

## Feed Administration of Estradiol-17 $\beta$ Stimulates Female Differentiation in Juvenile Grey Mullet *Mugil cephalus*

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**Ching-Fong Chang, Shih-Chien Lan and Bonnie Sun Pan (1995)** Feed administration of estradiol-17 $\beta$  stimulates female differentiation in juvenile grey mullet *Mugil cephalus*. *Zoological Studies* 34(4): 257-264. The objective of the study was to investigate the control of female differentiation by oral administration of varying doses of estradiol-17 $\beta$  (E2) in juvenile grey mullet *Mugil cephalus*. The concentrations of E2 in plasma, stomach and muscle were also measured after feeding experiment. Six-month-old juvenile grey mullet were divided into three groups, control ( $n = 55$ ), low ( $n = 35$ ) and high ( $n = 35$ ) doses of E2, respectively. The low-dose diet was 1 mg E2/kg feed (period I for 4 months) and then 15 mg/kg feed (period II for another 4 months). The high-dose diet was 8 mg E2/kg feed (period I for 4 months) and then 120 mg E2/kg (period II for another 4 months). Low- and high- doses of E2 stimulated development of a unisexual group of juvenile grey mullet, all in the female direction. The effects of E2 on feminization was dose-dependent. E2 was able to induce the beginning of sexual differentiation at a smaller body size and length in this species. E2 in muscle and blood was not detectable in the control or low-dose groups. Higher levels of E2 were detected in plasma, stomach and muscle immediately after the ending of treatment with high doses of E2. E2 in stomach and muscle in the high-dose group diminished to control levels after 45 days of feeding with the control diet.

**Key words:** Estradiol-17 $\beta$ , Feminization, Grey mullet, Monosex, Sex differentiation.

The number of grey mullet *Mugil cephalus* caught at sea has greatly diminished since 1987 in Taiwan. Grey mullet is an important food fish, cultured in Taiwan and other areas of the world (Nash and Shehaden 1980, Liao 1981). Female grey mullet are considered more valuable because they are a source of roe. Therefore, control of sexual differentiation, especially to produce an all-female population of this species, has a high potential value in aquaculture.

Yamamoto (1969) proposed a theory that sex steroids are the natural inducers of sexual differentiation in fish. Because generally the administration of sex steroids during the labile period results in varying sex ratios in a number of fish (see reviews by Yamamoto 1969, Hunter and Donaldson 1983, Yamazaki 1983). Oral

administration of an estradiol derivative for 9 months in 15-month-old grey mullet resulted in 94.7% females (Pan et al. 1992). Previous studies also showed that although a few grey mullet between 7 and 14 months had begun to sexually differentiate, this process mainly occurs after the age of 15 months (Chang et al. 1995). Differentiation into females occurred earlier than differentiation into males in grey mullet (Chang et al. 1995). Information on controlled sexual differentiation in grey mullet during early stages is lacking and needs to be further studied. Therefore, the objective of the present study was to investigate the experimental control of female differentiation by oral administration of different doses of estradiol-17 $\beta$  (E2). E2 concentrations in blood, stomach and muscle were also

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measured after completion of the feeding experiments.

## MATERIALS AND METHODS

### Fish

Six-month-old ( $n = 125$ , mean body weight =  $71.3 \pm 12.3$  g) juvenile grey mullet *M. cephalus* were reared in ponds ( $4.2 \times 3.3 \times 1.3$  m) in a seawater system at National Taiwan Ocean University. Fish were fed with a pelleted, formulated feed (Fwu Sow Feed Co., Taiwan).

### Experimental design

Juvenile grey mullet ( $n = 125$ ) were divided into three groups, control ( $n = 55$ ), low ( $n = 35$ ) and high ( $n = 35$ ) doses of E2, respectively. The control diet consisted of the feed without E2. The diet in the low-dose group consisted of 1 mg E2/kg feed (period I, from July 1991 to November 1991) followed by 15 mg E2/kg feed (period II, from November 1991 to March 1992). The diet in the high-dose group consisted of 8 mg E2/kg feed (period I) and then 120 mg E2/kg feed (period II). Experimental diets containing E2 were prepared by thoroughly mixing feed powder with E2 (Sigma Chem. Co., St. Louis, Mo) in ethanol/water and then pelleting the feed.

Fish were sampled at the end of both the first experimental period (period I, July 1991-November 1991) and the second experimental period (period II, November 1991-March 1992). The weights of whole body, liver, and gonads and the total body length were measured. Muscle, blood and stomach were also sampled 1.5 months after cessation of E2 administration (May 1992). The fish were anesthetized in 2-phenoxyethanol, and blood was taken in heparinized tubes from the caudal vasculature. The plasma was separated by centrifugation and stored at  $-70^{\circ}\text{C}$  for later steroid analysis. Gonadosomatic (liver weight/body weight  $\times 100\%$ ) indices were calculated.

Gonads were fixed in Bouin's fluid, embedded in paraffin and sectioned at  $6 \mu\text{m}$ . Transverse sections were stained with haematoxylin and eosin. Sexual differentiation into females and males was determined on the basis of germ cell development in the gonads. The criteria for judging sexual differentiation in gonadal histology were: appearance of oogonia and primary oocytes in female gonad, appearance of spermatogonia and tubular structure

in the male gonad, and absence of female or male characteristics in undifferentiated gonad.

### Assay

Weighed tissues (muscle and stomach) were homogenized and extracted with phosphate buffer. The supernatant was extracted 3 times with ethyl ether. E2 concentration in plasma and tissue (muscle and stomach) following ether extraction was measured by a radioimmunoassay as described by Chang and Yueh (1990). Means and standard errors of mean were calculated for the samples. All steroid assays were corrected for recovery. The recovery of E2 by ether extraction for E2 was 73.7%. The sensitivity of the assay for E2 was 10 pg per tube. Cross reactions of the antiserum with varying steroids against E2 were evaluated by England et al. (1974). Intra-assay and inter-assay ranges in coefficient of variation were 12.6% ( $n = 6$ ) and 15.5% ( $n = 5$ ), respectively.  $[2,4,6,7-^3\text{H}]\text{-Estradiol-17}\beta$  (99 Ci/mmol) was purchased from NEN Research Products (Boston, MA).

The measurements of moisture, crude protein and ash in feed followed the methods described in AOAC (1984). Total lipid was extracted with methanol/chloroform, and then the weight of lipid was measured (Folch et al. 1957). Total energy value in feed was determined with an IKA Calorimeter System (Model C 4000). The proximate composition and total energy in the feed are shown in Table 1.

### Data analysis

A Chi-square test of binomial proportion was used to determine whether there was a divergence from a 1:1 sex ratio after any treatment ( $\alpha = 0.05$ ). Analysis of variance followed by Bonferroni's test was used to analyze the differences of body weight, E2 concentrations in tissues, and trans-

**Table 1.** The proximate composition and total energy equivalence in feeds

	Moisture (%)	Ash (%)	Protein (%)	Lipid (%)	Total Energy (cal/g)
Control diet	7.56	10.68	22.5	8.74	4,201
E2 diet (mg E2/kg feed)					
1 mg/kg	7.35	10.50	22.9	11.85	4,297
8 mg/kg	6.96	10.45	23.1	8.25	4,351
15 mg/kg	10.30	11.05	25.8	8.86	4,117
120 mg/kg	8.60	10.15	23.9	9.86	4,339

formed gonadosomatic and hepatosomatic indices (arcsine transformation) among groups (Steel and Torrie 1980).

## RESULTS

Low doses of E2 did not affect fish growth during the treatment period, but significantly decreased growth after treatment with the highest

dose of E2 (120 mg/kg) in the second experiment (Table 2). Significantly higher hepatosomatic indices were observed in grey mullet treated with low and high doses of E2 (Table 2). No differences of the gonadosomatic index were observed between the treatment and control groups (Table 2). Low and high doses of E2 stimulated female differentiation after treatment during period I (Table 3). The treated fish were further treated during period II (15 mg E2/kg feed in the low-

**Table 2.** The body weight, gonadosomatic (GSI) and hepatosomatic (HSI) indices in grey mullet *mugil cephalus* after the first and second experimental periods of estradiol-17 $\beta$  (E2) administration

	Control	Low E2	High E2
First period			
Number	11	8	14
Body weight (g)	90.14 $\pm$ 25.78 <sup>a</sup>	81.82 $\pm$ 15.32 <sup>a</sup>	89.00 $\pm$ 19.54 <sup>a</sup>
GSI (%)	0.006 $\pm$ 0.002 <sup>a</sup>	0.005 $\pm$ 0.002 <sup>a</sup>	0.005 $\pm$ 0.002 <sup>a</sup>
HSI (%)	0.553 $\pm$ 0.156 <sup>b</sup>	0.772 $\pm$ 0.053 <sup>b</sup>	1.322 $\pm$ 0.109 <sup>a</sup>
Second period			
Number	44	25	18
Body weight (g)	142.17 $\pm$ 14.9 <sup>a</sup>	127.71 $\pm$ 13.49 <sup>a</sup>	95.05 $\pm$ 8.24 <sup>b</sup>
GSI (%)	0.105 $\pm$ 0.050 <sup>a</sup>	0.078 $\pm$ 0.021 <sup>a</sup>	0.079 $\pm$ 0.036 <sup>a</sup>
HSI (%)	0.695 $\pm$ 0.075 <sup>b</sup>	1.889 $\pm$ 0.066 <sup>a</sup>	2.167 $\pm$ 0.247 <sup>a</sup>

Values are expressed as the mean  $\pm$  standard error of mean.

<sup>a,b</sup>Values with different superscripts in a row differ from each other ( $p < 0.05$ ).

**Table 3.** The Effects of feed administration of estradiol-17 $\beta$  (E2) on sexual differentiation after the first (July to November 1992) and second (November 1992 to March 1993) experimental periods

	Number of Fish			
	Total	Female	Male	Undifferentiated
First period				
Control	11	1 (9.0%)	0	10 (91.0%)
Low dose (1 mg E2/kg feed)	8	3* (37.5%)	0	5 (62.5%)
High dose (15 mg E2/kg feed)	14	8* (57.0%)	0	6 (43.0%)
Second period				
Control	44	15 (34.1%)	9 (20.4%)	20 (45.5%)
Low dose (8 mg E2/kg feed)	25	19* (76.0%)	0	6 (24.0%)
High dose (120 mg E2/kg feed)	18	16* (88.9%)	0	2 (11.1%)

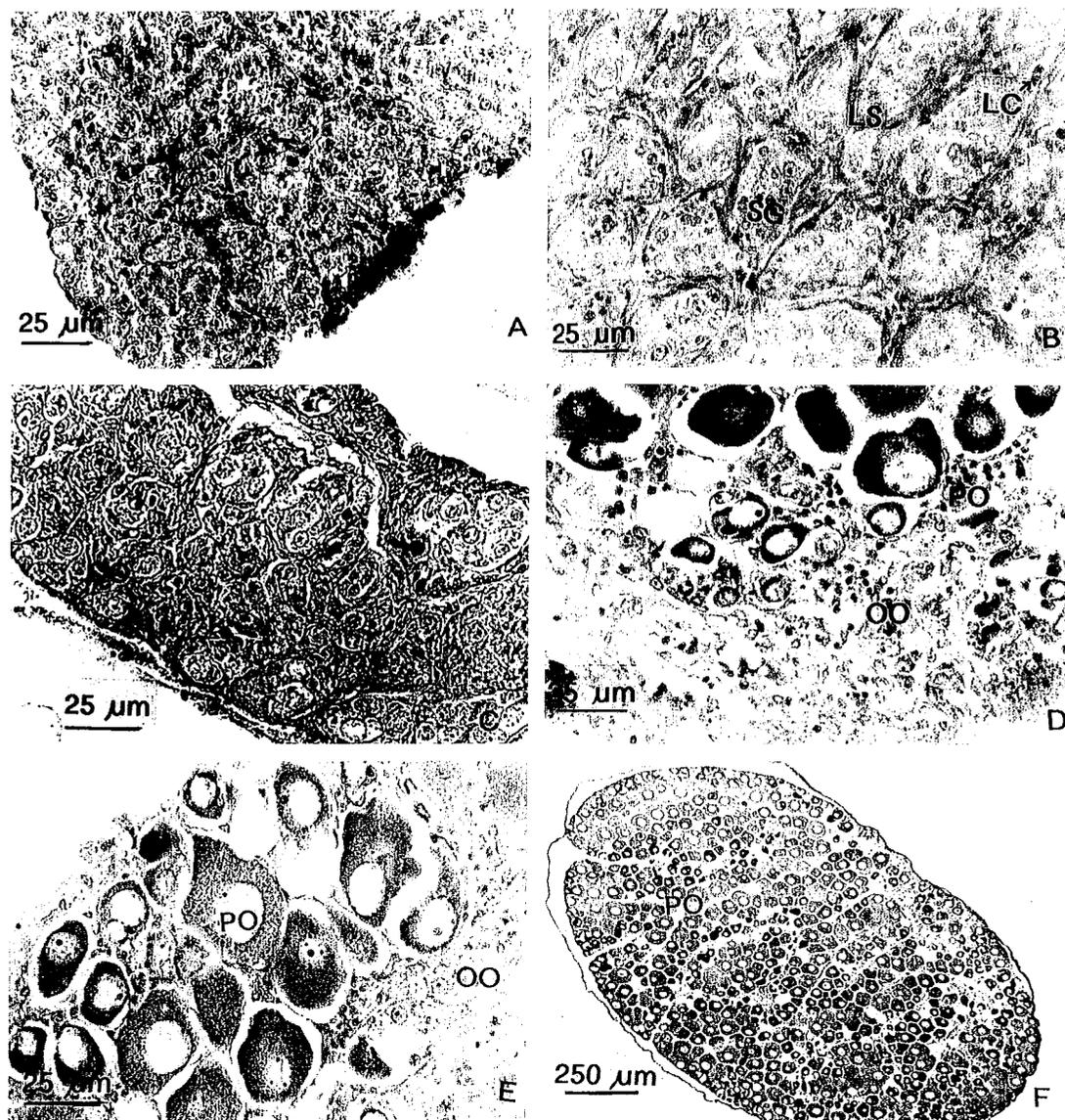
\*Values which differ from the 1:1 ratio of females to males ( $p < 0.05$ ). The criteria for judging a gonad female, male or undifferentiated were on the basis of the gonadal histology.

dose group; 120 mg E2/kg feed in the high-dose group). Both groups had a higher ratio of differentiated females after treatment during period II than the control group (Table 3). An absence of differentiated males, and a decreased ratio of undifferentiated fish were also observed in the E2-treated groups after 8 months of treatment (Table 3).

The representative gonadal histology in differentiated females, males and undifferentiated fish is shown in Fig. 1. Oogonia and developing primary oocytes appeared in differentiated females in the control (Figs. 1C, D) and low-dose groups

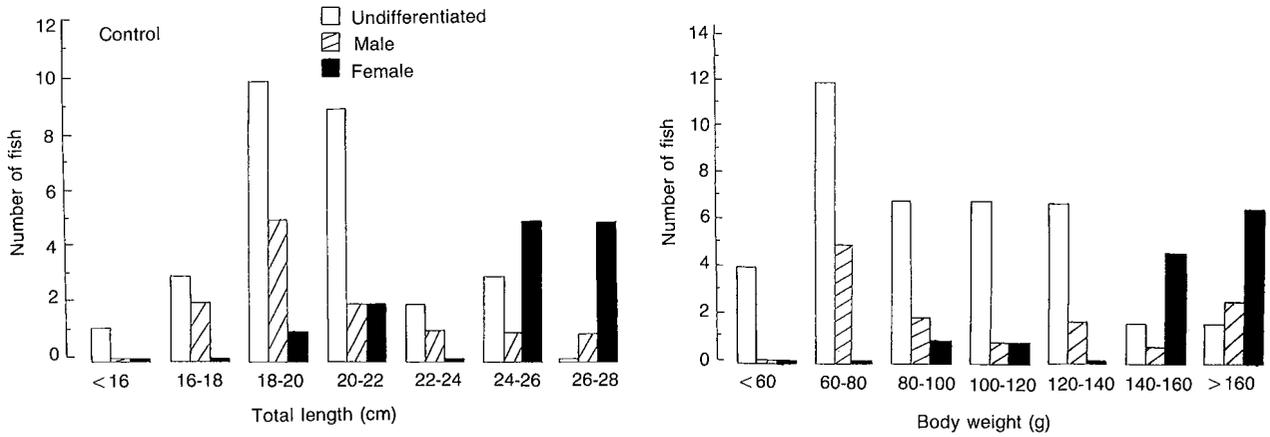
(Figs. 1D, E) after period I. Primary oocytes (Figs. 1E, F) were observed in differentiated females in the high-dose group (after periods I and II), and in the control and low-dose groups (after period II).

At least 50% of fish showing differentiation were used to estimate the ranges of body weight and length in which sexual differentiation occurred. The ranges of body weight and length in the control group were 140-160 g and 24-26 cm, respectively (Fig. 2); and were 80-100 g (body weight) and 20-22 cm (total body length) in both the low-dose and high-dose groups (Figs. 3, 4).

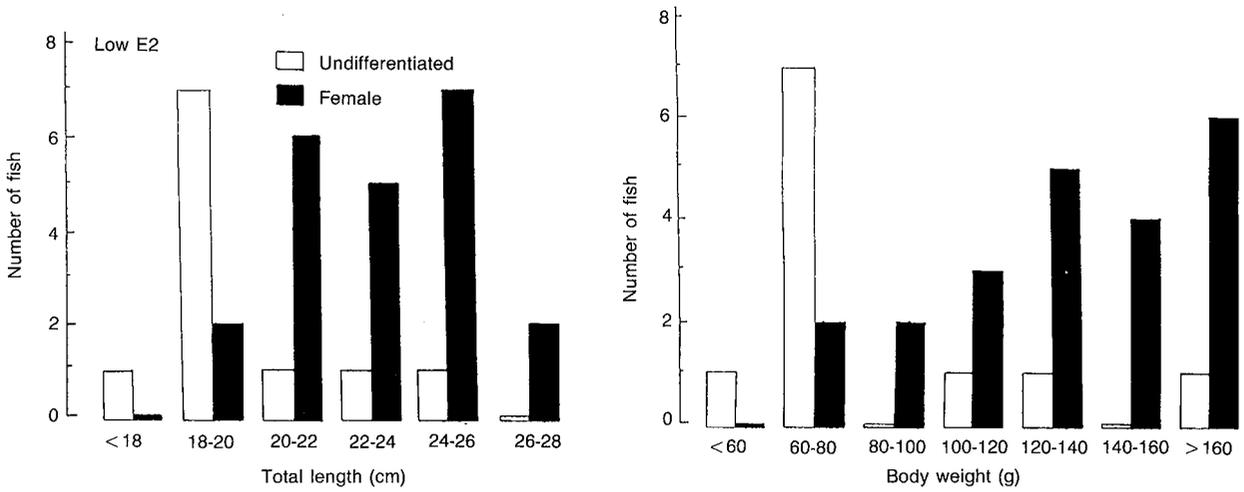


**Figs. 1.** Representative gonadal histology in undifferentiated, male and female grey mullet *Mugil cephalus*. (A) Undifferentiated gonad, (B) Testis with a lobular structure, (C) Ovary with oogonia, (D) Ovarian tissue in the developing primary oocytes, (E) Ovarian tissue in the developing perinucleolus stage, (F) Ovary in the stage of primary oocytes.

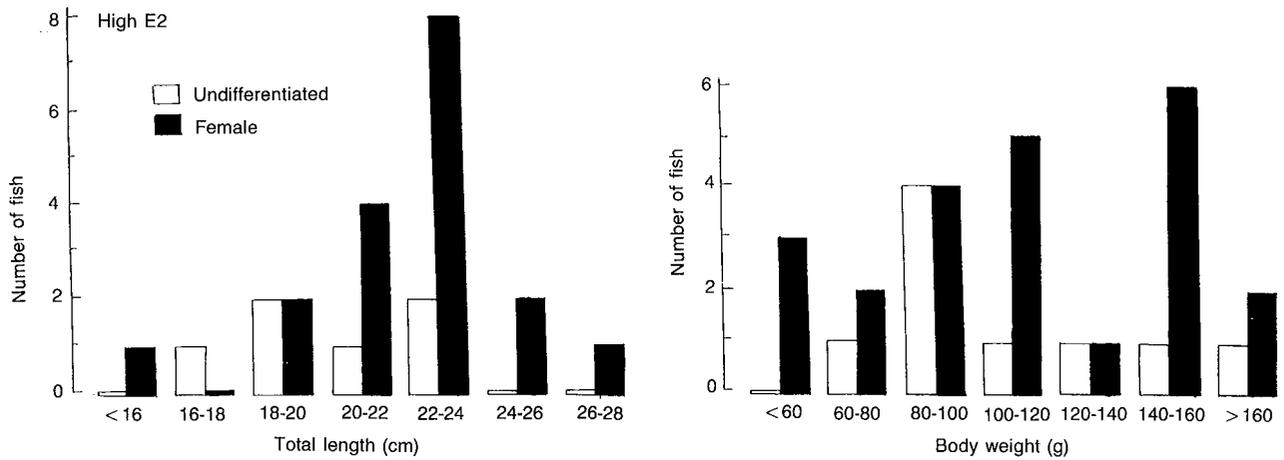
LC: Leydig cells; LS: Lobular structure; OO: Oogonia; PO: Perinucleolus primary oocyte; SG: Spermatogonia.



**Fig. 2.** The relationship between body weight, total body length and gonadal differentiation in the control group (first and second periods).



**Fig. 3.** The relationship between body weight, total length and gonadal differentiation in the low-dose of estradiol-17 $\beta$  (E2) group (first and second periods).



**Fig. 4.** The relationship between body weight, total body length and gonadal differentiation in the high-dose of estradiol-17 $\beta$  (E2) group (first and second periods).

**Table 4.** The estradiol-17 $\beta$  (E2) contents (mean  $\pm$  standard error of mean) in plasma, stomach, and muscle of the control and E2-fed groups

	Control	Low E2	High E2
Plasma (pg/ml)	I 547.3 $\pm$ 19.3 <sup>b</sup>	533.1 $\pm$ 54.1 <sup>b</sup>	1,354.1 $\pm$ 297.0 <sup>a</sup>
Stomach (pg/g)	I 56.3 $\pm$ 4.4 <sup>c</sup>	84.4 $\pm$ 1.5 <sup>b</sup>	130.6 $\pm$ 9.4 <sup>a</sup>
	II 14.2 $\pm$ 7.2 <sup>a</sup>	8.4 $\pm$ 3.4 <sup>a</sup>	11.4 $\pm$ 5.7 <sup>a</sup>
Muscle (pg/g)	I 19.3 $\pm$ 4.0 <sup>b</sup>	25.8 $\pm$ 3.0 <sup>b</sup>	36.3 $\pm$ 2.8 <sup>a</sup>
	II 17.5 $\pm$ 2.6 <sup>a</sup>	13.8 $\pm$ 4.3 <sup>a</sup>	23.4 $\pm$ 3.1 <sup>a</sup>

I. Samples collected immediately after the end of the second feeding experiment.

II. Samples collected after 45 days of feeding with the control diet following completion of the second experiment.

<sup>a,b,c</sup>Values with different superscripts in a row differ from each other ( $p < 0.05$ ).

The quantity of feed distributed to the fish ponds was recorded in order to estimate E2 intake. It was estimated that E2 intake was approximately 2.5 mg and 16.8 mg per fish over the whole treatment period for the low-dose and high-dose groups, respectively. E2 concentrations in muscle, stomach and blood of the low-dose group were not different from those of the control group (Table 4). Higher levels of E2 were detected in plasma, stomach and muscle of the high-dose group (Table 4). E2 concentrations in stomach and muscle in the high-dose group were significantly reduced 45 days after E2 feeding was discontinued (Table 4).

## DISCUSSION

Low and high doses of E2 stimulated female differentiation in grey mullet. Moreover, low doses of E2 (1 and 8 mg/kg) were high enough to alter the sex ratio in the female direction.

A wide range of natural and synthetic steroids have been successfully used to manipulate sex differentiation in fish, including medaka (Yamamoto 1969), cichlids (Hopkins et al. 1979, Hunter and Donaldson 1983), common carp (Sathyanarayana and Satyanarayana 1983, Komen et al. 1989) and salmon (Hunter and Donaldson 1983, Piferrer and Donaldson 1992). These steroids have exhibited biological activities which varied considerably among experimental animals. Estrone, E2, estriol, ethynylestradiol, diethylstilbestrol, and estradiol benzoate have been used for feminization (Hunter and Donaldson 1983). In general, synthetic steroids are

more potent than natural steroids (Yamamoto 1969, Hopkins et al. 1979, Hunter and Donaldson 1983).

Significantly higher ratios of differentiated females were observed in both low-dose and high-dose groups collected either after period I or period II. About 37-57% of grey mullet had shown gonadal differentiation after 4 months of E2 treatment (period I) compared to 9% of fish in the control group. Thereafter, a 15-fold increase of E2 concentrations in the low-dose (1 mg  $\rightarrow$  15 mg/kg feed) and high-dose (8 mg  $\rightarrow$  120 mg/kg feed) groups were used to continue treatment (period II). Primary oocytes occurred earlier in the high-dose group than in the low-dose or control groups (data not shown). The data show that high levels of E2 advanced oogenesis in grey mullet. Our data also supported the previous results that completion of sexual differentiation mainly occurs after the age of 15 months, because more than 50% of the control fish collected after period II had completed sexual differentiation (Chang et al. 1995).

The present data suggest that female differentiation occurs earlier than male differentiation in grey mullet. This phenomenon was also demonstrated in other species such as rainbow trout (Takashima et al. 1980), round goby (Moiseeva 1984), black sea mullet *Lisa saliens* (Mogil'naya and Moiseeva 1985), flounder (Tanaka 1987) and coho salmon (Feist et al. 1990).

Our data also show that high doses of E2 became toxic to grey mullet. High mortality and loss of body weight were observed after administration of 120 mg E2/kg for 1 month. The dead fish showed bulging eyes, soft abdomens, and bloated bellies because of the fluids that collected in the body cavity, as well as inflamed genital pores, green livers and hemorrhaging in the intestine mesentery. Piferrer and Donaldson (1992) also indicated that high doses of ethynylestradiol had toxic effects in salmon.

Sexual differentiation occurred in the majority of grey mullet in the control group when their body weights and lengths reached 140-160 g and 24-26 cm, respectively. E2 administration accelerated the sexual differentiation so that it occurred at a smaller body weight and length. Female differentiation also occurred with smaller body weights and lengths than did male differentiation. These data further support previous observation that female differentiation occurs earlier than male differentiation as judged by gonadal histology (Chang et al. 1995). E2 treatment, especially at high doses, resulted in an increased liver weight probably due to the vitellogenin biosynthesis in

liver stimulated by ingested estrogenic activity.

Muscle, stomach and mature gonads of grey mullet are served as food in Oriental society. However, gonads of the experimental fish were too small and were mainly used for histological analyses. E2 concentrations in muscle and blood in the low-dose group immediately after treatment did not differ from those in the control group. The metabolic clearance of ingested E2 might be too rapid; thus low concentrations of absorbed E2 were not immunologically detectable in plasma or tissue. Higher levels of E2 in the stomach were observed in both E2-treated groups. The data demonstrated that E2 was accumulated in the stomach probably due to absorption by the stomach or the presence of ingested feed in the stomach. It is unclear why stomachs in the control group also had a high concentration of E2. Higher levels of E2 were detected in tissue of the high-dose group. E2 levels in stomach and muscle significantly diminished after 45 days of resumed feeding with the control diet. Steroid derivatives were significantly diminished in fish after the interruption of steroid administration; therefore, metabolism of steroids is considered very fast in fish (Johnstone et al. 1983, Rothbard et al. 1990, Piferrer and Donaldson 1994). However, precautions still need to be taken to prevent steroid residues in food fish after manipulating sexual differentiation.

The proximate composition in the feeds of different groups was slightly different. This difference was probably due to the assay variation or the different moisture contents of the feed during diet preparation.

In conclusion, low doses (1 mg/kg [period I] and 15 mg/kg [period II]) and high doses (8 mg/kg [period I] and 120 mg/kg [period II]) of E2 stimulated gonadal differentiation of juvenile grey mullet in the female direction. E2 accelerated of sexual differentiation so that it occurred at smaller body weights and lengths in grey mullet. The effects of E2 on sexual differentiation was dose dependent.

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## 雌二醇促進幼烏魚雌性化

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本研究的目的為探討雌二醇對幼烏魚雌性化之作用。6月齡烏魚分為三組，對照組、低劑量與高劑量雌二醇 (estradiol-17 $\beta$ ) 組。低劑量組在第一個階段4個月實驗期間，烏魚投餵每公斤含1 mg 雌二醇之飼料；而後第二個階段4個月，改投餵飼料每公斤含15 mg 雌二醇。高劑量組第一個階段投餵飼料每公斤含8 mg 雌二醇，第二個階段改投餵飼料每公斤含120 mg 雌二醇。低劑量與高劑量雌二醇皆能促進烏魚雌性化，且隨著劑量增加，雌性化比例亦增加。雌二醇促使烏魚在較小的體重及體長時即可產生雌性化。低劑量組肌肉與血液所含之雌二醇濃度並未比對照組高，而高劑量組之血漿、胃與肌肉所含雌二醇濃度比對照組高；但停餵45天後，各組織所含雌二醇濃度顯著降低至對照組濃度之範圍。

關鍵詞：雌二醇，雌性化，烏魚，單性，性分化。

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