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烏魚性別分化過程中生殖腺與血漿性類固醇激素濃度之特性

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Gonadal Histology and Plasma Sex Steroids During Sex Differentiation in Grey Mullet, *Mugil cephalus*

Cheing-Fong Chang[1], Shih-Chieh Lan[1], And Hsin-Yiu Chou[1]

ABSTRACT

Gonadal histology and plasma levels of sex steroids were investigated during the period of sex differentiation and the annual cycle of grey mullet, *Mugil cephalus*.

Primordial germ cells were observed in the gonads of 3-month-old grey mullet and appeared to be undifferentiated in fish at 3 to 6 months of age. At 7 to 14 months of age 23.3% of the fish examined had differentiated into males and females whereas 70-90% of 15 - to 17-month-old fish and more than 95% of 18- to 24-month-old fish had completed gonadal differentiation. Differentiation into females occurred earlier than mals. In 15- to 17-month-old-fish, spermatogonia and primary oocytes in the perinucleolus stage were observed in the testes and ovaries, respectively. Testes underwent intensive spermatogenesis at 18 to 24 months whereas ovaries during this period contained both primary and well developes vitellogenic oocytes. There was no sexual dimorphism in the annual cycle of plasma estradiol-17β (E_2), testosterone (T) and 17α-hydroxyprogesterone (17α-OH P) in 7- to 24-month-old grey mullet. Plasma E_2 levels significantly decreased in 9- to 12-month-old presumptively female and male grey mullet. On the contrary, peak levels of plasma T were observed in both female and male grey mullet at 10 to 11 months. Plasma 17α-OH P levels did not show significant profiles in the annual cycle of grey mullet. The date conclude that few gonads at 7 to 14 months begin to differentiate, but sex differentiation mainly occurs at the age of 15 months. It is 9 to 12 months of age when the decreases of plasma E_2 and increases of T levels significantly occur in both female and male grey mullet, *M. cephalus*.

INTRODUCTION

The catch of the commercial grey mullet, *Mugil cephalus*, has been greatly reduced since 1987 in Taiwan. The grey mullet has become an important food fish cultured in

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Taiwan and other areas of the world (Nash and Shehadeh, '80; Liao, '81). Female grey mullet are considered to be more valuable because females could serve as a source to supply roe for food processing. The understanding of the timing of the gonadal differentiation and the development of germ cells in cultured grey mullet is of interest and also commercial value to fish farmers. However the characteristics of sex differentiation in grey mullet, *M. cephalus*, are still unclear (Brusle, '81).

The characteristics of sex differentiation have been studied in a number of gonochoristic fish including tilapia, *Oreochromis niloticus* (Nakamura and Nagahama *et al.*, '85); rainbow trout, *Salmo gairdnerri* (Takashima *et al.*, '80 van den Hurk *et al.*, '82); coho salmon, *Oncorhynchus kisutch* (Feist *et al.*, '90; Foyle, '93); African catfish, *Clarias gariepinus* (van den Hurk *et al.*,'89); round goby, *Neogobius melanostomus* (Moiseeva, '84); flounder, *Paralichthys olivaceus* (Tanaka, '87); channel catfish, *Ictalurus punctalus* (Goudie *et al.*, '83); medaka, *Oryzias latipes* (Yoshikawa and Oguri, '79); whitespotted char, *Salvelinus leucomaenis* (Nakamura, '82) and black sea mullet, *Liza saliens* (Mogil'naya and Moiseeva, '85). Furthermore, the administration of sex steroids during the labile period resulted in varying degrees of sex changes in a variety of fish (reviewed by Yamamoto, '69; Hunter and Donaldson, '83). These data imply that steroids play an important role in sex differentiation in fish. Yamamoto ('69) proposed the theory that sex steroids are the natural inducers od sex differentiation in fish.

The profiles of sex steroids during gonadal differentiation in fish were only scarecely studied. The testosterone (T) levels in the whole body extract of tilapia, *O. niloticus*, reflected the processes of sex differentiation (Rothbard *et al.*, '87; Nakamura and Nagahama, '89). T, 11-ketotesttosterone and androstenedione have also been indicated to involve sex differentiation in coho salmon, *O. kisutch* (Feist *et al.*, '90). The characteristics of plasma sex steroids in the processes of sex differentiation in fish should be further studied.

The present study was undertaken to investigate the histology of gonadal differentiation in cultured grey mullet. Profiles of the endocrine system in the processes of sex differentiation were also studied by measuring the plasma levels of estradiol-17β (E_2), T and 17α-hydroxyprogesterone (17α-OH P) levels during the annual cycle in juvenile grey mullet.

MATERIALS AND METHODS

Fish

Three- (mean body weight = 4.81 + 0.38 g) and six-month-old (mean body weight =71.33 + 12.31 g) juvenile grey mullet, *M. cephalus*, reared from May 1991 to December 1992 in a pond (4.2 × 3.3 × 1.3 m) with a seawater system at the University, were used for

the study of the juvenile reproductive cycle in gonadal histology and sex steroid profiles. Fish were fed with a commercial feed (Fwu Sow Feed Co., Taiwan).

Experimental design

For the histological experiment, juvenile fish were obtained at the age of 3 to 24 months. The fish at 3 months were weighed and decapitated, and decapitated bodies were fixed in Bouin's fluid, since gonads were macroscopically invisible in situ. After the body weight was measured, the gonads collected from 5 to 24 months of age were quickly dissected and fixed in Bouin's fluid. Pieces of the gonads were embedded in paraffin and sectioned at 6 μ m. Transverse sections were stained with haematoxylin and eosin. Sex differentiation was identified according to the development of germ cells.

To measure sex steroid porfiles,blood samples were collected biweekly from reared juvenile fish from July 1991 to December 1992. Seven-month-old juvenile fish(n=36, Mean body weight = 79.38$\pm$8.63g)were divided into two groups and marked to distinguish each individual for serial sampling. Each group of fish was reared in a pond (1.88$\times$1.24$\times$1.10 m) and bled monthly, but the bleeding schedule in two groups was consecutively separated by 2 weeks throughout the experimental period. The fish were anesthetized in 2-phenoxyethanol and blood was taken with heparinized tubes from the caudal vasculature. The plasma was separated by centrifugation and stored at-70°C for later steroid analysis. Total body and gonadal weight were measured for the calculation of gonadosomatic index (GSI =gonadal weight/body weight $\times$ 100%). The gender of grey mullet could not be determined at the beginning of blood collecting (July 1991)because the sex differentiation had not yet occurred. Also, distinguishing between the sexes is impossible unless gonadal histology is examined. Therefore, the differentiated males and females could only be discriminated at the end of the experiment by investigating gonadal histology. The presumptively male (n=7) and female (n=7) grey mullet were grouped, respectively, for the measurement of steroid concentrations.

Assay

E2, T, 17 α -OH P in plasma following ether extraction were measured by radioimmunoassay as described by Chang and Yueh ('90) . Bound steroids were separated from free steroids with a dextrancoated charcoal solution and centrifuged following overnight incubation of extracted steroids with antiserum. The radioactivity of the bound ^{3}H-ste-roid in the supernatant was counted in a Beckman liquid scintillation spectrophotometer (Beckman 1801) with scintillation fluid (Ready safe, Beckman Instruments Inc., Fullerton, CA). Standard were processed in the same manner as the unknown samples. Mean and standard error of mena were calculated in the samples. Mean and standard error of mean were calculated in the samples. All steroid assays were corrected

for recovery. The recovery of ether extraction for E2, T and 17 α-OH P was 73.3%, 77% and 86.7%, respectively. The sensitivities of the assay for E2, T, 17 α-OH P were 5, 6.25 and 12.5 pg per assay, respectively. Cross-reactions of the antisera against E2, T, 17 α-OH P were evaluated by England *et al.* ('74). Kime and Dolben ('85) and Flint *et al.* ('78), respectively. Intra-assay and inter-assay ranges in variation for the three steroids were 12.6-19.0% and 15.5-23.4%, respectively.[2,4,6,7-3H]-Estradiol-17B(99 Ci/mmole), [1,2,6,7,-3 H]-testosterone (89.1Cimmol) were purchased form NEN Research Products (Boston, MA).

RESULTS

Undifferentiated stage (≤6 months of age)

In serial tranverse sections, primordial germ cells were observed in the gonads of 3-month-old grey mullet (Fig. 1). The early gonads were connected with the peritoneal wall via an intestinary mesentery and located between gut and mesonephric duct (Fig. 1). The number of primordial germ cells gradually increased in some (26.7%) of the 6-month-old fish (Fig. 2, n=15). Gonads appeared to be undifferentiated in 39 juvenile fish at 3 to 6 months of age (Table 1). The diameters of primordial germ cells and nucleus were about 10 μ m and 5 μ m, respectively.

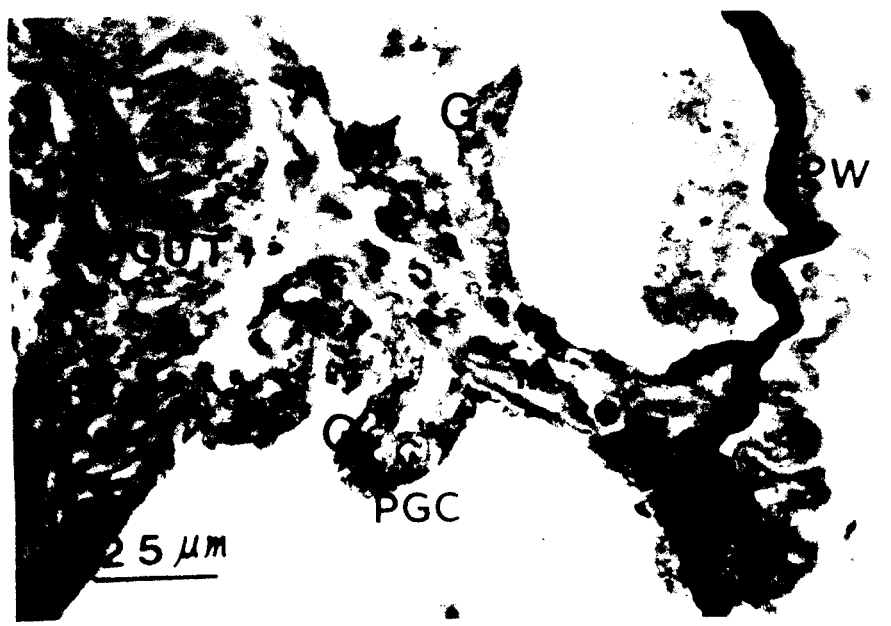

Fig. 1. Transverse section of an early gonad of a 3-month-old grey mullet showing primordial germ cell (PGC). G, gonad; M, mesentery; PW, peritoneal wall.

Fig. 2. Undifferentiated gonad of a 6-month-old grey mullet showing primordial germ cells (PGC) and blood vessel (BV).

Table. 1 Gonadal differentiation in 3- to 24-month old cultured grey mullet.

Age(month)	BW(g)	GSI%	Number of fish			
			Total	Femal	Male	Undifferentiaed
Undifferentiated Period						
3	6.07±0.05	–[a]	20	0	0	20
5	6.92±2.23	–	4	0	0	4
6	71.33±12.31	–	15	0	0	15
Differentiating Period						
7	87.63±12.13	0.02±0.04	8	3	0	5
10	114.10±18.64	0.02±0.01	7	1	0	6
11	82.85±21.24	0.01±0.01	5	0	0	5
13	123.28±12.91	0.03±0.01	19	2	1	16
14	93.81±7.69	0.02±0.01	9	1	1	7
Differentiated Period						
15	127.52±13.87	0.08±0.03	10	4	3	1
17	156.79±16.60	0.69±0.25	12	6	5	1
24	210.35±20.04	0.56±0.11	21	7	13	1
Total Number			128	24	23	81

[a]The gonad was macroscopically invisible in situ.
GSI, gonadosomatic index.
BW, body weight.

Differentiating stage (7-14 months of age)

There were only 23.3% (n=9) of grey mullet (from 7-to 14-month-old, n=48) that had differentiated into females (n=7) and males (n=2) according to the development of germ cells (Table 1). The majority of fish (76.5%) were still undifferentiated. Oogonia (Fig. 3A) and primary oocytes in the perinucleolus stage (Fig. 3B) were observed in the ovaries.

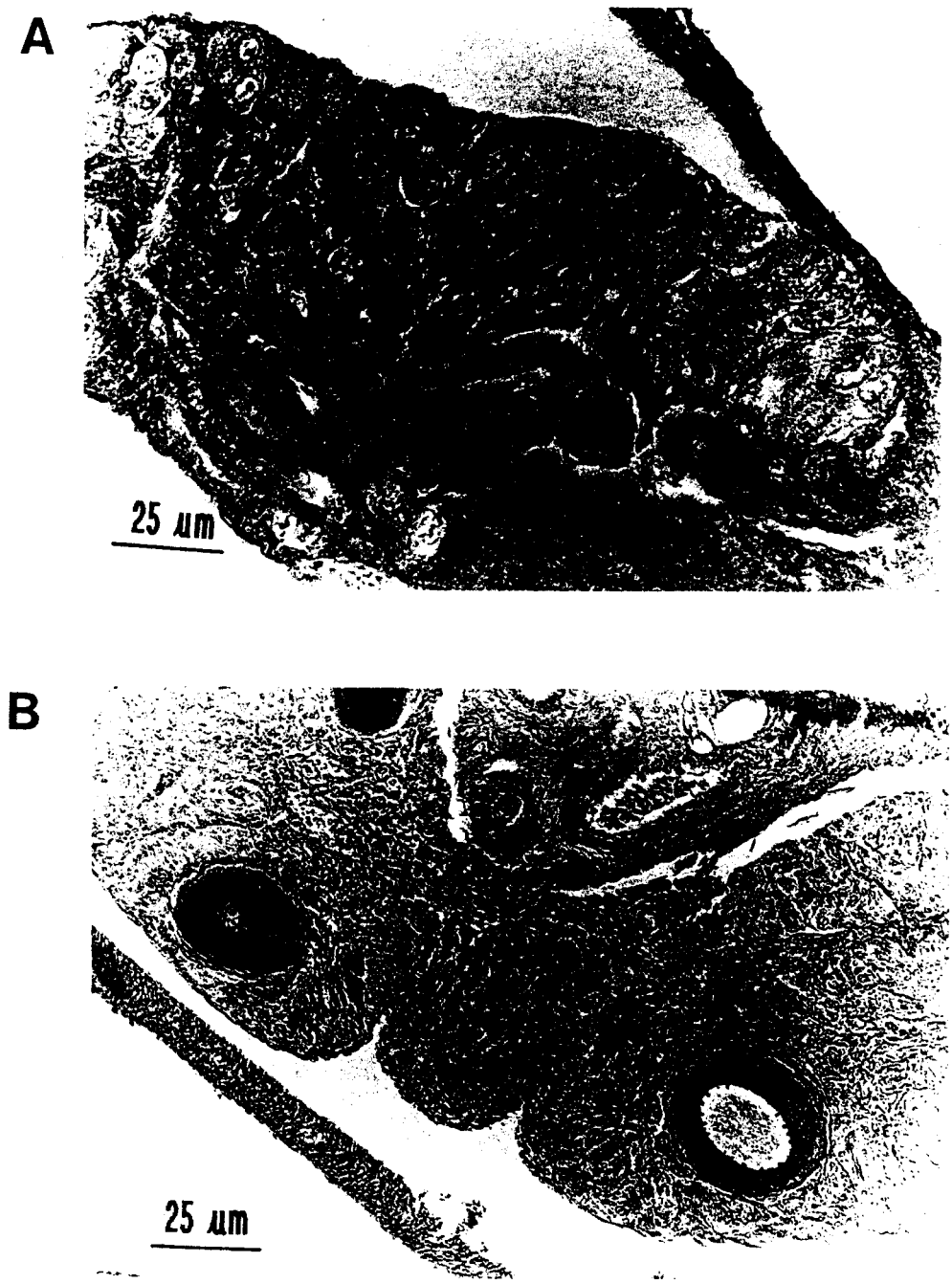

Fig. 3. Presumptive ovary of a differentiating grey mullet showing oogonia (A) and primary oocytes in the perinucleolus stage (B). BV, blood vessel; O, oogonia; PN, perinucleolus primary oocyte.

Spermatogonia and lobule structure were observed in the testes (Fig. 4). The development of germ cells in the peripheral tissue of the gonad occurred earlier than in the central part of the gonad. Differentiation into females occurred earlier than in males (Table 1).

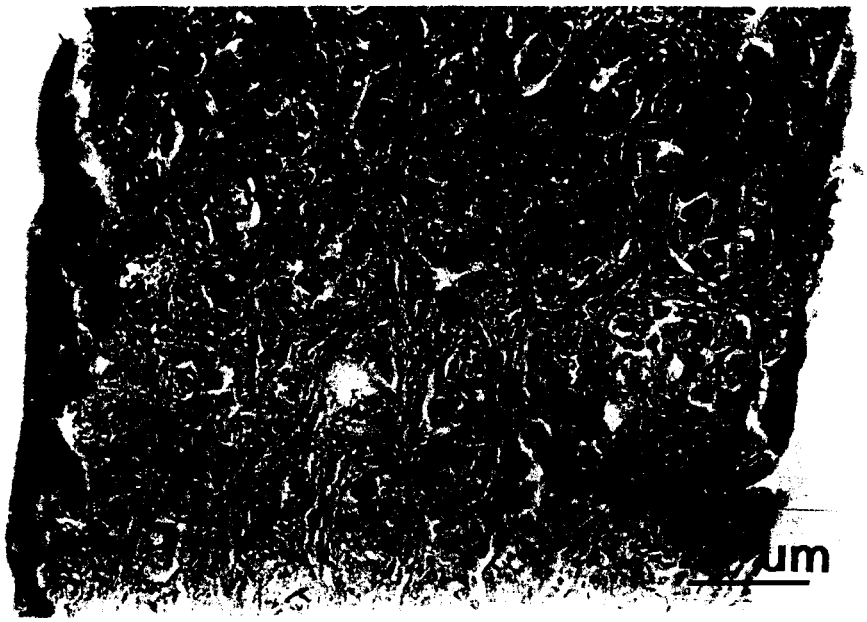

Fig. 4. Presumptive testis of a differentiating grey mullet showing lobular structure (L) and spermatogonia (SG).

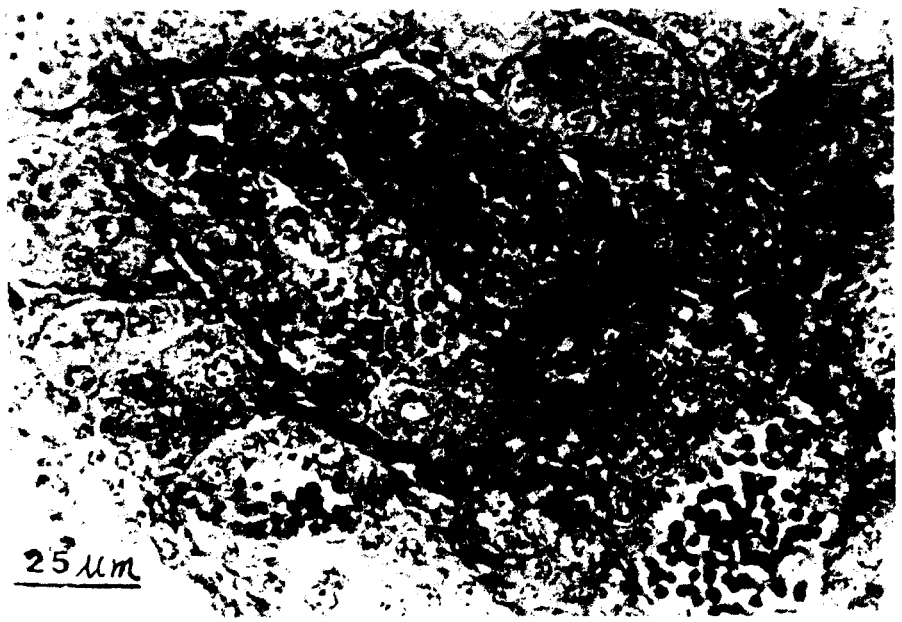

Fig. 5. Testis of male grey mullet showing spermatogonia (SG), spermatocyte (SC), and spermatid (ST).

Differentiated stage (≥ 15 months of age)

The majority of grey mullet differentiated into males and females in 15- (70%) and 17-month-old (90%) fish (Table 1). Spermatogonia and primary oocytes in the perinucleolus stage were observed in the testes and ovaries, respectively. More than 95% of 18-to 24-month-old grey mullet had completed gonadal differentiation. Testes contained spermatogonia, spermatocytes and spermatide (Fig. 5). Primary oocytes and vitellogenic oocytes were observed in the ovaries (Fig. 6). The sex ratio of total differentiated females and males was 1.04:1.

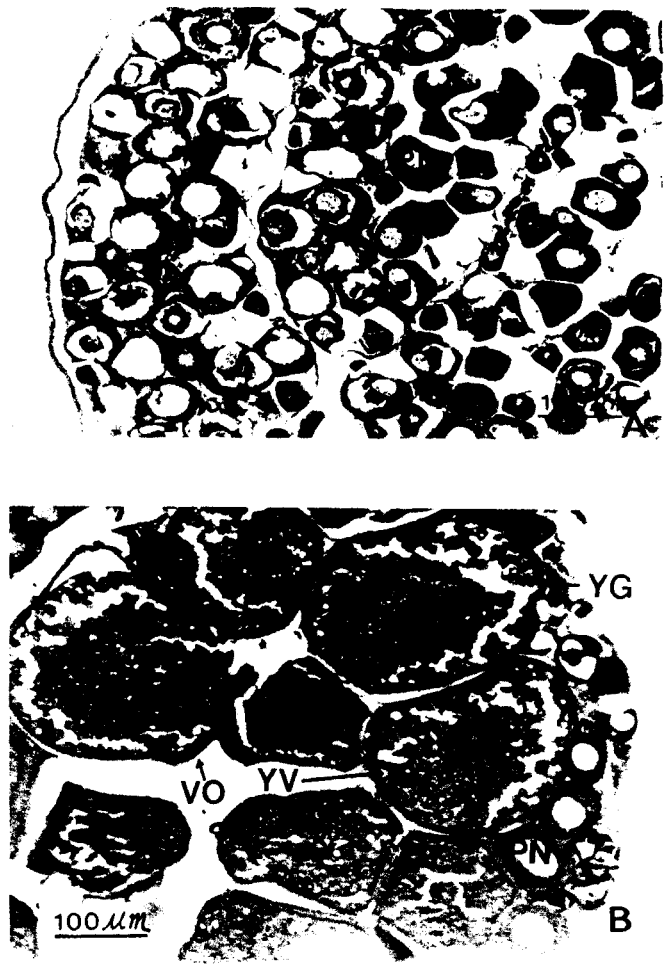

Fig. 6. Ovaries of female grey mullet showing primary oocyte at perinucleolus (PN) stage (A) and vitellogenic oocyte (VO) (B). YG, yolk globule; YV, yolk vesicle.

Steroid profiles

The lowest levels of plasma E2 occurred in both presumptive females and males at the age of 9 to 12 months (Fig. 7), then E2 levels increased to 300-500 pg/ml after the age of 13months. No different profiles of plasma E2 in the annual cycle were observed in female and male grey mullet (Fig. 7). Peak levels of plasma T were obtained in both presumptive female and male grey mullet at the age of 10 to 11 months (Fig. 8). There were also no different profiles of plasma T levels between females and males. Plasma 17 α-OH P levels in the annual cycle did not show significant profiles in juvenile female and male grey mullet (Fig. 9).

DISCUSSION

The present data showed that gonadal differentiation in grey mullet kept under laboratory conditions were first visible at 7 months of age . The majority of grey mullet began their gonadal differentiation after 1 year of age. The ovaries were recognized by the

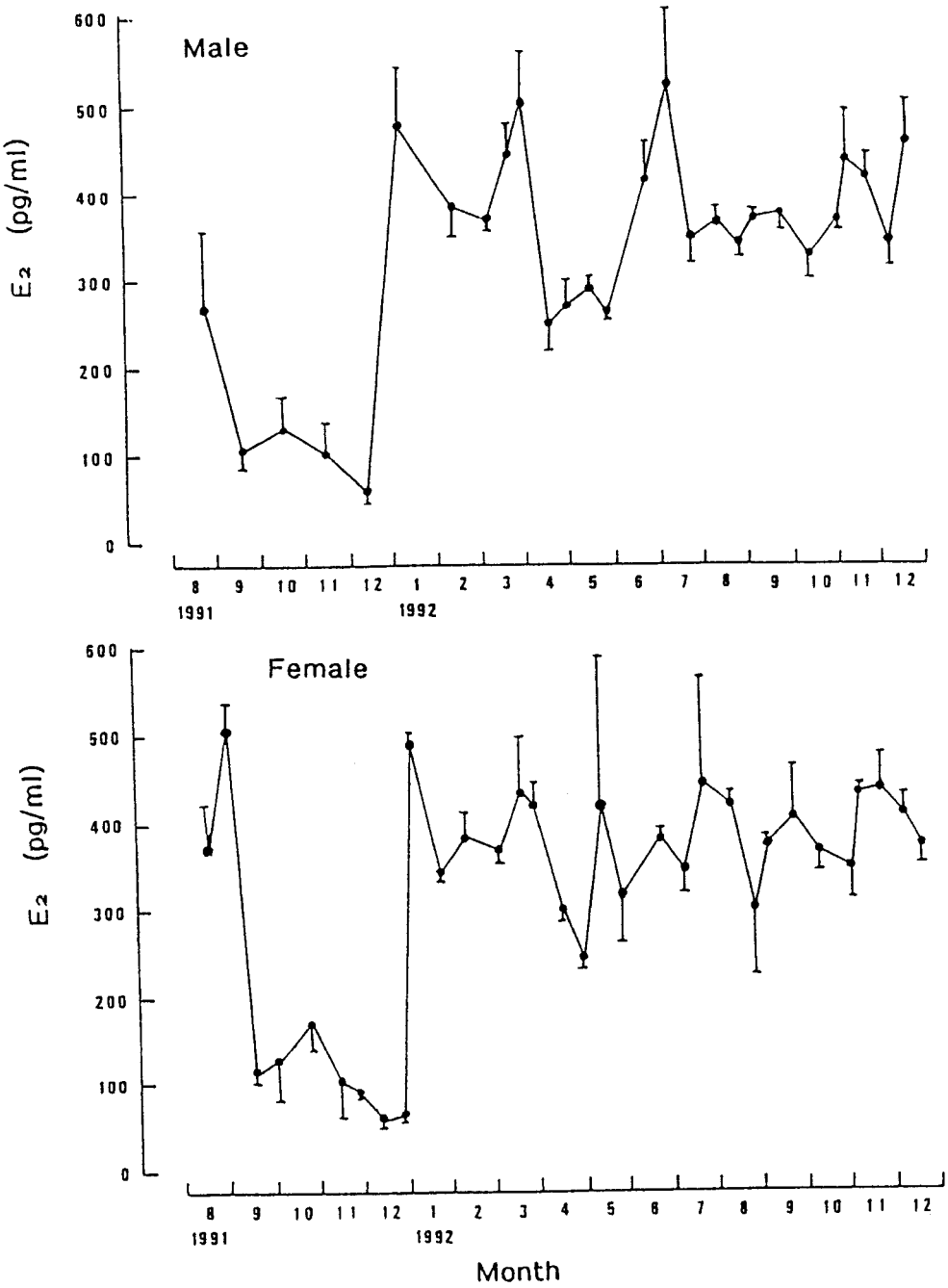

Fig. 7. The concentrations of plasma estradiol-17 β (E2) in male (n = 7) and female (n = 7) grey mullet from the age of 8 to 24 months.

formation of central cavity, the presence of oogonia and primary oocytes in the chromatin-perinucleolus stage. The testes were recognized by the formation of lobular structure and the presence of spermatogonia. The data also demonstrated that grey mullet is one of gonochoristic fish.

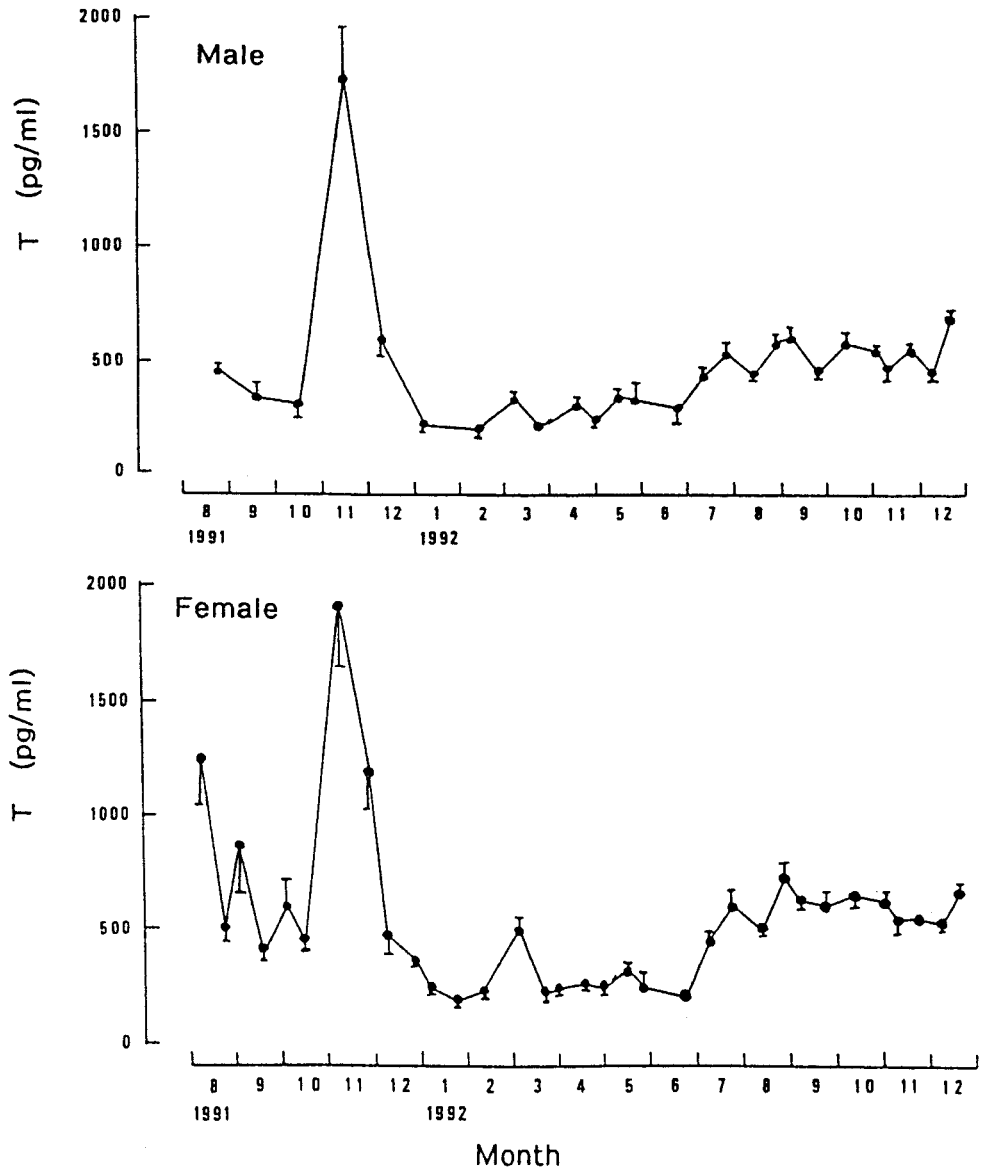

Fig. 8. The concentrations of plasma testosterone (T) in male (n =7) and female (n =7) grey mullet from the age 8 to 24 months.

Sex differentiation occurred much earlier in most species of fish as compared to grey mullet. The timing of sex differentiation in fish has been shown as follows : tilapia (*O. niloticus*), 23-26 days posthatching (Nakamura and Nagahama, '89); round goby (*N. melanostomus*), 15-20 days posthatching in females and 40-45 days posthatching in males (Moiseeva, '84); African catfish (*C. gariepinus*), 28 days posthatching in females and 42

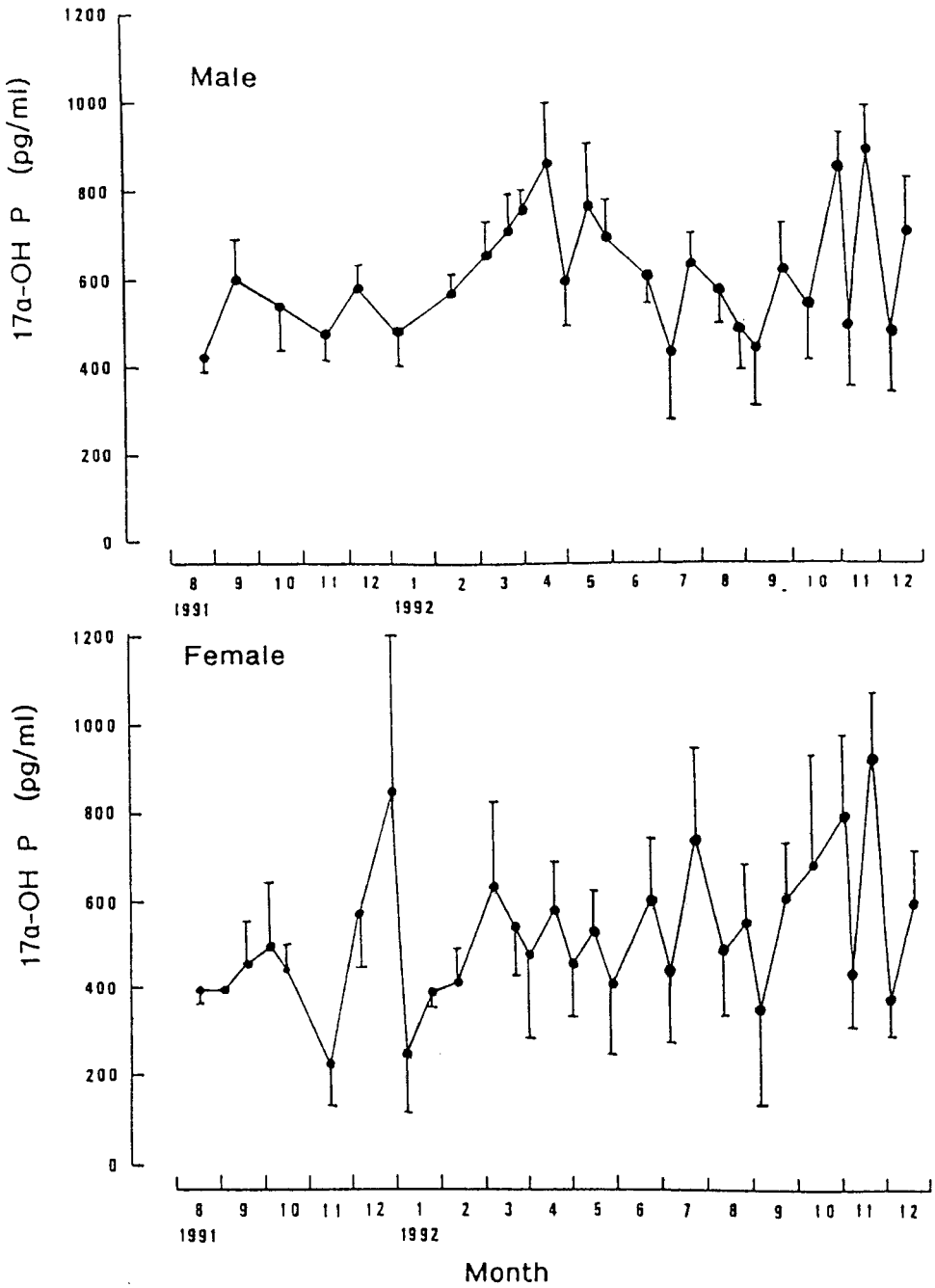

Fig. 9. The concentrations of plasma 17a-hydroxyprogesterone (17 α-OH P) in male (n =7) and female (n =7) grey mullet from the age 8 to 24 months.

days posthatching in males (van den Hurk *et al.*, '89); rainbow trout (*S. gaidneri*), 45-55 days postfertilization (van den Hurk and Slof, '81); coho salmon (*O. kisutch*), 27 days posthatching (Piferrer and Donaldson., '89), but 65-75 days postfertilization in females and 75-85 days postfertilization in males (Feist *et al.*, '90); common carp (*C. carpio*), 17 weeks posthatching (Parmentier and Trimmermans, '85). The oral administration of estradiol

derivative for 9 months to 15-month-old grey mullet reared in outdoor pond could result in 94.7% of female (Pan *et al.*, '92). These data supported our findings that majority of grey mullet completed gonadal differentiation about at the age of 15 months.

Ovarian differentiation preceded that of the testis in grey mullet. These phenomena were also demonstrated in other species such as rainbow trout (Takashima *et al.*, '80), round goby (Moiseeva, '84), flounder (Tanaka, '87), black sea mullet (*L. saliens*) (Mogil'naya and Moiseeva, '85) and coho salmon (Feist *et al.*, '90). Germ cells in future testes usually remain quiescent for a long time in grey mullet. The transition from spermatogonium to spermatid did not occur until about 18 months of age. The primacy of female development is suggested by the initial ovarian phase in some gonochoristic and all protogynous fish (Shapiro, '92).

The germ cells development and grew significantly after the occurrence of gonadal differentiation in grey mullet. The diameters of oogonia were 10-12.5 μ m with 5-7.5 μ m of nuceleus, primary oocytes in the perinucleous stage 15-85 μ m with 7.5-50 μ m of nucleus. The perinucleolus number rangerd from 1 to 18 in each primary oocyte. Vitellogenic oocytes ranged from 100 to 500 μ m in diameter. The diameters of spermatogonia were about 10 μ m with 7.5 μ m of nucleus, 4-5 μ m of primary spermatocyte, 2.5 μ m of secondary spermatocyte and 1- 1.5 μ m of spermatid.

The profiles of T and E_2 in the process of sex differentiation were first characterized in the plasma of grey mullet on the basis of repeated sampling for more than 1 year at 2 week intervals. High levels of T and the changes of T/E_2 ratio are suggested to be one of the characteristic profiles in the process of sex differentiation in grey mullet. Peak levels of whole body T content were also observed in tilapia at 6-8 weeks (Rothbard *et al.*, '87). Very low E_2 contents (less than 0.1 ng/individual) were also observed in tilapia at the age of 8 weeks (Rothbard *et al.*, '87). Peak levels of plasma T and E_2 were detected in 5-month-old common carp, and no sex dimorphism in the plasma levels of sex steroids was observed (Chang and Chen, '90). Higher contents of whole body T, 11-ketotestosterone and androstenedione appeared in the presumptively male coho salmon (Feist *et al.*, '90). No sexual dimorphism was shown in the E_2 contents in coho salmon (Feist *et al.*, '90). Androstenedione and T but not E_2 were significantly produced in incubated gonads taken shortly after the onset of feeding in rainbow trout and were higher from males than females (Fitzpatrick *et al.*, '93). High levels of E_2 and E_2/T ratio have also been suggested to involve in sex change in protandrous anemonefish, *Amphiprion melanopus* (Godwin and Thomas, '93) and black porgy, *Acanthopagrus schlegeli* (Chang *et al.*, '94).

The timing of the changed profiles in the plasma E_2 and T did not completely coinicide in grey mullet . The decreases in plasma E_2 were observed during the period of 9-12 months. Right after the occurrence of decreasing E_2, plasma T significantly increased to peak levels. Plasma E_2 rose from the nadir at 12 months of age follow after the decline of plasma T. The

age of 9-12 months might be a critical period to the occurrence of sex differentiation in grey mullet. The results from gonadal histology demonstrated that gonadal differentiation began to occur in few grey mullet at 7 to 14 months of age. The majority of gery mullet had completed their differentiation at around 15 months of age. The germ cell development was used as a major criterion to investigate sex differentiation in this study. The gonadal differentiation is considered as one of later stages in the process of sex differentiation. It is still too preliminary to speculate that the change in sex steroid levels from 9 to 12 months of age might be indicative of the onset of sex differentiation in grey mullet. Further research is underway to closely investigate the association of sex steroid to the process of sex differentiation in grey mullet.

Our data supported that steroid synthesizing capability in the early gonad in grey mullet started before gonadal differentiation . This is consistent to the suggestion in coho salmon by Feist *et al.* ('90). Steroidogenic activities have been demonstrated during early development in guppy (Takahashi and Iwasaki, '73; Fitzpatrick *et al.*, '93), rainbow trout (van den Hurk *et al.*, '82), tilapia (Rothbard *et al.*, '87) and coho salmon (Feist *et al.*, '90). However, it is still impossible to conclusively indicate whether the changes in sex steroids are a cause of sex differentiation or simply a result of it in grey mulllet. Also, the origin of the steroids in plasma remains problematic.

High levels of T might be important to induce sex differentiation in vertebrate brain (MacLusky and Naftolin, '81; Arnold and Gorski, '84). Androgens have been indicated to control sex differentiation in neuron numbers in certain brain nuclei (Nordeen *et al.*, '85). Androgens could stimulate the activity of brain aromatase in mammal (Roselli *et al.*, '84). In the brain aromatization of androgens to estrogens appears to be an essential step in the regulation of several neuroendocrine and behavioral responses (Wilson *et al.*, '81; McEwen *et al.*, '82; Callard, '83).

No significant sexual dimorphism of plasma T and T and E_2 was found in presumptively sex female and male grey mullet. However , a slight, but insiginificant, difference of steroid profiles may have had enough to play a role in the sex dimorphism of differentiation. The failure to detect the sex dimorphism of T and E_2 in grey mullet might also be due to the inappropriate bleeding schedule. Other steroids such as androstenedione, 11 β - hydroxyandrostenedione and 11-ketotestosterone should be further studied to examine the involvement of the sex differentiatioin and also sex dimorphism in grey mullet.

In summary, gonads were undifferentiated in grey mullet at 3 to 6 months of age. At 7 to 14 months of age 23.3 % of fish examined had differentiated into males and females whereas 70-90 % of 15- to 17-month-old fish and more than 95 % of 18- to 24-month-old fish had completed gonadal differentiation. Ovarian differentiation preceded that of the testis in grey mullet. It is 9 to 12 months of age when the decreases of plasma E_2 and increases of T levels significantly occurred in both females and males. No clear sexual

dimorphism of plasma profiles in T, E_2 and 17α-OH P was found in grey mullet. The saaociation of sex steroids with sex differentiation needs to be further studied in gonochoristic *M. cephalus*.

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烏魚性別分化過程中生殖腺與血漿性類固醇激素濃度之特性

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摘　要

本研究目的在探討烏魚在性別分化及年週期間，生殖腺組織特性及血漿性類固醇濃度變化。3月齡烏魚生殖腺可發現primordial germal cells，3月至6月齡烏魚尚未分化，7至14月齡約有23.3%的魚以完成分化，而15至17月齡則有70-90%完成分化。雌魚性別分化比雄魚早。在15-17月齡雌雄生殖腺分別以primary oocytes及spermatogonia為主，自18-24月齡精巢開始進行精子生成作用，卵巢開始進行卵黃生成與堆積作用。分化為雌魚或雄魚均在9-12月齡時，estradiol-17 β濃度顯著降低，而tetosterone則在10-11月齡顯著上升，17 α-hydroxyprogesterone則無顯著變化型態。

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