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INDUCTION OF OVULATION AND SPAWNING IN THE MEDITERRANEAN RED PORGY, *PAGRUS PAGRUS*, BY CONTROLLED DELIVERY AND ACUTE INJECTION OF GnRHa

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Abstract

Gonadotropin-releasing hormone analogue (GnRHa) in the form of saline injections or sustained-release microspheres was used to induce oocyte maturation, ovulation, and spawning in captive red porgy (*Pagrus pagrus*). Individually tagged vitellogenic females (n = 9 or 10) were treated at the beginning of the spawning season (March) with 20 µg/kg body weight (bw) GnRHa-loaded microspheres, a single injection of 20 µg/kg bw dissolved in saline, or physiological saline (control). Females were placed in tanks (one tank per treatment) connected to overflow egg collectors and monitored for 11 days. In addition to the eggs collected from the tank overflow, eggs were stripped from the fish on a daily basis. Only one spawn was obtained from the control fish, probably from a single female, given the small relative fecundity (700 eggs/kg bw). On the contrary, treatment with a GnRHa injection produced two spawns (9 and 11 days after treatment) and 50% of the fish ovulated. Treatment with GnRHa microspheres induced seven spawns (3 and 6-11 days after treatment) and 100% of the females ovulated. Females did not spawn all the eggs ovulated on a particular day, evident from the significant number of eggs obtained by manual stripping. Egg quality did not significantly differ among treatments, whereas number of spawned eggs and total relative fecundity were significantly higher in fish treated with GnRHa microspheres (ANOVA, $p<0.05$). The results demonstrate the potential of GnRHa-loaded microspheres to induce spawning in red porgy, as a method of overcoming spawning failures in commercial hatcheries.

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Introduction

The red porgy (*Pagrus pagrus* L. 1758) is a marine fish of great commercial importance to the fishing industry in both the Mediterranean Sea and the Atlantic Ocean (Vassilopoulou and Papacon-stantinou, 1992; Harris and McGovern, 1997). Due to its wide geographical distribution, high market demand, and good growth rates (Pajuelo and Lorenzo, 1996; Maragoudaki et al., 1999), there is strong interest in establishing captive brood stocks and commercially growing the red porgy (Kolios et al., 1997; Bodington, 2000). However, commercial production is limited, primarily because of problems with skin discoloration in growout facilities (Kolios et al., 1997; Cejas et al., 2003) but also because of inconsistent spawning (Zohar and Mylonas, 2001).

The red porgy is a protogynous hermaphroditic species with asynchronous ovarian development. In captivity, it reaches puberty at 3-5 years of age (Kokokiris et al., 1999). Depending on latitude, spawning occurs from February to mid-June (Vaughan et al., 1992; Pajuelo and Lorenzo, 1996; Kokokiris et al., 2001). Studies on spawning kinetics, fecundity, and egg quality in captivity recently demonstrated the existence of significant variations in spawning and egg production characteristics among seemingly identical groups, as well as within the same brood stock in different spawning seasons (Mylonas et al., 2004). Such variations in egg production can create significant problems in farm planning and there is a need to develop methods to induce ovulation and reliable spawning to optimize spawning performance in captivity.

Important advances in the manipulation of spawning in a variety of farmed fish have been made by using gonadotropin releasing hormone agonists (GnRHa; Crim and Bettles, 1997; Zohar and Mylonas, 2001). Injected GnRHa stimulates the synthesis and release of the endogenous luteinizing hormone (LH; Breton et al., 1990), the hormone controlling final oocyte maturation (FOM) and ovulation (Nagahama et al., 1994), through stimulation of steroidogenesis and production of the maturation inducing steroid (Goetz and Garczynski, 1997). GnRHa can be administered in the form of saline injections or sustained-release delivery (Weil and Crim, 1983; Crim and Bettles, 1997; Mylonas and Zohar, 2001). Sustained administration of GnRHa is advantageous compared to the injection approach, especially in fishes with asynchronous ovaries, because it stimulates long-term elevations of plasma LH, resulting in multiple spawnings. Some of these species include the gilthead sea bream (*Sparus aurata*; Zohar et al., 1995), yellowtail flounder (*Pleuronectes ferrugineus*; Larsson et al., 1997), turbot (*Scophthalmus maximus*; Mugnier et al., 2000), and dusky grouper (*Epinephelus marginatus*; Marino et al., 2003). The alternative method of multiple GnRHa injections is labor-intensive and can be harmful to brood fish due to the frequent handling required.

The objective of the present study was to evaluate the effectiveness of two GnRHa delivery systems in inducing ovulation and spawning in the red porgy and the resulting production of viable eggs.

Material and Methods

In March, at the beginning of the red porgy spawning period (Kokokiris et al., 2001), 3-year-old females (first spawning season) were selected after biopsy of the gonads indicated that they were sexually mature. For the biopsy, females were anesthetized with 2-phenoxyethanol (0.4 ml/l) and a 2 mm polyethylene tube was introduced through the genital pore. At the same time the fish were implanted intraperitoneally with passive integrated transponder tags (P.I.T.; Fish Eagle Co., UK). The biopsy samples were placed in 0.9 g/l NaCl solution at 4°C for up to 6 h and the diameters of the 30 largest oocytes were measured under a dissecting microscope. Only females with a mean oocyte diameter above 500 µm were selected for the experiment.

Females were randomly allocated to three treatments (n = 9 or 10), each in one 2-m^3 tank. The tanks were supplied with running, aerated seawater (salinity 38-40 psu, pH 7.8-8.0, temperature 17-18°C), renewed at a rate of 25% per hour. One group was injected with 20 µg/kg body weight (bw) of [des-Gly^{10},

$_{D}$Ala^{6}, Pro^{9}-NEt]-GnRHa (Bachem, California) in 0.9% NaCl as the vehicle. The second group was injected with a suspension of biodegradable microspheres at a GnRHa dose of 20 µg/kg. The third group was injected with saline as a control. The GnRHa microspheres were prepared according to Mylonas et al. (1995) using poly-[fatty acid dimer-sebasic acid] and were suspended in a solution containing 0.8% agar and 5% glucose at a concentration of 50 mg microspheres/ml. The microspheres were implanted into the dorsal musculature using a syringe with an 18-gauge needle. Two or three spermiating males were placed in each tank to enable spawning and egg fertilization. Overflow egg collectors were connected to the outlet of the tanks and spawned eggs were collected from the collectors daily.

The experiment lasted 11 days after treatment. Each day all fish were anesthetized, weighed, and checked for ovulation by applying gentle abdominal pressure to strip unreleased eggs. Eggs were collected into volumetric cylinders and their volume was recorded. Eggs from the egg collector were placed in 10-l buckets and their number and the percentage of fertilized eggs were estimated by sub-sampling and examination under a dissecting microscope. Fertilization (%) was calculated as the number of fertilized eggs undergoing normal cell division divided by the total number of spawned eggs.

Data on daily ovulated eggs, daily spawned eggs, total daily fecundity, and fertilization percentage were analyzed using one-way of variance (ANOVA) followed by the post-hoc Dunkan's multiple range test to compare mean levels at different times or treatments (STATISTICA, StatSoft Inc, USA). Bartlett's test was used to verify the homogeneity of variances. When necessary, data were log transformed before subjection to ANOVA.

Results

Control females spawned only once, 9 days after treatment (Fig. 1), and no eggs could be stripped from them (Fig. 2). Females given a single GnRHa injection spawned on days 9 and 11 (Fig. 1), while ovulating females were detected only on day 10 when five were found to have ovulated (Fig. 2). On the contrary, all females given GnRHa microspheres were found to have ovulated during the monitoring period. Females of this group spawned on days 3 and 6-11 (Fig. 1) and eggs were stripped from some females on days 3 and 7-10 (Fig. 2). The mean number of ovulated eggs produced by microsphere females was higher although not significantly different from that of the injected females (p>0,05; Table 1) while mean daily spawning fecundity was significantly higher (p = 0.01) in the microsphere group (Table 1). Individual tagging showed that three of the nine females given GnRHa micropsheres ovulated at least twice while the rest ovulated at least once. As daily spawns were recorded in the microsphere group, some of the ovulating females may have already spawned by the time of ovulation monitoring, so that no more eggs could be stripped. Therefore, it is possible that three or more sequential spawns occurred in some of the females in this group. Overall mean total relative fecundity (including spawned and ovulated eggs) was significantly higher in the microsphere group than in the injected group (p<0.01). There was no difference in fertilization rate (%) between eggs from these groups. The single spawn from the control group (Fig. 3) had a fertilization rate of 20%.

Discussion

Treatment with GnRHa, both as a single injection and as sustained-release microspheres, induced multiple spawning or ovulation in 3-year-old female red porgy and increased egg production compared to untreated fish. However, only the microsphere treatment induced ovulation in 100% of the females and, by stimulating multiple FOM cycles, resulted in significant increases in the daily number of eggs produced and the total relative fecundity. These results confirm previous findings that the red porgy is a serial spawner with spawning intervals of 1-3 days (Kokokiris et al., 2001; Mylonas et al., 2004). The effectiveness of GnRHa from sustained-release devices in inducing multiple spawning agrees with results in other reared marine species such as the gilthead sea bream (Zohar et al.,

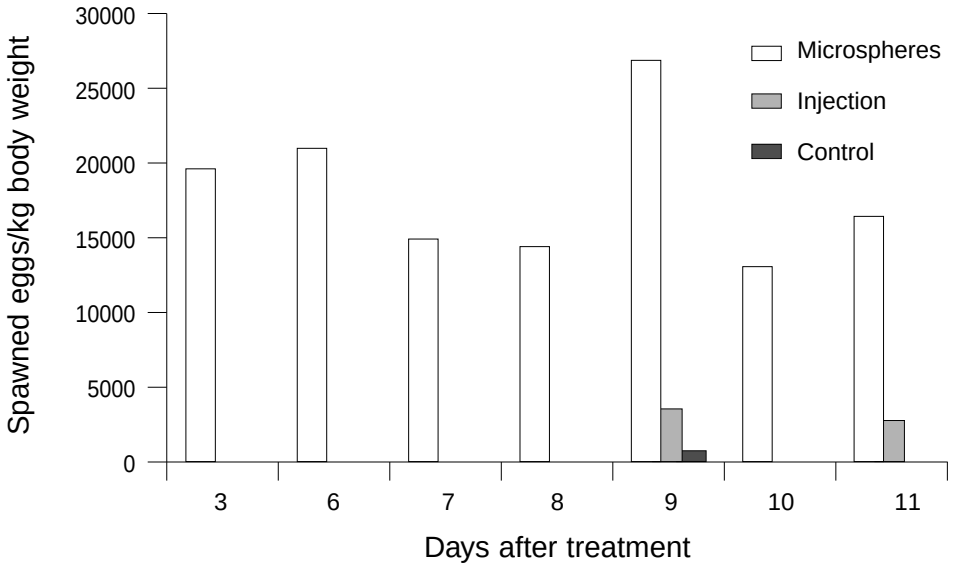

Fig. 1. Number of spawned eggs in egg collection trays from female red porgy treated with a GnRHa injection, controlled-release GnRHa microspheres, or saline control.

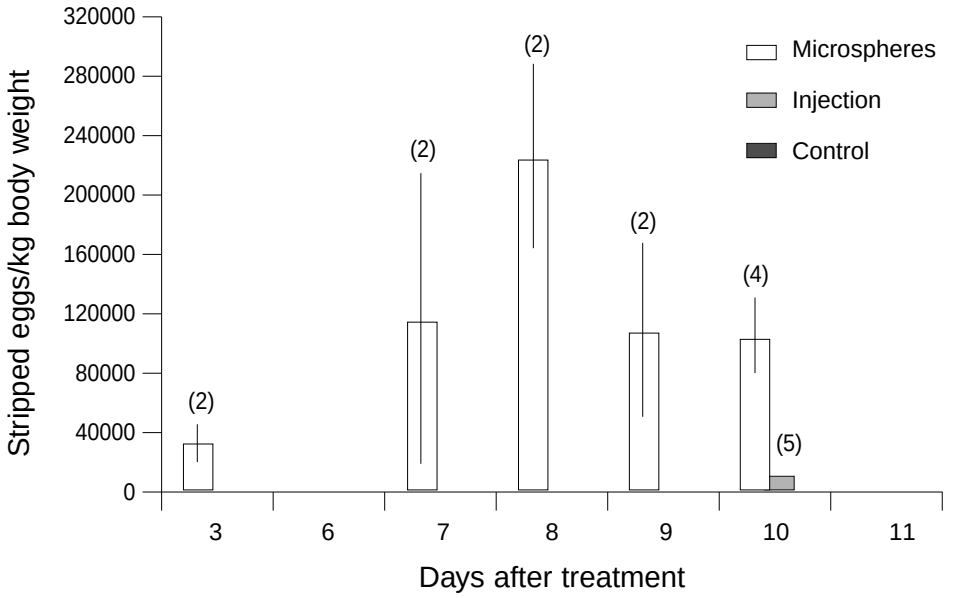

Fig. 2. Mean (±SEM) daily number of eggs obtained by stripping female red porgy treated with a GnRHa injection or controlled-release GnRHa microspheres. Numbers in parentheses indicate number of females that released eggs after gentle abdominal pressure. No eggs were stripped from females in the control.

Table 1. Mean (±SEM) daily egg production parameters of red porgy after treatment with GnRHa via injection or controlled-release microspheres. Egg numbers are expressed per kg body weight of the females in the population.

Treatment	*n*	*Body wt (kg)*	*Ovulation success (%)*[1]	*Spawned (x 1000/kg)*[2]	*Stripped eggs (x 1000/kg)*[3]	*Relative fecundity (x 1000/kg)*[4]	*Fertilization rate (%)*
Control	9	1.20±0.06	0	0.7[a]	0[a]	0.7[a]	20[a]
Injection	10	1.30±0.08	50	2.2±1.1[a]	1.5±1.5[a]	3.8±0.4[b]	37±25[ab]
Microspheres	9	1.15±0.03	100	18.2±2.4 [b]	23.0±8.5[a]	41.2±7.7[c]	61±6.5[b]

Values in a column with different superscripts are significantly different (p<0.05).
[1] Percentage of females that ovulated during the study.
[2] Number of eggs obtained from the egg collector after tank spawning.
[3] Number of eggs obtained by manual stripping during daily monitoring.
[4] Sum of ovulated and spawned eggs.

1995), white bass (*Morone saxatilis*; Mylonas et al., 1996), turbot (Mugnier et al., 2000), European sea bass (*Dicentrarchus labrax*; Forniés et al., 2001), and common dentex (*Dentex dentex*; Greenwood et al., 2001). Treatment with GnRHa has also been effective at inducing ovulation in the congeners red sea bream (*Pagrus major*; Matsuyama et al., 1995) and red snapper (*Pagrus auratus*; Pankhurst, 1994).

Compared to a single injection of GnRHa, treatment with GnRHa microspheres was more efficient in terms of number of fish that ovulated, number of ovulations, and fecundity. It is likely that plasma GnRHa remains elevated for a long time allowing a continuously elevated plasma LH that promotes multiple cycles of FOM, ovulation, and spawning (Zohar et al., 1995). The GnRHa microspheres used in this study were shown to produce elevated plasma GnRHa levels for periods of 3-8 weeks (Mylonas et al., 1995). In the European sea bass, a single GnRHa injection induced a short-term elevation of LH lasting only two days, whereas GnRHa implants and microspheres induced a sustained elevation of plasma LH for 35-44 days (Mañanos et al., 2002). A similar observation was made in the common dentex, where GnRHa implants caused sustained elevation of GnRHa for a longer period than did an acute injection (Greenwood et al., 2001).

Although the majority of eggs were obtained by manual stripping, spawning took place in all groups during the course of the experiment. However, it was more regular in females treated with GnRHa microspheres. It is uncertain whether eggs obtained by stripping would have eventually been naturally spawned if the fish were not handled every day during the study. However, in at least three species, the white grouper (*Epinephelus aeneus*), the tiger puffer (*Takifugu rubripes*), and the dusky grouper (*Epinephelus marginatus*), it has been proven necessary to strip eggs obtained by GnRHa induction (Hassin et al., 1997; Matsuyama et al., 1997; Marino et al., 2003). The need to strip ovulated eggs stored in the ovarian cavity may be due to stress caused by rearing conditions, stress

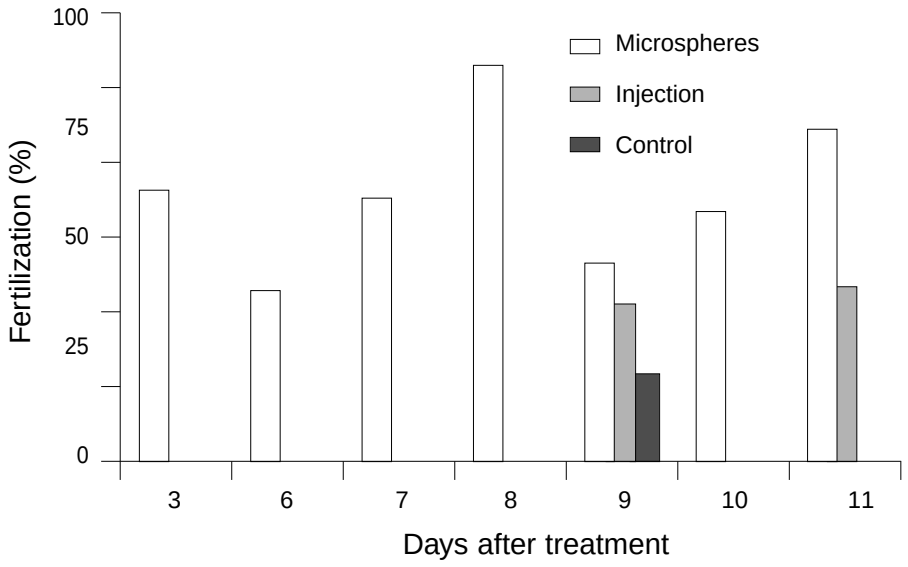

Fig. 3. Fertilization success of eggs spawned by female red porgy treated with a GnRHa injection, controlled-release GnRHa microspheres, or saline control.

due to handling and injecting, or a low number of males in the tank.

Injected females began to ovulate only 10 days after treatment while microsphere females began spawning within three days of treatment, suggesting that the sustained release of GnRHa accelerated spawning. It is likely that the dose of GnRHa administered via injection was inadequate to induce an earlier reaction. Fertilization rates did not significantly differ between eggs of microsphere and injected females and ranged in values similar to those reported for naturally spawning red porgy in captivity (36.9-68.9%; Mylonas et al., 2004).

In conclusion, applying GnRHa through sustained release microspheres appears to be an effective method for inducing FOM, ovulation, and spawning in red porgy females. Such treatment increases the percentage of females undergoing multiple FOM cycles, results in higher fecundity and better synchronization of ovulation, and can be an important tool for broodstock management in the commercial aquaculture of the red porgy.

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