RECENT PROGRESS IN CONTROLLED REPRODUCTION OF SOUTHERN FLOUNDER *PARALICHTHYS LETHOSTIGMA*

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ABSTRACT

Hormone-induced spawning of southern flounder *Paralichthys lethostigma* has produced substantial numbers of viable eggs, but wide variations in percent fertilization and percent hatching have been reported. Recently, sustained natural spawning of southern flounder broodstock, without hormone induction, has been achieved at the Center for Marine Science, University of North Carolina at Wilmington (UNCW), USA. Adults (avg. wt. = 1.12 kg; n = 25) were stocked in two 4.8-m^3 controlled-environment tanks in October 1998 and held under natural photothermal conditions until January 1999, when an artificial winter photoperiod of 10 L: 14 D was maintained through April 1999. Natural spawning was observed in early December 1998 and increased in frequency to a peak in March 1999, before declining in late April. Water temperature ranged from 13.9 to 24.5 C during the spawning period. Natural spawns over 142 d produced a total of 18.3 x 10^6 eggs, with an average percent fertilization of 28.0% (range = 0-100%), yielding 4.94 x 10^6 viable eggs. The percentage of eggs that remained buoyant in full-strength sea water averaged 41.3% (0-99%), while the percent hatching of buoyant eggs averaged 37.3% (0-100%) and survival of yolksac larvae to the first-feeding stage averaged 30.2% (0-100%). A preliminary comparison suggests that natural spawning may produce higher egg quality than hormone-induced spawning in terms of fertilization and hatching success. These results suggest that natural and hormone-induced spawning of photothermally conditioned fish will help produce the large numbers of eggs required to support commercial production. Additional research is needed to address the problem of variable egg quality.

INTRODUCTION

The southern flounder *Paralichthys lethostigma* is a high-value recreational and commercially harvested flatfish found in estuarine and shelf waters of the Atlantic and Gulf coasts from North Carolina, USA, to Mexico (Gilbert 1986). With the implementation of fishery quotas for the summer flounder in the early 1990s, landings of southern flounder have increased. Today, the southern flounder is the number one flatfish species landed in North Carolina (Copeland et al. 1999).

Interest in the southern flounder as an aquaculture candidate in the southeastern United States is related to the wide range of temperature and salinity tolerance of this species; 50-d-old juveniles can tolerate salinities as low as five ppt, while older juveniles can tolerate fresh water (Smith et al. 1999a). This suggests that this species could potentially be cultivated in inland fresh and brackishwater ponds as well as in coastal areas (Berlinsky et al.1996; Daniels et al.1996; Jenkins and Smith 1999; Smith et al.1999a). At present, reliable methods for controlled breeding and production of high quality eggs are needed to accelerate hatchery research and development of commercial grow-out systems.

In Japan, commercial farmers rely mainly on photothermal conditioning and natural spawning of Japanese flounder *P. olivaceus* broodstock to supply the large numbers of high quality eggs needed to support commercial hatchery operations (Ijima et al. 1986; Min 1988; Tsujigado et al. 1989). In the United States, natural spawns of the southern flounder have been rare, and researchers have therefore focused on hormone-induced spawning. Intramuscular implantation of a cholesterol-cellulose pellet containing gonadotropin releasing hormone-analogue (GnRH-a) has produced repetitive spawning and substantial numbers of viable eggs in Southern flounder, but wide variations in percent fertilization and percent hatching have been reported (Berlinsky et al. 1996).

Natural spawning without hormone induction of captive southern flounder broodstock was recently achieved in our laboratory. The objectives of this paper are to describe the environmental and culture conditions and the reproductive performance associated with natural spawning. A preliminary comparison of natural spawning and hormone-induced spawning is also made.

MATERIALS AND METHODS

Natural Spawns

The southern flounder broodfish used in this study originated from two sources. One group (laboratory-reared) originated from fish collected as juveniles at 100-105 mm SL in the summer of 1993 near Beaufort, North Carolina, USA. A second group (wild-caught) originated as adults captured by commercial fishermen during September 1998 in Pamlico Sound, North Carolina, USA. Wild-caught fish were held in 20-m diameter outdoor concrete tanks supplied with flow-through brackish water (12-20 g/L salinity) for 6 wk before transport to the Center for Marine Science, University of North Carolina at Wilmington (UNCW), where this study took place between December 1998 and April 1999. Fish were individually tagged and held for 3 wk in flow-through sea water (34 ppt) tanks under ambient conditions before stocking into a controlled-environment broodfish system.

The controlled-environment broodfish system consisted of two circular fiberglass tanks (diam. = 2.46 m; depth = 1 m; vol. = 4.76 m^3). Situated out of doors, the broodfish tanks were insulated and provided with a conical fiberglass cover fitted with a timer-controlled, fluorescent fixture, containing two 20-W daylight bulbs. Average light intensity at the water surface was 234 lux.

Both tanks were supported by a water-recirculating system, consisting of a high-rate sandfilter, fluidized bed biofilter, foam fractionater, and ultraviolet sterilizer. Water from each tank drained through an egg collector (diam. = 0.76 m; depth = 0.76 m; vol. = 0.24 m^3) before entering a reservoir tank (diam. = 1.54 m; depth = 1 m; vol. = 1.86 m^3), from which water was pumped to the biofilter system. Water flow to each tank was approximately 38 L/min and water was exchanged at a rate of approximately 10%/d. Immersion heaters placed in the reservoir tank controlled water temperature.

In October 1998, one broodfish tank (tank 1) was stocked with 13 fish (2.73/m^3) consisting of six laboratory-reared and seven wild-caught adults with an average weight of 0.948 kg (2.59 kg/m^3). At stocking, the sex ratio was 8 female: 3 males: 2 unknown. In November 1998, the second broodfish tank (tank 2) was stocked with twelve fish (2.52/m^3), consisting of only wild-caught adults, with an average weight of 1.28 kg (3.23 kg/m^3). The sex ratio was unknown at the time of stocking.

Gonadal maturity of individual brooders was assessed periodically by biopsy of anaesthetized (0.3 g/L 2-phenoxyethanol) fish, using a polyethylene cannula (1.57 mm o.d. x 1.14-mm i.d.) (Shehadeh et al. 1973). Ovarian samples were fixed in a solution of 10% formalin in sea water. General stage of oocyte development (i.e, pre-vitellogenic, cortical vesicle, vitellogenic and atretic) was determined from the microscopic appearance, and males were identified by the presence of milt when pressure was applied to the gonadal area.

Fish were fed to satiation once daily (approximately 0900), a diet that consisted primarily of Atlantic silversides *Menidia menidia* supplemented with squid, krill, and commercially-prepared diets containing 45% (INVE Aquaculture, Grantsville, Utah, USA) and 55% protein (Corey Feed Mills Ltd., New Brunswick, Canada) and 16% fat. The feeding rate of the broodstock averaged about 1% BW/d.

To obtain gonadal maturation and spawning in January, presumed to be the natural reproductive period of southern flounder in North Carolina waters (Berlinsky et al. 1996), fish were exposed to ambient light and temperature conditions until 9 January 1999, when timers were used to maintain a constant winter photoperiod of 10 L: 14 D, and a water temperature that did not fall below approximately 14.5 C (Fig. 1).

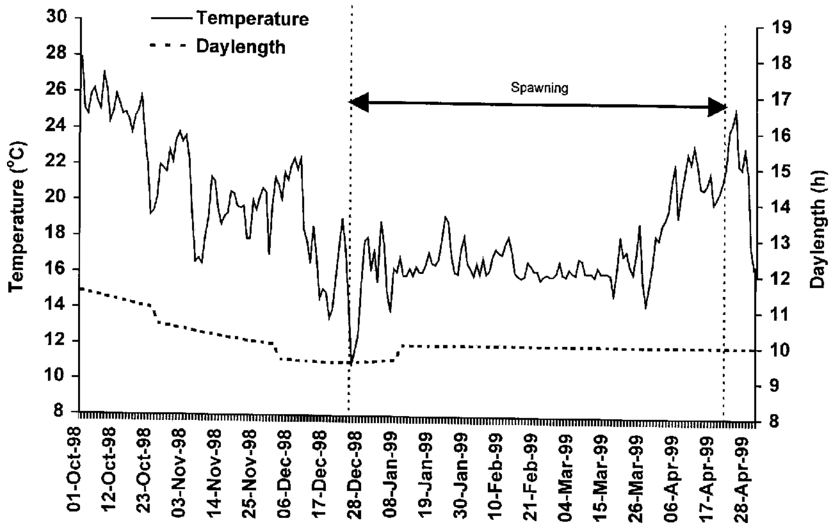

Figure 1. Photoperiod and temperature conditions during natural spawning of Southern flounder broodstock in 4.76 m^3 tanks.

Egg collectors were checked daily for spawned eggs. Once daily, eggs were siphoned from the collector, transferred to a separatory funnel in sea water (32-37 ppt), and buoyant eggs ("floaters") were separated from sinking eggs ("sinkers"). The numbers of eggs in each fraction were estimated using volumetric methods.

Floaters were transferred to 15-L airlift "in-tank" incubators placed inside the reservoir tank or situated in an indoor laboratory. In-tank incubators were stocked at a density of 1,000 eggs/L, while indoor incubators were stocked at densities of 300-600 eggs/L. Indoor incubators were provided with 1-µm-mesh filtered sea water (sterilized by ultraviolet light) at 16-19 C and supplied with diffused aeration. Using volumetric methods, survival of embryos were monitored through hatching (d 2 to d 3 after fertilization) and at the first-feeding stage (d 6 to d 7 after hatching), when 100% of the larvae possessed functional (fully pigmented) eyes, mouth, and alimentary tract.

Percent fertilization was determined as the percentage of viable embryos, while percent hatching was determined as the percentage of viable larvae hatched from fertilized eggs. Percent fertilization and percent hatching were expressed as percentages of total eggs and of buoyant eggs. Survival to the first-feeding stage was expressed as a percentage of total and of buoyant eggs.

Water Quality

Temperature, salinity, and dissolved oxygen were monitored daily, while pH, total ammonia-nitrogen, nitrite and nitrate were monitored weekly. Average daily values (and ranges) were as follows: salinity, 35.2 (32-37) g/L; dissolved oxygen, 7.64 (6.01-9.01) mg/L; pH 8.12(7.8-8.4); total ammonia-nitrogen, 0.029 (0-0.08) mg/L, nitrite-nitrogen, 0.022 (0.003-0.051) mg/L; nitrate-nitrogen, 2.75 (0.6-9.4) mg/L. Temperature, salinity, dissolved oxygen, and pH in the incubators were monitored once at the end of the incubation period. Average values (and ranges) were as follows: salinity, 35.5 (34-38) g/L; dissolved oxygen, 8.50 (7.59-9.27) mg/L; pH, 8.36 (8.2-8.5); temperature, 17.4 (16-18.6) C.

Hormone-induced Spawns

Hormone-induced spawning trials were conducted at the Tidewater Research Station (North Carolina State University) in Plymouth, North Carolina, USA. Adult Southern flounder (avg. wt. 1.2 kg) originated from the same source as the wild-caught brooders used for natural spawning trials at UNCW. Fish were stocked into tanks (diam. = 3.0 m; depth = 1.0 m; vol. = 7.4 m^3) supplied with recirculating sea water. Broodfish were exposed to artificial photoperiod and temperature conditions simulating ambient, reaching 9 h L:15 h D and 16 C by 15 December. Beginning in January 1999, females with maximum oocyte diameters of 500 μm were selected for hormone-induced spawning. To induce spawning, females were implanted with a 95% cholesterol and 5% cellulose pellet (Sherwood et al. 1988) containing [D-Ala[6] Des-Gly[10]] LHRH ethylamide (GnRH-a, Sigma Chemical Co., St. Louis, Missouri, USA) at a dose of 100 μg/kg (Berlinsky et al. 1996).

In some hormone-induced spawning trials, females were allowed to spawn volitionally in the tanks ("tank spawns"). In others trials, ovulated females were strip-spawned by applying gentle pressure to the abdomen. Eggs from a single female were collected in a glass beaker and mixed with the sperm from two males (Berlinsky et al. 1996), then left undisturbed in at least 100 ml of sea water for 1 h. The floating eggs were separated from the sinking eggs in a separatory funnel. Embryos were incubated in a 70-L round fiberglass tank containing 34 ppt filtered sea water at 16 C and at a maximum density of 500 eggs/L. Percent fertilization and percent hatching were determined as described above.

RESULTS

Natural Spawns

On 3-5 December 1998, fully hydrated ova were first observed in the egg collector from tank 1, consisting of both laboratory-reared and wild-caught adults. None of these individuals had been treated with hormones, indicating that natural spawning had occurred. Spawning increased in frequency to a peak in March 1999 before declining in mid-April. Fertilized eggs were first observed in tank 1 on 12 January 1999.

On 5 February 1999, fully hydrated ova were first observed in the egg collector from tank 2, consisting entirely of wild-caught adults. Natural spawning in tank 2 increased in frequency to a peak in late March and early April before declining in late April and fertilized eggs were first observed on 2 March 1999.

During a 142-d spawning period from 3 December 1998 to 23 April, eggs were collected on 70 days in tank 1 and on 53 days in tank 2 (Table 1). Numbers of eggs collected/d from each tank ranged from 5,490 to 601,250, averaging 159,596 in tank 1 and 133,104 in tank 2. For the duration of the 142–d spawning period, a total of 11,331,319 eggs were collected from tank 1 and 7,054,489 were collected from tank 2, for a total of 18,385,808 eggs from both tanks.

Egg buoyancy, Fertilization, and Hatching

Percent fertilization of naturally spawned eggs varied day to day from 0 to 97.2%, averaging 30.6% in tank 1 and 24.9% in tank 2, with an overall average of 28.0% for both tanks (Table 1). A total of 3,470,516 fertilized eggs were produced in tank 1, while 1,472,577 were

Table 1. Summarized data on natural spawning of southern flounder broodstock in two 4.76-m3 tanks (3 December 1998 to 23 April 2000). Each tank was stocked with 12-13 adults. Data are presented for each tank and for both tanks combined.

Tank No.	No. of days eggs observed	Total eggs spawned (No. spawned per day)	No. of floaters (No. per day)	Floaters (%) (range)	Fertilization rate (% overall) (range)	Fertilization rate ((% of floaters) (range)	No. of fertilized eggs (range)	Hatching rate (% of floaters) (range)
1	70	11,331,319 (5,490-601,250)	5,737,902 (0-412,750)	46.6 (0-97.5)	30.6 (0-99.0)	50.2 (0-100)	3,470,516 (0-177,300)	41.9 (0-87.1)
2	53	7,054,489 (22,750-419,000)	2,199,867 (0-244,000)	34.2 (0-96.5)	24.9 (0-95.2)	50.6 (0-100)	1,472,577 (0-233,996)	31.9 (0-99.1)
1 + 2	123	18,385,808	7,937,769	41.3	28.0	50.4	4,943,093	37.3

Estimated as percent hatching (% overall) = floaters (%) x percent hatching (% of floaters).
Estimated as survival to first-feeding (% overall) = floaters (%) x survival to first-feeding (% of floaters).

produced in tank 2, for a total of 4,943,092 for both tanks combined. On days that spawning was observed, an average of 34,811 fertilized eggs were collected from each tank.

During incubation of eggs in full-strength sea water (32-36 g/L), the percentage of eggs that remained buoyant (i.e.,"floaters") varied among spawns from 0 to 99.2%, with an average of 41.3% for both tanks (Table 1). For both tanks 1 and 2, percent hatching averaged 37.3% of floaters (15.4% overall), while survival of yolk-sac larvae to the first-feeding stage averaged 30.2% of floaters (12.5% overall).

Thermal Regime

From 5 January to 23 April 1999, the period during which the majority of spawns occurred, water temperatures varied over a wide range of 13.9 C to 24.5 C (Fig. 1). Fertilized eggs were also obtained under this temperature range, although availability of fertilized eggs increased to a peak during March and early April, while water temperature averaged around 17.5 C. The number of eggs spawned decreased as water temperatures increased steadily in April. Spawning appeared to be stimulated by a change in weather conditions; a warming or cooling trend was followed by an increase in egg release.

Growth and Sex Ratios of Broodstock

A gonadal examination of all broodstock made on 11 January 1999 revealed the following sex ratios: 8 females: 3 males: 2 unknown in tank 1 and 5 females: 2 males: 5 unknown in tank 2. A gonadal examination of all broodstock made on 27 April 1999 revealed four individuals in tank 1 and two individuals in tank 2 with atretic, hydrated eggs, indicating probable spawners. Assuming that these six individuals participated in spawning, an average of 3,064,301 eggs was released per female during the study. Two individuals in tank 1 and one individual in tank 2 from which ovarian tissue was sampled were also observed to emit a milky fluid from the urogenital opening on the dorsal (ocular) side when pressure was applied to the abdominal region. Microscopic examination of this fluid revealed highly motile 1-2 μm particles, presumably spermatozoa, and indicating hermaphroditic individuals.

Hormone-induced Spawns

In 1999, a total of 31 hormone-induced spawning trials were conducted, producing a total of 1,101,000 eggs of which 62% were floaters (Table 2). Of the floaters, 19% were fertilized (12% overall). The percent hatching of floaters was 16.5% (9.9% overall).

Of the 31 hormone-induced spawning trials, 14 tank spawns yielded 345,000 eggs, of which 55% were floaters, but no fertilization was obtained under this method (Table 2). Seventeen strip-spawning trials yielded 756,000 eggs, of which 68% were floaters, with 40% of these floaters being fertilized (27% overall). Percent hatching of the floaters averaged 30% (20.4% overall).

DISCUSSION

Natural Spawning

Through sustained, natural spawning of captive southern flounder broodstock, commercially significant quantities of viable embryos were produced in this study. Previous attempts to obtain natural spawning of this species met with limited success. Using photoperiods and

Table 2. Summarized data on hormone-induced spawns of southern flounder (*Paralichthys lethostigma*) at the Tidewater Research Station (North Carolina State University) in 1999, including eggs collected from both tank and strip spawns. Average percent hatching (range) was determined within 24 h of first observed hatch for floating eggs only.

Method of spawning	Batches	Total eggs spawned	Floaters	Floaters (%)	Fertilization rate (% overall)	Fertilization rate (% of floaters)	No. of fertilized eggs	Hatching rate (% of floaters)	Hatching rate (% overall)
Tank	14	345,000	190,000	55	0	0	0	0	0
Strip	17	756,000	514,000	68	27	40	206,000	30	20.4
Total	31	1,101,000	704,000	62	12	19	134,000	16.5	9.9

temperatures which simulated natural seasonal changes, Arnold et al. (1977) obtained natural spawning from 3 of 6 females for 13 consecutive days in 30-m^3 tanks producing 120,000 eggs with a percent fertilization of 30-50% and a percent hatching of 6-35% of the fertilized eggs. Controlled photoperiod and temperature were also effective in stimulating release of 200,000 eggs from 5 females over 2 spawning seasons, but no fertilization was obtained (Henderson-Arzapalo et al. 1988).

The results of this study demonstrate that wild-caught southern flounder adults, conditioned through photothermal manipulation for only 8 wk, can be spawned successfully without hormone induction during their first season in captivity. This is important to avoid a prolonged period of acclimation to captivity. In wild-caught turbot *Scopthalmus maximus*, efficient spawning occurred only after 2 yr of habituation to captivity (Devauchelle et al. 1988). In southern flounder, only females > 2 kg spawned naturally in captivity (Arnold et al. 1977) and successful hormone-induced tank spawning was attained with broodstock held in captivity for at least 1.5 yr and receiving photothermal conditioning for a minimum of 12 wk prior to spawning (Smith et al. 1999b).

The natural spawning period for southern flounder is believed to be December and January (Henderson-Arzapalo 1988; Berlinksy et al. 1996; Smith et al. 1999b). In this study, viable embryos were produced from January through late April 1999, indicating that the extended photoperiod regime was effective in extending the spawning season at least 2 mo beyond the natural spawning period for this species. Using a combination of photothermal conditioning and GnRH-a implants, southern flounder broodstock spawned volitionally in tanks over an extended period of 99 d from January to late April (Smith et al. 1999b).

It is likely that natural spawning of Southern flounder in this study was promoted by a number of factors. The fish were received from fishermen in good health and water quality parameters in broodtanks were maintained within optimal ranges throughout the study. The fish fed well and showed little or no evidence of disease for the duration of the study. In addition to the photothermal conditions, which maintained fish in a reproductive state from December through April, factors such as diet, tank size and color, and low light intensities apparently minimized stress and were conducive to natural spawning.

Based on our observation that egg release increased following a change in weather conditions, pulsatile temperature conditions may have been an important stimulus for sustained natural spawning in this study. Smith et al. (1999b) reported that a drop in water temperature from 17 to 14 C inhibited spawning in southern flounder, which resumed when temperature was returned to 17 C. In red drum, spawning is stimulated by gently raising or lowering the water temperature (Roberts 1990).

Sustained release GnRH-a pellet implants are highly effective in inducing ovulation of successive batches of eggs in the southern flounder (Berlinksy et al. 1996), allowing repetitive strip spawning. These workers produced substantial numbers (1.6 x 10^6) of eggs from multiple strip spawns of 12 females over a 2-wk period, but percent fertilization varied considerably (7-95%) between females and between spawns from individual fish. As an alternative to strip-spawning, photothermal conditioning coupled with GnRH-a implants have also resulted in successful tank spawning of southern flounder (Smith et al. 1999b). From a broodstock consisting of 3 females, these workers collected an average of 277,844 eggs during a 99-day period for a total of 17,782,000 of which 32.8% were fertilized (range = 0-82%), although survival through hatching was not reported. A combination of photothermal conditioning and GnRH-a implants apparently reduced stress and resulted in higher egg production and an extended spawning period (Smith et al., 1999b).

Results of this study demonstrate that photothermal conditioning of southern flounder can produce natural spawning, without the use of hormones, resulting in relatively high spawning success in terms of egg production and quality and the duration of the spawning period. Natural spawning of southern flounder broodstock produced a total of 18.4 million eggs, from 5 females, with an overall percent fertilization of

28% and an overall percent hatching of 9%. In comparison, 31 hormone-induced spawning trials produced 1,101,000 eggs, with lower overall percent fertilization and percent hatching of 12% and 9.9%, respectively. While spawning success varied widely under both natural and hormone-induced spawning trials, the data suggest that natural spawns generally resulted in higher egg quality.

Higher egg quality under natural spawning may be related to minimal handling of the broodfish, since stress can reduce spawning success (Kjesbu 1989). Inappropriate hormone dosages can also affect egg quality (Lam 1994), and strip spawning may cause varying egg quality due to over ripening or disturbances in the ovulation process (Bromage 1995).

A major disadvantage of natural spawning is the inability to time the spawns to suit the needs of the fish culturist. Both natural and GnRH-a induced spawns will help supply the large numbers of eggs necessary to support commercial hatcheries. Additional research is needed on hormonal, nutritional and environmental effects on egg quality.

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