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# Fertilization by intracytoplasmic sperm injection in Nile tilapia (*Oreochromis niloticus*) eggs

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**Abstract**

In this report we describe the steps used for the production of Nile tilapia by intracytoplasmic sperm injection (ICSI). To assure a constant and reliable source of eggs for ICSI we used a method where female Nile tilapia, *Oreochromis niloticus*, spawned when held individually in 80-l tanks (a single-breeding system). This allowed collection of eggs every 28 ± 9 days (mean ± S.D.) from 26 spawning events by 7 females that spawned two or more times. It was common to obtain more than 1000 eggs from a single stripping. The second step in this project involved the extension of egg viability to assure sufficient time for the ICSI procedure. This was accomplished by placing the eggs in Hanks' balanced salt solution after stripping, which extended the period of fertility for at least 3 h after collection. The third step was to minimize chromosomal damage after ICSI by localization of the metaphase plate within eggs. The maternal chromosomes were found to be ~43 μm from the micropyle (injection site) at the moment of fertilization. The final step was to apply these methods to evaluate the ICSI procedure for tilapia. From a total of 113 Nile tilapia eggs injected with fresh sperm, 7 (5%) were fertilized, 5 (4%) developed abnormally to neurula and 1 (1%) developed normally and reached adulthood. These results demonstrated for the first time that the ICSI procedures here described allow fertilization and subsequent development of Nile tilapia eggs into normal larvae, hatching and beyond. This provides opportunities for the study of basic processes involved in fertilization and zygotic development and expands the utility and range of sperm storage methods such as cryopreservation or freeze-drying.


*Keywords:* ICSI; Nile tilapia; *Oreochromis niloticus*; Fertilization; Sperm; Eggs; Artificial spawning

## 1. Introduction

Nile tilapia *Oreochromis niloticus* is an important cultured fish in more than 55 tropical and subtropical countries. Among the factors that contribute to the widespread use of tilapias in aquaculture are their resistance to poor water quality (e.g., low oxygen) and

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diseases, fast embryonic development (hatching in 5 days), maturity at an early age (4–6 months), and year-round spawning. These characteristics also facilitate studies of genetic improvement using tools such as hybridization (Lovshin, 1982), sex reversal (Guerrero, 1982), gynogenesis (Don and Avtalion,1988; Varadaraj, 1990; Peruzzi et al., 1993) and polyploidy (Valenti, 1975; Myers, 1986; El Gamal et al., 1999).

In studies of genetic manipulation, it is important to have a constant and reliable source of gametes and this is achieved by understanding and controlling the spawning conditions of the broodstock. In Nile tilapia, although the presence of males influences the spawning cycle of females, the absence of males does not stop it. Nile tilapia females have been shown to 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 a few minutes after being released from the female into water (reviewed by Yamamoto, 1975). Tilapia eggs (*O. niloticus* and *Oreochromis mossambicus*), however, are still viable after 15 min of being submerged in water (Myers and Hershberger, 1991). This hydration tolerance is beneficial when artificial spawning and some treatments such as temperature or pressure shock are applied, but may not be sufficient for other genetic studies. Fertilization has been extended in fish eggs by placing them in isotonic solutions. In medaka *Oryzias latipes*, eggs remained fertile for more than 2 h when kept in Ringers' solution (Yamamoto, 1975). The same has been found for zebrafish, *Danio rerio*, where eggs could be held for 2 h 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).

One technique with potential use for the improvement of aquaculture species is intracytoplasmic sperm injection (ICSI). This technique involves the fertilization of eggs by direct injection of a desired number of sperm into the egg cytoplasm. Although ICSI was first studied in aquatic species such as sea urchin and sea star (Lillie, 1914; Hiramoto, 1962; Dale et al., 1985), it has been applied exclusively for the past 27 years in mammals (Uehara and Yanagimachi, 1976; Hosoi and Iritani, 1993; 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), and has recently been applied for the first time in fish (Poleo et al., 2001). These initial ICSI studies addressed a small aquarium research model, the zebrafish. There are no reports addressing the use of ICSI for a cultured fish species.

At the time of fertilization, eggs of teleost fishes are arrested in metaphase II of meiosis (Stricker, 1999). The maternal chromosomes are aligned at the spindle and a stimulus is required to resume meiosis, which results in the extrusion of the second polar body and the formation of the female pronucleus. The location of the metaphase spindle in Nile tilapia eggs is not known. The position of this structure is important when performing ICSI if mechanical injury by the injecting pipette to the maternal chromosomes is to be avoided.

It has been suggested that resumption of meiosis in Nile tilapia occurs when the sperm comes into contact with the egg (Stefano Peruzzi, personal communication, Haskin Shellfish Research Laboratory, New Jersey). 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 (ionophores) that alter the membrane electrical potential and trigger the calcium wave. In fish, activation could be achieved by the use of a genetically inert sperm such as those 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 sperm from common carp or a related species such as goldfish *Carassius auratus* could also be used when performing ICSI.

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

## 2. Materials and methods

### 2.1. Animals

Twenty-five female Nile tilapias were maintained in 80-l aquaria individually (single-breeding system) (Mires, 1982; Gautier et al., 2000) with a natural photoperiod in recirculating systems at the LSU Agricultural Center, Aquaculture Research Station (ARS). The females were placed into 80-l aquaria at 26 °C. Salinity was maintained at 1.5 mg/l, and alkalinity and hardness at ~200 mg/l. Beginning 2 months after stocking, females were checked each morning for signs of maturation. Those that showed a swollen belly and projecting genital papilla were selected for spawning several hours later. Fish were anesthetized with tricaine methanesulfonate (methyl-*m*-aminobenzoate, MS222) (Argent Laboratories, Redmond, WA) before egg stripping. Their abdomens 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 and not counted as efficient spawning. The date of volitional spawning or stripping for each female was recorded and the spawning cycle (time between each spawn) was calculated for those fish that spawned twice or more.

Sperm were obtained by gently squeezing the sides of unanesthetized males. A 10-µl pipette tip connected to a mouth pipette was used to extract sperm cells which were diluted 1:1 in Hanks' balanced salt solution (280 mOsm/kg, pH 7.0) in 1.5-ml micro-centrifuge tubes and placed on ice. Sperm motility was estimated at 200-X by use of dark-field microscopy (Nikon Inc., Tokyo, Japan). The addition of 20 µl of distilled water was used to activate a 2-µl sperm suspension (Tiersch et al., 1994). Only sperm with high motility (>90%) were used for experiments.

### 2.2. Egg storage before fertilization

All eggs were collected and stored in HBSS at 25 °C until fertilization. Three fertilization trials were performed from 0 to 80 min at 10-min intervals and another trial at 0 and 3 h. At fertilization, most of the HBSS was decanted and sperm were 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 $Na_2HPO_4$, 0.44 mM $KH_2PO_4$, 1.30 mM $CaCl_2$, 1.00 mM $MgSO_4$, 4.20 mM $NaHCO_3$) (Westerfield, 1995) at 52 mOsm/kg and pH 7.0 was added for activation. After 10 min, 100 ml of EMB were added to the eggs and they were left undisturbed without movement in a convection incubator at 28 °C (Precision Scientific Group, Chicago, IL). Unfertilized eggs were removed and EMB was changed twice daily. After 48 h, the eggs were evaluated for percent fertilization. Those that developed to stage 11 (embryonic keel and somite formation) (Galman and Avtalion, 1989) were recorded as fertilized.

### 2.3. Staining of DNA

Plastic baskets were constructed to hold the eggs. The bottoms of 10-mm plastic cryogenic goblets (Southland Cryogenics, Carrollton, TX) were removed yielding a tube of about 1.5 cm in length. A hot plate (Thermolyne, Nuova II, Model SP18425, Dubuque, IO) 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-µm 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 following the procedures of Hart et al., (1992), with some modifications as described below. Eggs were placed in 4% paraformaldehyde (PFA) for 1 h, 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 fixed eggs were removed from the baskets and dissected. Using a 26-gauge needle, the animal pole was separated from the vegetal pole 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. Animal poles were incubated in 5 μg/ml of Hoechst 33342 (Sigma Chemical Corporation, St. Louis, MO) for 5 min and the samples were washed three times with PBS for 5 min to remove excess dye. Chambers for the observation of the stained animal pole were made with a glass microscope 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, PA) 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, WA).

### 2.4. Sperm injection

Injections were performed at the LSU Embryo Biotechnology Laboratory (16 km from the ARS) using a micromanipulator apparatus consisting of an inverted microscope (Diaphot Nikon Inc., Tokyo, Japan) equipped with two mechanical micromanipulator units (Leitz, Rockliegh, IL) that moved either the holding or injection pipettes. 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, CA) and prepared following standard procedures (Payne, 1995). Due to the variability in size among eggs of different females, 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, NY).

All the sperm injected in Nile tilapia eggs were placed in embryo medium before the injections, activating them and assuring loss of motility. The immobilized sperm cells dropped to the bottom of the dish. A selected sperm was then aspirated into the injection pipette and positioned close to the pipette opening. When eggs were correctly positioned with the animal pole facing outward (Fig. 1), 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.

### 2.5. Egg activation

Egg activation was evaluated using four 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.

### 2.6. Sperm injection after insemination with goldfish sperm

Sperm were collected from goldfish following the procedures described above for tilapia. A single Nile tilapia egg was placed in a small droplet (~200 μl) of

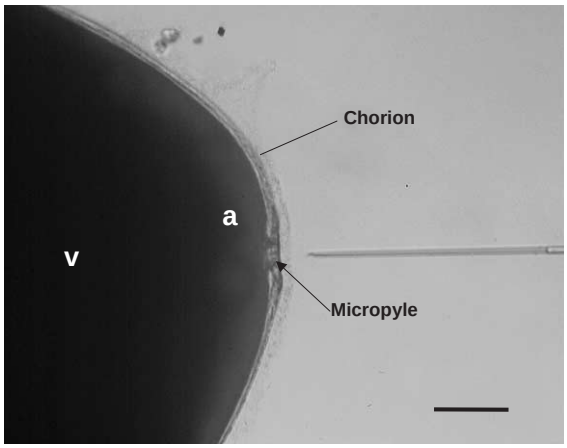


Fig. 1. A Nile tilapia egg shown in position for sperm injection. The tip of the injection pipette is pointing to the micropyle of the egg (scale bar is ~100 μm). The vegetal pole (v) represents more than 70% of the egg volume. The animal pole (a) is present at the narrow end of the egg.

EMB, incubated with goldfish sperm and injected with Nile tilapia sperm. The injections were performed using the methods described above. Eggs were individually held at 28 °C in 35-mm petri dishes (Corning Glass Works) after injection and fertilization was evaluated after 48 h. 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 each batch was artificially fertilized with fresh Nile tilapia sperm. Only eggs that came from batch with a fertilization percentage higher than 70% were included in the analysis.

### 2.7. DNA content analysis

Analysis of DNA content was performed using a FACSCalibur® flow cytometer (Becton Dickinson, San Jose, CA) 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). Samples of blood from channel catfish *Ictalurus punctatus* (1.98 pg of DNA/cell) (Tiersch et al., 1990) and zebrafish (3.15 pg of DNA/cell) (Poleo et al., 2001) were used as internal reference. Measurements of the DNA content of normal diploid Nile tilapia ($2.10 \pm 0.09$ pg of DNA/cell, $n=6$) were compared with those of larvae produced by ICSI.

### 2.8. Statistical analysis

Statistical analysis was performed using SAS software for Windows® version 8.1 (SAS Institute, Cary, NC). Fertilization and activation data were analysed and compared using one-way factorial analysis (ANOVA). Differences were determined by the Tukey's test. Percentages were arcsine-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.

## 3. Results

### 3.1. Single-breeding system

During the study period (February–November 2001), female Nile tilapia began to spawn regularly after being in the recirculating system for 2 months. All spawns were collected in the afternoon between 1300 h and 1800 h. 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 $28 \pm 9$ (mean ± S.D.) days with a minimum of 10 days and a maximum of 39 days. It was common to obtain more than 1000 eggs from stripping of individual females. This method enhanced monitoring and control of the broodstock.

### 3.2. Egg storage before fertilization

There was no significant difference among batches of eggs fertilized at 10-min intervals for 80 min. During this period, there was an average fertilization of $88 \pm 4\%$ (mean ± S.D.). Although variation increased, similar results were observed for comparison of fertilization at 5 min ($97 \pm 1\%$) and 3 h ($85 \pm 15\%$). Although it was not statistically tested, eggs could be fertilized after 5 h (77% fertilization).

### 3.3. Staining of DNA

At 30 s after insemination, the sperm head was localized at the sperm entry site (Fig. 2A) and it showed an average width of $1.8 \pm 0.1$ μm (stained fresh sperm were $2.2 \pm 0.2$ μm). The maternal chromosomes showed an average size of $2.3 \pm 0.1$ μm and were situated $40 \pm 5.5$ μm from the sperm head (Fig. 2B). At 10 min after insemination, the resumption of meiosis was observed by the splitting of the maternal chromosomes (Fig. 2C) and at 20 min

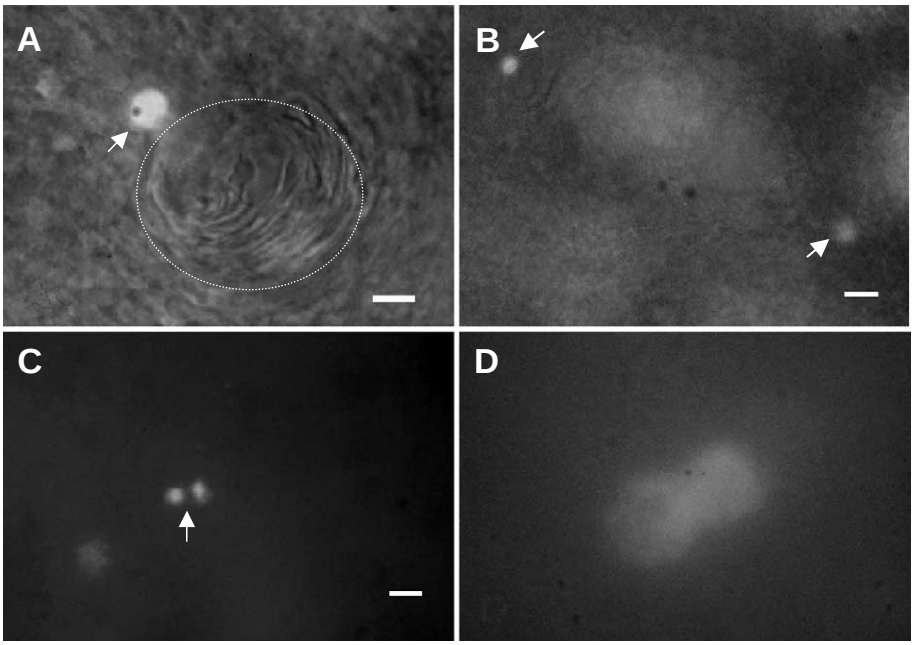


Fig. 2. Staining by Hoechst 33342 to reveal the location of maternal and paternal DNA. (A) 30 s after insemination: the sperm head (arrow) was present at the micropyle canal (circle) (scale bar, 2 μm). (B) 30 s after insemination (lower magnification than in (A)): the distance between the sperm head (left arrow) and the maternal chromosomes (right arrow) was ~40 μm (scale bar 3.6 μm). (C) 10 min after insemination: the maternal DNA (arrow) has begun to split (scale bar, 3.6 μm). (D) 25 min after insemination: male and female pronuclei were in contact.

after fertilization, the decondensed female and male pronuclei were visible (Fig. 2D).

### 3.4. Activation

There was significant difference ($P<0.001$) in egg activation (i.e., water hardening, formation of blastodisc) (Fig. 3) among the eggs that were injected with or without sperm and the eggs that were incubated in EMB alone or inseminated with goldfish sperm (Fig. 4). No significant difference in activation was found between eggs injected with sperm and eggs injected with buffer only (Fig. 4). No significant difference was found between eggs

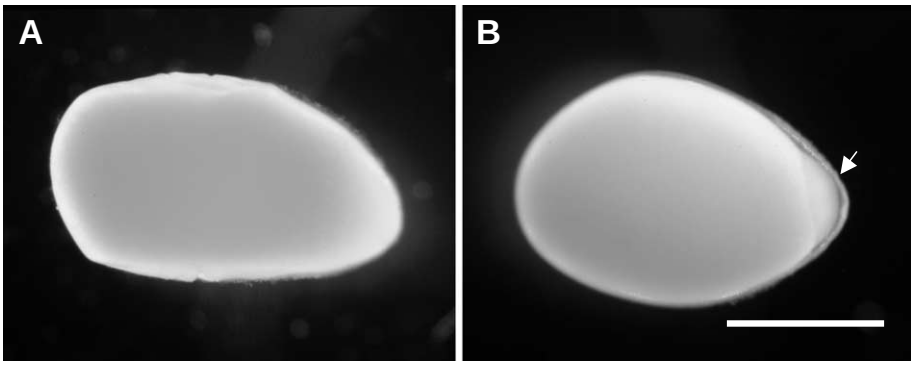


Fig. 3. Activation of Nile tilapia eggs. (A) 30 min after placement in embryo medium buffer (EMB): there were no signs of activation (water hardening and blastodic formation). (B) 30 min after insertion of the injecting pipette: the chorion was raised and the egg membrane was more rigid. The blastodisc was developed at the animal pole (arrow) (scale bar, 750 μm).

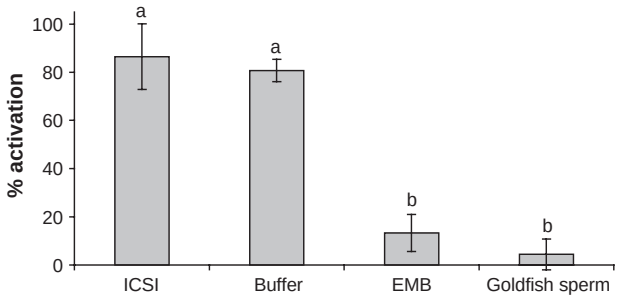


Fig. 4. Activation of Nile tilapia eggs. Eggs were injected with sperm (ICSI), injected only with injecting buffer (Buffer), placed in embryo medium buffer without sperm (EMB), or inseminated with sperm of goldfish, C. auratus. Shared letters above error bars (±S.D.) represent no significant difference.

incubated in EMB and eggs inseminated with goldfish sperm (Fig. 4).

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 (a sufficient amount of time to observe meiotic resumption in artificially fertilized eggs) after incubation with goldfish sperm. The same results were obtained when the eggs where incubated with EMB alone. Four of five eggs stained with Hoechst 33342 after being injected with buffer showed splitting of the maternal chromosomes, suggesting that the injections triggered activation.

### *3.5. Sperm injection*

There was no significant difference between ICSI performed in eggs previously fertilized with goldfish sperm and the eggs injected with Nile tilapia sperm (Table 1). Overall, of 113 eggs injected with sperm, 6 (5%) developed to Stage 11 (Galman and Avtalion, 1989) (Fig. 5). Of these, five were abnormal and one, named "Cristobal", developed and hatched (Fig. 6) and at the time of final writing this report (April, 2005) was still alive at the Aquaculture Research Station and is to our knowledge the first Nile tilapia produced by ICSI. The phenotypic sex of the fish is male, based on external urogenital characteristics. Eggs injected with buffer only (control injections) did not develop beyond blastodisc (Table 1). Flow-cytometric analysis of two abnormal embryos and a blood sample from the normal fish revealed that they were diploid.

## 4. Discussion

The single-breeding system of spawning individual female Nile tilapia was first published 20 years ago (Mires, 1982). The single-breeding system provides easy access to broodstock and allows for rapid and effective collection of ripe eggs. It also reduces the number of males needed, or eliminates them if sperm cryopreservation is employed, and provides greater control over the desired offspring by facilitating

Table 1
Percent fertilization and development in Nile tilapia eggs after intracytoplasmic sperm injection (ICSI), ICSI in eggs previously incubated with goldfish sperm, and control injections which received no sperm

| Treatment | Number of injections | Fertilized | Percent fertilization | Advanced neurula[a] | | Juvenile[b] |
|---|---|---|---|---|---|---|
| | | | | Abnormal | Normal | |
| ICSI | 59 | 2 | 3 | 1 | 1 | 1 |
| ICSI (goldfish sperm) | 54 | 4 | 7 | 4 | 0 | 0 |
| Control | 55 | 0 | 0 | 0 | 0 | 0 |

[a] Embryos that showed a complete neural tube, somites and optic vesicle; Stage 11 (Galman and Avtalion, 1989).
[b] Fish that developed beyond 2 months after fertilization.

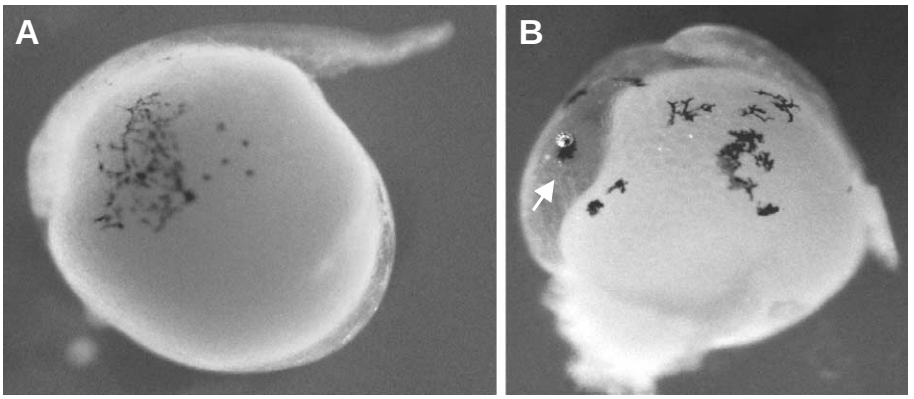


Fig. 5. (A) Normal Nile tilapia embryo produced by ICSI, 52 h after injection. (B) Abnormal Nile tilapia produced by ICSI, 52 h after injection in which the pigment cells appeared larger than normal. A large space between the yolk and the epithelium was also evident (arrow). This embryo did not survive beyond 72 h.

selective breeding and other genetic tools such as hybridization, polyploidy and sex reversal. Spawning could be maximized through the year because conditions such as photoperiod, temperature and water quality can be manipulated. The single-breeding system yielded a reliable supply of eggs in this study and, together with the use of an isotonic solution that extended the viability of the eggs up to 3 h, facilitated their use in ICSI. As observed in other studies (Jalabert and Zohar, 1982; Mires, 1982) the female tilapia did not require interaction with males to spawn. The spawning cycle reported here ($28 \pm 9$ days) agrees with a study in France (Gautier et al., 2000) where single females spawned every $31 \pm 6$ days and, as in this study and others (Myers and Hershberger, 1991; Baroiller et al., 1997), all fish spawned during the afternoon. A similar spawning cycle was observed (23 and 34 days) in *O. niloticus* females reared with males that were strip-spawned (Myers and Hershberger, 1991); however, females reared with males showed a shorter cycle (15 days) when deprived of their eggs the day following spawning (Baroiller et al., 1997).

Sperm injections have been demonstrated to cause physical damage to oocyte 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 (2% fertilization rate) (Poleo et al., 2001), intracytoplasmic sperm injection in Nile tilapia yielded a low fertilization rate (5%, this is the combination of data for eggs activated with goldfish sperm and normal injections in Table 1) and a high frequency of abnormal embryos that may have been the result of chromosomal damage. We found that the distance between the tilapia egg chromosomes and the sperm head at 30 s after insemination was about 40 μm. This value defined an approximate radius between the maternal DNA and the micropyle (natural sperm entry site), where injections could be 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 (Poleo et al., 2001), it is possible that the injection pipette could have damaged structures involved in sperm or pronuclear movement inside the egg. In mammals and echinoderms, it is known that the egg cytoskeleton plays important roles in sperm binding, sperm movement and embryonic development. Disruption of cytoskeletal structures involved in fertilization could interfere with developmental processes. More basic studies are needed to clarify where disruption, if any, took place. Future research focusing on failures after ICSI in fish will need to address other possible damage (such as membrane disruption), 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 induce activation as confirmed by water hardening and the subsequent formation of the blastodisc. However, the injection of a sperm was necessary for further development.

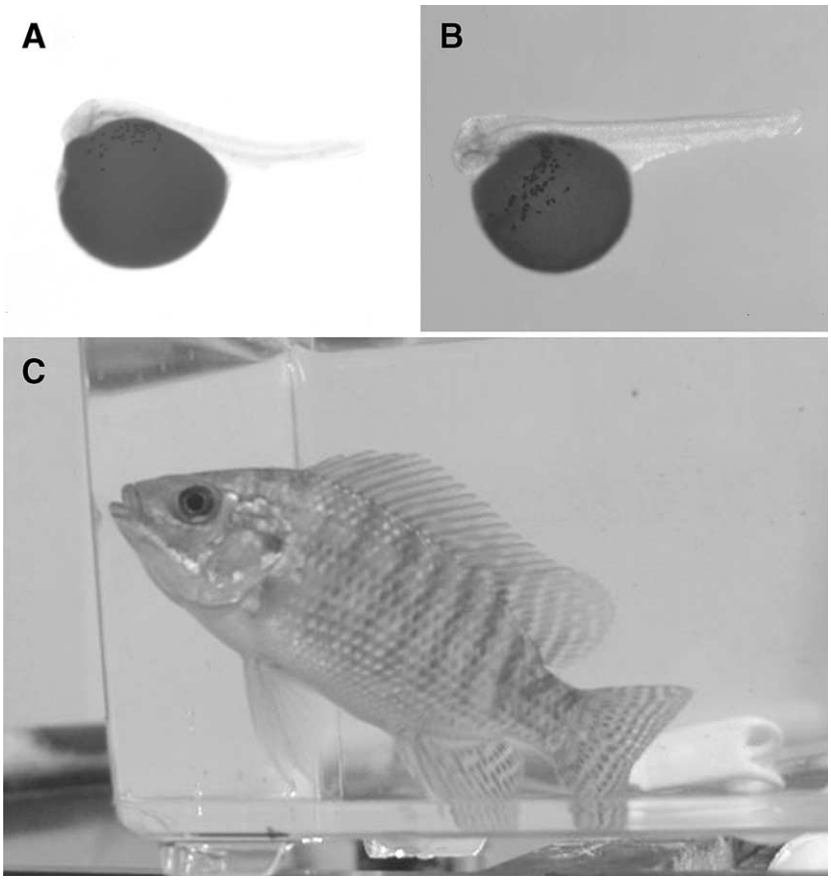


Fig. 6. ICSI in Nile tilapia. (A) Control, 3 days after artificial fertilization with fresh Nile tilapia sperm. (B) 3 days after sperm injection. (C) The same fish (in B) 3 months after hatching. It showed the male phenotype and was named "Cristobal".

Similar behavior can be observed in eggs of the African clawed frog *Xenopus laevis* (Wolf, 1974; Kroll and Amaya, 1996) and in hamster eggs (Uehara and Yanagimachi, 1976, 1977) where pricking activates the eggs but the injection of a sperm is needed for further development. This suggests that the sperm content is needed for cell division. In contrast, it has been shown in humans (Tesarik and Sousa, 1994; Dozortsev et al., 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 mechanisms of oocyte activation after ICSI in mammals are controversial, there is evidence to support that a factor or factors released from the sperm into the oocyte after injection causes oocyte activation (Swann, 1990; Dozortsev et al., 1995; Kuo et al., 2000).

In fish, egg activation seems to be species-specific. In zebrafish, 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; Wolenski and Hart, 1987; Hart and Fluck, 1995). Although this stimulus is sufficient to trigger the calcium wave (Lee et al., 1999) and resumption of meiosis, it is not sufficient to produce a parthenogenetic (haploid) embryo. However, injection of a single sperm cell into the cytoplasm of activated zebrafish eggs yielded normal fish (Poleo et al., 2001). The results shown in the present study suggest that activation in Nile tilapia eggs is also a process where at least two major steps may be involved. Piercing of the egg with a glass pipette induces water hardening and resumption of meiosis, but cell division does not occur. The contribution of factors contained in the

sperm thus 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 is produced with irradiated sperm (Corley-Smith et al., 1996) or sperm from other species yielding the production of haploid embryos (Peruzzi et al., 1993).

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 (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) indicates that the normal fish obtained after ICSI are not of parthenogenetic origin. However, it might be that the sperm injection prevents the extrusion of the second polar body resulting in a meiotic gynogenetic fish (a meiogyne). 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 as Nile tilapia possess a male heterogametic mechanism (XX–XY) of sex determination (Penman and McAndrew, 2000). Although two minor sex-influencing genes appear to alter this determination system in some lines of Nile tilapia when both are in the homozygous state for specific alleles, Müller-Belecke and Hörstgen-Schwark (1995) found that meiogynes in this species were all-female even though the maternal fish in question produced a low percentage (<10%) of male mitotic gynogenetic offspring. Further verification could be gathered by comparing specific genetic markers in the genomes of parents and the offspring by techniques such as DNA fingerprinting. Various techniques have been applied in fishes to evaluate the production of homozygosity by gynogenesis. For example, multilocus DNA fingerprinting has been used to reveal genetic relationships between strains of Nile tilapia (Harris et al., 1991; Naish et al., 1995) and the parental contribution of gynogenesis in tilapias (Carter et al., 1991; Jenneckens et al., 1999).

Nile tilapia proved to be a good model for the application of ICSI. The spawning characteristics of these fish permitted a reliable supply of eggs. The size and morphology of the eggs facilitated the injection procedure, and the easy manipulation of embryos and sacfry facilitated survival after ICSI. The limited but positive results shown here demonstrate that ICSI could be used as a genetic tool for Nile tilapia and perhaps for other aquatic species as well.

The results obtained in this work provide the first step towards the development of further uses of ICSI in fish including the production of hybrids that otherwise would not be possible: the study of the fertilization process, the preservation of endangered species, and the production of polyploidy. Sperm injection could also be used for the production of adrogenesis, 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. Injection of two sperms into an irradiated egg 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. The procedures described in this work 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.

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. However, the efficacy in production of transgenic animals is ambiguous. An 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.

Sperm preservation will become important for genetic improvement in aquaculture, although the current practice of cryopreservation can be costly for poor countries or small private companies. Intracytoplasmic sperm injection could expand opportunities for utilization of preservation techniques where sperm lose motility such as freeze drying. Freeze-dried sperm injected in oocytes of rodents have produced normal offspring (Wakayama and Yanagimachi, 1998; Kusakabe et al., 2001; Kaneko et al., 2003; Ward et al., 2003; Liu et al., 2004). Others reported that injection of mice sperm chemically dehydrated in 100% ethanol, 100% methanol, chloroform–methanol and Carnoy's fluid were capable of developing into male pronuclei (Tateno et al., 1998; Katayose et al., 1992). Preliminary studies in our laboratory have shown that injected immotile Nile tilapia sperm that had been cryopreserved trigger cell division (Poleo et al., in press). These works suggest that a new technology that would reduce maintenance and the cost of genetics repositories in the future could be developed for animals including fish.

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