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Estradiol-17β Stimulates Aromatase Activity and Reversible Sex Change in Protandrous Black Porgy, *Acanthopagrus schlegeli*

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ABSTRACT The objective was to investigate the effects of estradiol-17β (E_2) on gonadal development, spermiation, gonadal aromatase activity, and the concentrations of plasma sex steroids and vitellogenin in 2-year-old protandrous black porgy, *Acanthopagrus schlegeli*. Black porgy were divided into two groups, one fed a control diet and the other a diet mixed with E_2 (4.0 mg/kg feed) for 4½ months. Significantly lower GSI was observed in the E_2 group. Fish treated with E_2 showed completely suppressed spermiation, and 38% had developing vitellogenic oocytes in the gonad (an evidence of sex reversal in 2-year-old fish). Higher gonadal aromatase activity and plasma E_2 concentrations and lower concentrations of plasma 11-ketotestosterone (11-KT) were observed in the E_2 group. After finishing 4½ months of E_2 treatment in the early spawning season, the gonadosomatic index increased and spermiation resumed in the E_2 group (which was fed a control diet) during the mid- or late-spawning season. Steadily increasing levels of plasma T and 11-KT and decreasing gonadal aromatase activity and plasma vitellogenin concentrations were observed in the E_2 group. The present data show that E_2 induced a temporary and reversible sex change (only a small proportion of the fish). Elevated aromatase activity in gonads, elevated E_2 levels in plasma, and diminished levels of plasma 11-KT are associated with the occurrence of sex reversal in protandrous black porgy. *J. Exp. Zool. 280:165–173, 1998.*

Black porgy, *Acanthopagrus schlegeli* Bleeker, a widely distributed marine protandrous hermaphrodite, is of particular interest for commercial aquaculture in parts of Asia (Chang and Yueh, '90a). The fish are functional males for the first 2 years of life but begin to change sex during the third year. Only about 40% of cultured black porgy change to females, whereas the rest remain in the male phase during the spawning season in the 3- or 4-year-old fish (Chang et al., '94). Black porgy in Taiwan have an annual reproductive cycle with a pattern of multiple spawning occurring in later winter and early spring.

High levels of plasma estradiol-17β (E_2) during the prespawning and spawning season are likely correlated with the natural sex reversal of 3-year-old black porgy (Chang et al., '94). Oral administration of E_2 (4 mg per kg of feed) for at least 5 months induced sex reversal in 2-year-old black porgy with vitellogenic oocytes in the gonads (Chang et al., '95a). E_2 also induced sex reversal in 1-year-old black porgy; however, the ovary remained at the stage of primary oocytes (Chang et al., '94, '95a, b). We conclude that E_2 likely plays an important role in the natural and control sex reversal in protandrous black porgy.

Our previous data still could not answer the question of whether the reversed fish will undergo full ovarian development or reverse to males in the absence of any further treatment. Further experimentation still needs to be studied in order to understand better the mechanism and control of sex reversal in black porgy.

Androgens are converted to estrogens by an enzyme complex termed aromatase, which is located in the smooth endoplasmic reticulum and is made up of a NADPH-cytochrome P450 reductase and aromatase cytochrome P450. Aromatase activity was high in the vitellogenic oocytes but low in the early vitellogenic and mature oocytes in amago salmon *Oncorhynchus rhodurus* (Young et al., '83). Treating chicken embryos with aromatase inhibitors has suggested that aromatase is a key

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developmental switch in the sex determination of chickens (Elbrecht and Smith, '92). Aromatase activity in the ovaries was inhibited by 4-hydroxyandrostenedione and resulted in the ac-cumulation of testosterone (T), which induced transformation of the ovaries into testes in *Rana catesbeiana* tadpoles (Yu et al., '93). However, the relationship between gonadal aromatase activity and sex reversal in protandrous fish is less understood. Control of the aromatase activity is also little known in fish.

Therefore, the objective of the present study is to investigate the responses of gonadal development, spermiation, gonadal aromatase activity, and the concentrations of plasma sex steroids and vitellogenin following oral administration of E_2 in 2-year-old black porgy. The effects of interrupting the E_2 treatment in the E_2 group during the early spawning season was also studied.

MATERIALS AND METHODS

Animals

Two-year-old black porgy, *Acanthopagrus schlegeli* (n = 150, mean body weight = 238.5 ± 12.8 g) were obtained from pond culture in September 1991. All experimental fish were acclimated to the pond at the University culture station with a seawater system. The fish were fed with commercial feed (Fwu Sow Feed Co., Taichung, Taiwan). Water temperature ranged from 19° to 26°C during the experimental period.

Experimental design

Because a dose of 4.0 mg E_2/kg feed given to 1- and 2-year-old black porgy has been shown to induce sex reversal (Chang et al., '94, '95a, b), that dosage was also selected for this experiment. Black porgy were divided equally into two groups, a control group and one given 4.0 mg E_2/kg of feed. The E_2 treatment by oral administration ad libitum was maintained during the first experimental period (October 4, 1992–February 17, 1993). Treatment was interrupted in the E_2 group after February 17, 1993, and all the fish were fed control diets from February 18 to April 22, 1993 (second experimental period). Every two weeks, 8–12 fish per group were randomly collected and bled and tested for spermiation. Every month, eight fish per group were bled and sacrified.

Sampling procedures

The fish were anaesthetized in 2-phenoxyethanol, and blood was taken with an EDTA-containing tube from the caudal vasculature. Milt was obtained just after bleeding by hand stripping with an application of gentle pressure on the abdomen. The number of fish spermiating and the volume of collectible milt were recorded. The sperm concentrations in milt were measured with a hematocytometer. The plasma was separated by centrifugation and stored at –70°C for later analysis of sex steroids and vitellogenin. After blood samples were collected, the gonads were quickly dissected and weighed. Part of the gonads were fixed in Bouin's fluid for histology. Aromatase activity in the gonad was also measured. Total body and gonadal weight were measured for the calculation of gonadosomatic index (GSI = gonadal weight/body weight × 100%).

Gonadal histology

A piece from the central part of the gonad (a better representative of the bisexual gonad) was fixed in a Bouin's solution and embedded in paraffin and sectioned at 6 μm. Transverse sections were stained with hematoxylin and eosin. Developmental stages of germs cells were determined.

Measurement of aromatase activity

Monthly aromatase activity in the gonad was measured by a radiometric method because of the stereospecific loss of hydrogen from the C-1β position of 1β-^{3}H-androstenedione (^{3}H-A) during aromatization and the formation of H_2O (method modified from Fishman and Raju, '81; Chen and Tsai, '90). Gonads were homogenized with potassium phosphate buffer (100 mM KCl, 10 mM KH_2PO_4, 1mM EDTA, 10 mM dithiothreitol, pH 7.4; 1:10, w/v). The homogenate was centrifuged at 1,000*g* for 10 min at 4°C. The crude supernatant fraction was added with 100 μl cofactor solution (100 mM KCl, 10 mM K_2HPO_4, 1mM EDTA, 10 mM dithiothreitol, 5 mM glucose-6-phosphate, 1mM β-nicotinamide adenine dinucleotide phosphate, 10 U glucose-6-phosphate dehydrogenase, pH 7.4) and 0.6 μM ^{3}H-A (555 GBq-1.11 TBq; Du Pont Co., NEN Research Products, Boston, MA). The reaction solution was incubated at 28°C for 80 min and stopped by adding 10% trichloroacetic acid (containing 20 mg charcoal/ml). After centrifugation, the supernatant solution was subjected to a column (1.0 × 3.0 cm, i.d.) packed with the mixture of AG 50W-X4 resin (50–100 mesh and 100–200 mesh, Bio-Rad CO., Hercules, CA) and charcoal (6:1). The elution was performed with 5 ml distilled water and collected at 0.5 ml per fraction. The radioactivity of each fraction was

measured by a liquid scintillation counter (Beckman 1801). The total radioactivities of the fractions from 1.0 to 2.5 ml were calculated as the production of H_2O on the basis of the preliminary study. The protein concentration of the crude supernatant fraction was measured with a Bio-Rad protein assay kit (Bio-Rad Co.). Gonadal aromatase activity was expressed as fmol ^3H_2O/hr·mg protein.

Steroid and vitellogenin assay

Plasma E_2, T, and 11-ketotestosterone (11-KT) were measured in plasma samples collected biweekly. Radioimmunoassay was performed following diethyl ether extraction as described by Chang et al. ('95a). Vitellogenin was measured in plasma using a solid-phase enzyme-linked immunosorbent assay according to the method of Chang et al. ('96).

Data analysis

Student's *t* test was used to test whether the results of the control and treated groups were significantly different at the 5% level ($P < 0.05$). Results are given as a mean ± standard error of the mean.

RESULTS

Gonadosomatic index, spermiation, and gonadal histology

The E_2 group showed significantly lower GSI ($P < 0.05$) during the first experimental period than the control group did during the early spawning season (December–February, Table 1). Spermiation did not occur in the E_2 group during the first experimental period (Table 2). Spermiation in the control group mainly occurred in February and March with the concentration (1.55–2.06) × 10^{10} sperm/ml of milt (Table 2).

TABLE 1. The effects of oral administration of estradiol-17β (E_2) on gonadosomatic index in two-year-old black porgy[1]

	Gonadosomatic index (%)[2]	
Date	Control	E_2
1992		
Sep	0.06 ± 0.01	0.06 ± 0.01
Oct	0.19 ± 0.04	0.10 ± 0.01
Nov	0.25 ± 0.04	0.25 ± 0.09
Dec	0.80 ± 0.17	0.21 ± 0.10*
1993		
Jan	2.06 ± 0.64	0.20 ± 0.06*
Feb	5.97 ± 1.17	0.80 ± 0.39*
Mar	9.03 ± 1.39	2.30 ± 0.64*
Apr	1.52 ± 0.10	3.85 ± 0.78*

[1]The period of E_2 treatment was from October 4, 1992 to February 17, 1993.
[2]Mean ± SEM (n = 8 per datum)
*The values significantly differed between the control and E_2 groups ($P < 0.05$).

After interruption of E_2 treatment in February (second experimental period), GSI, percentage of fish spermiating, and milt volume increased significantly in the E_2 group (Tables 1, 2).

In the control group, gonad with mainly primary oocytes and a small portion of developing testicular tissue was observed in October (Fig. 1a), and then advanced male germ cells were observed in December (Fig. 1b). Well-developed testicular tissue was observed in February, but only a few primary oocytes appeared in the ovarian tissue in the control group (Fig. 1c). Regression of testicular tissue in the control group appeared in April (Fig. 1d). After 1–3 months E_2 treatment, testicular tissue was gradually regressed and the development of primary oocytes was stimulated in the E_2 group (Fig. 2a, b, c, d). The male germ cells were not easily identified in the regressing testicular tissue in December and January (Fig. 2d). After 4½ months of E_2 treatment (February), ovarian tissue with vitellogenic oocytes appeared in the gonads in 38% of E_2-treated fish (Fig. 2e). After interruption of E_2 treatment for 2 months in the E_2 group, testicular tissue was well-developed and became dominant, and spermatozoa mainly appeared in the testicular tissue (Fig. 2f).

Aromatase activity in gonads

Gonadal aromatase activity increased but did not differ in the two groups in October (Fig. 3). Aromatase activity was significantly ($P < 0.05$) stimulated by the E_2 treatment in the gonads of E_2 group as compared to the control group from November to February (Fig. 3). The control group had higher ($P < 0.05$) gonadal aromatase activity as compared to the E_2 group after the interruption of E_2 treatment in April (Fig. 3).

Plasma sex steroids and vitellogenin

Plasma E_2 concentrations were quite low (<100 pg/ml) in both groups, but the E_2 group had higher ($P < 0.05$) levels of E_2 during the E_2 treatment period than did the control group (Fig. 4). Increasing concentrations of plasma T were observed in the control group during the spawning season (Fig. 5). Lower concentrations of plasma T in December–February were observed in the E_2 group as compared to the control group (Fig. 5). A surge of plasma T levels was detected in the E_2 group just after interruption of E_2 treatment (Fig. 5).

In the control, plasma concentrations of 11-KT

TABLE 2. The effects of estradiol-17β (E_2) on numbers of fish spermiating, milt volume and sperm concentrations in black porgy (N = 8–12 per datum)

		Fish spermiating (%)		Milt volume (ml/spermiating fish)		Sperm no. ($\times 10^{-10}$/spermiating fish)	
		Control	E_2	Control	E_2	Control	E_2
1992							
Nov	28	12.5	0	0.16	—[2]	0.89	—
Dec	1	8.3	0	+[3]	—	—	—
	23	62.5	0	+	—	—	—
1993							
Jan	7	80.0	0	0.21 ± 0.06	—	1.78 ± 0.24	—
	19	87.5	0	0.54 ± 0.18	—	1.94 ± 0.28	—
Feb	2	100.0	0	1.56 ± 0.35	—	2.06 ± 0.14	—
	17	100.0	0	1.41 ± 0.27	—	1.64 ± 0.14	—
Mar	9	100.0	0	3.74 ± 0.73	—	1.55 ± 0.08	—
	23	87.5	25.0	4.28 ± 0.61	1.59 ± 1.21	1.56 ± 0.06	0.89 ± 0.00
Apr	8	100.0	100.0	2.92 ± 0.30	1.73 ± 0.44	1.78 ± 0.09	1.93 ± 0.16
	22	100.0	100.0	1.43 ± 0.33	2.01 ± 0.49	1.41 ± 0.27	2.45 ± 0.15

[1]The period of E_2 treatment was from October 4, 1992 to February 17, 1993. Spermiation did not occur in the control and E_2-treated groups from September 21 to November 6, 1992.
[2]Measurement of milt volume or sperm number is not applicable.
[3]Only small amount of milt could be collected.

significantly increased from September to February ($P < 0.05$); in contrast, only low levels of plasma 11-KT were detected in the E_2 group (Fig. 6). In the control group, 11-KT levels decreased in March and April (Fig. 6). Plasma 11-KT levels increased ($P < 0.05$) after the interruption of E_2 treatment in the E_2 group (Fig. 6).

Significantly higher ($P < 0.05$) concentrations of plasma vitellogenin were observed in the E_2 group (Fig. 7). Plasma vitellogenin levels significantly decreased after E_2 interruption and then reduced to the levels in the control group in March (Fig. 7).

DISCUSSION

The E_2 treatment completely suppressed testicular development and spermiation while it stimulated ovarian development in 2-year-old black porgy. All the control fish showed spermiation (as a functional male during the spawning season). The success of sex reversal is demonstrated in 38% of the E_2-treated fish in February on the basis of the presence of vitellogenic oocytes. However, after the interruption of E_2 treatment, the gonadosomatic index increased in the E_2 group from 0.80 in February to 3.85 in April as compared to the control group (from 5.97 to 1.52). After treatment stopped, testicular development and spermiation resumed in all the E_2-treated fish. Therefore, E_2 induced a temporary and reversible female (in only a small proportion of fish) in this study.

E_2 was able to induce sex reversal in 1- and 2-year-old black porgy after more than 5 months of treatment (Chang et al., '94; '95a, b). We further observed that 10% of 2-year-old reversed black porgies became mature (with transparent oocytes) in April (Chang et al., '95a). Compared to the previous studies, different responses of sex reversal were observed in this experiment. The difference in the experimental design and the shift of the spawning season might cause this inconsistency. The duration of the E_2 treatment was 4½ months intervals in this study, which was shorter than in the previous studies (Chang et al., 95a, b). The peak spawning season did not occur until March in the control fish in this study, as compared to the previous studies (February) (Chang et al., '95a, b). However, this study indeed provides another interesting observation that E_2 induced a reversible sex change in black porgy. The duration, timing and even the dose of E_2 treatment might be important to induce a complete sex reversal, and those factors need to be further studied in black porgy. Low doses of E_2 (0.25 or 1.0 mg/kg feed), on the contrary, stimulated testicular development and plasma 11-KT concentrations in 1-year-old black porgy (Chang et al., '95b).

Low concentrations of plasma E_2 in 2-year-old male black porgy (control groups) were consistent with the previous studies in black porgy (Chang and Yueh, '90; Chang et al., '94; '95a, b) and in *Sparidendex hasta* (Kime et al., '91). Higher levels of plasma E_2 were observed in the naturally reversing female during the prespawning season (Chang et al., '94, '95c). In this study, higher lev-

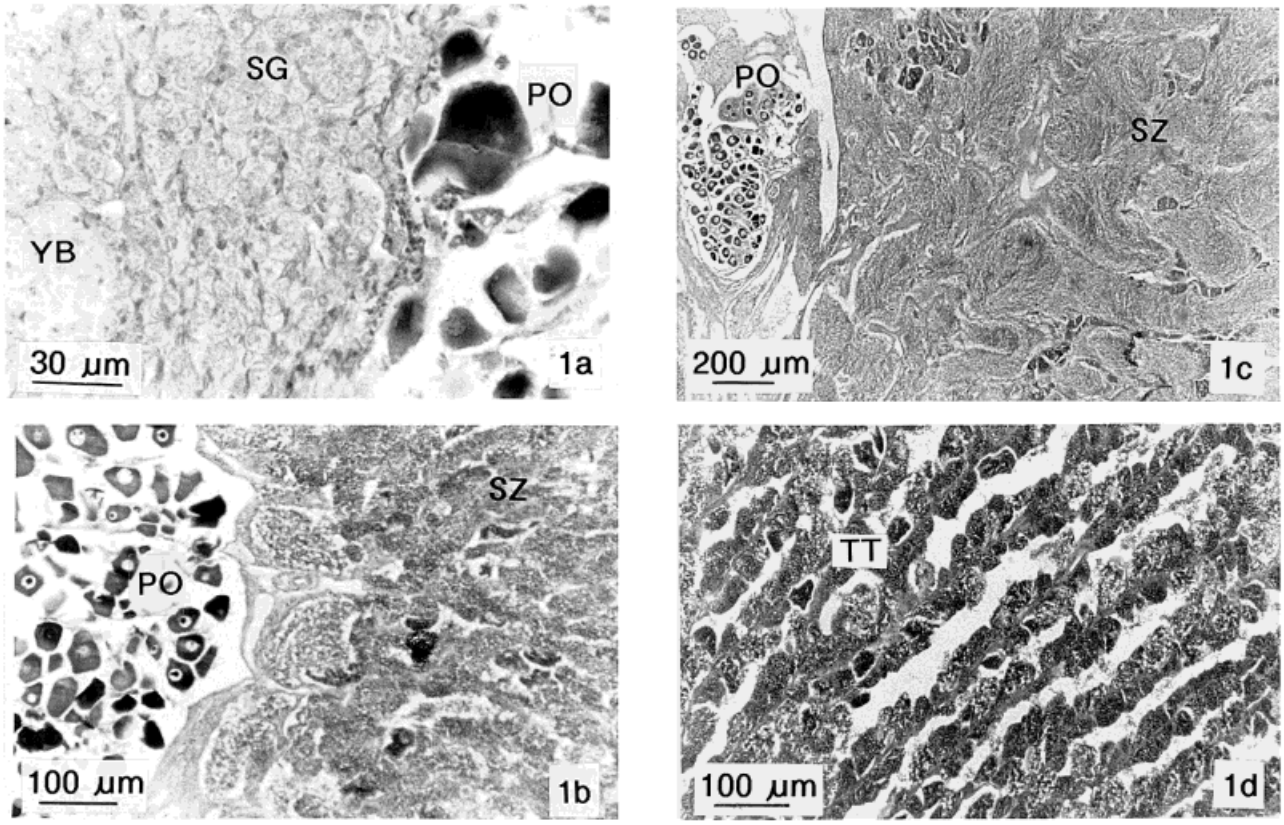
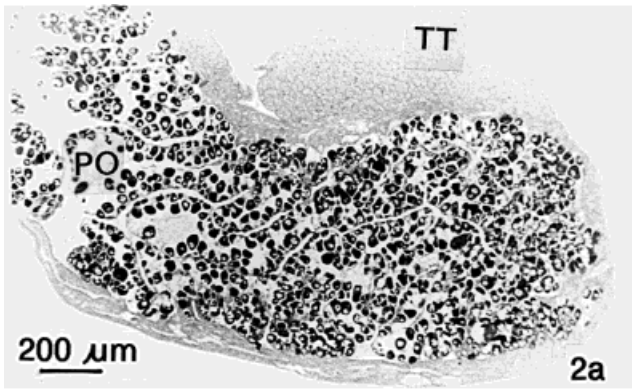
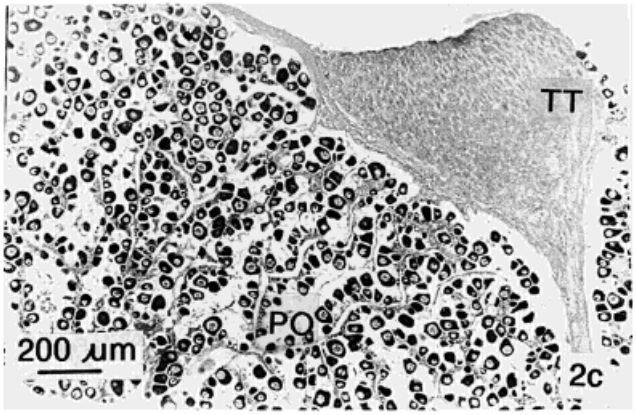


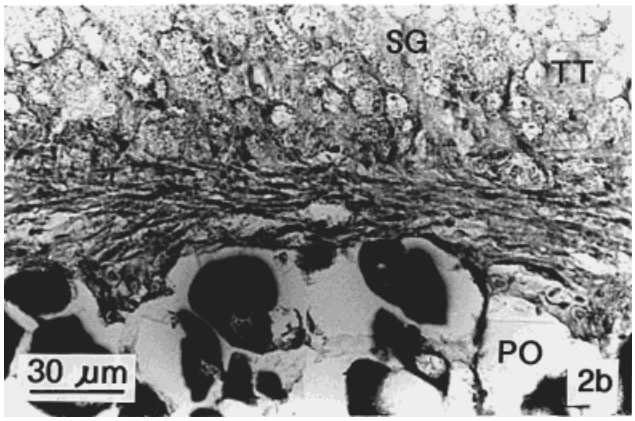
Fig. 1. Transverse sections of the gonads stained with haematoxylin and eosin in the control group. (**a**) Fish in October with spermatogonia (SG) in testicular tissue and primary oocytes (PO) with regressing tissue (yellow bodies, YB) in gonadal tissue. (**b**) Fish in December showing advanced male germ cells (spermatozoa, SZ) in testicular tissue and primary oocytes (PO) in ovarian tissue. (**c**) Fish in February showing spermiating spermatozoa (SZ) in testicular tissue and a few primary oocytes (PO) in ovarian tissue. (**d**) Fish in April showing regressing testicular tissue (TT).

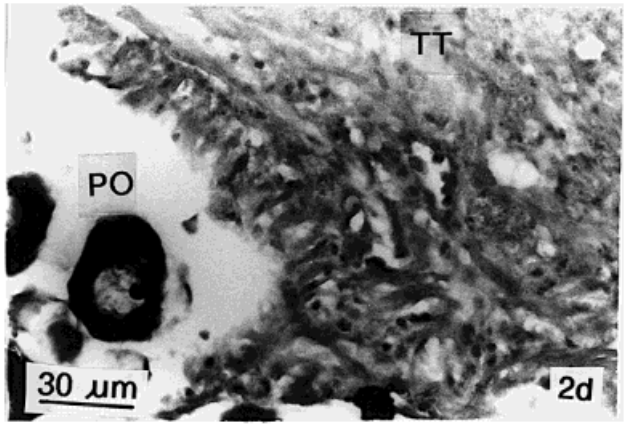
els of plasma E_2 were also observed in the E_2 group than the control group. It is possible that plasma levels of E_2 in the treated group were mostly the results of both anabolism (endogenous production) and catabolism. However, the levels of plasma in the E_2-treated group (this study) were significantly lower than those in naturally reversing females (Chang et al., '94). Sex reversal in the E_2 treated fish may be not completed because plasma E_2 levels were still quite low.

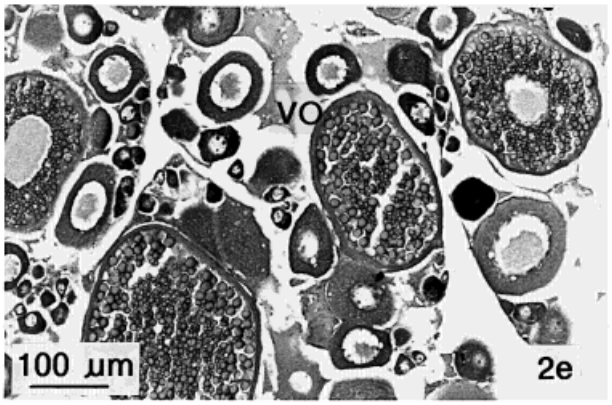
Higher levels of plasma E_2 were comparable to the higher gonadal aromatase activity in most of the samples in the E_2 group. Sex reversal was also associated with higher gonadal aromatase activity in this study. Previous studies also implied the deficiency of gonadal aromatase activity in male and bisexual black porgy because plasma E_2 levels were not induced in those fish by the injection of LHRH analog (Chang et al., '91). The current data further support the involvement of the "gonadal aromatase-E_2 biosynthesis" on sex reversal in the protandrous black porgy (Chang et al., '97). This may be the key step of the mechanism of sex reversal in the protandrous black porgy. Higher levels of plasma E_2 are more consistent with the E_2 treatment than with the gonadal aromatase activity. It is unclear why high aromatase activity was observed in the control group in October. The increase in aromatase activity in the control fish in April was probably due to the development of primary oocytes in the ovarian tissue when testicular regression occurred concomitantly. Gonadal aromatase activity has been shown to have a relationship to ovarian development in animals. Masculinization of the gonad by aromatase inhibitors has also been seen in gonochoristic chinook salmon (*Onchorhynchus tshawytsha*) (Piferrer et al., '94) and lizard (Wennstrom and Crews, '95). Aromatase activity had been indicated to play a key role in female expression in chickens (Elbrecht and Smith, '92) and tadpoles (Yu et al., '93). Aromatase activity in the ovarian











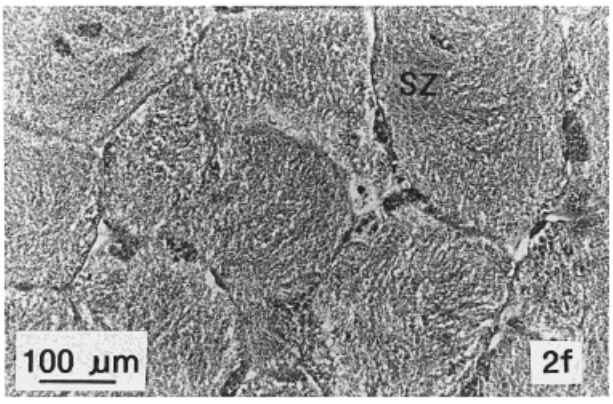


Fig. 2. Transverse sections of the gonads stained with haematoxylin and eosin in the estradiol-treated group. (**a**) Fish in November showing well-developed primary oocytes (PO) in ovarian tissue, and regressing testicular tissue (TT). (**b**) A magnified gonadal tissue from Figure 2 (a) showing the primary oocytes (PO) in ovarian tissue and spermatogonia (SG) in regressing testicular tissue (TT). (**c**) Fish in December and January showing well-developed primary oocytes (PO) in ovarian tissue and regressing tissue (TT). (**d**) A magnified regressing testicular tissue from Figure 2 (c). (**e**) Fish in February showing vitellogenic oocytes (VO) in the gonads. (**f**) Fish in April showing spermiating spermatozoa in the well-developed testicular tissue.

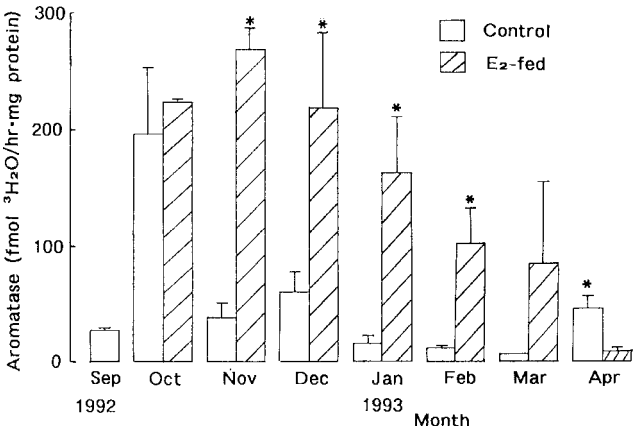


Fig. 3. The gonadal aromatase activity in the 2-year-old black porgy during oral administration of estradiol-17β (E_2) or control diet. The period of E_2 treatment is from October 4 of 1992 to February of 1993. *Significant difference ($P < 0.05$) between the control and E_2-treated group.

granulosa cells of amago salmon *O. rhodurus* is associated with the stage of oocytes growth (Young et al., '83). These data support the proposal of Pieau et al. ('94) that high levels of estrogen resulting from the activation of aromatase gene transcription would activate ovary-determining genes in vertebrates.

Gonadal aromatase activity was significantly stimulated by E_2 treatment in black porgy. There is less information available regarding the regulation of gonadal aromatase activity by sex steroids. It is not clear that the stimulation of gonadal aromatase activity was due to the direct effects of E_2 on the gonad or indirect effects stimulated by the action of gonadotropin secretion. Ovarian aromatase activity was stimulated by the

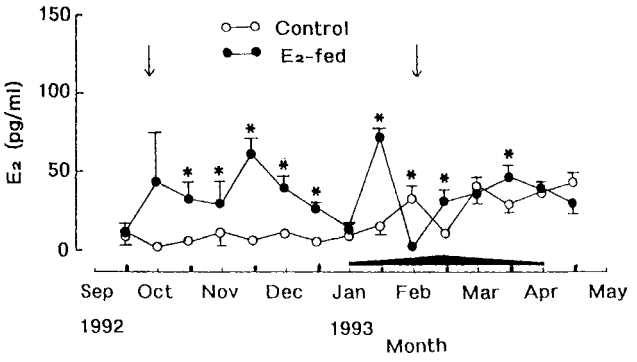


Fig. 4. The concentrations of plasma estradiol-17β (E_2) in the 2-year-old black porgy during oral administration of E_2 or control diet. Arrows indicate the period of the E_2 treatment. The black bar indicates the spawning season in the control. *Significant difference ($P < 0.05$) between the control and E_2-treated groups.

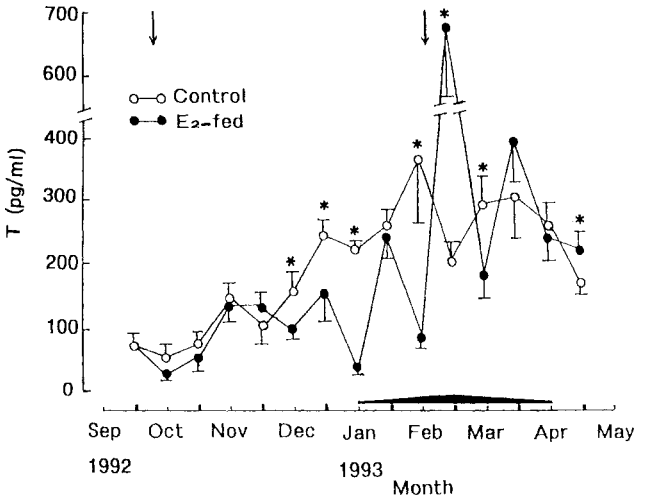


Fig. 5. The concentrations of plasma testosterone (T) in the 2-year-old black porgy during oral administration of estradiol-17β (E_2) or control diet. Details and symbols as for Figure 4.

injection of pregnant mare's serum gonadotropin in the medaka, *Oryzias latipes* (Nagahama et al., '91). Treatment of castrated males with T and 11-KT increased the aromatase activity in whole brains of Atlantic salmon (*Salmo salar* L.) parr (Mayer et al., '91). In rat brain, T but not E_2 stimulated aromatase activity, while E_2 and dihydrotestosterone acted synergistically to regulate aromatase activity (Roselli and Resko, '93).

Exogenous E_2 suppressed the concentrations of plasma 11-KT more than T in black porgy. Significantly lower levels of plasma 11-KT were ob-

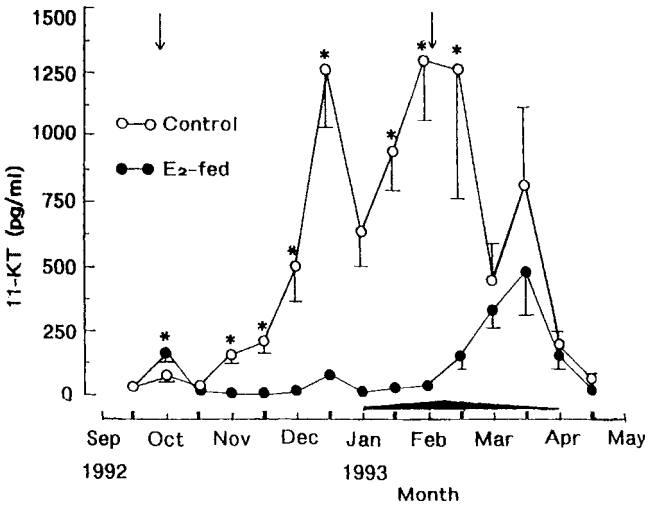


Fig. 6. The concentrations of plasma 11-ketotestosterone (11-KT) in the 2-year-old black porgy during oral administration of estradiol-17β (E_2) or control diet. Details and symbols as for Figure 4.

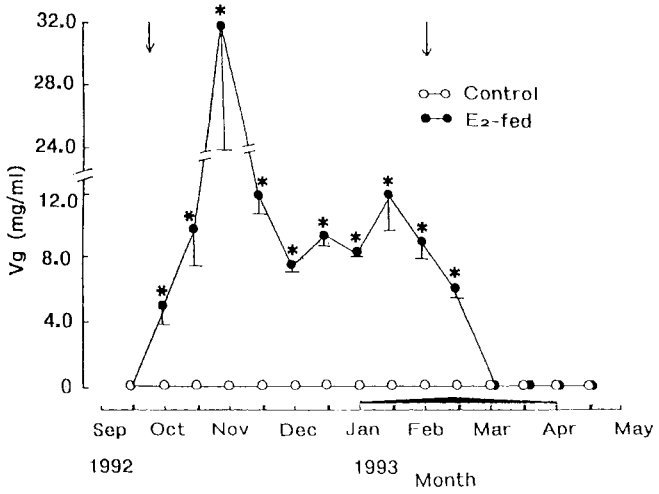


Fig. 7. The concentrations of plasma vitellogenin (Vg) in 2-year-old black porgy during oral administration of estradiol-17β (E_2) or control diet. Details and symbols as for Figure 4.

served in E_2-treated fish than in the control group, whereas plasma T levels were only transiently lower in December-February. Significantly lower levels of plasma 11-KT but not T were also observed in the 4.0 mg E_2-treated (reversed female) 1-year-old black porgy (Chang et al., '95b). Concentrations of plasma T were not different in the male and naturally reversing female (3-year-old) black porgy (Chang et al., '94, '95c). Both plasma 11-KT and T were decreased in the 4.0 mg E_2-treated (reversed female) 2-year-old black porgy (Chang et al., '95a, b). The results are also consistent with other species; protandrous sobaity, *S. hasta* (Kime et al., '91) and seabass, *Lates calcarifer* (Guiguen et al., '93) are reported to have higher levels of plasma 11-KT in males than in females. Significant increases in plasma T occurred when the E_2 feedings ceased. These data may indicate that E_2 feedings have a "feedback inhibition" on the hypothalamo-hypophyseal axis or "direct inhibition" on the T biosynthesis in the gonad. The possible negative feedback inhibition of E_2 on the hypothalamo-hypophyseal axis was also suggested in the previous study (Chang and Yuen, '90b).

The present study shows that levels of 11-KT but not of T have a positive association to GSI value. These data confirmed the previous findings that 11-KT associated with the testicular development and spermatogenesis in 1- and 2-year-old black porgy (Chang et al., '95a, b). 11-KT has also been implicated in spermatogenesis of Japanese eel, *Anguilla japonica* (Miura et al., '91a, b). However, 11-KT levels did not closely related to the percentage of spermiating fish and the amount of milt production. Periods of the maximal levels of 11-KT and spermiating performance in this experimental were February and March, respectively. Other steroids, such as 17, 20β-dihydroxy-4-pregnen-3-one or 17, 20β, 21-trihydroxy-4-pregnen-3-one, might be more closely associated with the spermiation in black porgy. Both 17, 20β-dihydroxy-4-pregnen-3-one and 17, 20β, 21-trihydroxy-4-pregnen-3-one were potent inducers *in vivo* for stimulating spermiation in black porgy (Yueh and Chang, '97). More detailed studies are necessary to clarify which steroids is physiologically implicated in the spermiation of black porgy.

In summary, E_2 stimulated a higher gonadal aromatase activity and sex reversal and also selectively suppressed plasma 11-KT levels. Interruption of E_2 treatment during the early spawning season resulted in the resumption of spermatogenesis, spermiation, and testicular development. The data show that E_2 induced a temporary and reverible sex change in a small proportion of the fish. Elevated aromatase activity in gonad and diminished levels of plasma 11-KT are associated with the occurrence of sex reversal in protandrous black porgy.

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