

# Body color change and serum steroid hormone levels throughout the process of sex change in the adult wrasse, *Pseudolabrus sieboldi*

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**Abstract** Gonadal sex steroid hormones are the principal factors that directly control the gonadal and morphological alterations during sex change in hermaphrodite fish; however, the physiological mechanism of action by which these hormones govern body coloration is poorly understood. The protogynous wrasse *Pseudolabrus sieboldi* is a good model for understanding the physiological mechanisms of gonadal and body color change during sex change in hermaphrodite fish. To obtain information on the relationship between sex steroids and body color change during the process of gonadal sex change, we analyzed body color, gonadal histology, and serum levels of sex steroids. Body color was analyzed using a quantitative analytical method based on the hue value. Compared to other body parts of the fish, the anal fin changed color the most, becoming increasingly redder in association with gonadal changes that converted ovaries to testes. Levels of serum 11-ketotestosterone (11KT) increased as the gonadal sex change proceeded, whereas no significant change was observed in

estradiol-17 $\beta$  (E2) levels. Moreover, we found a significant correlation between the hue value of the anal fin and serum 11KT levels, but not E2 levels. These results suggest that androgen, but not estrogen, plays a principle role in the changes in both gonadal morphology and body color in the transformation from female to male in this species. To our knowledge, this is the first quantitative demonstration of the relationship between body color and serum steroid levels during sex change in fish.

## Introduction

In teleosts, a number of species change their sex from female to male (protogynous), male to female (protandrous), or both directions (bi-directional sex change; Atz 1964; Reinboth 1970; Kuwamura and Nakashima 1998). These sex changes are dramatic events that convert both gametogenesis and behavior between female and male within a single individual. Additionally, in several species, morphological changes such as in body shape and coloration accompany the process of sex change (Shapiro 1979). Many attempts have been made to clarify the physiological mechanism of sex change, including the physiological roles of several substances such as monoamines (Larson et al. 2003), arginine vasotocin (Grober and Sunobe 1996), gonadotropin-releasing hormone (Kramer et al. 1993), gonadotropin (Koulish and Kramer 1989), and sex steroid hormones (reviewed by Devlin and Nagahama 2002). Of these substances, most attention has been focused on the role of sex steroids.

In some protogynous and protandrous species, high levels of estradiol-17 $\beta$  (E2), the major estrogen in vertebrates, and low levels of 11-ketotestosterone (11KT), the most active androgen in teleosts, have been detected in the

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circulation of fish in the female phase and vice versa for fish in the male phase. The androgen 11KT and 11 $\beta$ -hydroxylase, the key enzyme to synthesize 11KT, correlate with sex change in protogynous and protandrous species (Devlin and Nagahama 2002). Additionally, in many protogynous species, treatment with 11KT can result in female to male sex change (Devlin and Nagahama 2002). An exception is the rice-field eel *Monopterus albus*, in which 11KT administration failed to induce sex change (Tang et al. 1974). Treatment with 11KT also induces a body color change in the parrotfish *Sparisoma viride* (Cardwell and Liley 1991) and the wrasse *Thalassoma bifasciatum* (Grober et al. 1991) associated with the protogynous sex change. Therefore, gonadal androgen is generally accepted as the key hormone in the development of male characteristics, including gonad and body morphology, in many hermaphrodite species.

Aromatase is the enzyme that converts androgen to estrogen. Its enzymatic activity (Lee et al. 2001), immunolocalization (Sunobe et al. 2005), and the transcript level of the gonadal form of aromatase (*cyp19a1a*; Liu et al. 2004; Wong et al. 2006) have been linked to sexual dimorphism and sex change in hermaphrodites. In addition, administration of E2 has resulted in male to female changes in the protandrous black porgy *Acanthopagrus schlegelii* (Lee et al. 2001), while blocking estrogen synthesis using an aromatase inhibitor (AI) suppresses this protandrous sex change (Lee et al. 2002). AI induces the female to male sex change in protogynous species such as the goby *Coryphopterus nicholsii* (Kroon and Liley 2000), the wrasse *Halichoeres trimaculatus* (Higa et al. 2003), and the grouper *Epinephelus merra* (Bhandari et al. 2004). Moreover, experimental manipulation of E2 levels by AI and E2 administration leads to the induction of sex change in each direction in the coral goby, a species in which bi-directional sex changes occur (Kroon et al. 2005). These results indicate that estrogen plays a role in female development and maintenance, and that suppression of aromatase (i.e., estrogen biosynthesis) may lead to testicular differentiation of the male phenotype. Although it remains unclear whether the steroidogenic change initially comes from androgen or estrogen biosynthesis, sex steroids appear to be a significant factor controlling gonadal sex in hermaphrodite species.

The wrasse *Pseudolabrus sieboldi* exhibits diandric protogyny with small initial-phase (IP) males (primary males), IP females, and large terminal-phase (TP) males (Nakazono 1979). TP males arise from one of two processes: sex change in an IP female (secondary male) or a role and color change in an IP male. In autumn, *P. sieboldi* spawns almost daily, and there is a diurnal pattern of oogenesis in females and spermatogenesis in secondary males (Matsuyama et al. 1997, 1998). Recently, we clarified the synthetic pathway of sex steroids in the ovarian follicles and secondary testes of this species (Ohta et al. 2001; Sundaray et al. 2003), and

demonstrated that the pathways for E2 and 11KT synthesis branch from androstendione (AD). From AD, E2 is synthesized by aromatase and 17 $\beta$ -hydroxysteroid dehydrogenase (17 $\beta$ -HSD) activities in the ovarian follicle, while 11KT is synthesized in the secondary testes with 11 $\beta$ -hydroxylase, 17 $\beta$ -HSD, and 11 $\beta$ -HSD activities. These results clearly indicate that the steroidogenic pathway is converted from an ovarian to a testicular mode in association with gonadal sex changes and suggest that each sex steroid plays a role in the maintenance and/or change of sex.

The purpose of this study was to understand the relationship between body coloration and serum steroid levels during sex change in *P. sieboldi*. Nakazono (1979) previously obtained a few individuals with hermaphroditic gonads in March and June and suggested that sex change occurs during the non-spawning season. However, sampling was not conducted throughout the year, and consequently no information on sex change and body color was obtained from winter to spring just after the spawning season. Therefore, we examined adult IP and TP individuals of *P. sieboldi* over a range of body lengths throughout the year. First, the gonads were examined histologically, and we recorded the monthly occurrence of each gonad type, i.e., ovary, testis, or transitional (intersexual). Second, individual body color was analyzed quantitatively using a newly developed method. Finally, circulating levels of 11KT and E2 were measured, and the relationship of sex steroids with body color and gonadal histology was investigated.

## Materials and methods

### Fish and sampling

Every month throughout the year, adult 9–19 IP and 8–10 TP fish were sampled. From April to November 2000, the fish were caught by hook and line around the rocky shore near the Fisheries Laboratory of Kyushu University (33.8°N; 130.4°E), Fukuoka Prefecture, Japan. IP and TP fish were transferred to the Fisheries Laboratory and kept in a 1,000-l tank with filtered seawater under conditions of natural day length and water temperature. Because spawning occurs within 1 or 2 days after transfer to a tank during the spawning season (Matsuyama et al. 1998), we held fish for 5 days to acclimate before performing the experiments.

From December 2000 to March 2001, rough weather prevented us from collecting fish in the field; therefore, we collected specimens from stocks maintained at the Fisheries Laboratory during this period. Eighteen IP and ten TP fish were kept in a 1,000-l tank under the conditions described above. Five tanks were prepared, and all fish in a tank were removed each month, from December to April, for experimentation. Throughout the year, 329 adult individuals were

obtained and used for histological observation, and 308 fish whose fins were not injured were used for the body color analysis. We found that the sex change in *P. sieboldi* took place and proceeded during the non-spawning season. For serum steroid measurements, we used 43 fish including female, secondary male derived from female, and inter-sexual phase individuals collected between February and July.

### Experimental procedure

After anesthetization with 50 ppm of ethyl-4-aminobenzoate (benzocaine), each fish was placed in a transparent glass container filled with seawater. A 200-megapixel digital camera (C-920, Olympus, Japan) and four photograph lights were fixed to a camera stand (CS-5, LPL SHOJI K.K., Japan) in a dark room. The left lateral view of an individual fish were digitally photographed, together with a color separation guide (Kodak, NY, USA) that was also attached to the stand 600 mm under the camera. After taking the picture, blood samples were collected from the caudal vessel using syringes with 25-gauge needles and then centrifuged (2,000 rpm, 4°C, 20 min). The separated serum was stored at  $-30^{\circ}\text{C}$  until assayed for sex steroid levels. Gonads were fixed in Bouin's solution overnight, dehydrated, and embedded in Technovit resin (Kulzer, Wehrheim, Germany). For light microscopy, 4  $\mu\text{m}$  thick sections were cut and stained with 1% toluidine blue. To clarify the difference of male germ cell occurrence among the gonadal parts, cross sections of the anterior, middle, and posterior of left and right gonads (six parts in total) were examined for the occurrence of male germ cells in the transitional gonads. Although no significant differences were observed between each gonadal part with respect to the area of male germ cells/gonad, we cut a section of the right posterior part for all individuals (data not shown). The types of ovarian and testicular germ cells were classified as in previous studies (Matsuyama et al. 1997, 1998; Morita et al. 1998).

### Body color analysis

Images of each fish were stored as JPEG files and analyzed using Adobe Photoshop 5.5 (Adobe Systems Inc, CA, USA). To equalize the standard colors among the images, RGB values of the black and white squares of the color separation guide in each picture were adjusted to  $R = 5$ ,  $G = 5$ ,  $B = 5$  and  $R = 245$ ,  $G = 245$ ,  $B = 245$  by adjusting the input levels of red, green, and blue, respectively. In this equalization process, the hue values of the red and yellow squares of the color separation guide were always  $0^{\circ}$  and  $55^{\circ}$ , respectively. Subsequently, the hue value of each body part of the fish was determined using the “eyedropper” tool in

Adobe Photoshop. For the fins, the areas between the third spine and the first ray of the anal fin, between the second and third spine (anterior) and the eighth and ninth ray (posterior) of the dorsal fin, and between the rays on an extension of the lateral line of the tail fin were measured, avoiding line patterns and spots.

In *P. sieboldi*, the body color of IP females is mainly red and yellow, therefore, the value of the hue was sequentially expressed by establishing a range, from  $0^{\circ}$  to  $\pm 180^{\circ}$ , with red set as  $0^{\circ}$ .

### Serum steroid levels

Serum E2 levels were measured using the Estradiol EIA Kit (Cayman Chemical, MI, USA), while 11KT levels were determined using an enzyme-linked immunosorbent assay (ELISA) following the same procedure as for testosterone (Morita et al. 1997; Ohta et al. 2001). The intra- and inter-assay coefficients of variation were 9.6 and 2.8% for E2, and 10.7 and 7.2% for 11KT, and competition curves for serum collected from *P. sieboldi* were almost parallel to the standard curves (ANCOVA  $P > 0.05$ ).

### Statistics

Statistical analyses were carried out with the StatView software program (SAS Institute Japan, Japan). Mann–Whitney  $U$  test was performed to analyze differences in body color among body length within IP and TP individuals, between IP male and females, and between female and other sexual stages. Serum steroid levels for the different gonadal stages were compared using a one-way analysis of variance (ANOVA) followed by the Tukey–Kramer test. Pearson's correlation coefficient followed by Fisher's  $z$  transformation was used to analyze the relationship between hue values and steroid levels.

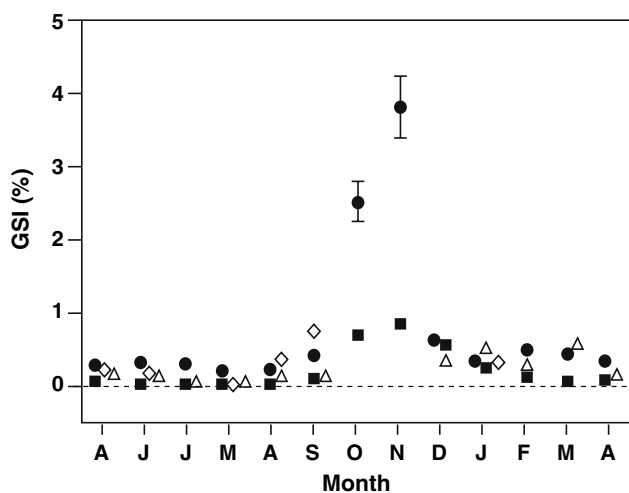
### Results

#### Monthly occurrence of different gonadal stages

Spawning was observed in the tanks in October and November. The gonadosomatic index (GSI; gonad weight/whole body weight  $\times 100$ ) of females increased dramatically during the spawning season and decreased rapidly thereafter (Fig. 1).

Immediately after the spawning season, vitellogenic oocytes in the ovary regressed and disappeared, whereas pre-vitellogenic oocytes remained and were observed throughout the year. Similarly, pre-vitellogenic oocytes were present in transitional gonads at an early stage of sex



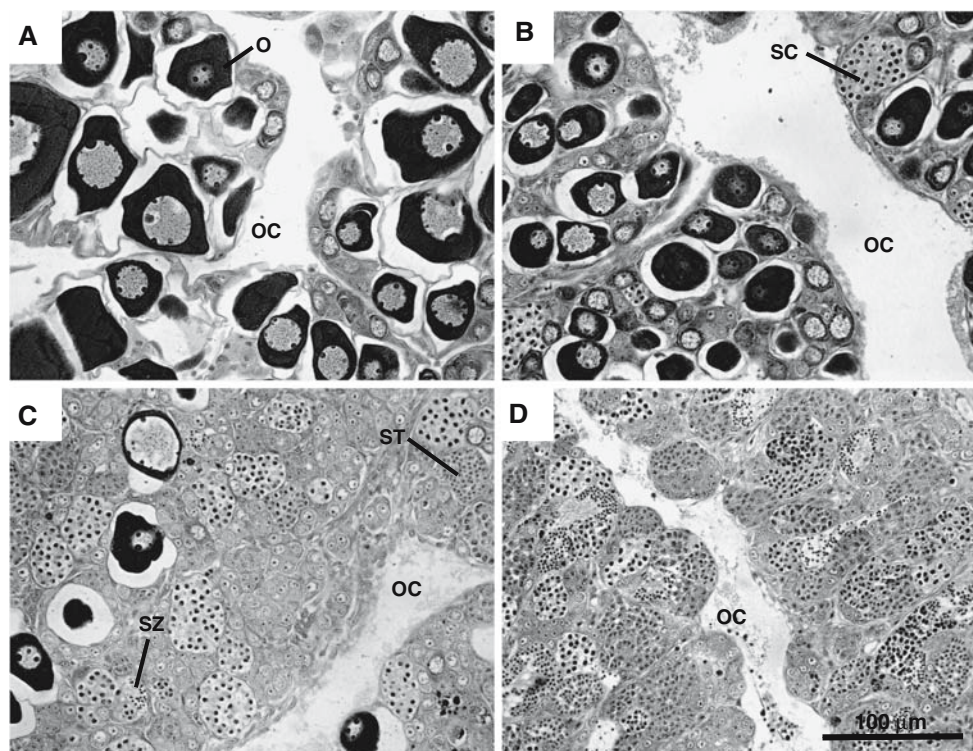


**Fig. 1** Monthly variation in the gonadosomatic index of *Pseudolabrus sieboldi*. Closed circles females, closed squares TP males derived from IP males and females, open triangles Stage 1 and 2, open diamonds IP males. Sample size corresponds to Table 1

change. There were no morphological differences in the pre-vitellogenic oocytes of the ovary (Fig. 2a) and the transitional gonads; therefore, the gonadal stage of sex change was evaluated based on the developmental stage of testicular tissue. Gonads in which the cysts of spermatocytes appeared sporadically but ubiquitously among pre-vitello-

genic oocytes are referred to as stage 1 (Fig. 2b), whereas gonads in which spermatids and/or spermatozoa had developed are referred to as stage 2 (Fig. 2c). The secondary testes of *P. sieboldi* (Fig. 2d), which are found in sex-changed females, can be generally distinguished from the primary testes derived from IP males by the presence of an ovarian cavity remnant, ovarian wall, and peripheral sperm duct (Nakazono 1979; Matsuyama et al. 1997). However, a number of testes obtained during the non-spawning season could not be identified as primary or secondary testes because of the drastic regression of gonadal structure after the spawning season. Consequently, the primary and secondary testes of TP individuals (TP primary males derived from IP primary males and TP secondary males derived from females) were pooled and are presented in Tables 1 and 2 as testes of TP males.

IP fish with ovaries and TP fish with testes were found in every month throughout the year (Table 1). Fish with stage 1 transitional gonads were first collected in December, just after the spawning season, and were collected continually until September, just before the next spawning season. Fish with stage 2 gonads were first collected in February and throughout the non-spawning season. No fish with transitional gonads were observed during the spawning season. Gonads in intersexual stages (stage 1 and 2), i.e., those undergoing a sex change, were seen in 34 (10.3%) of the



**Fig. 2** Histology of the gonads of *Pseudolabrus sieboldi* at each stage during sex change. Gonads were collected during the non-spawning season. **a** Ovary, **b** stage 1, **c** stage 2, **d** secondary testis of TP second-

ary male derived from female. *O* oocyte (pre-vitellogenic), *SC* spermatocytes, *ST* spermatids, *SZ* spermatozoa, *OC* ovarian cavity or ovarian cavity remnant

**Table 1** Monthly occurrence of fish with different stages of gonads in *P. sieboldi*

Gonadal stage	Month													Total
	A	M	J	J	A	S	O	N	D	J	F	M	A	
Ovary	13	18	15	12	13	13	14	13	14	10	13	16	7	171
Stage 1	1	2	–	1	1	1	–	–	1	4	2	1	–	14
Stage 2	7	4	3	1	1	–	–	–	–	–	2	–	2	20
Testis <sup>a</sup>	6	6	9	9	9	10	10	9	10	10	8	10	8	114
Testis <sup>b</sup>	1	1	–	2	2	1	–	2	–	1	–	–	–	10
Total	28	31	27	25	26	25	24	24	25	25	25	27	17	329

<sup>a</sup> Testis of TP males derived from IP males and females

<sup>b</sup> Testis of IP males

**Table 2** Total length of fish with different stage of gonad in *P. sieboldi*

Gonadal stage	Total length (mm)								Total
	81–100	101–120	121–140	141–160	161–180	181–200	201–220	221–240	
Ovary	3	72	74	20	2	–	–	–	171
Stage 1	–	6	6	2	–	–	–	–	14
Stage 2	–	–	5	10	4	1	–	–	20
Testis <sup>a</sup>	–	1	8	38	46	18	1	2	114
Testis <sup>b</sup>	–	7	3	–	–	–	–	–	10
Total	3	86	96	70	52	19	1	2	329

<sup>a</sup> Testis of TP males derived from IP males and females

<sup>b</sup> Testis of IP males

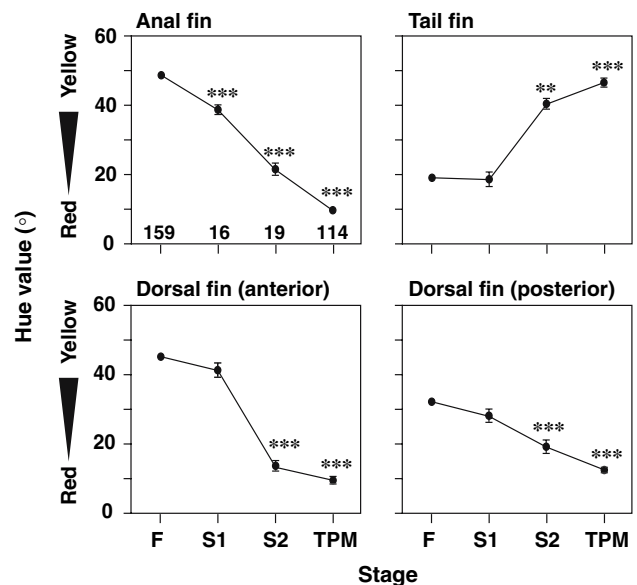
329 fish examined. The total length of IP fish with ovaries ranged from 98 to 162 mm, and TP fish with testes ranged from 120 to 230 mm (Table 2). The total length of fish with intersexual gonads ranged from 114 to 189 mm. Ten IP fish with primary testes were also collected and ranged in length from 106 to 134 mm.

**Body color analysis**

The anal fin, dorsal fin, and tail fin were selected for body color analysis because there were clear differences in the color of these structures between fish with ovaries and those with secondary testes. The anal fin and the anterior and posterior parts of the dorsal fin showed a change in hue from yellow to red. In contrast, the hue of the tail fin changed from red to yellow. Of the body parts examined, the hue of the anal fin changed most, with the hue value gradually decreasing with progressing gonadal stage during the sex change process (Figs. 3, 4). Differences in fin color did not correlate with body size within IP and TP individuals, and between IP males and females ( $P > 0.05$ ).

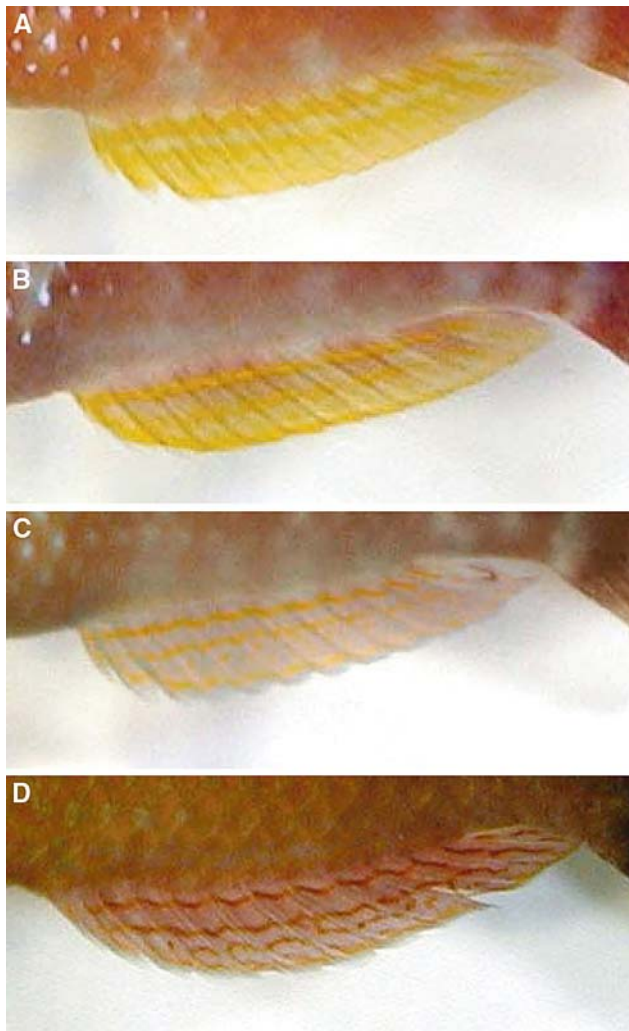
**Serum sex steroid levels**

During the sex change process, serum E2 levels fluctuated between 0.54 and 1.06 ng/ml; however, these changes were not significant (Fig. 5). In contrast, serum 11KT levels remained between 0.90 and 0.98 ng/ml in fish with ovaries and stage 1 gonads, but increased significantly at stage 2 (1.51 ng/ml), and peaked in fish with secondary testes (2.29 ng/ml).



**Fig. 3** Hue values of the anal, tail, and dorsal fins at each stage during sex change in *Pseudolabrus sieboldi*. F female, S1 stage 1, S2 stage 2, TPM TP male derived from IP male and female. Numbers indicate sample size. Each value is the mean ± SEM. Asterisks represent significant difference from female (\* $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\* $P < 0.001$ )

The relationship between the hue value of the anal fin and the serum steroid level was plotted for all fish (Fig. 6). The hue value of the anal fin decreased significantly with increasing serum 11KT levels (all four stages:  $r = -0.67$ ,  $P < 0.0001$ ,  $n = 43$ ; stage 1 and 2:  $r = -0.53$ ,  $P = 0.0087$ ,  $n = 23$ ). On the other hand, serum E2 levels did not show a significant change in association with the hue value of the

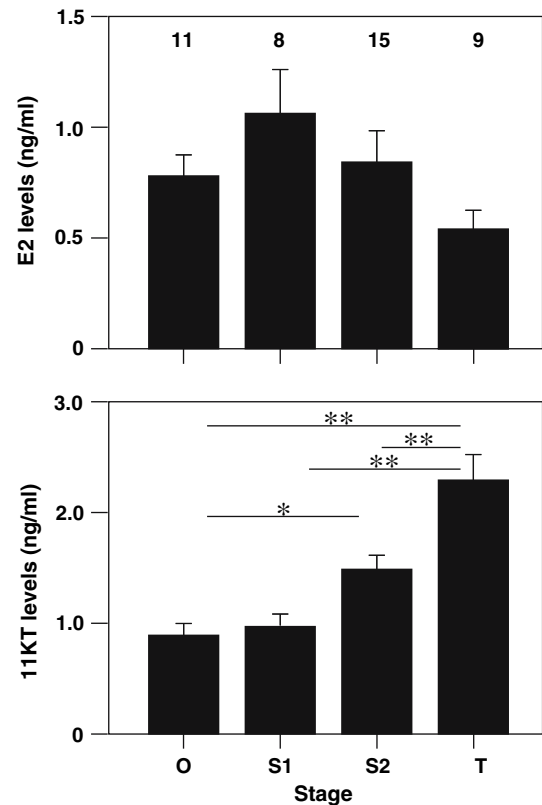


**Fig. 4** Anal fin colouration of *Pseudolabrus sieboldi* during the sex change process. Gonadal stages of individuals: **a** ovary, **b** stage 1, **c** stage 2, **d** secondary testis of TP secondary male derived from female

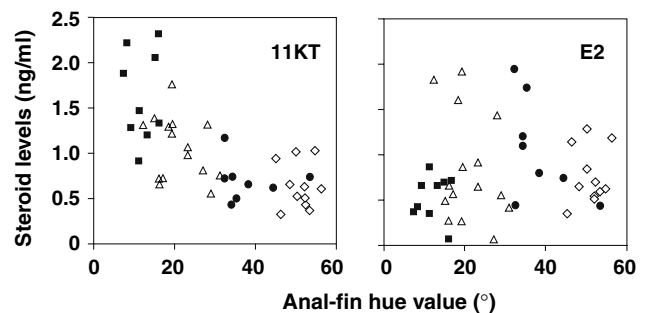
anal fin (all four stages:  $r = -0.07$ ,  $P < 0.6756$ ; stage 1 and 2:  $r = -0.06$ ,  $P = 0.7730$ ). Pearson correlation coefficients between serum steroid levels and hue value of four fins are shown in Table 3. The hue values of the tail fin and the anterior of dorsal fin also showed significant correlations with serum 11KT levels, whereas the posterior of dorsal fin significantly correlated with E2 levels during stage 1 and 2.

## Discussion

We were able to quantify body color change during sex change in *P. sieboldi*, and showed that the anal fin coloration of this species correlates with both the stage of sex change and serum 11KT levels. To our knowledge, this is the first direct demonstration of the correlation between the body coloration and serum androgen level in sex-changing



**Fig. 5** Serum steroid levels at each gonadal stage during sex change in *Pseudolabrus sieboldi*. O ovary, S1 stage 1, S2 stage 2, T secondary testis of TP secondary male derived from female. Numbers indicate sample size. Each value is the mean  $\pm$  SEM. Significant differences between groups are indicated by asterisks (\* $P < 0.05$ , \*\* $P < 0.01$ )



**Fig. 6** Relationship between anal fin hue value and serum steroid level. Open diamonds females, closed circles stage 1, open triangles stage 2, closed squares secondary males

species, and will provide a model system to analyze the physiological mechanism of sex change especially with respect to body coloration.

The spawning season of *P. sieboldi* spanned October and November, which is consistent with previous studies (Matsuyama et al. 1997, 1998; Morita et al. 1998). As suggested by Nakazono (1979), individuals undergoing sex change were obtained outside the 2-month spawning season. This also supports the hypothesis that sex change in



**Table 3** Correlation between serum steroids and hue of four different fins

Fin	11KT		E2	
	All stages <sup>a</sup>	Stage 1 and 2 <sup>b</sup>	All stages	Stage 1 and 2
Anal fin	−0.67 <sup>c</sup> (<0.001) <sup>d</sup>	−0.53 (0.009)	0.07 (0.676)	−0.06 (0.773)
Dorsal fin (a) <sup>e</sup>	−0.54 (<0.001)	−0.48 (0.017)	0.10 (0.503)	0.03 (0.887)
Dorsal fin (p) <sup>f</sup>	−0.33 (0.028)	−0.27 (0.193)	−0.30 (0.050)	−0.48 (0.016)
Tail fin	0.42 (0.005)	0.69 (<0.001)	−0.09 (0.557)	−0.19 (0.380)

<sup>a</sup> Gonadal stages of ovary, stage 1, 2 and secondary testis,  $n = 43$

<sup>b</sup>  $N = 23$

<sup>c</sup> Pearson coefficient

<sup>d</sup>  $P$  value

<sup>e</sup> Anterior of dorsal fin

<sup>f</sup> Posterior of dorsal fin

many protogynous species takes place outside the reproductive season (Shapiro 1987). In this study, we must take into consideration that fish obtained in the winter season were examined after being kept in tanks for 1–4 months. Our recent experiment demonstrated that *P. sieboldi* changes sex according to social status (Ohta et al. 2003). Therefore, it is possible that sex change was induced by the conditions in the tank. Body colorations at each sexual stage were not distinct from fish collected during other seasons (data not shown), and the GSI values at each gonadal stage were not markedly different between the fish collected in April 2000 and those sampled in April 2001 that had been reared in tanks for 5 months beginning in November 2000. Therefore, *P. sieboldi* is capable of changing sex in captivity as well as under natural conditions.

This is the first study to quantitatively analyze the changes in body color that accompany sex change in a fish. The results indicate an excellent correlation between the hue value of the anal fin and the stage of gonadal sex change. Generally, body color change in fish has been divided into two categories; one is a physiological color change, which is attributed to rapid motile responses of chromatophores, and the other is a morphological color change, which results from changes in morphology and density of chromatophores (Fujii 2000; Sugimoto 2002). The coloration of anal, dorsal and tail fin in *P. sieboldi* did not change rapidly, even when fish were pale or in a dark environment (data not shown). Therefore, the color changes that we observed in three fins were probably a morphological change. Further study of the change in chromatophore densities will provide direct evidence for the mechanism of the alteration in three fins coloration of *P. sieboldi*. Meanwhile, monitoring changes in the color of the anal fin is a useful approach for identifying the sex of *P. sieboldi* individuals. Accordingly, we could easily distinguish the sex-change status of a particular individual based on the color of its anal fin.

Field research has previously demonstrated that TP males show their ventral side to females at the beginning of their mating behaviour, when females enter the TP male's territory (Nakazono 1979). This behaviour and the fin colour change lead us to speculate that the colour of the anal fin is an important trait communicating the sexuality of a male to a female. It has been suggested that male-specific coloration is important in female mate choice in other protogynous wrasses (Warner and Schultz 1992; Kuwamura et al. 2000). However, these colorations may be unnecessary for starting the mating behaviours because sex changing individuals spawn with females even before the completion of their colour changes (Godwin et al. 1996; Sakai et al. 2002). Future experiments should be conducted to explore the role of fin colouration in the mating success of male *P. sieboldi*.

Here, we found that serum levels of 11KT increased in association with the development of testicular tissue during sex change in *P. sieboldi*. In our previous studies of *P. sieboldi*, we showed that the pathways to E2 and 11KT synthesis in ovarian follicles and testes branch from AD (Ohta et al. 2001, Sundaray et al. 2003). In ovarian follicles, E2 is synthesized from AD via estrone (E1), but not via testosterone (T). In the testes of *P. sieboldi*, 11KT is produced from AD via T. Therefore, T is not produced in the ovarian follicles, but is synthesized in testicular tissue, and 11KT is synthesized only after T production. Moreover, we have previously shown that 11KT induces spermatogenesis when 11KT is implanted intraperitoneally into female fish (Ohta et al. 2003). Although extra-follicular productions of T and 11KT are possible because of the presence of circulating 11KT in females (Ohta et al. 2001; this study), increased levels of 11KT probably play roles in promoting gonadal change from females to males in *P. sieboldi*. These effects of 11KT may be general in gonadal change of protogynous species, as seen in other species (Devlin and Nagahama 2002).

We found a significant correlation between serum 11KT levels and the hue values of the anal fin, tail fin, and anterior of dorsal fin. This result agrees with a previous study that the treatment of IP females with 11KT induces the change to TP males (Ohta et al. 2003). Therefore, 11KT probably plays a role in the transformation of IP females to TP males in *P. sieboldi* as in other protogynous species (Cardwell and Liley 1991; Grober et al. 1991). The body color change that occurs in the transition from IP female to TP male in protogynous hermaphroditic fish is traditionally thought to be similar to the expression of secondary sexual characteristics in other vertebrates (Brantley et al. 1993). Secondary characteristics of males are generally induced by androgen, not only in fish, but also in amphibians, reptiles, birds, and mammals (Borg 1994; Hews and Moore 1995; Emerson et al. 1997; Eens et al. 2000; Sinisi et al. 2003). In addition, the development of some male characteristics is mediated by the androgen receptor (AR), including the development of the gonopodium of mosquitofish (Ogino et al. 2004) and the male reproductive organs of mice (Yeh et al. 2002). Further studies are needed to determine whether the AR is involved in the body color change in fish. The results of such studies will also provide insight into the general mechanism behind the morphological development of secondary characteristics in male vertebrates, including body shape and coloration.

On the other hand, the hue value of the posterior of dorsal fin showed a significant correlation with E2 levels during stage 1 and 2. The role of estrogen for body color change is unknown. Further study must be performed to clarify the mechanism of color change in the posterior of dorsal fin.

During the process of gonadal sex change in *P. sieboldi*, serum E2 levels did not change drastically. This may be due to the fact that the serum levels of sex steroids in *P. sieboldi* were analyzed in the non-spawning season when their sex changes occur. In some protogynous and bi-directional species, serum E2 levels decrease dramatically at the onset of the gonadal sex change from female to male, in which fish from which circulating levels of female hormones are obtained have actively vitellogenic and maturational oocytes (wrasse *Thalassoma duperrey*; Nakamura et al. 1989, parrotfish *Sparisoma viride*; Cardwell & Liley 1991, goby *Gobiodon histrio*; Kroon et al. 2003). Indeed, serum E2 levels in females were 6–24 times higher in the spawning season (Ohta et al. 2001) than that in the non-spawning season that we examined. Such seasonal changes are also observed in many hermaphrodites (e.g., Yeung and Chan 1987a, b; Guiguen et al. 1993; Bhandari et al. 2004) that undergo sex changes during the non-breeding season. Moreover, recent studies have revealed that E2 is also involved in male spermatogenesis (Miura et al. 1999; Amar et al. 2001). Therefore, the dramatic decrease in E2 levels

does not appear necessary for the protogynous sex change in the gonads and body coloration.

In contrast, recent studies have revealed that the sex changes in some protogynous and bi-directional sex-changing species were induced by implanting females with the non-steroidal AI fadrozole (gobies; Kroon and Liley 2000; Kroon et al. 2005, wrasse; Higa et al. 2003, grouper; Bhandari et al. 2004). These sex changes may be induced by the change in the circulation profiles of E2. Our study revealed that the profiles of serum E2 levels are completely different between females and males in the spawning season (Ohta et al. 2001; Sundaray et al. 2003). Female E2 levels fluctuated in the range of 5.03–18.52 ng/ml throughout 1 day in the spawning season, whereas a surge of E2 was observed around 1500 hours in males, reaching 36.8 from 0.2 ng/ml. Thus, the constant levels of E2 may be necessary for female maintenance. The physiological role of estrogen in males, as well as the changes in the diurnal profiles of E2 levels in individuals undergoing sex change, should be analyzed in *P. sieboldi*. Additionally, it is noteworthy that serum 11KT levels in female *P. sieboldi* were relatively low throughout the year, including both spawning and non-spawning season (Morita et al. 1997; this study). This likely plays a role in preventing testicular development and TP body coloration during the female phase in *P. sieboldi*.

In conclusion, we quantitatively evaluated the change in body color accompanying gonadal sex change from female to male in *P. sieboldi*. Based on the analysis of serum steroids combined with the results of our earlier experiments, we propose that 11KT production in *P. sieboldi* acts as a promoter of sex change. These features of *P. sieboldi* have allowed the characterization of all steroid hormones produced in the gonads of this species, as well as their synthetic pathways (Ohta et al. 2001, Ohta and Matsuyama 2002; Sundaray et al. 2003). *P. sieboldi* is useful as a model species in which to investigate the endocrinological and molecular mechanisms of sex change in fish.

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## References

- Amar MA, Miura T, Miura C, Yamauchi K (2001) Involvement of sex steroid hormones in the early stages of spermatogenesis in Japanese huchen (*Hucho perryi*). Biol Reprod 65:1057–1066
- Arenas MI, Royuela M, Lobo MV, Alfaro JM, Fraile B, Paniagua R (2001) Androgen receptor (AR), estrogen receptor- $\alpha$  (ER- $\alpha$ ) and estrogen receptor- $\beta$  (ER- $\beta$ ) expression in the testis of the newt, *Triturus marmoratus marmoratus* during the annual cycle. J Anat 199:465–472



- Atz JW (1964) Intresexuality in fishes. In: Armstrong CN, Marshall AJ (eds) *Intersexuality in vertebrates including man*. Academic, London, pp 145–232
- Bhandari RK, Higa M, Nakamura S, Nakamura M (2004) Aromatase inhibitor induces complete sex change in the protogynous honeycomb grouper (*Epinephelus merra*). *Mol Reprod Dev* 67:303–307
- Bhandari RK, Alam MA, Soyano K, Nakamura M (2006) Induction of female-to-male sex change in the honeycomb grouper (*Epinephelus merra*) by 11-ketotestosterone treatments. *Zool Sci* 23:65–69
- Borg B (1994) Androgen in teleost fishes. *Comp Biochem Physiol C* 109:219–245
- Brantley RK, Wingfield JC, Bass AH (1993) Sex steroid levels in *Porichthys notaus*, a fish with alternative reproductive tactics, and a review of the hormonal bases for male dimorphism among teleost fishes. *Horm Behav* 27:332–347
- Cardwell JR, Liley NR (1991) Hormonal control of sex and color change in the stoplight parrotfish, *Sparisoma viride*. *Gen Comp Endocrinol* 81:7–20
- Devlin RH, Nagahama Y (2002) Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture* 208:191–364
- Emerson SB, Carroll L, Hess DL (1997) Hormonal induction of thumb pads and the evolution of secondary sexual characteristics of the Southeast Asian fanged frog, *Rana blythii*. *J Exp Zool* 279:587–596
- Eens M, Van Duyse E, Berghman L, Pinxten R (2000) Shield characteristics are testosterone-dependent in both male and female moorhens. *Horm Behav* 37:126–134
- Fujii R (2000) The regulation of motile activity in fish chromatophores. *Pigment Cell Res* 13:300–319
- Godwin JR, Thomas P (1993) Sex change and steroid profiles in the protandrous anemonefish *Amphiprion melanopus* (Pomacentridae, Teleostei). *Gen Comp Endocrinol* 91:144–157
- Godwin J, Crews D, Warner RR (1996) Behavioral sex change in the absence of gonads in a coral reef fish. *Proc R Soc Lond B* 263:1683–1688
- Grober MS, Sunobe T (1996) Serial adult sex change involves rapid and reversible changes in forebrain neurochemistry. *Neuroreport* 7:2945–2949
- Grober MS, Jackson IMD, Bass AH (1991) Gonadal-steroids affect LHRH preoptic cell number in a sex-role changing fish. *J Neurobiol* 22:734–741
- Guiguen Y, Jalabert B, Thouard E, Fostier A (1993) Changes in plasma and gonadal steroid hormones in relation to the reproductive cycle and the sex inversion process in the protandrous seabass, *Lates calcarifer*. *Gen Comp Endocrinol* 92:327–338
- Hassin S, de Monbrison D, Hanin Y, Elizur A, Zohar Y, Popper DM (1997) Domestication of the white grouper, *Epinephelus aeneus*: 1. Growth and reproduction. *Aquaculture* 156:305–316
- Hews DK, Moore MC (1995) Influence of androgens on differentiation of secondary sex characteristics in tree lizards, *Urosaurus ornatus*. *Gen Comp Endocrinol* 97:86–102
- Higa M, Ogasawara K, Sakaguchi A, Nagahama Y, Nakamura M (2003) Role of steroid hormones in sex change of protogynous wrasse. *Fish Physiol Biochem* 28:149–150
- Koulish S, Kramer CR (1989) Human chorionic gonadotropin (hCG) induces gonad reversal in a protogynous fish, the bluehead wrasse, *Thalassoma bifasciatum* (Teleostei Labridae). *J Exp Zool* 252:156–168
- Kramer CR, Caddell MT, Bubenheimer-Livolsi L (1993) sGnRH-A ((D-Arg<sup>6</sup>, Pro<sup>9</sup>, NET-) LHRH) in combination with domperidone induces gonad reversal in a protogynous fish, the bluehead wrasse, *Thalassoma bifasciatum*. *J Fish Biol* 42:185–195
- Kroon FJ, Liley NR (2000) The role of steroid hormones in protogynous sex change in the Blackeye goby, *Coryphopterus nicholsii* (Teleostei: Gobiidae). *Gen Comp Endocrinol* 118:273–283
- Kroon FJ, Munday PL, Pankhurst NW (2003) Steroid hormone levels and bi-directional sex change in *Gobiodon histrio*. *J Fish Biol* 62:153–167
- Kroon FJ, Munday PL, Westcott DA, Hobbs JP, Liley NR (2005) Aromatase pathway mediates sex change in each direction. *Proc Biol Sci* 272:1399–1405
- Kuwamura T, Nakashima Y (1998) New aspects of sex change among reef fishes: recent studies in Japan. *Environ Biol Fish* 52:125–135
- Kuwamura T, Karino K, Nakashima Y (2000) Male morphological characteristics and mating success in a protogynous coral reef fish, *Halichoeres melanurus*. *J Ethol* 18:17–23
- Larson ET, Norris DO, Summers CH (2003) Monoaminergic changes associated with socially induced sex reversal in the saddleback wrasse. *Neuroscience* 119:251–263
- Lee YH, Du JL, Yueh WS, Lin BY, Huang JD, Lee CY, Lee MF, Lau EL, Lee FY, Lee MF, Lau EL, Lee FY, Morry C, Nagahama Y, Chang CF (2001) Sex change in the protandrous back porgy, *Acanthopagrus schlegeli*: a review in gonadal development, estradiol, estrogen receptor, aromatase activity and gonadotropin. *J Exp Zool* 290:715–726
- Lee YH, Yueh WS, Du JL, Sun LT, Chang CF (2002) Aromatase inhibitors block natural sex change and induce male function in the protandrous black porgy, *Acanthopagrus schlegeli* Bleeker: possible mechanism of natural sex change. *Biol Reprod* 66:1749–1754
- Liu X, Liang B, Zhang S (2004) Sequence and expression of cytochrome P450 aromatase and FTZ-F1 genes in the protandrous black porgy (*Acanthopagrus schlegeli*). *Gen Comp Endocrinol* 138:247–254
- Matsuyama M, Morita S, Hamaji N, Kashiwagi M, Ohta K, Nagahama Y (1997) Diurnal spermatogenesis and spawning in the secondary male of a protogynous wrasse, *Pseudolabrus japonicus* (Teleostei, Labridae). *Zool Sci* 14:1001–1008
- Matsuyama M, Morita S, Nasu T, Kashiwagi M (1998) Daily spawning and development of sensitivity to gonadotropin and maturation-inducing steroid in the oocytes of the bambooleaf wrasse *Pseudolabrus japonicus*. *Environ Biol Fish* 52:281–290
- Miura T, Miura C, Ohta T, Nader MR, Todo T, Yamauchi K (1999) Estradiol-17 $\beta$  stimulates the renewal of spermatogonial stem cells in males. *Biochem Biophys Res Commun* 264:230–234
- Morita S, Matsuyama M, Kashiwagi M (1997) Seasonal changes of gonadal histology and serum steroid hormone levels in the bambooleaf wrasse *Pseudolabrus japonicus*. *Bull Jpn Soc Sci Fish/Nippon Suisan Gakkai Shi* 63:694–700
- Nakamura M, Hourigan TF, Yamauchi K, Nagahama Y, Grau EF (1989) Histological and ultrastructural evidence for the role of gonadal steroid hormones in sex change in the protogynous wrasse *Thalassoma duperrey*. *Environ Biol Fishes* 24:117–136
- Nakazono A (1979) Studies on the sexual reversal and spawning behavior of the five species of Japanese labrid fishes. *Rep Fish Res Lab Kyushu Univ* 4:1–64
- Ogino Y, Katoh H, Yamada G (2004) Androgen dependent development of a modified anal fin, gonopodium, as a model to understand the mechanism of secondary sexual character expression in vertebrates. *FEBS Lett* 575:119–126
- Ohta K, Matsuyama M (2002) Steroidogenic pathways to 17,20 $\beta$ -dihydroxy-4-pregnen-3-one and 17,20 $\beta$ ,21-trihydroxy-4-pregnen-3-one in the ovarian follicles of the bambooleaf wrasse, *Pseudolabrus sieboldi*. *Fish Sci* 68:41–50
- Ohta K, Mine Y, Yamaguchi A, Matsuyama M (2001) Steroidogenic pathway to estradiol-17 $\beta$  synthesis in the ovarian follicles of the protogynous wrasse, *Pseudolabrus sieboldi*. *Zool Sci* 18:937–945
- Ohta K, Sundaray JK, Okida T, Sakai M, Kitano T, Yamaguchi A, Takeda T, Matsuyama M (2003) Bi-directional sex change and its steroidogenesis in the wrasse *Pseudolabrus sieboldi*. *Fish Physiol Biochem* 28:173–174

- Reinboth R (1970) Intersexuality in fishes. *Mem Soc Endocrinol* 18:515–543
- Roberts DE, Schlieder Jr RA (1983) Induced sex inversion, maturation, spawning and embryogeny of the protogynous grouper, *Mycteroperca microlepis*. *J World Maricult Soc* 14:639–649
- Sakai Y, Karino K, Nakashima Y, Kuwamura T (2002) Status-dependent behavioral sex change in a polygynous coral-reef fish, *Hali-choeres melanurus*. *J Ethol* 20:101–105
- Shapiro DY (1979) Social behavior, group structure, and the control of sex reversal in hermaphroditic fish. *Adv Study Behav* 10:43–102
- Shapiro DY (1987) Sexual differentiation, social behavior and the evolution of sex change in coral reef fishes. *Bioscience* 37:490–497
- Sinisi AA, Pasquali D, Notaro A, Bellastella A (2003) Sexual differentiation. *J Endocrinol Invest* 26(3 Suppl):23–28
- Sugimoto M (2002) Morphological color changes in fish: regulation of pigment cell density and morphology. *Microsc Res Tech* 58:496–503
- Sundaray JK, Ohta K, Yamaguchi A, Suzuki K, Matsuyama M (2003) Diurnal rhythm of steroid biosynthesis in the testis of terminal phase male of protogynous wrasse (*Pseudolabrus sieboldi*), a daily spawner. *Fish Physiol Biochem* 28:193–195
- Sunobe T, Nakamura M, Kobayashi Y, Kobayashi T, Nagahama Y (2005) Aromatase immunoreactivity and the role of enzymes in steroid pathways for inducing sex change in the hermaphrodite gobiid fish *Trimma okinawae*. *Comp Biochem Physiol A Mol Integr Physiol* 141:54–59
- Tan-Fermin JD, Garcia LMB, Castillo Jr AR (1994) Induction of sex inversion in juvenile grouper, *Epinephelus suillus*, (Valenciennes) by injections of 17 alpha-methyltestosterone. *Jpn J Ichthyol* 40:413–420
- Tang F, Chan STH, Lofts B (1974) Effect of steroid hormones on the process of natural sex reversal in the rice-field eel, *Monopterus albus* (Zuiew). *J Comp Endocrinol* 24:227–241
- Warner RR, Schultz ET (1992) Sexual selection and male characteristics in the bluehead wrasse, *Thalassoma bifasciatum*: mating site acquisition, mating site defense, and female choice. *Evolution* 46:1421–1442
- Yeh S, Tsai M-Y, Xu Q et al (2002) Generation and characterization of androgen receptor knockout (ARKO) mice: An in vivo model for the study of androgen functions in selective tissues. *Proc Natl Acad Sci USA* 99:13498–13503
- Yeung WSB, Chan STH (1987a) The plasma sex steroid profiles in the freshwater, sex reversing teleost fish, *Monopterus albus* (Zuiew). *Gen Comp Endocrinol* 65:233–242
- Yeung WSB, Chan STH (1987b) A radioimmunoassay study of the plasma levels of sex steroids in the protandrous, sex-reversing fish *Rhabdosargus sarba* (Sparidae). *Gen Comp Endocrinol* 66:353–363