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

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Abstract

Female southern flounder (*Paralichthys lethostigma*) grow 2–3 times larger than males. Therefore, all-female production will maximize profit potential for the culture of this species. We have developed protocols to produce all-female southern flounder through induction of meiotic gynogenesis with heterologous sperm of black sea bass (*Centropristis striata*). Experiments were conducted to establish these practical methods using a total of 40 spawns from 32 broodstock. The first set of experiments determined the UV dose that genetically inactivated black sea bass sperm, yet retained adequate motility for activation of flounder eggs. Milt from several black sea bass was diluted 1:10 with Ringer's solution and UV irradiated with doses ranging from 0–130 J/cm^2. Two criteria were utilized to evaluate the UV irradiation effects: percentage of motile sperm and duration of sperm activity. Motility and duration of activity generally decreased with increases in UV dosage. At UV doses greater than or equal to 90 J/cm^2, motility was <1.5%. Fertilization rates were significantly lower at the highest UV dose of 130 J/cm^2 but were not different for the other treatments. Hatch rate was highest at 70 J/cm^2. A second set of experiments examined appropriate pressure shock protocols for retention of the 2nd polar body in southern flounder eggs after activation with black sea bass sperm. A pressure shock of 8500 psi was initiated at varying times of 1, 2, and 3 min post-fertilization and maintained for 6 min. Eggs that were handled similarly, but not pressure shocked, served as negative controls. Pressure shock applied at either 1 or 2 min post-fertilization resulted in higher rates of hatch and survival. Using these methods, six separate spawns produced offspring that survived through and beyond metamorphosis. The average fertility (±SEM) was 70.9+12.8%. Of the fertilized eggs, percentage hatch varied with pressure shock initiation times and ranged from 1.48+0.52% (1 min) to 0.61+0.11% (3 min). Gynogenetic flounder were sex-reversed to males by high temperature and, upon reaching maturity, expressed motile sperm that resulted in successful fertilization of flounder eggs. These results indicate that the use of UV irradiated sperm from black sea bass for activation of flounder eggs and pressure shock for polar body retention is an effective method to produce gynogenetic offspring.

Keywords: Gynogen; Meiotic gynogenesis; Hydrostatic pressure; Southern flounder; Black sea bass

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1. Introduction

Southern flounder (*Paralichthys lethostigma*) inhabit the coastal waters of the eastern United States from North Carolina, throughout the Gulf of Mexico into northern Mexico. Southern flounder exhibit a sexually dimorphic pattern of growth typical of the genus *Paralichthys*, where females grow 2 to 3 times larger in size than males (Grist, 2004). Because of minimum size restrictions on commercial harvest of southern flounder, the majority of harvested flounder are female and usually caught within the first 3 years of their life (Grist, 2004). Males rarely attain body sizes greater than the current minimum size restriction of 35 cm (14 in.) in North Carolina.

To realize the greatest economic benefit for aquaculture of this promising candidate species, efforts must focus on production of all-female fingerlings, eliminating production of slower-growing males. One means of creating 100% female populations in some teleost fishes is through artificial gynogenesis (reviewed by Pandian and Koteeswaran, 1998). Gynogenesis is the process of producing offspring composed only of the maternal genotype. This is often accomplished by using genetically inactivated spermatozoa to activate ova. Through exposure to ultraviolet (UV) irradiation these spermatozoa are unable to contribute paternal chromosomes to offspring. UV-irradiated heterologous sperm (from a different species) is commonly used to activate eggs, thereby acting as a double control (e.g., Chourrout, 1984; Ihssen et al., 1990; Arai, 2001; Felip et al., 2001). The use of heterologous sperm ensures that any surviving offspring are truly gynogens and not the result of normal fertilization, as is possible with homologous sperm.

Most fish expel a polar body containing a haploid set of maternal chromosomes upon fertilization (physical activation event), signifying the completion of meiosis. Meiotic gynogenesis occurs when an introduced factor prevents the expulsion of the second polar body, resulting in a diploid (2n) embryo containing two complete sets of maternal chromosomes. Several techniques have been employed for blocking the expulsion of the second polar body. These include temperature shock (e.g., Ueno and Arimoto, 1982; Koteeswaran et al., 1995; Luckenbach et al., 2004), pressure shock (e.g., Tabata, 1991; Malison et al., 1993; Kavumpurath and Pandian, 1994), electricity (Teskeredic et al., 1993), cytochalasin B (Refstie et al., 1977), and anesthetic treatments (Johnstone et al., 1989). The use of hydrostatic pressure for induction of gynogenesis usually ensures higher survival of offspring when compared to other methods (for review see Pandian and Koteeswaran, 1998). While the use of hydrostatic pressure requires more specialized equipment, the possibility of error is limited mainly to that of timing and accurate pressure measurement. Once a protocol is established, induction of gynogenesis with hydrostatic pressure is generally more reproducible and easily accomplished relative to other procedures, thereby limiting the handling stress to eggs.

Application of high temperatures at a critical post-metamorphic developmental stage in Paralichthid flounders has been shown to cause genotypic females (XX) to become phenotypic males (Yamamoto, 1999; Godwin et al., 2003; Luckenbach et al., 2003). Therefore, gynogenetic offspring can be effectively sex-reversed using temperature manipulations rather than treatment with endogenous androgens (Kitano et al., 1999; Yamamoto, 1999; Arai, 2001). Matings between sex-reversed gynogenetic males and normal females should theoretically generate 100% genotypically female offspring in flounder and this can be verified by progeny testing on the second generation (Yamamoto, 1999; Arai, 2001).

The purpose of this study was to develop an effective protocol using pressure shock to induce gynogenesis in the southern flounder. Black sea bass (*Centropristis striata*) sperm was used for fertilization of southern flounder eggs. Black sea bass were chosen because they are a marine teleost that spermiates well in captivity (Copeland et al., 2003) and when used to fertilize flounder eggs should not produce a viable, hybridized offspring. This research examines and reports procedures for the induction of gynogenesis in southern flounder and represents the first use of pressure shock for polar body retention in southern flounder.

2. Materials and methods

A series of experiments was conducted on 40 separate egg spawns from 32 female broodstock to determine the optimum UV dose and timing of post-fertilization pressure shock. 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

2.1.1. Systems

Rearing for broodstock maturation, larval production, and growout at NCSU and UNCW–CMS were done in temperature-controlled rooms containing closed recirculating systems equipped with bubble bead biofilters, foam fractionators, heat pumps, water pumps, high rate sand filters, fluidized bed biofilters, and UV sterilizers (see

Daniels and Watanabe, 2002; Copeland et al., 2003 for details). All recirculating systems at UNCW–CMS consist of insulated fiberglass tanks held outdoors under manipulated photoperiod (Watanabe et al., 2001, 2006).

2.1.2. Animals

Wild caught female flounder broodstock were purchased from Coastal USA Fish Company (Cedar Island, North Carolina, USA), transported to NCSU and UNCW–CMS and held for 1–6 years. Female flounder broodstock ranged from 3–7 years of age and 1.0–4.1 kg average weight. During the off-season, broodstock were held under an artificial photoperiod with the aim of spawning each October to December. Fish were photothermally cycled from a maximum of L:D 14:10 to 10:14 at either 16 °C (NCSU) or 18 °C (UNCW–CMS) and maintained at this photoperiod for the 3-month spawning period (Watanabe et al., 2001; 2006; Daniels and Watanabe, 2002). Broodstock were fed with frozen rainbow smelt (*Osmerus mordax*), squid (*Illex* spp.), shrimp (*Panaeus* spp.), and a pelleted feed (13 mm floating, Melick Aquafeed, Catawissa, PA, USA). The remaining broodstock were held under ambient light conditions (Watanabe et al., 2001; 2006).

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

2.2. Effects of UV irradiation on black sea bass sperm motility

Two separate trials were conducted in either triplicate or quadruplicate to assess effects of UV irradiation on black sea bass spermatozoa following the methods of Luckenbach et al. (2004). Broodstock were removed from tanks and anesthetized (MS-222) in 40 L containers under aeration before handling. Milt was hand stripped from black sea bass broodstock 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. Milt from multiple males was pooled to increase the volume and reduce the effects of individual variation. An undiluted sample of milt was placed on a slide and viewed under a compound light microscope (Olympus BH-2) at 400× magnification to ensure that the spermatozoa were not activated during collection. The sample was then activated with seawater at room temperature (19 °C) to check sperm motility.

Half of the remaining milt was diluted 1:10 using Ringer's Solution (0.75% NaCl, 0.04% CaCl, and 0.02% KCl in 1 L of ddH_20) kept on ice. A 3 μL sample of diluted milt was placed on a microscope slide and irradiated using a UV crosslinker (Fisher Biotech FB-UVXL-1000, Pittsburgh, PA, USA) at UV doses from 0 to 130 J/cm^2 (11 doses tested). UV irradiation was applied within 2 h after milt collection. The UV crosslinker delivers a desired UV dosage, which is detected by a sensor, and is not based on duration of exposure. Microscope slides were placed 10 cm from the UV source during irradiation. The slide was then transferred to the microscope where the sample was activated with 3 μL of room temperature seawater (19 °C). Once activated, a coverslip was placed over the sample and sperm motility was recorded using a camera and videocassette recorder to allow additional enumeration and validation of the sperm counts (see Luckenbach et al., 2004).

The total number of spermatozoa and the number of motile spermatozoa that appeared in the microscope frame were counted. After activation the total duration of sperm motility was measured for each sample using a stopwatch. Two separate experiments were conducted on milt collected from different black sea bass. This procedure was repeated in quadruplicate for the first experiment and triplicate for the second experiment.

2.3. Timing of pressure shock

Broodstock were anesthetized before handling as described above. Female flounder held at UNCW–CMS were implanted with a single pellet of LHRHa (94.79% cholesterol, 4.74% cellulose) at a dose of 50 μg/kg body weight. NCSU female flounder were implanted with 75 μg Ovaplant LHRH implants using a RalGun pellet injector (Syndel International, Inc, Vancouver, British Columbia, Canada). Female flounder were placed on a light table to determine ovulation stage and eligibility for spawning (Daniels and Watanabe, 2002). Eggs were stripped manually into a 1 L beaker using gentle abdominal pressure beginning 48 h after implanting.

Three treatments, done in triplicate with different females, were employed and consisted of flounder eggs that were fertilized using UV irradiated (dose of 70 J/cm^2) black sea bass sperm and hydrostatic pressure applied at either 1, 2, or 3 min post-fertilization. Eggs fertilized with UV-irradiated black sea bass sperm without pressure shock served as a negative control and was predicted to produce haploids. Black sea bass milt was diluted with Ringer's solution as described above and irradiated in a Petri dish at approximately 0.2 mm depth. The UV-irradiated milt was transferred to a 400-ml beaker containing eggs and mixed by swirling gently for 15 s. Gametes were activated with

35 ppt seawater at either 16 °C or 18 °C. Five hundred ml of 250 μm-filtered seawater was poured into a stainless steel hydrostatic pressure chamber (Aquatic Ecosystems, Apopka, Florida, USA). The gamete mixture was then added, filled further with seawater, and 8500 psi (Yamamoto, 1999) was then applied at 1, 2, or 3 min post-fertilization. Pressure was maintained in the chamber for 6 min and was then immediately released. The order of pressure application was randomized for replicates of each treatment to minimize any environmental effects on egg quality. The pressure shock apparatus consisted of a 20-ton industrial hydraulic press (Arcan, Northern Hydraulics Eden Prairie, Minnesota, USA) and was hand cranked to achieve 8500 psi.

Once removed from the pressure chamber, the eggs were transferred to a separatory funnel and total egg fecundity was determined by counting the relative proportion of floating versus sinking eggs. The eggs were then transferred to a plastic bag with 10 L of filtered seawater and acclimatized, and transferred to 70-L hatching tanks.

Percent fertilization and total egg number was determined on the portion of floating eggs approximately 4 h post-fertilization at the 16 to 32-cell stage using a dissecting microscope (Fisher Scientific FW00-20-1589) at 30× magnification. Percent fertilization was determined on samples containing 50–100 eggs. Viable but unfertilized eggs remain floating for some time during incubation and fertilization can be easily assessed at the 16–32 cell stage, therefore accurate estimates of fertility could be taken at this time.

Percent hatch was determined 2–4 days post-fertilization in a 1 L sample taken from the hatching tanks by visually counting the number of larvae. Only larvae that appeared to be developing normally, without haploid syndrome (bowed body or kyphosis; see Luckenbach et al., 2004), were included in these estimates. Larvae were reared according to Daniels and Watanabe (2002) using live food (enriched rotifers and *Artemia*) and then weaned to dry, manufactured feed beginning 14 dph.

2.4. UV optimization for gynogenesis

Southern flounder eggs, stripped from 2–3 females, were placed into a 1-L beaker and then divided evenly into 400-ml beakers representing different dosages of UV irradiation (0, 40, 50, 60, 70, 80, 90, and 130 J/cm^2). These UV doses were selected based upon the results of spermatozoa experiments described in Section 2.2 and that previously reported (Luckenbach et al., 2004). Milt was collected from black sea bass broodstock, diluted with Ringer's, exposed to the described UV doses using the protocol in Section 2.2. Afterward, the eggs and UV-irradiated milt were combined, activated with 150 ml seawater, and pressurized to 8500 psi at 1 min post-fertilization as described in Section 2.3. Handling of eggs and larvae was as described above.

2.5. Gynogen growout

Gynogens produced using the protocol developed from the above experiments were grown in recirculating systems at NCSU. Premetamorphic flounder were kept in separate 70-L fiberglass tanks until after metamorphosis. At 2 dph, larval survival was determined and all surviving larvae were combined into a single 70-L tank to increase density and enhance feeding behavior. The larvae were maintained at 20.5+1.0 °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+1 °C until the average total length was 20 mm. Juvenile flounder were then shifted to 28+1 °C to induce sex reversal of gynogens to males (Luckenbach et al., 2003). The juveniles were maintained

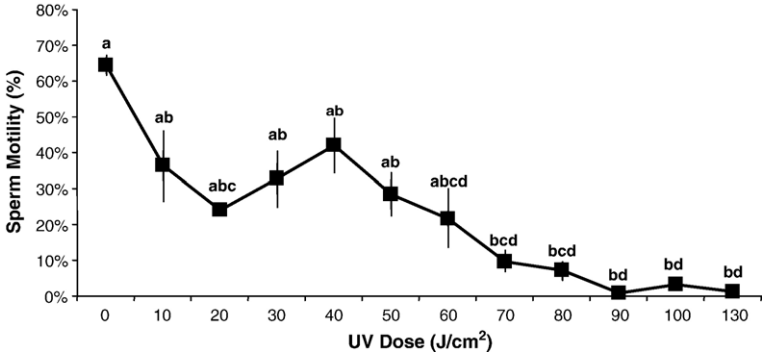

Fig. 1. Mean percent motility of black sea bass (*C. striata)* spermatozoa (mean+SEM) exposed to UV dosages ranging from 0 to 130 J/cm^2. Sperm was first diluted in Ringer's solution and then activated by seawater. Data represent the average sperm motility from replicate experiments (N=7) in which semen was pooled from four fish. Data points with different letters are significantly different (P<0.05).

Table 1
Duration of black sea bass sperm motility diluted with Ringer's solution and UV irradiated (0–130 J/cm^2) after activation with seawater

UV dose (J/cm^2)	Motility duration (min)
0 (control)	$3.21+0.28^{a}$
10	$2.86+0.58^{a,b}$
20	$2.34+0.15^{a,b,c}$
30	$2.97+0.40^{a,b}$
40	$3.04+0.10^{a}$
50	$2.68+0.25^{a,b}$
60	$1.69+0.51^{a,b,c,d}$
70	$1.31+0.30^{b,c,d}$
80	$0.93+0.39^{c,d}$
90	$0.53+0.26^{d}$
100	$0.36+0.18^{d}$
130	$0.49+0.32^{d}$

Semen samples were pooled from four males in replicate experiments ($N=4$). Treatments with different letters are statistically different ($P<0.05$).

at 28 °C until reaching 90 mm TL, which brackets the putative thermosensitive period of sex differentiation in this species (Luckenbach et al., 2005). After reaching 90 mm TL, fish were shifted back to 23 °C, which allows for more rapid growth relative to that observed at 28 °C (Godwin et al., 2003). Growth and survival of the gynogens were recorded every 2 weeks until 292 dph.

2.6. F1 strip spawn between gynogens and wild female

Gynogenetic offspring were photothermally conditioned for spawning using an accelerated photoperiod of 4 months, beginning at 270 dph. Spermiation was induced in putative gynogenetic male flounder by reducing the temperature to 16 °C and photoperiod to L:D 10:14. Sex-reversed gynogen male and normal female broodstock were strip spawned using standard in vitro techniques (Berlinsky et al., 1996). The eggs were fertilized for 3 min, 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, were arcsine transformed then 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 + SEM unless otherwise indicated. Differences were considered statistically significant when $P<0.05$.

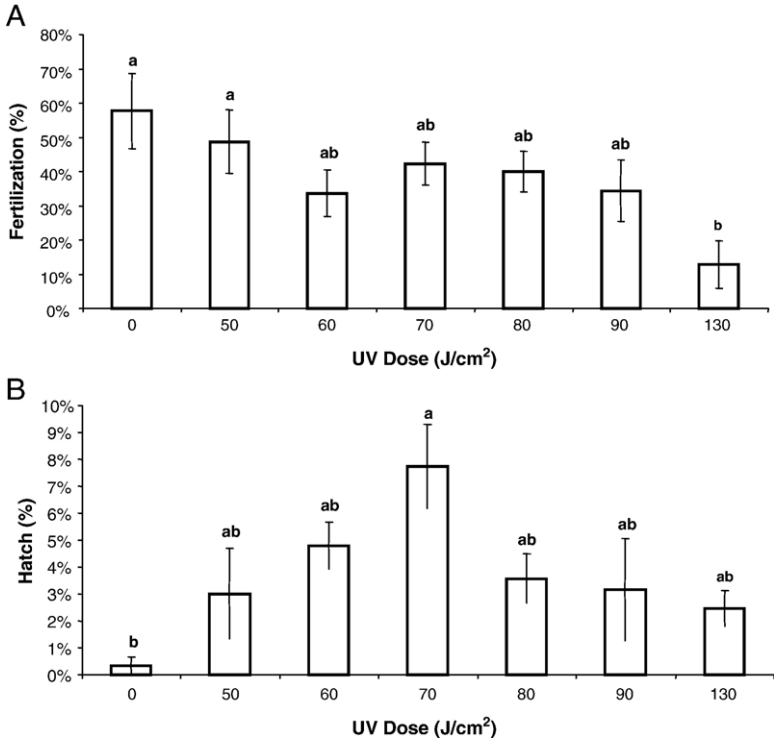

Fig. 2. Mean percent fertilization (A) and mean percent hatch (B) of southern flounder (*P. lethostigma*) eggs (mean + SEM) fertilized with black sea bass (*C. striata*) sperm UV irradiated at doses from 0 to 130 J/cm^2. Sperm was diluted in Ringer's solution and then activated with seawater. Each treatment was conducted in triplicate. Treatments with different letters are significantly different ($P<0.05$).

3. Results

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

Both percent sperm motility and duration of motility decreased as UV dose increased. Compared to the control group, a significant difference in sperm motility (0 J/cm^2) was first observed at a dose of 70 J/cm^2 (Fig. 1). At this dose sperm motility was reduced by approximately 75% (Fig. 1) and duration of motility was decreased by approximately 70% (Table 1). The Ringer's solution diluent did not reduce motility after activation with seawater (N=7). A noticeable reduction in motility was not observed until 2 days after milt was diluted in Ringer's solution and the dilute sperm could

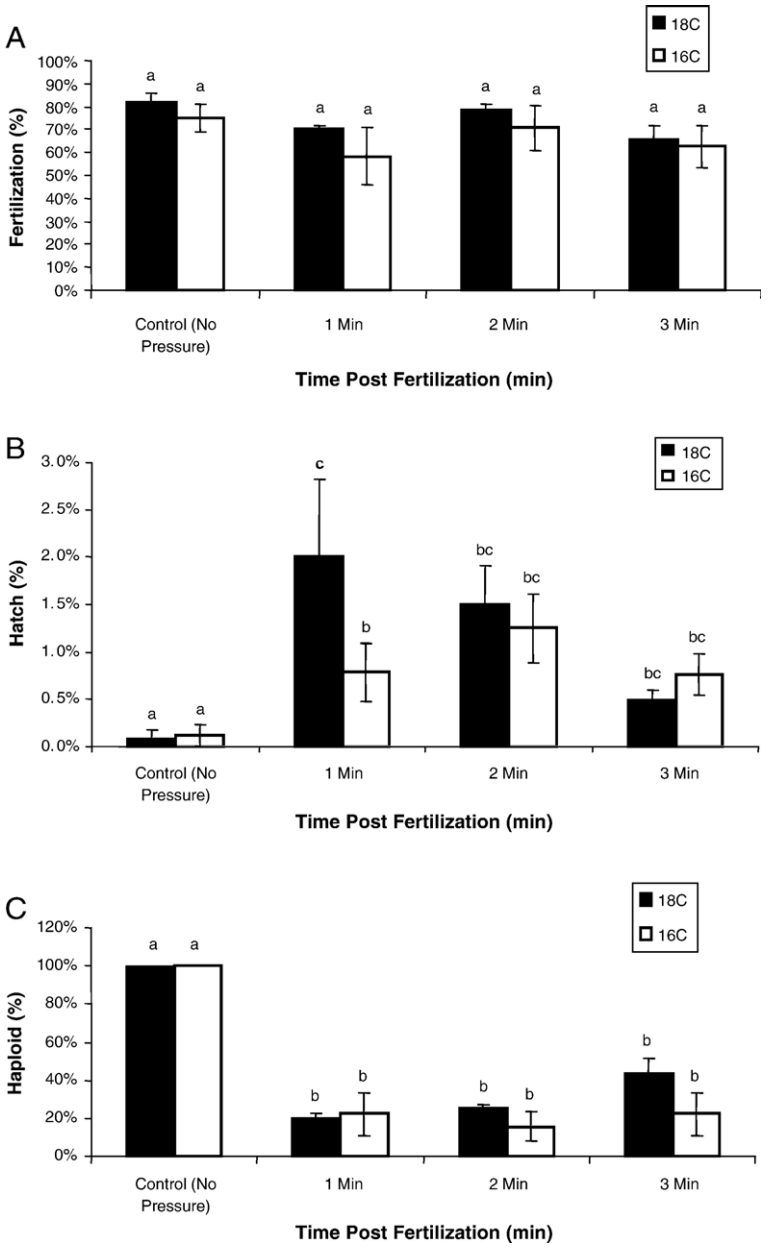

Fig. 3. Mean percent fertilization (A), hatch (B), and haploid syndrome (C) of southern flounder (*P. lethostigma)* eggs (mean+SEM) fertilized at 16 and 18 °C and at different times of pressure initiation post-fertilization. Eggs were subjected to 8500 psi after fertilization with UV irradiated (70 J/cm^2) black sea bass (*C. 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 2
Survival of southern flounder gynogens from hatch to 2 dph, 2 dph to completion of metamorphosis (45–60 dph), and from the completion of metamorphosis through 1 year of age

Trial	Survival (%)		
	Hatch-2 dph	2 dph-metamorphosis	Metamorphosis — 1 year
1	53.0	45.4	13.9
2	39.2	50.2	0
3	42.9	26.6	13.5
4	83.7	24.5	*
5	24.9	12.5	*
6	38.5	9.6	*

Each survival stage is calculated based upon the number of animals surviving from the previous stage. Eggs were fertilized with black sea bass sperm and subjected to 8500 psi pressure 1–2 min post-fertilization. Asterisks indicate that the gynogen cohort has not attained 1 year of age.

be activated with sea water up to 4 days when stored at 4 °C.

Preliminary results using copious amounts (50 μL per ml eggs) of irradiated black sea bass sperm revealed no significant differences between fertilization at any UV doses. This was likely due to the extremely high sperm to egg ratio during the *in vitro* fertilization process. Percent fertilization using lower amounts of irradiated semen (10 μL per ml eggs), was variable with the percent fertilization falling off as UV dosages increased (Fig. 2A).

3.2. UV optimization

There was a significant difference in fertilization between different female spawns ($N=4$) ($P=0.0003$) and UV treatment within the same female spawns ($P=0.007$). Fertilization was significantly lower at 130 J/cm^2, but was not different for the other treatments (Fig. 2A). Percent hatch was similar to controls at UV doses ranging from 50–130 J/cm^2, but the average was highest at a dose of 70 J/cm^2 (Fig. 2B). Percent fertility ranged from 0.03–86.80% (Fig. 2A) and percent hatch ranged from 0.00–13.30% (Fig. 2B).

3.3. Timing of pressure initiation

Separate pressure shock experiments were conducted with broodstock held at 16 and 18 °C (eggs activated with filtered broodstock water) to examine any possible effects of water temperature on optimal timing of pressure application. There was no significant difference in percent fertilization across treatments or temperatures (Fig. 3A). We also found no significant difference in percentage hatch at 16 and 18 °C (Fig. 3B). However, in 3 of the 4 replicates, there was a trend that the percent hatch at 16 °C was the highest at 1 min post-fertilization. At 18 °C, the highest percentage hatch occurred at 2 min post-fertilization for all replicates, although the difference was not significant. There was a highly significant difference between the percentage hatch at 1 min post-fertilization at 16 versus 18 °C ($P=0.0009$) and a significant interaction between temperature and treatment ($P=0.01$) (Fig. 3B). Regardless of temperature, hatch of gynogens was generally higher when pressure shock was applied at 1 or 2 min after fertilization. The percent hatch for all treatments was significantly different than controls that received no pressure shock. The percentage of larvae displaying haploid syndrome ranged from 0–100% within the treatments (Fig. 3C). Larvae from the control group (putative haploids) hatched in only one trial and all died within 2 dph.

3.4. Survival

Survival from 2 dph through metamorphosis ranged from 9.6–50.2% (Table 2). Of two cohorts surviving to date, survival from the completion of metamorphosis to 1 year was 13.5% and 13.9% respectively (Table 2). Three additional gynogen cohorts have not yet achieved 1 year in age.

3.5. Sex inversion and spawning of gynogens

Only one gynogen cohort has been verified to be successfully sex-reversed using high temperature treatment (28 °C). In this cohort, at least 50% of the animals could be manually induced to express milt that could be activated with sea water and were therefore confirmed males. One *in vitro* cross was conducted between these spermiating gynogens and a wild caught broodstock female. Survival of F1 larvae was >90% to first feeding and 33.5% through metamorphosis Day 34. Based on the known mechanism of sex determination in flounder of this genus (XX/XY; Yamamoto, 1999), these animals are presumed to be 100% genetically female. However, sex will later be confirmed by histology after sex differentiation occurs.

4. Discussion

In this study, we developed a protocol to produce gynogenetic southern flounder using UV-irradiated black sea bass sperm and pressure shock for second polar body retention. We determined that the black sea bass is a good sperm donor for achieving gynogenesis in the southern flounder due to their availability and performance in

captivity (Copeland et al., 2003). Importantly, the use of heterologous sperm ensures that surviving offspring are gynogens, as any resulting offspring of a putative black sea bass and flounder cross will die before or shortly after hatching, as reported for other species (Chourrout, 1984; Peruzzi et al., 1993; Arai, 2001). We demonstrate that black sea bass sperm that has been irradiated with UV light activates southern flounder eggs. Thinly spread dilute milt is likely critical to providing spermatozoa full exposure to UV light needed to inactivate the DNA while maintaining the ability to activate egg development. It was determined that Ringer's solution did not adversely affect the motility or duration of motility of black sea bass spermatozoa. We also determined that a 1:10 dilution ratio of sperm : diluent was adequate to allow UV penetration.

In initial studies on the effects of UV light exposure on black sea bass sperm, a dose of 130 J/cm^2 reduced motility by over 99.5%. Despite this, the same UV dosage applied to milt in the fertilization trials produced fertilization rates of 12.78%+6.88% (Fig. 2A). As demonstrated in other *in vitro* studies, sperm motility and fertilization success are not always positively correlated (e.g., see Scott and Baynes, 1980). Fertilization rates observed with 130 J/cm^2 UV-irradiated semen may have resulted from the physical mixing with the eggs or by the hydrostatic pressure causing contact of spermatozoa and eggs.

We found that 70 J/cm^2 is an effective UV dose for irradiation of black sea bass spermatozoa that maintains adequate motility to activate southern flounder eggs. This dosage was the only one that produced a statistically higher percent hatch compared to the control. The 70 J/cm^2 dosage also produced mean hatch rates that were approximately 3% higher than any of the other doses tested, although this effect was not statistically significant.

Hydrostatic pressure shock is considered an effective method for inducing gynogenesis in many fish species (Pandian and Koteeswaran, 1998; Ihssen et al., 1990; Peruzzi and Chatain, 2000). In southern flounder, however, this technique had not been previously investigated. We therefore experimentally investigated each step in the hydrostatic pressure induction process, because the optimum UV dose, timing of pressure initiation and amount of pressure varies widely among teleost species. We observed a possible temperature-dependent interaction on the time to initiate pressure. Although these numbers are not statistically different, the highest mean hatch was observed at 16 °C, where the best time for pressure shock initiation appeared to be 2 min post-fertilization based solely on trends in the data, while at 18 °C the best time appeared to be 1 min post-fertilization.

Low hatch and survival are typical of the gynogenesis procedure and our findings are comparable to those with other species (Felip et al., 2001), including flatfishes (Tvedt et al., 2006). The African catfish showed a range of 6–30% survival to swimming (Varadi et al., 1999). Peruzzi and Chatain (2000) demonstrated that very few European sea bass survived posthatch after pressure or temperature shock induced gynogenesis. Palti et al. (1997) achieved a low hatch in rainbow trout in a study aimed at improving gynogen production methods.

As reported in several other studies of gynogenesis (Cherfas, 1981; Felip et al., 1999; Luckenbach et al., 2004), high mortality of southern flounder larvae occurred at approximately 2 dph. Luckenbach et al. (2004) described a high percentage of damaged southern flounder eggs from cold shock treatment relative to that observed with pressure shock in this study. This is likely because the pressure shock method requires less handling of the eggs prior to incubation and exposure is brief compared to cold shock, which was sustained for ~ 45 min (Luckenbach et al., 2004). Direct comparison of results across these studies is difficult. Fertilization rates generally appear to be higher when using black sea bass semen as opposed to mullet semen, although a more concentrated and larger semen volume was used in the present study. Hatch rates, however, were generally lower in the present study compared to those recorded following cold-shock induced gynogenesis. Long-term survival to various larval stages (first-feeding, initiation, and completion of metamorphosis) was generally higher using hydrostatic pressure shock, but is particularly difficult to compare across studies due to the many factors that may influence later survival of gynogens (e.g., water quality, genetics).

During the course of these experiments, one of the gynogen cohorts spermiated, following temperature-induced sex reversal and growout to maturation at approximately 1 year of age. Semen from the sex-reversed gynogen males was stripped using described techniques, activated in sea water, and normal morphology and motility were visually confirmed under microscopic examination.

These results show that it is possible to use black sea bass sperm and hydrostatic pressure to induce gynogenesis in southern flounder. Based on the results reported in this study, a recommended protocol would use irradiated (70 J/cm^2) heterologous black sea bass sperm to activate southern flounder eggs followed by application of hydrostatic pressure (8500 psi) at 1–2 min post-fertilization for 6 min duration. For the first time we have produced gynogenetic southern flounder that will survive to adulthood and that can be sex-reversed to produce spermiating males of the XX genotype for use as broodstock. Once gynogenetic male broodstock with fully capacitated spermatozoa become available (2 years maturation) it will be possible to mate them to normal females to produce F1 generations of

100% females. Availability of all-female populations will eliminate production of the slower-growing males and improve production efficiency.

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