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D-Trp^{6}-LH-RH微粒膠囊促進黑鯛之產精作用

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Microencapsulated LH-RH Analog Accelerated Spermiation in Protandrous Black Porgy, *Acanthopagrus schlegeli*

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ABSTRACT

The objective of this study was to investigate the regulation of reproduction and gonadal steroids in two-year-old protandrous black porgy through treatment with microencapsulated D-Trp^6-luteinizing hormone-releasing hormone (LH-RH analog). Sixteen black porgy were equally divided into two groups and injected with saline and microencapsulated LH-RH analog, respectively. Spermiation and plasma levels of testosterone(T), estradiol-17β(E_2), and 17α-hydroxyprogesterone(17α-OH P) were measured just before and after treatment at intervals of 1-2 weeks for four months. Microencapsulated LH-RH analog accelerated the onset of spermiation by three weeks. Percentages of spermiating fish were higher in the microencapsulated analog group (100%) than the control group (50%). During the experimental period the total amount of milt produced was higher in the microencapsulated analog group (84 ml) than in the control group (7 ml). Peak levels of plasma T occurred before the onset of spermiation in both the LH-RH analog group and control group. Two other minor peaks of plasma T were also noted during the spawning season. Levels of plasma E_2 were higher in the control group as compared to the LH-RH analog group. Plasma 17α-OH P levels remained at undetectable levels in fish of both groups. Non-significant differences in gonadosomatic indices were observed; in addition, there were no sexual reversal fish in either group.

INTRODUCTION

Superactive analogs of mammalian luteinizing hormone-releasing hormone (LH-RH) have been widely used to induce ovulation and spermiation in a number of teleosts, including: ayu, *Plecoglossus altivelis* (Aida, 1983; Hirose *et al.*, 1983); black porgy, *Acanthopagrus*

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schlegeli (Yueh *et al.*, 1990; Chang and Yuen 1990a; Chang *et al.*, 1991); walleye, *Stizostedion vitreum* (Pankhurst *et al.*, 1988); 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); and milkfish, *Chanos chanos* (Lee *et al.*, 1986).

The black porgy is a marine protandrous hermaphrodite that is a vaulable species in Asia. The majority of fish are male during the first two years of life but they begin to transform sexually to female after the third year. Previous research has established that two doses of LH-RH analog given by injection can result in high plasma levels of testosterone (T) but not of estradiol -17β (E_2) in two-year-old black porgy during both spawing and non-spawing seasons (Chang *et al.*, 1991). The effects of continuous stimulation with low doses of LH-RH in black porgy during the prespawning period may be of commercial value. Accelerated ovulation and spermiation were observed in rainbow trout and salmon using a pelleted form of LH-RH analog (Crim *et al.*, 1983; Weil and Crim,1983; Crim and Glebe, 1984). Therefore the objective of this study was to investigate the regulation of reproduction and gonadal steroids following treatment with microencapsulated D-Trp^6-LH-RH (LH-RH analog) in two-year-old black porgy by measuring levels of spermiation, T, E2, and 17α-hydroxyprogesterone (17α-OH P).

MATERIALS AND METHODS

Fish: Two-year-old protandrous black porgy (mean body weight = 337±15g) were obtained from pond culture in October, 1988. All experimental fish were acclimated to the University culture station pond and fed commercial feed (Fwu Sow Feed Co., Taichung, Taiwan).

Microencapsulation of LH-RH Analog: Microcapsules of LH-RH analog in poly(DL-lactide-coglycolide) were prepared using a phase-separation process developed by P. Orsolini at Cytotech (Martigny, Switzerland) and supplid by Debiopharm (Lausanne, Switzerland). This polymer is biodegradable and biocompatible with living tissue. Microcapsules of LH-RH analog in aliquots of 4 mg with a 1.82% concentration loading of LH-RH analog were designed to release 2.5μg/day for 30 days.

Experimental design: Experiments were conducted from November, 1988 to April, 1989. Sixteen fish were equally divided into two groups and injected with saline and microencapsulated D-Trp^6-LH-RH (4 mg), respectively. Microcapsules were mixed with 2% carboxymethyl cellulose and 1% Tween-20. Injections of microcapsules were made once during the four months experimental period according to a previous study (Roberts *et al.*, 1989). Blood samples were collected at 1-2 week intervals before and after the treatment. The water temperature of the culture tank ranged from 16.5-20 °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 from caudal vasculature in heparinized tubes. Plasma was obtained by centrifugation and stored at -20°C until analysis. Numbers of spermiating fish and volumes of collected milt were recorded. Milt was obtained by the gentle application of pressure on the abdomen just before or after bleeding.

Total body and gonadal weights at the end of the experiment for the calculation of gonadosomatic index (GSI = gonadal weight /body weight x100). Gonads were fixed in bouin's fluid and transverse sections were stained with haematoxylin and eosin.

Assay: Following solvent extraction, E_2, T, and 17α-OH P in plasma were measured by radioimmunoassy as described by Chang and Yueh (1990b). The radioactivity of the bound [^{3}H]-steroid in supernatant was counted with a liquid scintillation spectrophotometer (Beckman 1801) containing counting fluid (NE266, Nuclear Enterprises, Edinburgh, Scotland). [2,4,6,7-^{3}H]Estradiol-17β(85-110Ci/mmol), [1,2,6,7-^{3}H]testosterone (80-105 Ci/mmol) and 17α-hydroxy [1,2,6,7-^{3}H] progesterone(55-85Ci/mmol) were purchased from the Amersham Co. (Arlington Heights,IL).

Data Analysis: Standard error of mean (SEM) was calculated. A Student's t-test and analysis of variance followed by Duncan's multiple range test were used to compare differences (Steel and Torrie, 1980).

RESULTS

Treatment with microencapsulated LH-RH analog accelerated the onset of spermiation in treated black porgy compared with control fish (Fig. 1). After the administration of microencapsules of LH-RH analog, spermiation occurred three weeks earlier than in control fish; by week 4, all fish were spermiating in the treated group. By week 6-9 and week 11, only one and four fish in the control group were spermiating, respectively (Fig. 1). A significantly larger amount of milt (84 ml) was collected from fish injected with LH-RH

Table 1. Responses of 2-year-old black porgy injected with microencapsulated D-Try^6-LH-RH (LH-RH-A) or saline.

	N	GSI[1]	No. of fish spermiated	Week of spermiated	Total milt volume(ml)
Control	8	0.68 ± 0.49	4	10	7.2
Microencapsulated LH-RH-A	8	0.52 ± 0.22	8	13	83.8

[1]Gonads were dissected at the end of experiment and expressed as gonadosomatic index (GSI, mean ± SEM). No difference ($p>0.05$) in GSI was observed between two groups.

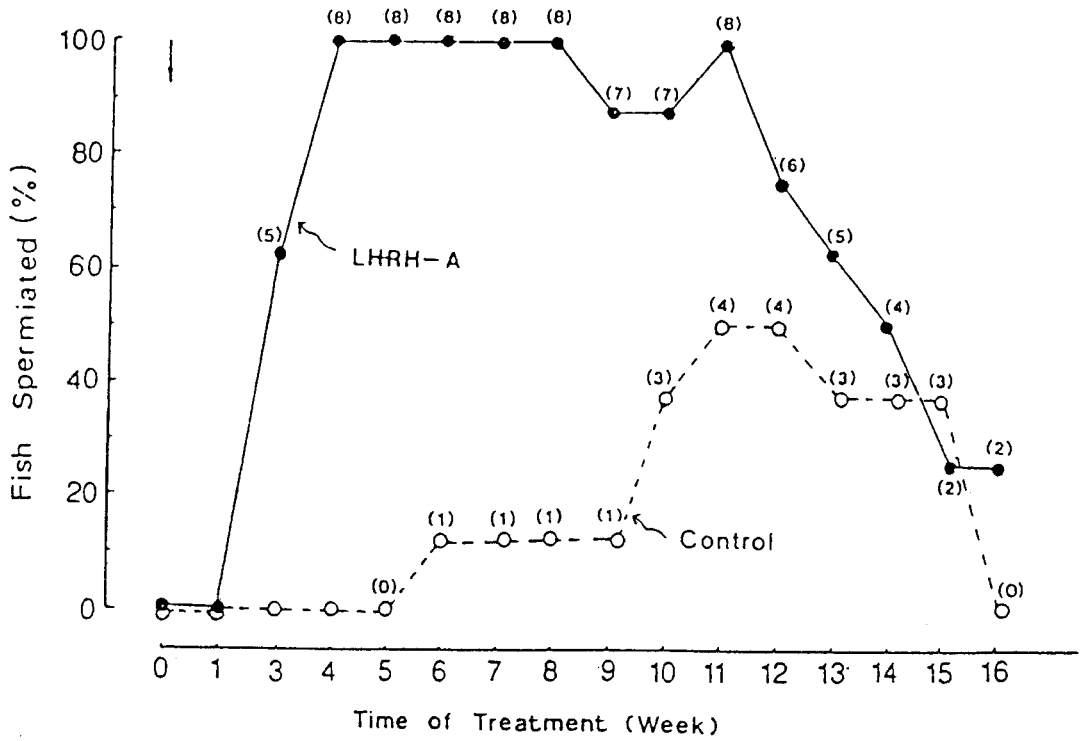

Fig. 1. Percentage of black porgy which spermiated following treatment with microencapsulated D-Trp^6-LH-RH (LH-RH-A) or saline. Numbers in parentheses indicate numbers in sample. Arrow (↓) indicates injection schedules.

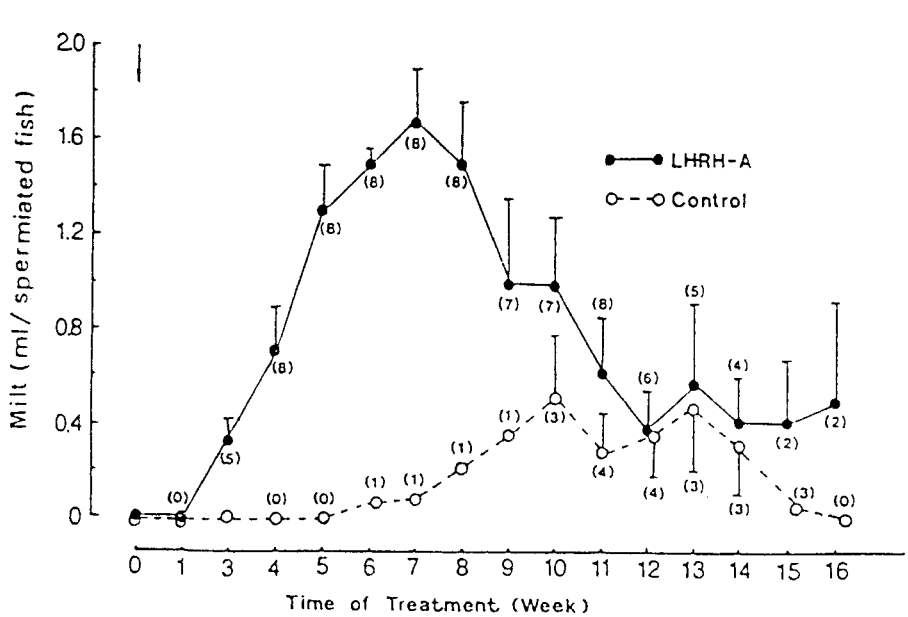

Fig. 2. Average milt volume (mean + SEM) per spermiating black progy following treatment with microencapsulated D-Trp^6-LH-RH (LH-RH-A) or saline. See Fig. 1 legend for details.

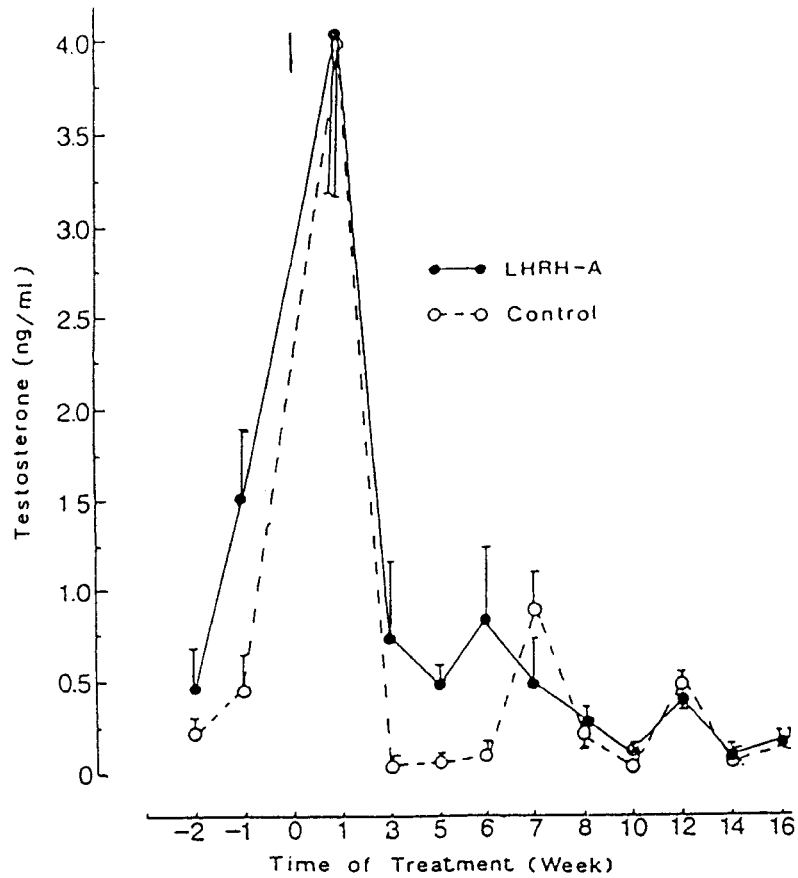

Fig. 3. Plasma testosterone profiles (n= 8, mean ± SEM) in microencapsulated D-Trp^6-LH-RH (LH-RH-A) or control groups. See Fig. 1 legend for details.

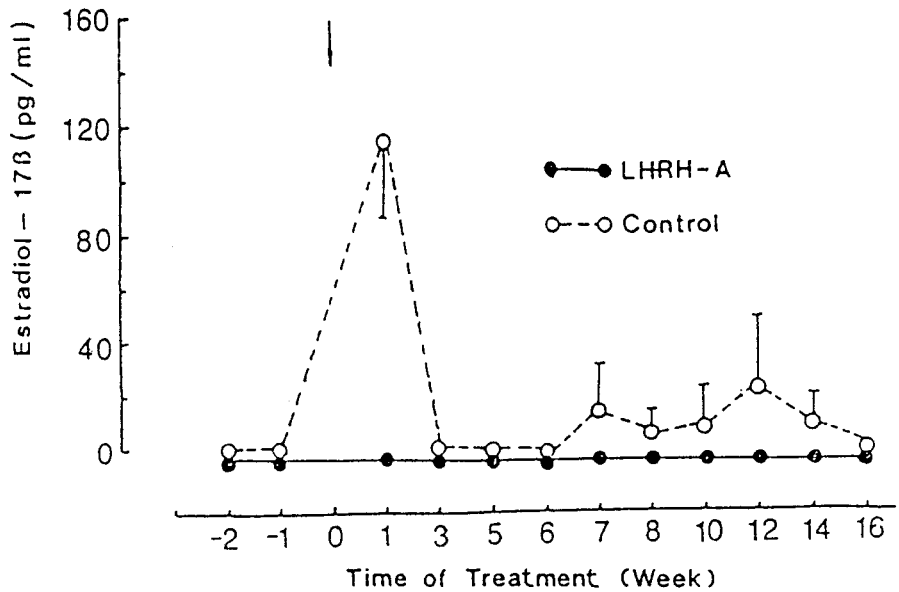

Fig. 4. Plasma estradiol-17β profiles (n= 8, mean ±SEM) in microencapsulated D-Trp^6-LH-RH (LH-RH-A) or control groups. See Fig. 1 legend for details.

analog as compared to control fish (7 ml) (Table 1). Milt volume was highest during weeks 5-9 in the micro-encapsulated LH-RH analog group (Fig. 2).

By week 1, significant peak levels of plasma T were observed in fish of both the micro-encapsulated LH-RH analog and control groups (Fig. 3). High levels of plasma T occurred before the onset of spermiation. During the spawning season, two additional small peaks of plasma T were noted in the control group (Fig. 3). Similar profiles of plasma T were observed in plasma E_2 levels of the control group (Fig. 4). However, no peak of plasma E_2 was observed in the microencapsulated LH-RH analog-treated fish (Fig. 4). Plasma 17α-OH P levels remained at undetectable levels throughout the experimental period for fish in each group. The sensitivity of the assay for 17α-OH P was 12.5 pg per tube; each sample volume for the assay was 0.1 ml.

Ovarian and testicular tissues were observed in the gonads of both the control and microencapsulated LH-RH analog groups. A primary yolk globule stage (vitellogenic oocytes) was observed in the ovarian tissue of two control fish (25%). A primary oocyte stage (perinucleolus oocytes) was observed in the ovarian tissue of the other control fish (75%) and all of the LH-RH analog-treated fish. Degenerating testicular tissues were observed in both groups.

DISCUSSION

The treatment of black porgy with microencapsulated LH-RH analog was very effective in inducing precocious spermiation. Microencapsulated LH-RH analog accerlerated the onset of spermiation by at least three weeks. The percentage of spermiating fish was higher in the microencapsulated LH-RH analog group (100%) than in the control group (50%). The total amount of milt collected during the expeimental period was also larger in the microencapsulated LH-RH analog group than in the control group. Our previous studies had shown that LH-RH analog enhanced spermiation but did not significantly decrease sperm counts in black porgy (Yueh *et al.*, 1990). Therefore, we suggest that sperm counts probably also did not decline following stimulation with microencapsulated LH-RH analog in this experiment.

Injected microencapsules were estimated to have released 2.5 μg/day of LH-RH analog for 30 days (Redding *et al.*, 1984; Mason-Garcia *et al.*, 1985). Due to the low ambient water temperature (below 20°C), the rate and duration of LH-RH analog release may have been slower and longer in the black porgy than that reported by Mason-Garcia *et al.* (1985) in rats. The sustained release of LH-RH analog significantly stimulated spermiation in black porgy during the non-spawning season. However, the injection of two doses of LH-RH analog into black porgy during the non-spawning season failed to accerlerate spermiation (Chang *et al.*, 1991). Therefore, treatment with microencapsulated LH-RH analog could be a useful tool

for regulating the spawning season in this fish. Our data were consistent with earlier reports that injections of LH-RH analogs, pituitary extracts, or various gonadotropins stimulated spermiation in several teleosts (Billard *et al.*, 1982; Weil and Crim, 1983; Kyle *et al.*, 1985; Ueda *et al.*, 1985; Courtois *et al.*, 1986; Kobayashi *et al.*, 1986; Chang *et al.*, 1991). LH-RH analog released from microencapsules should stimulate thesecretion of gonadotropin in black porgy. Gonadotropin should further stimulate the production of the steroid mediators that induce spermiation.

There is now abundant evidence indicating that T, 11-ketotestosterone and 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-diOH P) are directly or indirectly involved in spermatogenesis and spermiation (Sundararaj and Nayyar, 1967; Pandey, 1969; Billard *et al.*, 1982; Fostier *et al.*, 1982; Scott *et al.*, 1984; Ueda *et al.*, 1983, 1984; Kobayashi *et al.*, 1986). Due to the lack of availability of 11-ketotestosterone, 17α, 20β-diOH P and black porgy gonadotropin assays for this experiment, only T, E_2, and 17α-OH P were measured. T and 17α-OH P can act as precursors of 11-ketotestosterone and 17α, 20β-diOH P, respectively. Levels of plasma 17α-OH P were not detectable in the black porgy used in this experiment or in previous studies (Chang and Yueh, 1990a and b; Chang *et al.*, 1991). The physiological significance of 17α, 20β-diOH P and 17α-OH P in black porgy requires further study.

Simultaneous peak levels of plasma T and E_2 occured about two weeks before the onset of spermiation in the control group similar to the profiles previously observed in one-year-old black porgy (Chang and Yueh, 1990b); peak levels of sex steroid may be important to the development of gonadal tissues. There was no difference in plasma levels of T between the LH-RH analog-treated and control groups, although milt volumes were larger in the LH-RH analog group. Two minor peaks of plasma T ($p<0.5$) and E_2 ($p>0.5$) were observed in control black porgy during the spawning season. T is not considered to directly involved in the regulation of spermiation (Billard *et al.*, 1982). None of the black porgy were able to produce a plasma E_2 peak following treatment with microencapsulated LH-RH analog. We also found that injections of two doses of LH-RH analog (10 μg/kg BW and 50 μg/kg BW) black porgy during the non-spawning season and functional male phase stimulated high levels of plasma T but not E_2 (see also Chang *et al.*, 1991). The reason for the much lower levels of plasma E_2 in the microencapsulated LH-RH analog was less clear. Bisexual tissues were observed in the gonads of both the microencapsulated LH-RH analog and control fish. Based on the appearance of vitellogenic oocytes in the gonadal tissues, two control fish seemed to be in the early stages of sex reversal. The development of ovarian tissues may be responsible for the higher levels of plasma E_2 observed in the control group.

In conclusion, microencapsulated LH-RH analog can accelerate spermiation in two-year-old protandrous black porgy . Sex reversal from male to female did not occur following treatment with microencapsulated LH-RH analog.

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D-Trp^6-LH-RH 微粒膠囊促進黑鯛之產精作用

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摘　要

本研究之目的在探討以 D-Trp^6-LH-RH 微粒膠囊對 2 年黑鯛生殖與血液性類固醇激素濃度之影響。16 尾黑鯛分為兩組，分別注射一次 D-Trp^6-LH-RH 微粒膠囊或生理食鹽水，實驗期間為 4 個月。由研究結果得知 D-Trp^6-LH-RH 微粒膠囊可促使黑鯛提早 3 星期產精，且每尾皆有產精作用，每尾之產精量也遠比對照組多;而對照組只有 4 尾(50%)有產精。黑鯛血液睪固酮濃度在 D-Trp^6-LH-RH 微粒膠囊與對照組並無顯著之差別，但經 D-Trp^6-LH-RH 微粒膠囊處理之黑鯛血液雌二醇濃度比對照組低，而兩組之血液 17α-hydroxyprogesterone 濃度在實驗期間都是很低。