ABSTRACT

MORGAN, ANDREW JACKSON. Genetic and Temperature Manipulation of Southern Flounder (*Paralichthys lethostigma*) for the Production of Monosex Populations. (Under the direction of Harry V. Daniels.)

Experiments were conducted to establish methods to produce an all female population of southern flounder (*Paralichthys lethostigma*) gynogens. The first series of experiments was to optimize the protocol for using UV irradiated black sea bass (*Centropristis striata*) sperm for the induction of meiogynogenesis. Milt was collected from multiple black sea bass, pooled, and diluted 1:10 (v:v) using Ringer's solution. The diluted milt was irradiated using an UV Crosslinker with doses ranging from 0-130 J/cm^2. The optimal UV dose of 70 J/cm^2 was determined by observing a 10 % motility, motility duration of approximately 1 minute, and giving the highest percent hatch (7.73$\pm$1.57 %) which was statistically different (P=0.05) than the control. Pressure shock was used to retain the 2nd polar body in southern flounder eggs. Application of 8500 psi for 6 minutes was initiated at varying times 1, 2, and 3 minutes post fertilization with no pressure acting as the negative control. Based upon percentage hatch and survival, the optimal time to apply pressure was either 1 or 2 minutes post fertilization, which was temperature dependent (1 minute at 18^{o}C and 2 minutes at 16^{o}C.) The overall average hatch varied with initiation times and ranged from 1.48 $\pm$ 0.52% (1 min) to 0.61 $\pm$ 0.11% (3 min) of the fertilized eggs (average 70.9 $\pm$ 12.8% fertility.) The development of these techniques can allow aquaculturists to take advantage of the better growth rates of female southern flounder by producing all female offspring.

Three preliminary experiments were also conducted to establish methods to produce cloned, monosex populations of southern flounder through mitotic gynogenesis. The first experiment determined the timing of the first mitotic division of the southern flounder egg. Eggs were fertilized with UV irradiated (70 J/cm^2) black sea bass sperm and allowed to normally divide between 17-20^oC. There was no correlation between spawning temperature, the timing of the first mitotic division, or the rate of mitosis. The timing of first cleavage varied from 63-88 minutes post fertilization. Once eggs began the first cellular division, 90 % of the eggs had completed this stage within 40 minutes. Using these data, southern flounder eggs were fertilized with UV irradiated (70 J/cm^2) black sea bass sperm to create mitogynogens. Percent fertilization was 44.7% and percent hatch was 0%, although some eggs advanced to the blastula stage. Triploidy hatch percentages were 18.0-42.7 % of the normal diploid control percent hatch.

A third series of experiments were designed to test for sex differences among populations from North Carolina and Texas southern flounder raised under the same conditions and shifted to higher temperatures. At the end of the study, there was no significant difference in lengths or weights between the treatments. The flounder that remained in 23^oC indicated a trend for larger growth for the Texas flounder while the 28^oC tended to grow larger for the North Carolina flounder (not significant.) Final sex ratios were 100% male for all treatments. An additional experiment was conducted to determine the window of temperature dependent sex determination in North Carolina southern flounder. A sample was shifted to 28^oC at 60 mm and 90 mm in duplicate to determine when sex differentiation occurred. The North Carolina southern flounder in

this study grew better in 23^{o}C until 60 mm and then their growth rate increased in 28^{o}C until approximately 125 mm.

Genetic and Temperature Manipulation of Southern Flounder (*Paralichthys lethostigma*) **for the Production of Monosex Populations**

by
ANDREW J. MORGAN

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APPROVED BY:

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Russell Borski John Godwin

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Harry V. Daniels

Chair of Advisory Committee

Biography

I was born 30 November 1972 and grew up on a farm near Caldwell, WV. I graduated from Greenbrier East High School in 1991 and immediately went to college at Marshall University. Upon graduating from Marshall, I married GeorgeAnne and was commissioned into the Chemical Corps of the US Army. Since then and now, we have been blessed by two daughters, Shawna and Kaitlin.

The Army is not about staying in one place for very long, so in the past 10 years we have lived near Ft. McClellan, Alabama, Ft. Hood, Texas, Ft. Leonard Wood, Missouri, Ft. Bragg, North Carolina, and currently in Cary, North Carolina.

After commanding the 21st Chemical Company (Airborne), 82nd Airborne Division, I was selected to become a professor at West Point. The US Army sent me to obtain a master's degree at North Carolina State University. After graduation I will move my family to the United States Military Academy located in West Point, New York where I will be a Chemistry professor for the next three years.

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First and foremost I would like to thank my wife, GeorgeAnne, and daughters, Shawna and Kaitlin, for being so patient and supportive while I stumbled through graduate school.

Thank you to my advisor, Dr. Harry Daniels, and to my committee, Dr. Russell Borski and Dr. John Godwin, for all the assistance and incredible guidance that you gave me to get through my research, writing my thesis, and graduate school.

My research would not have been possible without the invaluable help from friends and colleagues alike. Thank you to Laura, Marc, and Adam for everything.

Many trips to Wilmington, NC showed me that there is a lot of great research going on at the University of North Carolina-Wilmington Center for Marine Science. Thank you to Dr. Wade Watanabe for all the help and guidance you provided on many of my trips. I would also like to thank Chris and Kim for all the hours and eggs we went through during my research.

Last, but definitely not least, thank you to Ryan for many hours of assistance, guidance, listening, and setting me on the right path. Mostly thank you for friendship. It has been rumored that you are the best flounder man and I now know it not a rumor but a fact.

I would also like to thank the US Army, West Point, and the Chemical Corps for allowing me to attend and funding my time at North Carolina State University.

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CHAPTER 1 ABSTRACT

Optimization of UV dose and pressure shock for induction of meiogynogenesis in southern flounder (*Paralichthys lethostigma*) using black sea bass (*Centropristis striata*) sperm

Three experiments were conducted to establish methods to produce an all female population of southern flounder gynogens. Because female flounder grow 2-3 times larger than males, practical protocols to produce all female fingerlings are required. Through the course of these experiments, 40 spawns from 32 different broodstock were used.

The first experiment optimized the UV dose for black sea bass (*Centropristis striata*) sperm. Milt was collected from multiple black sea bass, pooled, and diluted 1:10 using Ringer's solution. The diluted milt was irradiated using an UV Crosslinker with doses ranging from 0-130 J/cm^2. Two criteria were established to determine the UV irradiation effects on the spermatozoa; the total number of sperm that were activated with full strength sea water and the swimming activity duration. The motility and duration of activity gradually decreased with an increase in UV dose. At UV doses above 90 J/cm^2, the spermatozoa lost all motility. The optimal UV dose was 70 J/cm^2 by observing a 10% motility rate along with motility duration of approximately 1 minute.

Pressure shock was used to retain the 2nd polar body in southern flounder eggs. Heterologous, UV irradiated milt was used to act as a double control (no pressure and no hybridization.) Application of 8500 psi for 6 minutes was initiated at varying times 1, 2, and 3 minutes post fertilization with no pressure acting as the negative control. Based upon percent hatch and survival, the optimal time to apply pressure was either 1 or 2 minutes post fertilization. Application of pressure shock timing is dependent on water

temperature. Percent hatch was higher at 18^{o}C when pressure shock was initiated at 1 minute post fertilization and higher at 2 minutes post fertilization for 16^{o}C.

Six trials produced viable offspring that survived through metamorphosis. The overall average hatch varied with initiation times and ranged from 1.48 $\pm$ 0.52% (1 min) to 0.61 $\pm$ 0.11% (3 min) of the fertilized eggs (average 70.9 $\pm$ 12.8% fertility). Because of the use of heterologous sperm, all spawns had a high percentage of haploid syndrome (kyphosis and low survival to first feeding.)

While both percent hatch and survival are far lower than natural spawns, these results indicate that UV irradiated sperm from black sea bass and pressure shock is a practical method to produce meiogynogenetic offspring. These techniques can allow aquaculturists to take advantage of the better growth rates of female southern flounder.

CHAPTER 1

1. Introduction

Southern flounder (*Paralichthys lethostigma*) inhabit the East Coast (south of North Carolina) and Gulf Coast (from Florida to northern Mexico) regions with a break along the southern portion of Florida. Because of minimum size restrictions on commercial harvest of southern flounder, it is becoming apparent that the majority of harvested flounder are female and usually caught in the first three years of their life (Grist 2004). Male southern flounder rarely attain the 14-inch size restriction (Grist 2004). The aquaculture industry is intensively investigating methods for the commercial production of southern flounder. Southern flounder exhibit the sexually dimorphic growth patterns typical of the genus *Paralichthys* (Yamamoto 1999), where females attain 200-300% larger size than males grown under similar conditions. The sexually dimorphic growth appears at the onset of sexual maturity and prior to the completion of the growout phase. Optimal grow out for southern flounder would eliminate the slower-growing males and focus on all-female fingerlings to reach the greatest economic benefit.

One means of creating 100% female populations in teleost fishes that exhibit sexually dimorphic growth is through gynogenesis. Gynogenesis is the process of producing offspring using only maternal inheritance of chromosomes. This process does not require hormone treatments and is the most environmentally benign procedure for all-female production. Gynogenesis has evolved naturally in the Amazon molly, *Poecilia formosa.* The Amazon molly uses a form of gynogenesis by using the spermatozoa of a different species to initiate fertilization of eggs, but naturally retains the second polar body. The

result is a diploid embryo with both sets of chromosomes acquired from the maternal egg (Rasch et al. 1982).

Gynogenesis has been investigated in many species (reviewed by Pandian and Koteeswaran 1998). One proven technique to produce a monosex population is by meiogynogenesis. There are several techniques to inducing meiogynogenesis, all of which involve disrupting the second meiotic spindle during the completion of meiosis. Most fishes possess a natural diploid egg that expels a polar body containing a haploid set of maternal chromosomes upon fertilization (physical activation event) and signifies the completion of meiosis. Meiogynogenesis occurs when an external block prevents the expulsion of the second polar body and results in a diploid (2n) set of maternal chromosomes. There are many techniques for blocking the expulsion of the second polar body. These include temperature shock (Uneo and Arimoto 1982, Kavumpurath and Pandian 1992**,** Koteeswaran et al. 1995, Luckenbach et al. 2004), electricity (Teskeredzic et al. 1993), cytochalasin B (Reftsie et al. 1977), Colchicine baths (Wu and Chen 1986), and anesthetic treatments (Johnstone et al. 1989.) The most effective, simplest, and most reproducible method of induction of meiogynogenesis is the use of hydrostatic pressure (Tabata et al. 1991, Malison et al. 1993, Kavumpurath and Pandian 1994, Yamamoto 1999).

The use of hydrostatic pressure for induction of gynogenesis involves the use of genetically inert spermatozoa, which have had the DNA destroyed through UV exposure, to activate the eggs which then undergo a doubling of the number of maternal chromosomes by retention of the second polar body. UV- irradiated heterologous sperm (from a different species) is commonly used to activate the eggs and act as a double

control (Chourrout 1984, Ihssen et al. 1990, Felip et al. 2001, Arai 2001, Felip et al. 2001, Luckenbach et al. 2004). The use of heterologous sperm ensures that any surviving offspring are gynogenetically produced and not the result of normal diploid fertilization.

The application of high temperatures at a critical development stage of Paralichthids causes XX females to become phenotypic males (Yamamoto 1999, Godwin et al. 2003, Luckenbach et al. 2003.) To obtain a breeding population of phenotypic males the genotypic female offspring could then be sex reversed using proven methods of temperature manipulation (Tabata 1991, Yamamoto 1999, Luckenbach et al. 2003) into phenotypic males and used as brood stock to produce 100% female offspring (Yamamoto 1999.)

Gynogenesis is required to produce XX males, as southern flounder do not have any visually distinguishable sex characteristics. All male offspring produced using gynogenesis and heterologous sperm are presumed to be homogametic with 100% maternal chromosomal inheritance (Yamamoto 1999, Arai 2001.) This procedure would be verified by progeny testing on the second generation and should produce nearly 100% female offspring in a mating cross between a sex-reversed gynogenetic male and a natural female.

The purpose of this study was to develop an optimum protocol using pressure shock to induce meiogynogenesis in the southern flounder. Black sea bass (*Centropristis striata)* sperm was used as a heterologous source to determine if other marine species could produce gynogenetic offspring (Luckenbach et al. 2004.) Black sea bass were chosen based on the availability of captive, spermiating males that perform well in captivity (Copeland et al. 2003.) Captive black sea bass produce copious amounts of sperm that

successfully activated flounder eggs but would not produce viable offspring. In addition, any animals produced that do not successfully retain the polar body will be unviable and die before or shortly after hatching (Arai 2001).

2. Materials and Methods.

A series of experiments was conducted on 40 spawns from 32 female broodstock to determine the optimum ultraviolet (UV) radiation dose and timing of pressure initiation post fertilization. These experiments were conducted from October 2003-March 2004 and Oct 2004 – January 2005 at North Carolina State University (NCSU) (Raleigh, NC, USA) and the University of North Carolina-Wilmington Center for Marine Science (UNCW-CMS) (Wilmington, NC, USA).

2.1 Systems and Animals:

Systems. All systems for broodstock maturation, larval rearing, and growout at NCSU and UNCW-CMS were closed recirculating systems consisting of a temperature-controlled room under fluorescent light. The recirculating systems consist of various sized insulated fiberglass and polyethylene tanks, bubble bead biofilters, foam fractionators, heat pumps, water pumps, high rate sand filters, fluidized bed biofilters, and UV sterilizers (Daniels and Watanabe 2002, Copeland et al. 2003). All systems at UNCW-CMS are recirculating systems in insulated fiberglass tanks held outdoor under manipulated photoperiod.

Animals. Wild caught female flounder broodstock were purchased from Coastal USA Fish Company (Cedar Island, North Carolina, USA) and transported to NCSU or UNCW-

CMS and maintained in captivity for 1-6 years. The off-season spawning broodstock were under an artificial photoperiod with an aim of spawning October to December and were maintained in this photoperiod cycle for two years. Fish were photothermally cycled from a maximum of L:D 14:10 to 10:14 at either 16^{o}C or 18^{o}C and maintained at this photoperiod for the 3 month period (Watanabe et al. 2001, Daniels and Watanabe 2002.) Broodstock were fed with frozen lake smelt (*Osmerus mordax*), squid (*Illex* spp.), shrimp (*Panaeus* spp.), and a pelleted feed (13 mm floating, Melick Aquafeed, Catawissa, PA, USA). The remaining broodstock at NCSU and UNCW-CMS were held under ambient light conditions (Watanabe et al. 2001.)

Male black sea bass were trapped off Carolina Beach, NC, transported to UNCW-CMS, and maintained in captivity 1-4 years (Copeland et al. 2002) in recirculating seawater systems (Watanabe et al. 2003). A small subset of males was transported to NCSU. Broodstock were fed with frozen lake smelt (*Osmerus mordax*), squid (*Illex* spp.), and shrimp (*Panaeus* spp.)

2.2 General Methods

Animal Handling. All animals were removed from tanks by hand net and anesthetized in 40 L containers (MS-222) under aeration before handling.

Milt Collection. The milt was hand stripped using gentle abdominal pressure and collected with glass Pasteur pipettes while avoiding any contact with water. The milt was transferred to a 5 ml test tube kept on ice. The milt from multiple males was collected and pooled to increase the volume and reduce the effects of individual variation.

Egg Collection and handling. Female flounder were placed on a light table (Daniels and Watanabe 2002) to determine ovulation stage and readiness for stripping. Eggs were

stripped into a 1 L beaker using gentle abdominal pressure and the eggs maintained at 16 or 18^{o}C (broodstock temperatures.) Once fertilized, the eggs were then transferred to a separatory funnel to determine floating egg and sinking egg ratios for total egg fecundity using a graduated cylinder. Once removed from the pressure chamber, the eggs were placed in a plastic bag with 10 L of filtered water from the broodstock tank. The bag was filled with oxygen, sealed, and floated in the 70 L hatching tank for acclimatization. The eggs were released into the tank once the temperature was equal to the tank temperature.

Percent fertilization and total number of eggs were determined approximately 4 hours post fertilization at the 16-32-cell stage using a dissecting microscope (Fisher Scientific FW00-20-1589) at 3X magnification. A 200 ml sample was taken from the 70 L hatching tank to determine number of eggs. Percent fertilization was determined on a 50-100-egg sample under a dissecting microscope. Fertilization was recorded as a percent of total eggs and percent of normally dividing eggs.

Percent hatch was determined 2-4 days post fertilization on a 1 L sample taken from the hatching tanks by visually counting the number of larvae. The numbers were recorded as total hatched larvae and larvae that appeared to be developing normally (without haploid syndrome). Larvae were reared according to Daniels and Watanabe (2002) using live food (enriched rotifers and *Artemia*) and then weaned to dry, manufactured food beginning 14 dph.

2.3 Effects of UV radiation on Black Sea Bass sperm motility.

Three separate trials were conducted in either triplicate or quadruplicate. An undiluted sample of sperm was placed on a slide and viewed under a compound light microscope (Olympus BH-2) at 400X to ensure that the spermatozoa had not been

activated and were still viable. The sample was then activated with seawater at room temperature (19^{o}C). Activated spermatozoa were videotaped using a CCD video camera (Sony XC-77), videocassette recorder (Panasonic Omnivision VHS model PV-7450), and monitor (Panasonic CT-1383) (Luckenbach et al. 2004).

Half of the remaining milt was diluted 1:10 using Ringer's Solution (0.75% NaCl, 0.04% CaCl, and 0.02% KCl) in double distilled water kept on ice. A 3uL sample of milt and Ringer's Solution dilutant was placed on a slide and irradiated with a UV crosslinker (FisherBiotech FB-UVXL-1000, Pittsburgh, PA, USA) at each UV dose. UV irradiation was applied within two hours post collection. The slide was placed 10 cm from the UV bulbs. The slide was then transferred to the microscope where the dilute milt was activated with 3 uL of room-temperature seawater (19^{o}C.) Once activated, a cover slip was placed on the slide and the resulting image was recorded with the VCR connected through the camera.

Counts were made of the total number of spermatozoa and the number of motile spermatozoa that appeared on the microscope. The total duration of motility was measured using a stopwatch. This procedure was repeated in quadruplicate for UV doses from 0 to 100 (11 doses) and 130 J/cm^2, and followed by a second 0 J/cm^2 UV dose. Two separate milt collections were used to verify the procedure. The videotape was later reviewed to ensure that accurate numbers were used in all calculations.

2.4 Meiotic gynogenesis.

Female flounder broodstock ranged from 3-7 years of age and 1-4.1 kg average weight. UNCW-CMS female flounder were implanted with a single pellet of LHRHa (94.79% cholesterol, 4.74% cellulose) at 50 ug/kg body weight. NCSU female flounder

were implanted with 75 *u*g Ovaplant LHRH implants using a RalGun pellet injector (Syndel International, Inc, Vancouver, British Columbia, Canada) at a dose of 50 *u*g/kg body weight. Eggs were stripped manually 48 hours after implanting.

Strip spawning was conducted in a temperature-controlled room under fluorescent light. The eggs were stripped and subdivided into four 400 ml beakers for each treatment. The three treatments consisted of flounder eggs that were fertilized using irradiated (70 J/cm^2) black sea bass sperm. The diluted milt was irradiated in a petri dish at approximately 0.2 mm depth. The UV-irradiated sperm was transferred to 400 ml beaker of eggs and uniformly distributed by swirling gently for 15 seconds. The spermatozoa were activated with 35 ppt seawater at either 16^{o}C or 18^{o}C (taken from the spawning tank.) 500 ml of filtered seawater (filtered to 250 *u*m) was placed in a stainless steel hydrostatic pressure chamber (Aquatic Ecosystems, Apopka, Florida, USA) prior to fertilization. The eggs/activated milt mixture was then placed into the pressure chamber, the pressure chamber filled with spawning water, and 8500 psi was applied at the appropriate initiation time (1, 2, or 3 min post fertilization). Eggs fertilized with UV-irradiated black sea bass sperm without pressure served as the negative control (presumptive haploids).

Pressure was applied using a 20-ton industrial hydraulic press (Arcan, Northern Hydraulics Eden Praire, Minnesota, USA) and hand cranked to achieve the 8500 psi. Each treatment remained in the pressure chamber for 6 minutes, and the pressure was immediately released from the fertilized eggs. The order of pressure application was randomized for each replicate to minimize any environmental effects on egg quality.

This experiment was conducted on broodstock that were maintained in 16^{o}C or 18^{o}C water to determine the effects of spawning temperature on the timing of the expulsion of the second meiotic polar body. Each trial was triplicated with different female broodstock over time.

2.5 UV Optimization for Meiogynogenesis.

Eggs were stripped into a 1 L beaker and then divided evenly into 400 ml beakers. These beakers were designated 0, 40, 50, 60, 70, 80, 90, and 130 J/cm^2 to determine the optimal UV exposure (Luckenbach et al. 2004). The dilute milt was exposed to UV-radiation in the increasing dose. The eggs and sperm were activated and pressurized to 8500 psi at 1 minute post fertilization as described in section 2.4. Throughout different trials, the order that the treatments were subjected to the pressure chamber was randomized to minimize random environmental effects on the eggs.

2.6 Meiogynogenetic Flounder Grow out Procedures.

Culture experiments were conducted in recirculating NCSU systems. The larvae were kept in separate 70 L fiberglass tanks until they were post metamorphic. At 2 dph, the survival was determined and all surviving treatments were combined into a single 70 L tank to increase density and enhance feeding behavior. The larvae were maintained at 20.5 $\pm$1^{o}C throughout larval culture and were fed according to methods described by Daniels and Watanabe (2002.) Once metamorphosis was complete, the flounder fingerlings were transferred to 1.2 m diameter, 250 L fiberglass tanks and kept at 23 $\pm$1^{o}C until the average length was 20 mm (Yamamoto 1999). At an average of 20 mm, the juvenile flounder were shifted to 28$\pm$1^{o}C (Yamamoto 1999, Luckenbach et al. 2003) in the same tank to cause a temperature induced sex reversal to males. The juvenile

flounder were maintained at 28°C until reaching 90 mm TL (Luckenbach, et al. 2003) and then shifted back to 23°C to ensure that the juvenile flounder were exposed to high temperatures during the period of sex determination. Densities during rearing were maintained at approximately 3.3 fish/L through metamorphosis and generally less than 40 fish/m^2 after the completion of metamorphosis. Density calculations were changed from fish/L to fish/m^2 upon the completion of metamorphosis and the larvae changed from pelagic stage to benthic juveniles. Once all the gynogens were shifted to 23°C, the flounder were reared in accordance with Daniels and Watanabe (2002).

At 179 dph, 33 natural-spawned juvenile flounder of similar size were added to the tank containing the gynogen cohort to improve feeding behavior and growth. The natural spawned flounder were marked with a caudal fin clip and subcutaneous elastomer injection (Northwest Marine Technology, Shaw Island, WA, USA) for identification. Both the natural spawned flounder and presumptive gynogens were transferred to a 1.8 meter polyethylene grow out tank and maintained on a accelerated photoperiod (duration of 4 months) to spawn in November. The growth and survival were recorded every two weeks until 292 dph using a standard metric ruler and Ohaus Scout II (SC2020) metric scale. At 292 dph the natural spawned flounder were removed from the grow-out tank to lower density and prevent cannibalism of the gynogen cohort.

2.6 100% female offspring production.

Gynogenetic offspring were reared to maturity according to Daniels and Watanabe (2002) in a closed recirculating system. The gynogens were photothermally conditioned using an accelerated photoperiod for 4 months (beginning at 270 dph) to initiate spermiation of the phenotypic males by reducing the temperature to 16°C and

photoperiod to L:D 10:14. Photoperiod and temperature manipulation were performed as described in paragraph 2.1. Sex reversed gynogen males and female broodstock were stripped using described techniques and the eggs fertilized with collected milt. The egg and spermatozoa mixture was swirled for 3 minutes, placed in a separatory funnel, and the floating eggs released into a 250 l hatching tank.

2.7 Statistical Analysis.

Percent sperm motility and duration of motility, fertilization, hatch, and UV optimization doses were analyzed using both one-way analysis (ANOVA) and split plot analysis. All statistical analyses were performed using SAS 9.1 software (SAS Institute, Cary, NC, USA). Data are expressed as mean $\pm$ SE unless otherwise indicated.

3. Results

3.1 Effects of UV radiation on black sea bass sperm motility.

Both percent motility and duration of motility decreased as UV dose increased. The motility was reduced by approximately 75% (Fig 1.1) and duration by approximately 70% (Table 1.1) at 70-80 J/cm^2. The target sperm motility of 10 % (Luckenbach et al. 2004) was achieved at these same UV doses. The Ringer's solution dilutant did not reduce motility after activation with seawater (N=7). A noticeable reduction in motility was not observed until 2 days after dilution and the sperm remained viable up to 4 days.

Preliminary results using copious amounts (3-4 ml of sperm diluted in Ringer's solution) of irradiated black sea bass sperm revealed no significant differences between fertilization at any UV doses. Percent fertilization using lower amounts of irradiated

sperm were variable with the percent fertilization falling off at the higher UV doses (Figure 1.2A.)

3.2 UV Optimization

The UV doses that were found to meet the criteria of sperm motility duration and percentage motility (40-90 and 130 J/cm^2) were used to fertilize southern flounder eggs. There was a significant difference in fertilization between spawns (P=0.0003) and UV treatment (P=0.007) (Figure 1.2A). Percent hatch was optimized at 70 J/cm^2, which was significantly different from the control (0 J/cm^2)(P=0.05) (Figure 1.2B).

In preliminary experiments using UV irradiated, diluted sperm and non-irradiated sperm conducted to ensure that black sea bass spermatozoa were able to activate southern flounder eggs, the eggs were activated and a small percentage of the eggs fertilized with UV irradiated sperm hatched, although all died within 2 dph. No larvae hatched from the non irradiated sperm fertilization trials. Percent fertility ranged from 0.03-86.8% (Figure 1.2A) and percent hatch ranged from 0-13.3% (Figure 1.2B). The percentage of larvae displaying haploid syndrome ranged from 0-100% (Figure 1.3C). Larvae hatched in only one trial of both temperatures for the control (all died within 2 dph.)

3.3 Timing of pressure initiation

Based upon the results of the UV irradiation experiments, southern flounder eggs were fertilized with dilute sperm irradiated with 70 J/cm^2. Percent fertility ranged from 44.7-86.7% (Figure 1.2A), hatch ranged from 0-3.5% (Figure 1.2B), and the percentage of larvae that displayed haploid syndrome (Figure 1.3C) was widely variable. Survival to 2 dph ranged from 0-64% (Figure 1.4A.) The percentage of larvae displaying haploid syndrome (kyphosis) ranged from 0-98% (Figure 1.3C).

Experiments were conducted with broodstock held at 16 and 18^{o}C. There was no significant difference in percentage hatch at either temperature (Figure 1.3B) due to the treatment effect caused by varying pressure initiation times. In 3 of the 4 replicates, there was a trend that the percent hatch at 16^{o}C was the highest at 1 minute post fertilization. At 18^{o}C, there was a trend that the highest percentage hatch occurred at 2 minutes post fertilization for every replicate, even though the difference was not significant. There was a highly significant difference between the percentage hatch at 1 minute post fertilization at 16 versus 18^{o}C (P=0.0009) and a significant interaction between temperature and treatment (P=0.01) (Figure 1.3B). All treatments were significantly different from the control (no pressure.)

3.4 Survival

Survival from 2 dph through metamorphosis ranged from 9.6 – 50.2% (Table 1.2). Of two surviving cohorts, survival from the completion of metamorphosis to one year was 13.5 and 13.9% respectively (Table 1.2). We currently have 3 gynogen cohorts that have not achieved one year in age.

3.5 100% Female offspring

One strip spawn was conducted between sex reversed southern flounder gynogens and a wild caught female broodstock. Extremely small quantities (<10 *u*L) of sperm was collected. Percent fertilization was 5.0% and percentage hatch was 20% of fertilized eggs. Survival of F1 larvae was >90% to first feeding.

4. Discussion

The effectiveness of hydrostatic pressure in inducing gynogenesis has been proven in many species (Pandian and Koteeswaran 1998, Ihssen et al. 1990, Peruzzi and Chatain 2000) and in Paralichthids specifically (Tabata 1991 and Yamamoto 1999.)

Each step in the hydrostatic pressure induction process was investigated as the optimum UV dose, timing of pressure initiation, and amount of pressure varies widely among teleost species. We determined that the black sea bass is a good sperm donor for achieving gynogenesis in the southern flounder due to availability of captive, spermiating males that perform well in captivity (Copeland et al. 2003, Luckenbach et al. 2004). Importantly, the use of heterologous sperm ensures that surviving offspring are gynogens as any resulting offspring of a black sea bass and flounder will die before or shortly after hatching as reported by other researchers (Chourrout 1982, Peruzzi et al. 1993, Arai 2001.) Black sea bass sperm that has been irradiated with UV light activates southern flounder eggs and produces viable gynogens that will survive to adulthood. These broodstock can be used as successfully sex reversed XX male broodstock to produce 100% female offspring.

Thinly spread dilute milt is likely critical to providing most of the spermatozoa more exposure to the UV light in order to inactivate the DNA while maintaining the ability to penetrate the egg. It was determined that Ringer's solution did not adversely affect the motility or duration of motility of black sea bass spermatozoa. The initial dilution of 1:50 proved to be too dilute to observe a significant number of spermatozoa under the microscope. We determined that a 1:10 dilution ratio of sperm : dilutant was adequate to allow UV penetration and corresponded to the final sperm ratios determined as optimal

by Luckenbach et al. (2004). In initial studies on the effects of UV light exposure on black sea bass sperm, 130 J/cm^2 reduced the motility >99.5%. This same UV exposure to sperm in fertilization trials still achieved a 12.78 $\pm$ 6.88% fertilization (Figure 1.2A.) To investigate this inconsistency, we kept sperm kept in a freezer for 7 days until they could not activate with sea water. This sperm achieved a low level of fertilization in southern flounder eggs (<5%). This fertilization with inactive sperm could be due to the physical mixing of the eggs and irradiated spermatozoa or a result of the hydrostatic pressure forcing dead spermatozoa into the eggs resulting in fertilization. These results are comparable to the results achieved using heterologous mullet sperm to activate southern flounder eggs (Luckenbach et al. 2004.) Based on our results, black sea bass appears to be an adequate species for producing gynogenetic offspring in the southern flounder.

The similar percentage hatch and fertilization achieved are characteristic of UV irradiated sperm. Many researchers have described the advantages of hydrostatic pressure over cold shock for the induction of diploid gynogenesis (Ihessen et al. 1990, Palti et al. 1997, Pandian and Koteeswaran 1998, Peruzzi and Chatain 2000) and our results validate the effectiveness of hydrostatic pressure for inducing gynogenesis in southern flounder. Our observations indicate the survival to metamorphosis is much higher using hydrostatic pressure than that found using cold shocking techniques (Luckenbach et al. 2004.) Likewise, the use of pressure has allowed us to rear gynogens to maturity for the first time. We believe this is due to a less harsh environment and less handling of the eggs. Luckenbach et al. (2004) described a high percentage of damaged eggs from the cold shock treatment and these damaged eggs were not observed using pressure where very few eggs were visibly damaged. There were high levels of mortality

approximately 2 dph (Cherfas 1981, Felip et al. 1999, Luckenbach et al. 2004) as seen in other gynogenesis experiments, but low mortality was observed at metamorphosis and transition to artificial feed. This is different from results seen in a previous study on southern flounder (Luckenbach et al. 2004.) High mortality was observed post metamorphosis, but we believe this is due to the high temperature treatments in our research to produce XX male broodstock. We recommend using hydrostatic pressure to induce gynogenesis in the southern flounder in order to produce large numbers of larvae that have higher survival rates from 2 dph to adulthood, which will be used as broodstock to produce an all female population.

We observed that there might be a temperature dependent interaction on the time to initiate pressure. Based on our experience, at 16^{o}C the optimal initiation time is 2 minutes post fertilization and at 18^{o}C, the optimal time is 1 minute post fertilization. Gynogenesis induction in *Paralichthys olivaceus* was optimized at 2 minutes post fertilization using hydrostatic pressure (Tabata 1991, Yamamoto 1999), but these researchers did not describe a temperature dependent interaction on pressure initiation.

In order to retain the second polar body in meiogynogens, a high level of hydrostatic pressure (8500 psi) was applied shortly after fertilization for a duration of 6 minutes. Although we observed a low survival after the completion of metamorphosis, we believe this to be mainly due to the higher temperature needed to sex reverse flounder and produce XX males.

Low percentages of hatch and survival are typical of this procedure and are comparable to results found with other species (Felip et al. 2001.) The African catfish showed a range of 6-30% survival to swimming (Varadi et al. 1999.) Peruzzi and

Chatain demonstrated that very few European sea bass lived after hatching using pressure and cold shock induction of gynogenesis. Palti et al. (1997) achieved a low hatch in rainbow trout in a study aimed at improving gynogen production methods.

We have shown that it is possible to use hydrostatic pressure to induce gynogenesis in the southern flounder. For the first time we have shown that gynogenetic southern flounder will survive to adulthood. We are currently in the process of using these animals and taking advantage of their natural temperature dependent sex determination (Luckenbach et al. 2003) to produce sex reversed XX males. These animals have been used as broodstock to produce an monosex, all female population of southern flounder. Our low percents fertilization and hatch indicate that the sperm that we were stripping was comprised mostly of immature spermatids instead of capacitated spermatozoa. This corresponds to the percentage of sperm cells that activated with sea water (approximately 25%). Further research on gynogen sperm will be conducted during the next spawning season when fully capacitated spermatozoa are readily available

5. Summary

In this study an optimized protocol to develop meiogynogenetic flounder using UV irradiated, heterologous black sea bass sperm was developed. We conclude that black sea bass are acceptable sperm donors for the induction of gynogenesis in southern flounder. The methods described here produce southern flounder gynogens that will survive to adulthood (>15 months.)

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CHAPTER 2 ABSTRACT

Preliminary Investigations for Induction of Mitogynogenesis and Triploidy in Southern Flounder (*Paralichthys lethostigma)* Using Black Sea Bass (*Centropristis striata)* Sperm.

Three preliminary experiments were conducted to establish methods to produce cloned, monosex populations of southern flounder (*Paralichthys lethostigma).* The establishment of cloned populations would facilitate advanced experiments in breeding and other genetic studies.

The first experiment determined the timing of the first mitotic division of the southern flounder egg (N=7). Eggs were fertilized with UV irradiated (70 J/cm^2) black sea bass (*Centropristis striata)* sperm and allowed to normally divide at temperatures ranging from 17-20^{o}C. The timing of first cleavage varied from 63-88 minutes post fertilization. Once eggs began the first cell division, 90 % of the eggs had completed this stage within 40 minutes. There was no correlation between spawning temperature, the timing of the first mitotic division, or the rate of mitosis.

A second experiment were also conducted to produce cloned populations (mitogynogenetic) (N=1). Southern flounder eggs were fertilized with UV irradiated (70 J/cm^2) black sea bass sperm. Once the first mitotic division was observed, the eggs were placed in a hydrostatic pressure chamber and pressurized at 8500 psi for 6 minutes. Percent fertilization was 44.7% and percent hatch was 0%, although some eggs did advance to the blastula stage.

Triploidy was induced in the southern flounder to determine the effects of hydrostatic pressure on egg hatch using homologous flounder sperm. Percent fertilization ranged

from 19.9-53.3 % and hatch ranged from 3.96-10.68 %. These hatch percentages are 18.0-42.7 % of the normal diploid control.

CHAPTER 2

1. Introduction.

Gynogenesis has been investigated in many species (reviewed by Ihssen et al. 1990 and Pandian and Koteeswaran 1998). One proven means to produce a clonal, monosex population is by mitotic gynogenesis. There are several methods to induce mitotic gynogenesis, all of which involve disrupting the first mitotic division of the fertilized egg using an external shock. Mitogynogenesis occurs when an external block prevents the first mitotic cellular division and results in a diploid (2n) set of identical maternal chromosomes that are identical at all loci.

Historically, several methods of preventing this cellular division have been explored, beginning with hydrostatic pressure shocking of trout eggs to produce gynogens (Chourrout 1984, Parsons and Thorgaard 1985). These early studies on trout also usually involved ploidy level manipulation to prevent sexual maturation. Hydrostatic pressure has been used to induce gynogenesis and control ploidy levels in the European sea bass *(Dicentrarchus labrax)* (Francescon et al. 2004), mud loach *(Misgurnus mizolepis)* (Nam et al. 2004), Nile tilapia (*Oreochromis niloticus)* (Ezaz et al. 2004), Japanese flounder (*Paralichthys olivaceus)* (Tabata 1991, Yamamoto 1999), and the red sea bream (*Pagrus major)* (Kato et al. 2001.)

Temperature shock (reviewed by Pandian and Koteeswaran 1998) has also been used to induce mitogynogenesis and control ploidy levels in many species including the African catfish *Clarias gariepinusi* (Galbusera et al. 2000), common carp *Cyprinus carpio* (Sumantadinta et al. 1990), goldfish *Carassisus auratus* (Nagoya et al. 1990), and the Mozambique mouthbrooder *Oreochromis mossambicus* (Varadaraj 1990).

The production of a cloned population of southern flounder by using mitogynogenesis would greatly enhance breeding programs (Yamamoto, 1999) by increasing the amount of inbreeding in genetic lines that are highly desirable for culture conditions. One of the potential uses of mitogynogenetic southern flounder is the production of cloned populations that are homozygous at all alleles of their chromosomes (Quillet, 1994). Upon sexual maturation, adult broodstock will produce homozygous gametes that can be used to produce clonal, monosex F2 populations when using sex reversed flounder males (Luckenbach et al. 2003). Heterozygous mitogynogen crosses would yield cloned populations with a higher survival (Yamamoto 1999.) Mitotic gynogens also opens the future for many possible genetic studies. This inbreeding would fix genetic traits within that genetic line and likely help establish a production facility within the United States. The purpose of this study was to determine the range of conditions in which mitogynogenetic flounder and triploids could be produced.

2. Materials and Methods.

The first experiment was conducted to determine the timing and pressure requirements to produce mitogynogenetic southern flounder using heterologous black sea bass sperm. A second experiment was conducted to determine the effects of hydrostatic pressure on growth and development of southern flounder eggs and to determine the success rate of retaining the 2nd polar body by producing triploids. These experiments were conducted from October 2004 – February 2005 at North Carolina State University (NCSU) (Raleigh, NC, USA) and the University of North Carolina-Wilmington Center for Marine Science (UNCW-CMS) (Wilmington, NC, USA).

2.1 Systems and Animals.

Systems. All systems for broodstock maturation, larval rearing, and growout at NCSU and UNCW-CMS were closed recirculating systems consisting of a temperature-controlled room under fluorescent light. The recirculating systems consist of various sized insulated fiberglass and polyethylene tanks, bubble bead biofilters, foam fractionators, heat pumps, water pumps, high rate sand filters, fluidized bed biofilter, and UV sterilizers (Daniels and Watanabe 2002, Copeland et al. 2003). All systems at UNCW-CMS are recirculating systems in insulated fiberglass tanks held outdoor under manipulated photoperiod.

Animals. Wild caught female flounder broodstock were purchased from Coastal USA Fish Company (Cedar Island, North Carolina, USA) and transported to NCSU or UNCW-CMS and maintained in captivity for 1-6 years. Spawning broodstock were held under a L:D 10:14 photoperiod at 16 or 18^{o}C (Daniels and Watanabe 2002.) The off-season spawning broodstock were under an artificial photoperiod with an aim of spawning October to December and were maintained in this photoperiod for two years. Fish were conditioned for spawning during a three-month period by manipulating photoperiod and temperature (Watanabe et al. 2001). Broodstock were fed with frozen lake smelt (*Osmerus mordax*), squid (*Illex* spp.), shrimp (*Panaeus* spp.), and a pelleted feed (13 mm floating, Melick Aquafeed, Catawissa, PA, USA). The remaining broodstock at NCSU and UNCW-CMS were held under ambient light conditions (Watanabe et al. 2001.)

Male black sea bass (*Centropristis striata*) were trapped off Carolina Beach, transported to UNCW -CMS and maintained in captivity 1-4 years (Copeland et al. 2002). The wild caught males were maintained in UNCW-CMS recirculating seawater

systems (Watanabe et al. 2003). A small subset was transported to NCSU. Broodstock were fed with frozen lake smelt (*Osmerus mordax*), squid (*Illex* spp.), and shrimp (*Panaeus* spp.)

2.2 General Methods

Animal Handling. All animals were removed from tanks by hand net and anesthetized in 40 L containers (MS-222) under aeration before handling.

Milt Collection. The milt was hand stripped using gentle abdominal pressure and collected with glass Pasteur pipettes while avoiding any contact with water. The milt was then transferred to a 5 ml test tube kept on ice. Milt from multiple males was collected and pooled to increase the volume and reduce the effects of natural genetic variation.

Egg Collection and handling. Female flounder were placed on a light table (Daniels and Watanabe 2002) to determine ovulation stage and readiness. Eggs were stripped into a 1 L beaker using gentle abdominal pressure and the eggs maintained at 16 or 18^{o}C (broodstock temperatures.) Once fertilized, the eggs were then transferred to a separatory funnel to determine floating egg and sinking egg ratios for total egg fecundity. Once removed from the pressure chamber, the eggs were placed in a plastic bag with 10 L of filtered water from the broodstock tank, filled with oxygen, sealed and floated in the 70 L hatching tank for acclimatization. The eggs were released into the tank once the temperature was equal to that of the tank temperature.

Percent fertilization and total number of eggs were determined approximately 4 hours post fertilization at the 16-32-cell stage using a dissecting microscope (Fisher Scientific FW00-20-1589) at 3X magnification. A 200 ml sample was taken to determine number of eggs. Percent fertilization was determined on a 50-100-egg sample under a dissecting

microscope. Fertilization was recorded as a percent of total eggs and percent of normally dividing eggs.

Percent hatch was determined 2-4 dph on a 1 L sample from the hatching tanks and visually counting the number of larvae. The numbers were recorded as total hatched larvae and larvae that appeared to be developing normally (without haploid syndrome). Larvae were reared according to Daniels and Watanabe (2002) using live food (enriched rotifers and *Artemia*) then weaned to dry, manufactured food beginning 14 dph.

2.3 Timing of the 1st mitotic cellular division of southern flounder eggs fertilized with UV irradiated black sea bass sperm.

Female flounder broodstock ranged from 3-7 years of age and 1.0-4.1 kg average weight. Female flounder were implanted with a single pellet of LHRHa (94.79% cholesterol, 4.74% cellulose) at 50 ug/kg body weight. Eggs were stripped manually 48 hours after implanting into a 1 L beaker and separated evenly into 400 ml beakers at temperatures ranging from 17-20^{o}C (broodstock). Black sea bass sperm was collected and diluted 1:10 in Ringer's solutions (0.75% NaCl, 0.04% CaCl, and 0.02% KCl) in double distilled water. Black sea bass sperm was irradiated at 70 J/cm^2 and 2-3 ml of UV irradiated sperm was added to a 400 ml beaker of eggs containing 40 ml of eggs. The mixture was gently swirled for 15 seconds to evenly distribute the sperm. The sperm and egg mixture was activated using 200 ml of filtered broodstock water and swirled for 3 minutes.

A 50 ml random sample of fertilized eggs was taken every 2-3 minutes beginning at 30 minutes post fertilization and continuing until >90% of the fertilized eggs were observed to be complete with the first mitotic cell division. The eggs were observed

using a dissecting microscope (Fisher Scientific FW00-20-1589) with ocular magnification of 3 times. The eggs were returned to the incubation beaker and a new sample was taken to prevent accelerated cellular division from the heat of the microscope lamp. The results were recorded as total number of eggs, percent undergoing the first mitotic division (genetic spindles appeared and the nuclear mass began to separate), and percent complete with the first mitotic cellular division (cell membranes were completely formed and two distinct cells were visible).

2.4 Southern flounder mitogynogenesis using black sea bass sperm.

Eggs were fertilized using irradiated (70 J/cm^2) black sea bass sperm. The diluted milt was irradiated in a petri dish at approximately 0.2 mm depth. At the onset of mitotic division, the eggs were transferred to a stainless steel hydrostatic pressure chamber (Aquatic Ecosystems, Apopka, Florida, USA). Pressure was applied using a 20-ton industrial hydraulic press (Arcan, Northern Hydraulics Eden Praire, Minnesota, USA) and hand cranked to achieve 8500 psi. Each treatment remained in the pressure chamber for 6 minutes, then the pressure was immediately released.

2.5 Induction of triploidy in southern flounder.

Female flounder broodstock ranged from 3-4 years of age and 1.8-2.6 kg average weight. Female flounder were implanted with a single pellet of LHRHa (94.79% cholesterol, 4.74% cellulose) at 50 *u*g/kg body weight. Eggs were stripped manually 48 hours after implanting. This experiment was conducted on broodstock that were maintained in 18^{o}C water.

Spawning was conducted in a temperature-controlled room under fluorescent light. Eggs were stripped into a 1 L beaker and maintained at 18^{o}C. The eggs were subdivided

into two 400ml beakers for each treatment. Eggs fertilized with southern flounder sperm and not subjected to pressure served as the control (diploids). The treatment consisted of flounder eggs that were fertilized using southern flounder sperm (collected from 2-3 males) and pressurized to 8500 psi for 6 minutes. The spermatozoa were activated with 35 ppt seawater (taken from the spawning tank.) Filtered seawater (500 ml filtered to 250 *u*m) was placed in a stainless steel hydrostatic pressure chamber (Aquatic Ecosystems, Apopka, Florida, USA) prior to fertilization. The eggs/activated milt was then placed into the pressure chamber, the pressure chamber filled with spawning water, and 8500 psi was applied 2 minutes post fertilization.

Pressure was applied using a 20-ton industrial hydraulic press (Arcan, Northern Hydraulics Eden Praire, Minnesota, USA) and hand cranked to achieve 8500 psi. The treatment remained in the pressure chamber for 6 minutes and was immediately released.

2.6 Statistical Analysis.

All analysis was conducted using one-way analysis (ANOVA) and linear regression. All statistical analyses were performed using SAS 9.1 software (SAS Institute, Cary, NC, USA). Data are expressed as mean $\pm$ SE unless otherwise indicated.

3. Results.

3.1 Timing of the 1st mitotic cellular division.

The first mitotic division varied between 63 and 88 minutes post fertilization (mean 76.25 $\pm$ 2.95 minutes) (Table 2.1). The duration from the time the first egg started mitosis until the last egg sampled began mitotic division ranged from 8-40 minutes (mean 22.88 $\pm$ 3.11 minutes) (Table 2.1). There was no correlation between temperature and the

beginning of the first mitotic division. The expected outcome of increased temperature advancing mitotic division and shortening duration time was not observed.

3.2 Southern flounder mitogynogenesis using black sea bass sperm.

Only one trial was conducted due to prioritization and commitment of eggs to other research interests. Percent fertility was 44.7% and percent hatch was 0%.

3.3 Induction of triploidy in southern flounder using heterologous black sea bass sperm.

Percent fertility ranged from 19.9-53.3% and hatch ranged from 3.96-10.68% (Table 2.2). The hatch was 18-42.7% of the diploid control. Larvae hatched approximately 2 dpf. Presumptive triploids were reared through metamorphosis. Growth was poor past metamorphosis and survival was low (<1%) and most juveniles died by 120 dph.

4. Discussion.

This study reports the results of preliminary experiments to determine the critical period for using hydrostatic pressure to induce mitogynogenesis in southern flounder with the goal of producing a cloned population for genetic studies. The effectiveness of hydrostatic pressure to induce mitogynogenesis has been established in many species (Chourrout 1984, Parsons and Thorgaard 1985, Francescon et al. 2004, Nam et al. 2004, Ezaz et al. 2004, Kato et al. 2001) and in other Paralichthids specifically (Yamamoto 1999.) It has been shown that ploidy in southern flounder can not be determined through visual inspection (Luckenbach et al. 2004), so the induction of triploidy would allow us to determine the amount of degradation experienced by eggs due to hydrostatic pressure compared to the use of heterologous sperm.

In this study, the first mitotic cellular division occurred between 63 and 88 minutes post fertilization and was complete 8-40 minutes later. Given the large differences in the initiation of first cleavage (25 minutes) and the wide variation in the completion (32 minutes), it is extremely unlikely that a single application of the 6 minute pressure shock can be recommended for all southern flounder. This variation in timing of first cleavage has been observed by other researchers in other teleost fishes (Park and Johnson 2002, Francescon et al. 2004, Hershberger and Hostuttler, in press) and has been attributed to the probable role of parental variation on post fertilization events (Hershberger and Hostuttler, in press.) In our only attempt to produce gynogenic clones, we experienced some success with a 44.7% fertilization. No larvae hatched, although a few larvae did progress to blastula stage. In future studies, it should be recognized that the timing of pressure shock may need to be different for each female and can only be determined upon observation of the fertilized eggs. Once first cleavage is observed, the eggs should be subjected to the pressure treatment.

Our attempts to induce triploidy in southern flounder using homologous flounder sperm shows that pressure shock causes a wide range of percents fertilization and hatch. The wide range of results in fertilization and hatch is comparable to the results achieved in past flounder rearing trials under hatchery conditions. The percentage of the control (normal flounder diploids) hatch does not allow us to draw any definitive conclusions about the effects of hydrostatic pressure versus using heterologous or homologous sperm for fertilization, although a lower hatch was observed. Fertilization percentages were similar to those observed using our meiotic gynogenesis protocol, but lower than historical hatchery percentages for normal diploid flounder production. Survival to 2 dph

and to metamorphosis was higher than meiogynogens, but survival past metamorphosis was extremely low (<1%). We were not able to conduct nuclear area measurements (Luckenbach et al. 2004) of triploids due to low survival.

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CHAPTER 3 ABSTRACT

Growth and Temperature Dependent Sex Differentiation of North Carolina and Texas Southern Flounder (*Paralichthys lethostigma)*

Southern flounder (*Paralichthys lethostigma*) inhabit the East Coast, south of North Carolina and Gulf Coast from Florida to northern Mexico with a break along the southern portion of Florida. These populations appear to have minimal interbreeding and usually do not intermingle due to the physical separation along the southern edge of Florida. The physical separation that occurs in this species may have led to divergence of local adaptations affecting the temperature dependent sex determination (TSD) that occurs in domestic southern flounder.

Separate populations from North Carolina and Texas southern flounder were raised from the egg stage onward under the same conditions and shifted to 28^{o}C at 30-40 mm TL to determine if the TSD response was similar in the two groups. A control for both populations was maintained in 23^{o}C. The amount of growth (increase in TL and total weight) in rank order was TX23 (396% and 7444%)> TX28 (346% and 5254%)> NC28 (339% and 5130%) > NC23 (296% and 3375%). The sex ratios of the Texas flounder reared at 23^{o}C were determined to be 100% male (N=10). Only 1 flounder remained for histological sex determination in each of the North Carolina 23^{o}C and the Texas 28^{o}C groups and both of these were also male. Preliminary sampling of North Carolina 23^{o}C treatment (N=6) indicated that all offspring were 100% male.

CHAPTER 3

1. Introduction.

Southern flounder (*Paralichthys lethostigma*) inhabit the East Coast, south of North Carolina and Gulf Coast from Florida to northern Mexico with a break along the southern portion of Florida. These populations have a minimal intermixing of genetic material and usually do not intermingle due to the physical separation along the southern edge of Florida. The temperature dependent sex determination (TSD) of southern flounder has been documented (Luckenbach et al. 2003) along with other flatfish of the genus *Paralichthys* such as the Japanese flounder (*Paralichthys olivaceus)* (Yamamoto 1999).

It is hypothesized that the physical separation that occurs in this species may have led to divergent local adaptations that may affect the TSD that occurs in domestic southern flounder. If the separate populations do show local adaptations, they may perform differently when reared under similar environmental conditions. The purpose of this experiment was to determine if the growth and sexual differentiation of southern flounder had any local environmental adaptations by comparing growth and conducting a temperature shift study (Luckenbach et al. 2003) with North Carolina and Texas flounder populations.

2. Materials and Methods.

A series of experiments was conducted on distinct populations of southern flounder (Texas and North Carolina populations) from natural spawns. The purpose of these experiments was to assess growth, temperature dependent sex determination, and final sex ratios based upon temperature shifts at various temperatures and time periods. These

experiments were conducted from March 2004-January 2005 at North Carolina State University (NCSU) (Raleigh, NC, USA).

2.1 Systems and Animals.

Systems. All systems for broodstock maturation, larval rearing, and growout at NCSU were closed recirculating systems consisting of a temperature-controlled room under fluorescent light. The recirculating systems consist of various sized insulated fiberglass and polyethylene tanks, bubble bead biofilters, foam fractionators, heat pumps, water pumps, fluidized bed biofilter, and UV sterilizers (Daniels and Watanabe, 2002.)

Animals. Texas southern flounder for the first series of experiments were received from University of Texas at Austin Marine Science Institute (Port Aransas, Texas, USA) from a natural spawn of wild caught females. Wild caught North Carolina female flounder broodstock were purchased from Coastal USA Fish Company (Cedar Island, North Carolina, USA) and transported to NCSU and maintained in captivity for 1-3 years. Spawning broodstock were held under a L:D 10:14 photoperiod at 16^{o}C (Daniels and Watanabe, 2002). Broodstock were fed with frozen lake smelt (*Osmerus mordax*), squid (*Illex* spp.), shrimp (*Panaeus* spp.), and a pelleted feed (13 mm floating, Melick Aquafeed, Catawissa, PA, USA).

2.3 General Methods:

Egg Collection and handling. Percent fertilization and total number of eggs were determined approximately 4 hours post fertilization at the 16-32-cell stage using a dissecting microscope (Fisher Scientific FW00-20-1589) at 3X magnification. A 200 ml sample was taken from the hatching tank to determine number of eggs. Percent fertilization was determined on a 50-100 egg sample under a dissecting microscope.

Fertilization was recorded as a percentage of total eggs and percent of normally dividing eggs.

Percent hatch was determined 2-4 days post fertilization (dpf) on a 1 L sample from the 250 L aerated hatching tanks by visually counting the number of larvae and recorded this as total hatched larvae. Larvae were reared according to Daniels and Watanabe (2002) using live food (enriched rotifers and *Artemia*) and then weaned to dry, manufactured food beginning 14 days post hatch (dph). Growth of southern flounder was measured as total length (TL) and total weight (TW) at the completion of metamorphosis and termination of the experiment.

Histology. Gonadal tissues were preserved in Bouin's fixative for at least 48 h, washed in 50% ethanol, and stored in 70% ethanol until processed for histology. Tissues were embedded in paraffin, sectioned to 5-7 *u*m, and placed on a microscope slide for staining. Tissue slides were stained with hematoxylin and counterstained with eosin. Distinctive gonadal tissue was identified and classified as either male or female.

2.3 Determination of Temperature Dependent Sex Determination periods and relative body size due to local adaptations of Texas and North Carolina southern flounder.

The Texas southern flounder eggs maintained in duplicate 1.2 m diameter, 250 L fiberglass tanks. The North Carolina southern flounder eggs were obtained from a natural spawn of a single female two days later from NCSU broodstock. The North Carolina eggs were also kept in the same closed re-circulation system in identical duplicate tanks. All eggs were hatched and reared through metamorphosis in identical

tanks in the same recirculating system. Larval culture and care of the juvenile flounder was accomplished according to Daniels and Watanabe (2002.) The two tanks of Texas flounder were combined into a single tank at 64 dph when the survival was low (<50%) and total numbers caused stunted growth and development.

Both treatments were raised using similar protocols. Larval rearing temperatures were $18\pm1^{o}C$ through metamorphosis. All larvae were raised to 30-40 mm (Luckenbach et al. 2003) and then shifted to $23^{o}C$ and $28^{o}C$ in duplicate tanks. The limiting factor for the total number of juvenile flounder in each tank was the total number of Texas flounder (210 total). The same numbers of juvenile North Carolina flounder were shifted to the higher temperatures simultaneously.

The original goal of the experiment was to shift the flounder to higher temperatures at similar body sizes. Based on an increased initial growth rate of the North Carolina flounder, the initial shift was conducted when the average size of the North Carolina flounder was 40 mm and the Texas flounder was 30 mm total length. All flounder were shifted at the same time, regardless of size, to standardize growth comparisons as well as sexual differentiation.

The flounder were sampled for increase in total weight and length every two weeks until 170 dph. After this period the flounder were sampled every month to determine growth and decrease stress from handling and sampling. A preliminary sample of sex ratios was taken at 259 dph and final sex ratios were determined at 327 dph. Lengths were measured to the nearest millimeter using a standard ruler and weights to the nearest tenth of a gram using an Ohaus Scout II (SC2020) metric scale.

2.4 Temperature shift of North Carolina southern flounder.

Duplicate trials were conducted to compare final sex ratios of southern flounder and identify the range of body size where the critical period for TSD effectively closes and sex is determined in the southern flounder. All flounder were natural spawned and hatched in identical tanks. Larval flounder were reared according to Daniels and Watanabe (2002) in 1.2 m diameter, 250 L insulated fiberglass tanks. At an average size of 60 and 90 mm, 30 randomly selected juvenile flounder were shifted from 23^{o}C to 28^{o}C in duplicate tanks. The controls were duplicate tanks of southern flounder that remained in 23^{o}C. Length and weight measurements were taken every 2 weeks using a standard metric ruler and Ohaus Scout II (SC2020) metric scale. The purpose of this experiment was to determine the critical period when the southern flounder was capable of sexual differentiation.

2.5 Statistical Analysis.

All analysis was conducted using one-way analysis (ANOVA) using SAS 9.1 software (SAS Institute, Cary, NC, USA). Data are expressed as mean $\pm$ SE unless otherwise indicated.

3. Results.

3.1 Southern Flounder growth due to local adaptations of Texas and North Carolina.

The North Carolina strain averaged 38.6 mm and 0.66 g at 125 dph (Table 3.1). The Texas strain averaged 30.74 mm and 0.37 g at 127 dph. At the termination of the experiment, the final weights were taken at 289 dph. The flounder that remained in 23^{o}C

grew larger for the Texas flounder while the North Carolina flounder grew larger at 28°C (not significant as shown in Table 3.1). The amount of growth (increase in TL and total weight) in rank order was TX23 (396% and 7444%)> TX28 (346% and 5254%)> NC28 (339% and 5130%) > NC23 (296% and 3375%) as shown in table 3.2. These data are reported at 289 dph and consist of N values ranging from 36-79.

3.2 Sex ratios.

Final sex based upon preliminary subsamples suggested that the flounder reared at 23°C (controls) had a high percentage of males. The sampled flounder (N=6) from the duplicate tanks were all male. The sample sex ratios of the Texas flounder reared at 23°C were determined to be 100% male (N=10). Only 1 flounder remained for histological sex determination in both the North Carolina 23°C and the Texas 28°C and both of these were also male. Preliminary sampling of North Carolina 23°C treatment (N=6) suggested that the offspring were 100% male. The final numbers of fish were low due to death of most of the fish in the last month of growout. These data are reported from 316 dph when all surviving southern flounder were processed for histology.

3.3 Temperature shift of North Carolina southern flounder.

The North Carolina southern flounder that were shifted to 28°C at 60 mm grew 24.02 mm and 11.33 g larger than the controls that remained at 23°C. The North Carolina southern flounder that were shifted to 28°C at 90 mm grew 14.71 mm and 7.39 g larger than the controls that remained at 23°C. No sex ratio data were gathered for the 60 and 90 mm shift treatment as all flounder died before the termination of the experiment.

4. Discussion.

The early growth of the Texas strain of southern flounder was lower than that of the North Carolina strain. Even though the North Carolina flounder were 2 days younger than the Texas fish, by 127 dph the North Carolina juveniles were 7.9 mm and 0.29 g larger (Table 3.1). This larger early growth by the North Carolina flounder was surpassed by the Texas flounder during the 162 days of the growth study (Table 3.3.) These values are expressed as the amount of growth from the size at stocking. Due to the low numbers of flounder when the experiment was terminated, the data only suggest that Texas flounder grow slightly better at 23^{o}C while North Carolina flounder grow slightly better in 28^{o}C until the ending body size of the experiment (~107 – 130 mm.)

Histological examination suggested sex ratios were 100% male for all treatments that were sampled (NC and TX 23^{o}C and TX 28^{o}C.) As this was the first study to assess whether sex ratios differ across temperatures between the Texas and North Carolina cohorts, our indications are that both groups experience the same unstable ability to become phenotypic males. Although the larvae were reared at 18^{o}C through metamorphosis, either the larval or juvenile rearing conditions or an unexpected instability in the parental inheritance may have contributed to these final sex ratios. These data are inconsistent with past constant temperature rearing studies (Yamamoto 1999, Luckenbach et al. 2003) that showed nearly 50% females when reared under optimal conditions. Luckenbach et al. (2003) determined that an approximately 50% female sex ratio was achieved when rearing to 90 mm at 23^{o}C.

The growth rate of the flounder shifted to 28^{o}C at 60 mm was initially higher than that of the controls (23^{o}C) but their growth rate decreased later in the experiment. This is

indicative of the flounder that were shifted to 28^{o}C at the beginning of the experiment (125 mm TL.) This is similar to the results in past southern flounder growth trials (Luckenbach et al. 2003.) The North Carolina southern flounder in this study grew better in 23^{o}C until 60 mm and then their growth rate increased in 28^{o}C until approximately 125 mm. The flounder that were shifted to 28^{o}C at 90 mm also grew larger than the controls at 23^{o}C, but not as large as those shifted at 60 mm.

Further research needs to be conducted in this area to answer several questions. A designed temperature shift study aimed at determining any differences between populations should be repeated as described above. Both populations should be shifted based on the same body size instead of time. The growth could then be determined from stocking Day 0 and compared to time in each treatment instead of age.

Another area that should be addressed is whether temperature is a direct influencing factor on sex determination or a down stream regulator as it may be perceived as a environmental stressor. A designed study to determine optimal conditions to rear southern flounder larvae to neutralize their tendency to become 100% male would have to be conducted prior to repeating studies of local adaptations to temperature tolerance.

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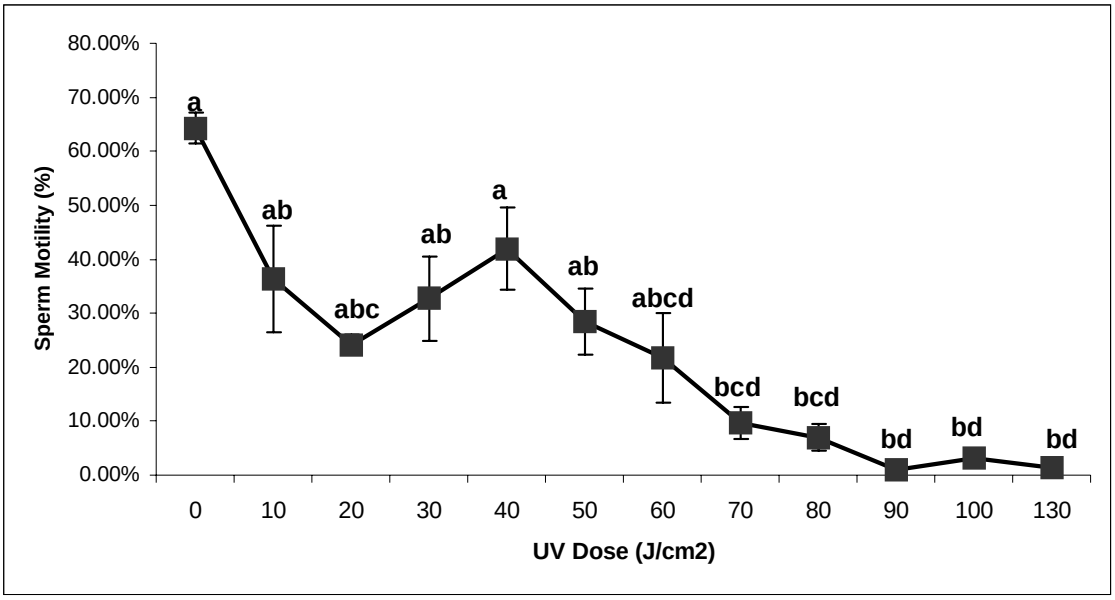

Figure 1.1. Mean percent motility of black sea bass (*Centropristis striata)* spermatozoa (mean $\pm$ SEM) exposed to UV dosages ranging from 0 to 130 J/cm^2 after being diluted in Ringer's Solution. Sperm motility was determined after activation by seawater. Data represent the average sperm motility from replicate experiments (N=7) in which semen was pooled from four male fish. Data points denoted with different letters are significantly different (P=0.05).

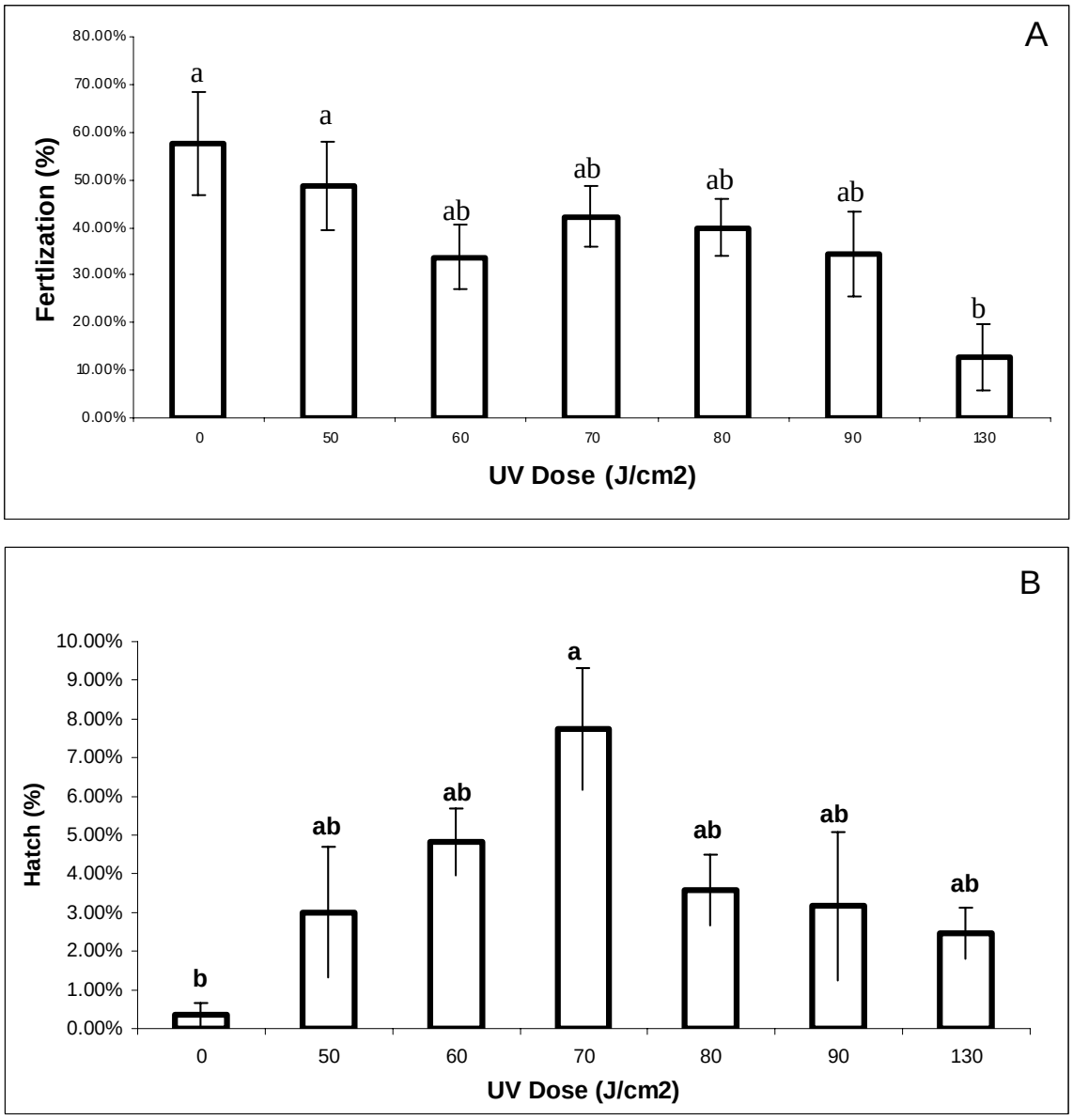

Figure 1.2. Mean percent fertilization (A) and mean percent hatch (B) of southern flounder (*Paralichthys lethostigma)* eggs (mean ± SEM) fertilized with black sea bass (*Centropristis striata)* sperm UV irradiated at doses from 0 to 130 J/cm^2 after dilution in Ringer's Solution. Each treatment was conducted in triplicate. Treatments with different letters are significantly different (*P*=0.05).

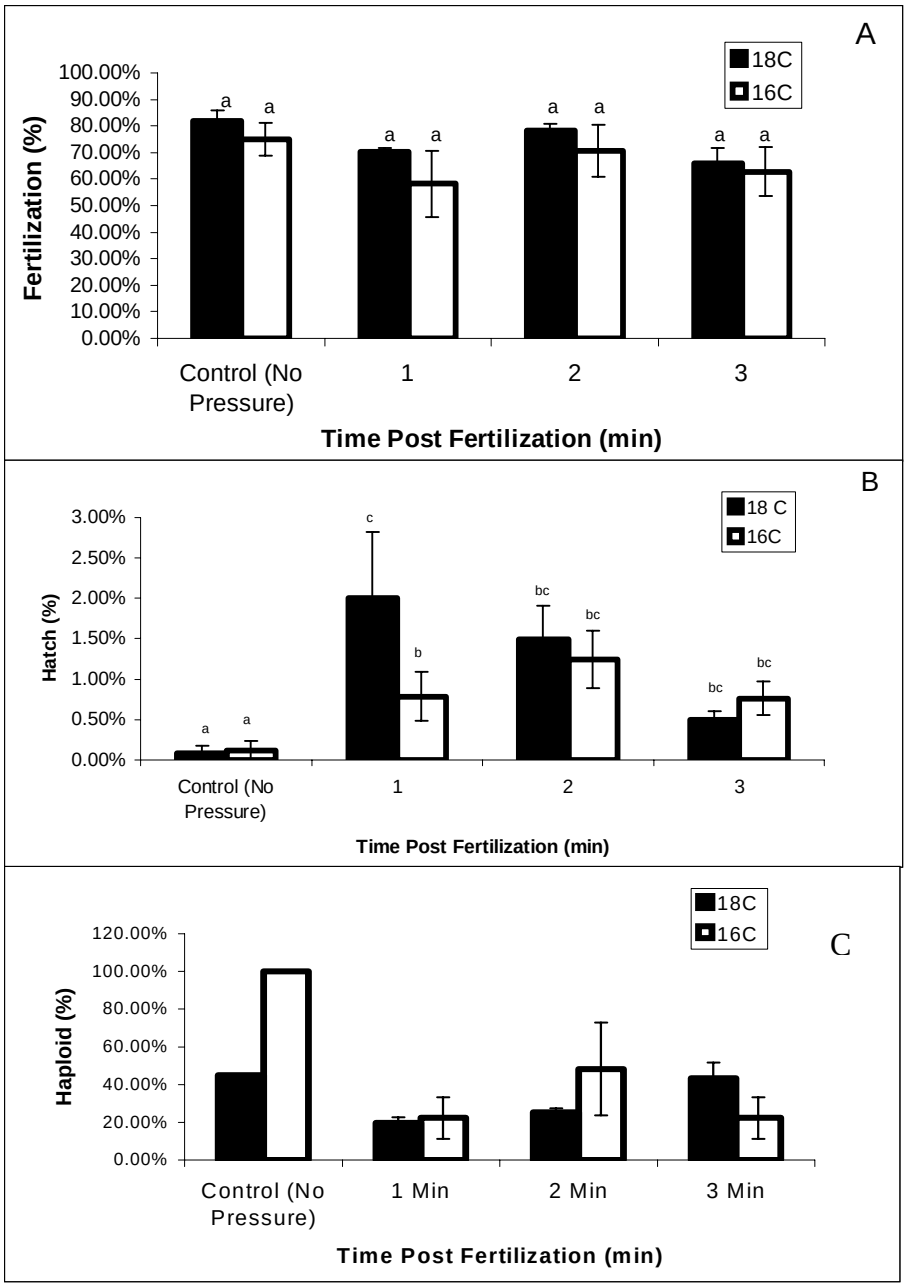

Figure 1.3. Mean percent fertilization (A), percent hatch (B), and percent haploid syndrome (C) of southern flounder (*Paralichthys lethostigma)* eggs (mean ± SEM) fertilized at 16 and 18^{o}C and at different times of pressure initiation post fertilization. Eggs were subjected to 8500 psi after fertilization with UV irradiated (70 J/cm^{2}) black sea bass (*Centropristis striata)* sperm. Eggs in the control treatment were fertilized but not subjected to pressure. Treatments with different letters are statistically different (*P*=0.05).

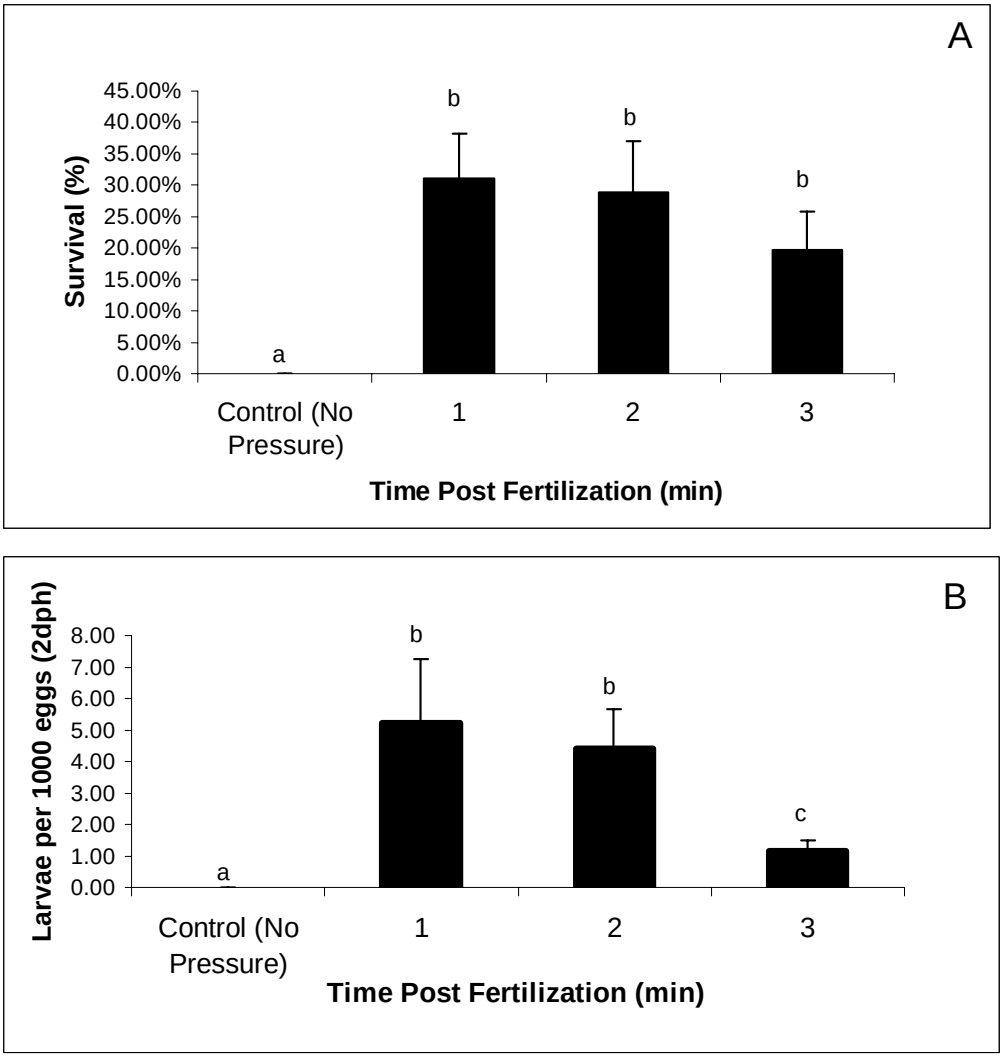

Figure 1.4. Mean survival (A) and survival per 1000 eggs (B) at 2 dph of southern flounder (*Paralichthys lethostigma)* eggs (mean ± SEM) fertilized at 16 and 18°C and at different times of pressure initiation post fertilization. Eggs were subjected to 8500 psi after fertilization with UV irradiated (70 J/cm^2) black sea bass (*Centropristis striata)* sperm. Eggs in the control treatment were fertilized but not subjected to pressure. Treatments with different letters are statistically different (*P*=0.05).

Table 1.1. Duration of black sea bass sperm motility (min) diluted with Ringer's solution and UV irradiated (0– 130 J/cm^2) after activation with seawater. Semen samples were pooled from 4 males in replicate experiments (N=4 for preliminary and N=3 for final). Treatments denoted with different letters are significantly different (P=0.05).

UV Dose (J/cm^2)	Preliminary	Final
0 (Control)	4.80±0.64 [a]	3.21±0.28 [a]
10		2.86±0.58 [a,b]
20		2.34±0.15 [a,b,c]
30		2.97±0.40 [a,b]
40	3.83±0.32 [a,b]	3.04±0.10 [a]
50		2.68±0.25 [a,b]
60		1.69±0.51 [a,b,c,d]
70	2.15±0.40 [b,c]	1.31±0.30 [b,c,d]
80		0.93±0.39 [c,d]
90		0.53±0.26 [d]
100	0.86±0.25 [c,d]	0.36±0.18 [d]
130	0.28+0.19 [d]	0.49+0.32 [d]

Table 1.2. Survival of southern flounder gynogens from 2dph to completion of metamorphosis and from the completion of metamorphosis through one year. Eggs were fertilized with black sea bass sperm and subjected to 8500 psi pressure 1-2 minutes post fertilization. Asterisks indicate that the gynogen cohort has not attained 1 year of age.

Trial	Survival (%)	
	2dph-completion of metamorphosis	metamorphosis – 1 year
1	45.4	13.9
2	50.2	0
3	26.6	13.5
4	24.5	*
5	12.5	*
6	9.6	*

Table 2.1. Timing of the first mitotic cellular division of southern flounder eggs (*Paralichthys lethostigma)* fertilized with UV irradiated (70 J/cm^2) black sea bass (*Centropristis striata)* sperm for 8 replicated experiments.

Cohort	Temperature (^{o}C)	1st Cleavage observed (min)	Duration of Mitosis (min)
3-Oct-03	18	88	22
5-Jan-04	20	63	40
6-Jan-04	19.5	80	23
7-Jan-04	19.5	85	25
8-Jan-04	19.5	78	25
12-Jan-04	20	75	8
14-Jan-04	20	73	20
11-Feb-04	17	68	20
	Mean	76.25	22.88
	SE	2.95	3.11

Table 2.2. Fertilization, hatch, and survival to 2 dph and metamorphosis for 2 triploid flounder replicate experiments during ploidy experiments using homologous flounder sperm.

Cohort	Fertilization (%)	Hatch (%)	% of Control	Survival (2 dph)	Survival (metamorphosis)
22-Oct-04	17.90	3.96	18.00	80.0	N/A
5-Nov-04	53.30	10.68	42.70	87.8	26.8

Table 3.1. Initial and final length and weights, N values, and percentage increase in size of duplicate tanks with North Carolina and Texas southern flounder from natural spawns cultured at different temperatures.

	TX 23			NC 23		
	TL (mm)	TW (g)	N value	TL (mm)	TW (g)	N value
Initial Size	30.30±0.64	0.36±0.03	36	38.93±0.05	0.68±0.02	72
Final Size	120.08±4.92	26.80±3.55		115.29±4.84	22.95±3.34	
% Increase	396	7444		296	3375	
	TX 28			NC 28		
	TL (mm)	TW (g)		TL (mm)	TW (g)	
Initial Size	31.18±0.36	0.39±0.01	56	38.27±1.46	0.64±0.06	79
Final Size	107.81±2.06	20.49±1.29		129.73±9.36	32.83±5.30	
% Increase	346	5254		339	5130	

Table 3.2. Growth rates of duplicate tanks with North Carolina and Texas southern flounder from natural spawns cultured at different temperatures.

DPH	TX23 Length (mm/day)	TX23 Weight (g/day)	TX28 Length (mm/day)	TX28 Weight (g/day)	NC23 Length (mm/day)	NC23 Weight (g/day)	NC28 Length (mm/day)	NC28 Weight (g/day)
142	0.64	0.03	0.98	0.06	0.39	0.03	1.00	0.08
155	0.57	0.05	0.39	0.04	0.55	0.04	0.36	0.05
170	0.24	0.03	0.33	0.05	0.26	0.04	0.33	0.05
198	0.58	0.09	0.70	0.14	0.58	0.09	0.66	0.11
226	0.54	0.16	0.47	0.16	0.60	0.17	0.47	0.23
258	0.55	0.22	0.30	0.14	0.31	0.14	0.44	0.18
289	0.65	0.36	0.31	0.16	0.54	0.30	0.67	0.46

Table 3.3. Increase in length and weights (times initial size) of duplicate tanks with North Carolina and Texas southern flounder from natural spawns cultured at different temperatures expressed as a percentage of growth.

	Growth Increase (%)	
	TL	TW
TX 23C	396	7444
TX 28C	346	5254
NC 23C	296	3375
NC 28C	339	5130
60 mm	361	5211
90 mm	337	4613

APPENDIX

Table 1. Fertilization, hatch, percent haploid, and survival of southern flounder gynogens to 2 dph for 10 meiogynogenetic flounder replicate experiments during pressure initiation time optimization using heterologous black sea bass sperm irradiated at 70 J/cm^2.

	Trial	Fertilization (%)	Hatch (%)	% Haploid	Survival (2dph)
1	Cont	86.70%	0.00%	0%	0%
	1 Min	74.00%	3.50%	26%	52%
	2 Min	79.60%	1.57%	29%	64%
	3 Min	70.30%	0.50%	63%	12%
2	Cont	86.50%	0.34%	45%	0%
	1 Min	68.60%	3.34%	17%	51%
	2 Min	70.30%	2.62%	20%	21%
	3 Min	64.90%	0.81%	27%	48%
3	Cont	78.00%	0.00%	0%	0%
	1 Min	14.50%	0.00%	0%	0%
	2 Min	19.60%	0.00%	0%	0%
	3 Min	20.00%	0.00%	0%	0%
4	Cont	84.20%	0.00%	0%	0%
	1 Min	67.40%	0.74%	13%	48%
	2 Min	82.10%	0.73%	23%	44%
	3 Min	78.30%	0.32%	34%	20%
5	Cont	18.30%	0.00%	0%	0%
	1 Min	26.50%	0.00%	0%	0%
	2 Min	49.20%	0.00%	0%	0%
	3 Min	41.20%	0.00%	0%	0%
6	Cont	70.10%	0.00%	0%	0%
	1 Min	71.60%	0.43%	22%	36%
	2 Min	80.50%	1.06%	29%	29%
	3 Min	50.00%	0.34%	50%	11%
7	Cont	62.90%	0.00%	0%	0%
	1 Min	42.90%	0.51%	33%	31%
	2 Min	51.90%	1.07%	25%	56%
	3 Min	44.70%	0.92%	33%	43%
8	Cont	80.00%	0.00%	0%	0%
	1 Min	82.90%	1.39%	33%	53%
	2 Min	83.90%	1.94%	22%	62%
	3 Min	75.00%	1.03%	33%	17%
9	Cont	0.522	0.00%	0%	0%
	1 Min	0.41	0.00%	0%	0%
	2 Min	0.415	0.00%	0%	0%
	3 Min	0.581	0.00%	0%	0%
10	Cont	82.10%	0.35%	0%	0%
	1 Min	48.90%	0.45%	0%	41%
	2 Min	76.10%	0.74%	98%	12%
	3 Min	68.50%	0.34%	0%	46

Table 2. Fertilization, hatch, percent haploid, and survival of southern flounder gynogens to 2 dph for 10 meiogynogenetic flounder replicate experiments during UV optimization using heterologous black sea bass sperm and pressurized at 1 minute post fertilization (18°C) or 2 minutes post fertilization (16°C).

	Trial	Fert (%)	Hatch (%)	% Haploid		Trial	Fert (%)	Hatch (%)	% Haploid
6-Jan-04	Cont	84.10%	0.10%	100%	13-Oct-04	Cont	41.20%	0.12%	100%
	40 J/cm	77.10%	0.24%	0%		40 J/cm	29.60%	3.82%	65%
	50 J/cm	78.00%	0.90%	38%		50 J/cm	46.80%	3.17%	50%
	60 J/cm	80.00%	1.63%	31%		60 J/cm	27.30%	5.39%	32%
	70 J/cm	65.90%	2.24%	42%		70 J/cm	55.90%	9.48%	15%
	80 J/cm	80.40%	0.12%	0%		80 J/cm	40.00%	5.87%	20%
	90 J/cm	75.90%	1.31%	50%		90 J/cm	44.70%	8.84%	43%
7-Jan-04	Cont	63.40%	0.00%	0%	21-Oct-04	Cont	50.00%	0.00%	0%
	40 J/cm	59.10%	2.45%	48%		50 J/cm	30.80%	0.00%	0%
	50 J/cm	67.90%	1.31%	45%		60 J/cm	24.10%	0.00%	0%
	60 J/cm	63.20%	1.05%	75%		70 J/cm	24.40%	0.46%	0%
	70 J/cm	56.20%	0.98%	67%		80 J/cm	30.10%	0.00%	0%
	80 J/cm	50.80%	1.68%	56%		90 J/cm	16.70%	1.02%	0%
	90 J/cm	55.30%	0.00%	0%		130 J/cm	0.03%	0.00%	0%
12-Jan-04	Cont	86.80%	0.21%	100%	4-Nov-04	Cont	77.10%	1.28%	96%
	40 J/cm	84.40%	0.36%	67%		50 J/cm	84.50%	0.78%	0%
	50 J/cm	84.80%	1.38%	63%		60 J/cm	57.90%	2.95%	11%
	60 J/cm	81.10%	0.67%	40%		70 J/cm	56.10%	3.65%	15%
	70 J/cm	83.60%	1.36%	30%		80 J/cm	57.10%	3.70%	17%
	80 J/cm	85.70%	0.78%	29%		90 J/cm	64.20%	0.57%	23%
	90 J/cm	76.20%	0.53%	25%		130 J/cm	32.30%	3.76%	6%
14-Jan-04	Cont	72.50%	0.00%	0%	12-Nov-04	Cont	88.70%	0.00%	0%
	40 J/cm	60.00%	1.70%	17%		50 J/cm	43.20%	0.35%	0%
	50 J/cm	45.70%	2.78%	29%		60 J/cm	38.30%	3.98%	40%
	60 J/cm	68.60%	1.92%	17%		70 J/cm	43.20%	4.48%	33%
	70 J/cm	52.30%	2.78%	13%		80 J/cm	47.90%	1.45%	80%
	80 J/cm	60.00%	2.97%	22%		90 J/cm	29.60%	1.84%	50%
	90 J/cm	63.60%	1.76%	0%		130 J/cm	8.50%	1.58%	0%
28-Jan-04	Cont	16.70%	0.00%	0%	8-Dec-04	Cont	31.30%	0.00%	0%
	40 J/cm	41.80%	0.42%	100%		50 J/cm	38.50%	7.71%	13%
	50 J/cm	48.10%	1.34%	75%		60 J/cm	20.80%	6.94%	0%
	60 J/cm	42.10%	0.90%	50%		70 J/cm	31.90%	13.30%	19%
	70 J/cm	34.50%	1.75%	40%		80 J/cm	24.70%	3.28%	0%
	80 J/cm	31.30%	1.91%	20%		90 J/cm	16.70%	1.40%	0%
	90 J/cm	17.80%	3.46%	75%		130 J/cm	10.30%	2.04%	0%