart and Fluck 1995). In other fishes such as the medaka, *Oryzias latipes*, chum salmon, *Oncorhynchus keta*, Pacific herring, *Clupea pallasii*, (reviewed by Iwamatsu 1992) and Nile tilapia, *Oreochromis niloticus* (Stefano Peruzzi, Haskin Shellfish Research Laboratory, New Jersey, personal communication), depolarization of the membrane and the calcium wave are triggered by sperm contact.

FERTILIZATION IN ZEBRAFISH

Zebrafish belong to the family Cyprinidae (minnows and carps) and are native to rivers in Asia (Pakistan, India, Bangladesh and Nepal). Zebrafish have been distributed around the world due to commercial value as an aquarium fish (Figure 2-1). Recently, zebrafish have become important as a vertebrate research model in more than 270 laboratories around the world (Zebrafish Information Network, <http://zfin.org/index.html>). The zebrafish embryo offers a useful system for investigating mechanisms that control development in vertebrates. The embryos can be obtained in large numbers, are optically clear, and permit easy visualization of developmental events (Figure 2-2). The available genetic and molecular tools permit detailed molecular studies to be undertaken. Because the embryos develop externally they can be treated with chemicals or can be easily manipulated and grown without the use of complicated media. Because of its popularity as a research model, the zebrafish has been used as tool to uncover the mechanism of fertilization in fishes.

Normal fertilization involves the events that lead to the union of a single (haploid) set of chromosomes from each parent to restore the diploid genome. Newly

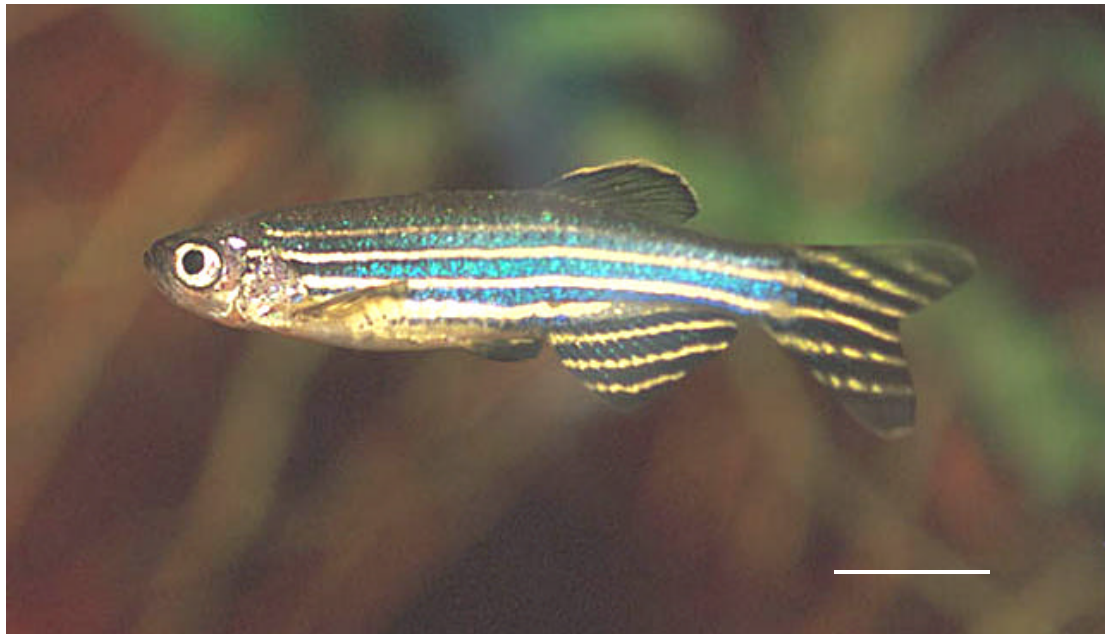


Figure 2-1. The zebrafish *Danio rerio* is native to India, Pakistan, and Nepal. Currently the zebrafish is distributed around the world due to its value as a research model and aquarium fish. This picture was downloaded from FishBase a global information system on fishes (www.fishbase.org). Bar is 0.6 cm.

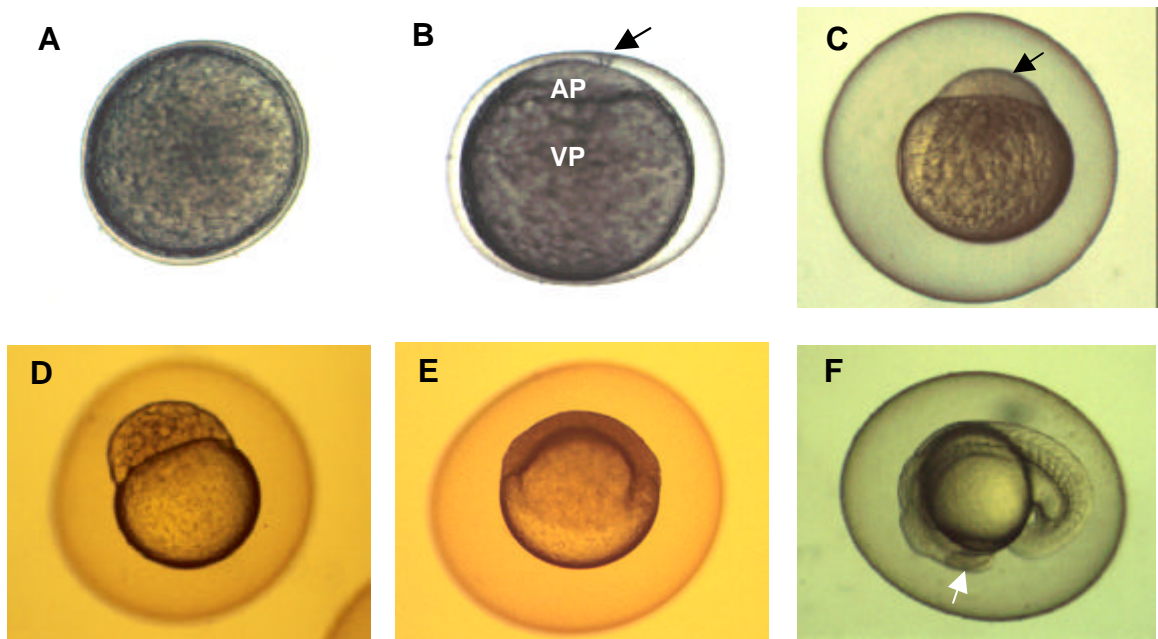
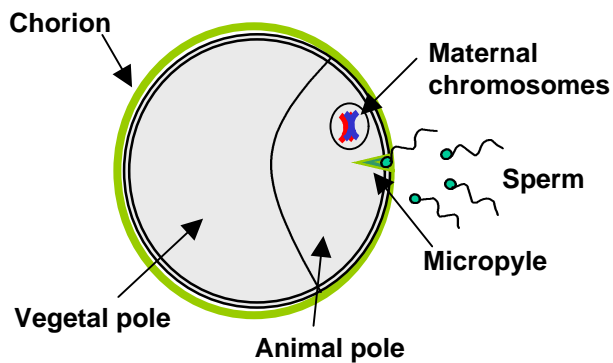


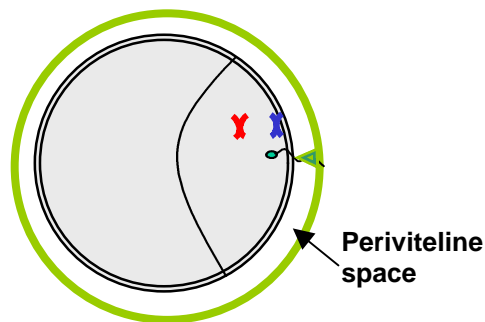
Figure 2-2. Early development in zebrafish. Development can be followed easily due to the transparency of the eggs. It is interesting to note the velocity of embryonic development. A) unactivated and unfertilized egg. B) 30 seconds after insemination, the animal pole (AP) and vegetal pole (VP) are clearly defined: arrow shows the micropyle. C) 45 min after fertilization, blastodisc formation (arrow). D) 3 h after fertilization, blastula stage. E) 5 h after fertilization, gastrula stage. F) 20 h after fertilization, embryo shows many of the adult structures: eyes (arrow), all parts of the brain, and movement begins. Hatching occurs ~48 h after fertilization at 28°C.

spawned fish eggs are at rest in metaphase II of the meiotic cycle. After coming in contact with the spawning medium, the cell cycle is resumed by undergoing a series of programmed steps (Figure 2-3A). This results in physiological changes (Figure 2-3B) including extrusion of an extra set of chromosomes as a polar body (Figure 2-3C). The haploid female pronucleus must come into contact with the male (sperm) pronucleus in order to fuse and form the diploid zygote (Figure 2-3D). The first step during activation is a rapid rise of intracellular calcium that propagates as a wave through the egg cortex from the micropyle region at the animal pole to the vegetal pole (Lee et al. 1999) (Figure 2-4A,B).

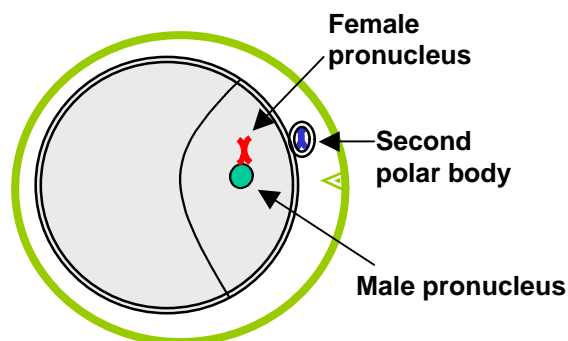
The calcium wave triggered when the eggs come in contact with the water acts as the signal that resumes meiosis but unlike in other vertebrates it does not depend on the sperm (Figure 2-5). Immediately after the calcium wave, a phenomenon seen in most vertebrates, called the cortical reaction, begins. Cortical granules migrate to the egg membrane and by exocytosis release their contents to the perivitelline space probably by contraction of the cytoskeleton (Becker and Hart 1999). This initiates the rise of the chorion which detaches from the micropyle around 30 seconds after activation (Hart and Yu, 1980). At this time sperm can no longer reach the membrane through the micropyle, suggesting that zebrafish eggs have a restricted window of opportunity to be fertilized. It is thought that the discharge of the granule contents prevents fertilization by multiple sperm cells (polyspermy) (Becker and Hart 1999) and protects the developing egg by changing properties of the chorion (Iwamatzu et al. 1995). Another area of the egg that undergoes drastic changes is the sperm entry site. The actin filaments that form the cortical cytoskeleton reorganize at the sperm entry site



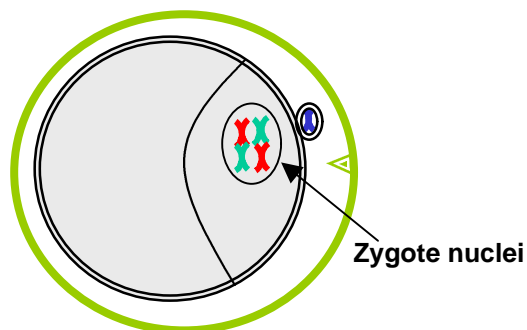
A) Sperm can enter the egg only through the micropyle (canal). Within 5 sec after insemination the sperm head can traverse the micropyle and bind to the egg membrane.



B) Within 30 sec of sperm entry into the egg, several physiological events occur. The chorion increases in size by the incorporation of water in the perivitelline space, a process called “water hardening”. Meiosis is resumed and the maternal chromosomes begin to divide within 1 minute.

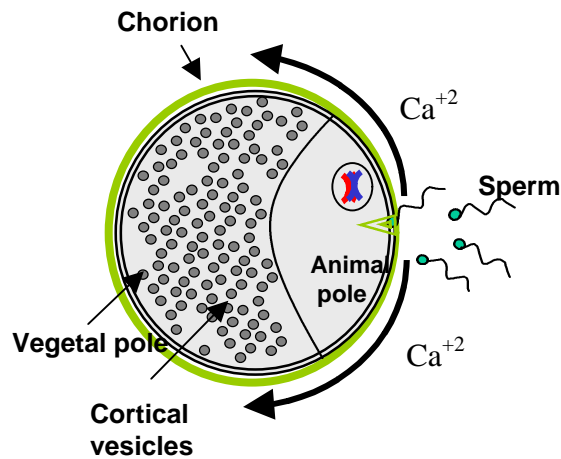


C) Within 5 min, half of the maternal chromosomes (1N) form the second polar body which is extruded and eventually degenerates. The other half (1N) forms the female pronucleus. The DNA of the sperm pronucleus unwinds (decondenses).

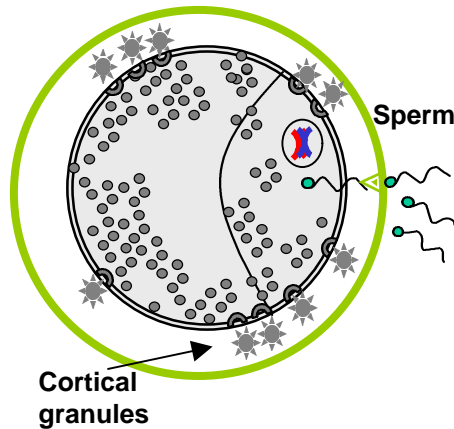


D) Within 15 minutes, the male and female pronuclei join to form the nucleus of the zygote (yielding two set of chromosomes). After DNA synthesis (yielding a doubling of the DNA and the equivalent of four chromosome sets), the first cell division occurs ~ 50 min after

Figure 2-3. Events before, during and after fertilization in zebrafish, *Danio rerio*.



A) Upon activation in zebrafish eggs a wave of free calcium travels from the micropyle region at the animal pole to the vegetal pole. This is the first step in a cascade of reactions that triggers the cell cycle.



B) The cortical granules are released by exocytosis which causes a rearrangement of the egg surface. A uniform elevation of the chorion (water hardening) occurs due to accumulation of cortical granule contents (Becker and Hart 1999). Cortical granules change the chemical structure of the chorion. It is thought that these physiological changes protect the egg from physical damage.

Figure 2-4. Calcium wave and cortical reaction in zebrafish eggs.

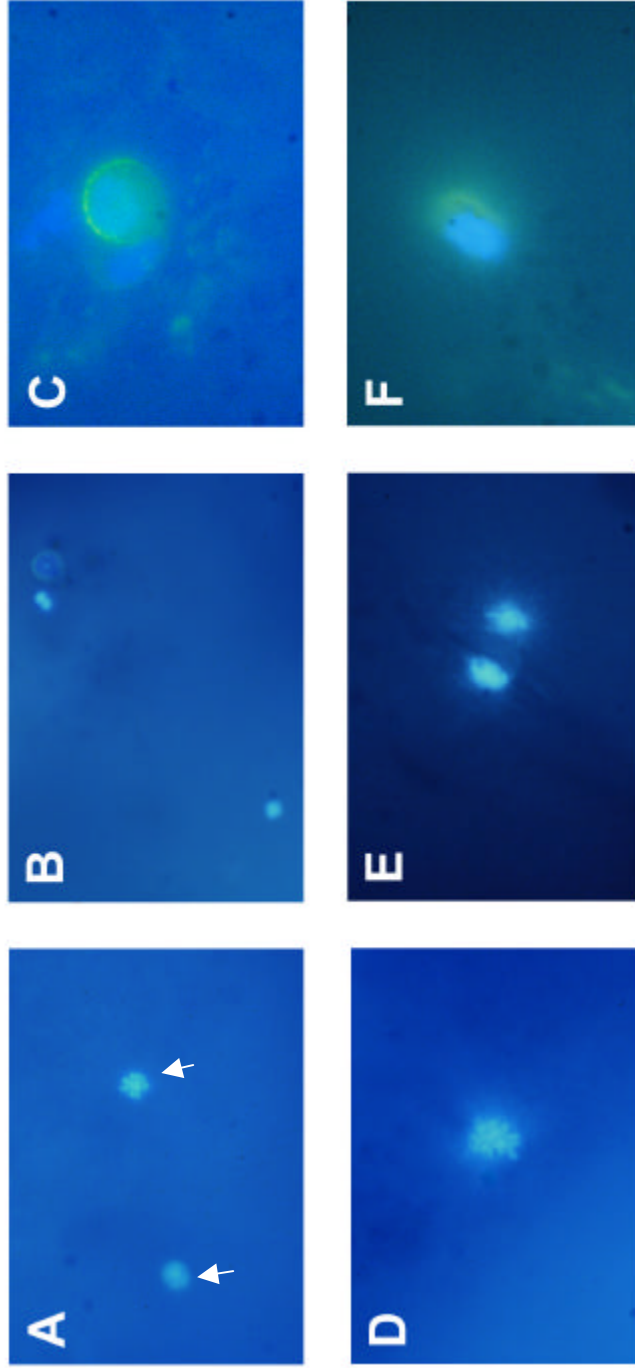


Figure 2-5. Fertilization and activation of zebrafish eggs. Gametes were tracked during fertilization and activation by staining with the DNA-specific dye Hoechst 33342. Panels A, B and C show female and male gametes during the first 10 min after insemination. A) 30 sec after insemination the sperm head (left arrow) is on the surface of the egg membrane. The female chromosomes (right arrow) are positioned close to the sperm entry site at the moment of insemination. B) 5 min after insemination female chromosomes start to split, half will form the female pronucleus and the other half will be extruded as the second polar body. C) 10 min after insemination the second polar body has been extruded from the egg. It is shown surrounded by actin filaments (green). Double staining was performed using Hoechst 33342 to stain DNA and Alexa Fluor® 488 phalloidin to stain filaments of actin. Panels D, E and F show eggs during the first 10 min after activation by placement in fresh water. These panels correspond with the upper panels and demonstrate that the cell cycle is resumed despite the absence of sperm (i.e. polar body formation and extrusion).

and form a fertilization cone. Once the sperm reaches the egg membrane (within 15-20 seconds of insemination) it seems to bind to a microvilli cluster and fusion occurs. It has been proposed that the sperm nuclear membrane becomes structurally linked with actin filaments (Figure 4-2, Chapter 4) that are probably involved in the movement of the sperm nucleus to the inner cytoplasm (Hart et al. 1992).

TILAPIA

Tilapias belong to the family Cichlidae and although they are endemic to the African continent and Palestine (Jordan Valley and coastal rivers) (Philippart and Ruwet 1982), they are now distributed around the world due to their popularity for culture. Three different have been identified based upon feeding and spawning behaviors. The genus *Tilapia* contains fish that eat coarse vegetation such as grasses, young shoots and leaves (macrophagy), and after spawning they guard their broods (substrate-spawners). The genera *Oreochromis* and *Sarotherodon* feed on unicellular algae and bacteria (microphagy) and care for the young by keeping them in the mouth (mouthbrooders) (Trewavas 1982). The most cultured is the genus *Oreochromis* which includes Nile tilapia, *Oreochromis niloticus* (Figures 2-6, 2-7). Although tilapia are the focus of numerous studies, specific information of the basic processes of fertilization is limited in these fishes. It is known that Nile tilapia eggs are activated by contact with sperm and that they remain fertile for around 15 min after contact with water (Myers and Hershberger 1991). This short window for fertilization could be overcome by immersing the fish eggs in ovarian fluid or isotonic solutions such as Ringers' or Hanks' balanced salt solutions (isotonic method of fertilization) (Yamamoto 1961).



Figure 2-6. One of the female Nile tilapia *Oreochromis niloticus* used for egg production in this study. Scale bar is 4 cm



Figure 2-7. Political world map showing some of the countries where Nile tilapia *Oreochromis niloticus* are cultured (black dots). The information is from FishBase a global information system on fishes (www.fishbase.org).

OVERCOMING THE FERTILIZATION BARRIERS IN FISH

Fertilization mechanisms in animals have evolved to restrict situations that could be detrimental for survival, such as interspecific crosses and polyspermy. Differences in breeding behavior, physical structure of gametes, or in the molecules involved in fertilization are some of the reproductive barriers that are widespread in nature. Closely related fishes often have different spawning behaviors and their gametes present physical and molecular barriers such as sperm entry sites that accommodate only single sperm of a specific size (Amanze and Iyengar 1990, Wolenski and Hart 1987) or species-specific extracellular molecules involved in sperm-egg recognition.

Different strategies have been developed by researchers and culturists to overcome these fertilization barriers. With artificial insemination, fish can be spawned year round and hybridization has become a common practice in aquaculture. With ICSI, it is theoretically possible to produce polyploid fish by injecting more than one sperm or to produce hybrids by injecting sperm from other species. Because the sperm do not need to be motile in order to fertilize an egg, ICSI could assist in developing preservation techniques other than cryopreservation.

Despite the actual value of ICSI in the field of assisted reproduction and in the study of basic fertilization mechanisms this technique has not been applied in fishes. The studies in this dissertation focus on developing standard ICSI procedures to be applied in fish. In the first part of this work, the zebrafish *Danio rerio* was used as a model for ICSI. The conditions of gametes (activated, non-activated) and time of the injection after egg collection were evaluated when performing ICSI (Chapter 3). Improvement of the ICSI technique was also attempted by injecting in different areas of

the zebrafish egg (Chapter 4). Nile tilapia were used as model for ICSI in aquaculture species (Chapter 5). This work also evaluated ICSI with sperm that had been cryopreserved, preserved in methanol or freeze-dried (Chapter 6). Finally, this study compared the characteristics of three genera of fishes for the application of ICSI (Chapter 7).

REFERENCES

- Amanze, D. and A. Iyengar. 1990. The micropyle: a sperm guidance system in teleost fertilization. *Development* 109:495-500.
- Becker, K. A. and N. H. Hart. 1999. Reorganization of filamentous actin and myosin-II in zebrafish eggs correlates temporally and spatially with cortical granule exocytosis. *Journal of Cell Science* 112:97-110.
- Brun, R. 1974. Studies on fertilization in *Xenopus laevis*. *Biology of Reproduction* 11:513-518.
- Catt, J. and S. L. Rhodes. 1995. Comparative intracytoplasmic sperm injection (ICSI) in human and domestic species. *Reproduction, Fertility, and Development* 7:161-167.
- Catt, S. L., J. W. Catt, G. Evans, and W. M. C. Maxwell. 1996. Birth of male lamb after intracytoplasmic sperm injection of a presumptive male sperm. *Veterinary Research* 139:494-495.
- Chan, A. W. S. 1999. Transgenic animals: current and alternative strategies. *Cloning* 1:25-46.
- Cochran, R., B. Meintjes, B. Reggio, D. Hylan, J. Carter, C. Pinto, D. Paccamonti, and R. A. Godke. 1998. Live foals produced from sperm-injected oocytes derived from pregnant mares. *Journal of Equine Veterinary Science* 18:736-740.
- ODale, B., L. J. DeFelice, and G. Ehrenstein. 1985. Injection of soluble sperm fraction into sea-urchin eggs triggers the cortical reaction. *Experientia* 41:1068-1070.
- Gomez, M. C., C. E. Pope, R. Harris, A. Davis, S. Mikota, and B. L. Dresser. 2000. Birth of kittens produced by intracytoplasmic sperm injection of domestic cat oocytes mature in vitro. *Reproduction, Fertility and Development* 12:423-433.
- Goto, K., A. Kinoshita, Y. Takuma, and K. Ogawa. 1990. Fertilization of immobilized, killed spermatozoa. *The Veterinary Record* 127:517-520.

- Hart, N. H., and S. F. Yu. 1980. Cortical granule exocytosis and cell surface reorganization in eggs of *Brachydanio*. *The Journal of Experimental Zoology* 213:137-159.
- Hart, N. H., K. A. Becker, and J. S. Wolenski. 1992. The sperm entry site during fertilization of zebrafish egg: localization of actin. *Molecular Reproduction and Development* 32:217-228.
- Hart, N. H. and R. A. Fluck. 1995. Cytoskeleton in teleost eggs and early embryos: contributions to cytoarchitecture and motile events. *Currents Topics in Developmental Biology* 31:343-381.
- Hiramoto, Y. 1962. Microinjection of the live spermatozoa into sea urchin eggs. *Experimental Cell Research* 27:416-426.
- Hosoi, Y. and A. Iritani. 1993. Rabbit microfertilization. *Molecular Reproduction and Development* 36:282-284.
- Iwamatsu, T. 1992. Egg activation. *The Fish Biology Journal Medaka* 4:1-10
- Iwamatsu, T., Y. Shibata, and T. Kanie. 1995. Changes in chorion proteins induced by exudates released from the egg cortex at the time of fertilization of teleost, *Oryzias latipes*. *Development, Growth and Differentiation* 37:747-759.
- Kimura, Y. and R. Yanagimachi. 1995. Mouse oocytes injected with testicular spermatozoa or round spermatids can develop into normal offspring. *Development* 121:2397-2405.
- Koch, R.A., and C. C. Lambert. 1990. Ultrastructure of sperm, spermiogenesis, and sperm-egg interactions in selected invertebrates and lower vertebrates which use external fertilization. *Journal of Electron Microscopy Techniques* 16:115-154.
- Kolbe, T. and W. Holtz. 2000. Birth of a piglet derived from oocyte fertilized by intracytoplasmic sperm injection (ICSI). *Animal Reproduction Science*. 64:97-101.
- Kroll, K. L. and E. Amaya. 1996. Transgenic *Xenopus* embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. *Development* 122:3173-3183.
- Lee K.W., S. E. Webb, and A. L. Miller. 1999. A wave of free cytosolic calcium traverses zebrafish eggs on activation. *Developmental Biology* 214:168-180
- Lillie, F. R. 1914. Studies of fertilization. *The Journal of Experimental Zoology* 16: 523-590.

- Mann, J. 1988. Full-term development of mouse eggs fertilized by spermatozoon microinjected under the zona pellucida. *Biology of Reproduction* 38:1077-1083.
- Markert, C. 1983. Fertilization of mammalian eggs by sperm injection. *The Journal of Experimental Zoology* 228:195-201.
- Myers, J. M. and W. K. Hershberger. 1991. Artificial spawning of tilapia eggs. *Journal of the World Aquaculture Society* 22:77-82.
- Otha, T. and T. Itwamatsu. 1983. Electron microscopic observations on sperm entry into eggs of the rose bitterling, *Rhodeus ocellatus*. *The Journal of Experimental Zoology* 227:109-119.
- Palermo, G., H. Joris, P. Devroey, and A. C. Van Steirteghem. 1992. Pregnancies after intracytoplasmic injection of single spermatozoon into and oocyte. *Lancet* 340: 17-18.
- Perry, A. C. F., T. Wakayama, H. Kishikawa, T. Kasai, M. Okabe, Y. Toyoda, and R. Yanagimachi. 1999. Mammalian transgenesis by intracytoplasmic sperm injection. *Science* 284:1180-1097.
- Philippart, J-CL. and J-CL. Ruwet. 1982. Ecology and distribution of tilapias. Pages 15-59 in R.S.V. Pullin and R.H.Lowe-McConnell, editors. *The Biology and Culture of Tilapia*, International Center for Living Aquatic Resources Management, Manila, Philippines.
- Pope, C. E., C. A. Johnson, M. A. McRae, G. L. Keller, and B. L. Dresser. 1998. Development of embryos produced by intracytoplasmic sperm injection of cat oocyte. *Animal Reproduction Science*. 53:221-236.
- Ren, D., B. Navarro, G. Perez, A. C. Jackson, S. Hsu, Q. Shi, J. L. Tilly, and D. E. Clapham. 2001. A sperm ion channel required for sperm motility and male fertility. *Nature* 413:603-609.
- Uehara, T., and R. Yanagimachi. 1976. Microsurgical injection of spermatozoa into hamster eggs with subsequent transformation of sperm nuclei into male pronuclei. *Biology of Reproduction* 15:467-470.
- Shaoyi, Y., M. Zhongrui, Y. Huiyi, T. Miao, L. Shukhong, H. Guoping, L. Guanshan, G. Lin, J. Guanquin, H. Ruifen, L. Lidong, X. Shiling, Y. Leining, X. Dequan, and Z. Nianci. 1991. Further investigation on nuclear transplantation in different orders of teleost: the combination of nucleus of tilapia (*Oreochromis nilotica*) and the cytoplasm of loach (*Paramisgurnus dabryanus*). *International Journal of Developmental Biology* 35:429-435.

- Shivers, C. A. and J. M. James. 1970. Capacitation of frog sperm. *Nature* 227:183-184.
- Sparrow, D. B., B. Latinkic, and T. J. Mohun. 2000. A simplified method of generating transgenic *Xenopus*. *Nucleic Acid Research* 28:E12-E12 (<http://nar.oupjournals.org/egi/reprint/28/4/e12.pdf>).
- Sutovsky, P., R. Oko, L. Hewitson, and G. Schatten. 1997. The removal of sperm perinuclear theca and its association with the bovine oocyte surface during fertilization. *Developmental Biology* 188:75-84.
- Steinhardt, R. A., D. Epel, E. J. Carroll, and R. Yanagimachi. 1974. Is calcium ionophore and universal activator for unfertilized eggs? *Nature* 252:41-43.
- Stricker, S. A. 1999. Comparative biology of calcium signaling during fertilization and egg activation in animals. *Developmental Biology* 211:157-176.
- Tanimoto, S., Y. Kudo, T. Nakazawa, and M. Morisawa. 1994. Implication that potassium flux and increase in intracellular calcium are necessary for the initiation of sperm motility in salmonid fishes. *Molecular Reproduction and Development* 39:409-414.
- Takai, H. and M. Morisawa. 1995. Change in intracellular K⁺ concentration caused by external osmolality change regulates sperm motility of marine and freshwater teleost. *Journal of Cell Science* 108:1175-1181.
- Topfer-Petersen, E. 1999. Molecules on the sperm's route to fertilization. *The Journal of Experimental Zoology* 285:259-266.
- Trewavas, E. 1982. Tilapias: Taxonomy and Speciation. Pages 3-13 in R.S.V. Pullin and R.H.Lowe-McConnell, editors. *The Biology and Culture of Tilapia*, International Center for Living Aquatic Resources Management, Manila, Philippines.
- Wilmut, I., A. E. Schnieke, J. McWhir, A. J. Kind, K. H. Campbell. 1997. Viable offspring derived from fetal and adult mammalian cells. *Nature*. 385:810-813.
- Wolenski, J. and N. Hart. 1987. Scanning electron microscope studies of sperm incorporation into the zebrafish (*Brachydanio*) egg. *The Journal of Experimental Zoology* 243:259-273.
- Yamamoto, T. 1961. Physiology of fertilization in fish eggs. *International Review of Cytology* 12:361-405.

CHAPTER 3

FERTILIZATION OF ZEBRAFISH EGGS BY INTRACYTOPLASMIC SPERM INJECTION (ICSI)*

Injection of one or more sperm cells into the cytoplasm of an egg is called intracytoplasmic sperm injection or ICSI. Research in ICSI in higher vertebrates began 25 years ago when hamster eggs injected with sperm developed to pronuclei and early cleavage stages (Uehara and Yanagimachi 1976). In the past decade this technique has gained interest due to its diverse applications, and it has been evaluated with varying levels of success in different mammalian species (Mann 1988, Goto et al. 1990, Cochran et al. 1998) including humans (Palermo et al. 1992). In aquatic organisms, only work in echinoderms (Hiramoto 1962, Dale et al. 1985) and amphibians has been reported (Kroll and Amaya 1996, Sparrow et al. 2000).

Levels of success found for ICSI depend on intrinsic characteristics of the organism studied. Fish spermatozoa, in contrast to mammalian spermatozoa, are immotile when in seminal plasma or in an isotonic or isoionic solution (Morisawa and Morisawa 1994). In nature, the sperm become motile upon entering the external environment (fresh water or salt water) and depending on the species, sperm activation is triggered by changes in osmotic pressure or ion concentration (Detweiler and Thomas 1998). Regardless of the activation mechanism of teleost sperm, they typically have access to the membrane of the egg only through a specific location or sperm entry site (a microvillar cluster), beneath an inner aperture called the micropyle (Wolenski and Hart 1987).

* Portions of this chapter have been published in the journal *Biology of Reproduction* 2001, Vol. 65, pp961-966

Egg activation begins with a rapid rise in intracellular calcium levels that propagates throughout the egg (Lee et al. 1999). In mammals and amphibians, contact with sperm triggers the activation of the egg. In fish, egg activation seems to be species-specific. In zebrafish, *Danio rerio*, activation does not occur when sperm react with the egg surface, but rather when the eggs come in contact with the spawning medium (Hart and Yu 1980, Hart and Fluck 1995, Iwamatsu 1997). Accordingly, activation of eggs could be important when ICSI is performed.

The present study investigated the potential of performing ICSI in teleost fishes using the zebrafish as a model. The objectives were: 1) to evaluate the micropyle as a sperm injection site; 2) to evaluate standard ICSI techniques for use in zebrafish; 3) to evaluate the effect of gamete conditions (activated or non-activated) on fertilization and development; 4) to evaluate the effect on fertilization of the timing of ICSI after egg collection, and 5) to verify ploidy and paternal contribution in ICSI-fertilized embryos.

MATERIALS AND METHODS

ANIMALS

Two lines of zebrafish (pigmented, wild-type, and gold, long-fin) were obtained from a commercial supplier (Scientific Hatcheries, Huntington Beach, California). The gold, long-fin line is recessive for pigmentation (Dallas Weaver, Scientific Hatcheries, personal communication) that results in a visible lack of pigmentation compared to the wild-type (Figure 3-1A). Fish were maintained in ten separate 80-L aquaria linked in a recirculating system. Filtration was accomplished using an upwelling bead filter. Temperature was maintained at 26°C and a photoperiod of 10 h of darkness and 14 h light was established. Salinity of the water was maintained at 1 g/L by the addition of

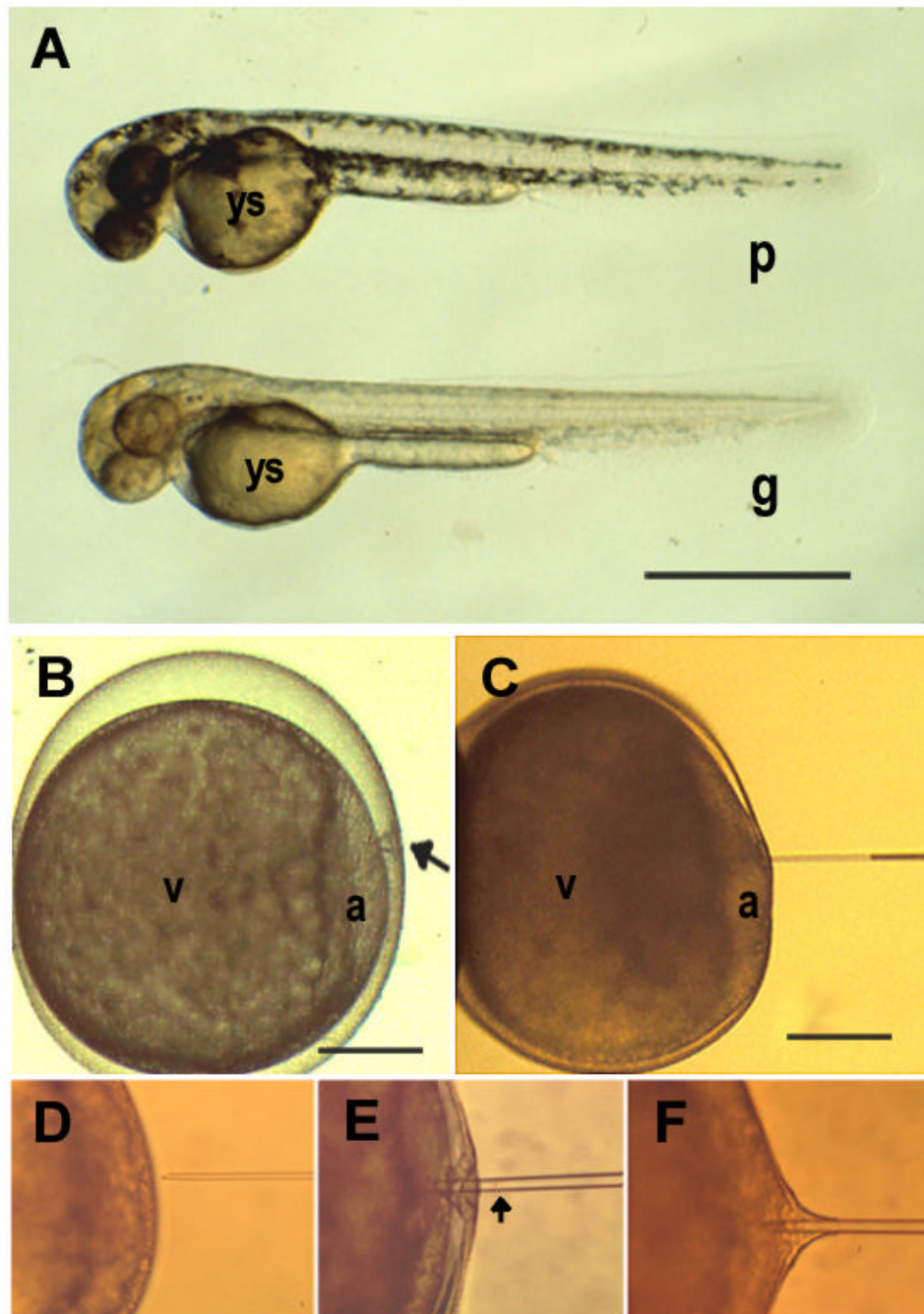


Figure 3-1. A) Zebrafish embryos at 48 h after fertilization: yolk sac (ys), wild-type, pigmented (p), and gold, long-fin (g). B) Activated zebrafish egg: the micropyle is indicated by the arrow. C) Non-activated zebrafish egg during microinjection with sperm: animal pole (a), vegetal pole (v). D and E Injection procedure (note pipette to right of egg): arrow in E shows the location of the sperm. F) Retraction of pipette (note adherence by egg). Scale bars in A and B, 200 μ m; C, 1 mm.

NaCl (Mix-N-Fine, Cargill, Minneapolis, Minnesota). Hardness (measured as CaCl_2) and alkalinity (measured as NaHCO_3) were monitored weekly using a standard water quality test kit (Model FF-1A, Hach Company, Loveland, Colorado) and were each maintained at 100 mg/L.

GAMETE COLLECTION AND PREPARATION (APPENDIX A, SOP-1)

The night before ICSI experiments, adult fish were placed in 6-L breeding tanks in a ratio of 3 females to 2 males (Westerfield 1995). Collection of gametes was performed on the day of the ICSI trials. The zebrafish were anesthetized by immersion in water containing 0.17 mg/mL tricaine methanesulfonate (methyl-m-aminobenzoate, MS 222, Argent Laboratories, Inc., Redmond, Washington). Sperm were obtained by gently squeezing the sides of the males followed by dilution in Hanks' balanced salt solution (HBSS) (Appendix A, SOP-2) (Westerfield 1995) and storage on ice until use. Eggs were obtained by gently pressing on the sides of females, starting behind the pectoral fins and moving toward the tail. Non-activated eggs were kept in HBSS containing 0.5% BSA (Sakai et al. 1997) until use. The first 35 injection trials in this study were used to establish basic techniques. These trials were not included in the analysis of injection time after egg collection ($n = 153$); however, they were included in the overall results ($n = 188$).

Activation of gametes was induced by transferring them to embryo medium buffer (EMB) (13.7 mM NaCl, 5.40 mM KCL, 0.25 mM Na_2HPO_4 , 0.44 mM KH_2PO_4 , 1.30 mM CaCl_2 , 1.00 mM MgSO_4 , 4.20 mM NaHCO_3) (Appendix A, SOP-2) (Westerfield 1995) at 52 mOsmol/kg. Gamete activation was analyzed in a 2 x 2 factorial design comparing the use of activated and non-activated sperm and eggs. Eggs

from individual females were assigned across treatments and sperm from pigmented males only were used in all experiments.

SPERM INJECTION

Injections were performed following accepted methods for ICSI in other vertebrates (Payne 1995) with some modifications described below. Eggs were placed in 100 μ L of EMB or HBSS in the lid of a 100-mm plastic cell culture dish (Corning Glass Works, Corning, New York). Microinjections were performed using an inverted microscope (Diaphot Nikon Inc., Tokyo, Japan) equipped with two mechanical micromanipulator units (Leitz, Rockliegh, Illinois) (Figure 3-2). Injecting pipettes (~15 μ m internal diameter) and holding pipettes (~300 μ m internal diameter) were made from borosilicate glass capillary tubes. A sharp spike was created at the tip of the injecting pipette.

Non-activated sperm were diluted in HBSS containing 5% polyvinylpyrrolidone (PVP) (360,000 mw) (Sigma Chemical Corporation, St. Louis, Missouri), which was used to prevent the sperm from sticking to the inner wall of the injecting pipette. Activated sperm were produced by dilution in EMB containing 5% PVP. Zebrafish sperm became motile after contact with the EMB and lost motility within 60 sec of activation. The activated sperm used for injection were injected in a non-motile condition. A single sperm was injected with ~7 pL of injection buffer (HBSS plus 5% PVP, or EMB plus 5% PVP, depending of the treatment). For injections, the egg was held with the animal pole facing outward as the injecting pipette was pushed through the micropyle into the cytoplasm (Figure 3-1).

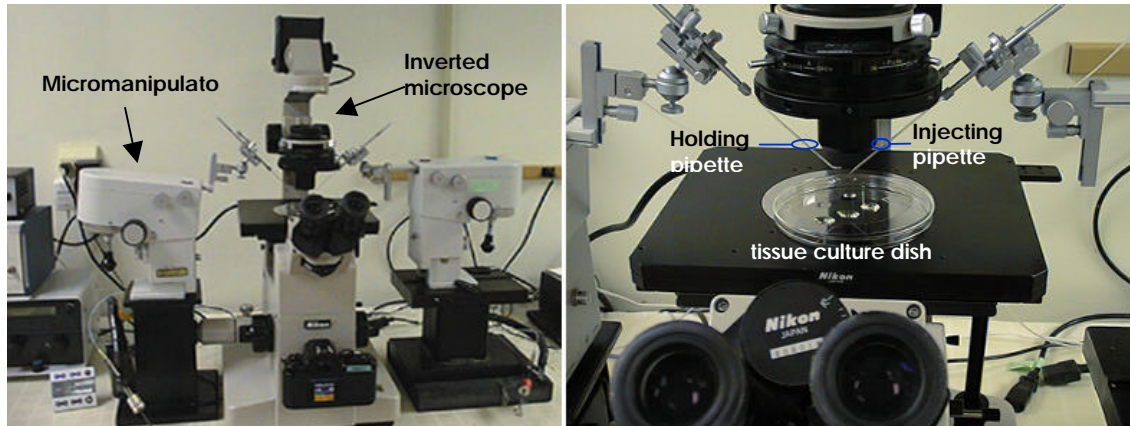


Figure 3-2. Microinjection equipment used during the course of this dissertation. Included two mechanical micromanipulators that moved the holding and injecting pipettes in three dimensions. The eggs were held in a 100-mm tissue culture dish during injections.

Zebrafish eggs undergo many physical changes when activated. One is the expansion of the chorion that causes detachment of the micropyle from the egg cytoplasm. Injections in activated eggs were performed only when the micropyle was connected to the egg plasma membrane. After injection, eggs were transferred immediately to a 35-mm tissue culture dish (Corning Glass Works), containing EMB and were incubated at 28°C. After 3 h, the eggs were inspected under a dissecting microscope for development. Embryos that presented a well-developed blastodisc were counted as fertilized and were left for further development at 28°C.

Two different sets of control treatments were performed. To evaluate the possibility of parthenogenetic activation by piercing (e.g., gynogenesis), eggs were injected only with injection buffer. The second control was done to observe possible damage to embryonic development caused by injection and toxicity of PVP. Because the incorporation of sperm into the zebrafish egg cytoplasm occurs within 2 to 3 min of insemination (Wolenski and Hart 1987), eggs were injected with ~7 pL of EMB containing 5% PVP at 5 min after insemination using fresh sperm (no ICSI).

Egg quality was evaluated for each experiment (i.e. for each female) by observation of the percentage of fertilization by artificial insemination. A known number of stripped eggs were incubated with fresh sperm in 500 µL of EMB. After 10 min, 4 mL of EMB were added and the eggs were incubated at 28°C. Percentage fertilization and development were verified at 3 h and at 24 h after insemination. Embryos that presented a well-developed blastodisc at 3 h after insemination were counted as being fertilized.

DNA CONTENT ANALYSIS

Analysis of DNA content was performed using a FACSCalibur® flow cytometer (Becton Dickinson, San Jose, California) equipped with an air-cooled 480 nm argon laser. The FACSComp® software (Becton Dickinson) was used to calibrate the instrument settings. Embryos were disrupted by passage through a 3-mL syringe fitted with a 25-gauge needle while suspended in 0.5 mL of lysis buffer containing 25 µg buffered RNase, 0.1% sodium citrate and 0.1% Triton X-100 (Tiersch and Chandler 1989). Samples were mixed and filtered through 20-µm nylon mesh. For analysis, an aliquot of 250 µL was diluted in lysis-staining buffer, which included 25 µg of propidium iodide. A sample of frozen blood from rooster, *Gallus gallus*, was used as an internal reference (2.5 pg of DNA/cell) (Tiersch and Wachtel 1991). Measurements of the DNA content of normal diploid cells of zebrafish (3.15 ± 0.06 pg of DNA/cell; n = 10) and haploid cells of zebrafish (calculated as 1.59 pg) were compared with those of larvae produced by ICSI.

STATISTICAL ANALYSIS

Difference in the incidence of fertilization among treatments was evaluated using a logistic regression model. The statistical model used was:

$$\log p/1-p = \alpha + \beta_1 S + \beta_2 E = [-1.439 + 0.8224 (S) + (-1.0821) (E)] = a$$

where α is the intercept; β_1 and β_2 are the logistic regression coefficients; S is sperm (designated as “1” for activated sperm, or “0” for non-activated); E is eggs (designated as “1” for activated, or “0” for non-activated). The probability of fertilization was calculated by the formula:

$$p = e^a / 1 + e^a$$

Where p is the probability of fertilization and a is the value predicted by the model. Differences were considered to be significant at $P < 0.05$.

RESULTS

SPERM INJECTION SITE

Localization of the micropyle, before activation or when the eggs were suspended in extender solution, was difficult because there was no clear demarcation between the animal and vegetal poles. This difficulty in distinguishing the position of the animal pole in extender solution increased the difficulty of localization of the micropyle in non-activated eggs. Also, due to the size of the eggs (~800 μm) and dense cytoplasmic content, the tip of the injecting pipette was sometimes not evident inside the eggs. This created difficulty in monitoring of the injection of the sperm into the egg.

GAMETE CONDITIONS

The different gamete treatments were evaluated using eggs from two different zebrafish lines (gold, long-fin and wild-type, pigmented) and sperm from wild-type pigmented males (Tables 3-1 and 3-2). There was no significant difference in fertilization by ICSI between the two zebrafish lines ($P = 0.997$; logistic regression), and therefore, these results were combined for further analysis. Overall, there was no significant difference in fertilization ability of activated and non-activated sperm by use of the ICSI procedure ($P = 0.057$). However, there was a significant difference in fertilization between activated and non-activated eggs ($P = 0.010$). When activated sperm were injected into activated eggs, 13 of 83 eggs (16%) were fertilized, 12 (14%) developed into gastrulae, 5 (6%) developed abnormally as larvae, and 1 (1%) developed

Table 3-1. Effect of sperm injections from wild-type, pigmented males into eggs from wild-type, pigmented females with activated and non-activated gametes. All embryos were pigmented.

Eggs	Sperm			
	Activated		Non-activated	
	Number	Percent	Number	Percent
Activated				
Total injections	18	100	41	100
Fertilized	3	17	1	2
Blastula	3	17	1	2
Gastrula	2	11	1	2
Abnormal embryo	1	6	0	0
Normal embryo	1	6	0	0
Non-activated				
Total injections	15	100	10	100
Fertilized	7	47	2	20
Blastula	7	47	2	20
Gastrula	7	47	1	10
Abnormal embryo	3	20	.0	0
Normal embryo	0	0	0	0

Table 3-2. Effect of injections of sperm from wild-type, pigmented males into eggs from gold, long-fin females with activated and non-activated. All embryos that developed beyond 42 h were pigmented indicating genetic contribution from injected sperm.

Eggs	Sperm			
	Activated		Non-activated	
	Number	Percentage	Number	Percentage
Activated				
Total injections	65	100	15	100
Fertilized	10	15	3	20
Blastula	10	15	3	20
Gastrula	10	15	3	20
Abnormal embryo	4	6	1	7
Normal embryo	0	0	1	7
Non-activated				
Total injections	14	100	10	100
Fertilized	3	21	2	20
Blastula	3	21	2	20
Gastrula	2	14	2	20
Abnormal embryo	1	7	0	0
Normal embryo	0	0	1	10

normally and hatched. When non-activated sperm were injected into activated eggs, 4 of the 56 eggs (7%) were fertilized, 4 (7%) developed into gastrulae, 1 (2%) developed abnormally, and 1 (2%) developed normally and hatched. When activated sperm were injected into non-activated eggs, 10 (34%) of the 29 eggs were fertilized, of these 9 (31%) developed into gastrulae, and 4 (14%) developed abnormally. When non-activated sperm were injected into non-activated eggs, 4 of the 20 eggs (20%) were fertilized, of these 3 (15%) developed into gastrulae, and 1 (5%) developed normally and hatched.

After combining the data, from a total of 188 eggs injected with sperm, 31 developed into blastulae (16%), 28 (15%) developed into gastrulae, 10 (5%) developed abnormally as larvae, and 3 (2%) developed normally and hatched (Figure 3-3). Two of the normal fish were killed for analysis of DNA content at 7 days after hatching. The third fish grew to begin feeding (7 days after hatching) and died when the holding tank was accidentally drained. The probabilities of fertilization calculated for the different treatments are shown in Table 3-3.

From the 21 eggs injected only with injection buffer (EMB plus 5% PVP or HBSS plus 5%PVP), none developed beyond the one-cell stage and thus none met the criteria for fertilization. From the 8 eggs injected with injecting medium after artificial insemination, 7 (90%) developed normally beyond the one-cell stage to hatching. This was not different from the overall fertilization percentage ($62 \pm 31\%$) that we observed for the 4,200 control eggs that were artificially fertilized (eggs of 42 females).

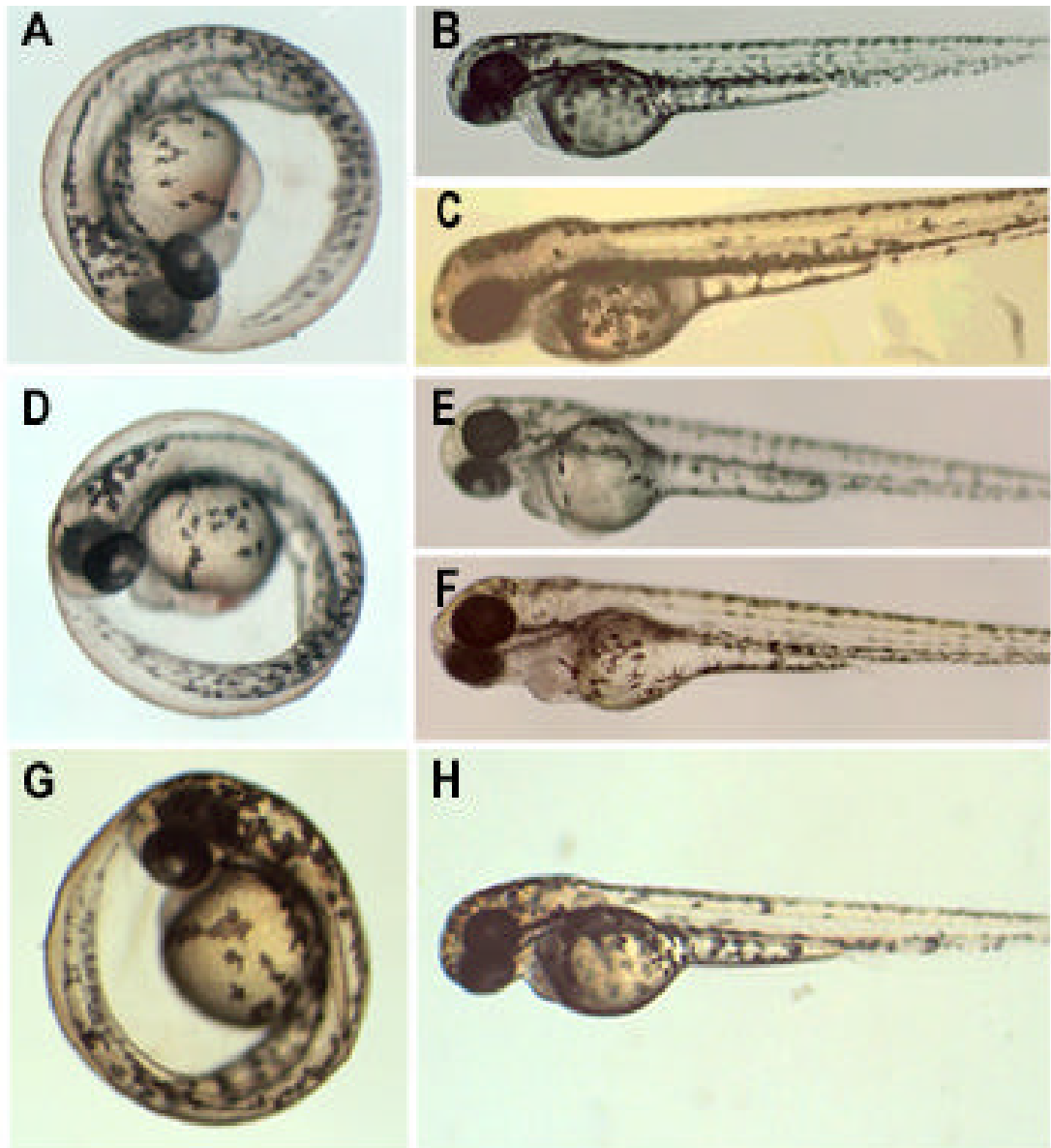


Figure 3-3. Comparison of embryonic stages. A, B and C) Normal zebrafish embryos obtained by artificial insemination. D-H) Embryos produced by ICSI. D) ICSI embryos obtained by injecting sperm from wild-type, pigmented male into eggs from wild-type, pigmented females. A, D and G) Fully developed embryos inside the chorion (48 h after fertilization). B, E and H) Hatched embryo (56 h). C, F) 5-day-old larvae. G-H) Zebrafish embryo produced by injecting sperm from wild-type, pigmented males into eggs from gold, long-fin females. The resulting fish shows the dominant pigmented phenotype.

Table 3-3. Probability of fertilization by ICSI using gametes in different conditions.

Eggs	Sperm	
	Activated	Non-activated
Activated	15.5%	7.4%
Non-activated	35.1%	19.2%

Model: $-1.439 + 0.8224 (\text{Sperm}) + (-1.0821) (\text{Eggs}) = a$.

Probability; $p = e^a / 1 - e^a$

EFFECT OF TIME AFTER EGG COLLECTION

The time after egg collection was recorded for the injection of 153 eggs (Figure 3-4). Injections resulted in 28% fertilization in the first 20 min after egg collection, 18% fertilization from 21 min to 40 min after egg collection, and 9% fertilization from 41 min to 60 min after egg collection. With respect to ICSI, normally developed, hatching larvae were produced from eggs injected at 15, 21 and 60 min after collection.

DEVELOPMENT

Ten of the 13 ICSI embryos that developed beyond the gastrula stage were abnormal. All 11 embryos that survived beyond 48 h showed wild-type pigmentation, and all abnormal embryos showed pigmentation as well, indicating successful incorporation of the male genome. The abnormal zebrafish showed signs of developmental retardation (Figure 3-5). The anterior-posterior axis was compromised as indicated by a curvature of the caudal fin (Figure 3-5B-E). The eyes were poorly developed and were smaller than normal. One embryo showed no caudal development (Figure 3-5B). The yolk sacs in abnormal embryos persisted longer and thus appeared to be larger than those of normal embryos (Figure 3-5A). DNA content of the abnormal larvae was 3.1 pg of DNA per cell indicating that they were diploid.

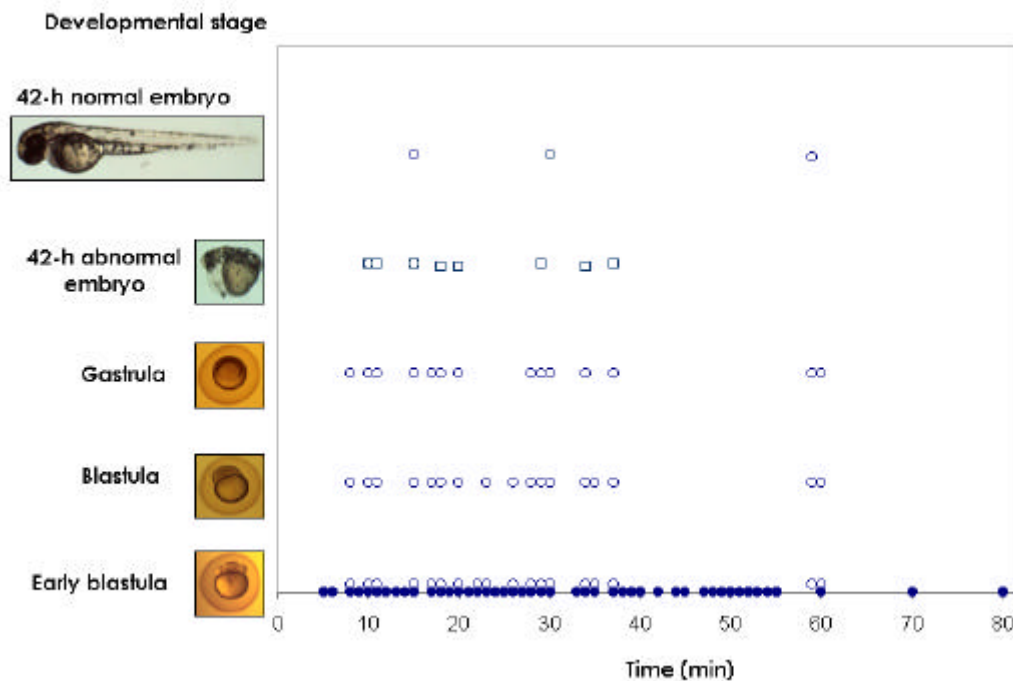


Figure 3-4. Effect of time after egg collection on fertilization rate after ICSI (n = 153). Eggs were placed in HBSS containing 0.5% BSA immediately after collection. Closed circles indicate unfertilized eggs after ICSI. Open circles indicate fertilized eggs. Squares indicate fish with developmental abnormalities. Pictures show the developmental stages that each embryo passed through.

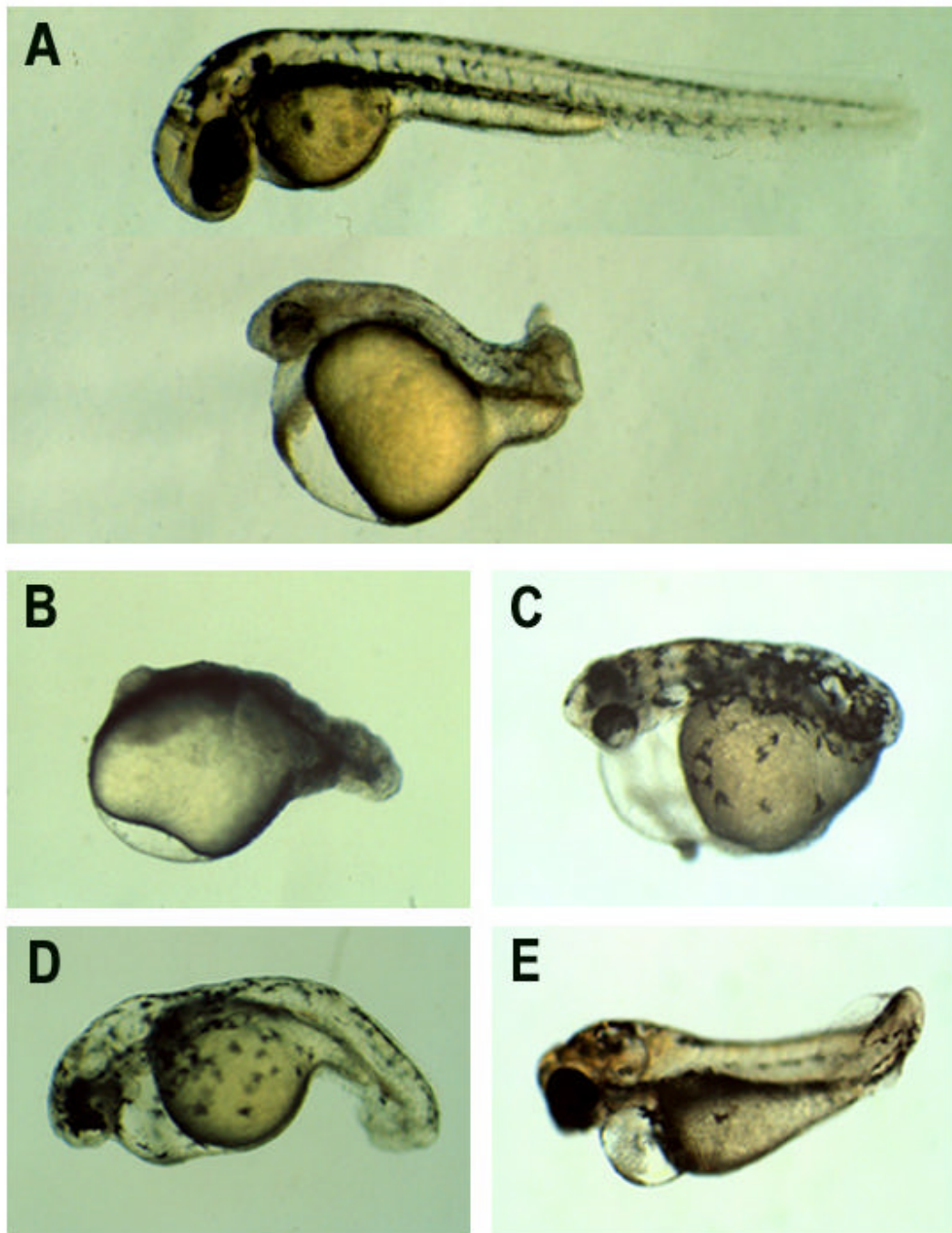


Figure 3-5. Development of zebrafish embryos after ICSI. A) Normal (control) embryo (top) at 48 h after artificial insemination and abnormal ICSI embryos at the same time. B-E) The anterior-posterior axis is affected, and the eyes are reduced in size. B) 24 h after ICSI the head is not present. C, D and E) Abnormal larvae (72 h after ICSI) showing pigmentation produced by injection of sperm from pigmented males into eggs from gold females.

DISCUSSION

The present work evaluated the possibility of applying standard ICSI technology to fishes. Basically the same equipment used for ICSI in mammals was used in zebrafish. However, the size of the zebrafish eggs dictated the sizing of the injecting and holding pipettes and affected ease of manipulation. The zebrafish egg has a diameter of $\sim 800\ \mu\text{m}$ comprised mostly by yolk in the vegetal pole. This contrasts with the smaller overall size of mammalian eggs (e.g., the human egg is $\sim 100\ \mu\text{m}$ in diameter), although it is important to note that the animal pole of the zebrafish presents a comparably sized target ($\sim 100\ \mu\text{m}$) for injection.

In a condition typical for most fishes, fertilization occurs only in a single location (the microvillar cluster) (Wolenski and Hart 1987), which is located beneath the micropyle. Because sperm normally traverse the micropyle to gain entry to the egg, it was selected as the path for the injection pipette. This differs from ICSI in mammals where piercing of the egg is not limited to a specific location of the zona pellucida. Although the micropyle is situated at the animal pole, it was difficult to localize in zebrafish eggs immediately after collection. However, after 10 to 15 min in extender solution or after egg activation, the border between the poles was better differentiated and localization of the micropyle became easier.

In fishes, which spawn by external fertilization, sperm capacitation (if it occurs) would coincide with contact with water and activation of motility by changes in osmotic pressure or ion concentration. This mechanism suggested that fish sperm would need to be activated before injection into the egg. Our results show that activation of sperm is not a prerequisite for fertilization by ICSI. However, we observed a significantly higher

probability of fertilization when activated sperm were injected into non-activated eggs (although, we cannot exclude the possibility that partial activation of the eggs occurred while in the extender). We also observed fertilization with non-activated sperm when injections were performed in activated eggs. In addition, the eggs clearly had a limited window during which fertilization was possible after collection (i.e., less than 2 h).

Although there are many reports of successful ICSI in domestic animals, there are also reports of failures and reduced developmental competency of the embryos. This was observed in the present study which yielded abnormal development and low fertilization rates after ICSI. While it is necessary to note that these were preliminary trials intended only to evaluate the technical potential for ICSI in fishes (improved techniques await development), there are several biological possibilities that must be considered. One is the possibility that abnormal embryos were haploid carrying only a maternal genetic contribution. However, the phenotypes observed did not correspond with those shown by the haploid syndrome in zebrafish (Corley-Smith et al. 1996). Also, flow cytometric analysis of nuclear DNA content confirmed diploidy. The injection of sperm with a dominant pigmentation marker provided additional proof that the diploid embryos resulted from fertilization by the injected sperm and were not derived through gynogenesis (e.g., parthenogenesis resulting in all-maternal inheritance).

Other explanations of the abnormalities that resulted after ICSI include that the injections may have disrupted a meshwork of actin filaments in the fertilization cone just beneath the plasma membrane of the sperm entry site (Hart et al. 1992). It has been suggested that these filaments are responsible in zebrafish eggs for the stabilization of

the microvilli cluster of the sperm entry site, for the formation of the fertilization cone, for the binding and fusion of the sperm plasma membrane with the microvilli, and for the movement of the sperm nucleus into the inner cytoplasm (Hart et al. 1992). Future studies should address the effect of disruption in this area, the development of procedures to minimize injury to the injection site, and evaluate the potential for injection in other sites.

This study demonstrates for the first time that ICSI is possible in fishes, but further studies are needed to improve efficiency. The development of this technology in fishes could yield valuable applications. The use of cryopreservation of sperm will become a standard practice in fishes in the near future (Tiersch 2000). Although useful, cryopreservation can be costly and developing countries or small facilities may not be able to afford long-term storage in liquid nitrogen.

Alternatives include the use of freeze-dried sperm samples (Katayose et al. 1992) or those preserved in alcohol (Tateno et al. 1998). These sperm would not need to be motile if ICSI procedures were available to produce fertilization of fish eggs. Given the technical requirements for ICSI, the procedure would (with present technology) be best suited to reconstitution of desired lines and the production of small broodstock populations to be used to produce large numbers of fish. This approach could even have application in restoring endangered or extinct stocks by injection of cryopreserved sperm into irradiated eggs from a related stock or species to produce androgenesis (all-paternal inheritance of nuclear DNA) (Purdom 1969, Parson and Thorgaard 1985).

Other applications could be in the production of transgenic fish. One problem in gene transfer in fishes is the occurrence of mosaicism (incomplete incorporation of the transferred DNA in various cells or tissues). A new approach with a greater level of success in mice (Perry et al. 1999) and the African clawed frog, *Xenopus laevis* (Kroll and Amaya 1996), has used ICSI to inject membrane-disrupted sperm heads that are briefly incubated with linearized DNA (Perry et al. 1999, Sparrow et al. 2000). This method could be used in fishes to generate stable and uniform gene transfer.

In conclusion, various combinations of conditions of the gametes allowed fertilization by ICSI. However, it appears that sperm injection should be pursued using non-activated eggs and activated sperm. This combination yielded the highest probability of fertilization and the use of non-activated eggs allowed more time to position the eggs before injection. The data presented here also suggest that injections will be more likely to fertilize an egg at 5 to 40 min after collection. Thus, although we were able to induce fertilization and embryonic development by sperm injection in zebrafish, the efficiency was low. Improved methods of injection are needed and identification of specific constraints is necessary to improve our understanding of ICSI and the fertilization process in fish eggs.

REFERENCES

- Brun, R. 1974. Studies on fertilization in *Xenopus laevis*. *Biology of Reproduction* 11:513-518.
- Catt, J. W. and S. L. Rhodes. 1995. Comparative intracytoplasmic sperm injection (ICSI) in human and domestic species. *Reproduction, Fertility, and Development* 7:161-166.
- Chang, M. C. 1951. 1951. Fertilizing capacity of spermatozoa deposited into the fallopian tubes. *Nature* 168:697-698.

- Cochran, R., M. Meintjes, B. Reggio, D. Hylan, J. Carter, C. Pinto, D. Paccamonti, and R. A. Godke. Live foals produced from sperm-injected oocytes derived from pregnant mares. *Journal of Equine Veterinary Science* 18: 736-740.
- Corley-Smith, G. E., C. J. Lim, and B. P. Brandhorst. 1996. Production of androgenetic zebrafish (*Danio rerio*). *Genetics* 142:1265-1276.
- Dale, B., L. J. DeFelice, and G. Ehrenstein. 1985. Injection of a soluble sperm fraction into sea-urchin eggs triggers the cortical reaction. *Experientia* 41:1068-1070.
- Detweiler, C. and P. Thomas. 1998. Role of ions and ion channels in the regulation of Atlantic croaker sperm motility. *The Journal of Experimental Zoology* 281:139-148.
- Goto, K., A. Kinoshita, Y. Takuma, and K. Ogawa. 1990. Fertilization of bovine oocyte by the injection of immobilized, killed spermatozoa. *The Veterinary Record* 24:517-520.
- Goodrowe, K. L., S. L. Walker, D. P. Ryckman, G. F. Mastromonaco, M. A. Hay, H. L. Bateman, and W. T. Waddell. 2000. Piecing together the puzzle of carnivore reproduction. *Animal Reproduction Science* 60-61:389-403.
- Hart, N. H., K. A. Becker, J. S. Wolenski. 1992. The sperm entry site during fertilization of the zebrafish egg: Localization of actin. *Molecular Reproduction and Development* 32:217-228.
- Hart, N. H. and R. A. Fluck. 1995. Cytoskeleton in teleost eggs and early embryos: contributions to cytoarchitecture and motile events. *Current Topics in Developmental Biology* 31:343-381.
- Hart, N. H. and S. F. Yu. 1980. Cortical granule exocytosis and cell surface reorganization in eggs of *Brachydanio*. *The Journal of Experimental Zoology* 213:137-159.
- Hiramoto, Y. 1962. Microinjection of the live spermatozoa into sea urchin eggs. *Experimental Cell Research* 27:416-426.
- Iwamatsu, T., N. Yoshizaki, and Y. Shibata. 1997. Changes in the chorion and sperm entry into the micropyle during fertilization in the teleostean fish, *Oryzias latipes*. *Development Growth and Differentiation* 39:33-41.
- Katayose, H., J. Matsuda. and R. Yanagimachi. 1992. The ability of dehydrated hamster and human sperm nuclei to develop into pronuclei. *Biology of Reproduction* 47: 277-284.

- Keefer, C. L. 1989. Fertilization by sperm injection in the rabbit. *Gamete Research* 22:59-69.
- Kimura, Y. and R. Yanagimachi. 1995. Mouse oocytes injected with testicular spermatozoa or round spermatids can develop into normal offspring. *Development* 121:2397-2405.
- Kroll, K. L. and E. Amaya. 1996. Transgenic *Xenopus* embryos form sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. *Development* 122:3173-3183.
- Lee, K. W., S. E. Webb, and A. L. Miller. 1999. A wave of cytosolic calcium traverses zebrafish eggs on activation. *Developmental Biology* 214:168-180.
- Mann, J. R. 1988. Full-term development of mouse eggs fertilized by a spermatozoon microinjected under the zona pellucida. *Biology of Reproduction* 38:1077-1083.
- Markert, C. 1983. Fertilization of mammalian eggs by sperm injection. *The Journal of Experimental Zoology* 228:195-201.
- Morisawa, M. and S. Morisawa. 1994. Acquisition and initiation of sperm motility. Pages 137-151 in C. Gagnon, editor. *Controls of Sperm Motility: Biological and Clinical Aspects*. CRC press, Boca Raton, Florida.
- Naish, S.J., S. D. Perreault, and B. R. Zirkin. 1987. DNA Synthesis following microinjection of heterologous sperm and somatic cell nuclei into hamster oocytes. *Gamete Research* 18:109-120.
- Neuber, E., E. Havari, J. A. Sanchez, R. D. Powers, and L. J. Wangh. 1999. Efficient human sperm pronucleus formation and replication in *Xenopus* egg extract. *Biology of Reproduction* 61:912-920.
- Palermo, G., H. Joris, P. Devroey, and A. C. Van Steirteghem. 1992. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet* 340:17-18.
- Parsons, J. E. and G. H. Thorgaard. 1985. Production of androgenetic diploid rainbow trout. *Journal of Heredity* 76:177-181.
- Payne, D. 1995. Intracytoplasmic sperm injection: instrumentation and injection technique. *Reproduction, Fertility, and Development* 7:185-196.
- Perry, A. C. F., T. Wakayama, H. Kishikawa, T. Kasai, M. Okabe, Y. Toyoda, and R. Yanagimachi. 1999. Mammalian transgenesis by intracytoplasmic sperm injection. *Science* 284:1180-1097.

- Purdom, C. E. 1969. Radiation-induced gynogenesis and androgenesis in fish. *Heredity* 24:431-444.
- Shivers, C. A. and J. M. James. 1971. Capacitation of frog sperm. *Nature* 227:183-184.
- Sakai, N., S. Burgess, and N. Hopkins. 1997. Delayed *in vitro* fertilization of zebrafish eggs in Hank's saline containing bovine serum albumin. *Molecular Marine Biology and Biotechnology* 6:84-87.
- Sparrow, D. B., B. Latinkic, and T. J. Mohun. 2000. A simplified method of generating transgenic *Xenopus*. *Nucleic Acid Research* 28:E12-E12 (<http://nar.oupjournals.org/cgi/reprint/28/4/e12.pdf>).
- Sutovsky, P., R. Oko, L. Hewitson, and G. Schatten. 1997. The removal of sperm perinuclear theca and its association with the bovine oocyte surface during fertilization. *Developmental Biology* 188:75-84.
- Tateno, H., T. Wakayama, W. S. Ward, and R. Yanagimachi. 1998. Can alcohol retain the reproductive and genetic potential of sperm nuclei? Chromosome analysis of mouse spermatozoa stored in alcohol. *Zygote* 6: 233-238.
- Tiersch, T. R. 2000. Introduction. Pages xix-xxvi in T. R. Tiersch and P. M. Mazik editors. *Cryopreservation in Aquatic Species*. World Aquaculture Society. Baton Rouge, Louisiana.
- Tiersch, T. R. and S. S. Wachtel. 1991. On the evolution of genome size of birds. *Journal of Heredity* 82:363-368.
- Tiersch, T. R. and R. W. Chandler. 1989. Chicken erythrocytes as an internal reference for analysis of DNA content by flow cytometry in grass carp. *Transactions of the American Fisheries Society* 118:713-717.
- Topfer-Petersen, E. 1999. Molecules on the sperm's route to fertilization. *The Journal of Experimental Zoology* 285:259-266.
- Uehara, T. and R. Yanagimachi. 1976. Microsurgical injection of spermatozoa into hamster eggs with subsequent transformation of sperm nuclei into male pronuclei. *Biology of Reproduction* 15:467-470.
- Wang, I. J. 1989. Injection of *Xenopus* eggs before activation, achieved by control of extracellular factors, improves plasmid DNA replication after activation. *Journal of Cell Science* 93:1-8.
- Westerfield, M. 1995. *The Zebrafish Book. Guide for the Laboratory Use of Zebrafish (Danio rerio)*. Third edition. University of Oregon Press, Eugene.

Wolenski, J. and N. Hart. 1987. Scanning electron microscope studies of sperm incorporation into the zebrafish (*Brachydanio*) egg. *The Journal of Experimental Zoology* 243:259-273.

CHAPTER 4

INTRACYTOPLASMIC SPERM INJECTION IN SITES OTHER THAN THE MICROPYLE IN ZEBRAFISH EGGS

Since intracytoplasmic sperm injection (ICSI) has become an established practice in mammalian species, there has been concern associated with possible oocyte damage that this technique could cause, compromising embryonic development (Catt and Rhodes 1995). Direct injection of sperm into the cytoplasm of an oocyte is a disruptive action that could damage the chromosomal constitution of embryos, the cytoskeletal architecture (e.g. metaphase spindle) (Staessen and Van Steirteghem 1997) and the integrity of the membrane (Motoishi et al. 1996, Dumoulin et al. 2001). To avoid such damage, different strategies have been developed involving manipulation of the injection pipette, injection solutions and site of injection. Typically, mammalian oocytes are injected perpendicular to the position of the second polar body, to position the injection pipette as far as possible from the region where the meiotic spindle is located.

It is important to point out that the structure of the egg or oocyte dictates in many cases the technique of injection. In mammals, the size and symmetry of the oocyte facilitate the visualization of organelles and positioning of the oocyte for sperm injection. Sperm injections can be performed in any location in the cytoplasm as long as it is adjacent to the metaphase spindle and damage to this structure is avoided (reviewed by Mansour 1998).

Fish eggs that are spawned externally present a different architecture when compared to mammalian oocytes. These eggs possess a large deposition of yolk at one end (vegetal pole) that supplies energy to the embryo during early development, and the

cell cytoplasm in the other pole (animal pole). In normal fertilization the sperm enters the egg cytoplasm through the sperm entry site beneath the micropyle situated at the animal pole. This site was used in Chapter 3 to perform sperm injections in zebrafish eggs. Although the fertilization rate after ICSI was 16%, most of the fertilized eggs developed abnormally or did not develop beyond the gastrula stage. Due to the characteristics of the zebrafish egg, the position of the metaphase spindle could not be predicted and possible damage to this structure could have been the cause of failure after ICSI. The spindle could be localized by staining the chromosomes with a DNA-specific dye. Bisbenzimidazole or Hoechst dyes have been intensively used to label DNA of gametes in mammals. These dyes are cell-permeant and fluoresce bright blue when excited with ultraviolet light at ~350 nm. In previous work, Hoechst 33342 together with a dye that specifically binds actin filaments (rhodamine phalloidin) were used to disclose the fertilization mechanism during the first events in fertilization in zebrafish (Hart et al. 1992).

It has been reported that gamete fusion can occur at different places in teleost eggs that have been previously dechorionated (Yanagimachi 1957, Sakai 1961, Iwamatsu and Ohta 1978, Iwamatsu 1983). Sperm can fuse to the membrane in all areas of the egg and form structures similar to the ones formed at the micropyle such as the microvilli cluster. Sperm nuclei in eggs that have been fertilized by multiple sperm (polyspermy) decondense and develop into male pronuclei. This observation suggests that during ICSI, sperm can be injected within the animal pole in places other than the micropyle. The objectives of this experiment were: 1) to localize the position of the maternal chromosome after fertilization in zebrafish eggs; 2) to evaluate injection of

sperm in a location other than the micropyle, and 3) to evaluate damage to the mechanism of fertilization by following resumption of meiosis after injection.

MATERIALS AND METHODS

ANIMALS

Two zebrafish lines were used. A gold, long-fin line recessive for pigmentation was used for egg collection and a wild-type, pigmented line was used for sperm collection. Care of broodstock, and collection and preparation of gametes were carried out following the procedures described in Chapter 3 (Appendix A, SOP-1)

DNA STAINING

The large size (~800 μm) and opacity of zebrafish eggs did not allow observation of the maternal DNA in intact eggs. Therefore, a DNA-specific dye (Hoechst 33342) was used to localize the position of the maternal chromosomes in the animal pole of zebrafish eggs. Eggs were fixed at selected time intervals (30 sec to 25 min) after insemination in 4% paraformaldehyde (PFA) (Appendix A, SOP-3) dissolved in a phosphate-buffered saline (PBS)(Appendix A, SOP- 3) at pH 7.0 for 12 hours at 4°C. Eggs were washed twice in PBS for 10 min and twice in PBS containing 0.1% Tween-20 (Mallinckrodt Baker, Paris, Kentucky) (PBST). The animal poles were separated from the vegetal poles (yolk) of the eggs by use of two 25-gauge syringe needles. Dissected animal poles were incubated in PBST containing 5 $\mu\text{g/mL}$ of Hoechst 33342 (2, 5' benzimidazole, 2' -(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl) (Sigma Chemical Corporation, St. Louis, Missouri). Observation chambers were made with a glass slide and three cover slips (Appendix A, SOP-4). Two cover slips were attached with Superglue (Ross Superglue gel, American Glue Corporation, Taylor

Michigan) to the glass slide at ~1.5 cm apart. The stained tissue was placed between the two cover slips, with the animal pole facing upward, and a third cover slip was placed on top of the other two, forming a bridge over the tissue to minimize compression of egg fragments. Slides were observed with ultraviolet light microscopy (Nikon, Microphot-SA, Tokyo, Japan). The widths of the labeled sperm DNA and the egg chromosomes were calculated as well as the distance between them by use of image analysis software (Optimas 5.1a, Bioscan Inc, Edmund, Washington).

STAINING OF THE EGGS FOR ACTIN

Eggs were fixed in 5% formaldehyde in actin-stabilizing buffer (ASB) (Hart et al. 1992) for 10 hours at 4°C. The fixation buffer was prepared fresh from stock solutions to a final concentration of 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM ethylene glycol-bis(β-aminoethyl ether) (EGTA), 10 mM piperazine-N, N'-bis[2-ethanesulfonic acid] (Pipes) (pH 7.3), 5 mM MgCl₂, 100 mM KCl and 5% formaldehyde. Eggs were washed twice in PBS and twice in PBST for 10 min. The animal poles were dissected as described above and incubated in PBS containing 1% bovine serum albumin (BSA) for 30 min to reduce nonspecific background. Blocking solution was removed and samples were incubated in 165 nM Alexa fluor® 488 phalloidin (Molecular Probe, Inc., Eugene, Oregon) for 20 min at room temperature. Samples were incubated once in PBS containing 5 ug/mL Hoechst 33342 for 5 min.

Fluorescent dyes can fade (photobleach) when they are exposed to excitation light. To retard photobleaching, samples were washed three times in Slow Fade® equilibration buffer (Molecular Probe, Inc.) for 5 min and were placed on the slide with the micropyle facing upward. The tissues were covered with one drop of Slow Fade®

antifade reagent, mounted and sealed with nail polish (Love My Nails®, Greenwich, Connecticut). Slides were observed with fluorescence microscopy (Nikon, Microphot-SA).

SPERM INJECTION

The procedures used for injection were described in Chapter 2 with some modifications as described below. Two set of injections were performed, one through the micropyle and the other ~100 μ m away from the micropyle (Figure 4-1). Sperms cells were always injected after being activated by placing 2 μ L of sperm suspension in 2 μ L of embryo medium containing 10% polyvinylpyrrolidone (360,000 mw) (PVP). Eggs were injected in a non-activated state by leaving them in Hanks' balanced salt solution (HBSS) at 280 mOsmol/kg. The eggs were immediately activated after injection by placing them in embryo medium buffer (EMB). All injections were performed within 1 hr of egg collection.

Injects with buffer only (control injections) were done to rule out the possibility of partenogenetic development (gynogenesis). To observe possible damage after ICSI, 7 eggs from three different females were injected with sperm through the micropyle and fixed with 5% formaldehyde in actin-stabilizing buffer 10 min later. Staining of DNA and actin filaments were carried out following the protocols described above.

Eggs from individual females were used for each trial of ICSI. Egg quality was assessed by artificially inseminating eggs ~50 from each batch. Those eggs used for ICSI that came from batches with fertilization percentage higher than 60% were included in the analysis.

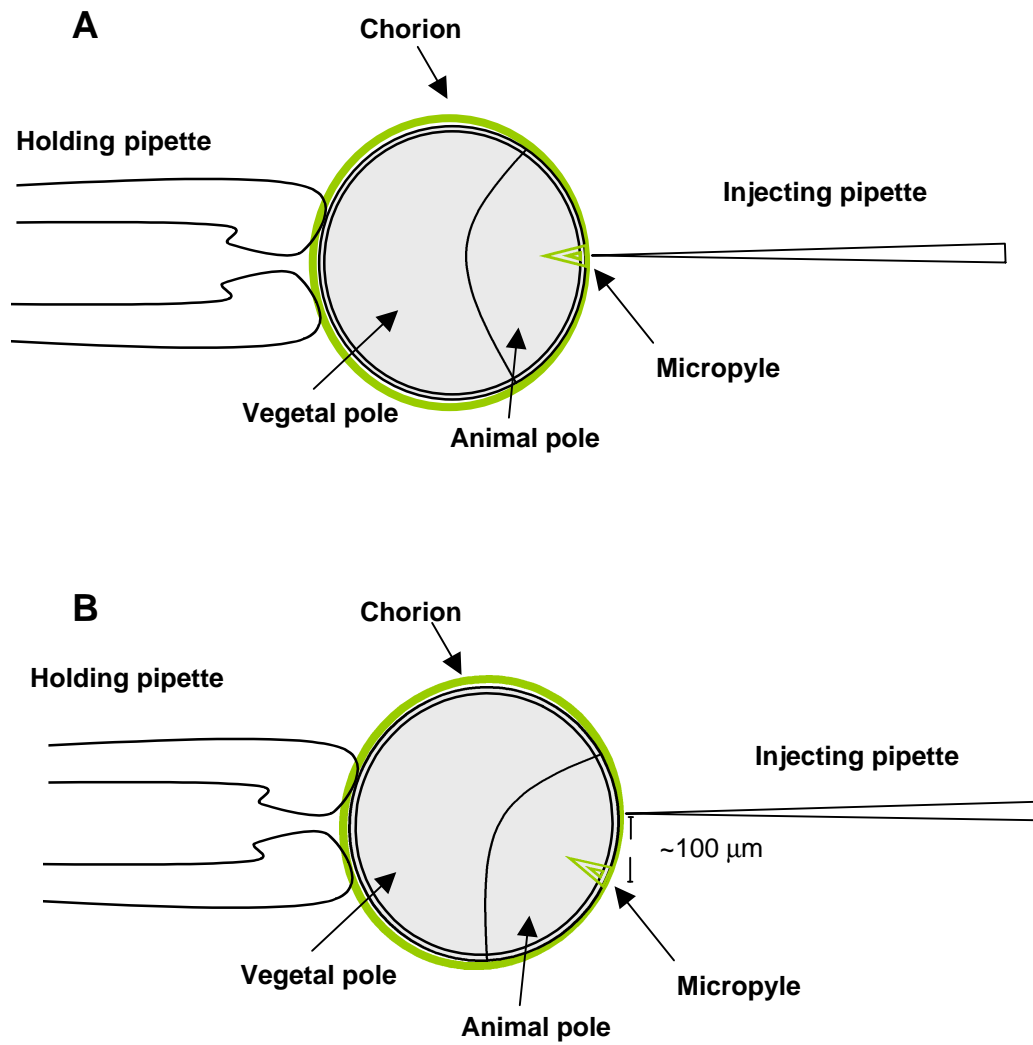


Figure 4-1. Injection procedure. A) Sperm injection through the micropyle. B) Sperm injection ~100 μm from the micropyle.

DNA CONTENT ANALYSIS

Analysis of cellular DNA content was performed using a FACSCalibur[®] flow cytometer (Becton Dickinson, San Jose, California) equipped with an air-cooled 480 nm argon laser. The FACSComp[®] software (Becton Dickinson) was used to calibrate the instrument settings. Embryos were disrupted by passage through a 3-mL syringe fitted with a 25-gauge needle while suspended in 0.5 mL of lysis buffer containing 25 µg of buffered RNase, 0.1% sodium citrate and 0.1% Triton X-100 (Tiersch and Chandler 1989). Samples were mixed and filtered through 20-µm nylon mesh. For analysis, an aliquot of 250 µl was diluted in lysis-staining buffer, which included 25 µg of propidium iodide (Sigma Chemical Corporation). A sample of frozen blood from rooster, *Gallus gallus*, was used as an internal reference (2.5 pg of DNA/cell) (Tiersch and Wachtel 1991). Measurements of the DNA content of normal diploid zebrafish (3.15 ± 0.06 pg of DNA/cell) and haploid zebrafish (1.59 pg) were compared with those of larvae produced by ICSI.

STATISTICAL ANALYSIS.

Statistical analysis was performed using SAS software for Windows (Version 8.01, SAS Institute, Cary, North Carolina). Differences in the incidence of fertilization among treatments during ICSI were evaluated using a logistic regression using the GENMOD procedure. Logistic regression can be modeled as a class of generalized linear model where the response probability distribution function is binomial and the link function is logit. The following program was used:

```
proc genmod;  
model r/n = trt / dist=binomial link=logit type3;  
run;
```

Differences were accepted as significant when $P < 0.05$.

RESULTS

STAINING

The staining method permitted tracking of the paths of the maternal and paternal chromosomes after fertilization, as well as their localization within the egg. At 30 sec after insemination, zebrafish sperm heads at the egg membrane were $2.6 \pm 0.2 \mu\text{m}$ wide ($n = 6$) (Figure 4-2A, B) (the width of stained fresh sperm was $2.9 \pm 0.3 \mu\text{m}$). The distance between the micropyle, or sperm entry site (injection site), and maternal chromosomes at 30 seconds after insemination was $37.6 \pm 9.5 \mu\text{m}$ ($n = 10$) (Figure 4-2A). Five minutes after insemination, the sperm head was internalized in the egg and was surrounded by actin filaments (as shown by Hart et al. 1992) (Figure 4-2B), and the maternal DNA had begun to divide (Figure 4-2C). One set of chromosomes ($1n$) formed the second polar body and the other formed the female pronucleus ($1n$). At 15 min after insemination, the male and female pronuclei decondensed and moved together (Figure 4-2D) and at 25 min they joined to form the zygote nucleus (Figure 4-2E). At 25 min, the second polar body was detected outside of the cytoplasm as a DNA structure surrounded by actin filaments (Figure 4-2F).

Of the 7 eggs injected with sperm through the micropyle, 5 showed sperm in the cytoplasm and all showed second polar body formation (Figure 4-3A,B).

SPERM INJECTION

Of 45 sperm injections through the micropyle, 5 eggs (11%) were fertilized and one developed normally and was pigmented. Flow cytometric analysis showed that this embryo was diploid. Of the 42 sperm injections carried out at sites other than micropyle, 4 eggs (9%) were fertilized and none developed normally (Figure 4-3).

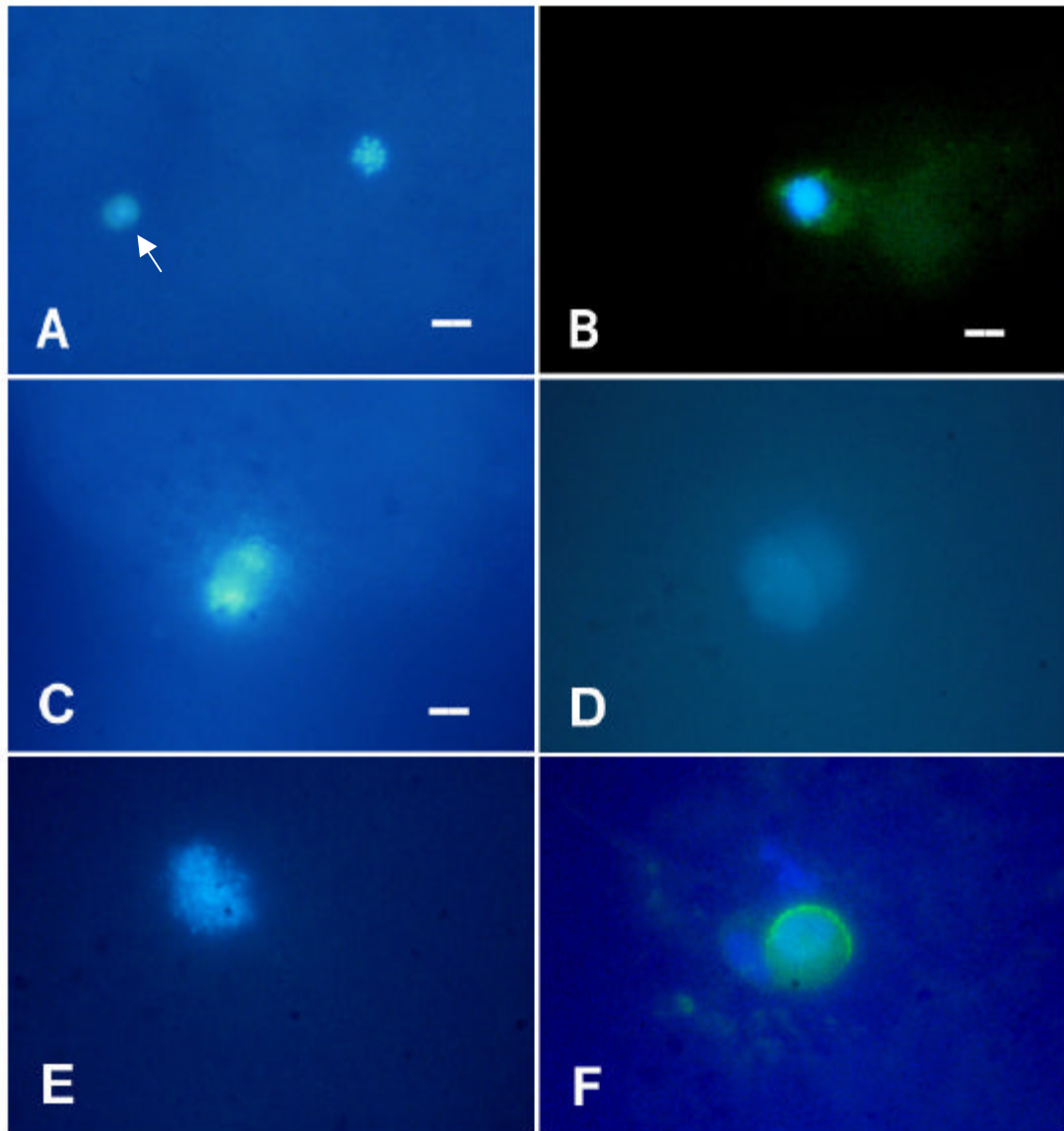


Figure 4-2. Fertilization in zebrafish. A) 30 sec after insemination, the sperm (arrow) is still present at the sperm entry site and the maternal chromosomes are about 40 μm from it; bar 6.8 μm . B) 5 min after insemination, DNA was labeled with Hoechst 33342 and actin filaments with Alexa fluor® 488 phalloidin, sperm head (blue) is surrounded by a meshwork of actin filaments (green); bar 2 μm . C) 5 min after insemination, the maternal chromosomes begin to split; bar 6 μm . D) At 15 min after insemination, female and male pronuclei appear decondensed and united. E) At 25 min after insemination, zygote pronuclei can be seen. F) Second polar body labeled with Hoechst and phalloidin.

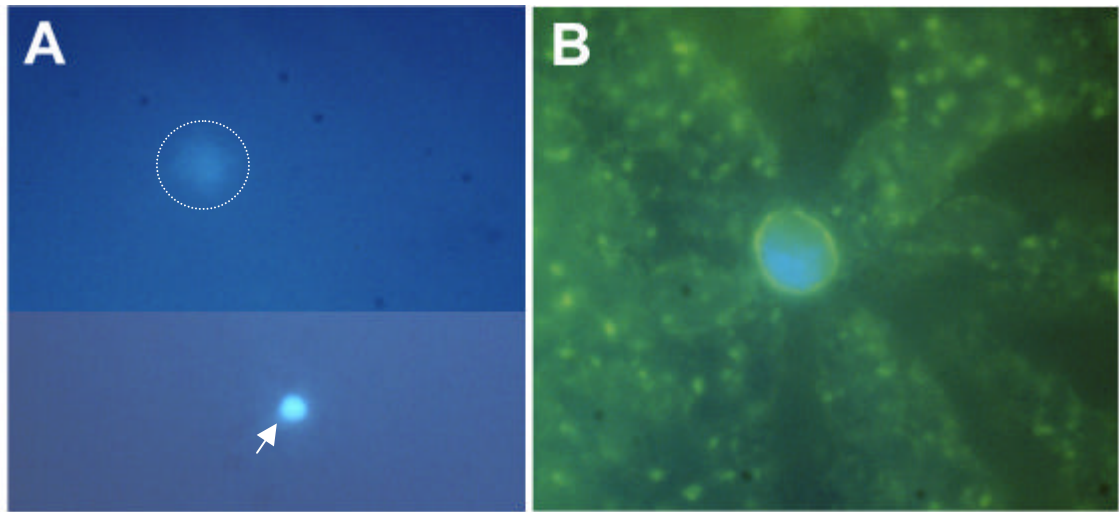


Figure 4-3. Sperm injected into zebrafish eggs. A) 10 min after injection, eggs were fixed in 4% paraformaldehyde. In 5 of 7 fixed eggs, the sperm was observed (arrow) in proximity to the maternal chromosomes (circle). Two pictures of the same frame but at different levels were taken and aligned for this image. B) DNA of the polar body surrounded by actin filaments. The entire egg surface was labeled with fluorescence. Extrusion of the polar body was observed in 7 of 7 injected eggs.

No significant difference ($P = 0.8264$; logistic regression) in fertilization was observed between the two treatments. No development was observed in the egg with control injections (no sperm) (Table 4-1).

Table 4-1. Development of zebrafish embryos produced by injecting sperm cells through the micropyle or 100 μm from the micropyle. Control injections were made without sperm.

	Number of eggs	Blastula	Gastrula	Advanced neurula		Pigmented larvae*
				Abnormal	Normal	
Micropyle	47	5 (11%)	5 (11%)	3 (6%)	1 (2%)	1 (2%)
Control	31	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Non-micropyle	43	4 (9%)	3 (7%)	2 (5%)	0 (0%)	0 (0%)
Control	38	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

* Offspring showing the paternal pigmented phenotype. Sperm from wild-type pigmented males were injected into eggs of gold, long-fin females.

Eighty nine percent of the embryos produced by ICSI were abnormal (Figure 4-4A) compared with the 3% abnormality found in artificially inseminated eggs. DNA content of the normal larvae was 3.0 pg of DNA per cell indicating that it was diploid.

DISCUSSION

The aim of this experiment was to improve ICSI in zebrafish. Localizing the maternal chromosomes after fertilization by use of a fluorescent dye (Hoechst 33342) allowed evaluation of the possibility of chromosomal injury after ICSI.

Although the maternal DNA was in proximity to the micropyle ($\sim 40 \mu\text{m}$), the results suggest that if injections were performed carefully through the micropyle, neither the maternal chromosomes nor the mechanisms involved in maternal DNA division should be damaged. This also suggested that the explanation for the low rate of fertilization and the compromised development of the embryos observed here and in Chapter 3 lies elsewhere. It was found that 28% of the injected eggs did not show

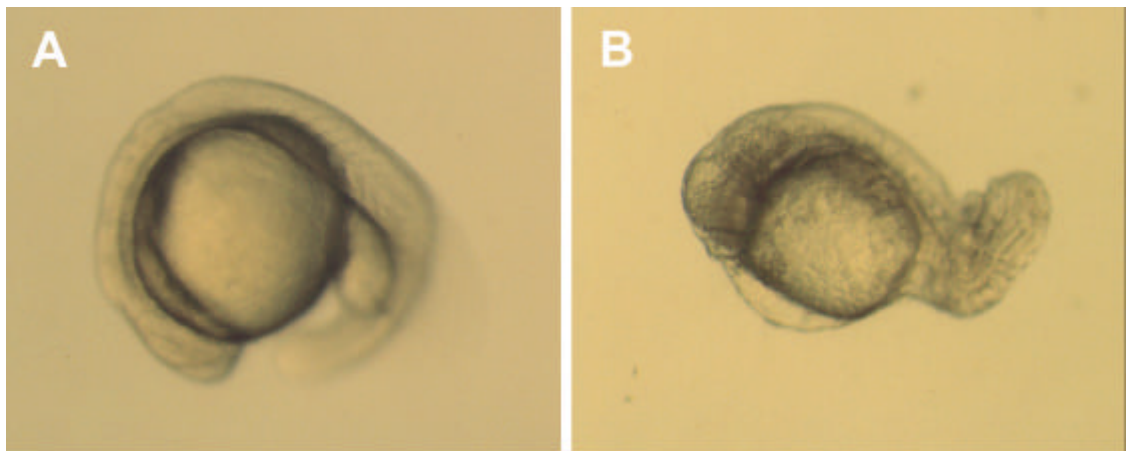


Figure 4-4. Zebrafish embryos 20 h after fertilization. A) Normal embryo after artificial insemination; B) Abnormal embryo after ICSI.

sperm in the cytoplasm, suggesting that the low fertilization rate could be the result of sperm expulsion through the injection tract or a failure in membrane penetration by the injecting pipette, preventing sperm incorporation. Similar results have been found in humans where a percentage of oocytes (~13%) that failed to fertilize after ICSI did not show sperm in the cytoplasm (Flaherty et al. 1995, Sakkas et al. 1996, Lopes et al. 1998, Rawe et al. 2000). Although failure in sperm delivery could have reduced fertilization, it does not account for abnormal development. Abnormal fish occur after normal insemination, however, the percentage of abnormality observed in ICSI fertilized eggs was greater (89%) than the percentage of abnormalities found in fish produced by artificial insemination (3%). This clearly suggests that ICSI is the source of these developmental problems.

Injection at sites away from the micropyle did not show improvement in the fertilization rate nor reduction of the developmental abnormalities. Even though the fertilization rate did not improve by injecting at a site other than the micropyle, the fact that embryos were produced by this method agrees with findings in medaka, *Oryzias latipes*, Pacific herring, *Clupea pallasii* and rose bitterling, *Rhodeus ocellatus ocellatus* (Yanahimachi 1957, Sakai 1961, Iwamatsu and Ohta 1978, Iwamatsu 1983) that show that sperm can fertilize eggs by entry at sites other than the micropyle in dechorionated eggs.

It is possible that the sperm contributes to the fertilization process only when placed in a specific region of the cytoplasm where the cytoskeleton machinery involved in binding of the sperm head is localized. It has been shown that the zebrafish sperm head is surrounded by a shell of actin filaments that are thought to become structurally

linked to the sperm nuclear membrane after the gamete membranes fuse (Hart et al. 1992). The actin meshwork seems to form part of the mechanism of sperm movement inside the eggs. If this mechanism is disrupted, or if this mechanism only works in a specific area of the egg, sperm cells injected away from this area would not be able to contribute to the fertilization process. In humans, defects of male pronucleus migration has also been found lead to fertilization failures after ICSI (Schatten 1994). It is thought that this occurs from the inability of microtubules to assemble around the paternal centrosome.

The principal reason for fertilization failure after ICSI in humans (~40%) is the lack of oocyte activation (Rawe et al. 2000). It is thought that sperm present an oocyte-activating factor that is able to trigger calcium oscillations and initiate activation of development in oocytes (Swann 1990, Dozortsev et al. 1995). A soluble sperm protein called oscillin was suggested as the physiological trigger of activation (Parrington et al. 1996). More recently nitric oxide has been suggested as the possible activation factor (Kuo et al. 2000). The absence or lack of activity of this factor or factors could lead to fertilization failure.

Although zebrafish eggs are partially activated by the spawning medium, they need something else associated with the sperm cell that has the ability to activate development. This is suggested by the lack of parthenogenesis observed when eggs were placed in water without insemination or when pierced with a glass needle. However, development occurs after ICSI, and also after insemination with sperm that are genetically inert or incompatible (irradiated sperm, or sperm from other species such as Nile tilapia) (Corley-Smith et al. 1996, Appendix A). It would be informative to use

genetically inert sperm cells as egg activators during ICSI procedures in fish. This method of activation is discussed in Chapter 5 and in Appendix B.

From the studies performed thus far, basic knowledge from mammals can assist development of ICSI in fishes. Future work in fish will need to address many of the same questions that researchers have addressed when performing ICSI in mammals such as egg activation, sperm nuclear decondensation and migration, centrosome arrangement and cell injury.

References

- Catt, J. W. and S. L. Rhodes. 1995. Comparative intracytoplasmic sperm injections (ICSI) in human and domestic species. *Reproduction Fertility and Development* 7:161-167.
- Corley-Smith, G. E., J. L. Chinten, and P. B. Bruce. 1996. Production of androgenetic zebrafish (*Danio rerio*). *Genetics* 142:1265-1276.
- Dumoulin, J. C. M., E. Coonen, M. Bras, J. M. Bergers-Janssen, R. C. M. Ignoul-Vanvuchelen, L. C. P. van Wissen, J. P. M. Geraedts, and J. L. H. Evers. 2001. Embryo development and chromosomal anomalies after ICSI: effect of the injection procedure. *Human Reproduction* 16:306-312.
- Dozortsev, P., A. Rybouchkin, and P. DeSutter. 1995. Human oocytes activation following intracytoplasmic sperm injection; the role of the sperm. *Human Reproduction* 9:2139-2144.
- Flaherty, S. P., D. Payne, N. J. Swann, and C. D. Matthews. 1995. Aetiology of failed and abnormal fertilization after intracytoplasmic sperm injection. *Human Reproduction* 10:2623-2629.
- Hart, N., B. A. Becker, and J. S. Wolenski. 1992. The sperm entry site during fertilization of the zebrafish egg: localization of actin. *Molecular Reproduction and Development* 32:217-228.
- Iwamatsu, T. and T. Ohta. 1978. Electron microscopic observation on sperm penetration and pronuclear formation in the fish egg. *The Journal of Experimental Zoology* 205:157-180.

- Iwamatsu, T. 1983. A new technique for dechoriation and observation on the development of the naked egg in *Oryzias latipes*. *The Journal of Experimental Zoology* 228:83-89.
- Kuo R. C., G. T. Baxter, S. H. Thompson, S. A. Stricker, C. Patton, J. Bonaventura, and D Epel. 2000. NO is necessary and sufficient for egg activation at fertilization. *Nature* 406:633-636.
- Lopes, S., A. Juriscova, and R. Casper. 1998. Gamete-specific DNA fragmentation in unfertilized human oocytes after intracytoplasmic sperm injection. *Human Reproduction* 13:703-708.
- Mansour, R. 1998. Intracytoplasmic sperm injection: a state of the art technique. *Human Reproduction* 4:43-56.
- Motoishi, M., M. Goto, K. Tomita, S. Ookutsu, and Y. Nakanishi. 1996. Examination of the safety of intracytoplasmic sperm injection procedures by using bovine zygotes. *Human Reproduction* 11:618-620.
- Parrington J., K. Swann, V. I. Shevchenko, A. K. Sesay, and F. A. Lai. 1996. Calcium oscillations in mammalian eggs triggered by a soluble sperm protein. *Nature* 379:364-368.
- Rawe, V. Y., S. Brugo Olmedo, F. N. Nodar, G. D. Doncel, A. A. Acosta, and A. D. Vitullo. 2000. Cytoskeletal organization defects and abortive activation in human oocytes after IVF and ICSI failure. *Molecular Human Reproduction* 6:510-516.
- Sakai, Y. 1961. Method for removal of chorion and fertilization of the naked egg in *Oryzias latipes*. *Embryologia* 5:357-379.
- Sakkas, D., F. Urner, P. G. Bianchi, I. Wagner, N. Jaquenoud, G. Manicardi, and A. Campana. 1996. Sperm chromatin anomalies can influence decondensation after intracytoplasmic sperm injection. *Human Reproduction* 11:837-843.
- Schatten, G. 1994. The centrosome and its mode of inheritance: the reduction of the centrosome during gametogenesis and its restoration during fertilization. *Developmental Biology* 165:299-335.
- Staessen, C. and A. C. Van Steirteghem. 1997. The chromosomal constitution of embryos developing from abnormally fertilized oocyte after intracytoplasmic sperm injection and conventional in-vitro fertilization. *Human Reproduction* 12:321-327.
- Swann, K. 1990. A cytosolic sperm factor stimulates repetitive calcium increases and mimic fertilization in hamster eggs. *Development* 110:1295-1302.

- Tiersch, T. R. and S. S. Wachtel. 1991. On the evolution of genome size of birds. *Journal of Heredity* 82:363-368.
- Tiersch, T. R. and R. W. Chandler. 1989. Chicken erythrocytes as an internal reference for analysis of DNA content by flow cytometry in grass carp. *Transactions of the American Fisheries Society* 118:713-717.
- Wolenski, J. and N. Hart. 1987. Scanning electron microscope studies of sperm incorporation into the zebrafish (*Brachydanio*) egg. *The Journal of Experimental Zoology* 243:259-273.
- Yanagimachi, R. 1957. Studies on fertilization in *Cuplea pallasii*. VI. Fertilization of the egg deprived of the membrane. *Japanese Journal of Ichthyology* 6:41-47.

CHAPTER 5

FERTILIZATION OF NILE TILAPIA EGGS BY INTRACYTOPLASMIC SPERM INJECTION (ICSI)

Nile tilapia *Oreochromis niloticus* is an important cultured fish in more than 55 countries located in the tropics and subtropics. Among the factors that contribute to the widespread use of tilapias in aquaculture are resistance to poor water quality (e.g. low oxygen) and diseases, fast embryonic development (hatching in 5 days), maturity at an early age (4-6 months), and year-round spawning. These characteristics have enhanced studies of genetic improvement using tools such as hybridization (Lovshin 1982), sex reversal (Guerrero 1982), gynogenesis (Don and Avtalion 1989, Varadaraj 1990, Peruzzi et al. 1993) and polyploidy (Valenti 1975, Myers 1986, El Gamal et al. 1999). The results obtained in Chapter 3 of this dissertation suggested that intracytoplasmic sperm injection (ICSI) could be used in fish as another tool for genetic improvement (Poleo et al. 2001). The importance of Nile tilapia in aquaculture and its spawning characteristics make it a good candidate for ICSI. This Chapter addresses the possibility of applying ICSI in Nile tilapia, but first it is important to consider some aspects of the spawning and fertilization processes in this species.

Nile tilapia males build a nest in the substrate to attract ripe females with a courtship display. The female lays eggs in the nest a few at a time and collects them in her mouth after they have been fertilized by the male. The eggs hatch in the mouth around 5 days after fertilization and remain there until the yolk sac is absorbed. The fry begin swimming in and out of the mouth to feed, and stay with the mother until they become too large (~14 mm total length) to fit in the mouth. After the offspring are dispersed the female can spawn again within 4 weeks (reviewed by Beveridge and

McAndrew 2000). Although the presence of males influences the spawning cycle of females, the absence of males does not stop it. Nile tilapia females can spawn regularly when placed in individual tanks (Mires 1982, Gautier et al. 2000). This method of spawning, identified here as the “single-breeding” system, is useful when artificial spawning is required.

It is known that unfertilized eggs of teleosts typically lose their fertilization capability within few minutes after being immersed in water (reviewed by Yamamoto 1975). Unfertilized eggs of Nile tilapia become incapable of fertilization within 15 min after being immersed in fresh water (Myers and Hershberger 1991). In zebrafish *Danio rerio* the window for fertilization is shorter, with eggs losing fertilization capability within 1 min in fresh water (Wolenski and Hart 1987). It has also been found that fertilization capability can be extended if eggs are placed in isotonic solutions. In medaka *Oryzias latipes* eggs remained fertile for more than 2 hours when kept in Ringers’ solution (Yamamoto 1975). The same has been found for zebrafish where eggs could be held for 2 hours using Hanks’ balanced salt solution (HBSS) containing bovine serum albumin (BSA) (Sakai et al. 1997) or in ovarian fluid from coho salmon *Oncorhynchus kisutch* (Corley-Smith et al. 1996).

At the time of fertilization, eggs of teleost fishes are arrested in metaphase II of the meiotic cycle. The maternal chromosomes are aligned at the spindle and a stimulus is required to resume meiosis which results in extrusion of the second polar body and formation of the female pronucleus. The location of the metaphase spindle in Nile tilapia is not known. The position of this structure is an important piece of information

when performing ICSI if mechanical injury by the injecting pipette to the maternal chromosomes is to be avoided.

As mentioned in Chapter 2, resumption of the meiotic cycle in Nile tilapia occurs when the sperm comes into contact with the egg. This suggests that eggs would need to be activated before or after performing ICSI if fertilization is to be achieved. In some mammals, artificial activation after ICSI is performed by using electric current or chemicals that alter membrane electrical potential (ionophores) and trigger the calcium wave. In fish, activation could be achieved by the use of a genetically inert sperm such as sperm treated with ultraviolet light (Don and Avtalion 1988, Corley-Smith et al. 1996) or by using sperm from another species. Sperm of common carp *Cyprinus carpio* have been used to activate tilapia eggs for the induction of gynogenesis (Varadaraj 1990, Peruzzi et al., 1993). The use of common carp sperm or sperm from a related species such as goldfish *Carassius auratus* could also be used when performing ICSI.

The goal of this Chapter was to develop a basic technique for ICSI in Nile tilapia. The objectives were: 1) to evaluate the method of spawning individual females in tanks as a source of eggs for ICSI; 2) to determine the window of fertilization when placing Nile tilapia eggs in an isotonic solution; 3) to localize the maternal DNA of unfertilized eggs; 4) to evaluate ICSI in Nile tilapia eggs, and 5) to evaluate the use of goldfish sperm to activate eggs before ICSI is performed.

MATERIALS AND METHODS

ANIMALS

Twenty five Nile tilapia females were maintained individually in recirculating systems at the LSU Aquaculture Research Station with a natural photoperiod. Females

were placed into 80-L tanks (Figure 5-1) at 26°C. Salinity was maintained at 1.5 parts per thousand (ppt), and alkalinity and hardness at ~200 mg/L. Females that showed a swollen belly and projecting genital papilla were selected for spawning. Fish were anesthetized with tricaine methanesulfonate (methyl-m-aminobenzoate, MS222) (Argent Laboratories, Redmond, Washington) before stripping of eggs. The abdomens of fish were dried with paper towels and squeezed gently, starting behind the pectoral fins and moving towards the tail. Ripened eggs were easily stripped and showed a uniform size. The presence of blood, connective tissue or size variation indicated that the eggs were not ripe and they were not used for experiments (Appendix A, SOP-6). The date of spawning for each female was recorded and the spawning cycles (time between each spawn) was calculated for regular spawners.

Sperm were obtained by gently squeezing the sides of unanesthetized males. A 10- μ L pipette tip connected to a mouth pipette was used to extract sperm which were diluted 1:1 in HBSS (~280 mOsmol/kg) in 1.5-mL microcentrifuge tubes and placed on ice. Sperm motility was estimated by use of dark-field microscopy (Appendix A, SOP-5). Only sperm with high motility (>90%) were used for experiments.

EGG STORAGE BEFORE FERTILIZATION

All eggs were collected and stored in HBSS (Appendix A, SOP-2) at 25°C until fertilization. Three fertilization trials were performed from 0 to 80 min at 10-minute intervals and another trial at 0 and 3 hours. At fertilization, most of the HBSS was removed and sperm was mixed with the eggs. A total of 2 mL of embryo medium buffer (EMB) (13.7 mM NaCl, 5.40 mM KCL, 0.25 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 1.30 mM CaCl₂, 1.00 mM MgSO₄, 4.20 mM NaHCO₃) (Appendix A, SOP-2)

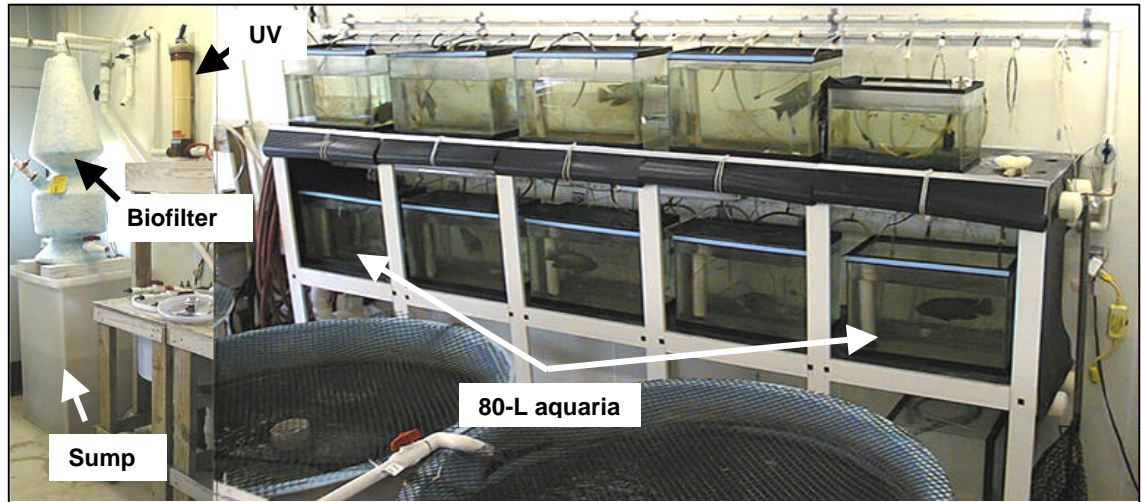


Figure 5-1. System for holding of female Nile tilapia composed of nine 80-L aquaria linked to a biofilter and an ultraviolet sterilizer (UV). This system is located in room 142 at the LSU Aquaculture Research Station.

(Westerfield 1995) was added for activation. After 10 min, 100 mL of EMB was added to the eggs which were left undisturbed in an incubator at 28°C. Unfertilized eggs were removed and EMB was changed twice daily. After 48 hours, eggs were evaluated for percent fertilization. Those that developed to stage 10 (neurula) (Galman and Avtalion 1989) were recorded as fertilized.

STAINING OF DNA

Plastic baskets were constructed to hold the eggs. The bottoms of plastic cryogenic goblets (Southland Cryogenics, Carrollton, Texas) were removed yielding a tube of about 1.5 cm in length. A hot plate (Thermolyne, Nuova II, Model SP18425, Dubuque, Iowa) was turned to its highest setting and aluminum foil was placed on the surface. One end of the tube was placed on top of a square (1 cm) of 85- μ m mesh and was placed onto the surface of the plate, so that the mesh was sandwiched between the upright tube and the aluminum foil. The free end of the tube was held onto the surface of the plate until melting was visible. The tube was lifted off the plate and the foil removed. Mesh surrounding the tube was trimmed with scissors.

Eggs were fertilized and fixed at specific time intervals. DNA staining was performed by following the procedures of Hart et al. (1992), with some modifications as described below. Eggs were placed in 4% paraformaldehyde (PFA) (Appendix A, SOP-3) for 1 hour, changed to a fresh PFA solution and held overnight at 4°C. Eggs were washed twice in phosphate-buffered saline (PBS) (0.80% NaCl, 0.02% KCl, 0.02% KH_2PO_4 , and 0.115% Na_2HPO_4) and twice in PBS containing 0.1% Tween-20 (PBST) for 10 min each time. The eggs were held in 4 mL of PBST at 4°C for no more than 5 days until analysis.

The eggs were removed from the baskets and dissected. Using a 26-gauge needle, the animal pole was separated from the vegetal pole. The chorion was removed and as much lipid as possible was removed from beneath the animal pole. The cleaned animal pole was placed in a 1.5-mL microcentrifuge tube. At least four eggs were dissected in this manner for each time interval. Animal poles were incubated in 5 $\mu\text{g/mL}$ of Hoechst 33342 (Sigma Chemical Corporation, St. Louis, Missouri) for 5 min and the samples were washed three times with PBS for 5 min to remove excess dye.

Chambers for the staining procedure were made following the procedure described in Appendix A SOP-4 with a slide and three cover slips. The slides were viewed under an ultraviolet light microscope (Nikon Microphot-SA microscope, Nikon, Tokyo, Japan). Observations were recorded with a video camera (Microimage Video Sytem, model A206A, Boyertown, Pennsylvania) onto a computer. The widths of the stained and unstained sperm and egg DNA were calculated, as well as the distance between them, by use of image analysis software (Optimas 5.1a, Bioscan, Edmund, Washington).

EGG INJECTIONS

Injections were performed using a micromanipulator apparatus consisting of an inverted microscope (Diaphot Nikon Inc., Tokyo, Japan) equipped with two mechanical micromanipulator units (Leitz, Rockliegh, Illinois). A manipulator moved either the holding or injection pipette. Each pipette was connected to a screw syringe by fine-bore Teflon tubing filled with light mineral oil (Sigma Chemical Corporation). The injection pipette and the holding pipette were made from borosilicate glass capillary tubes (Sutter Instrument Company, Novato, California). The injection pipette had an internal

diameter of ~15 μm . Due to the variability in size between eggs of females of Nile tilapia, the holding pipettes were constructed for each batch of eggs with an internal diameter ranging from ~200 to ~400 μm . Eggs were placed in a 200- μL drop of HBSS in the lid of a 100-mm plastic culture dish (Corning Glass Works, Corning, New York). When eggs were correctly positioned with the animal pole facing outward (Figure 5-2), the injection pipette was pushed through the micropyle canal into the cytoplasm and a sperm was injected with a small volume (~7 pL) of EMB containing 10% polyvinylpyrrolidone (PVP) to prevent the sperm from sticking to the wall of the pipette.

EGG ACTIVATION

Egg activation was evaluated using different treatments. Eggs injected with Nile tilapia sperm, eggs injected without sperm (injection buffer only), eggs placed in EMB, and eggs inseminated with goldfish sperm. An egg was considered to be activated when rising of the chorion (water hardening) and formation of the blastodisc were observed.

A group of 20 eggs placed in EMB, 20 eggs inseminated with goldfish sperm and 5 eggs injected without sperm were also fixed and stained with Hoechst 33342 to evaluate resumption of meiosis as seen by division of the maternal DNA.

EGG INJECTION AFTER INSEMINATION WITH GOLDFISH SPERM

Sperm were collected from goldfish by the procedure described above for tilapia. A single Nile tilapia egg was put in a small droplet (~200 μL) of EMB, incubated with goldfish sperm and injected with Nile tilapia sperm. The injections were performed using the methods described above. Eggs were individually held in 35-mm

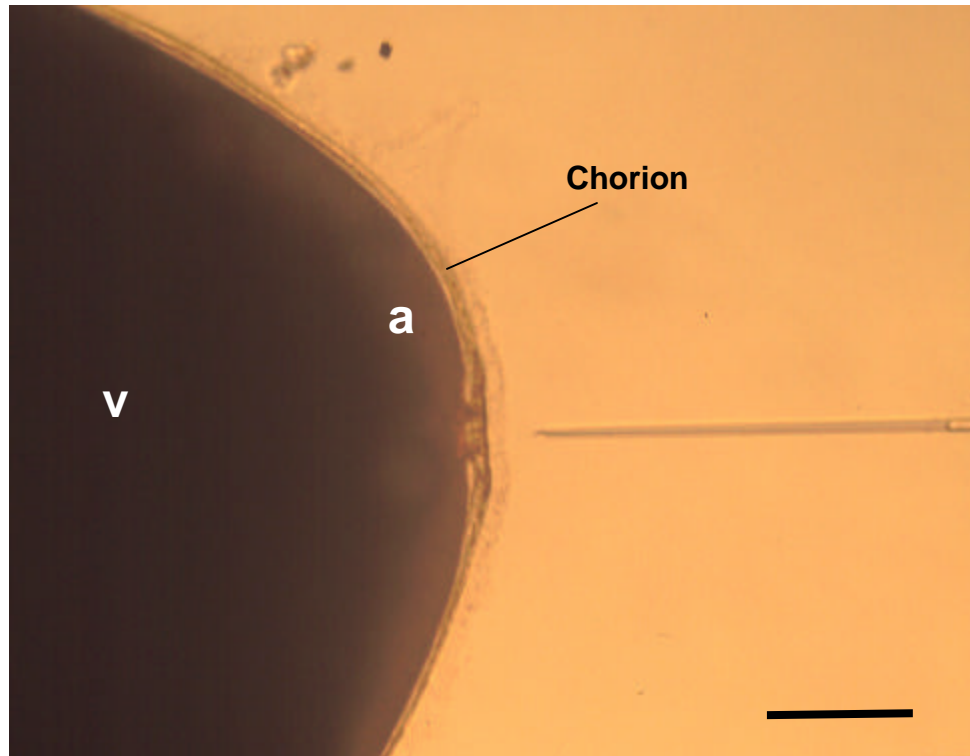


Figure 5-2. Nile tilapia egg shown in position for sperm injection. The tip of the injecting pipette is pointing to the micropyle of the egg. Scale bar ~100 μm . The vegetal pole (v) represents more than 70% of the egg volume. The animal pole (a) is present at the narrow end of the egg.

petri dishes after injection (Corning Glass Works) at 28°C and fertilization was evaluated after 48 hours. Embryos that reached stage 11 (embryonic keel and somite formation) (Galman and Avtalion 1989) were counted as fertilized. In order to assess egg quality, a group of ~50 eggs from every batch was artificially inseminated with fresh Nile tilapia sperm. Egg used for ICSI coming from batch of eggs with a percentage of fertilization higher than 70% were included in analysis.

DNA CONTENT ANALYSIS

Analysis of DNA content was performed using a FACSCalibur[®] flow cytometer (Becton Dickinson, San Jose, California) equipped with an air-cooled 480 nm argon laser. The FACSComp[®] software (Becton Dickinson) was used to calibrate the instrument settings. Embryos were disrupted by passage through a 3-mL syringe fitted with a 25-gauge needle while suspended in 0.5 mL of lysis buffer containing 25 µg of buffered RNase, 0.1% sodium citrate and 0.1% Triton X-100 (Tiersch and Chandler 1989). The yolk sac of embryos was removed at 4 days after fertilization and the remaining tissues were disrupted by passing them three times through a 25-gauge syringe. The solution was filtered through 20-µm nylon mesh. Blood from a 3-month-old ICSI fish was also collected from the caudal vessels. For analysis, an aliquot of 250 µL of disrupted embryo or 1 µL of blood was diluted in lysis-staining buffer, which included 25 µg of propidium iodide (Sigma Chemical Corporation). A sample of blood from channel catfish *Italurus punctatus* (1.98 pg of DNA/cell) (Tiersch et al. 1990) and zebrafish *Danio rerio* (3.15 pg of DNA/cell) (Poleo et al. 2001), were used as an internal reference. Measurements of the DNA content of normal diploid Nile tilapia

(2.10 ± 0.09 pg of DNA/cell, $N = 6$) were compared with those of larvae produced by ICSI.

STATISTICAL ANALYSIS

Statistical analysis was performed using SAS software for Windows® version 8.1 (SAS Institute, Cary, North Carolina). Fertilization and activation data were analysed and compared using one-way factorial analysis (ANOVA). Differences were determined by the Tukey's test. Percentages were arcsin transformed before analysis. Differences were accepted as significant at $P < 0.05$. Differences in the incidence of fertilization among treatments during ICSI were evaluated using a logistic regression.

RESULTS

During the study period (February-November, 2001), female Nile tilapia began to spawn regularly after being in the recirculating system for 2 months. All spawning occurred in the afternoon between 1300 hours and 1800 hours. Records showed that the duration of the spawning cycle for a total of 26 spawns from 7 females that spawned regularly (more than twice) was 28 ± 9 days with a minimum of 10 days and a maximum of 39 days. It was common to obtain more than 1,000 eggs from stripping of individual females.

EGG STORAGE BEFORE FERTILIZATION

There was no significant difference ($P = 0.2518$) for eggs fertilized at 10-min intervals for 80 min (Figure 5-3). During this period, there was an average fertilization of $88 \pm 4\%$ (mean \pm SD). Although variation increased, similar results were observed for comparison of fertilization at 5 min ($97 \pm 1\%$) and 3 hours ($85 \pm 15\%$)

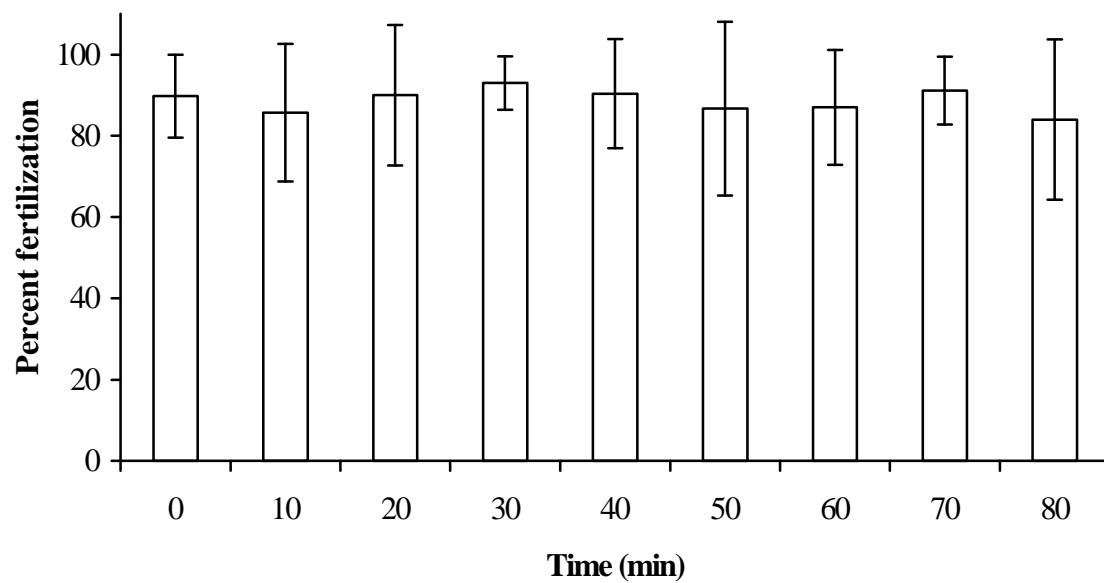


Figure 5-3. Fertilization of Nile tilapia eggs stored in Hanks' balanced salt solution (HBSS) from 0 to 80 min.

(Figure 5-4). Although it was not statistically tested, eggs could be fertilized after 5 hours (77% fertilization)

STAINING OF DNA

At 30 seconds after insemination, the sperm head was localized at the sperm entry site (Figure 5-5A) and it showed an average width of $1.8 \pm 0.1 \mu\text{m}$ (stained fresh sperm, 2.2 ± 0.2). The maternal chromosomes showed an average size of $2.3 \pm 0.06 \mu\text{m}$ and were situated around $40 \pm 5.5 \mu\text{m}$ from the sperm head (Figure 5-5B). At 10 min after insemination, the resumption of meiosis was observed by the splitting of the maternal chromosomes (Figure 5-5C) and at 20 min after fertilization the decondensed female and male pronuclei were visible (Figure 5-5D).

ACTIVATION

There was significant difference ($P < 0.001$) in egg activation (i.e., water hardening, formation of blastodisc) (Figure 5-6) among the eggs that were injected with or without sperm and the eggs that were incubated in EMB alone or inseminated with gold fish sperm (Figure 5-7). No significant difference ($P = 0.4463$) in activation was found between eggs injected with sperm and eggs injected with buffer only (Figure 5-7). No significant difference ($P = 0.2501$) was found between eggs incubated in EMB and eggs inseminated with goldfish sperm (Figure 5-7). Twenty Nile tilapia eggs that were previously incubated with goldfish sperm were stained with Hoechst 33342. The stain revealed that the cell cycle was not triggered, as splitting of the maternal DNA was not observed 10 min after incubation with goldfish sperm. The same results were obtained when the eggs were incubated with EMB alone (Figure 5-8). Four of five

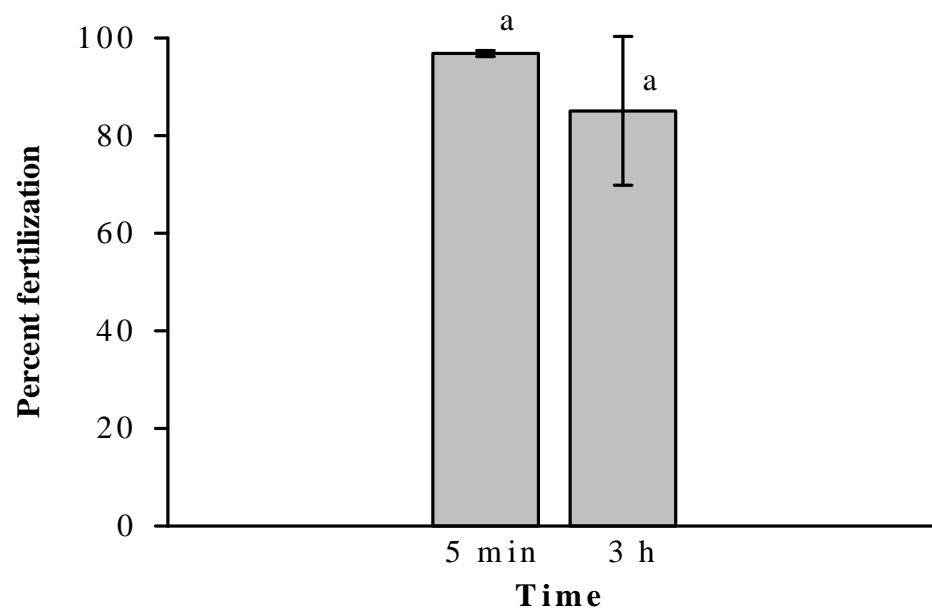


Figure 5-4. Fertilization of Nile tilapia eggs that were stored in Hanks' balanced salt solution (HBSS) for 5 min or 3 hours.

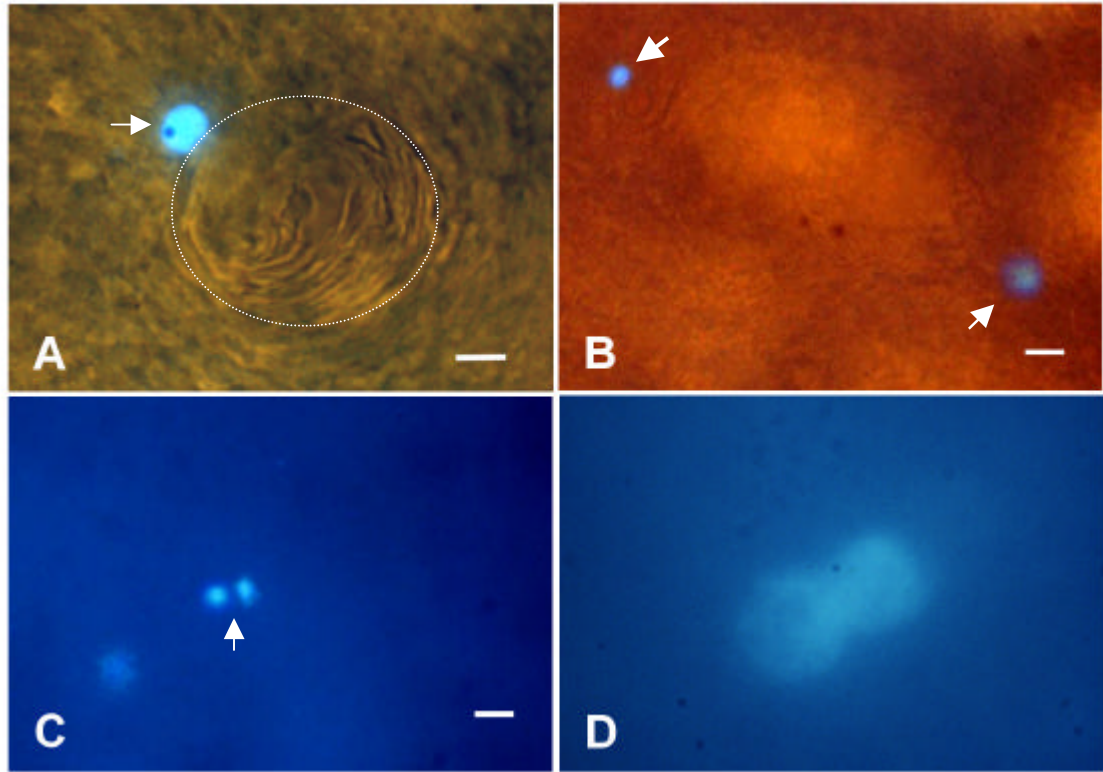


Figure 5-5. A) 30 seconds after insemination, the sperm head (arrow) was present at the micropyle canal (circle dot); bar 2 μm . B) 30 seconds after insemination, lower magnification than in A: the distance between the sperm head (left arrow) and the maternal chromosomes (right arrow) was $\sim 40 \mu\text{m}$; bar 3.6 μm . C) 10 min after insemination, the maternal DNA (arrow) has begun to split; bar 3.6 μm . D) 25 min after insemination, male and female pronuclei were in contact.

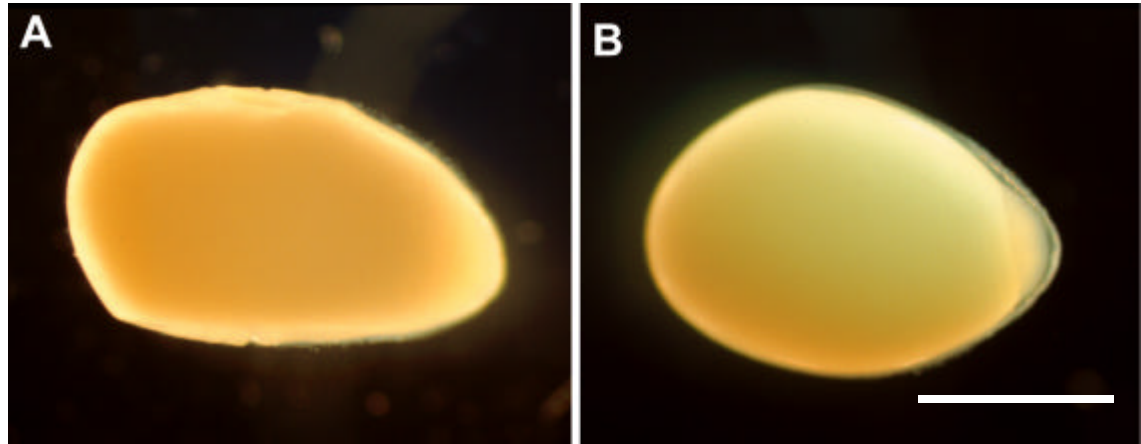


Figure 5-6. Activation in Nile tilapia eggs. A) 30 min after placement in embryo medium buffer (EMB). There are no signs of activation (water hardening and blastodic formation). B) 30 min after insertion of the injecting pipette, the chorion is raised and the egg membrane is more rigid. The blastodisc is developed at the animal pole (arrow); scale bar is ~ 750 μ m.

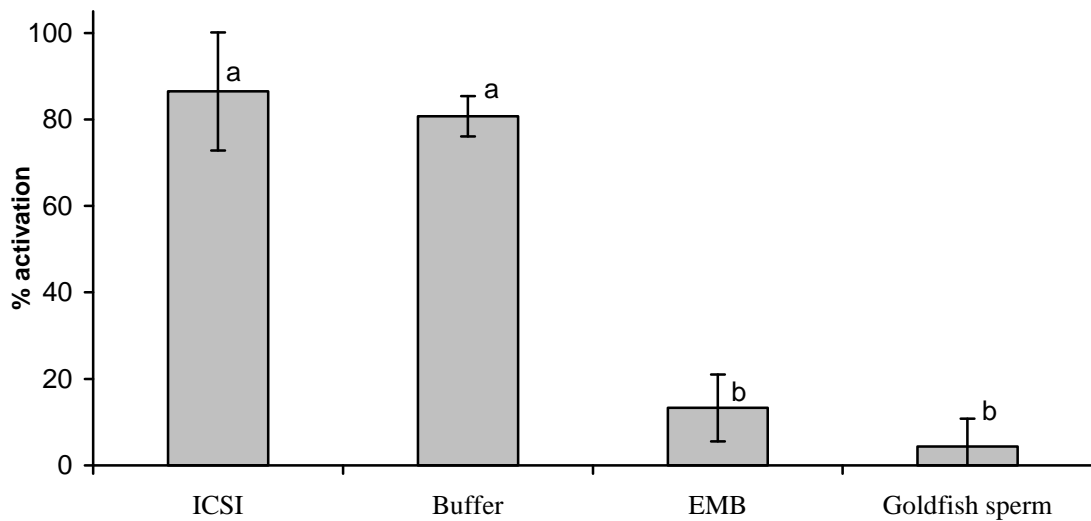


Figure 5-7. Activation in Nile tilapia eggs. Eggs were injected with sperm (ICSI), injected only with injecting buffer (Buffer), placed in embryo medium buffer without sperm (EMB), or inseminated with goldfish sperm. Shared letters above error bars (\pm SD) represent no significant difference.

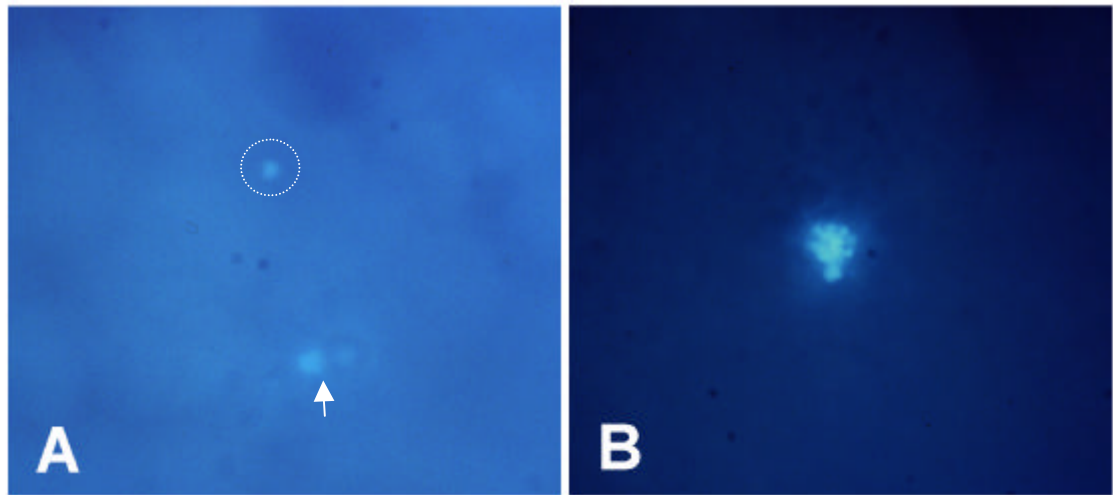


Figure 5-8. Activation in Nile tilapia eggs after DNA was labeled with Hoechst 33342. A) 10 min after insemination with Nile tilapia sperm, meiosis II is resumed as indicated by the division of the female chromosomes (arrow). The incorporated sperm head is shown in the circle. B) 10 min after the eggs were placed in embryo medium without injection, meiosis II was not resumed.

eggs stained with Hoechst 33342 after being injected with buffer showed splitting of the maternal chromosomes.

EGG INJECTION

There was no significant difference ($P = 0.490$) between ICSI performed in eggs previously fertilized with goldfish sperm and the eggs injected with Nile tilapia sperm (Table 5-1). Overall, of 113 eggs injected with sperm, only 6 (5%) developed into late neurula stage 11 (Galman and Avtalion 1989) (Figure 5-9). Of these, five were abnormal and one developed and hatched (Figure 5-10) and at the time of writing this dissertation (3 months after fertilization) was still alive at the Aquaculture Research Station, and is to our knowledge the only living Nile tilapia produced by ICSI (Figure 5-10). The sex of the fish was determined at three months after fertilization by observing the external urogenital area which showed no oviduct confirming the male phenotype. Eggs injected with buffer only (control injections) did not develop beyond blastodisc (Table 5-1). Flow cytometric analysis of two abnormal embryos and a blood sample from the normal fish, revealed that they were diploid.

Table 5-1. Intracytoplasmic sperm injection in Nile tilapia

	Number of injections	Percent fertilization	<u>Advanced neurula*</u>		Adult**
			Abnormal	Normal	
Fertilization with goldfish sperm	54	7	4	0	0
Nile tilapia sperm	59	3	1	1	1
Control	55	0	0	0	0

* Embryos that showed a complete neural tube, somites and the optic vesicle, stage 11 (Galman and Avtalion 1989)

** Fish that developed beyond 4 months after fertilization.

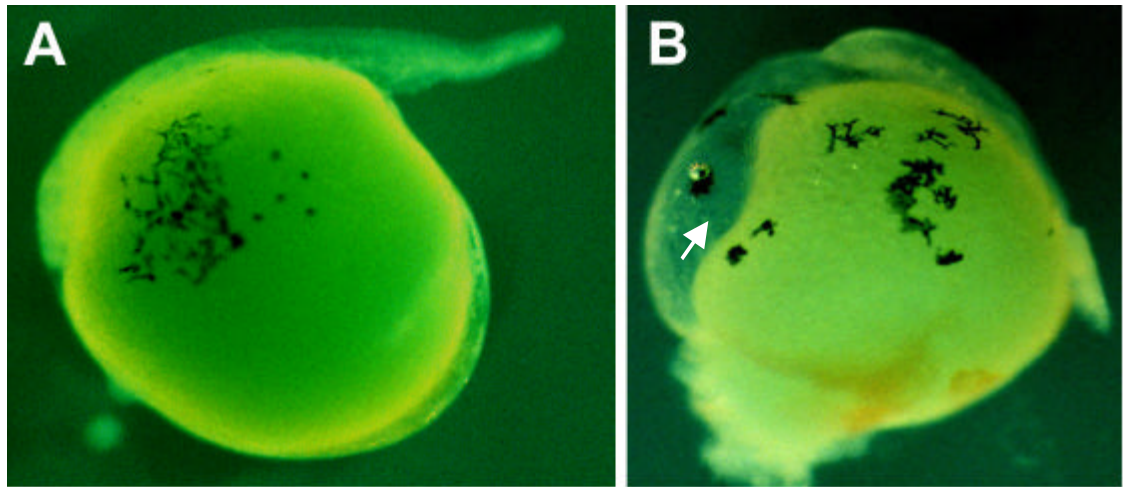


Figure 5-9. A) Normal Nile tilapia embryo produced by ICSI, 52 h after injection. B) Abnormal Nile tilapia produced by ICSI, 52 h after injection. Pigment cells looked larger than normal. A large space between the yolk and the epithelium is seen (arrow). This embryo did not survive beyond 72 h.

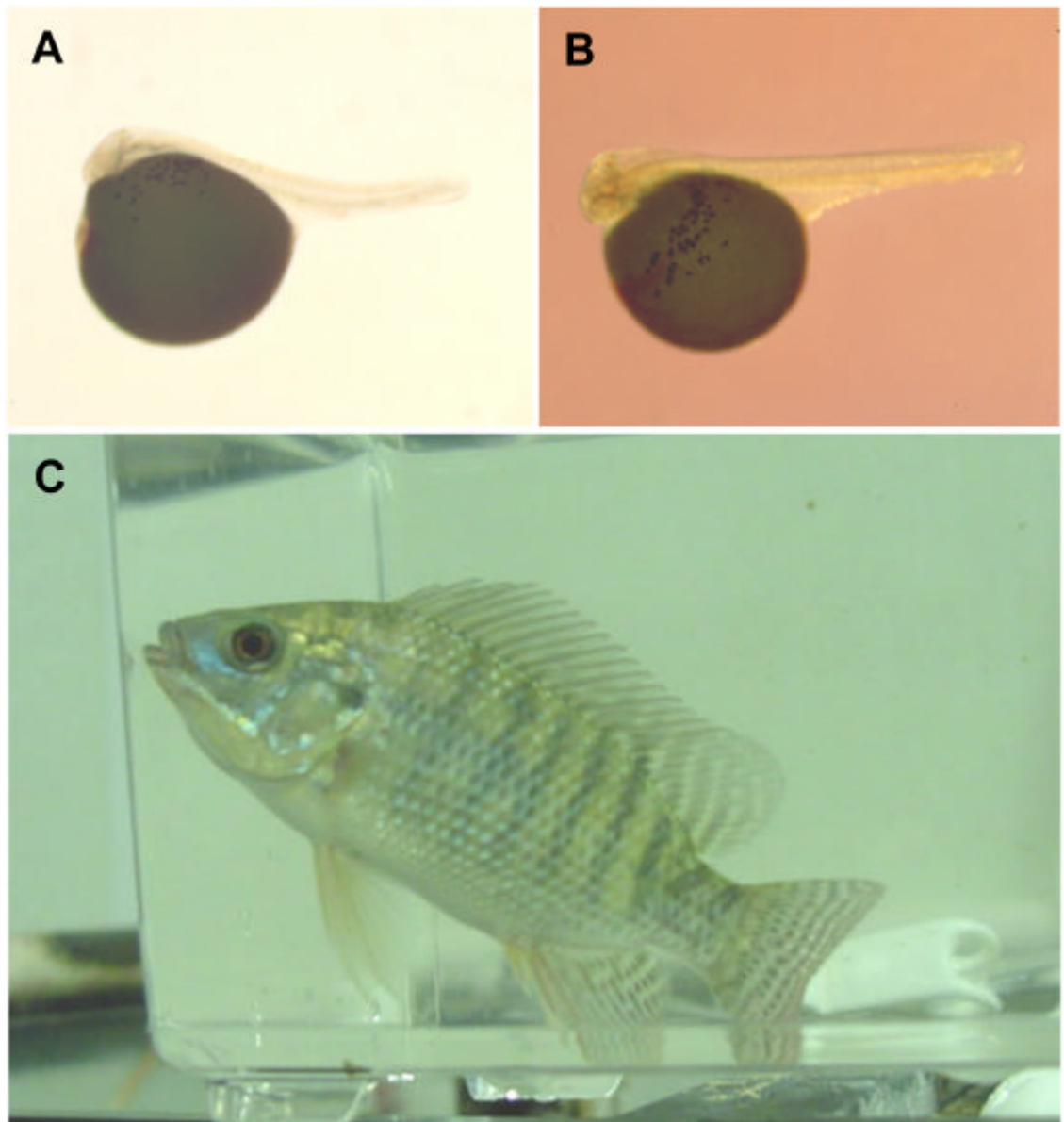


Figure 5-10. ICSI in Nile tilapia. A) 3 days after artificial insemination with fresh Nile tilapia sperm, control. B) 3 days after sperm injection C) same fish (in B) 3 months after hatching. It showed male phenotype.

DISCUSSION

The single breeding system of female Nile tilapias was yielded a reliable supply of eggs. The female tilapia did not require interaction with males to spawn (Jalabert and Zohar 1982, Mires 1982). The spawning cycle reported here (28 ± 9 days) agrees with a study in France (Gautire et al. 2000) where single females spawned every 31 ± 6 days and, as in this study, all fish spawned during the afternoon.

This method of spawning individual female Nile tilapias has been in the literature for 20 years. It is not utilized commercially, although application in areas where tilapia cannot be grown in ponds due to environmental conditions or restrictive laws would result in production of fry. Currently, tilapia hatcheries in the United States obtain fry by stocking in ponds at a rate of 3 fish per m^2 at a sex ratio of 1 male to 3 females, or in tanks at 5 fish per m^2 at a sex ratio of 1 male to 2 to 7 females (Malcolm Dickson, Aquaculture and Fisheries Consultancy Services www.pathfoot.demon.co.uk) (Costa-Pierce and Rakocy 1997).

Pond breeding systems can yield 380 fry per female per month and tank systems up to 1,500 fry per female per month when well managed. Aside from these high levels of production the single breeding system requires less space, provides easy access to broodstock and rapid and effective treatments for disease. It also reduces the number of males needed, or eliminates them if sperm cryopreservation is employed. The single breeding system also provides greater control over the desired offspring by facilitating selective breeding and other genetic tools such as hybridization, polyploidy and sex reversal. Tagging of fish is not necessary and feeding could be less costly and more efficient than in ponds or communal tanks. Spawning could be maximized through the

year because conditions such as photoperiod, temperature and water quality can be manipulated.

The single breeding system allows for a rapid and effective collection of ripe eggs. This together with the use of an isotonic solution extended the viability of the eggs to 3 hours making possible their use in ICSI. This procedure required at least 5 to 10 min to collect eggs, a 10 min trip from the Aquaculture Research Station to the Embryo Biotechnology laboratory at St. Gabriel and 10 more min for the preparation of the ICSI equipment.

Sperm injections has been demonstrated to cause physical damage to oocytes structures of different mammalian species including humans. For example, disruption of the metaphase spindle could cause chromosomal fragmentation, and impair embryonic development (Dumoulin et al. 2001). As seen for zebrafish (Chapters 3 and 4), intracytoplasmic sperm injection in Nile tilapia yielded a low fertilization rate (9%) and a high frequency (83%) of abnormal embryos that may have been the result of chromosomal damage. It was found that the distance between the tilapia egg chromosomes and sperm head at 30 seconds after insemination was about 40 μm . This value gave an approximate radius between the maternal DNA and the micropyle (natural sperm entry site), where injections were performed, and suggested that if the injections were done carefully, the maternal chromosomes should not be damaged.

The reasons for the low rate of fertilization and the production of abnormal fish are unknown. As suggested for zebrafish (Chapter 3), it is possible that the pipette could have damaged mechanisms involved in sperm movement. In mammals and echinoderms it is known that the cytoskeleton of the egg plays an important role in

sperm binding, sperm movement and embryonic development. Disruption of cytoskeletal structures involved in fertilization could interfere with the developmental process. More basic studies need to be performed to clarify where the disruption, if any, took place. Future research focusing on failures after ICSI in fish will need to address other possible damage (such as membrane breakage), the site of sperm delivery, and the mechanism of egg activation.

Piercing of Nile tilapia eggs with a glass pipette seemed to be sufficient to activate them as confirmed by water hardening and the subsequent formation of the blastodisc. However the injection of a sperm was necessary for further development. This suggests that the sperm content is needed for cell division. Activation of eggs after piercing with a glass needle has been documented in the African clawed frog *Xenopus laevis* (Wolf 1974) and the medaka fish *Oryzias latipes* (Yamamoto 1954). It has been shown in humans (Tesarik et al. 1994; Dosortsev 1995) and in mice (Wakayama and Yanagimachi 1998) that it is not the injection process, but the introduction of sperm that causes oocyte activation. Although the mechanism of oocyte activation after ICSI in mammals is controversial, there is evidence to support that a factor or factors released from the sperm into the oocytes after injection cause oocyte activation (Swann 1990, Dozortzev et al. 1995, Kuo et al. 2000).

What is meant by egg activation in fish? The results shown in this dissertation suggest that activation in zebrafish and Nile tilapia is a process where at least two major steps may be involved. For example, zebrafish eggs are partially activated when they come into contact with the spawning medium. This stimulus is enough to trigger the calcium wave (Lee et al 1999) and resumption of meiosis (Chapter 4), but it is not

sufficient to produce a parthenogenetic (haploid) embryo. In Nile tilapia piercing of the egg with a glass pipette induces water hardening and resumes meiosis, but cell division does not occur. The contribution of factors contained in the sperm might be necessary for the second major step of cell division. This is suggested by the production of fish by ICSI and in experiments where insemination with irradiated sperm (Corley-Smith et al 1995) or sperm from other species resulted in the production of haploid embryos (Appendix A).

Nile tilapia eggs showed little to no sign of activation after incubation with goldfish sperm. This result agreed with the results obtained by staining of the egg chromosomes with Hoechst 33342. No goldfish sperm were observed in the membrane or in the cytoplasm of the eggs, suggesting that goldfish sperm did not contribute to the fertilization of the Nile tilapia eggs and would not affect fertilization after ICSI. The possibility that sperm from other species could improve ICSI cannot be ruled out. An alternative could be the use of sperm from common carp *Cyprinus carpio* (Peruzzi et al., 1993) or the use of irradiated sperm from Nile tilapia. However, the use of irradiated sperm carries the risk of genetic contamination, which is difficult to detect unless there is a genetic or phenotypic marker that allows recognition of the paternal DNA contribution.

The lack of fertilization observed after injection without sperm (controls) suggested that the normal fish obtained should be the product of ICSI and not of parthenogenetic development. However, it might be that the sperm injection prevented the extrusion of the second polar body resulting in a meiotic gynogenetic fish (all-maternal heritance). This possibility was ruled out by the sex of the resulting fish

(male) which could only result from the combination of a male and female gamete because Nile tilapia possess a male heterogametic mechanism (XX-XY) of sex determination (Penman and McAndrew 2000).

Nile tilapia proved to be a good model for the application of ICSI. The spawning characteristics of these fish permit a reliable supply of eggs. The size and morphology of the eggs facilitated the injection procedure and the easy manipulation of embryos and sac fry led to greater survival after ICSI.

The limited but positive results shown here demonstrate that ICSI could be used as another genetic tool for improvement in aquaculture. Sperm preservation will become important for genetic improvement in aquaculture, although the current practice of cryopreservation can be costly for poor countries or small companies. Intracytoplasmic sperm injection is necessary for preservation techniques where sperm have lost motility. Chapter 6 investigated the feasibility of using ICSI with three different sperm preservation techniques: cryopreservation, fixation in methanol and freeze-dried with ICSI.

REFERENCES

- Beveridge M. C. M. and B. J. McAndrew. 2000. Reproductive biology, mating systems and parental care. Pages 33-58 in Beveridge M. C. M. and B. J. McAndrew, Editors. *Tilapias: Biology and Exploitation*. Kluwer Academic Publishers. Dordrecht, The Netherlands.
- Corley-Smith, G. E., C. J. Lim, and B. P. Brandhorst. 1996. Production of androgenetic zebrafish (*Danio rerio*). *Genetics* 142:1265-1276
- Costa-Pierce, B. A. and J. E. Rakocy. 1997. *Tilapia aquaculture in the Americas*. Volume 1 in Costa-Pierce, B. A. and J. E. Rakocy, Editors. World Aquaculture Society and The American Tilapia Association. Baton Rouge, Louisiana.

- Don, J. and R. R. Avtalion. 1988. Production of F1 and F2 diploid gynogenetic tilapias and analysis of the 'Hertwig Curve' obtained using ultraviolet irradiated sperm. *Theoretical and Applied Genetics* 76:253-259.
- Dozortsev, D., A. Rybouchkin, P. De Sutter, C. Qian, and M. Dhont. 1995. Human oocyte activation following intracytoplasmic sperm injection: the role of the sperm cell. *Human Reproduction* 10:403-407.
- Dumoulin, J. C. M., E. Coonen, M. Bras, J. M. Bergers-Janssen, R. C. M. Ignoul-Vanvuchelen, L. C. P. van Wissen, J. P. M. Geraedts, and J. L. H. Evers. 2001. Embryo development and chromosomal anomalies after ICSI: effect of the injection procedure. *Human Reproduction* 16:306-312.
- El Gamal, A. A., K. B. Davis, J. A. Jenkins, and E. L. Torrans. 1999. Induction of triploidy and tetraploidy in Nile tilapia, *Oreochromis niloticus* (L.). *Journal of the World Aquaculture Society* 30:269-275.
- Galman, O.R. and R.R. Avtalion. 1989. Further study of the embryonic development of *Oreochromis niloticus* (Cichlidae, Teleostei) using scanning electron microscopy. *Journal of Fish Biology* 34:653-664.
- Gautier, J-Y., B. Lefaucheux, M. Foraste, B. Jalabert, and J-F. Baroiller. 2000. Periodicity and duration of the papillary, sexual and behavioral cycle in the tilapia *Oreochromis niloticus*. Page 48 in K. Fitzsimmons and J. Carvalho Filho, Editors. *Proceeding from the Fifth International Symposium on Tilapia Aquaculture*. Rio de Janeiro, Brazil.
- Guerrero, R. D. III. 1982. Control of tilapia reproduction. Pages 309-316 in P. R. V. Pullin and R. H. Lowe-McConnell, Editors. *The biology and culture of tilapia*. ICLARM conference proceeding 7, International Center for Living Aquatic Resources Managament, Manila, Philipines.
- Hart, N. H., K. A. Becker, J. S. Wolenski. 1992. The sperm entry site during fertilization of the zebrafish egg: Localization of actin. *Molecular Reproduction and Development* 32:217-228.
- Jalabert B. and Y. Zohar. 1982. Reproductive physiology in cichlid fishes, with particular reference to *Tilapia* and *Sarotherodon*. Pages 129-140 in P. R. V. Pullin and R. H. Lowe-McConnell, Editors. *The biology and culture of tilapia*. ICLARM conference proceeding 7, International Center for Living Aquatic Resources Managament, Manila, Philipines.
- Kuo R. C., G. T. Baxter, S. H. Thompson, S. A. Stricker, C. Patton, J. Bonaventura, and D. Epel. 2000. NO is necessary and sufficient for egg activation at fertilization. *Nature* 406:633-636.

- Lee, K., S. E. Webb, and A. L. Miller. 1999. A wave of free cytosolic calcium traverses zebrafish eggs on activation. *Developmental Biology* 214:168-180.
- Lovshin, L. L. 1982. Tilapia hybridization. Pages 279-308 in P. R. V. Pullin and R. H. Lowe-McConnell, Editors. *The biology and culture of tilapia*. ICLARM conference proceeding 7, International Center for Living Aquatic Resources Management, Manila, Philippines.
- Mires, D. 1982. A study of the problems of the mass production of hybrid tilapia fry. Pages 317-329 in P. R. V. Pullin and R. H. Lowe-McConnell, Editors. *The biology and culture of tilapia*. ICLARM conference proceeding 7, International Center for Living Aquatic Resources Management, Manila, Philippines.
- Myers, J. M. 1986. Tetraploid induction in *Oreochromis* spp. *Aquaculture* 57:281-287.
- Myers, J. M. and W. K. Hershberger. 1991. Artificial spawning of tilapia eggs. *Journal of the World Aquaculture Society* 22:77-82.
- Naruse, K., K. Ijiri, A. Shima, N. Egami. 1985. The production of cloned fish in the medaka (*Oryzias latipes*). *The Journal of Experimental Zoology* 236:335-341.
- Penman D. J. and B. J. McAndrew. 2000. Genetics for the management and improvement of cultured tilapias. Pages 227-266 in M. C. M. Beveridge and B. J. McAndrew, Editors. *Tilpias: Biology and Exploitation*. Kluwer Academic Publisher, London.
- Peruzzi, S., A. G. Scott, J. C. J. Domaniewski, and G. F. Warner. 1993. Initiation of gynogenesis in *Oreochromis niloticus* following heterologous fertilization. *Journal of Fish Biology* 43:585-591.
- Poleo, G. A., R. S. Denniston, B. C. Reggio, R. A. Godke, and T. R. Tiersch. 2001. Fertilization of eggs of zebrafish, *Danio rerio*, by intracytoplasmic sperm injection. *Biology of Reproduction* 65:961-966.
- Swann, K. 1990. A cytosolic sperm factor stimulates repetitive calcium increases and mimic fertilization in hamster eggs. *Development* 110:1295-1302.
- Sakai, N., S. Burgess, and N. Hopkins. 1997. Delayed in vitro fertilization of zebrafish eggs in Hank's saline containing bovine serum albumin. *Molecular Marine Biology and Biotechnology* 6:84-87.
- Tesarik, J. and M. Sousa. 1994. Comparison of Ca^{2+} responses in human oocytes fertilized by subzonal insemination and by intracytoplasmic sperm injection. *Fertility and Sterility* 62:1197-1204

- Tiersch, T. R. and R. W. Chandler. 1989. Chicken erythrocytes as an internal reference for analysis of DNA content by flow cytometry in grass carp. *Transactions of the American Fisheries Society* 118:713-717.
- Tiersch, T. R., B. A. Simco, K. B. Davis, R. W. Chandler, S. S. Wachtel, and G. J. Carmichael. 1990. Stability of genome size among stocks of the channel catfish. *Aquaculture* 87:15-22.
- Valenti, R. J. 1975. Induced polyploidy in *Tilapia aurea* (Steindachner) by means of temperature shock treatment. *Journal of Fish Biology* 7:519-528.
- Varadaraj, K. 1990. Production of diploid *Oreochromis mossambicus* gynogens using heterologous sperm of *Cyprinus carpio*. *Indian Journal of Experimental Biology* 28:701-705.
- Wakayama, T. and R. Yanagimachi. 1998. Development of normal mice from oocytes injected with freeze-dried spermatozoa. *Nature Biotechnology*. 16:639-641.
- Westerfield, M. 1995. *The Zebrafish Book. Guide for the Laboratory Use of Zebrafish (Danio rerio)*. 3rd ed. University of Oregon Press, Eugene.
- Wolenski, J. S. and N. H. Hart. 1987. Scanning electron microscope studies of sperm incorporation into zebrafish (*Brachydanio*) egg. *The Journal of Experimental Zoology* 243:259-273.
- Wolf, D. P. 1974. The cortical response in *Xenopus laevis* ova. *Developmental Biology* 40:102-115.
- Yamamoto, T. 1975. Fertilization in *Oryzias* Egg. Pages 80-96 in T. Yamamoto editor. *Medaka (killifish): Biology and Strains*. Keigaku Pun. Co., Tokyo.

CHAPTER 6

INTRACYTOPLASMIC SPERM INJECTION WITH CRYOPRESERVED, FIXED AND FREEZE DRIED SPERM IN NILE TILAPIA

Sperm storage began to be applied commercially shortly after the first calf produced from cryopreserved semen was born in 1951 (reviewed by Curry 2000). Currently, industries such dairy are based on the use of artificial insemination and frozen sperm. Although cryopreservation presents advantages over normal fertilization, not all farm animal industries have incorporated it. This is the case in aquaculture where cryopreservation has been studied in an estimated 200 species of fish (Rana 1995) but its use is not widespread (Tiersch 2000). This will probably change in the near future given that aquaculture is the fastest growing sector of agriculture and over-fishing and human development is threatening dozens of species of fish. The establishment of repositories for genetic material is seen as a way of maintaining biological diversity, developing broodstocks and reducing space used in hatcheries for maintenance of males (Holt 1997, Tiersch 2000, Cloud 2000).

At present, cryopreservation is the only reliable way to preserve sperm indefinitely, but it is expensive because it requires a constant supply of liquid nitrogen. A good alternative would be the use of freeze-dried sperm or sperm preserved in alcohol, each of which could be stored at 4°C or at room temperature (25°C). The concept of freeze-dried sperm was first introduced in the literature 50 years ago with unsuccessful attempts to obtain viable avian sperm after vitrification and dehydration (Polge et al. 1949). It was not until 1957 that the first live births of rabbits were reported from freeze-dried spermatozoa (reviewed by Larson and Graham 1976). However attempts in other laboratories were not successful (Saacke and Almquist 1961;

Larson and Graham 1976). At that time, the aim of preservation was to maintain the integrity or viability of the sperm cell for later use in *in vitro* fertilization. After physical and chemical treatments, motility of the sperm was compromised limiting the use of this technique. The introduction of the concept of sperm microinjection in mammalian species (Yanagimachi and Uehara 1976) revitalized the notion of sperm preservation. It was shown that freeze-dried sperm injected in mice oocytes could produce normal offspring (Wakayama and Yanagimachi 1998). Others reported that injection of sperm chemically dehydrated in 100% ethanol, 100% methanol, chloroform-methanol and Carnoy's fluid were capable of developing into pronuclei (Tateno et al. 1998, Katayose et al. 1992). Although these treatments destroyed the cell membrane, the sperm did not need to be motile if intracytoplasmic sperm injection (ICSI) was used. After these treatments, sperm are considered to be conventionally "dead" but possess genetically normal nuclei, capable of supporting development.

The goal of this Chapter was to evaluate the possible use of sperm preservation methods in combination with ICSI for use in Nile tilapia *Oreochromis niloticus*. The objectives of this work were: 1) to evaluate injection of cryopreserved sperm; 2) to evaluate injection of sperm fixed in a 70% solution of methanol; and 3) to evaluate the injection of freeze-dried sperm.

MATERIALS AND METHODS

ANIMALS

Females were placed individually into 80-L tanks at 26°C. Salinity was maintained at 1.5 mg/L, and alkalinity and hardness at around 200 mg/L as CaCO₃. Females that showed a swollen belly and projecting genital papilla were selected for

spawning. Fish were anesthetized with tricaine methanesulfonate (methyl-m-aminobenzoate, MS222) (Argent Laboratories, Redmond, Washington) before stripping of eggs. The abdomen of fish were dried with paper towels and squeezed gently, starting behind the pectoral fins and moving towards the tail. Ripened eggs were stripped easily and showed a uniform size (Appendix A, SOP-6). The presence of blood, connective tissue or size variation indicated that the eggs were not ripe and they were not used for experiments. An effort was made to sperm from double-homozygous pearl Nile tilapia (Figure 6-1) (Til-Tech Aquafarm, Roberts, Louisiana). This phenotype is a white coloration resulting from interaction of alleles at two loci (Lutz 1999) and injection of double-homozygous sperm in wild-type Nile tilapia eggs should yield all-pearl offspring.

Sperm were collected by gently squeezing the sides of unanesthetized males. A 10- μ L pipette tip connected to a mouth pipette was used to extract sperm which were diluted 1:1 in Hanks' balanced salt solution (HBSS) (~280 mOsmol/kg) (Appendix A, SOP-2) in 1.5-mL microcentrifuge tube and placed on ice. Motility was estimated by placing 10 μ L of sperm dilution on a glass microscope slide and mixing with 20 μ L of distilled water (Appendix A, SOP-5). The sperm dilution was observed by use of dark-field microscopy (Optiphot 2, Nikon, Tokyo, Japan). Sperm that actively moved forward were considered to be motile. Only samples of sperm showing 90% or greater motility were used for studies.

SPERM INJECTIONS

Injections were performed using a micromanipulator apparatus consisting of an inverted microscope (Diaphot Nikon, Tokyo, Japan) equipped with two mechanical

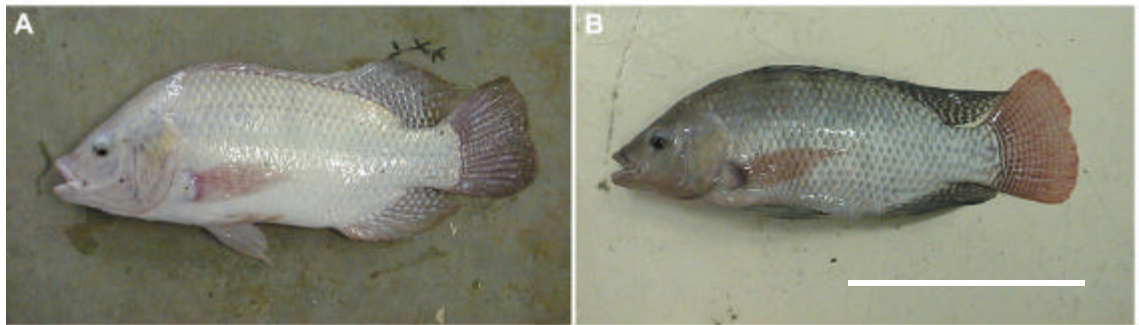


Figure 6-1. A) Pearl Nile tilapia. This line of fish presents a white coloration in the skin, all other phenotypic characteristics remain the same as wild-type fish B) Wild-type Nile tilapia; bar 12 cm.

micromanipulator units (Leitz, Rockliegh, Illinois). A manipulator moved either the holding or injection pipette. Each pipette was connected to a screw syringe by fine-bore Teflon® tubing filled with light mineral oil (Sigma Chemical Corporation, St. Louis, Missouri). The injection pipette and the holding pipette were made from borosilicate glass capillary tubes (Sutter Instrument Company, Novato, California). The injection pipette had an internal diameter of ~15 µm. Due to the variability in size among eggs of Nile tilapia, the holding pipettes were prepared for each batch of eggs with internal diameters ranging from ~200 µm to ~400 µm. When eggs were correctly positioned with the animal pole facing outward, the injection pipette was pushed through the micropyle into the cytoplasm and a sperm cell was injected with a small volume (~7 pL) of embryo medium buffer (EMB) containing 5% polyvinylpyrrolidone (PVP) (360000 MW) (Sigma Chemical Corporation) to prevent sperm from sticking to the wall of the pipette. As a control, eggs were injected with ~7 pL of EMB containing 5% PVP, and egg quality was evaluated by artificial insemination of ~50 eggs with fresh sperm. Eggs with 70% or greater fertilization were included in the analysis. Eggs were evaluated 4 hours after injection and those that presented well developed blastomeres were counted as fertilized.

DNA CONTENT ANALYSIS

Analysis of DNA content was performed using a FACSCalibur® flow cytometer (Becton Dickinson, San Jose, California) equipped with an air-cooled 480 nm argon laser. The FACSComp® software (Becton Dickinson) was used to calibrate the instrument settings. Embryos were disrupted by passage through a 3-mL syringe fitted with a 25-gauge needle while suspended in 0.5 mL of lysis buffer containing 25 µg of

buffered RNase, 0.1% sodium citrate, and 0.1% Triton X-100 (Tiersch and Chandler 1989). The yolk sac of 4-days-old embryos were removed and the remaining tissue was disrupted by passage three times through a 25-gauge syringe. The solution was filtered through 20- μ m nylon mesh. Blood was collected from the caudal vein of a fish 3 months after production by ICSI. For analysis, an aliquot of 250 μ l of disrupted embryo or 1 μ L of blood was diluted in lysis-staining buffer, which included 25 μ g of propidium iodide. Blood cells from channel catfish *Ictalurus punctatus* (1.98 pg of DNA/cell) (Tiersch et al. 1990) and zebrafish *Danio rerio* (3.15 pg of DNA/cell) (Poleo et al. 2001) were used as internal references. Measurements of the DNA content of normal diploid Nile tilapia (2.10 ± 0.09 pg of DNA/cell) were compared with those of larvae produced by ICSI.

CRYOPRESERVATION OF SPERM

Sperm cryopreservation was performed following the procedure of Rana and McAndrew (1989) with some modifications as described below. Sperm were diluted 1:10 in HBSS containing a final concentration of 10% methanol and stored in 0.5-mL straws. A 10-mL syringe coupled to rubber tubing was used to fill the straws. The open end of the straws were sealed with polyvinyl chloride powder and cooled to -80°C in a computerized freezer (Kryo 10 series, Planer products, Sunbury-on-Thames, United Kingdom) at a rate of 40°C per minute. The straws were plunged into liquid nitrogen for storage. After a week, the content of single straws were used for a set of injections. Straws were thawed in a 40°C water bath for 8 seconds. Both ends of the straws were cut and the sperm solution was expelled out into a 1.5-mL microcentrifuge tube and held on ice. For injections, within 5 min of thawing an aliquot was diluted 1:20 in

HBSS to reduce the methanol concentration to 0.5%. Washing by centrifugation was not carried out to avoid further damage to the sperm. Artificial insemination was conducted using cryopreserved sperm with eggs of Nile tilapia. To observe the possible effect of the low concentration of methanol (0.5%), eggs were artificially inseminated, and after 5 min, were injected with buffer containing 0.5% methanol and 5% PVP.

METHANOL FIXATION OF SPERM

Sperm were collected as described above. The sperm solution was diluted in methanol to a 70% final concentration and kept at 4°C for 1 day. Before injection, an aliquot of the 70% solution was diluted 1:200 in HBSS to reduce the methanol concentration to 0.35%. Artificial insemination was carried out using fixed sperm with eggs of Nile tilapia.

FREEZE-DRYING TREATMENT OF SPERM

Sperm were collected and cryopreserved as described above. Straws were removed from liquid nitrogen and sperm were immediately placed in a pre-cooled (-80°C) 800-mL flask (Labconco Corporation, Kansas City, Missouri), which was connected to a lyophilizer (Lyph-Lock 18, Freezedry/shell freeze system, model 77555-01, Labconco). Samples were dried overnight and stored in a sealed container at 4°C. Samples were prepared for injection by rehydrating them with 500 µL of distilled water and diluting 1:1 with EMB containing 10% PVP. Artificial insemination was performed by incubating rehydrated sperm with Nile tilapia eggs.

DNA STAINING

Freeze-dried sperm were hydrated with water containing 5 µg/mL of Hoechst 33342 (Sigma). A 10-µL drop was put on a slide and observed under ultraviolet light.

A sample of fresh sperm was used to compare the size and appearance of the nuclei. Observations were performed using ultraviolet light microscopy (Nikon, Microphot-SA, Tokyo, Japan). The diameters of the sperm were calculated using image analysis software (Optimas 5.1a, Bioscan, Edmund, Washington).

STATISTICAL ANALYSIS

Statistical analysis was performed using SAS software for Windows (Version 8.01, SAS Institute, Cary, North Carolina). Differences in the incidence of fertilization among treatments during ICSI were evaluated using a logistic regression using the GENMOD procedure. Logistic regression can be modeled as a class of generalized linear models where the response probability distribution function is binomial and the link function is logit. The following program was used:

```
proc genmod;  
model r/n = trt / dist=binomial link=logit type3;  
run;
```

Differences were accepted as significant when $P < 0.05$.

RESULTS

SPERM INJECTION

Of the 47 eggs injected with fresh sperm, 11 (23%) were fertilized (8-cell, Stage 4) (Galman and Avtalion 1989) 7 (15%) developed to blastula (Stage 8), 5 (11%) developed abnormally to neurula (Stage 11) and 4 (8%) developed normally and hatched (Figure 6-2). Two of the hatched embryos developed to active feeding. One of the fish remains alive at the time of writing of this dissertation (Figure 6-2). DNA content of four of the abnormal embryos indicated that they were diploid. Flow cytometric analysis of a blood sample from the surviving ICSI fish taken two months

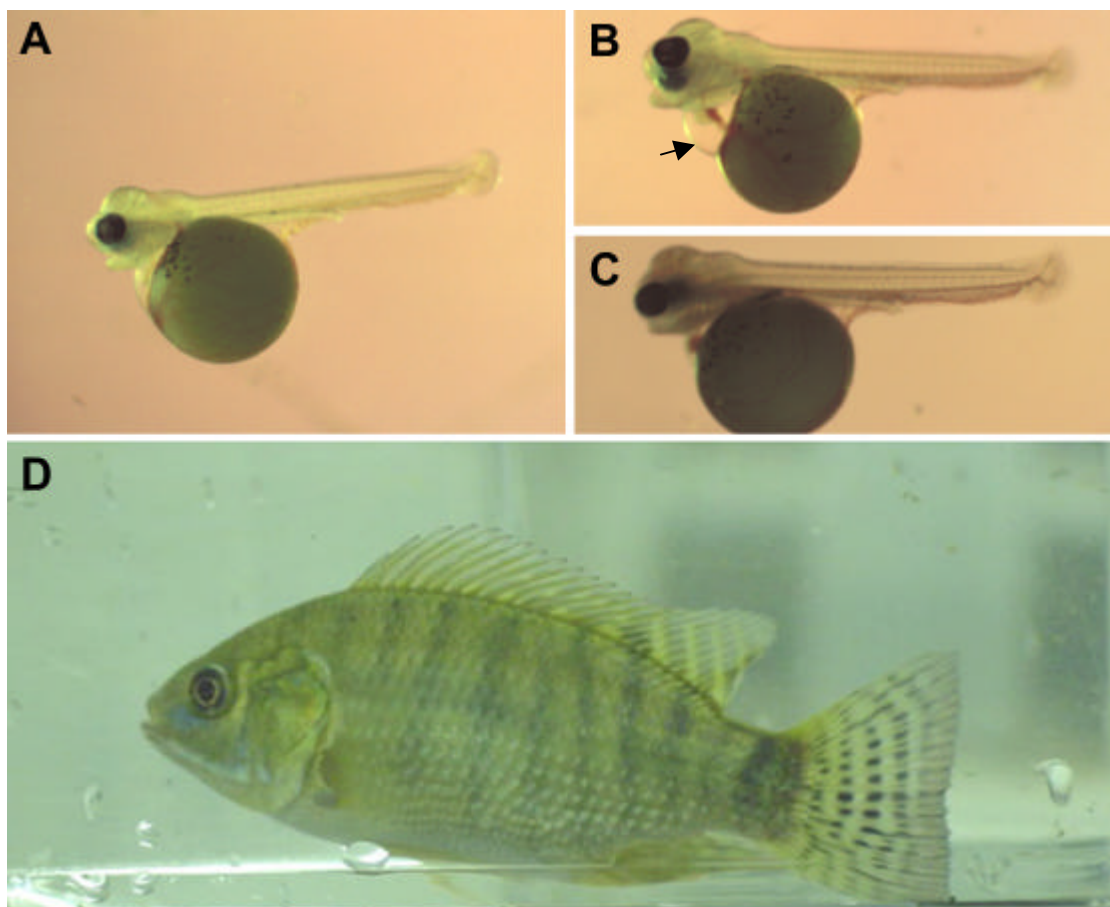


Figure 6-2. Intracytoplasmic sperm injection using fresh Nile tilapia sperm. A) 3 days after fertilization, Nile tilapia sac fry produced from artificial insemination (Control). B) 3 days after fertilization, Nile tilapia sac fry produced by ICSI. The pericardial cavity (arrow) was larger than normal, and fish died 7 days after hatching. C) 3 days after fertilization, Nile tilapia sac fry produced by ICSI showing characteristics of normal sac fry. D) The same fish (shown in C) 3 months later.

after fertilization indicated that it was diploid. It is important to point out that the normal fish produced from ICSI was a female showing wild-type pigmentation and not the pearl phenotype expected. Artificial insemination of eggs from wild-type Nile tilapia with sperm from the suspected double-homozygous Pearl Nile tilapia resulted in a population of offspring showing both phenotypes, demonstrating that this male was not double homozygous.

Before freezing, the sperm presented 95% motility compared with 60% after thawing. Artificial insemination using cryopreserved sperm resulted in $86 \pm 1\%$ of fertilization. Of the 45 eggs injected with cryopreserved sperm, 9 (20%) were fertilized (Stage 4), 9 developed to late morula (Stage 7) and did not develop beyond this stage (Figure 6-3). No significant difference ($P = 0.6$) was observed between fertilization using fresh sperm and cryopreserved sperm.

Of the 45 eggs injected with sperm that were fixed in 70% methanol, none were fertilized (Table 6-1).

Table 6-1. Effect of cryopreservation and methanol fixation on the ability of Nile tilapia sperm to fertilize after ICSI. Control injections were made without sperm.

	Number of Injections	Percent fertilization	Blastula	Gastrula	Advanced neurula ⁺⁺		Sac fry ⁺	Fry [*]
					Abnormal	Normal		
Fresh sperm	47	23	11	9	5	4	4	2
Control	45	0	0	0	0	0	0	0
Cryopreserved Sperm	45	20	9	0	0	0	0	0
Control	41	0	0	0	0	0	0	0
Methanol	40	0	0	0	0	0	0	0
Control	45	0	0	0	0	0	0	0

⁺⁺ Embryos that showed a complete neural tube, somites and optic vesicle (stage 11) (Galman and Avtalion 1989).

⁺ Hatched embryo that has not absorbed the yolksac.

^{*} A free-swimming fish with active feeding.

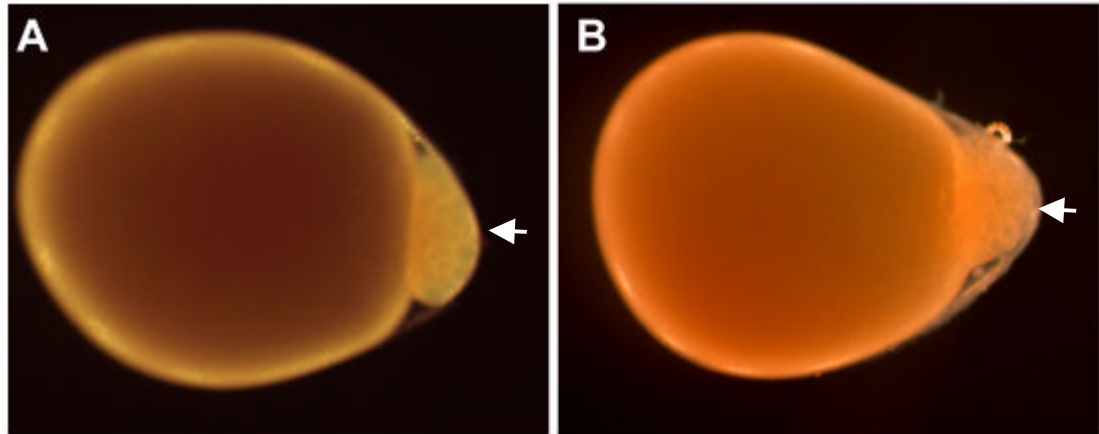


Figure 6-3. Intracytoplasmic sperm injection using cryopreserved Nile tilapia sperm. A) Morula stage, 6 h after artificial insemination (Control). B) Morula stage, 6 h after ICSI. The embryos stopped development at this stage. Cells are at the animal pole (arrows).

Injections with freeze-dried sperm were not possible due to the difficulty of visualizing the sperm in the injection buffer. Sperm that were freeze-dried had no tails, and the heads were difficult to localize by light microscopy. Staining of the sperm DNA with Hoechst 33342 showed that the nuclei retained a round morphology and normal size ($2.3 \pm 0.2 \mu\text{m}$) when compared with fresh sperm ($2.3 \pm 0.2 \mu\text{m}$). However, the nuclei appeared diffuse (Figure 6-4).

DISCUSSION

The aim of this chapter was to evaluate for the use with ICSI sperm that were cryopreserved, preserved in methanol, or freeze-dried. The percentage of ICSI embryos that reached neurula stage (19%) was almost four times greater for Nile tilapia than in Chapter 5 (5%) probably due to the experience gained in manipulation of the gametes. The phenotype expressed in this study by the fish obtained by ICSI (female with wild-type pigmentation) could have been the result of use of sperm from a male heterozygous for the pearl phenotype or to the induction of gynogenesis by the injection. Although gynogenesis is possible, the probability of this occurring was low. It has been reported that the production of mitotic gynogenetic *Oreochromis aureas* yielded a mean survival rate of $0.4 \pm 0.3\%$ for fish that developed beyond yolk sac and of these 50% showed abnormal development (Don and Avtalion 1988). In other reports where Nile tilapia were used in gynogenetic experiments, none of the fish developed beyond sac fry (Peruzzi et al. 1993).

The low percentage of viability and the high percentage of abnormalities (disjunction of the jaws from the body and head enlargement) found in gynogenetic fish have often been attributed to the expression of defective recessive alleles and an

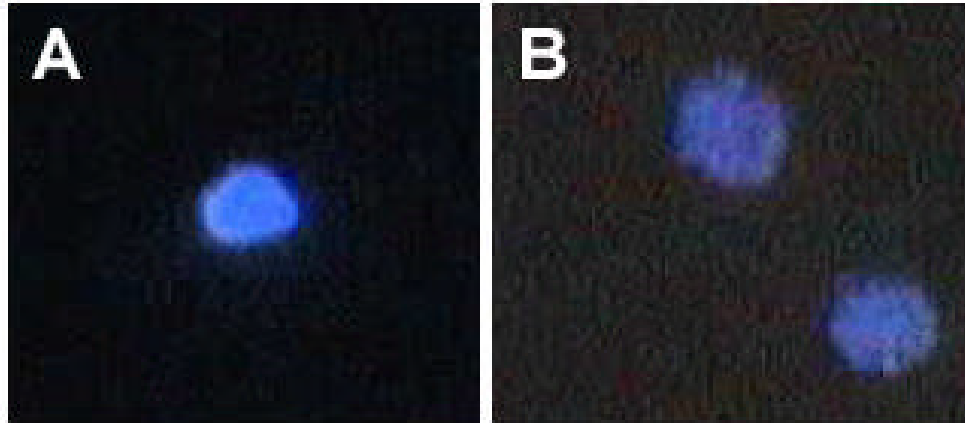


Figure 6-4. A) Fresh and B) Freeze-dried Nile tilapia sperm stained with Hoechst 33342. Note the diffuse appearance of the freeze-dried nuclei.

increase of the level of inbreeding (Purdom 1969, Onozato 1984, Suzuki et al. 1985, Don and Avtalion 1988). In this work 9% of the injected eggs developed beyond sac fry without showing major malformations, suggesting that the fish were the result of ICSI. Further evidence could be gathered by comparing specific genetic markers in the genomes of parents and offspring by techniques such as DNA fingerprinting. Various techniques had been applied in fishes to verify the production of homozygosity by gynogenesis. Multilocus DNA fingerprinting has been used to reveal genetic relationships between strains of Nile tilapia (Harris et al. 1995, Naish et al. 1995) and the parental contribution in gynogenesis in tilapias (Carter et al. 1991, Jenneken et al. 1999). This technique involves labeling with a small nucleotide sequence (e.g., GTG) of genomic DNA that has been digested with specific restriction endonucleases. Usually individual genomes present a unique pattern of bands which allow differentiation. Another technique that had been used to verify gynogenesis in fish is random amplified polymorphic DNA (RAPD) (Van Eenennaam et al. 1996, Jenneken et al. 1999). This technique uses the polymerase chain reaction with short oligonucleotide primers (~10 base pairs) that will randomly amplify a series of DNA fragments that form a pattern of bands in an electrophoresis gel that can be unique to each fish studied. The use of these techniques could provide proof of the origin of fish produced after ICSI.

Development of eggs injected with fresh sperm (23%) or cryopreserved sperm (20%) to late morula (Stage 7) occurred within 10 hours of injection. All the eggs fertilized using cryopreserved sperm stopped development at blastula. This does not reflect parthenogenetic activation because none of the 82 control eggs injected without

sperm developed beyond blastodisc formation. It is possible that the sperm selected for injections were damaged, compromising further development. Although the effectiveness of cryopreservation has been established for artificial insemination, a percentage of the cryopreserved cells usually suffer some type of injury as indicated by decreased motility after freezing. However, lack of motility does not necessarily define the fertilization capability of sperm as demonstrated throughout this dissertation when eggs injected with non-motile sperm produced normal fish. Nevertheless, ice crystal formation can disrupt the sperm membrane resulting in loss of cytoplasmic contents that may be necessary for egg activation. Ice crystals can also damage the chromatin resulting in fragmented DNA. Injection of eggs with sperm containing fragmented chromatin resulted in failure of sperm decondensation, fertilization and embryonic development in humans (Sakkas et al. 1996; Dumoulin et al. 2001). Also, there is a possibility that the low concentration of methanol (0.5% of injection volume) injected with the sperm affected development. However, control injections of 0.5% methanol in eggs previously inseminated with fresh sperm did not compromise development.

Injection of sperm preserved in 70% methanol did not fertilize any of 45 Nile tilapia eggs, suggesting that the sperm were not genetically viable. It is known that high concentrations of methanol or ethanol (>70%) do not alter the primary structure of DNA (Sambrook et al. 1989) or the protamine molecules involved in the stability and packaging of the sperm nuclei in hamsters (Lee et al. 1991). However, methanol could affect the interaction between protamines and DNA, or affect other factors involved in activation of the eggs. Human and hamster sperm stored in methanol, ethanol and chloroform-methanol were capable of decondensation and development into pronuclei

after injection into hamster oocytes (Katayose et al. 1992), although this does not assure the genetic integrity of the sperm nuclei and future embryonic development. Other experiments where mouse oocytes were injected with sperm stored in ethanol showed that storage for more than one day changed the structure of the sperm chromosomes compromising the development of the embryo (Tateno et al. 1998). These results suggested that ethanol might cause subtle effects in DNA-protamine interactions inhibiting proper DNA decondensation. In mammals, sperm nuclei are stabilized by extensive disulfide bonding between the DNA and protamines. In fish, protamines are also present in sperm nuclei and are thought to stabilize the nuclei (Dixon and Smith 1968, Shimizu et al. 2000). However there are few disulfide bonds, which could make fish sperm less resistant to alcohol treatment (Yanagida et al. 1991)

Alcohols could also affect factors involved in egg activation and embryonic development. Mouse oocytes do not usually require artificial activation after ICSI. However oocytes injected with sperm fixed in ethanol required artificial activation to stimulate further development (Kimura et al. 1998, Tateno et al. 1998). It might be useful to artificially activate Nile tilapia eggs after injection with sperm preserved in methanol to see if the same response is observed. This would await development of procedures for artificial activation.

The production of mice by injection of freeze-dried sperm has tested the conventional definitions of “live” and “dead” sperm (Wakayama and Yanagimachi 1998). Although mouse sperm are considered to be dead when freeze-dried, their fertilization potential is not eliminated and the sperm factors involved in oocyte activation remain intact. This was shown by the fertilization of mouse oocytes by ICSI

without artificial stimulus. No injections of freeze-dried sperm were possible in this study due to technical problems related to manipulation of the rehydrated sperm cells. However, the information gathered could serve as starting point for future experiments. With the protocol used here, the freeze-dried sperm were not motile and were not able to fertilize Nile tilapia eggs likely due to loss of the tail. Although the size of the stained sperm heads seemed to be the same as those of fresh sperm, the nuclei appeared more diffuse. Freezing-drying altered the refractive properties of the sperm preventing visualization with conventional light microscopy. To perform injections using freeze-dried sperm from Nile tilapia, a microscope with phase-contrast filters would be desirable. The use of dyes to stain the sperm or the buffer solution to gain more contrast could also improve manipulation.

In summary, although these results are limited, those obtained with cryopreserved sperm suggest that with a more refined technique, ICSI could yield normal fish from preserved sperm. This could be the first step toward the development of new technology that would reduce maintenance and cost of genetics repositories in the future. However more basic information on the requirements for fertilization are needed to understand and develop new sperm preservation techniques for fish.

REFERENCES

- Carter, R. E, G. C. Mair, D. O. F. Skibinski, D. T. Parkin, and J. A. Beardmore. 1991. The application of DNA fingerprinting in the analysis of gynogenesis in tilapia. *Aquaculture* 95:41-52.
- Cloud, J. G., R. Armstrong, P. Wheeler, P. A. Kucera, and G. H. Thorgaard. 2000. The northwest salmonid germplasm repository. Pages 338-342 in T. R. Tiersch and P. M. Mazik, editors. *Cryopreservation in aquatic species*. World Aquaculture Society, Baton Rouge Louisiana.

- Curry, M. R. 2000. Cryopreservation of semen from domestic livestock. *Reviews of Reproduction* 5:46-52.
- Dixon G. H. and M. Smith. 1968. Nuclei acid and protamine in salmon testes. Pages 9-34 in J. N. Davidson and W. E. Cohn, editors. *Progress in Nucleic Acid Research and Molecular Biology*. Academic Press, New York and London.
- Don, J., and R. R. Avtalion. 1988. Production of F1 and F2 diploid gynogenetic tilapias and analysis of the "Hertwig curve" obtained using ultraviolet irradiated sperm. *Theoretical and Applied Genetics* 76:253-259.
- Dumoulin, J. C. M., E. Coonen, M. Bras, J. M. Bergers-Janssen, R. C. M. Ignoul-Vanvuchelen, L. C. P. van Wissen, J. P. M. Geraedts, and J. L. H. Evers. 2001. Embryo development and chromosomal anomalies after ICSI: effect of the injection procedure. *Human Reproduction* 16:306-312.
- Galman, O. R. and R. R. Avtalion. 1989. Further study of the embryonic development of *Oreochromis niloticus* (Cichlidae, Teleostei) using scanning electron microscopy. *Journal of Fish Biology* 34:653-664.
- Harris, A. S. S. Bieger, R. W. Doyle, and J. M. Wright. 1991. DNA fingerprinting of tilapia, *Oreochromis niloticus*, and its application to aquaculture genetics. *Aquaculture* 92:257-263.
- Holt, W. V. 1997. Alternative strategies for the long-term preservation of spermatozoa. *Reproduction Fertility and Development* 9:309-319.
- Jenneckens, I., A. Muller-Belecke, G. Horstgen-Schwark, J. N. Meyer. 1999. Proof of successful development of Nile tilapia (*Oreochromis niloticus*) clones by DNA fingerprinting. *Aquaculture* 173:377-388.
- Katayose, H., M. Junichiro, and R. Yanagimachi. 1992. The ability of dehydrated hamster and human sperm nuclei to develop into pronuclei. *Biology of Reproduction* 47:277-284.
- Kimura, Y., R. Yanagimachi, S. Kuretake, H. Bortkiewicz, A. C. F. Perry, and H. Yanagimachi. 1998. Analysis of mouse oocyte activation suggests the involvement of sperm perinuclear material. *Biology of Reproduction* 58:1407-1415.
- Larson, E. V. and E. F. Graham. 1976. Freeze-drying of spermatozoa. *Developments in Biological Standardization* 36:343-348.
- Lee, C., J. Mazrimas, and R. Balhorn. 1991. Analysis of protamines isolated from alcohol preserved epididymides. *Molecular Reproduction and Development*. 30:154-158.

- Lutz, C. G. 1999. The Nile pearl: An unusual color morph in *Oreochromis niloticus*. *Aquaculture Magazine* 25:74-77.
- Naish, K. A, M. Warren, F. Bardakci, D. O. F. Skibinski, G. R. Carvalho, G. C. Mair. 1995. Multilocus DNA fingerprinting and RAPD reveal similar genetic relationship between strains of *Oreochromis niloticus* (Pisces: Cichlidae). *Molecular Ecology* 4:271-274.
- Onozato, H. 1984. Diploidization of gynogenetically activated salmon eggs using hydrostatic pressure. *Aquaculture* 43:91-97.
- Peruzzi, S., A. G. Scott, J. C. J. Domaniewski, and G. F. Warner. 1993. Initiation of gynogenesis in *Oreochromis niloticus* following heterologous fertilization. *Journal of Fish Biology* 43:585-591.
- Poleo, G. A., R. S. Denniston, B. C. Reggio, R. A. Godke, and T. R. Tiersch. 2001. Fertilization of eggs of zebrafish, *Danio rerio*, by intracytoplasmic sperm injection. *Biology of Reproduction* 65:961-966.
- Polge, C., A. V. Smith, and A. S. Parks. 1949. Revival of spermatozoa. *Nature* 164:666.
- Purdom, C. E, 1969. Radiation-induced gynogenesis and androgenesis in fish. *Heredity* 24:431-444.
- Rana, K. J. and B. J. McAndrew. 1988. The viability of cryopreserved tilapia spermatozoa. *Aquaculture* 76:335-345.
- Rana, K. J. 1995. Cryopreservation of fish spermatozoa. Pages 151-165 in J. G. Day and M. R. Mclellan, editors. *Methods in Molecular Biology*. Humana Press Inc, Totowa, New Jersey.
- Saacke, R. G. and J. O. Almquist. 1961. Freeze-drying of bovine spermatozoa. *Nature* 192:995-996.
- Sakkas, D., F. Urner, P. G. Bianchi, I. Wagner, N. Jaquenoud, G. Manicardi, and A. Campana. 1996. Sperm chromatin anomalies can influence decondensation after intracytoplasmic sperm injection. *Human Reproduction* 11:837-843.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: A laboratory manual*. Volumes 1-3 in C. Nolan, editor. Cold Spring Harbor, Laboratory Press, New York.
- Shimizu. Y., K. Mita, M. Tamura, K. Onitake, and M. Yamashita. 2000. Requirement of protamine for maintaining nuclear condensation of medaka (*Oryzias latipes*)

- spermatozoa shed into water but not for promoting nuclear condensation during spermatogenesis. *International Journal of Developmental Biology* 44:195-199.
- Suzuki R, T. Oshiro, and T. Nakanishi. 1985. Survival, growth and fertility of gynogenetic diploids induced in the Cyprinid loach, *Misgurnus anguillicaudatus*. *Aquaculture* 48:45-55.
- Tateno, H., T. Wakayama, W. S. Ward, and R. Yanagimachi. 1998. Can alcohol retain the reproductive and genetic potential of sperm nuclei? Chromosome analysis of mouse spermatozoa stored in alcohol. *Zygote* 6:233-238.
- Tiersch, T. R. and R. W. Chandler. 1989. Chicken erythrocytes as an internal reference for analysis of DNA content by flow cytometry in grass carp. *Transactions of the American Fisheries Society* 118:713-717.
- Tiersch, T. R., B. A. Simco, K. B. Davis, R. W. Chandler, S. S. Wachtel, and G. J. Carmichael. 1990. Stability of genome size among stocks of the channel catfish. *Aquaculture* 87:15-22.
- Tiersch, T. R. 2000. Introduction. Pages xix-xxvi in T. R. Tiersch and P. M. Mazik, editors. *Cryopreservation in aquatic species*. World Aquaculture Society, Baton Rouge Louisiana.
- Van Eenennaam, A. L, J. P. Van Eenennaam, J. F. Medrano, and S. I. Doroshov. 1966. Rapid verification of meiotic gynogenesis and polyploidy in white sturgeon (*Acipenser transmontanus* Richardson). *Aquaculture* 147:177-189.
- Wakayama, T. and R. Yanagimachi. 1998. Development of normal mice from oocytes injected with freeze-dried spermatozoa. *Nature Biotechnology* 16:639-641.
- Yanagida, K., Yanagimachi, R. Perreault S. D, and R. G. Kleinfeld. 1991. Thermostability of sperm nuclei assessed by microinjection into hamster oocytes. *Biology of Reproduction* 44:440-447.

CHAPTER 7

EVALUATION OF INTRACYTOPLASMIC SPERM INJECTION IN EGGS OF THREE TELEOST FISHES

Chapters 3, 4, 5 and 6 of this dissertation showed that intracytoplasmic sperm injection (ICSI) can yield fertilization and normal development beyond hatching in zebrafish *Danio rerio* and Nile tilapia *Oreochromis niloticus*. These results have opened the door to new areas of research, ranging from basic reproductive biology to the production of hybrids that otherwise would not be possible. Although these results have demonstrated the feasibility of ICSI in fishes, the diversity of physiological and morphological characteristics among fish gametes suggests that techniques will need to be modified for various taxa. In this Chapter, gametes from fishes representing three families of teleosts will be compared for suitability of use in ICSI: zebrafish (Family Cyprinidae), channel catfish, *Ictalurus punctatus* (Ictaluridae) and Nile tilapia, (Cichlidae). The objectives were: 1) to evaluate manipulation of gametes; 2) to evaluate differences in spawning characteristics; 3) to evaluate micropyle localization; 4) to evaluate time required for sperm injection, and 5) to compare fertilization rates after ICSI among the three fishes.

MATERIAL AND METHODS

SPAWNING

Spawning of zebrafish was carried out following the protocols described in Chapter 3 and in Appendix A, SOP-1. Nile tilapia spawning was performed following the protocols described in Chapter 5 and in Appendix A, SOP-6.

Experiments on channel catfish were performed from March to May of 2001. This early spawning season was achieved by increasing the temperature of broodstock

ponds by adding water from a geothermal well (Lang 2001). Females were injected with synthetic luteinizing hormone-releasing hormone (LH-RH-ethylamide) (Sigma Chemical Corporation, St. Louis, Missouri) at a concentration of 100 µg/kg of fish (Busch and Steeby 1990). Females and males were paired in 80-L spawning tanks and behavior was monitored during the next 3 days (Bates and Tiersch 1998). When ~100 mL of eggs were discharged, females were removed and anesthetized in water containing tricaine methanesulfonate (methyl-m-aminobenzoate, MS 222) (Argent Laboratories, Inc., Redmond, Washington). The fish were dried and stripped by pressing the abdomen from the head to the anal region. Eggs were collected in greased plastic bowls containing Hanks' balanced salt solution (Tiersch et al. 1994). Channel catfish eggs develop an adhesive matrix after coming in contact with water that clusters and anchors them to the spawning chamber. This characteristic causes practical difficulties as the eggs become adhesive after being activated. To avoid the binding of the eggs to the holding pipette during injections, it was coated with vacuum grease (Dow Corning Corporation, Midland, Michigan) and all petri dishes were coated with silicon solution (Sigmacote®, Sigma Chemical Corporation).

MORPHOLOGY AND DEVELOPMENT

Observations were performed using a dissecting microscope (model FX-35DX, Nikon Inc., Tokyo, Japan) and an inverted microscope (Diaphot, Nikon). Photographs were taken using a 35-mm camera (Nikon, model FX-35DX) attached to the microscopes and loaded with 200 ASA color slide film (Kodak Ektachrome or Fuji Fujichrome). Measurements of the eggs were performed using a calibrated slide (objective micrometer, Olympus Tokyo). For each of the species studied, eggs were

observed immediately after spawning, and embryo development was followed after fertilization at 1-hour intervals for the first 6 h and at every 24 h thereafter (Figures 7-1, 7-2 and 7-3).

RESULTS AND DISCUSSION

The size of the tools required for the injection of sperm and holding of eggs was dictated by the size of the eggs (Figure 7-4). The injection pipette was 10 to 15 μm in diameter for all the fish studied (dictated by the size of the sperm). However, the diameter of the holding pipette was changed depending on the diameter of the eggs. For zebrafish (egg diameter of 0.8 mm) (Figure 7-4) the size of the holding pipette was between 100 and 200 μm in diameter. Nile tilapia eggs are ovoid (Figure 7-4) and vary in size from female to female, which has been correlated with the size of the fish, with longer females producing larger eggs (Jalabert and Zohar 1983, Myers and Hershberger 1991). In this work, eggs from Nile tilapia varied from 1.5 mm to 2.5 mm. The holding pipettes were made individually for each batch of eggs (between 100 μm to 300 μm in diameter). Sperm injection in smaller eggs (~1.5 mm) was easier because handling during injection and observation of the animal pole was simpler. For channel catfish eggs (~4 mm diameter) (Figure 7-4) the holding pipette was 2 mm in diameter.

The characteristics that must be considered when performing ICSI in fish include spawning behavior, gamete size and activation characteristics, adhesive properties of eggs, transparency of eggs and chorion, size and localization of the micropyle (sperm entry site), and the value of the species studied (Table 7-1).

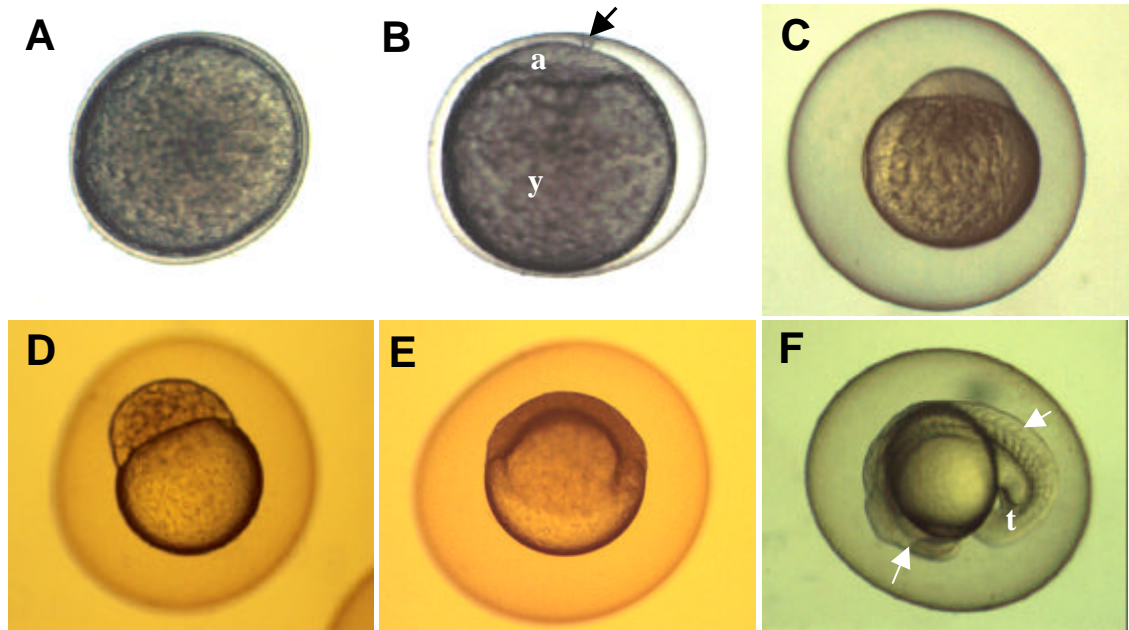


Figure 7-1. Embryonic development of zebrafish *Danio rerio*. A) Unfertilized egg. B) Activated egg showing the animal pole (a), micropyle (arrow) and the yolk (y) which occupies most of the egg. C) One-cell embryo, 1 h after fertilization. D) Blastula stage, 2.5 h after fertilization. E) Gastrula stage, 5 h after fertilization. F) Embryo 20 h after fertilization, at this time the head (bottom arrow), somites (top arrow) and the tail bud (t) are distinguishable.

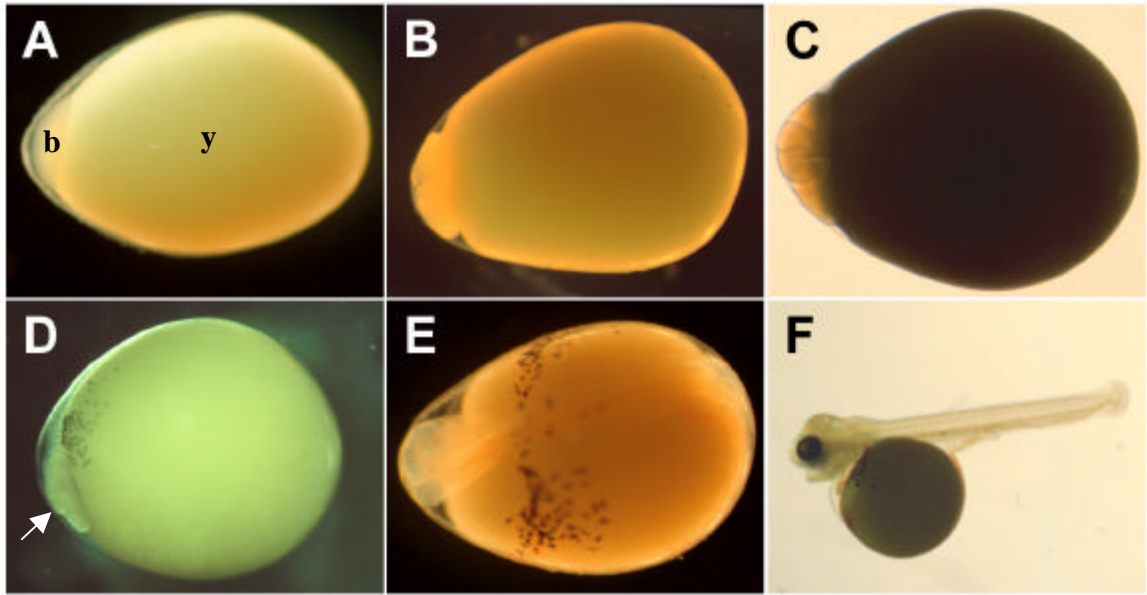


Figure 7-2. Embryonic development of Nile tilapia *Oreochromis niloticus*. A) Activated egg showing yolk (y) in the vegetal pole, and blastodisc (b). B) 2-cell embryo, 2 h after fertilization. C) 8-cell embryo, 4 h after fertilization. D) Embryo 48 h after fertilization. The head is forming, the optic bud is evident (arrow) and chromatophores have begun to appear over the dorsal part of the yolk. E) Embryo 52 h after fertilization. Chromatophores are larger. F) Hatched sac fry, 3 days after fertilization.

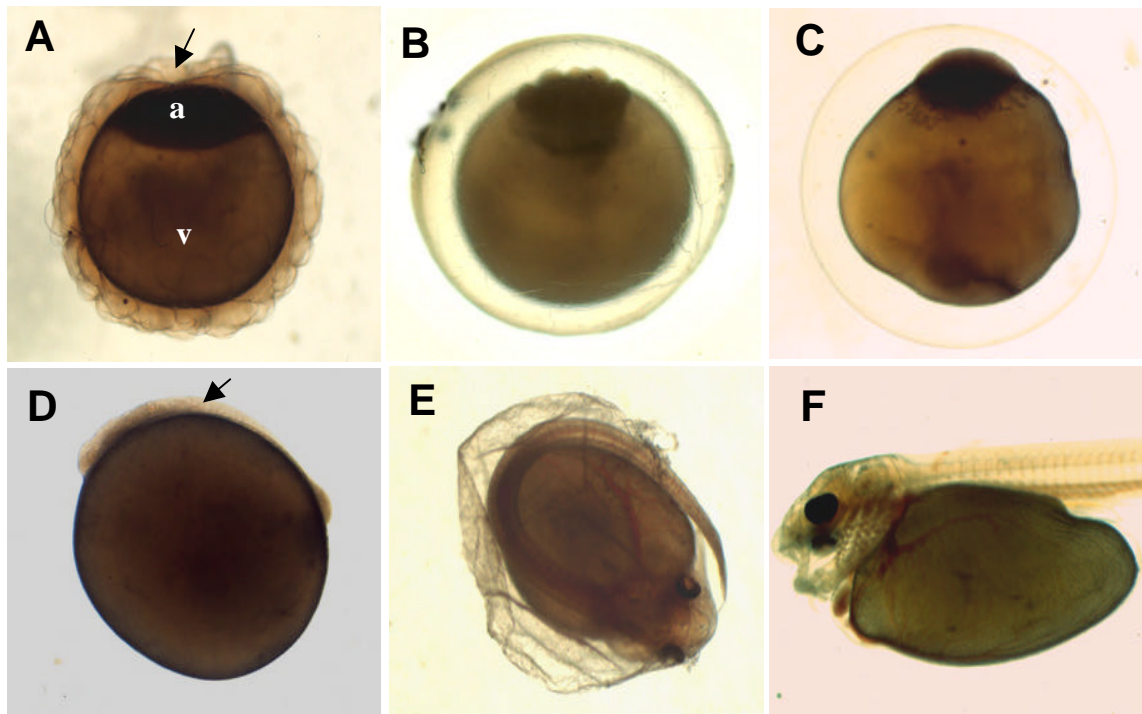


Figure 7-3. Embryonic development of channel catfish *Ictalurus punctatus*. A) Unfertilized egg. Animal pole (a), vegetal pole (v), micropyle (arrow) and the chorion covering the egg are easy to differentiate. B) Early blastula, 5 h after fertilization. Blastomeres are visible at the animal pole. C) Blastula stage, 9 h after fertilization. D) Embryo 30 h after fertilization, the antero-posterior axis has developed forming the embryonic keel. Somites (arrow) can also be seen at this stage. E) Sac fry during hatching, 5-days after fertilization. F) Sac fry 6 days after fertilization.

Zebrafish



0.8 mm

Nile tilapia



1.5-2.0 mm

Channel Catfish



4 mm

Figure 7-4. Size and morphology of unfertilized eggs from zebrafish, Nile tilapia and channel catfish.

Table 7-1. Characteristics of three teleost species for performance of intracytoplasmic sperm injection (ICSI).

Species	Holding				Gamete activation mechanism	Adhesive eggs	Transparent chorion	Micropyle identification	ICSI use
	Spawning period	Egg size (mm)	Holding pipette diameter (mm)						
Zebrafish	Year-round	0.8	0.1-0.2		Sperm, osmotic Eggs, osmotic	No	Yes	Difficult	Research model
Nile tilapia	Year-round	1.7-2.0	0.1-0.3		Sperm, osmotic Eggs, the sperm	No	Yes	Easy	Genetic improvement
Channel catfish	Mid April to June*	4.0	2.0		Sperm, osmotic Eggs, the sperm	Yes	No	Easy	Genetic improvement

* Normal spawning season can be extended to include March and beginning of April by heating of broodstock ponds (Lang 2001).

Zebrafish and Nile tilapia are relatively easy to spawn artificially. With the right conditions of temperature and photoperiod, gametes can be obtained year-round without the use of hormones. Channel catfish spawn seasonally, and eggs are available only for a few months and the use of hormone is typically required.

Activation of eggs is important when ICSI is performed. Activation begins with an increase of cytoplasmic concentration of calcium which triggers a cascade of events, which resumes the cell cycle. Activation can be seen externally by the rise of the chorion and formation of the blastodisc. Zebrafish eggs are partially activated by changes of osmotic pressure when they contact the spawning medium (Wolenski and Hart 1987, Lee et al. 1999). Tilapia eggs are activated when they come into contact with the sperm (Peruzzi personal communication, Haskin Shellfish Research Laboratory, New Jersey) or by piercing of the eggs through the micropyle with a glass needle (Chapters 5 and 6). In channel catfish the mechanism of activation has not been studied in detail, however changes in the chemical and morphological characteristics of the chorion are observed when the eggs come into contact with water, and perhaps sperm (Tucker 1985).

Each species of fish presented differences in the shape and chemical characteristics of the egg that were related to the spawning behavior. Some fish eggs present a coat of adhesive material that helps the eggs to adhere to substrate. This adhesiveness is an obstacle for ICSI because it hinders manipulation. Of the three species of fish described here, only channel catfish eggs were adhesive. However, this was overcome by the use of silicon and vacuum grease. The culture dishes where the eggs were held during the injection procedure were pre-coated using a commercially

available solution of silicon. The tools used to manipulate the eggs were coated with vacuum grease, including the holding pipette. These reduced stickiness of the egg facilitating manipulation.

Localization of the micropyle is essential during ICSI because it is used to guide the pipette into the cytoplasm of the egg. The homogeneous texture and shape of ripe eggs from zebrafish (Figure 7-5) together with the small size of the micropyle made localization difficult. The morphological structure of the egg and the large size of the micropyle facilitated the localization of the micropyle in Nile tilapia and channel catfish (Figure 7-5).

Injections in Nile tilapia eggs were easier to perform than in eggs of zebrafish and channel catfish. The average time required for each injection in Nile tilapia eggs was 5 min in comparison to 8 min for zebrafish and 10 min for channel catfish. The number of injections per session that could be performed in Nile tilapia was greater (~36) because the eggs can be held for longer time (~3 h) in Hanks' balanced salt solution (HBSS) (extender solution) without losing their fertilization capability. In zebrafish, approximately 8 injections could be done per batch of eggs in 1 hour. The difficulties for injecting channel catfish eggs were not the localization of the micropyle or the time the eggs could be held without losing fertilization capability (2-3 h in HBSS), but the adhesive characteristic of the eggs (mentioned above) and the large egg size which necessitated use of low magnification making sperm observation more difficult.

Sperm injections yielded fertilization and development in zebrafish and Nile tilapia but not in channel catfish (Table 7-2). From a total of 233 eggs injected through

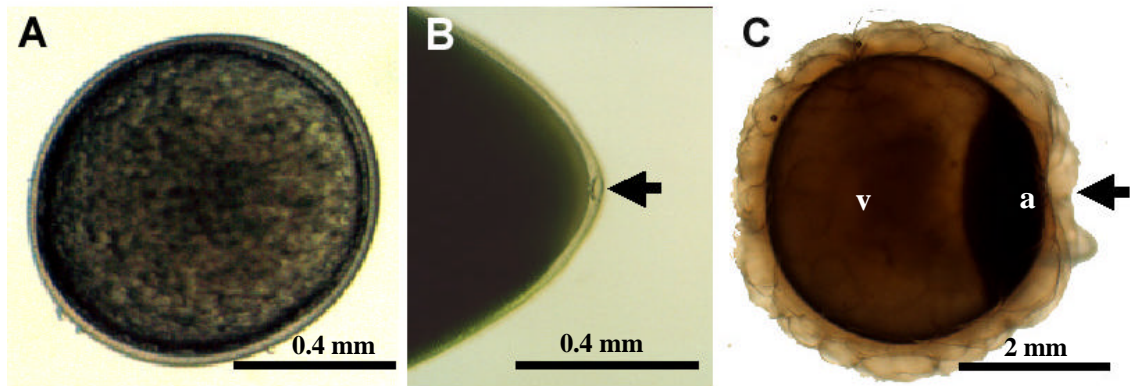


Figure 7-5. Micropyle localization for eggs of zebrafish, Nile tilapia and channel catfish. A) Non-activated zebrafish eggs. The animal and vegetal pole as well as the micropyle were difficult to localize. B) Non-activated Nile tilapia egg. The micropyle (arrow) is located at the narrow end of the egg. C) Non-activated channel catfish egg. The animal pole (a) the vegetal pole (v) and the micropyle (arrow) were easily distinguished.

the micropyle in zebrafish, 4 (2%) developed normally and hatched. Of the 160 eggs injected in Nile tilapia 5 (3%) developed normally and hatched. Two of these were still alive at the Aquaculture Research Station at the time this dissertation was written

Injectations of 188 channel catfish eggs yielded no fertilization. One possible reason for the lack of fertilization was that channel catfish eggs were not activated. Further studies will need to examine in detail the mechanisms of fertilization this species to see if artificial activation will be needed for ICSI.

Table 7-2. Results of ICSI in zebrafish, Nile tilapia and Channel catfish.

	Number of eggs	Advanced neurula	Normal development
Zebrafish	233	12 (5%)	4 (2%)
Nile tilapia	160	15 (9%)	5 (3%)
Channel catfish	188	0	0

The potential value of the fish involved will dictate the amount of time and funding available for research in ICSI. Zebrafish is extensively used as a vertebrate model for basic studies in fertilization, development and genetics in more than 260 laboratories around the world (Zebrafish Information Network, <http://zfin.org/index.html>). The amount of genetic analysis performed in zebrafish has brought this fish to an important place in the study of vertebrate biology, physiology and human disease. This is an important characteristic that justifies investment for the development of ICSI techniques that could potentially help to gather new information. Tilapias, after carps, are the most cultured fishes in the world. Global production of tilapias was estimated at 1.7 millions metric tons in 1999 (FAO, FAOSTAT, Fisheries Data, available at the web site <http://apps.fao.org>). The introduction of these fish in farming was based on their resistance to disease, tolerance to a wide range of

environmental conditions, good growth rate, and adaptability to intensive culture. The importance of tilapias has justified the amount of effort expended to increase production efficiency. The culture of channel catfish is an important economic activity for at least 13 states in the United States of America including Louisiana. Harvests were estimated approached 270 thousand metric tons during 2000 with a total farmgate value of 500 millions dollars (U. S. Department of Agriculture, 2001). This makes channel catfish the subject of research for the improvement of production, disease resistance and carcass quality.

Among the three fishes evaluated here, all present advantages and disadvantages that could be overcome depending on the amount of research and time available. A similar situation has been found in mammals where ICSI has been applied. Although the basic principles of fertilization are shared by all vertebrates, each species has evolved intrinsic characteristics. These characteristics require that researchers develop specific techniques for each species. Indeed, what works in humans often does not work in rodents or cattle, and that appears to be the case within fishes where success in one Family was not transferable to other families. Future work should address fishes within Families or Genera to evaluate what similarities exist among more closely related species.

REFERENCES

- Bates, M. and T. R. Tiersch. 1998. Preliminary studies of artificial spawning of channel catfish as male-female pairs or all-females groups in recirculating systems. *Journal of the World Aquaculture Society* 29:325-334.
- Busch, R. L. and Steeby, J. A. 1990. An evaluation of a leuteinizing hormone-releasing hormone analog to induce spawning of channel catfish *Ictalurus punctatus*. *Journal of the World Aquaculture Society* 21: 10-15.

- Jalabert, B. and Y. Zohar. 1983. Reproductive physiology in Cichlid fishes, with particular reference to *Tilapia* and *Sarotherodon*. Pages 129-140 in R. S. V. Pullin and R. H. Lowe-McConnell, editors. *The biology and culture of tilapias*. ICLARM Conference Proceedings 7. International Center for Living Aquatic Resources Management, Manila, Philippines.
- Lang, P. 2001. Induction of early out-of-season spawning in channel catfish (*Ictalurus punctatus*). Master's Thesis. Louisiana State University. Baton Rouge, Louisiana.
- Lee, K. W., S. E. Webb, and A. L. Miller. 1999. A wave of free cytosolic calcium traverses zebrafish eggs on activation. *Developmental Biology* 214:168-180.
- Myers, J. M. and W. K. Hershberger. 1991. Artificial spawning of tilapia eggs. *Journal of the World Aquaculture Society* 22:77-82.
- U. S. Department of Agriculture. 2000. National Agricultural Statistics Service (NASS), Agricultural Statistics Board, (<http://www.usda.gov/nass/>).
- Tiersch, T. R., C. A. Goudie, and G. J. Carmichael. 1994. Cryopreservation of channel catfish sperm: storage in cryoprotectants, fertilization trials, and growth of channel catfish produced with cryopreserved sperm. *Transactions of the American Fisheries Society* 123:580-586.
- Tucker, C. S. 1985. Channel catfish culture. Page 245 in C. S. Tucker, editor. *Development in Aquaculture and Fisheries Science*, Volume 15. Elsevier Scientific Publication Company.
- Wolenski, J. S. and N. H. Hart. 1987. Scanning microscope studies of sperm incorporation into zebrafish (*Brachydanio*) egg. *The Journal of Experimental Zoology* 243:259-272.

CHAPTER 8

SUMMARY AND CONCLUSION

Fertilization produced by injection of a single sperm cell into the egg cytoplasm, referred as intracytoplasmic sperm injection (ICSI), has become a routine tool in the treatment of human infertility, and has been proposed for assisted reproduction in livestock and the conservation of endangered mammalian species. However, this technique had not been previously applied in fish until the present work sought to develop basic protocols.

In the first part of this work (Chapters 3 and 4) zebrafish *Danio rerio* was chosen for the application of ICSI due to the availability of basic background information, the production of transparent embryos, ease in breeding, and short generation time. Activated and non-activated zebrafish eggs were injected with activated and non-activated sperm. It was found that all combinations yielded fertilization. Nonetheless, the injection of activated sperm into non-activated eggs showed the highest probability of fertilization (35%). This combination was thus used in successive chapters of the dissertation. Although fertilization was achieved and three normal zebrafish were produced by ICSI, the efficiency was low (16%) and a high percentage of developmental abnormalities was observed (77%) in those fish that developed to the neurula stage. One possible cause for the low fertilization rate might be damage caused to the egg chromosomes during the injection procedure. In mammalian species, sperm injection is performed to avoid the passage of the pipette through the region containing the meiotic spindle (Payne 1995). This can be easily achieved due to the location of the spindle in relation to the extruded and visible first polar body that facilitates the orientation of the oocytes.

In fish, the position of the metaphase spindle at the moment of fertilization is difficult to estimate *in vivo*, and thus the eggs need were fixed and the DNA labeled. A fluorescence dye (Hoechst 33342) showed that the maternal chromosomes were located ~ 40 µm from the site of injection (micropyle). To avoid possible injuries to the egg chromosomes, injections were performed ~100 µm distant from the micropyle. Although fertilization and embryonic development occurred, no improvement in fertilization rate or reduction of abnormalities were observed. In order to improve fertilization percentage after ICSI in zebrafish, future work will need to address factors such as sperm ejection after ICSI, egg activation, quality of sperm DNA and damage to the egg cytoskeleton.

The second part of this dissertation (Chapters 5 and 6) used Nile tilapia *Oreochromis niloticus* an aquaculture model for the development of ICSI. This research required a constant and predictable supply of viable eggs. With Nile tilapia the production of eggs was achieved through a “single-breeding” system where single females, kept in individual tanks, produced eggs every 28 ± 9 days. The viability of the eggs was extended for as long as 3 hours after collection by storage in Hanks’ balanced salt solution.

Of the 160 injections of fresh sperm carried out in Nile tilapia, 9 (6%) of the eggs developed abnormally while 5 (3%) developed normally and hatched, two of which reached adulthood. Sperm injections in Nile tilapia yielded 3% normal fish compared with 2% in zebrafish. Although there was no great technical difference between the two species of fish used, sperm injections in Nile tilapia proved to be easier. The long viability window of Nile tilapia eggs, together with their easy

manipulation and rapid localization of the micropyle, reduced the time required for injections, facilitating the overall procedure.

For ICSI to succeed, eggs have to be activated during or after the sperm are injection. In humans, ineffective activation accounts for ~40% of the failures after ICSI (Rawe et al. 2000). Egg activation during ICSI is species specific. While some species such as cattle require artificial activation (Goto et al. 1990), the injected sperm is sufficient to trigger the process in other species such as human and mouse (Palermo et al. 1992, Kimura and Yanagimachi 1995). In fishes, sperm from other species have been used to activate eggs for the production of gynogenetic fish (all-maternal inheritance) (Peruzzi et al. 1993). In this dissertation, sperm from goldfish *Carassius auratus* was evaluated as a potential activator of Nile tilapia eggs. The sperm did not activate the eggs nor did it improve fertilization after ICSI. However, these negative results cannot rule out the possibility of using incompatible sperm from other species or genetically inert sperm treated with ultraviolet light to activate Nile tilapia eggs when applying ICSI.

An important use of ICSI could be the development of new technologies of sperm preservation that require the delivery of non-motile sperm into the cytoplasm of the egg (Tateno et al. 1998, Wakayama 1998, Tiersch 2000). In this dissertation attempts were made to use ICSI with cryopreserved sperm, sperm fixed in methanol, and freeze-dried sperm. Nine of 45 eggs injected with cryopreserved sperm were fertilized but none developed beyond blastula stage. Injections with sperm fixed in methanol did not yield fertilization and no injections were possible with freeze-dried sperm due to the technical difficulties encountered as a result of the physical changes

undergone by the sperm. These sperm were more difficult to visualize, making it difficult to manipulate the sperm. The results shown here are preliminary and will need further study before any conclusions can be reached.

The ICSI conditions used in this study were perhaps far from ideal. Injection procedures, gamete conditions before and after injection, and gamete storage conditions will need further refinement to increase efficiency. Spindle damage, sperm and oocyte chromatin fragmentation, lack of activation after sperm injection, sperm expulsion through the injection track and inability of microtubules to assemble around the sperm centrosome are some of the reasons for fertilization failure in humans oocytes. These would also need to be addressed if improvements in fish are to be obtained.

The results obtained in this dissertation provide the first step towards the development of further uses of ICSI in fish including the production of hybrids, study of the fertilization process, preservation of endangered species, and production of polyploidy (Table 8-1).

Sperm injection could also be used for the production of androgenesis, normally produced by fertilization of an irradiated egg and the subsequent inhibition of the first mitotic division by heat or pressure shock (Myers et al. 1995, Corley-Smith et al. 1996). This results in a completely homozygous fish that receives all nuclear DNA from the male. However, homozygous fish produced in this fashion often die because of the presence of lethal recessive alleles. By injecting two sperm into an irradiated egg we could produce androgenetic fish with fewer deleterious effects of lethal recessive alleles as the fish would not be completely homozygous. This approach could be applied in the

restoration of endangered or extinct stocks by injecting sperm into irradiated eggs from related stocks or species.

Table 8-1. Potential applications of intracytoplasmic sperm injection (ICSI) in fish

ICSI Application	Reason
Breeding animals with poor quality sperm	Poor sperm motility or sperm damage
Hybridization	Fertilization not otherwise possible
Androgenesis (all-paternal inheritance)	Rapid inbreeding, species recovery
Conservation genetics	Use of low-cost sperm preservation methods
Breeding programs	Endangered species
Polyploidy	Research model, sterility induction
Fertilization studies	Research model
Developmental biology	Research model
Gene transfer	Developmental biology, stock improvement

Genetically modified organisms, despite the surrounding controversy, are important research tools. Different techniques have been developed to incorporate foreign DNA in specific organisms, mostly mammals. Among these techniques, the use of sperm as a carrier to introduce foreign DNA has been tested. Basically this technique works by incubation of intact spermatozoa with a desired DNA plasmid and use of the modified sperm to inseminate eggs. However, the efficacy in production of transgenic animals is ambiguous. A new approach with a greater level of success utilizes ICSI to inject membrane-disrupted sperm heads that are briefly incubated with linearized DNA (Perry et al. 1999, Sparrow et al. 2000). One of the problems with gene transfer in fish is the random incorporation of foreign DNA throughout the body

(mosaicism). The injection of sperm containing plasmids incorporated in the nuclei could be used in fish to generate non-mosaic organisms.

Fish are a remarkably diverse grouping of animals showing many characteristics not present in other vertebrates. Unlike mammals, aquatic organisms such as mollusks and fish can support extra sets of chromosomes. This attribute has been of interest in aquaculture for the genetic improvement of cultured species. Polyploidy in fish is usually achieved by inhibiting the extrusion of the second polar body during meiosis II (triploidy) or by inhibiting the first mitotic division after fertilization (tetraploidy). The treatments of choice have been the application of chemicals, changes in temperature (cold or heat shock) and pressure shock. Triploid fish sometimes present a higher survival rate than diploid fish especially with certain hybrid crosses. This characteristic could be potentially useful in ICSI research to increase survival rates of fish.

Intracytoplasmic sperm injection could be used as an alternative method for the production of polyploid fish by the injection of multiple sperm into an egg or by injecting a sperm after or before normal fertilization.

The procedures developed in this dissertation for ICSI in fish would be most useful in the reconstitution of desired lines and the development of small broodstock populations for the production of a larger number of fish (Figure 8.1).

REFERENCES

- Goto, K., A. Kinoshita, Y. Takuma, and K. Ogawa. 1990. Fertilization of immobilized, killed spermatozoa. *The Veterinary Record* 127:517-520.
- Kimura, Y. and R. Yanagimachi. 1995. Mouse oocyte injected with testicular spermatozoa or round spermatids can develop into normal offspring. *Development* 121:2397-2405.

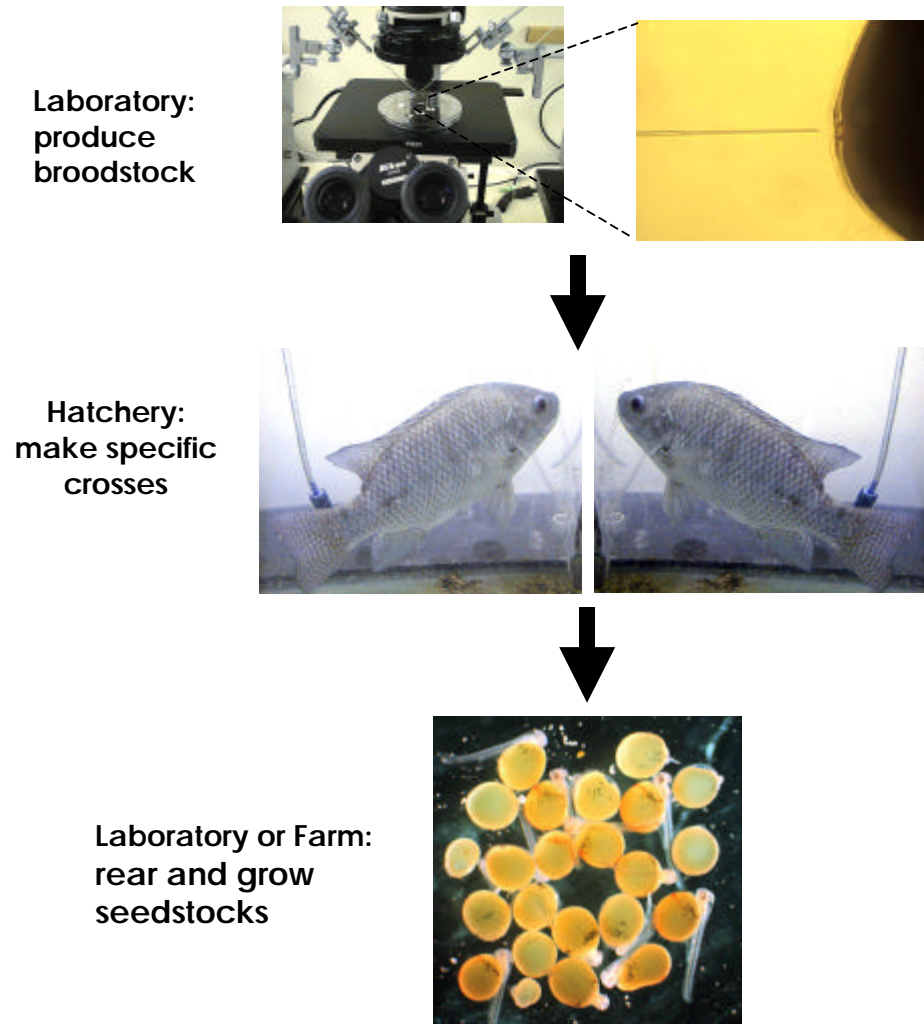


Figure 8-1. With the techniques described in this dissertation, ICSI could be use to create small broodstock populations to be used to produce offspring for research or commercial production.

- Myers, J. M., D. J. Penman, Y. Basavaraju, S. F. Powel, P. Baoprasertkul, K. J. Rana, N. Bromage, B. J. McAndrew. 1995. Induction of diploid androgenetic and mitotic gynogenetic Nile tilapia (*Oreochromis niloticus* L.). *Theoretical and applied genetics* 90:205-210.
- Palermo, G., Joris, H., Devroey, P., and Van Steirteghem, AC. 1992. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet* 340: 17-18.
- Payne, D. 1995. Intracytoplasmic sperm injection: instrumentation and injection technique. *Reproduction Fertility and Development* 7:185-196.
- Perry, A.C., T. Wakayama, H. Kishikawa, T. Kasai, M. Okabe, Y. Toyoda, and R. Yanagimachi. 1999. Mammalian transgenesis by intracytoplasmic sperm injection. *Science* 284:1180-1183.
- Peruzzi, S., A. G. Scott, J.C.J. Domaniewski, and G. F. Warner. 1993. Initiation of gynogenesis in *Oreochromis niloticus* following heterologous fertilization. *Journal of Fish Biology* 43:585-591.
- Rawe, V. Y., S. Brugo Olmedo, F. N. Nodar, G. D. Doncel, A.A. Acosta, and A. D. Vitullo. 2000. Cytoskeletal organization defects and abortive activation in human oocytes after IVF and ICSI failure. *Molecular Human Reproduction* 6:510-516.
- Sparrow, D.B., B. Latinkic, and T. J. Mohum. 2000. A simplified method of generating transgenic *Xenopus*. *Nucleic Acid Research* 28:E12.
- Tateno H., T. Wakayama, W. S. Ward, and R. Yanagimachi. 1998. Can alcohol retain the reproductive and genetic potential of sperm nuclei? Chromosome analysis of mouse spermatozoa stored in alcohol. *Zygote* 6:233-238.
- Tiersch, T. R. 2000. Introduction. Pages xix-xxvi in T. R. Tiersch and P. M. Mazik, editors. *Cryopreservation in Aquatic Species*. World Aquaculture Society, Baton Rouge, Louisiana.
- Wakayama, T. and R. Yanagimachi. 1998. Development of normal mice from oocytes injected with freeze-dried spermatozoa. *Nature Biotechnology* 16:639-641.

APPENDIX A

STANDARD OPERATING PROCEDURES

SOP-1. COLLECTION OF ZEBRAFISH GAMETES AND ARTIFICIAL INSEMINATION

Sperm collection

Materials needed:

Tricaine solution in two 250-mL beakers (0.17 mg/mL tricaine methanesulfonate (methyl-m-aminobenzoate, MS 222, Argent Laboratories, Redmond, Washington)

Stereo microscope (5 X magnification)

10 μ L pipette

20 mL beaker of Hanks' balanced salt solution (HBSS).

Kimwipes

1.5-mL microcentrifuge tube.

100-mm dish lid (Corning Glass Works, Corning, New York).

Procedure

1. Put 10 μ L of HBSS in the microcentrifuge tube and put on ice.
2. Remove two fish from the holding container with net and place into the 250-mL beaker containing the tricaine solution.
3. Remove the fish with a spoon when gill movement has slowed, and fish are upside down
4. Place the fish upside down in the lid of a 100-mm plastic cell culture dish
5. Gently wipe the region of the anal fin with the corner of a Kimwipe.
6. Place the dish with the fish under the objective lenses of the dissecting scope (Nikon, model SMZ-U, Japan) with the light illuminating the fish, especially the region of the anal fin.
7. With the 10 μ L pipette tip, gently push aside the anal fins to expose the urogenital area.
8. Using your fingers gently squeeze the sides of the fish at a point just anterior to the anal fins, collecting the sperm, with the pipette. When finished, return the fish to the recovery container.
9. When you have collected the sperm place it in the HBSS in a microcentrifuge tube in the ice bucket.
10. Repeat until you have collected sperm from the required number of males.
11. Keep the sperm and HBSS in the ice. This helps prolong the viability of the sperm.
12. Check the motility of the sperm (SOP-5)

Egg collection

Materials needed:

Tricaine solution in two 250-mL beakers
One package, 35-mm petri dishes (Corning Glass Works)
50 μ L micropipettes (Drummond Wiretrol)
Embryo medium buffer (EMB) (SOP-2)
HBSS

Procedure

1. Place two females into the tricaine solution in each of the two 250-mL beakers.
2. Remove one of the fish with the plastic spoon when gill movement has slowed.
3. Rinse the fish in the water in the finger bowl.
4. Gently place the fish on a paper towel to dry briefly.
5. Using the spoon, transfer the fish into a 35-mm tissue culture dish containing 500 μ L of HBSS.
6. Place one finger of one hand on the dorsal side of the fish.
7. Using one finger of the other hand express the eggs by gently pressing on the ventral side of the fish, starting just behind the pectoral fins and moving toward the tail. Only gentle pressure is needed. If the fish has eggs they will come out easily. If gentle pressure fails to produce eggs do not continue to squeeze harder (this could injure the fish).
8. If eggs are obtained, use a metal spatula to gently move them away from the fish and slide the fish out of the dish.
9. Put the fish into a recovery container to revive.
10. Repeat this for the remaining fish.

***in vitro* fertilization**

Procedure

1. Remove excess HBSS in the plastic dish containing the eggs.
2. With a pipette take the amount of sperm needed and expel it onto the eggs (~10 μ L per 100 eggs)
3. Gently mix the sperm and eggs with the tip of the pipette.
4. Using the 1-mL pipette, add 1 mL of water to the gametes mixture. This activates the sperm so that they can fertilize the eggs. The time of fertilization begins when the water is added, not when the sperm is added.
5. Allow 5 minutes for fertilization to complete, add more egg water (~2 mL).

REFERENCE

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

SOP-2. PREPARATION OF HANKS' BALANCED SALT SOLUTION (HBSS) AND EMBRYO MEDIUM BUFFER (EMB).

Method 1:

Full-strength HBSS	Full strength EMB
0.137 M NaCl	13.7 mM NaCl
5.4 mM KCl	5.4 mM KCL
0.25 mM Na ₂ HPO ₄	0.25 mM Na ₂ HPO ₄
0.44 mM KH ₂ PO ₄	0.44 mM KH ₂ PO ₄
1.3 mM CaCl ₂	1.3 mM CaCL ₂
1.0 mM MgSO ₄	1.0 mM MgSO ₄
4.2 mM NaHCO ₃	4.2 mM NaHCO ₃

Osmolality should be ~280 mOsmol/Kg and pH should be 7.2

Method 2:

HBSS and EMB can be made from stock solutions that can be refrigerated at 4°C

Solution 1	Solution 2	Solution 3	Solution 4
8.0 g NaCl	0.358 g Na ₂ HPO ₄ (anhydrous)	0.72 g CaCl ₂	1.23 g MgSO ₄ - 7H ₂ O
0.4 g KCl	0.60 g KH ₂ PO ₄	In 50 mL distilled water	In 50 mL distilled water
In 100 mL distilled water	In 100 mL distilled water		

Solution 5 (make fresh)

0.35 grams NaHCO₃
10.0 mL distilled water

To make 100 mL of HBSS combine in the following order:

10 mL of solution 1
1 mL of solution 2
1 mL of solution 3
86.0 mL of distilled water
1 mL of solution 4
1 mL of solution 5

To make 100 mL of EMB combine in the following order:

1 mL of solution 1
0.1 mL of solution 2
1 mL of solution 3
95.9 mL of distilled water
1 mL of solution 4
1 mL of solution 5
Adjust pH to 7.2

REFERENCE

Westerfield, M. 1995. *The Zebrafish Book. Guide for the Laboratory Use of Zebrafish (Danio rerio)*. 3rd edition. University of Oregon Press, Eugene.

SOP-3. Preparation of 4% PARAFORMALDEHYDE (PFA)

Materials needed:

Fume hood
Hot plate
pH meter
Filter paper Whatman N°1

Procedure

1. Add 4 g of paraformaldehyde in 100 mL of PBS (0.8% NaCl, 0.02% KCl, 0.02% KH_2PO_4 , 0.115% Na_2HPO_4).
2. Stir on a hot plate at 50°C until it dissolved.
3. Put the beaker on ice and adjust pH to 7.0
4. Filter with filter paper (Whatman N°1)
Because PFA is not stable at room temperature, this solution is good only for a week. To keep it longer, aliquots can be frozen at -20°C and used when need.

Note:

Paraformaldehyde is toxic. Manipulation must be done under a fume hood. Vapors from PFA are toxic by inhalation, may cause cancer and heritable genetic damage. Contact with the skin can causes severe irritation (MSDS page 141).

MATERIAL SAFETY DATA SHEET (MSDS)

SECTION 1. - - - - - CHEMICAL IDENTIFICATION

CATALOG #: 76240

NAME: PARAFORMALDEHYDE

SECTION 2. - - - - - COMPOSITION/INFORMATION ON INGREDIENTS - - - - -

CAS #: 30525-89-4

MF: (CH₂O)_N

SYNONYMS

ALDACIDE * FLO-MOR * FORMAGENE * PARAFORM *

SECTION 3. - - - - - HAZARDS IDENTIFICATION

LABEL PRECAUTIONARY STATEMENTS

FLAMMABLE (USA)

HIGHLY FLAMMABLE (EU)

TOXIC

MAY CAUSE CANCER.

MAY CAUSE HERITABLE GENETIC DAMAGE.

TOXIC BY INHALATION.

HARMFUL IN CONTACT WITH SKIN AND IF SWALLOWED.

MAY CAUSE SENSITIZATION BY INHALATION AND SKIN
CONTACT.

CAUSES SEVERE IRRITATION.

TARGET ORGAN(S):

NERVES

KEEP AWAY FROM SOURCES OF IGNITION - NO SMOKING.

IN CASE OF ACCIDENT OR IF YOU FEEL UNWELL, SEEK

MEDICAL ADVICE

IMMEDIATELY (SHOW THE LABEL WHERE POSSIBLE).

IN CASE OF CONTACT WITH EYES, RINSE IMMEDIATELY
WITH PLENTY OF

WATER AND SEEK MEDICAL ADVICE.

WEAR SUITABLE PROTECTIVE CLOTHING, GLOVES AND
EYE/FACE

PROTECTION.

SECTION 4. - - - - - FIRST-AID MEASURES

IN CASE OF CONTACT, IMMEDIATELY FLUSH EYES OR SKIN
WITH COPIOUS

AMOUNTS OF WATER FOR AT LEAST 15 MINUTES WHILE
REMOVING CONTAMINATED

CLOTHING AND SHOES.

IF SWALLOWED, WASH OUT MOUTH WITH WATER
PROVIDED PERSON IS CONSCIOUS.

CALL A PHYSICIAN.

IF INHALED, REMOVE TO FRESH AIR. IF NOT BREATHING
GIVE ARTIFICIAL RESPIRATION. IF BREATHING IS DIFFICULT, GIVE
OXYGEN.

ASSURE ADEQUATE FLUSHING OF THE EYES BY
SEPARATING THE EYELIDS
WITH FINGERS.

SECTION 5. - - - - - FIRE FIGHTING MEASURES
EXTINGUISHING MEDIA

CARBON DIOXIDE, DRY CHEMICAL POWDER OR
APPROPRIATE FOAM.

WATER SPRAY.

SPECIAL FIREFIGHTING PROCEDURES

WEAR SELF-CONTAINED BREATHING APPARATUS AND
PROTECTIVE CLOTHING TO

PREVENT CONTACT WITH SKIN AND EYES.

UNUSUAL FIRE AND EXPLOSIONS HAZARDS

THIS MATERIAL, LIKE MOST MATERIALS IN POWDER FORM,
IS CAPABLE OF

CREATING A DUST EXPLOSION.

UNDER FIRE CONDITIONS, MATERIAL MAY DECOMPOSE
TO FORM FLAMMABLE AND/OR EXPLOSIVE MIXTURES IN

AIR.

FLAMMABLE SOLID.

EMITS TOXIC FUMES UNDER FIRE CONDITIONS.

SECTION 6. - - - - - ACCIDENTAL RELEASE MEASURES

EVACUATE AREA.

SHUT OFF ALL SOURCES OF IGNITION.

WEAR SELF-CONTAINED BREATHING APPARATUS, RUBBER
BOOTS AND HEAVY

RUBBER GLOVES.

COVER WITH DRY-LIME, SAND, OR SODA ASH. PLACE IN
COVERED CONTAINERS

USING NON-SPARKING TOOLS AND TRANSPORT OUTDOORS.

VENTILATE AREA AND WASH SPILL SITE AFTER MATERIAL
PICKUP IS COMPLETE.

SECTION 7. - - - - - HANDLING AND STORAGE

REFER TO SECTION 8.

SECTION 8. - - - - - EXPOSURE CONTROLS/PERSONAL
PROTECTION- - - - -

WASH CONTAMINATED CLOTHING BEFORE REUSE.

WASH THOROUGHLY AFTER HANDLING.

WEAR APPROPRIATE NIOSH/MSHA-APPROVED RESPIRATOR,
CHEMICAL-RESISTANT

GLOVES, SAFETY GOGGLES, OTHER PROTECTIVE CLOTHING.

SAFETY SHOWER AND EYE BATH.

USE ONLY IN A CHEMICAL FUME HOOD.

DO NOT BREATHE DUST.
DO NOT GET IN EYES, ON SKIN, ON CLOTHING.
TOXIC.
SEVERE IRRITANT.
SENSITIZER.
KEEP CONTAINER CLOSED.
KEEP AWAY FROM HEAT, SPARKS, AND OPEN FLAME.
STORE IN A COOL DRY PLACE.

SOP-4. OBSERVATION OF THE ANIMAL POLE OF EGGS

Materials needed:

Super glue (any brand)
Glass microscope slide
Cover slips
Nail polish (any brand)

Procedure

1. Glue two cover slips about 1.5 cm apart (Figure A-1) on the glass slide.
2. Place the stained tissue between the cover slips with the animal pole facing upward, and place a third cover slip on top of the other two, forming a bridge over the tissue (Figure A-1).
3. Brush nail polish along the edges of the cover slip and allow to dry.
4. View the slides under an ultraviolet light microscope (Nikon Microphot-SA microscope, Nikon Inc, Garden City, New York).
5. Record observations with a video camera (Microimage Video Sytem, model A206A, Boyertown Pennsylvania) onto a computer or take photographs using a 35-mm camera attached to the microscope loaded with 800 ASA color slide film (Kodak Ektachrome).
6. Calculate the widths of the stained and unstained sperm and egg DNA and the distance between them by use of image analysis software (Optimas 5.1a, Bioscan Inc, Edmund, Washington).

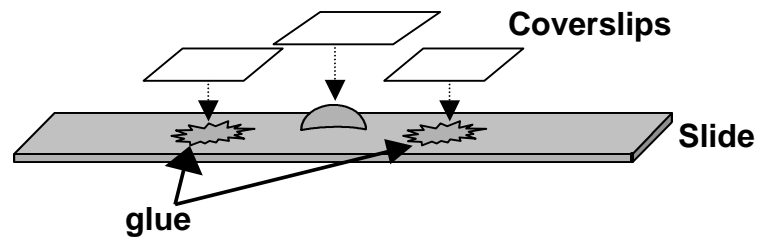


Figure A-1. Diagram of chambers used for the observation of the animal pole of zebrafish and Nile tilapia eggs.

SOP-5. ESTIMATION OF SPERM MOTILITY

Materials Needed:

10 μ L pipette and tips

Glass microscope slide

Microscope with darkfield filter and 10 or 20x objective.

Distilled water

Procedure

1. Place 10- μ L of sperm solution on a glass microscope slide.
2. Add 20 μ L of distilled water to the sperm solution and mix gently with the tip of the pipette
3. Rapidly put the slide under the microscope and using the darkfield filter observe the sperm
4. Sperm that exhibit vigorous forward movement are counted as motile.
5. The percentage of motility is a subjective estimate that required training from a person with previous experience.

SOP-6. COLLECTION OF NILE TILAPIA GAMETES AND ARTIFICIAL INSEMINATION

Sperm collection

Materials needed:

Microscopy for sperm motility estimation
100- μ L pipette and tips
Hanks' balanced salt solution (HBSS)
Paper towels
1.5-mL microcentrifuge tube
Ice

Procedure

1. Remove a fish from the holding tank.
2. Cover the head with paper towel to reduce the movement of the fish and provide a dry place to hold it during the procedure.
3. Place the fish upside down.
4. Gently wipe the region of the anal fin with a paper towel.
5. Gently push aside the anal fin to expose the urogenital area.
6. Using your fingers gently squeeze the sides of the fish at a point just anterior to the anal fins, collecting the sperm with the pipette. The sperm fluid has a white coloration, do not mistake it with urine which is clear. When finished return the fish to the recovery container.
7. When you have collected sperm from fish, add them to the microcentrifuge tube and add equal amount of the HBSS, place the microcentrifuge tube in the ice bucket.
8. Repeat until you have collected the required amount of sperm.
9. Keep the sperm and HBSS solution in the ice. This helps prolong the viability of the sperm.
10. Estimate motility (SOP-5)

Egg collection

Materials needed:

Tricaine (0.17 mg/mL tricaine methanesulfonate (methyl-m-aminobenzoate, MS 222, Argent Laboratories, Inc., Redmond, Washington)
Glass bowl
HBSS
Embryo medium buffer (EMB) or tank water for fertilization.
Paper towel
Rubber gloves
Plastic bucket

Procedure

1. Females that show a swollen belly and projecting genital papilla (Figure A-2A) are ready for spawning.
2. Place a ripe female into the tricaine solution in a bucket.
2. Remove the fish, using gloves, when gill movement has slowed, to avoid touching the tricaine solution.
3. Gently wipe the region of the anal fin with a paper towel.
4. With one hand gently press the abdomen of the fish starting behind the pectoral fins and moving towards the tail (Figure A-2B). Ripened eggs are easily stripped and showed a uniform size (Figure A-2C). The presence of blood, connective tissue or size variation indicate that the eggs are not ripe
5. Only gentle pressure is needed. If the fish has eggs, they will come out easily. If gentle pressure fails to produce eggs do not continue to squeeze harder. This could injure the fish.
10. Put the fish back into the tank to revive.



Figure A-2. Egg collection from Nile tilapia. A) An extended genital pore or papilla (arrow) indicates that the fish is ready to spawn. B) Gina Cheuk shown stripping eggs from anesthetized female Nile tilapia by gently squeezing the abdomen from the head to the anal fin. C) Ripe egg present a uniform shape and reduce blood and connective tissue in the ovarian fluid.

***in vitro* fertilization**

Procedure

1. Remove as much of the HBSS as possible in the glass bowl containing the eggs
2. With a pipette take the amount of sperm needed and expel it onto the eggs (10 μ L per 100 eggs)
3. Gently mix the sperm and eggs with the tip of the pipette.
4. Add 10 mL of EMB or tank water to the gametes mixture. This activates the sperm so that they can fertilize the eggs. The time of fertilization occurs when the water is added, not when the sperm is added.
5. Allow 5 minutes for fertilization to complete, then add more egg water. (approximately 50 mL).

APPENDIX B

FERTILIZATION OF ZEBRAFISH EGGS USING SPERM FROM OTHER SPECIES

In *in vitro* experiments in mammals, such as ICSI or nuclear transfer, oocytes are normally artificially activated after the procedure. Activation triggers the mechanism of the cell cycle and is carried out by incubating the oocytes with different chemicals (ionophores, ethanol, etc.) or by using electrical current. In fish, sperm previously treated either with ultraviolet light or with radiation is used as an egg activator. Treated sperm has been used for the induction of parthenogenesis (Corly-Smith et al. 1996) and gynogenesis (Onozato 1984). In previous work (Peruzzi et al., 1993) sperm from other species such as Common carp, *Cyprinus carpio* have been used to serve as activator of Nile tilapia eggs in the production of gynogenetic fish. Researchers have suggested that it is better to use sperm from other species rather than irradiated homologous sperm because it is less likely to result in undetected paternal genetic contamination (Peruzzi et al. 1993). Insemination of eggs before or after the ICSI procedure could help to increase the rate of success.

MATERIALS AND METHODS

ANIMALS

In order to test if sperm of other fish species could fertilize or induce parthenogenesis in zebrafish, sperm from representation of three different families of fishes, Ictaluridae (*Ictalurus punctatus* and *Ictalurus furcatus*), Cyprinidae (*Carassius auratus*), and Cichlidae (*Oreochromis niloticus*) were incubated with eggs of zebrafish.

FLOW CYTOMETRIC ANALYSIS

Analysis of DNA content was performed using a FACSCalibur[®] flow cytometer (Becton Dickinson, San Jose, California) equipped with an air-cooled 480 nm argon

laser. The FACSCComp® software (Becton Dickinson) was used to calibrate the instrument settings. Embryos were disrupted by passage through a 3-mL syringe fitted with a 25-gauge needle while suspended in 0.5 mL of lysis buffer containing 25 µg of buffered RNase, 0.1% sodium citrate and 0.1% Triton X-100 (Tiersch and Chandler 1989). Embryos were disrupted by passing them three times through a 25-gauge syringe. The solution was filtered through 20-µm nylon mesh. For analysis, an aliquot of 250 µl of disrupted embryo or 1 µL of blood was diluted in lysis-staining buffer, which included 25 µg of propidium iodide. A sample of blood from rooster, *Gallus gallus*, was used as an internal reference (2.5 pg of DNA/cell) (Tiersch and Wachtel 1991). Measurements of the DNA content of normal diploid zebrafish (3.15 pg of DNA/cell) were compared with those of embryos produced by insemination with goldfish and Nile tilapia sperm.

RESULTS

When using sperm of Nile tilapia, *Oreochromis niloticus*, $38 \pm 29\%$ of the eggs were fertilized (Figure B-1C). The 66 embryos that developed through 24 h showed haploid syndrome (Figure B-1F). That was confirmed by flow cytometric analysis (Figure B-2B) in which the genome size from putative haploids was to be half of the size of normal zebrafish.

When using sperm of goldfish, *Carassius auratus*, $73 \pm 10\%$ of the eggs were fertilized, developed and hatched. Morphologically, the embryos looked different than normal zebrafish embryos (Figure B-1G and H), the yolk sac was elongated and the swim bladder never formed. The embryos did not show the characteristic haploid syndrome and flow cytometric analysis showed that the embryos were not haploid

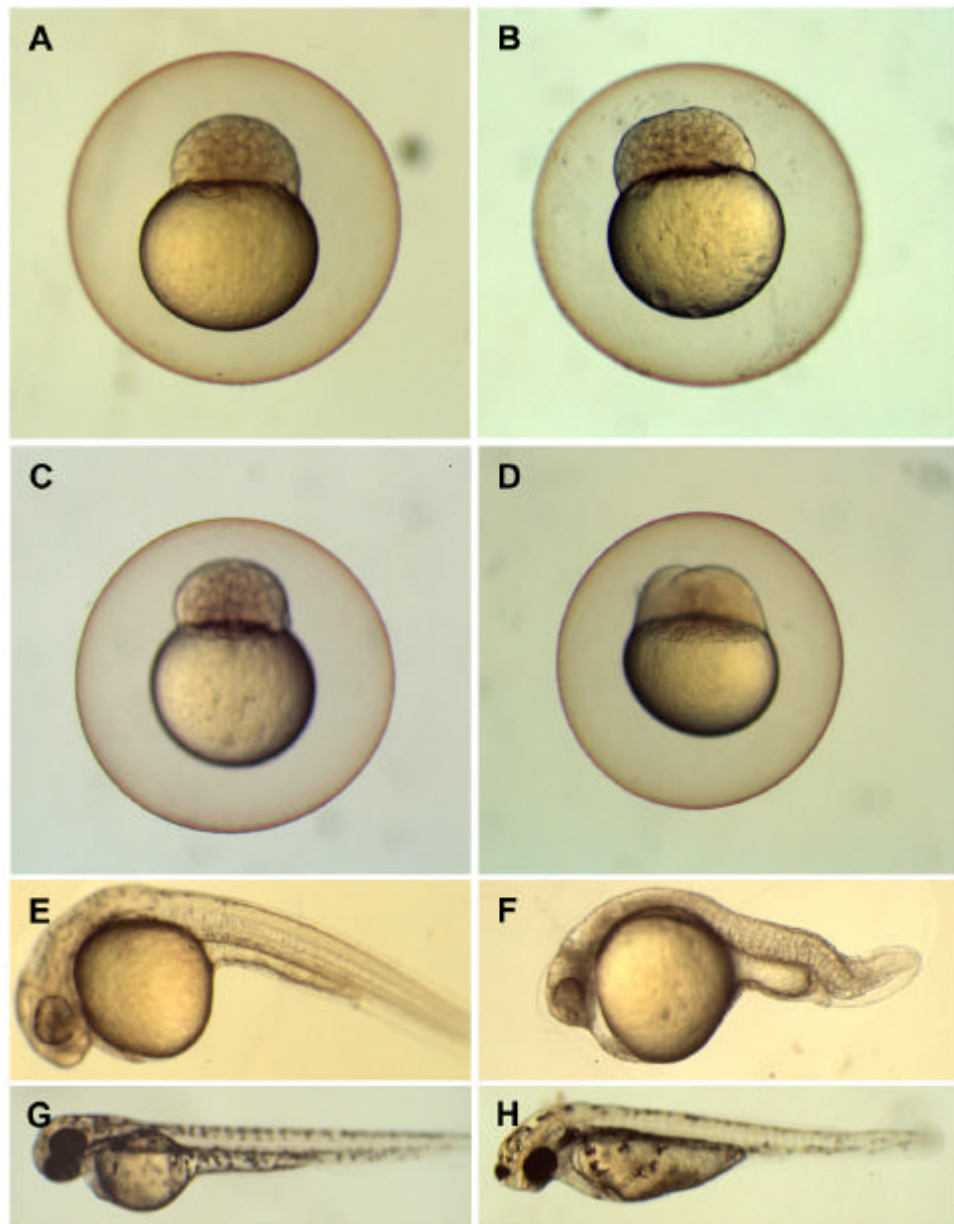


Figure B-1. Fertilization of zebrafish eggs using sperm from three different families of fish (Ictaluridae, Cyprinidae and Cichlidae). A) Zebrafish embryo 3 h after fertilization (Control). B) Embryo 3 h after fertilization produced using Nile tilapia (*Oreochromis niloticus*) sperm. C) Embryo 3 h after fertilization produced using goldfish (*Carassius auratus*) sperm, D) Undeveloped egg inseminated with channel catfish (*Ictalurus punctatus*) sperm. E) Zebrafish embryo 48 h after fertilization (Control). F) Embryo 48 h after fertilization produced by inseminating zebrafish eggs with goldfish sperm. Yolk sac is elongated compared with controls as well as chromatophores. G) Normal zebrafish embryo 48 h after fertilization. H) Embryo produced by inseminating zebrafish eggs with goldfish sperm.

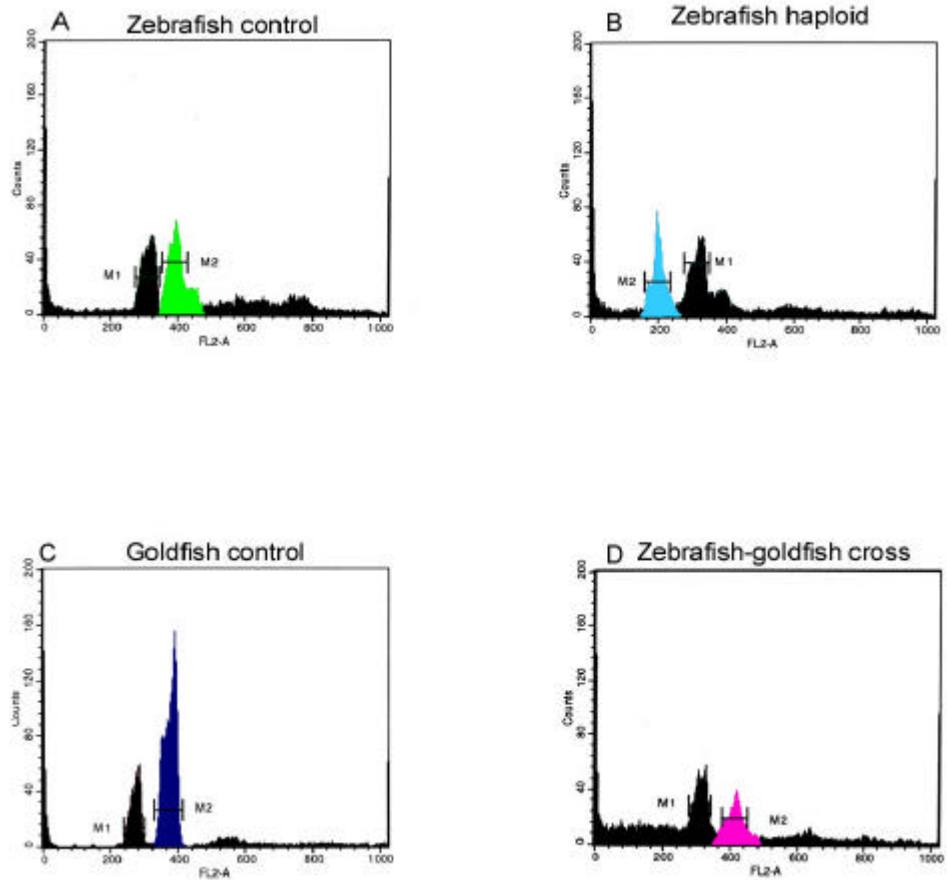


Figure B-2. DNA content measured by flow cytometry. DNA values are reported in arbitrary units expressed as fluorescence channel number. A) Wild-type zebrafish (M2, green peak) showing genome size of 3.15 pg/cell. B) Haploid zebrafish (M1, blue peak) showing DNA content of ~1.58 pg/cell were produced by using sperm from Nile tilapia. C) Goldfish (M2, blue) showing genome size of 3.5 ± 0.06 . D) Embryos produced by inseminating zebrafish eggs with goldfish sperm (M2, purple) showing a genome size of ~3.37 pg/cell that was the combination of zebrafish (1N) and goldfish (1N) genomes. Chicken blood was used as internal references (M1) 2.5. pg of DNA/cell.

(Figure B-2D). The DNA content found in the cross between zebrafish and goldfish was (~ 3.37 pg/cell) different from normal zebrafish (~ 3.15 pg/cell) and normal goldfish (3.55 ± 0.06 pg/cell). This suggested that the embryos were hybrid because the sum of half of the genome size of normal goldfish and zebrafish (~ 3.35 pg/cell) was close to genome size from the offspring of the zebrafish-goldfish cross (~ 3.37 pg/cell).

When using sperm from catfish, *Ictalurus punctatus* and from blue catfish, *Ictalurus furcatus*, eggs were not fertilized.

DISCUSSION

From these experiments it can be concluded that sperm of Nile tilapia can be used to activate eggs of zebrafish because it did appear to trigger the cell cycle and its DNA did not contribute to the genome.

Although the main goal for this objective was to find a sperm source that could function as an activator during our ICSI procedures, these findings could also have other applications. Tilapia sperm in zebrafish eggs could be used for the production of gynogenetic zebrafish or for the production of haploid fish which could be used for genetic studies. It could also be used in basic science for the study of the fertilization mechanisms such as how the sperm-egg binding occurs and which components are involved in the fertilization process. Zebrafish eggs are activated when they contact the spawning media. However, this is not enough to trigger further development of the eggs. This suggests that the sperm content or the sperm binding play an important role in fertilization.

The possibility of fertilizing fish eggs with sperm from closely related species also could be used for the restoration of endangered species by androgenesis (all-

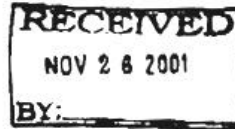
paternal inheritance). Genetically inert eggs (treated either with ultraviolet light or with gamma radiation) could be fertilized with sperm from a related species. A pressure or temperature shock can be applied to block the first cell division resulting in embryos with two identical sets of chromosomes from the father (Myers et al. 1995, Thorgaard et al. 1998).

It is known that activation of the egg during ICSI is necessary for the subsequent steps of development to occur. The use of sperm from another species that can activate the cell cycle of the egg but not contribute genetically to the embryo could improve fertilization when ICSI is performed. Further studies in zebrafish could include the use of Nile tilapia sperm as an egg activator.

REFERENCES

- Corley-Smith, G E. 1996. Production of androgenetic zebrafish (*Danio rerio*). *Genetics* 142:1265-1276.
- Myers, J. M., D. J. Penman, Y. Basavaraju, S. F. Powel, P. Baoprasertkul, K. J. Rana, N. Bromage, and B. J. McAndrew. 1995. Induction of diploid androgenetic and mitotic gynogenetic Nile tilapia (*Oreochromis niloticus* L.). *Theoretical and Applied Genetics* 90:205-210.
- Onozato, H. 1984. Diploidization of gynogenetically activated salmonid eggs using hydrostatic pressure. *Aquaculture* 43:91-97.
- Peruzzi, S., J C. Scott, C. J. Domaniewski, and G. F. Warner. 1993. Initiation of gynogenesis in *Oreochromis niloticus* following heterologous fertilization. *Journal of Fish Biology* 43:585-591.
- Thorgaard, G, P A. Wheeler, and R. D. Fields. 2000. Utilization of androgenesis for strain recovery from cryopreserved sperm. Pages 305-309 in T. R. Tiersch and P. M. Mazik, editor. *Cryopreservation in Aquatic Species*. World Aquaculture Society, Baton Rouge, Louisiana.

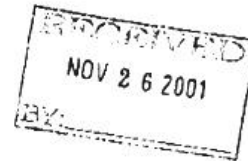
APPENDIX C
LETTER OF PERMISSION



AGRICULTURAL EXPERIMENT STATION
Aquaculture Research Station
2410 Ben Hur Rd.
Baton Rouge, LA 70820
(225)765-2848
Fax: (225)765-2877
Web: www.lsuagcenter.com


November 19, 2001.

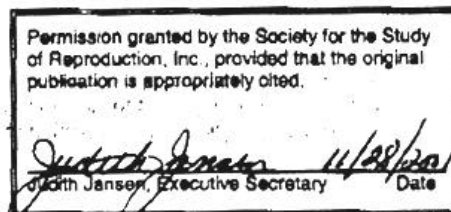
Dr. Virendra B. Mahesh, Editor-in-Chief
Biology of Reproduction
Department of Physiology and Endocrinology
Medical College of Georgia
Augusta, GA 30912



Dear Dr. Mahesh,

I am preparing my dissertation and I would like to request your permission to reproduce material from the manuscript entitled "Fertilization of eggs of zebrafish, *Danio rerio*, by intracytoplasmic sperm injection" where I am a first author, published in the *Biology of Reproduction* 2001; 65:961-966.

Regards,

German A. Poleo



VITA

Germán Antonio Poleo was born on February 23, 1966, in Caracas, Venezuela. He attended Universidad Central de Venezuela where he received in 1992 his bachelor's degree in biology with a major in cell biology. The title of his thesis was "Purificación y Estudio de las Propiedades Fisico-Químicas y Biológicas de la Lectina de *Pterocarpus rohrii*" (Isolation and study of the physical-chemical and biological properties of the Lectin *Pterocarpus rohrii*). In January of 1994 he was hired by Dr. Gustavo Benaim as Research Associate in the Instituto de Biología Experimental of the Universidad Central de Venezuela where he served until December of 1994. In October of 1994 he married Lucia Valentina Giordani. In January of 1995 he was hired as Assistant Professor by the Department of Physiology of the Jose Maria Vargas Medical School in Caracas and served there until August of 1995. In August of 1995 they moved to Ottawa, Canada, where he was enrolled in the graduate program at Ottawa University. He graduated in September of 1998 with a master of science degree in anatomy and neurobiology. The title of his thesis was "*Fibroblast Growth Factor 8 and Cell Proliferation in Zebrafish Fins.*" In 1998 their first child, Emilia, was born. The family moved to Baton Rouge, Louisiana, where Germán enrolled in the Graduate Program of the School of Forestry, Wildlife, and Fisheries at Louisiana State University, where he is currently a candidate for the degree of Doctor of Philosophy in Wildlife and Fisheries Science. Their second child, Andres, was born in Baton Rouge, Louisiana, on June 9, 2001.