Fish Physiology and Biochemistry vol. 14 no. 6 pp 481-488 (1995)
Kugler Publications, Amsterdam/New York

Estradiol-17β suppresses testicular development and stimulates sex reversal in protandrous black porgy, *Acanthopagrus schlegeli*

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Accepted: May 17, 1995

Keywords: estradiol-17β, sex reversal, testicular development, 11-ketotestosterone, black porgy

Abstract

Two year old black porgy (*Acanthopagrus schlegeli*) fed a diet containing 4.0 mg kg^{-1} of estradiol-17β (E_2) for 5 months had significantly lower GSI than the control group during the spawning season. E_2 suppressed testicular development, spermiation and plasma testosterone (T) and 11-ketotestosterone (11-KT) and stimulated ovarian development, vitellogenesis and sex reversal. Spermiation in the control group occurred in January and February with the concentrations of $1.08-1.36 \times 10^{10}$ sperm ml^{-1} of milt. Higher plasma T and 11-KT, but lower E_2 levels were detected in the spermiating fish (control group). Higher plasma E_2 levels were detected in the sex reversing black porgy during the pre-spawning season. A sharp rise in plasma 11-KT and a drop in T levels were detected in spermiating fish (control group) from January to February. Plasma 11-KT levels correlated with the testicular development and spermiation. The data suggest that E_2 plays an important role in controlling the sex reversal of black porgy.

Introduction

Sex steroids have significant effects on gonadal development and reproduction in teleosts. Estradiol-17β (E_2), testosterone (T) and 11-ketotestosterone (11-KT) have received the most attention; T and 11-KT appear to be involved in spermatogenesis and spermiation (Sundararaj and Nayyar 1967; Hansson *et al.* 1976; Cochran 1992), while E_2 induces vitellogenesis synthesis and oocyte development (de Vlaming *et al.* 1980; Kishida *et al.* 1992). There is less information on the gonadal development and its relation to endocrine data in the protandrous male black porgy.

Black porgy, *Acanthopagrus schlegeli* Bleeker, a marine protandrous hermaphrodite, is widely distributed and is a species of particular interest for commercial aquaculture in parts of Asia (Chang and Yueh 1990). Fish are males for the first 2 years of life but begin to change to females during the third year. Only about 40% of cultured black porgy change to females, while the rest remained in the male phase during the third spawning season (Chang *et al.* 1994). Black porgy in Taiwan have an annual reproductive cycle with a pattern of multiple spawning occurring in later winter and early spring.

High plasma E_2 levels during the prespawning and spawning season are likely correlated with the natural sex reversal of 3 year old black porgy (Chang *et al.* 1994). Oral administration of E_2 (4 mg kg^{-1} of feed) for 5 months induced sex reversal in one year old black porgy; however, the

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Table 1. The effects of oral administration of estradiol-17β (E_2) on gonadosomatic index in 2 year old black porgy

		Gonadosomatic index (%)[a]	
		Control	E_2
1991	Sep	0.40 ± 0.04	0.35 ± 0.05
	Oct	0.32 ± 0.04	0.30 ± 0.07
	Nov	0.20 ± 0.04	0.52 ± 0.07
	Dec	0.42 ± 0.09	0.60 ± 0.09
1992	Jan	1.37 ± 0.15*	0.92 ± 0.09
	Feb[b]	3.01 ± 0.27*	1.23 ± 0.39

[a]Mean ± SEM (n = 8); [b]all the control and E_2-treated fish in February were males and females, respectively, on the basis of the gonadal observation; *The values significantly differed between the control and 4.0 mg E_2 groups ($p < 0.05$).

ovarian gonad remained at the stage of primary oocyte (Chang *et al.* 1994). The effects of E_2 on the gonadal development in 2 year old protandrous black porgy remain unknown. Therefore, the objectives of the present study are to investigate the responses of gonadal development, spermiation, the concentrations of plasma sex steroids and vitellogenin following oral administration of E_2 in 2 year old black porgy.

Materials and methods

Animals

Two year old black porgy (n = 120, mean body weight = 147.1 ± 14.5 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).

Experimental design

E_2 at a dose of 4.0 mg kg^{-1} feed given to 1 year old black porgy for 5 months induced sex reversal (Chang *et al.* 1994) and was also selected for this experiment. Black porgy were divided equally into 2 groups, control and E_2-fed; the E_2 treatment by oral administration *ad libitum* was maintained during the whole experimental period (from September 1991 to February 1992). The bisexual gonad, consisting of mainly spermatogonia and primary oocytes, was confirmed in fish at the start of the experiment (September 1991). Every two weeks, 8–10 fish per group were randomly collected, bled and tested for spermiation. Every month, 8 fish per group were bled and sacrificed.

Sampling procedures

The fish were anaesthetized in 2-phenoxyethanol and blood was collected from the caudal vasculature in an EDTA-treated tube.

Milt was obtained just after bleeding by hand stripping and the number of fish spermiating and the volume of collectable milt were recorded. The sperm concentration in the milt was measured with a hematocytometer. The plasma was separated by centrifugation and stored at −70°C for later analysis of steroid and vitellogenin concentration. After blood samples were collected, the gonads were dissected, weighed and fixed in Bouin's fluid. Total body and gonadal weight were measured for the calculation of gonadosomatic index (GSI = gonadal weight/body weight × 100%).

Gonadal histology

Pieces taken from the central part of the gonads (a better representative for the bisexual gonad) were embedded in paraffin and sectioned at 5μm. Transverse sections were stained with haematoxylin and eosin and the developmental stages of germ cells were determined.

Assays

Plasma E_2, T and 11-KT concentrations were measured by radioimmunoassay following solvent extraction without chromatography separation. For each steroid assay, plasma (100 μl) was mixed with phosphate buffer (200 μl, pH 7.0) and extracted

Table 2. The effects of estradiol-17β (E_2) treatment on numbers of fish spermiating, milt volume and sperm concentrations in black porgy (n = 8–10). Spermiation did not occur in the control and E_2-treated groups from September 15 to December 15 of 1991.

	Fish spermiating (%)		Milt volume (ml per spermiating fish)		Sperm number × 10^{-10} per spermiating fish	
	Control	E_2	Control	E_2	Control	E_2
1991						
Dec 30	12.5	0	+[a]	0	–[b]	–
1992						
Jan 15	90	0	0.10 ± 0.01	0	1.10 ± 0.02	–
30	90	0	0.21 ± 0.02	0	1.36 ± 0.01	–
Feb 15	100	0	0.52 ± 0.01	0	1.08 ± 0.01	–

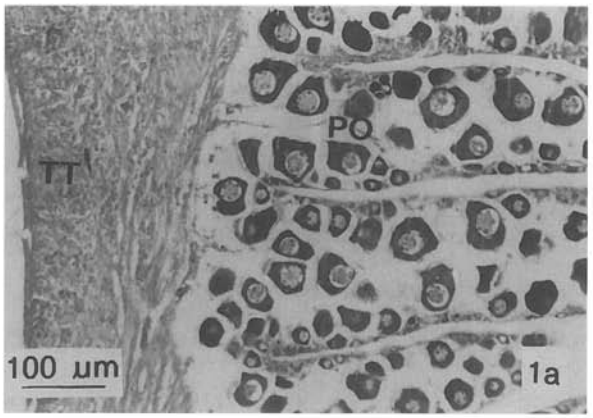
[a]Only small amount of milt could be collected; [b]number not available.

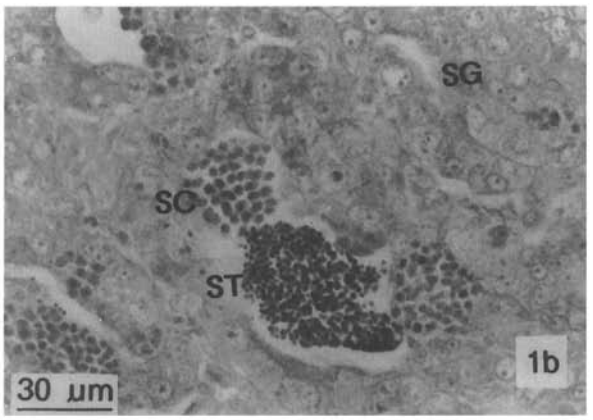
once with 3.5 ml of ethyl ether. Bound steroids were separated from free steroids by the addition of dextran-coated charcoal followed by centrifugation. The bound [^{3}H]-steroid in the supernatant fluid was counted in a liquid scintillation spectrophotometer (Wallac 1409, Pharmacia, Finland) after being mixed with a scintillation cocktail (Ready Safe, Beckman Inc., Fullerton, California). [2,4,6,7-^{3}H]-E_2 (3.15–4.26 TBq mmol^{-1}) and [1,2,6,7-^{3}H]-T (3.15–3.89 TBq mmol^{-1}) were purchased from NEN Research Products (Boston, Massachusetts). [1,2-^{3}H] 11KT (1.92 TBq mmol^{-1}) was a gift from Dr. P. Thomas (University of Texas, Austin, Texas), and had been purchased from Amersham Co. (Arlington Heights, Illinois). Extraction efficiencies for E_2, T and 11-KT were 74, 77 and 78%, respectively. The sensitivities of the assay for E_2, T and 11-KT were 5, 6.25 and 12.5 pg per tube, respectively. Cross-reactions of the antisera with E_2, T and 11-KT were evaluated by England *et al.* (1974) and Kime and Dolben (1985), respectively. Intra-assay and inter-assay variation for the steroids were 11.5–17.0 and 15.5–20.1%, respectively.

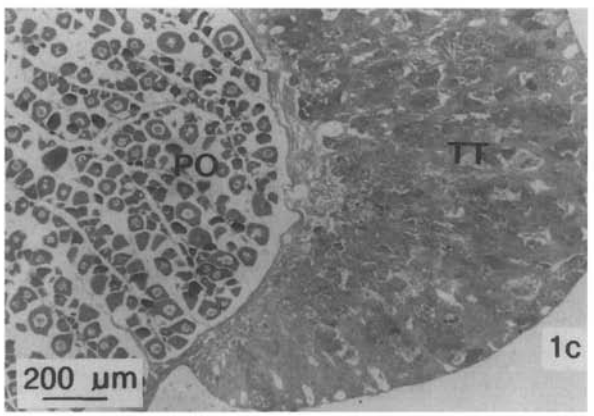
Vitellogenin concentrations in the plasma collected in February 1992 were measured with a solid-phase enzyme linked-immunosorbent assay (ELISA) modified from the methods described in prawns (Chang and Shih 1995). Vitellogenin standard was obtained from the purification of Sepharose-CL 2B and hydroxylapatite chromatography. Purified vitellogenin (1 μg 100 μl^{-1} in sodium bicarbonate buffer) was coated on the 96 well plates overnight at 4°C. After washing, the wells were blocked with a blocking buffer for 1 h at 37°C. The diluted plasma and specific antiserum were added to the wells, and incubated overnight at 4°C. Goat anti-rabbit Ig antibodies conjugated with alkaline phosphatase (Sigma) were diluted and added to each well. Dissolved *p*-nitrophenylphosphate (Sigma) solution (pH 9.8) was added to each well as a substrate. Optical densities were read at 410 nm using an ELISA reader (EIA MP 700 Microplate Reader, Dynatech Lab.). The sensitivity of ELISA was 4 ng per assay, and intra-assay and inter-assay variation was 5.8 (n = 9) and 9.4% (n = 5), respectively.

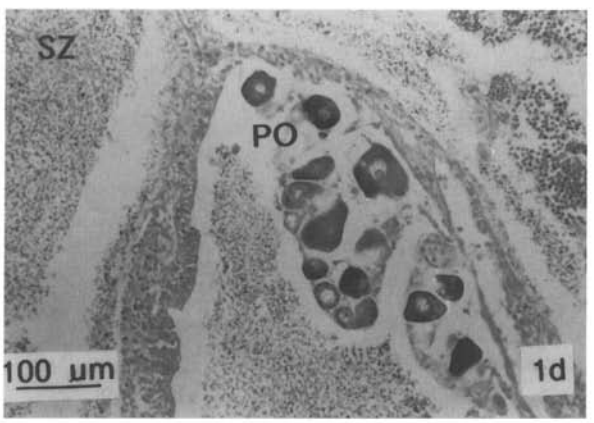
Data analysis

Standard error of mean (SEM) was calculated. Analysis of variance followed by Duncan's multiple range test was used to test the significance of difference in sex steroid concentrations and transformed gonadosomatic indices (arcsine transformation) between the control and E_2-treated groups (Steel and Torrie 1980).









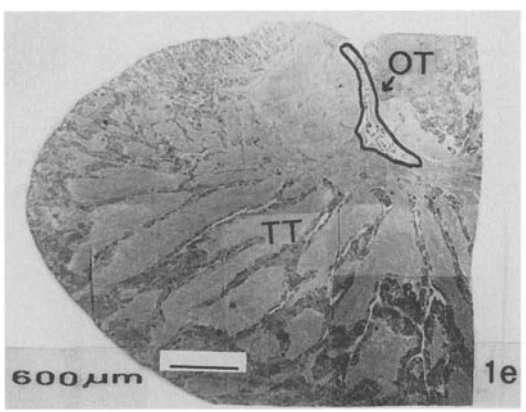
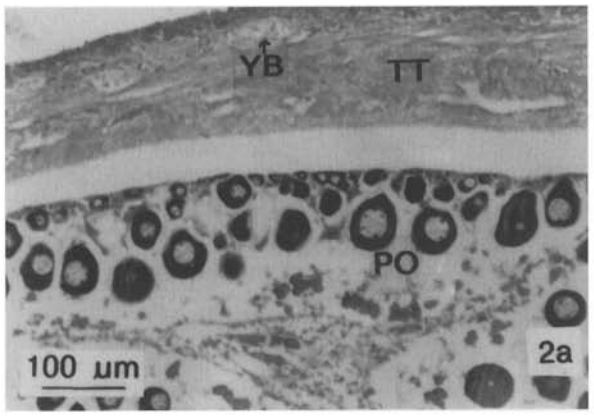


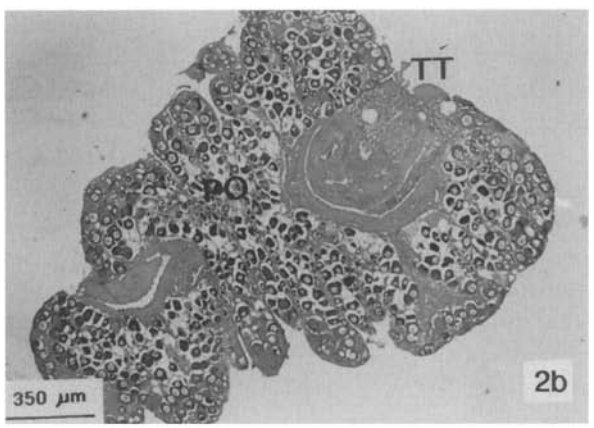
Fig. 1. Transverse sections of gonads of the control group stained with haematoxylin and eosin. (a) A fish in October with developing testicular tissue and primary oocytes (PO) in ovarian tissue, (b) a fish in November showing advanced germ cells (spermatogonia, SG; spermatocytes, SC; Spermatid, ST), (c) a fish in December showing well developed testicular tissue (TT) and primary oocytes (PO) in ovarian tissue, (d) a fish in February showing spermiating spermatozoa (SZ) and a few primary oocytes (PO) and (e) a fish in February showing a relative size of ovarian (OT)/testicular (TT) tissues.

Results

Gonadosomatic index and spermiation

Significantly lower GSI ($p<0.05$) was observed in the E_2-treated group compared with the controls during the spawning season (January and February; Table 1). Spermiation did not occur in the E_2-treated group during the experimental period (Table 2), but in the control group was evident mainly in January and February with concentrations of $1.08-1.36\times10^{10}$ sperm ml^{-1} of milt (Table 2).





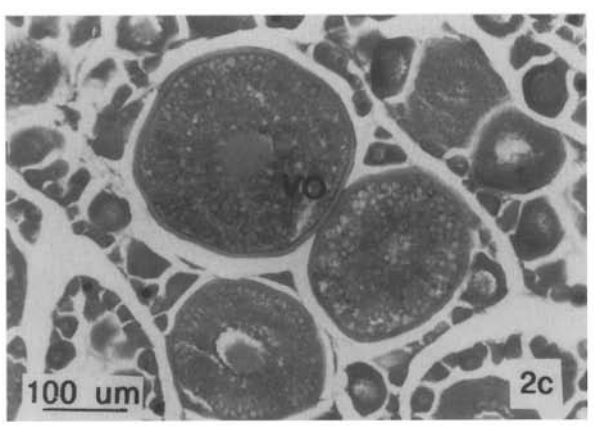


Fig. 2. Transverse section of gonad of E_2-treated fish stained with haematoxylin and eosin. (a) A fish in October showing primary oocytes (PO) and regressing testicular tissue (TT) with yellow body (YB), (b) a fish in December showing the primary oocytes (PO) in the ovarian tissue and regressed testicular tissue (TT), (c) a fish in February showing well developing vitellogenic oocytes (VO) in the gonad.

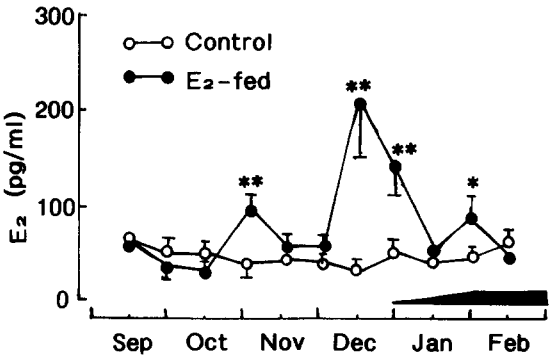


Fig. 3. Plasma E_2 concentrations in 2 year old black porgy fed E_2 or a control diet. The black bar indicates the spawning season. *, ** represent significant differences between the two groups at 5% and 1% levels, respectively.

Gonadal histology, plasma sex steroids and vitellogenin

In the control group, the gonad in October comprised mainly primary oocytes for small amounts of developing testicular tissue (Fig. 1a). Advanced male germ cells were observed in November-December (Fig. 1b, c). Testicular tissue was well developed in February, but only a few primary oocytes appeared in the ovarian tissue in the control group (Fig. 1d, e). After 1 and 3 months of E_2 treatment, testicular tissue and germ cells were regressing (Fig. 2a, b); after 5 months (February), ovarian tissue with vitellogenic oocytes was apparent in the gonads of all the fish ($n=8$; Fig. 2c).

In the control group, plasma E_2 concentrations were low but higher levels of plasma T and 11-KT were observed (Fig. 3–5). On the contrary, in the E_2-treated group, higher concentrations of plasma E_2, and lower levels of plasma T and 11-KT were observed (Fig. 3–5). Significantly higher plasma T levels were only detected in October, November and January in the control group as compared to those in the E_2-treated group; peak plasma T levels were detected in January (Fig. 4). Plasma 11-KT and T levels were suppressed 2 and 4 weeks after E_2 treatment (Fig. 4, 5). In the control group, plasma 11-KT levels significantly increased during the course of spermatogenesis (from September to December, Fig. 5). A sharp rise of plasma 11-KT and the drop of T were further detected in sper-

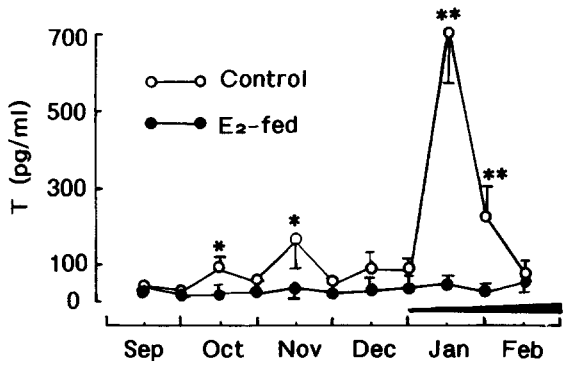


Fig. 4. Plasma T concentrations in 2 year old black porgy fed E_2 or a control diet. Details and symbols as for Figure 3.

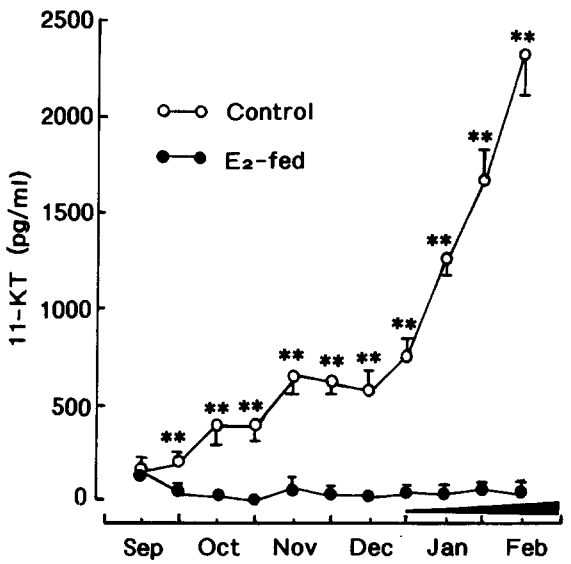


Fig. 5. Plasma 11-KT concentrations in 2 year old black porgy fed E_2 or a control diet. Details and symbols as for Figure 3.

miating black porgy (control group) from January to February (Fig. 4, 5). Significantly higher plasma vitellogenin concentrations (as measured in February, after 5 months of treatment, $n = 8$) were observed in the E_2-treated group (25.26 ± 2.03 mg ml^{-1}) than controls (0.01 ± 0.00 mg ml^{-1}).

Discussion

E_2 treatment completely suppressed testicular development and spermiation while stimulating ovarian development in 2 year old black porgy. The success of sex reversal is demonstrated by the presence of vitellogenic oocytes in the E_2-treated group. E_2 was able to induce sex reversal in one year old black porgy, but only the primary oocytes (previtellogenic oocytes) were observed (Chang *et al.* 1994). E_2 has also been shown to have significant effects on ovarian development in other teleosts (Fostier *et al.* 1983). The administration of E_2 during the labile period resulted in varying the sex ratios towards the female direction in gonochoristic fish (reviewed by Yamamoto 1969; Hunter and Donaldson 1983; Yamazaki 1983). E_2 also induced oocyte development in gonochoristic species (Ng and Idler 1983). Plasma vitellogenin concentrations were higher after E_2 treatment of black porgy thus agreeing with studies in other teleosts (de Vlaming *et al.* 1980; Kishida *et al.* 1992). The increased levels of plasma vitellogenin in the E_2-treated group may be due to the stimulation of ingested or endogenous estrogenic activity.

Significantly higher levels of plasma T and 11-KT were detected in the spermiating black porgy (control group); this is the first observation of high plasma 11-KT levels in black porgy during the spawning season. The plasma 11-KT and T concentrations were similar to those found in the closely related protandrous sobaity, *Sparidendex hasta* (Kime *et al.* 1991). Plasma T levels fell precipitously in January concomitant with a sharp rise in 11-KT levels in February. An increase in plasma T levels also preceded 11-KT in male brown trout, *Salmo trutta* (Kime and Manning 1982) and rainbow trout, *Oncorhynchus mykiss* (Scott *et al.* 1980). Plasma 11-KT levels appeared to be more closely associated with testicular development and spermatogenesis than T in protandrous black porgy.

The putative effects of 11-KT on spermatogenesis in black porgy were consistent with those in gonochoristic teleosts (Sundararaj and Nayyar 1967; Pandey 1969; Billard 1978; Miura *et al.* 1991a; Cochran 1992). Plasma levels of 11-KT, but not T, were also closely associated with the milt volume in black porgy; milt volume increased without affecting sperm concentrations during testicular development. Exogenous T stimulated spermatogenesis in hypophysectomized adult guppy, *Poecilia reticulata* (Pandey 1969), and 11-KT allowed complete spermatogenesis in immature testes of Japanese eel, *Anguilla japonica*, *in vitro* (Miura *et al.* 1991b); Miura *et al.* (1991b) further indicated

that 11-KT activated Sertoli cells to stimulate premitotic spermatogonia and spermatogenesis in Japanese eel. T, but not other androgens, promoted differentiation of spermatogonia in *Fundulus* testis *in vitro* (Cochran 1992). The quantities of collected milt are positively correlated with the plasma levels of 11-KT in rainbow trout (Fostier *et al.* 1982). 17α,20β-dihydroxy-4-pregnen-3-one may also be involved in the process of spermiation in teleosts (Ueda *et al.* 1985).

The low plasma E_2 concentrations in 2 year old male black porgy (control group) were consistent with the previous studies (Chang and Yueh 1990; Chang *et al.* 1991, 1994; Lee *et al.* 1993). Higher plasma E_2 levels were detected in the sex reversing black porgy (E_2-treated group) during the prespawning season than in the control group. Moreover, higher peak plasma E_2 levels were observed in the naturally reversing females (3 year old) during the early prespawning season (Chang *et al.* 1994, 1995). The plasma E_2 levels in male and reversing black porgy were similar to those of male and female sobaity (Kime *et al.* 1991). It is not clear how much of the high E_2 levels in the treated group was due to dietary intake and how much was from true endogenous production. The metabolism of ingested E_2 might be very rapid, thus absorbed E_2 may not be detected in plasma by RIA. The maximum estimated E_2 intake was about 3.6 μg per fish, per day calculated on the basis of the quantity of feed distributed to fish pond.

The success of the experimental induction of sex reversal by oral administration of E_2 to 2 year old black porgy and the higher levels of plasma E_2 in the sex reversed fish further support the importance of E_2 in the sex change of protandrous black porgy (Chang *et al.* 1994). Guiguen *et al.* (1993) suggest that E_2 plays an important role in the protandrous sex reversal process in the seabass, *Lates calcarifer*. The E_2 to androgens ratio also significantly increased during the course of sex reversal in the protandrous anemonefish, *Amphiprion melanopus* (Godwin and Thomas 1993). However, administration of androgens failed to induce a functional sex change in the protogynous ricefield eel, *Monopterus albus* and bluehead wrasse, *Thalassoma bifasciatum* (Tang *et al.* 1974; Kramer *et al.* 1988). The physiological importance of a decrease in androgens such as 11-KT in the sex reversal of protandrous black porgy is still unclear. Plasma T did not differ in male and naturally reversing black porgy (Chang *et al.* 1994, 1995). Decreasing plasma concentrations of 11-KT and 11-hydroxytestosterone were closely associated with the occurrence of sex reversal in the protandrous sobaity (Kime *et al.* 1991).

This study shows that E_2 treatment induces ovarian development and testicular regression. We further observed that 2 of 20 reversed black porgy (10% of treated fish left over from the end of the experiment in February) became mature (with transparent oocytes) in April. Further studies are needed to understand the control of sex reversal in black porgy. For example, the duration and timing of E_2 treatment for inducing reversal is not known, and we could not determine whether the reversed fish will undergo full ovarian development with ovulation in the following year in the absence of any further treatment or reverse to males. It is also unclear whether reversed fish at one year of age, as shown in the previous study (Chang *et al.* 1994), would mature and ovulate in their second spawning season or not.

Acknowledgements

The authors thank Drs. D.E. Kime (University of Sheffield) and G.D. Niswender (Colorado State University) for the antisera specific for T, 11-KT and E_2, respectively. Thanks also to Dr. P. Thomas (University of Texas) for the gift of [1, 2-^{3}H]11-KT. This work was supported in part by the National Science Council (CNS 81-0211-B019-512-BE).

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