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Original article

A microencapsulated analog of LH-RH accelerates maturation but without stimulating sex reversal in the protandrous black porgy, *Acanthopagrus schlegeli*

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Summary — The objective of this study was to regulate the reproduction and levels of gonadal steroids in 3-year-old protandrous black porgy *(Acanthopagrus schlegeli)* by treatment with microencapsulated D-Trp^6-luteinizing hormone-releasing hormone (LH-RH analog) during the prespawning season. Twenty-four previously male black porgy were equally divided into 2 groups and injected with vehicle (control group) or with microencapsulated LH-RH analog (LH-RH analog group), respectively. Spermiation and plasma levels of testosterone (T), estradiol-17β (E2) and 17α-hydroxyprogesterone (17α-OH P) were measured, after treatment, at intervals of 1–2 weeks for 4 months. Oocyte diameters were also measured after 4, 10, 12, 14 and 16 weeks of treatment. The microencapsulated LH-RH analog accelerated the onset of spermiation by at least 5 weeks. Oocyte diameters were also significantly increased in the LH-RH analog group. The microencapsulated LH-RH analog did not increase the number of sex-reversing females compared with the number in the control group. High levels of plasma E2 were found in the sex-reversing females in the LH-RH analog and control groups during the prespawning and spawning season. Low levels of plasma E2 were observed in the non-reversed males in both the LH-RH analog and the control groups. Similar profiles of plasma T levels were detected in male and in reversing female black porgy in the LH-RH analog and control groups. Plasma 17α-OH P levels were low and constant throughout the experimental period in fish in each group. These findings indicate that the microencapsulated LH-RH analog accelerated gonadal maturation in the black porgy during the prespawning season. Plasma levels of E2 seem to be closely related to the occurrence of natural sex reversal in the protandrous black porgy, *A schlegeli*.

black porgy / estradiol-17β / LH-RH analog / maturation / sex reversal / spermiation

Résumé — Effets d'un analogue de LHRH microencapsulé sur la maturation gonadique et le changement de sexe chez le protandre pagre noir *(Acanthopagrus schlegeli)*. *Le but de cette étude est d'analyser les effets d'un analogue de LHRH (Trp^6-LHRH) microencapsulé sur la stéroïdogenèse et la*

reproduction de pagres noirs âgés de 3 ans lorsque ce traitement est administré dans la période précédant le frai. Vingt-quatre pagres noirs qui étaient mâles précédemment ont été traités soit avec du LHRH microencapsulé (n = 12, lot traité) ou conservés comme témoins (n = 12). Les paramètres mesurés sont la spermiation et les niveaux sanguins de testotérone, œstradiol et 17α-hydroxyprogestérone (mesures toutes les 1 à 2 sem pendant 4 mois) ainsi que les diamètres ovocytaires (après 4, 10, 12, 14 et 16 sem de traitement). La spermiation est avancée d'au moins 5 sem par le traitement au LHRH. La croissance ovocytaire est également stimulée par le traitement au LHRH. En revanche le traitement n'a pas d'influence sur la fréquence d'individus changeant de sexe. Pendant le frai, des niveaux élevés d'œstradiol sont faibles chez les mâles qui ne changent pas de sexe. Les niveaux de testostérone observés chez les mâles et les femelles sont analogues dans les lots témoins et traités. Dans les 2 lots, les niveaux de 17α-hydroxyprogestérone sont faibles et constants. Ces résultats montrent que l'administration d'un analogue de LHRH dans la période précédant le frai accélère la maturation gonadique chez le pagre noir. Il existe également un lien entre des niveaux d'œstradiol élevés et le changement de sexe.

maturation gonadique / changement de sexe / protandre / analogue de LHRH

INTRODUCTION

Sex patterns in fishes include gonochorism and hermaphroditism. Protandrous and protoynous hermaphroditisms have been known in a number of teleosts (Chan and Yeung, 1983). Genetic factors, sex inductors, histocompatibility-Y chromosome antigen, endocrine system and extrinsic factors have been suggested to involve the sex determination and sex patterns in fish (Chan and Yeung, 1983; Reinboth, 1988).

In protandrous fish, each individual develops testes and spawns first as a male and later changes sex and functions as a female. The primacy of female development by the initial ovarian phase in the development of gonads is suggested not only in gonochoristic and protogynous fish but also in protandrous fish (Shapiro, 1992). Shapiro (1992) further suggests that male development is inserted into a female development sequence as a temporary phase, initiated and terminated by a male inductor. The physiological role of hormones in the sex change from male to terminal female has not been observed in protandrous fish (Reinboth, 1988; Shapiro, 1992).

Black porgy, *Acanthopagrus schlegeli*, is a marine protandrous hermaphrodite and has been a commercially valuable species. Fish are functional males for the first 2 spawning seasons but begin to change to terminal females during or after the third spawning season (Chang *et al*, 1994). The effects of exogenous luteinizing hormone-releasing hormone (LH-RH) on the induction of sex reversal and the changes in concentrations of sex steroid during sex reversal in protandrous fish are poorly understood but of considerable interest. The number of LH-RH cells was significantly increased in the forebrain preoptic area of terminal phase males in protogynous bluehead wrasse, *Thalassoma bifasciatum* (Grober and Bass, 1991; Grober *et al*, 1991). The treatment of LH-RH analog further induced sex reversal in bluehead wrasse (Kramer *et al*, 1993). The possible involvement of LH-RH and sex steroids in sex reversal of protandrous black porgy deserves to be further studied.

Superactive analogs of LH-RH have been widely used to induce ovulation and spermiation in a number of teleosts. These teleosts include ayu, *Plecoglossus altivelis* (Aida, 1983; Hirose *et al*, 1983), black porgy, *A schlegeli* (Yueh *et al*, 1990; Chang and Yueh, 1990a; Chang *et al*, 1991, Lee *et al*, 1993), walleye, *Stizostedion vitreum* (Pankhurst *et al*, 1986), catfish, *Clarias*

macrocephalus (Ngamvongchon *et al,* 1986), carp (Ngamvongchon *et al,* 1987), Atlantic salmon, *Salmo salar* (Weil and Crim, 1983; Crim and Glebe, 1984), seabass, *Lates calcarifer* (Harvey *et al,* 1985), rabbitfish, *Siganus guttatus* (Harvey *et al,* 1985), and milkfish, *Chanos chanos* (Lee *et al,* 1986).

Two doses of LH-RH analog given by injection can induce spermiation and oocyte maturation in functional male and female black porgy during the spawning season but not during the prespawning season (Chang *et al,* 1991). The effects of continuous stimulation with low doses of LH-RH analog in black porgy during the prespawning period may have commercial relevance. Accelerated ovulation and spermiation were observed in rainbow trout and Atlantic salmon treated with a LH-RH analog in pelleted form (Crim *et al,* 1983; Weil and Crim, 1983; Crim and Glebe, 1984).

Therefore, the objective of this study was to monitor reproduction and levels of gonadal steroids after treatment with microencapsulated D-Trp^6-LH-RH (LH-RH analog) of 3-year-old black porgy during the prespawning season. Thus, spermiation and oocyte growth were observed and correlated with levels of testosterone (T), estradiol-17β (E2) and 17α-hydroxyprogesterone (17α-OH P) in plasma. The possible role of LH-RH and E2 in the sex reversal was also investigated.

MATERIALS AND METHODS

Fish

Three-year-old black porgy, *A schlegeli*, at intersexual stage (mean body weight, 623 ± 33 g) were obtained from pond culture in October 1990. All the fish had been confirmed as functional males (with spermiation) at 2 years of age during the spawning season (January–March 1990). 'Intersexual stage' was defined as when the gonad consisted of testicular and ovarian tissues; perinucleolar stage was the most advanced oocytes in the ovarian tissues on the basis of the preliminary data. The experimental fish are not at the stage of changing sex because vitellogenic oocytes are not detected. Experimental fish were acclimated at the University culture station and fed commercial formulated feed (Fwu Sow Feed Co, Taiwan).

Microencapsulation of LH-RH analog

[D-Trp^6] LH-RH (LH-RH analog) was synthesized by solid-phase methods and supplied by Debiopharm (Lausanne, Switzerland). Microencapsulation of the LH-RH analog in poly (DL-lactide-coglycolide) was performed by a phase-separation process by P Orsolini at Cytotech (Martigny, Switzerland) and the microencapsulated analog was supplied by Debiopharm (Lausanne, Switzerland). The polymer is biodegradable and nontoxic to living tissues. An aliquot of microcapsules (4 mg) with a concentration of LH-RH analog of 1.82% (w/w) was given to each fish. The total quantity of LH-RH analog in 4 mg of microcapsules was 72.8 μg.

Experimental design

Twenty-four fish (intersexual stage) were equally divided into 2 groups and injected intramuscularly (im) with vehicle (n = 12) or microencapsulated LH-RH analog (n = 12; 4 mg), respectively. Microcapsules were suspended in a vehicle solution of 2% carboxymethyl cellulose and 1% Tween-20. Injections of microcapsules were given intramuscularly only one at the beginning of the experiment. The experiment was conducted from November 1990 (prespawning season) to March 1991 (spawning season, January–March). Each fish was labeled with a nose tag and blood samples were collected from fish in the afternoon (2.00–5.00 pm) at 1- to 2-week intervals before and after treatment for 16 weeks. Seawater temperatures in the culture tank were recorded and ranged from 16–21°C during the experimental period. Fish were anesthetized in a bath of 0.4 ml/l of 2-phenoxyethanol prior to handling. Blood samples were collected in heparinized tubes from caudal vasculature. The plasma was obtained by centrifugation and

stored at –30°C until analysis. The number of fish that spermiated and the volume of collectable milt were recorded. Milt was obtained by hand stripping with application of gentle pressure on the abdomen just after bleeding. Intra-ovarian oocytes ($n > 15$ per fish) were also collected by weeks 4, 10, 12, 14 and 16 from genital pores of sex-reversing females by use of silastic tubing. The diameters of oocytes were measured with an ocular micrometer.

Gonads were dissected at the end of the experiment to confirm the stage of sex reversal of each fish. Those fish which completed the sex reversal at the end of the experiment were called as 'sex-reversing females' in this experiment. 'Sex-reversing females' were defined as functional males in the second spawning season; they later reversed and became females during the third spawning period. The occurrence of sex reversal is considered from the late prespawning to spawning season. However, the exact timing of the onset of sex change is impossible to know by the observation of fish. The completion of sex reversal in black porgy was only able to be judged on the basis of 'collectable oocytes', 'lack of spermiation' and 'ovarian gonad' during the spawning season.

Analysis

Plasma E2, T and 17α-OH P were measured by radioimmunoassay after solvent extraction as described by Chang and Yueh (1990b). The sensitivities of the assays for E2, T and 17α-OH P were 5, 12.5 and 12.5 pg per assay, respectively. Intra-assay variation for E2, T and 17α-OH P was 12.6, 14.0 and 19.0%, respectively. Inter-assay variation for E2, T and 17α-OH P was 16.7, 15.5 and 23.4%, respectively. The radioactivity of the bound [^{3}H]steroid in the supernatant was counted in a liquid scintillation spectrophotometer (Ready Safe; Beckman Instruments Inc, Fullterton, CA). [2, 4, 6, 7-^{3}H]Estradiol-17β (99 Ci/mmol), [1, 2, 6, 7-^{3}H]testosterone (89.1 Ci/mmol) and 17α-hydroxy-[1,2-^{3}H]progesterone (57.4 Ci/mmol) were purchased from NEN Research Products (Boston, MA).

The standard error of the mean (SEM) was calculated for each set of data. Student's *t*-test and analysis of variance followed by Duncan's multiple range test were used to assess the significant differences (Steel and Torrie, 1980).

RESULTS

Treatment with the microencapsulated LH-RH analog accelerated the onset of spermiation in the 3-year-old black porgy as compared to that in control fish (fig 1). After administration of microcapsules of the LH-RH analog, spermiation occurred 5 weeks earlier than in control; by weeks 2 and 5, 20 and 80% of fish in the treated group were spermiating, respectively. In contrast, by week 7, only one fish was spermiating in the control group (fig 1). The percentage of fish that spermiated and the volume of milt ceased to differ between the 2 groups by week 16. A significantly larger amount of collectable milt was obtained from fish injected with LH-RH analog than from control fish from weeks 4 to 12 ($P < 0.05$; fig 1). The total collectable milt each group obtained from the whole experimental period was 230 and 56 ml in the LH-RH analog and control group, respectively.

Recognition of the completion of sex reversal in the experimental fish was based on the presence of collectable intra-ovarian oocytes and on the observation of ovarian gonads obtained at the end of the experiment. Eight and 7 functional males were detected in the LH-RH analog and control groups, respectively. Collectable oocytes were obtained in all the fish that did not show spermiation during the experimental period. The ovarian gonads were further identified in those fish at the end of the experiment. Therefore, 4 and 5 sex-reversing females were obtained in the LH-RH analog and control groups, respectively. The diameters of collectable oocytes in females ranged from 0.45 to 0.88 mm and from 0.28 to 0.5 mm in the LH-RH analog and control groups, respectively. Transparent and mature oocytes were observed in the case of 1 reversing females in the LH-RH analog by week 4. No intra-ovarian oocytes were collectable *via* the genital pore by week 4 in sex-reversing females in the control group (fig 2). Significantly larger

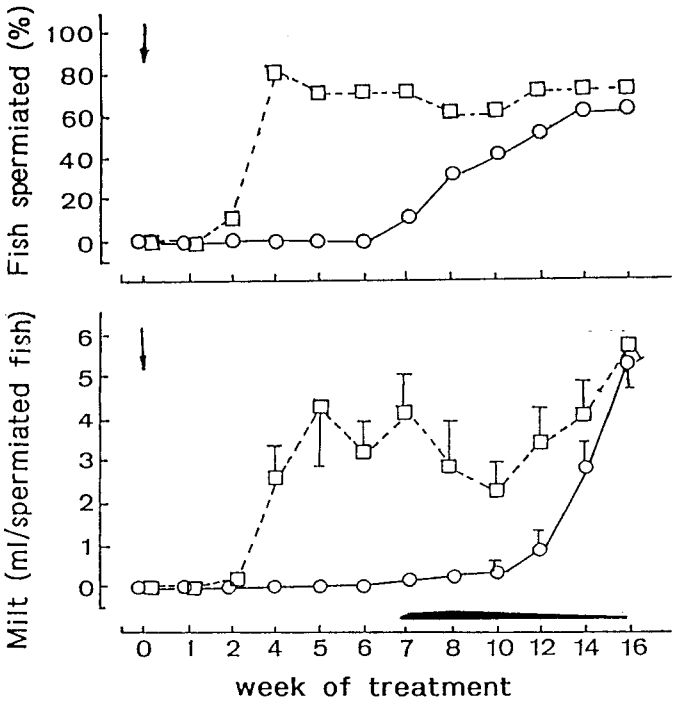

Fig 1. Percentages of black porgy that spermiated and average milt volume (ml) per spermiated fish after treatment with microencapsulated D-Trp^6-LH-RH (LH-RH-A) (□---□)or vehicle (O—O). Horizontal black bar indicates the spawning season. An arrow (↓) indicates when the injection was given.

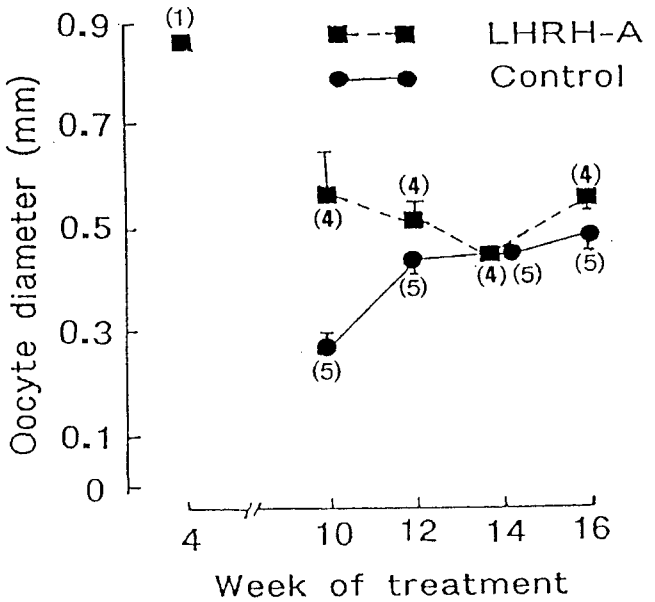

Fig 2. The changes of oocyte diameters collected in weeks 4, 10, 12, 14 and 16 in sex-reversing females in the group treated with microencapsulated D-Trp^6-LH-RH (LH-RH-A) (■---■) and in the control group (●—●). The injection was given by week 0. No oocyte was collectable *via* the genital pore by week 4 in the control group. Numbers in parentheses indicate the number in the samples of sex-reversing females.

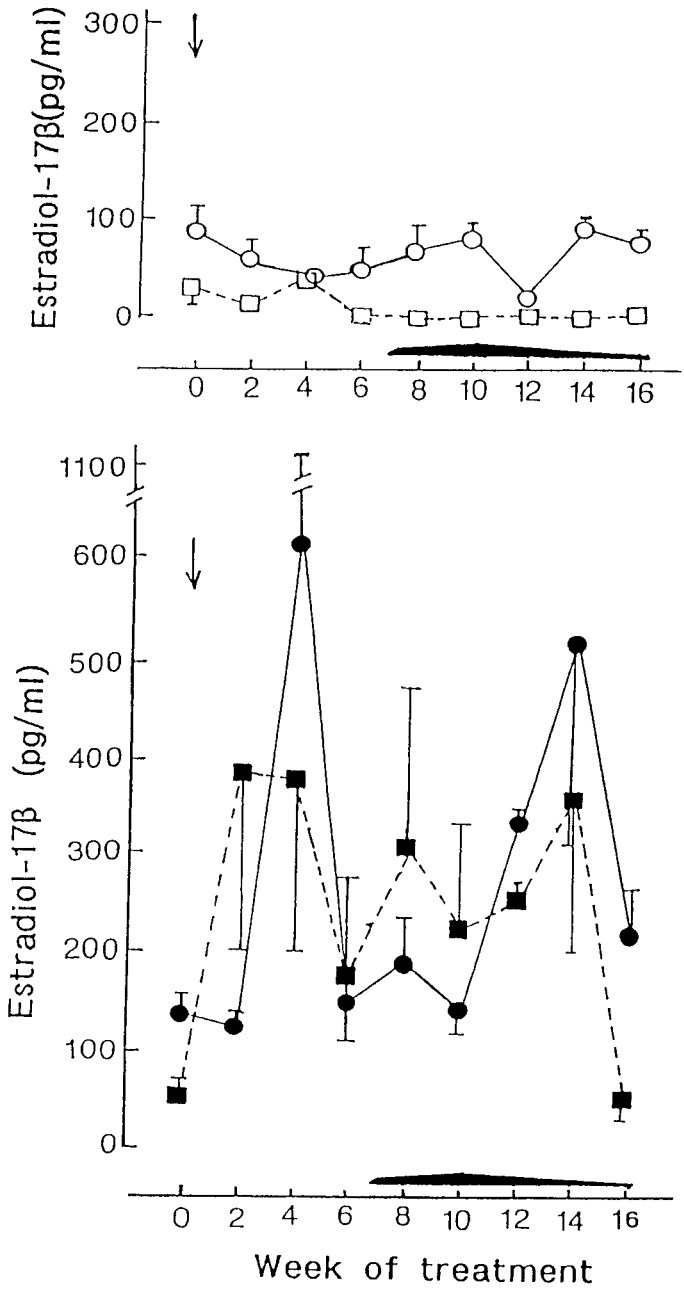

Fig 3. Profiles of plasma estradiol-17β levels in males (♂–>♂) (top) and sex-reversing females (♂–>♀) (bottom) in the group treated with microencapsulated D-Trp^6-LH-RH (LH-RH-A) (squares) and in the control group (circles). See legend to figure 1 for further details.

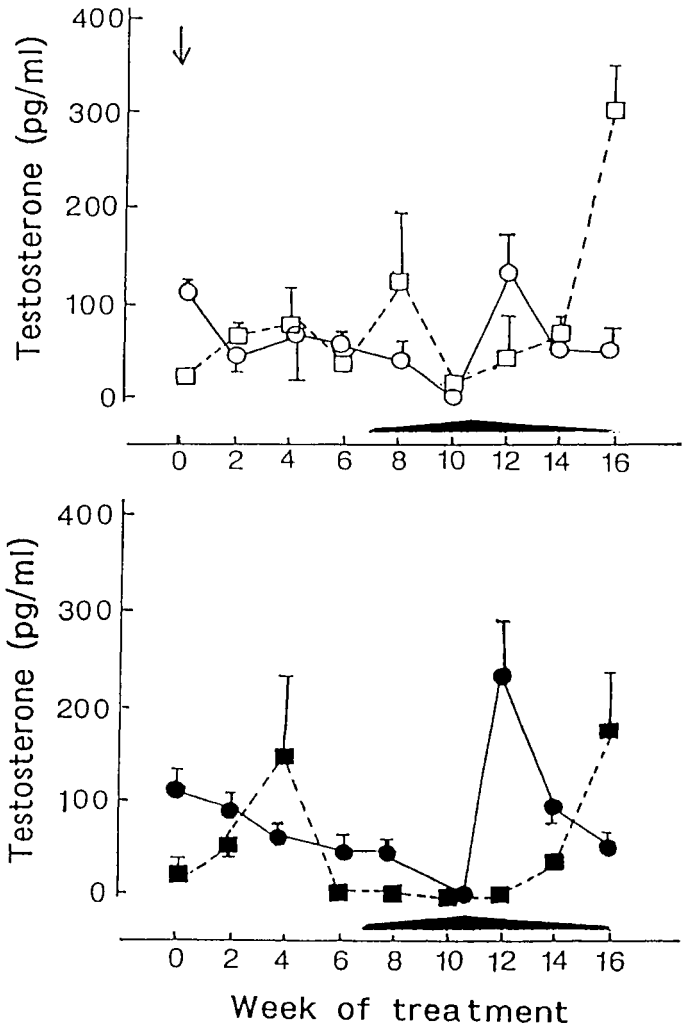

Fig 4. Profiles of plasma testosterone levels in males (♂->♂) (top) and sex-reversing females (♂->♀) (bottom) in the group treated with microencapsulated D-Trp^6-LH-RH (LH-RH-A) (squares) and in the control group (circles). See legend to figure 1 for further details.

oocytes collected by week 10 were also observed in the LH-RH analog than in the control group (fig 2; $P < 0.05$). No difference in oocyte diameters collected after week 12 was found between the 2 groups (fig 2).

High concentrations of plasma E2 were observed in the sex-reversing females of the LH-RH analog and control groups during the experimental period (fig 3). By contrast, low levels of plasma E2 were observed in the non-reversed males in both the LH-RH analog and control groups (fig 3). Levels of plasma E2 increased before the spawning season in the sex-reversing females. Similar profiles of plasma T were detected in males and in reversing females in the LH-RH analog and control groups (fig 4). Mean values of plasma E2 and T during the experimental period in males and reversing females in the control and LH-RH analog groups are shown in table I. The average concentrations of E2 per fish calculated from

Table I. Plasma estradiol-17β (E2) and testosterone (T) in male and reversing female black porgy in the control and microencapsulated LH-RH analog (LH-RH-A) groups.

	Steroids (pg/ml)	
	E2	*T*
LH-RH-A		
Males ($n = 8$)	8 ± 5	74 ± 28
Females ($n = 4$)	190 ± 50*	51 ± 22
Control		
Males ($n = 7$)	67 ± 8	60 ± 20
Females ($n = 5$)	270 ± 60*	79 ± 18

Each single value for a fish was calculated from the average of 9 values per fish measured from the whole experimental period (16 weeks) as shown in figures 3 and 4. * Values significantly differed from the data in males in the same group ($P < 0.05$).

the whole experimental period were significantly higher in reversing females than in males (table I). Plasma 17α-OH P levels (< 30 pg/ml) were very low and constant throughout the experimental period in fish of both groups.

DISCUSSION

Treatment of black porgy with microencapsulated LH-RH analog effectively induced precocious spermiation during the prespawning season. The microencapsulated LH-RH analog accelerated the onset of spermiation by at least 5 weeks compared with the control group. The total amount of milt collected during the experimental period was also larger in the LH-RH analog group than in the control group. Previous study showed that the increase of milt volume cause by the LH-RH analog did not significantly decrease the sperm count in black porgy (Yueh *et al*, 1990).

There is an increase in oocyte diameters collected by week 10 from the LH-RH analog group as compared to the control group. An oocyte with a diameter of approximately 0.5 mm in black porgy could be classified as maturing and at the end of vitellogenic stage. Mature and transparent oocytes in black porgy could be larger than 0.8 mm in diameter. Stimulation of oocyte vitellogenesis by microencapsulated LH-RH analog during the prespawning season is therefore suggested.

The LH-RH analog, delivered to fish by injection, by oral administration or implantation of slow-release devices, has been used to induce spawning (reviewed by Donaldson and Hunter, 1983; Thomas and Boyd, 1989; Solar *et al*, 1990). LH-RH analogs disappeared from the circulation within 30–60 min of injection in goldfish (Sherwood and Harvey, 1986) and within 1 to 2 h in seabream (reviewed by Zohar, 1989). The plasma concentration of LH-RH analog in sablefish *(Anoplopoma fimbria)* reached a peak 1 h after oral administration (Solar *et al*, 1990). However, 2 doses (10 and 50 μg/kg body weight) of D-Ala^6, des-Gly^{10} LH-RH ethylamide (without microencapsulation) failed to accelerate spermiation in 2-year-old black porgy during the prespawning period (Chang *et al*, 1991). The limited elevation of LH-RH in circulation after such treatment might be insufficient to induce maturation and spawning in fish during the prespawning season that have not reached the final stages of spermatogenesis and vitellogenesis. A system for the slow release of LH-RH analog provides a feasible solution to the short-lived bioactivity of the injected peptide. Cholesterol pellets containing LH-RH analogs successfully accelerated or induced ovulation in rainbow trout (Crim *et al*, 1983), Atlantic salmon (Crim and Glebe, 1984), seabass (Harvey *et al*, 1985) and milkfish (Lee *et al*, 1986). LH-RH analog that was micro-

encapsulated with biodegradable polymers was successfully used in the present experiment to enhance spermiation and oocyte growth of black porgy.

The levels of LH-RH analog released into plasma were not measured in this study due to the shortage of plasma samples. The application of microencapsulated LH-RH analog in rats has been described (Redding *et al,* 1984; Mason-Garcia *et al,* 1985). The release of LH-RH analog from microcapsules can last for at least 1 month in rats (Mason-Garcia *et al,* 1985). The released LH-RH analog should stimulate the production of steroid mediators that induce vitellogenesis and maturation (Chang *et al,* 1991). In this study, oocyte growth (*via* vitellogenesis) was stimulated by microencapsulated LH-RH analog as shown in week 10. Moreover, oocytes did reach maturation on week 4 in an early sex-reversing fish. The milt volume remained high from weeks 4 to 10 in males injected with microencapsulated LH-RH analog. Perhaps reproductive seasonality or other factors, but not LH-RH analog, might play a more important role in the rising phase of milt production after week 10 because milt volume gradually increased after week 10 in both treated and control groups. Therefore, the data tended to suggest that LH-RH analog was released from microcapsules for no longer than 10 weeks after implantation.

Plasma levels of E2 were much higher ($P < 0.05$) in sex-reversing females than in non-reversed male black porgy during the experimental period which was consistent with the previous data (Chang *et al,* 1994). Elevated concentrations of plasma E2 were also observed in the reversing females during the early prespawning season in this study. E2 seems to be closely related to the process of natural sex reversal in the protandrous black porgy. Our most recent studies showed that exogenous E2 successfully induced sex reversal in 1-year-old protandrous black porgy (Chang *et al,* 1994). The present data cannot directly provide an answer to the question of whether the E2 changes are driving the natural sex reversal or, on the other hand, whether the sex reversal results in changes of hormonal levels. As the experimental period covered the prespawning the spawning periods of the fish, it is also difficult to exclude the possibility that the changes in E2 levels during the experimental period associated with female spawning activity. No significant differences in the profiles of plasma levels of T were found between the males and reversing females in the 2 groups. The physiological role of androgens in the natural sex reversal of black porgy is unclear. E2 and 11-oxygenated androgens were significantly different between the sexes in protandrous sobaity, *Sparidentex hasta* during the spawning season (Kime *et al,* 1991). Guiguen *et al* (1993) suggest that E2 plays an important role in the protandrous sex reversal process in the seabass *L calcarifer.* The E2 to androgen ratio also significantly increased during the course of sex reversal in protandrous anemonefish, *Amphiprion melanopus* (Godwin and Thomas, 1993).

Induction of sex reversal from males to females did not occur significantly more frequently in 3-year-old black porgy after treatment with microencapsulated LH-RH analog than in the control group. However, the induction of gonadal reversal by the treatment of LH-RH analog was demonstrated in protogynous bluehead wrasse, *T bifasciatum* (Kramer *et al,* 1993). Luteinizing hormone but not LH-RH analog induced gonadal reversal in protogynous ricefield eel, *Monopterus albus* (Yeung *et al,* 1993). The increase of LH-RH cell number in the preoptic area of terminal phase males was observed in the protogynous bluehead wrasse, *T bifasciatum* (Grober and Bass, 1991; Grober *et al,* 1991). Elevated levels of 11-ketotestosterone (11-KT) were observed in the transition between the primary and terminal phases in the protogynous stoplight

parrotfish, *Sparisoma viride* (Cardwell and Liley, 1991) and wrasse *T dupperrey* (Nakamura *et al,* 1989). The discrepancy of the LH-RH action in the sex reversal is possibly due to the different mechanism of sex reversal in protandrous and protogynous fish and also species differences.

Plasma levels of E2 were also significantly lower ($P < 0.05$) in the males of the LH-RH analog group (8 ± 5 pg/ml) than in the males of the control group (67 ± 8 pg/ml). However, there was no difference in plasma levels of E2 between sex-reversing females in the LH-RH analog and control groups. The microencapsulated LH-RH analog seems to suppress the levels of plasma E2 in the male phase of 3-year-old black porgy. In our previous study we found that exogenous LH-RH analog cause high levels of plasma E2 in females but not in fish at the intersexual stage or in the male phase (Chang *et al,* 1991).

Administration of microencapsulated LH-RH analog did not stimulate the production of T, E2 and 17α-OH P in the black porgy. Elevated concentrations of plasma T were observed in black porgy by injection of D-Ala^6, des-Gly^{10} LH-RH ethylamide (without microencapsulation) during the prespawning season (Chang *et al,* 1991). The effects of prolonged stimulation by the released LH-RH analog might affect the production of gonadal steroids in black porgy. T, 11-KT, 17α-OH P and 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-diOH P) are involved in spermatogenesis, spermiation and oocyte maturation in fish (Billard *et al,* 1982; Young *et al,* 1986). Because of the unavailability of assays for 17α, 20β-diOH P, 11-KT and black porgy gonadotropin, only T, E2 and 17α-OH P were measured in the present study. Further research is necessary to investigate the profiles of 17α, 20β-diOH P, 11-KT and other androgens in black progy.

In summary, the microencapsulated LH-RH analog accelerated the onset of spermiation and stimulated the milt production in black porgy. Oocyte growth and maturation were also stimulated by the treatment of LH-RH analog. Different plasma levels of E2, but not T, were observed between males and reversing females. Microencapsulated LH-RH did not enhance the sex reversal in black porgy. High values of plasma E2 seem to be closely associated with the occurrence to natural sex reversal in protandrous black porgy.

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