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REPRODUCTIVE BIOLOGY OF THE SHI DRUM (UMBRINA CIRROSA) IN CAPTIVITY AND INDUCTION OF SPAWNING USING GNRHA

Constantinos C. Mylonas[1]*, Yiannos Kyriakou[2], Irini Sigelaki[1], Georgios Georgiou[2], Daphne Stephanou[2] and Pascal Divanach[1]

[1] *Hellenic Center for Marine Research, Institute of Aquaculture, P.O. Box 2214, Iraklion, Crete 71003, Greece*

[2] *Department of Fisheries and Marine Research, 13 Aeolos Street, 1416 Nicosia, Cyprus*

Abstract

The reproductive biology of the shi drum (*Umbrina cirrosa*) in culture was histologically examined and sperm quality was monitored during an entire reproductive period. Already in April, the ovary contained oocytes in all stages of maturation, from primary oocytes to full vitellogenesis, as expected from a group-synchronous multiple-batch spawning fish. Vitellogenesis of the first batch of oocytes occurred very rapidly and their mean diameter (500 µm) did not increase significantly ($p > 0.05$) as the reproductive period proceeded. The spermiation index peaked in May-June, but fish never produced copious amounts of milt upon abdominal pressure. The spermatozoa motility percentage remained unchanged throughout the spawning season (80%) and a significant percentage (40%) maintained viability after overnight storage at 4°C. Sperm density and motility duration increased during the reproductive period and varied 13-26 x10^9 spermatozoa/ml and 26-40 s, respectively. Spontaneous spawning was not observed during the two-year study. Injection of post-vitellogenic females with an agonist of gonadotropin-releasing hormone (GnRHa) was successful in inducing a single spawning after two days, with fertilization, hatching and 4-day larval survival rates of 65%, 42-76% and 46-80%, respectively. The results underline the failure of female shi drum in culture to undergo final oocyte maturation and, although GnRHa injection was effective in inducing spawning of viable eggs, multiple treatments did not induce multiple spawns, as was expected from fish with multiple-batch group-synchronous ovarian biology.

* Corresponding author. Tel.: +30-2810285443, fax: +30-2810241882, e-mail: mylonas@imbc.gr

Introduction

Species diversification in Mediterranean aquaculture has been identified as one way to address the problem of diminishing market prices for the two major cultured species, the European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*; Basurco and Abellan, 1999). Among species being examined for aquaculture is the shi drum (*Umbrina cirrosa*), a member of the family Sciaenidae (Nelson, 1994). This is a euryhaline teleost found throughout the Mediterranean Sea (Fischer et al., 1987). It has a good growth rate and market value (Fabi and Fiorentini, 1993; Mylonas et al., 2000) and, under optimal stocking densities, can reach a mean body weight of 700 g in 15 months (unpublished data). This species has other important advantages compared to the commonly cultured Mediterranean fishes. It spawns in the summer, and therefore does not compete for larvae rearing facilities with the commonly cultured winter spawning species. Also, the larvae can be grown without *Artemia* nauplii and weaned to artificial diets less than 35 days after hatching (Mylonas et al., 2000).

Early studies of the reproductive biology of the shi drum indicated that this is a multiple-batch group-synchronous spawning fish with a June-August spawning season (Barbaro et al., 1996) and an optimal spawning temperature of 22-26°C (Cardellini et al., 1999). Spermatogenesis and vitellogenesis proceed normally under culture conditions, but there is a complete failure of females to undergo final oocyte maturation (FOM). As a result, spawning has been accomplished almost exclusively through the use of hormonal manipulations (Melotti et al., 1995; Cardellini et al., 1998; Barbaro et al., 1999; Cardellini et al., 1999; Mylonas et al., 2000; Barbaro et al., 2002).

Failure of females to undergo FOM, ovulation and spawning is a common reproductive dysfunction of cultured fishes (Zohar, 1989). Manipulation of reproductive processes and induction of FOM can be achieved by a variety of exogenous hormones (Zohar and Mylonas, 2001). Agonists of gonadotropin-releasing hormone (GnRHa) have been extensively employed due to their high poten-cy, lack of species specificity and stimulation of endogenous gonadotropin release (Crim and Bettles, 1997; Peter and Yu, 1997). Treatment with GnRHa can be in the form of simple injections or sustained-release delivery systems (Donaldson, 1996; Crim and Bettles, 1997; Zohar and Mylonas, 2001). Due to the short half-life of GnRHa, a simple injection induces a brief elevation in plasma luteinizing hormone (LH), the gonadotropin responsible for FOM (Nagahama et al., 1994). As a result, some fishes require multiple GnRHa injections for effective treatment. Sustained-release delivery systems, on the other hand, induce long-term stimulation of LH release (Crim et al., 1988; Mylonas et al., 1998a; Mylonas et al., 1998b) with only a single treatment, and have proven effective in inducing FOM, ovulation and spawning in many fishes (Mylonas and Zohar, 2001).

The objectives of the present study were to (a) further examine the reproductive biology of the shi drum in culture and histologically describe the process of gametogenesis, (b) monitor sperm quality during the reproductive period and (c) use GnRHa in different modes of application to induce FOM, ovulation and spawning. The study attempted to induce multiple spawnings, as is natural in this species, and further knowledge by using a different type of GnRHa-delivery system (Barbaro et al., 2002).

Materials and Methods

Animal husbandry and sampling. The experiments were carried out in the aquaculture facilities of the Department of Aquaculture and Marine Research at Meneou, Cyprus, using 4 or 5-year-old females, and 2 to 4-year-old males produced from wild-caught broodstock (Mylonas et al., 2000). Fish were maintained during the year in mixed-sex populations in 40 m³ round tanks, supplied with surface sea water (Fig. 1), exposed to the ambient photoperiod and fed daily with commercial extruded feed, supplemented with squid, raw fish and mussels. Experimental animals were individually marked with a passive integrated transponder tag (P.I.T. tag; Fish Eagle
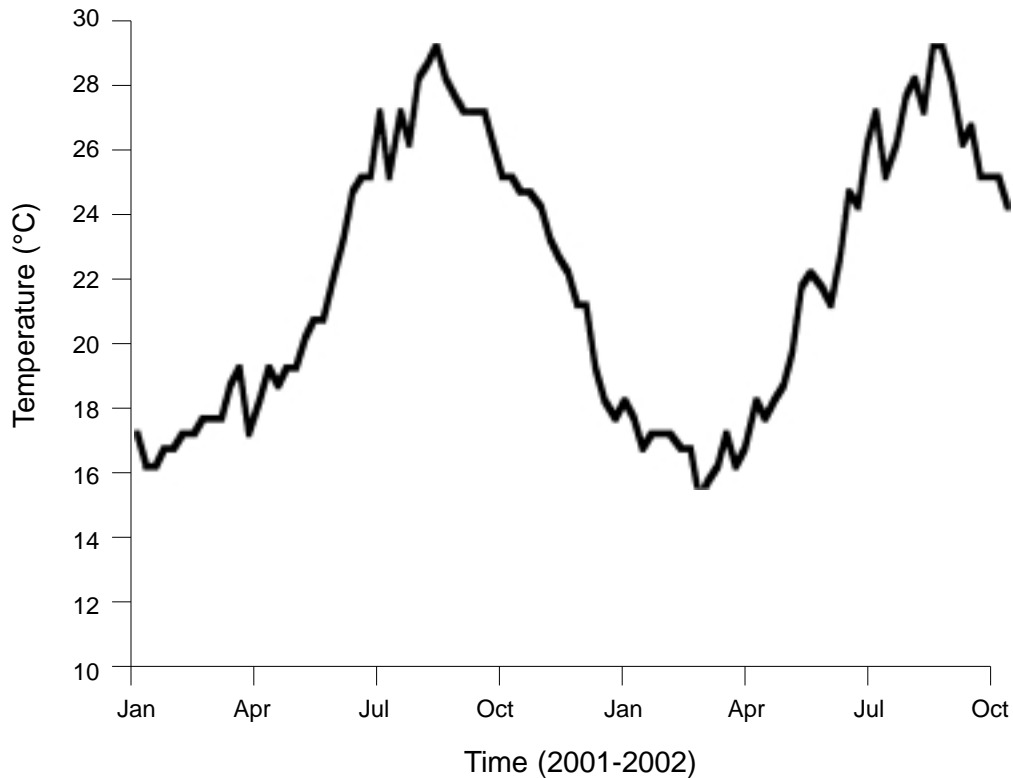
Fig. 1. Weekly water temperature in shi drum rearing tanks during experiments in 2001 and 2002.

International, UK). For handling, fish were first lightly anesthetized in the tank using clove oil (Wagner et al., 2002) at 0.01 ml/l and then placed in a separate bath and deeply anesthetized with 0.3 ml phenoxyethanol per liter. Fish were handled according to the European Union Directive (86/609EEC) for the protection of animals used for experimental and other scientific purposes.

Gametogenesis was studied in 2002 by monitoring reproductive maturation stages during monthly samplings, beginning in March. As the ovipore was too narrow in March, the first ovarian biopsies were obtained in April at the same time that spermiation was detected in some males. The spermiation condition was determined using a subjective scale: 0 = no milt released, 1 = only

a drop of milt released after multiple stripping attempts, 2 = milt was easily expressed after the first stripping attempt, 3 = copious amounts of milt flowed after the slightest pressure (Rainis et al., 2003). Two males were sacrificed at every sampling to obtain gonad tissue for histological evaluation. Ovarian biopsies were examined under a stereoscope to determine the stage of gonad development and measure the diameter of the largest oocytes. A portion of the ovarian biopsy and dissected testicular tissue was fixed for histological evaluation in a 4% formaldehyde:1% gluteraldehyde buffered saline (McDowell and Trump, 1976), dehydrated in a 75-95% ethanol series and embedded in glycol methacrylate resin (Technovit 7100, Heraeus Külzer GmbH, Germany). The blocks were

then cut into 3 µm sections on a microtome (Biocut 2035, Reichert Jung, Germany), stained with methylene blue/azure II/basic fuchsin (Bennett et al., 1976) and examined under a light microscope.

Sperm quality evaluation. After being completely anesthetized, fish were rinsed with clean sea water to prevent any anesthetic from coming into contact with the collected milt. The genital pore was carefully blot dried and gentle abdominal pressure was applied to force the milt out of the testes. Care was taken to prevent contamination of the samples with feces or urine. During the sampling procedure, we first evaluated the spermiation condition, and then collected a 10-100 µl sample using a microcapillary pipette (stored in a 500 µl microcentrifuge tube on ice) to evaluate motility, density and survival. Sperm quality was evaluated within one hour of sample collection. Sperm density was estimated after 10,501 x dilution in distilled water (Fauvel et al., 1999) by counting the number of spermatozoa in a Neubauer hemocytometer (in duplicates) under 400 x magnification. The percent of spermatozoa exhibiting forward motility immediately after addition of salt water (sperm motility, %) and the duration of forward movement of at least 5% of the viewed spermatozoa (motility duration, min) were evaluated (in duplicate) twice after collection, first immediately after milt collection and then (to evaluate sperm survival) after overnight (24 h) storage at 4°C (without the addition of any extenders). Sperm motility and motility duration were determined on a microscope slide (400 x magnification) immediately after mixing 1 µl of milt with a drop of salt water.

Spawning induction experiments. During the 2001 reproductive season, reproductively mature male and female shi drum were allocated to three groups and treated on July 17 with a saline injection (control), an injection of GnRHa (10 mg/kg) or a GnRHa implant (50 µg/kg). The GnRHa was desGly[10], DAla[6], Pro[9]-NEthylamide mGnRH (Bachem, Switzerland) and the GnRHa implants were prepared as monolithic 2 mm disks using ethylene-vinyl acetate copolymer (Mylonas and Zohar, 2001). At the time of hormone treatment, females had

oocytes with a mean maximum diameter of 550 µm and all males were spermiating. Fish of each treatment (n = 4 females, 5 males) were placed in separate 10-m^3 tanks supplied with surface sea water of ambient temperature (Fig. 1) and fitted with overflow egg collectors. The egg collectors were monitored daily and the eggs were collected and evaluated. The GnRHa injection group received a second injection 10 days after the first.

Based on 2001 results and gonad maturation in 2002, the spawning induction experiments in 2002 were initiated earlier, in June. Fish were again allocated into three treatment groups (n = 4 females, 5 males): a control that received a saline injection, a group that received three 10 µg/kg GnRHa injections at intervals of 10 days (GnRHa-10) and a group that received two 10 µg/kg GnRHa injections at an interval of 20 days (GnRHa-20). At the time of treatment, females had oocytes with a maximum diameter of >500 mm and all males were spermiating. Fish of each treatment were placed in separate 10-m^3 tanks fitted with overflow egg collectors. Spawns were monitored daily and the eggs were collected and evaluated.

Evaluation of egg quality. Eggs from the egg collectors were initially placed in a 10-l bucket to estimate fecundity and fertilization percentage in subsamples of 10 ml. They were left in the bucket for 5 min without agitation to separate the floating (fertilized) from the sinking (dead) eggs. For each spawn, two replicates of 96-well microtiter plates (mct) were loaded with fertilized eggs according to the procedure of Panini et al. (2001), with some modifications. Briefly, viable eggs from the 10-l bucket were taken in a 250 µm mesh filter and rinsed with sterilized sea water. About 200 eggs were placed in a petri dish and, with the assistance of a dissecting microscope, transferred individually with a 200 µl pipette to the wells of the mct plates. The mct plates were covered with a plastic lid, placed in a controlled-temperature incubator and maintained for four days at 21°C. Embryonic and early larval development was evaluated daily, the number of hatched larvae and viable larvae were recorded on day 4.

Statistical analysis. To detect significant changes in oocyte diameter or sperm quality (except spermiation condition) during the reproductive season, data were subjected to one-way analyses of variance (ANOVA), followed by Duncan's New Multiple Range test (DNMR) at a minimum level of significance of $p \leq 0.05$. Data from individual fish on spermiation condition were analyzed by a non-parametric statistic test (Kruskal-Wallis). Statistics were analyzed with a linear statistics software (SuperAnova, Abacus Concepts Inc., USA). Results are presented as means ± standard error of the mean (s.e.m), unless otherwise indicated.

Results

At the first sampling in April 2002, females were already in full vitellogenesis, with the largest oocytes having a mean diameter of 450 µm (Fig. 2). The ovary contained oocytes in different stages of maturation, from primary to fully vitellogenic oocytes (Fig. 3a). The mean diameter of the largest batch of vitellogenic oocytes did not increase statistically (ANOVA, DNMR, $p<0.05$) as the reproductive period proceeded, though mean values in July and August were greater than 500 µm. Fully vitellogenic oocytes had a similar appearance to those of other marine fishes, having a thick
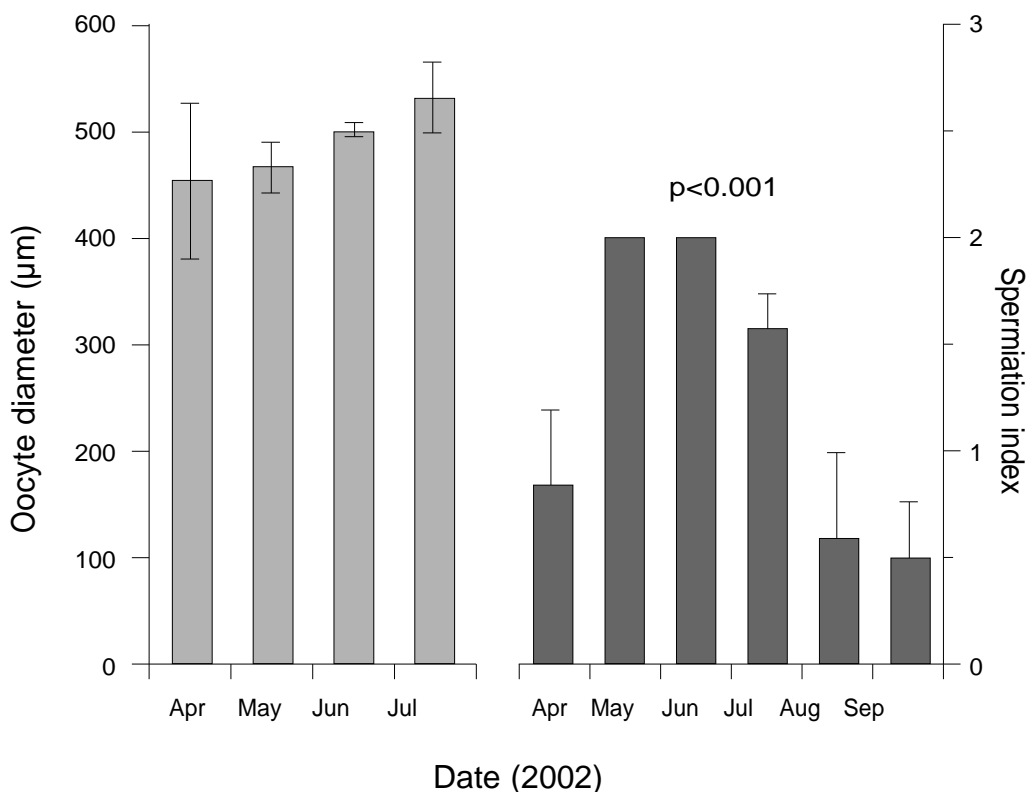

Fig. 2. Mean (± s.e.m.) oocyte diameter (n = 4-10) and spermiation index (n = 5-14) of shi drum. There were no statistical differences between sampling dates for oocyte diameter (ANOVA, DNMR, $p>0.05$) but there were significant differences in spermiation condition (Kruskal-Wallis, $p<0.001$).

zona radiata (zr), central nucleus (germinal vesicle, gv) and lipid droplets and yolk globules (y) dispersed throughout the cytoplasm (Fig. 3b). In the June sampling, most females contained oocytes at the early stage of FOM with the gv migrating towards the periphery and the lipid droplets accumulating in the center of the oocyte and beginning to coalesce (Fig. 3c). At the same time, we observed the onset of atresia in vitellogenic oocytes, with the breaking of the zr and disorganization of the cytoplasm (Fig. 3d). In late July, the majority of the ovary consisted of primary oocytes and the atretic remnants of vitellogenic material (Fig. 3e).

Spermiating fish were first observed in April when a very small volume of milt could be expressed from few males (Fig. 2). All males were spermiating in May and June, but a spermiation index of 3 was never observed. From July onwards, there was a decrease in spermiation and only some of the males produced expressible milt in August and September. Histologically, testes in April contained spermatocysts in all stages of spermatogenesis and a few cysts contained spermatozoa (Fig. 4a). In May, the central part of the testes contained only spermatozoa, whereas the cortical area contained large numbers of spermatocysts with spermato-
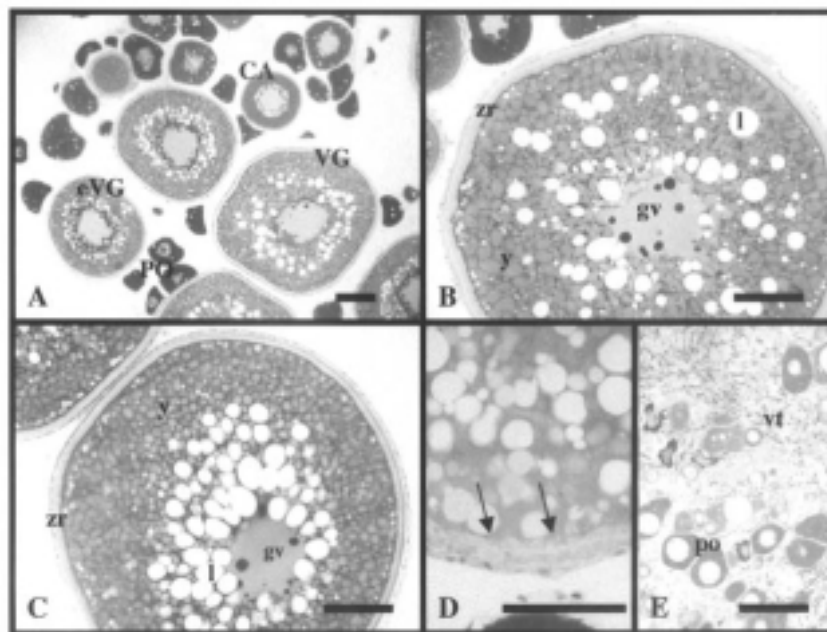

Fig. 3. Histological sections of oocytes in ovarian biopsies of shi drum throughout the 2002 reproductive season. (A) A biopsy from a female in April had oocytes in all stages of oogenesis: primary (po), cortical alveoli (CA), early vitellogenesis (eVG) and mid-vitellogenesis (VG). (B) Also in April, some females contained fully vitellogenic oocytes with a thick zona radiata (zr), a central germinal vesicle (gv), and lipid droplets (l) and yolk globules (y) dispersed throughout the cytoplasm. (C) In June, most females contained oocytes at the early stage of final oocyte maturation when the gv migrates towards the periphery and the lipid droplets accumulate in the center of the oocyte and begin to coalesce. (D) Also in June, we observed the onset of atresia in vitellogenic oocytes with the breaking of the zona radiata (zr) and disorganization of the cytoplasm. (E) In late July, the majority of the ovary consisted of primary oocytes and the atretic remnants of vitellogenic material (vt). The bar at the bottom of each photograph represents 100 μm.

cytes and spermatids (Fig. 4b). At the end of July, testes still contained a large number of spermatozoa, but the somatic cells were markedly hypertrophied and spermatogonia proliferated and lined the walls of the sperm tubules (Fig. 4c). Two-year-old males were immature and their testes contained only spermatogonia throughout the reproductive period (Fig. 4d).

Motility immediately after stripping and after 24 hours of storage at 4°C were similar throughout the spawning season, and were about 80 and 40%, respectively (Fig. 5). On the contrary, sperm density and motility duration increased with time and were significantly greater in July than in May (ANOVA, DNMR, $p<0.05$).

During the 2001 spawning experiments, no spawning was observed in the control fish that were not treated with hormones (Table 1). On the other hand, both GnRHa injected and implanted fish spawned two days after treatment. Injected fish had only one spawn with 65% fertilization success. Hatching percentage was 42% and 4-day larval survival was 80%. The GnRHa-implanted fish spawned a second time, three days after treatment, but neither spawn resulted in fertilized eggs. The group that received a second GnRHa, ten days after the first, spawned on days 2 and 3, but no eggs were fertile.
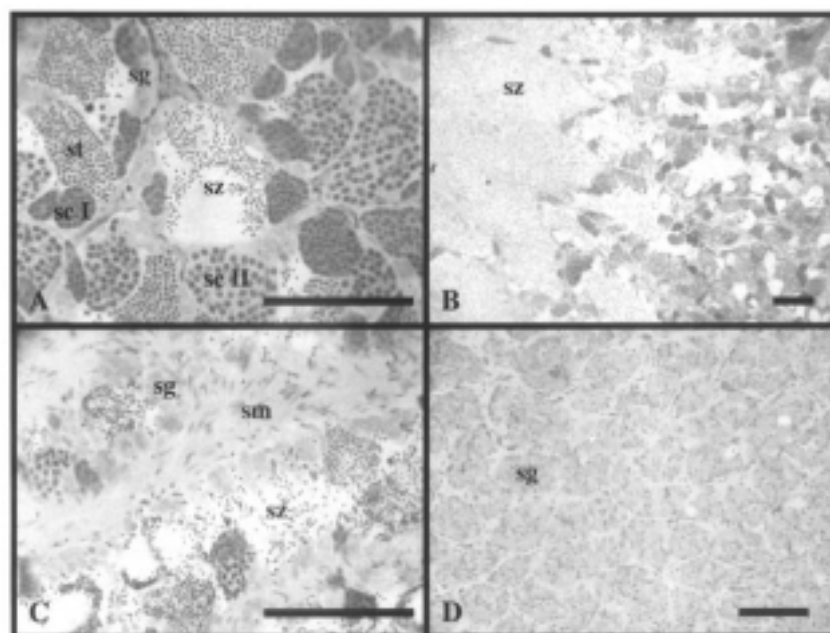
Fig. 4. Histological sections of testes from shi drum during the 2002 reproductive season. (A) Testis in April contained all stages of spermatogenesis including spermatogonia (sg), spermatocytes (sc) I and II, spermatids (st) and a few cysts with mature spermatozoa (sz). (B) At the end of May, males were fully spermiating with the central part of the testes full of spermatozoa (sz) while the cortical area contained cysts at earlier stages of spermatogenesis as well. (C) At the end of July, the testes still contained spermatozoa, but the somatic cells (sm) had begun to proliferate and hypertrophy and large numbers of spermatogonia (sg) lined the sperm tubules. (D) The testes of immature 2-year-old males contained only spermatogonia during the reproductive period. The bar at the bottom of each photograph represents 100 µm.

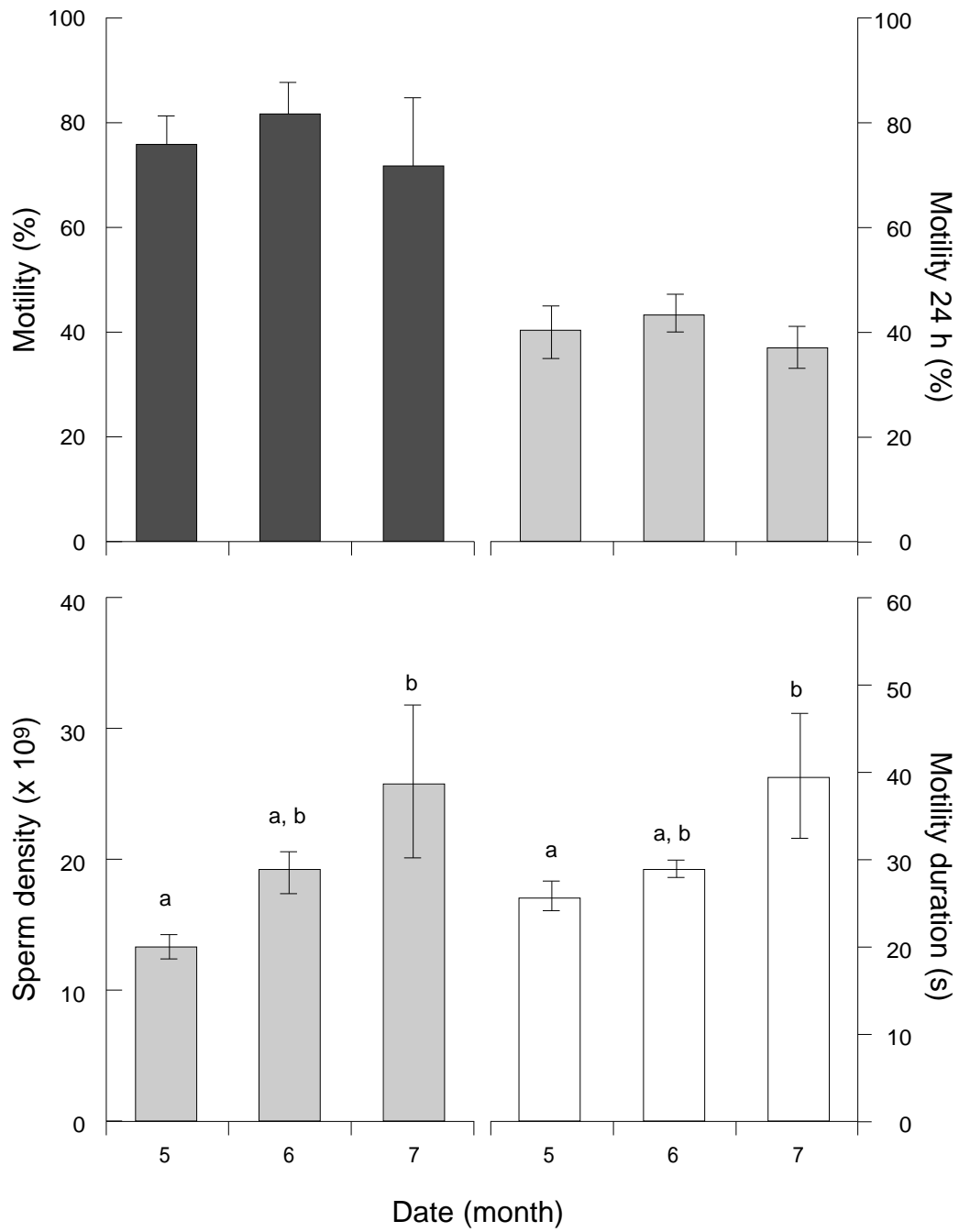Fig. 5. Mean (± s.e.m.) sperm quality parameters of shi drum (n = 6) during the 2002 reproductive season. Different superscripts indicate significant differences among means (ANOVA, DNMR, $p<0.05$).

Table 1. Hormonal induction of spawning in shi drum (*Umbrina cirrosa*) females during 2001. The first GnRHa injection and GnRHa implant were given on July 17. The second GnRHa injection was given on July 27.

	Mean wt (kg)	Oocyte diameter (mm)	Spawns (no.)	Day of spawning	Fecundity (eggs/tank)	Fertilization (%)	Hatching (%)	4 day-survival (%)
Control	2.1	500	none	—	—	—	—	—
GnRHa injection (1st injection)	2.7	500	1	2	236,000	65	42	80
GnRHa injection (2nd injection)			2	2	65,000	0	—	—
GnRHa implant	3.4	500	2	2	267,000	0	—	—
				3	239,000	0	—	—

The control fish also did not spawn during the 2002 spawning experiments. Fish given a GnRHa injection spawned after two days, with the GnRHa-10 group producing 640,000 eggs per tank with 65% fertilization (Table 2). Unfortunately, the egg collector of the GnRHa-20 group overflowed and the exact fecundity and fertilization of this group is unknown. Hatching in eggs produced after the first GnRHa injection ranged 74-76% while the 4-day larval survival was 46-54%. Subsequent injections in the GnRHa-10 group did not induce further spawning, whereas a second injection in the GnRHa-20 group induced another spawning, but with no fertile eggs.

Discussion

Since biopsies could not be taken before April, it is still uncertain when vitellogenesis begins in the shi drum. The facts that females already contained fully vitellogenic oocytes in April and that the mean diameter of the largest batch of oocytes did not increase much after April, suggest that vitellogenesis occurs rapidly compared to other multiple-batch spawning fish. For example, in the red porgy (*Pagrus pagrus*), vitellogenesis begins in December and peaks in March (Kokokiris et al., 2001), whereas in the common dentex (*Dentex dentex*) it starts in February and peaks in April-May (Pavlidis et al., 2000). The difference in the duration of vitellogenesis between these two Sparid fishes and the shi drum is probably due to differences in their reproductive period and the prevailing water temperatures. In red porgy and common dentex the reproductive season is winter-spring at water temperatures of 5-20°C, whereas shi drum reproduce in spring-summer at water temperatures of 17-28°C. It is reasonable to expect that gametogenesis may occur at a faster rate at such higher temperatures. Supporting this hypothesis is the observation that in the greater amberjack *(Seriola dumerili)*, a fish with a spring-summer reproductive period, gametogenesis is completed within a very short period between May and June (Mandich et al., 2003).

As expected from a group-synchronous

Mylonas et al.

Table 2. Hormonal induction of spawning of shi drum (*Umbrina cirrosa*) during 2002. The GnRHa-10 group was given an injection every 10 days and the GnRHa-20 every 20 days. The first GnRHa injection in both groups was given on June 26.

	Mean wt (kg)	Oocyte diameter (µm)	Spawns (no.)	Day of spawning	Fecundity (eggs/tank)	Fertilization (%)	Hatching (%)	4 day-survival (%)
Control		500	none	—	—	—	—	—
GnRHa-10	3.46	500	2	2	640,000	65.6	76	54
GnRHa-10 (2nd injection)		550	none	—	—	—	—	—
GnRHa-10 (3rd injection)		450	none	—	—	—	—	—
GnRHa-20	3.21	500	2	2	610,000*	4.6*	74	46
GnRHa-20 (2nd injection)		500	2	2	63,000	0	—	—

* The egg collector overflowed, therefore the exact fecundity and fertilization are unknown.

multiple-spawning fish, the ovary contained oocytes in different stages of maturation throughout the reproductive period. Early FOM was associated with the accumulation of lipid droplets around the nucleus and the beginning of coalescence, as observed in the European sea bass (Mayer et al., 1988; Asturiano et al., 2000), striped bass (*Morone saxatilis;* Mylonas et al., 1997a) and greater amberjack (Mylonas et al., 2004). Since early FOM oocytes had an oocyte diameter of 500-550 µm and females with this size oocytes successfully ovulated and spawned, we conclude that this is the maximum size of vitellogenic oocytes in the shi drum. Supporting this view, atretic oocytes of a similar size were found in samples obtained in June at the onset of the spawning season. This size is similar to previously measured post-vitellogenic oocytes of shi drum (Barbaro et al., 1996), two Sciaenidae species, the mulloway (*Argyrosomus hololepidotus*; Battaglene and Talbot, 1994) and the red drum (*Sciaenops ocelatus*; Colura, 1987), and other summer spawning marine fishes such as the dusky grouper (*Epinephelus marginatus*; Marino et al., 2003) and greater amberjack (Mylonas et al., 2004).

Spermatogenesis also occurred very rapidly. All males were spermiating within one month after the first detection of individuals expressing some milt, although none reached the highest spermiating index (i.e., 3 - producing copious amounts of milt with very little abdominal pressure), perhaps indicating dysfunctional spermiation in captivity. The presence of a large number of spermatocysts at early stages of spermatogenesis at the onset of the spawning season demonstrates that fish can potentially produce a much higher number of spermatozoa. A similar problem was encountered in cultured flatfishes where milt is limited, of high viscosity and low motility, and presents problems in fertilizing eggs (Suquet et al., 1992a; Clearwater and Crim, 1998; Vermeirssen et al., 1998). The lower spermiation index in July, coupled with the absence of a significant increase in mean oocyte diameter, suggests that shi drum hatcheries should begin spawning induction

between May and June to ensure the availability of adequate amounts of milt. Similarly, the spermiating males dropped from 70% in July to 8% in August in another cultured shi drum study (Barbaro et al., 1996).

Sperm motility immediately after milt collection did not change throughout the spawning season and remained around 80%. This value is similar to some fishes (Sorbera et al., 1996; Lahnsteiner and Patzner, 1998; Suquet et al., 1998; Fauvel et al., 1999; Lahnsteiner and Patzner, 1999; Liley et al., 2002; Mylonas et al., 2003a), but higher than for others (Lahnsteiner et al., 1996; Clearwater and Crim, 1998; Geffen, 1999; Mazorra de Quero et al., 2000). Similarly, sperm density ranged 13-26 x10^9 spermatozoa/ml and was within the range reported for other marine fishes (reviewed in Suquet et al., 1994). For example, sperm density (x 10^9 spermatozoa/ml) was 5-20 in rabbitfish (*Siganus guttatus*; Garcia, 1993), 20-55 in turbot (*Scophthalmus maximus*; Suquet et al., 1992b), 5-55 in the European sea bass (Sorbera et al., 1996), 66 in America shad (*Alosa sapidissima*; Mylonas et al., 1995a) and 60-00 in striped bass (Mylonas et al., 1997b). From May to July, sperm density increased significantly, contrary to what was anticipated. Spermiation is caused by an LH-controlled increase in seminal fluid production (Clemens and Grant, 1965), which increases the intra-testicular pressure and enables the spermatozoa to be transported to the sperm ducts (Billard, 1986). Therefore, a decrease in sperm density of expressible milt is considered a physiological phenomenon during the early spermiation period (Fostier et al., 1982; Büyükhatipoglu and Holtz, 1984; Aas et al., 1991; Jackson and Sullivan, 1995). The sperm density in some fishes, however, increases during the spawning period (Pironet and Neira, 1998; Suquet et al., 1998; Fauvel et al., 1999; Rakitin et al., 1999) and, in some, the resulting high viscosity is associated with dramatic reductions in spermatozoa motility (Vermeirssen et al., 1998; Mazorra de Quero et al., 2000; Vermeirssen et al., 2000). Although drastic reductions were not observed in the shi drum, the reduction in

spermiation index suggests the possibility that the increase in sperm density resulted from a reduction in seminal fluid production after June.

Sperm motility after overnight storage at 4°C declined significantly, but remained close to 40% throughout the spawning season. Survival of spermatozoa for a long period after stripping, without chemical extenders or complicated cryopreservation techniques (Rana, 1995), is important for hatcheries using *in vitro* fertilization techniques (Libertini et al., 1998). Extended survival times allow hatchery managers to collect milt from all males at once, evaluate it and store it, and then use it to inseminate eggs from females induced to ovulate at different hours or days. Survival of sperm stored at 4°C can vary from one day in salmonids (Billard et al., 1995) to 6-8 days in the common carp (*Cyprinus carpio*; Saad et al., 1988) and up to 14 days in the red porgy (Mylonas et al., 2003a). Loss of sperm viability and motility is the result of depletion of important substrates, lack of oxygen, enzymatic degradation, cell lysis or bacterial growth (Billard et al., 1995; Lahnsteiner et al., 1997). Therefore, addition of oxygen and antibiotics may extend the survival of stored shi drum sperm.

The duration of forward motility of shi drum spermatozoa ranged 26-40 s and was rather short compared to most marine fishes. For example, motility duration was 48 s in Mediterranean horse mackerel (*Trachurus mediterraneus*) and 1.5 min in red mullet (*Mullus barbatus*; Lahnsteiner and Patzner, 1998), 42 s-2.9 min in European sea bass (Fauvel et al., 1999; Rainis et al., 2003), 1.4 min in striped bass (Holland et al., 1996), 1.7 min in turbot (Chauvaud et al., 1995), 3-4 min in red porgy (Mylonas et al., 2003a) and 2.9-6.0 min in Atlantic herring (*Clupea harengus*; Evans and Geffen, 1998). Since studies of spermiating fish in the wild as well as similar studies of cultured shi drum do not exist, it is unclear whether the very brief spermatozoa motility duration observed in the present study is normal or is the effect of a reproductive dysfunction in captivity. What seems certain, however, is that such a limited motility dura-

tion could have a significant negative effect on fertilization success. The significant increase in motility duration observed in shi drum in July was surprising as the increase in sperm density and reduction in spermiation index observed at this time indicated the approaching end of the spermiation season. During this time, aging of the spermatozoa would be expected (Dreanno et al., 1999). In other studies of marine fishes, spermatozoa forward motility remained unchanged during the spermiation period, indicating that as long as any milt could be expressed, spermatozoa quality was unchanged (Fauvel et al., 1999; Mylonas et al., 2003a, Rainis et al., 2003).

The complete absence of spawning in control females during the present study, as well as the occurence of atretic oocytes in biopsies obtained at the onset of the spawning season, underline the seriousness of the reproductive dysfunction observed in cultured shi drum. Failure of female broodfish to undergo FOM at the completion of vitellogenesis is a common reproductive dysfunction of cultured fishes (Zohar, 1989) and is probably caused by the absence of an appropriate spawning environment, as well as stressors imposed by captivity. Studies of gilthead seabream and striped bass demonstrated that this is caused by an endocrinological failure at the level of the hypothalamus-pituitary, resulting in absence of release of LH (Zohar et al., 1995; Mylonas et al., 1998a). Similar studies have not yet been undertaken in the shi drum, but the success of GnRHa in inducing spawning (Melotti et al., 1995; Cardellini et al., 1999; Mylonas et al., 2000; Barbaro et al., 2002) suggests that a similar dysfunction may exist in this species.

Controlled-release GnRHa-delivery systems have been successfully employed in a variety of marine fishes to induce FOM, ovulation and spawning (Mylonas and Zohar, 2001). Treatment of females at the onset of the spawning period results in increasing (Sokolowska et al., 1984; Breton et al., 1990; Mylonas et al., 1998a) or constantly high plasma LH levels (Zohar, 1996). As a result of the sustained elevation in plasma LH, multiple spawns of good quality eggs were induced in

various fishes with asynchronous (Barbaro et al., 1997; Berlinsky et al., 1997; Larsson et al., 1997; Mugnier et al., 2000) or multiple-batch group-synchronous patterns of oocyte maturation (Almendras et al., 1988; Mylonas et al., 1995b; Mylonas et al., 1997c; Morehead et al., 1998; Mugnier et al., 2000; Marino et al., 2003). In these species, it appears that constantly high or increasing levels of plasma LH during the spawning period provide an appropriate endocrine signal for the ovary to undergo the cyclical processes of oocyte maturation and ovulation, while at the same time maintain the process of vitellogenesis in subsequent batches. Controlled-release GnRHa-delivery systems have showed some success in the shi drum (Barbaro et al., 1996; Cardellini et al., 1999; Mylonas et al., 2000; Barbaro et al., 2002) but, although GnRHa implants in the present study induced high-fecundity spawns two and three days after treatment, the eggs appeared to be unfertilized. On the contrary, during both years a GnRHa injection was successful in inducing a single spawn with a good percentage of fecundity and fertilization. Therefore, it appears that a pulse of GnRHa may be a more appropriate stimulus for the induction of FOM in the shi drum, compared to the sustained presence of GnRHa achieved by the implants. A similar observation was made in the European sea bass, which also has a multiple-batch group-synchronous ovarian morphology (Forniés et al., 2001; Mylonas et al., 2003b). In these studies, although GnRHa implants induced multiple spawns, spawning synchronization and egg quality improved with the use of injections of GnRHa spaced 7-14 days apart.

One explanation for the differences of shi drum and the European sea bass in spawning success after GnRHa implantation, compared to other multiple spawning species, may be a greater interval between natural spawning events. For example, the ovulation/spawning interval was one day for Asian sea bass (*Lates calcarifer*; Almendras et al., 1988), summer flounder (*Paralichthys dentatus*; Berlinsky et al., 1997), yellowtail flounder (*Pleuronectes ferrugineus;* Larsson et al., 1997) and gilthead seabream (Barbaro et al.,

1997), two days for turbot (Mugnier et al., 2000) and dusky grouper (Marino et al., 2003), and 2-4 days for the striped trumpeter (*Latris lineata*; Morehead et al., 1998) and American shad (Mylonas et al., 1995a). In the European sea bass, the spawning interval was suggested to be about seven days (Asturiano et al., 2000; Mylonas et al., 2003b). Recruitment of subsequent batches of oocytes and completion of vitellogenesis between spawnings may require a period of relatively low plasma LH, while FOM and ovulation requires acute increases in plasma LH (Peter and Yu, 1997). No information on the natural spawning kinetics in shi drum is available. The failure of GnRHa-delivery systems to induce multiple spawns after treatment (Mylonas et al., 2000; Barbaro et al., 2002) while further spawns were induced by a GnRHa injection (e.g., after ten days in 2001 and twenty days in 2002) suggest that shi drum may require a two-level gonadotropic stimulation. Further research should examine the spawning kinetics of individual shi drum and follow the hormonal profiles of relevant reproductive hormones (e.g., LH and sex steroids) after stimulation with GnRHa in different modes of administration.

Fertilization success of the first spawn after the GnRHa injection was similar to that in other shi drum studies (Melotti et al., 1995; Barbaro et al., 1996; Libertini et al., 1998; Barbaro et al., 2002). However, a second injection and GnRHa implant did not result in any fertilized eggs, a common observation in species requiring the hormonal treatment for the induction of FOM (Garcia et al., 2001; Barbaro et al., 2002; Mylonas et al., 2003b). Low fertilization percentages could be explained by the fact that males never reached spermiation index 3 during the spawning season (thus the volume of milt was limited), or that the duration of spermatozoa motility was extremely brief. However, the complete absence of fertilization points to poor synchronization of gamete release between males and females, or a failure of females to release eggs immediately after ovulation. Release of ovulated eggs long after ovulation may mean that the eggs could

not be fertilized, even with ample sperm. This is because once the eggs are ovulated into the ovarian cavity they loose their nutrients and oxygen. The "latency period" during which viability is maintained after ovulation varies greatly among species. For example, this period extends to a few weeks in salmonids (Springate et al., 1984), 4-6 hours in Atlantic halibut (*Hippoglossus hippoglossus*; Bromage et al., 1994), 1-2 hours in groupers of the genus *Epinephelus* (Tucker, 1994), and only 30 min in the white bass (*Morone chrysops*; Mylonas et al., 1996). Based on available information, we believe that the absence of fertilization success in the present study might be due to the failure of females to release eggs at the appropriate time after ovulation.

In conclusion, the present study demonstrates that gametogenesis in the shi drum is a rapid process and can proceed normally in captivity. However, sperm quality may be reduced and there is complete failure of females to undergo spontaneous FOM, ovulation and spawning. The limited success of GnRHa therapy to induce spawning suggests that the reproductive dysfunction in females is related to the absence of LH release at the onset of the reproductive season. Further research should be undertaken to develop methods, through both environmental manipulations and pharmacological treatments, (a) to improve sperm quantity and quality and (b) to enhance egg production by inducing multiple spawns of fertile eggs.

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