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Effect of exogenous hormones on ovulation and gonadal steroid plasma levels in starry flounder, *Platichthys stellatus*

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Abstract The present study was designed to examine the potential for inducing ovulation in starry flounder (*Platichthys stellatus*) using gonadotropin-releasing hormone analog (GnRHa) and human chorionic gonadotropin (hCG) to assess whether starry flounder are differentially responsive to GnRHa and hCG. Female starry flounder were injected or implanted with different doses of hCG or GnRHa pellets to examine their ovulation-inducing potential and effects on plasma levels of testosterone (T), 17β-estradiol (E2), and 17,20β-dihydroxy-4-pregnen-3-one (17,20βP). Blood samples were collected for up to 10 or 25 days post-injection or post-implantation in two separate experiments designed to mimic the early and middle stages of spawning, respectively. Fish treated with the GnRHa pellets (100 μg) showed a significant increase in the total number of stripped eggs relative to the controls. GnRHa administration had no effect on the floating rate or fertilization rate of ovulated eggs in the both experiments, whereas hCG injection affected both of these rates. Plasma T levels were not significantly different between the exogenous hormone-treated and control fish. In contrast, the plasma E2 level was elevated in those fish treated with GnRHa, regardless of injection or implantation, and was accompanied by increased numbers of stripped eggs in both experiments. Treatment with GnRHa resulted in higher 17,20βP levels compared to the controls, and there was a positive relationship between elevated plasma 17,20βP and an increase in ovulated eggs in response to GnRHa treatment. The implantation of starry flounder with GnRHa-containing pellets was effective at inducing sustained ovulation compared to hCG treatment.

Keywords Starry flounder · *Platichthys stellatus* · Ovulation · Gonadotropin-releasing hormone analog · Human chorionic gonadotropin · Gonadal steroid

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Introduction

Starry flounder (*Platichthys stellatus*) is widely distributed throughout the northern Pacific Ocean, including coastal waters of Korea, Japan, Russia, Canada, and North America. There is growing interest in developing starry flounder aquaculture in Korea and China; however, current techniques are limited by unreliable egg production, similar to many other marine flatfish species. Therefore, the mass yield of viable eggs is the first consideration in developing the commercial culture of this species. In cultured stocks of starry flounder, most females exhibit reproductive dysfunction, including a failure to ovulate and low-quality egg production. A variety of approaches have been used to address these problems, most of which involve artificial manipulation of the endocrine system. These treatments act at different levels in the hypothalamic–pituitary–gonadal axis, and they typically involve treatment with pituitary homogenates, purified gonadotropin preparations, and, more recently, synthetic agonists of gonadotropin-releasing hormone (GnRHa) (reviewed by Zohar and Mylonas 2001).

The injection of piscine or mammalian gonadotropins (GtHs) has been used successfully to induce ovulation in a wide range of species (Donaldson and Hunter 1983; Pankhurst 1998; Poortenaar and Pankhurst 2000; Zohar and Mylonas 2001; King and Pankhurst 2004; Wang et al. 2010). In addition, GnRHa has now been successfully tested in a range of marine and freshwater species and has been shown to be effective in improving egg quantity and quality (Lee et al. 1986, 1987; Poortenaar and Pankhurst 2000; Zohar and Mylonas 2001; King and Pankhurst 2004). GnRHa is a principal stimulator of follicle-stimulating hormone and luteinizing hormone (LH) release in teleost fish. GnRHa stimulates LH release by the pituitary, which in turn promotes the maturational competence of oocytes and production of maturation-inducing steroids (MISs) from the ovary. In many fish, the progesterone 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) does this. The MISs secreted by the induced follicle promote synthesis of the maturation-promoting factor responsible for the resumption of meiotic division and, consequently, oocyte ovulation (Nagahama 1994; Zohar and Mylonas 2001).

If a specific GtH assay was developed for a target fish species, GtH would be a suitable indicator of gonadal development in fish. However, GtH assays are currently limited in most fish; therefore, sex steroids remain the most useful endocrine markers. Testosterone (T) and 17β-estradiol (E2) are commonly measured indicators of ovarian development, and 17,20βP is the putative MIS (Poortenaar and Pankhurst 2000).

Treatment of fish with exogenous hormones by injection typically results in the short-term induction of ovulation and changes in plasma steroids (Ramos 1986; Haddy and Pankhurst 2000; Poortenaar and Pankhurst 2000; Levavi-Sivan et al. 2004; Denson et al. 2007). Treatment of fish with slow-release forms of GnRHa, injection with GnRHa microspheres, or implantation with GnRHa-containing pellets typically results in the slow but stable induction of ovulation over a long period (Lee et al. 1986; Harmin and Crim 1992; Berlinsky et al. 1997; Mylonas and Zohar 2001; King and Pankhurst 2004). Dose-sustained GnRHa release stimulates multiple ovulations, but in some species it also stimulates ovarian development in immature fish (Poortenaar and Pankhurst 2000). Therefore, GtH preparations have now been largely replaced by GnRHa treatment.

In the absence of reliable natural ovulation in cultured stocks, starry flounder egg production currently depends on induced ovulation using exogenous hormones. Thus, it is important to characterize the response to these treatments. Protocol development for brood stock management has included studies of the effects of GnRHa on the stimulation of milt

production and spermiation in male starry flounder (Lim et al. 2002; Moon et al. 2003; Lim and Kim 2007). Treatment of male starry flounder with GnRHa increased the milt volume, confirming the role of GtH in stimulating milt volume increase in this species over long periods. However, the effects of GnRHa on ovulation and egg quality remain unknown.

The aim of this study was to determine the potential for inducing ovulation in starry flounder using GnRHa and human chorionic gonadotropin (hCG) to assess whether starry flounder are differentially responsive to GnRHa and hCG, based on the dose and delivery mode over short and long periods. The response to GnRHa and hCG treatment was assessed in terms of the total number of eggs ovulated, the floating rate, and the fertilization rate of the eggs produced. Endocrine effects were assessed by measuring the plasma levels of E2, T, and 17,20βP.

Materials and methods

Fish and sampling

Female flounder were randomly selected from stocks grown at the East Sea Mariculture Research Center, National Fisheries Research and Development Institute, Uljin, Korea. During the experiment, the fish were maintained at a natural seawater temperature (mean ± SD, 11.0 ± 1.0 °C) and photoperiod and fed daily with moist pellets for olive flounder.

In our experiments, the fish were anesthetized with 200 ppm of ethyl 3-aminobenzoate methanesulfonate salt (Sigma, St. Louis, MO, USA), weighed, tagged for identification with PIT tags (Biomark Inc., Idaho, USA), and injected or implanted as described below. Blood samples were obtained by caudal puncture using pre-heparinized 23G needles. Subsequent blood samples were taken at various times post-treatment as described below. Plasma was separated from the blood by centrifugation at 18,000 × *g* for 5 min at 4 °C and frozen at −70 °C until hormone analysis. The numbers of total eggs and floated eggs were also measured.

Experiment 1: effect of GnRHa and hCG injection early in the spawning season

The first experiment lasted 15 days, when cultured fish are typically in the early stages of spawning (Lim et al. 2007). The mean weight of the fish used was 1503.8 ± 245.0 g. The fish were divided into five treatment groups, anesthetized, and treated with: (1) saline (control), (2) GnRHa salmon (Sigma) at 100 μg kg^{-1} body weight (BW), (3) GnRHa salmon at 200 μg kg^{-1} BW, (4) hCG (Sigma) at 50 IU kg^{-1} BW, and (5) hCG at 100 IU kg^{-1} BW ($n = 6$ fish per treatment). All treatments were administered by intramuscular injection (i.m.). Blood was collected from each fish immediately before injection (day 0) and at 1, 3, 5, 6, 8, and 10 days post-injection (p.i.). At 5 days p.i., each fish received a second injection with the same dose.

Experiment 2: effect of GnRHa treatment during the middle of the spawning season

The second experiment was conducted for 30 days, when cultured fish are typically in the middle stages of spawning (Lim et al. 2007). The fish in this experiment had a mean weight

of 1473.9 ± 116.2 g. Fish were implanted with either a blank pellet (control) or pellets containing either 100 or 200 μg kg^{-1} BW ($n = 7$ fish per treatment).

GnRHa pellets were produced according to Lee et al. (1986). LHRH salmon was dissolved in 50 % ethanol and mixed with cholesterol powder. The mixture was dried, combined with molten cocoa butter, and compressed into pellets. The pellets were individually prepared according to the BW of each fish and implanted into the dorsal muscle.

The fish were bled at the time of implantation (day 0) and at 1, 3, 5, 10, 15, 20, and 25 days post-implantation (p.im.).

Hormone measurements

Plasma steroids were extracted with ethyl acetate and the concentrations were measured using a radioimmunoassay for T, 11KT, and 17,20βP as described by Aida et al. (1984). Rabbit anti-E2-6-CMO-BSA, anti-T-6-CMO-BSA, and anti-17,20β-P-3-CMO-BSA sera were purchased from Cosmo Bio Co. Ltd. (Tokyo, Japan). Non-radioactive steroid standards were purchased from Steraloids Inc. (Wilton, NH, USA). Radio-labeled T and E2 ([2,4,6,7-^{3}H]-T and [2,4,6,7-^{3}H]-E2) were purchased from Amersham Biosciences (Piscataway, NJ, USA). Radio-labeled 17,20β-P was prepared from [1,2,6,7-^{3}H]-17α-OHP (Amersham Biosciences) by enzymatic conversion as described by Young et al. (1983) and separated from the parent compound by thin-layer chromatography. The assay sensitivities were 12.5, 10, and 10 pg ml^{-1} for E2, T, and 17,20β-P, respectively.

Ovulation and egg quality measurements

The fish were examined for ovulation by serial waves of abdominal pressure. Any eggs expressed were collected and measured with a 1000-ml glass cylinder, and their total and floating volumes recorded. The eggs were stripped daily for 15 and 30 days after treatment in both experiments, respectively. The number of eggs per ml was estimated (900 eggs per ml) based on counts of 1-ml subsamples ($n = 10$), and the buoyant rate of the ovulated eggs was used to assess egg quality. A subsample of eggs was transferred to 250-ml glass beakers and fertilized in triplicate by adding pooled milt with filtered seawater. The eggs and milt were mixed for approximately 5 min and incubated at 10 °C. The fertilization rate was determined after 8 h (8–16 cells) by the microscopic examination of approximately 30 eggs per batch.

Statistics

All data are expressed as the mean ± standard error of the mean (SEM); *t* tests or one-way ANOVA followed by Tukey's mean comparison test was used to analyze the data with the SPSS computer package. The level of statistical significance was set at $P < 0.05$.

Results

Experiment 1: effect of GnRHa and hCG injection early in the spawning season

The number of stripped eggs per 100 g BW increased in fish injected with 100 μg of GnRHa compared with the other fish, although the difference was not significant. The

Table 1 Effect of exogenous hormone injection on the number of stripped eggs, floating rate, and fertilization rate

Treatment	Number of stripped eggs (100 g^{-1} BW)	Floating rate (%)	Fertilization rate (%)
Control	1555 ± 1214	26.5 ± 0.6	96.5 ± 3.5^{c}
GnRHa 100	3907 ± 1302	52.4 ± 11.5	93.1 ± 2.6^{bc}
GnRHa 200	1890 ± 613	51.1 ± 11.8	92.6 ± 2.9^{bc}
hCG 50	1405*	25.0 ± 0	69.0 ± 0^{a}
hCG 100	1882 ± 554	31.8 ± 18.2	82.4 ± 4.2^{b}

Different superscripts indicate significant differences between treatments ($P < 0.05$)

* One time stripped

floating rate ranged from 25 to 54.1 ± 11.5 %, and there were no differences between the experimental groups; however, the fertilization rate was lower in the 50- and 100-IU hCG group compared to the control group (Table 1). Fish injected with 50 IU of hCG ovulated only once during the experimental period. The plasma E2 levels were significantly greater in fish treated with 100 and 200 μg of GnRHa compared to the controls at 1 and 3 days p.im.; there was no difference at 6 and 10 days p.im. The plasma T levels in fish injected with GnRHa and hCG were not different from the controls at 1–5 days p.im. The plasma 17,20βP levels of the fish in the control group remained lower throughout the experiment. However, fish treated with 100 μg of GnRHa were significantly elevated from 1 day p.im., compared to the control group, except at 3 days p.im. (Fig. 1).

Experiment 2: effect of GnRHa treatment during the middle of the spawning season

The number of stripped eggs per 100 g BW in experiment 2 showed the same pattern as in experiment 1, with a significant increase in the 100-μg GnRHa pellet-treated group. There was no effect of GnRHa treatment on the floating rate and fertilization rate between the experimental groups (Table 2).

At 1 and 3 days p.im., the plasma E2 levels were significantly greater in those fish implanted with 100 μg of GnRHa pellets compared to the controls, and the values decreased rapidly from 10 days p.im. The plasma T levels in those fish treated with GnRHa pellets were not different from those of the controls at any time, except at 20 and 25 days p.im., when the 100- and 200-μg group values were lower than those of the controls. The plasma 17,20βP levels in the 100-μg GnRHa implanted fish were significantly elevated at days 1, 3, and 5 p.im., compared to the control group (Fig. 2).

Discussion

Under culture conditions, fully grown starry flounder oocytes can be retained in the ovary without ovulation, and the stripped eggs show low fertility. Fish farmers have used various hormones to promote ovulation in hatcheries, but the results have been far from satisfactory in many cases. GnRHa and hCG treatment has been tested successfully in starry flounder males and has been shown to be an effective strategy for enhancing milt quantity

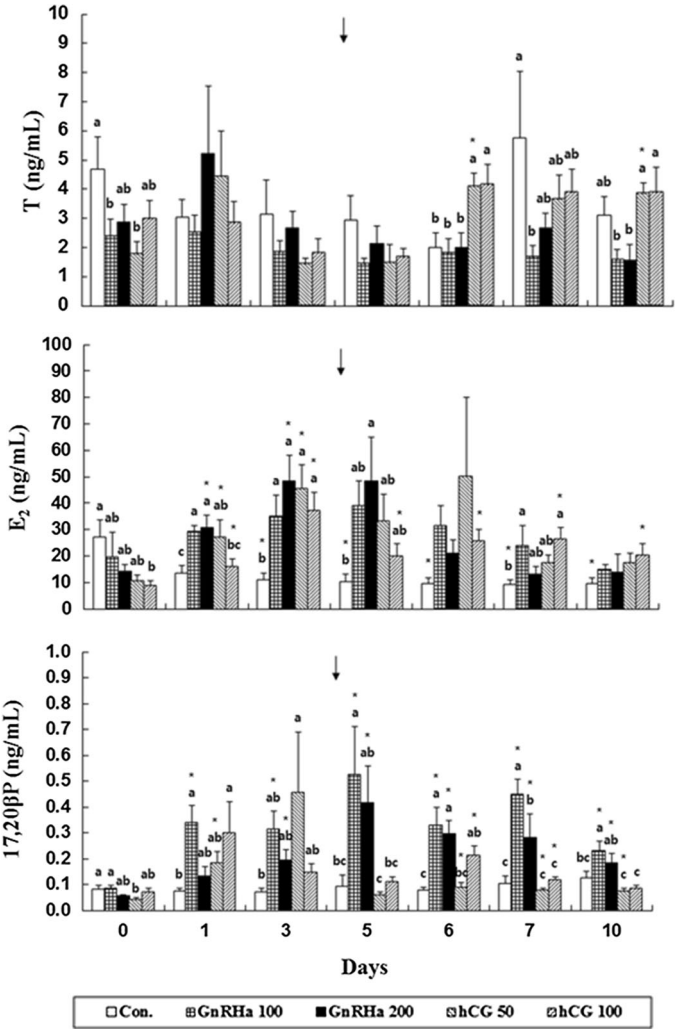

Fig. 1 Changes in plasma levels of 17β-estradiol (E2), testosterone (T), and 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) in starry flounder treated with exogenous hormone injections early in the spawning period. The *superscripts* indicate significant differences between treatments at that sampling time ($P < 0.05$). The *arrow* indicates the timing of the second injection

Table 2 Effect of exogenous hormone implantation on the number of stripped eggs, floating rate, and fertilization rate

Treatment	Number of stripped eggs (100 g^{-1} BW)	Floating rate (%)	Fertilization rate (%)
Control	4118 ± 1886^{b}	51.2 ± 6.7	84.1 ± 3.5
GnRHa 100	$16{,}913 \pm 2307^{a}$	41.9 ± 6.2	85.3 ± 5.5
GnRHa 200	7421 ± 2149^{b}	42.8 ± 5.2	84.3 ± 4.5

Different superscripts indicate significant differences between treatments ($P < 0.05$)

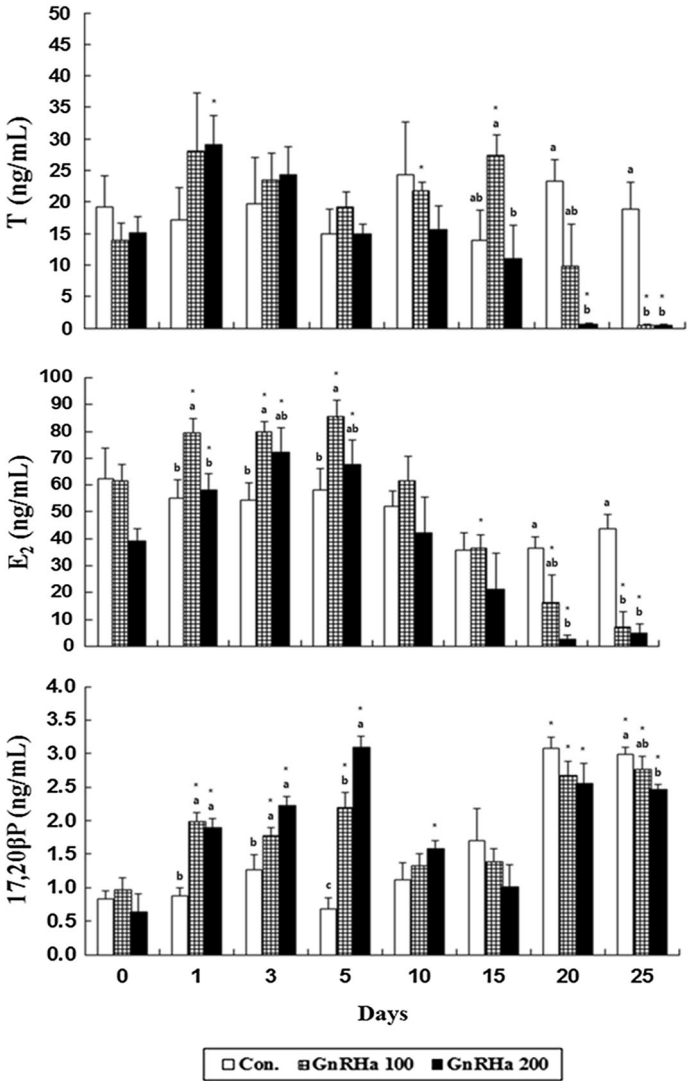

Fig. 2 Changes in the plasma levels of 17β-estradiol (E2), testosterone (T), and 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) in starry flounder treated with exogenous hormone injections in the middle of the spawning season. The *superscripts* indicate significant differences between treatments at that sampling time ($P < 0.05$)

(Lim et al. 2002; Moon et al. 2003; Lim and Kim 2007). hCG and GnRHa, administered short or long term via injection or pellet implant, increased the milt volume in previous experiments with starry flounder (Lim et al. 2002; Moon et al. 2003; Lim and Kim 2007). However, the effect of exogenous hormones on starry flounder ovulation is unknown. In the present study, GnRHa administered either by injection or pellet implantation induced successful ovulation in starry flounder. Most ovulations caused by GnRHa administration occurred on consecutive days and were accompanied by significant increases in egg

quantity. The results of the present study and of a previous study on oocyte development (Lim et al. 2007) suggest that female starry flounder are multiple ovulators and have multiple groups of synchronous oocyte batches. The finding of repeated ovulation in response to treatment with GnRHa is consistent with findings in other flatfish, yellowtail flounder (*Pleuronectes ferrugineus*) (Larsson et al. 1997), summer flounder (*Paralichthys dentatus*) (Berlinsky et al. 1997), and greenback flounder (*Rhombosolea tapirina*) (Poortenaar and Pankhurst 2000). However, it is unclear whether oocytes are recruited at certain developmental stages because of a lack of solid information on starry flounder ovarian cycling. In some species, hCG induces ovulation (Lam 1982; Peter et al. 1988) and produces high fertility, but not in summer flounder (like starry flounder in this study). Therefore, it may be dose, origin of exogenous hormone, and/or maturity level related. In many teleost species, mammalian GtHs have potencies that are orders of magnitude lower than teleost GtHs (Pankhurst 1998). Thus, large and repeated doses are required to induce ovulation upon hCG treatment (Smigielski 1975; Lam 1982; Donaldson and Hunter 1983; Berlinsky et al. 1997). Poortenaar and Pankhurst (2000) used a much greater concentration (1000 IU hCG kg^{-1} BW) to induce ovulation in greenback flounder compared with the present study (50–100 IU hCG kg^{-1} BW). Similarly, a lower dose of hCG by injection (250 IU kg^{-1} BW) was ineffective at inducing ovulation in summer flounder (Berlinsky et al. 1997). However, a relatively low dose of hCG (275 IU kg BW) induced ovulation in cobia (*Rachycentron canadum*) (Caylor et al. 1994).

In the present study, egg quantity and hormone secretion were significantly increased by GnRHa treatment regardless of the dose, though there was no treatment effect of GnRHa on the floating rate and fertilization rate between the experimental groups in experiment 2. This suggests that the optimal effective dose of GnRHa varies between species and that the degree of maturity and GnRHa pellet matrix are important for successful ovulation (Sherwood et al. 1988). Similar to greenback flounder, GnRHa (200 μg, ini) pellet implantation resulted in fewer ovulations and a smaller number of eggs ovulated compared to treatment with 100 μg of GnRHa (ini) and pellet implantation in this study (Poortenaar and Pankhurst 2000). Previous studies have shown that the optimal effective dose of GnRHa is species-specific: 1–5 μg in milkfish (*Chanos chanos*) (Tamaru et al. 1988), 6.3–23.6 μg in black sea bass (*Centropristis striata*) (Berlinsky et al. 2005), 70 μg in chum salmon (*Oncorhynchus keta*) (Park et al. 2007), and 300–400 μg in gray mullet (*Mugil cephalus*) (Lee et al. 1987).

Egg quality is an important term of reference following the successful induction of spawning in fish. Despite significant increases in the total number of stripped eggs, egg quality was not affected by the implantation of GnRHa pellets or injection of GnRHa in starry flounder, because the floating rate and fertilization rate were not significantly different between the control and GnRHa-treated groups. However, the hCG-injected groups showed lower egg fertility in this study. This could be due to the use of unsuitable doses of hCG for spawning induction, as reported previously for common sole (*Solea solea*) (Ramos 1986) or over-ripening of the egg after exogenous hormone treatment such as in Japanese flounder (*Limanda yokohamae*) (Hirose et al. 1979). Premature seasonal hormone treatment may induce precocious egg maturation and ovulation, causing low egg fertility in coho salmon (*Oncorhynchus kisutch)* (Hunter et al. 1981; Fitzpatrick et al. 1984).

The injection of exogenous hormones typically results in the short-term induction of ovulation, as well as changes in plasma steroids. Treatment of fish with slow-release forms of exogenous hormones such as microspheres or pellets typically produces slower but more sustained ovulation (Poortenaar and Pankhurst 2000; Zohar and Mylonas 2001). In this study, plasma T levels did not significantly increase upon exogenous hormone treatment during the early and middle parts of the spawning season. As mentioned in Poortenaar and

Pankhurst (2000), this may be the result of the rapid aromatization of T and/or detrimental effects of stress on plasma steroid levels. Stress has been reported to depress plasma levels of gonadal steroids in many species (Pankhurst 2011). The plasma E2 level was elevated in fish induced to spawn by GnRHa treatment in the present study. The elevated level of E2 likely resulted from the steroidogenic activity of developing vitellogenic follicles. In this study, the plasma levels of 17,20βP were consistently elevated in association with reproductive events in both experiments, similar to wild greenback flounder (Barnett and Pankhurst 1999). Several studies have indicated that the maturational steroid 17,20βP is involved in regulating spermiation and ovulation in most teleosts (reviewed in Pankhurst 1998; Zohar and Mylonas 2001). The plasma levels of 17α,20α-dihydroxy-4-pregnen-3-one, 11-deoxycortisol, and 5β-reduced pregnane were found to be at least as high as that of 17,20βP in previous studies (Canario and Scott 1990; Scott et al. 1991). Additional studies are required to confirm the existence of such steroids in starry flounder plasma, and the potency of these candidate MISs in inducing oocyte maturation in vivo. In the present study, GnRHa treatment was more potent and efficient than hCG treatment. As mentioned by Zohar and Mylonas (2001), this is because GnRH provides more balanced stimulation of reproductive events and, presumably, better integration of these events with other physiological functions by directly or indirectly affecting the release of other hormones necessary for successful final oocyte maturation, spermiation, and spawning.

The implantation of starry flounder with GnRHa-containing pellets was effective at inducing sustained ovulation compared to hCG treatment. Although future studies of the optimal doses of GnRHa, use of anti-dopaminergic drugs, and timing of treatment are needed to produce high-quality eggs in starry flounder, it is clear that GnRHa pellets can be applied to starry flounder hatchery processes without compromising egg quality or fertility.

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