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Expression Levels of Hormone Receptors and Bone Morphogenic Protein in Fins of Medaka

Arin Ngamniyom[1]*, Wichian Magtoon[2], Yoshitaka Nagahama[3] and Yuichi Sasayama[4]

[1]*Department of Life Science, Graduate School of Natural Science and Technology, Kanazawa University, Kanazawa, Ishikawa 920-1192, Japan*
[2]*Faculty of Science, Srinakharinwirot University, Bangkok 10110, Thailand*
[3]*Laboratory of Reproductive Biology, National Institute for Basic Biology, Okazaki, Aichi 444-8585, Japan*
[4]*Division of Biodiversity, Institute of Nature and Environmental Technology, Kanazawa University, Kanazawa, Ishikawa 920-1192, Japan*

In the genus *Oryzias*, the morphologies of the dorsal and anal fins are typical secondary sex characters. In the Japanese medaka (*Oryzias latipes*) and Thai medaka (*Oryzias minutillus*), androgen receptor (AR) expression levels in the dorsal, anal, and pectoral fins were higher in males than in females. Conversely, in both species estrogen receptor (ER) β expression levels in the dorsal and anal fins were higher in females than in males. AR and ERβ expression levels in the dorsal and anal fins of sex-undeterminable individuals of Thai medaka were intermediate between those in normal male and female Thai medaka. There was no difference in the bone morphogenic protein (Bmp) 2b expression level between male and female Japanese medaka. In contrast, the Bmp2b expression level in the dorsal fin of sex-undeterminable individuals was lower than in normal male and female Thai medaka. It is thus clear that androgen and estrogen regulate the sex-dependent characters of fin morphology in both *Oryzias* species. In sex-undeterminable individuals of Thai medaka, the low levels of Bmp2b expression in the dorsal fin are evidence that androgen and estrogen are necessary for adequate expression of Bmp2b in the normal development of at least the dorsal fin.

Key words: androgen receptor, estrogen receptor, bone morphogenic protein, Japanese medaka, Thai medaka

INTRODUCTION

The Japanese medaka (*Oryzias latipes*, Teleostei) is a model organism frequently utilized for experiments in various fields such as reproductive biology (Carlson et al., 2002) and the study of sex determination (Matsuda et al., 2002). The medaka was recently used as a sensitive bio-indicator of exogenous active endocrine chemicals (Urushitani et al., 2007). In the genus *Oryzias*, males can be distinguished from females by the secondary sex characters of fins (Okada and Yamashita, 1944). The dorsal and anal fins of males are usually longer than those of females. In contrast, the pectoral fins of females are longer than those of males. Papillar processes are present on the anal and pectoral fins only in males. In addition, leucophores are well developed on the caudal fin in males. In males, the numbers of papillar processes on the anal and pectoral fins and of leucophores on the caudal fin increase during the breeding season (Egami and Ishii, 1956; Arai and Egami, 1961).

* Corresponding author. Phone: +81-76-264-6307;
Fax : +81-768-74-1644;
E-mail: arin1681@stu.kanazawa-u.ac.jp
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The Thai medaka (*Oryzias minutillus*) is widely distributed in Thailand (Magtoon and Uwa, 1985). This species inhabits shallow ponds, ditches, and paddy fields (Magtoon et al., 1992). The secondary sex characters of fins in male Thai medaka are similar to those in male Japanese medaka, and the dorsal and anal fins are likewise longer in males than in females (Ngamniyom et al., 2007).

In all vertebrates, androgen plays a crucial role in the development and the maintenance of the male phenotype (Brinkmann et al., 1999). This hormone works on target cells via androgen receptors (Jenster et al., 1995). In male Japanese medaka, the papillar processes on the anal and pectoral fins, and the leucophores on the caudal fin, are controlled by androgen (Egami and Ishii, 1956; Arai and Egami, 1961; Kawamoto, 1969).

Just as androgen functions in males, estrogen functions in females via estrogen receptors and plays an important role in maturation of the reproductive organs (Nimrod and Benson, 1998; Nilsson et al., 2001). Three ER isoforms (ERα, ERβ, and ERγ) have been reported in vertebrates (Chang et al., 1999; Sabo-Attwood et al., 2004). In teleosts, ERβ is abundantly expressed in gonad, brain, liver, gastrointestinal tract, cartilage, and bone tissues (Tchoudakova et

al., 1999; Socorro et al., 2000; Menuet et al., 2002; Hawkins and Thomas, 2004). This suggests that among ERs, ERβ is also the main receptor in medaka; thus, in this study we examined expression levels of ERβ.

Bone morphogenic protein 2 (Bmp2) plays a major role in the development of bone and cartilage in mammals (Zhao et al., 2006). In the zebrafish (*Danio rerio*), a teleost, Crotwell et al. (2004) found that Bmp2 is expressed in developing dorsal, anal, and caudal fins. In mammals, expression levels of Bmp2 are affected by androgen and estrogen (Ide et al., 1997; Yamamoto et al., 2002); therefore, we also examined Bmp2 expression levels in fins.

Ngamniyom et al. (2007) reported the occurrence of many sex-undeterminable individuals of Thai medaka in suburbs of Bangkok, Thailand. In these individuals, the secondary sex characters of the fins are not useful in distinguishing males from females, since the morphology of the dorsal and anal fins of these individuals is intermediate between that of males and females.

In this study, we sought to clarify the molecular-biological background of the secondary sex characters of fins between normal males and females by examining mRNA expression levels of the androgen receptor (AR), estrogen receptor (ER) β, and Bmp2b in the dorsal, anal, pectoral, and caudal fins in Japanese and Thai medaka. In addition, we examined how these genes are expressed in the fins of sex-undeterminable individuals of Thai medaka by comparing the expression levels to those of normal males and females.

MATERIALS AND METHODS

Fish

Adult Japanese medaka were purchased from a commercial source in Kanazawa city, Ishikawa, Japan. Their standard length was 24–26 mm. Males and females were kept separately in aquaria with a controlled 14:12 hr light/dark photoperiod cycle at 26°C for 1 week, and fed ad libitum with TetraMin (Tokyo, Japan). Their sexes were judged from the morphology of the secondary sex characters of the dorsal and anal fins, according to the criteria of Okada and Yamashita (1944).

Adult Thai medaka were captured in ponds and ditches in suburbs of Bangkok, Thailand, from September to November 2007. Their standard length was 11–14 mm. Average water temperature in those areas was 26±1°C. Males were distinguished from females by examination of the secondary sex characters of the dorsal and anal fins (see Ngamniyom et al., 2007). To examine the sex ratio (male to female) in Thai medaka, captured individuals were fixed in 70% ethanol (Table 1). Individuals with uncertain sex were determined to be sex-undeterminable on the basis of their fin morphology. For semi-quantitative RT-PCR, additional typical males and females were captured from localities 1, 2, and 3, where the sex ratio was about 1:1. Additional sex-undeterminable individuals were captured from localities 4 and 5, where the sex ratio was unbalanced, with many sex-undeterminable individuals (Table 1). Specimens for RT-PCR were immediately put into a solution of RNAlater (Qiagen, Japan). To check their gonads, microscopic procedures were conducted.

To clarify differences between males and females, we examined only adult male and female Japanese and Thai medaka, since secondary sex characters are sometimes obscure in immature individuals.

In Japanese medaka, the natural breeding season lasts from May through August (Shima and Mitani, 2004). Thai medaka were captured from September to November because they are easy to capture in the dry season, which is the non-breeding season for Thai medaka and corresponds to the non-breeding season for Japanese medaka. To make conditions uniform in both species, experiments were conducted during the non-breeding seasons.

Table 1. Sex ratio and percentage of sex-undeterminable individuals in samples of Thai medaka.

Locality	Number of specimens			Sex ratio	Percentage sex-underminable
	male	female	sex-underminable	male : female	
1	19	21	–	1.0:1.1	–
2	20	14	1	1.4:1.0	2.9
3	30	36	2	1.0:1.2	3.0
4	6	13	7	1.0:2.2	26.9
5	5	10	6	1.0:2.0	28.6

Preparation of fins

The dorsal, anal, pectoral, and caudal fins were dissected out from 50 male and 50 female Japanese medaka. In the anal fin of males, the posterior part with papillar processes, comprising the 2nd to the 8th fin rays counted from the posterior end, was separated from the anterior part, according to the criteria of Iwamatsu et al. (2003). Five fins were pooled in each tube, and a sample of 10 tubes was collected. In Thai medaka, the dorsal fin, the whole anal fin (as there are no papillar processes in this species), a pectoral fin, and the caudal fin were removed from 70 males, 70 females, and 70 sex-undeterminable individuals; seven fins was pooled in each tube, and a sample of 10 tubes was collected.

Semi-quantitative RT-PCR

Total RNA from each sample of Japanese medaka or Thai medaka was extracted in Isogen isolation reagent (Wako, Tokyo, Japan) according to the manufacturer's protocol and treated with DNase1 (Takara, Tokyo, Japan) for 30 min at 37°C. Total RNA (100 ng) was reverse-transcribed with AMV reverse transcriptase XL (Takara, Tokyo, Japan) according to the manufacturer's instructions. The extract solution (0.5 μl) was used as a PCR template.

Primers were designed on the basis of data from Japanese medaka, and were also applied to Thai medaka. Primers for amplification of AR (5'-CAGGAGGAGTTCCTGTGCAT-3' and 5'-GGTGGTGGTAAGGTGAAGGA-3') were designed from a sequence (accession number AB076399) in the DNA Database of Japan (DDBJ). ERβ primers were 5'-CTGTTAGATGCCTCGGACCTT-3' and 5'-GATTGGCTGGTTTCGTG-3' (Inui et al., 2003). Bmp2b primers (5'-AAGGGCAAAACAACCCAGCAG-3' and 5'-GTTCCATCCCACATCGCTGAA-3') were designed from a sequence (accession number DQ915174) in DDBJ. As a loading control and reference, β-actin mRNA was amplified for each RT reaction; primers used were 5'-AGGGAGAAGATGACC-3' and 5'-CGCAGGACGCCATACCAA-3' (Scholz et al., 2004).

PCR conditions for the amplification of cDNA were 95°C for 30 sec for denaturation; 62°C (AR), 64°C (ERβ and Bmp2b), or 58°C (β-actin) for 1 min for annealing; and 72°C for 1 min for extension. Cycle numbers for Japanese medaka were 30 cycles for AR, ERβ, and Bmp2b; those for Thai medaka were 34 cycles for AR and 30 cycles for ERβ and Bmp2b. In both species, 20 cycles were performed for β-actin. PCR products were electrophoresed on 2% agarose gel, stained with ethidium bromide, and visualized on a UV-transilluminator. Amplification levels were quantified by using Scion Image software for Windows (Scion, Maryland, USA). Amplification levels of AR, ERβ, and Bmp2b for each fin type were divided by the corresponding amplification level of β-actin to obtain relative expression levels.

One-way ANOVA with Tukey's multiple comparison test, the unpaired Student *t*-test, and the Mann Whitney *U* test were used to examine differences statistically. Data were analyzed by using the statistical package for the social sciences (SPSS) for Windows,

version 13 (SPSS, Chicago, USA).

RESULTS

Levels of AR, ERβ, and Bmp2b mRNA expression in Japanese medaka

In males, AR mRNA expression levels were significantly higher in the dorsal fin and the anterior and posterior parts of the anal fin than in the pectoral and caudal fins (Fig. 1A). In contrast, the ERβ mRNA expression level was significantly higher in the caudal fin than in the anterior and posterior parts of the anal fin (Fig. 1A). No significant difference in the Bmp2b mRNA expression level was found among the fins (Fig. 1A).

In females, AR and ERβ mRNA expression levels were significantly higher in the dorsal and anal fins than in the pectoral and caudal fins (Fig. 1B). No significant difference

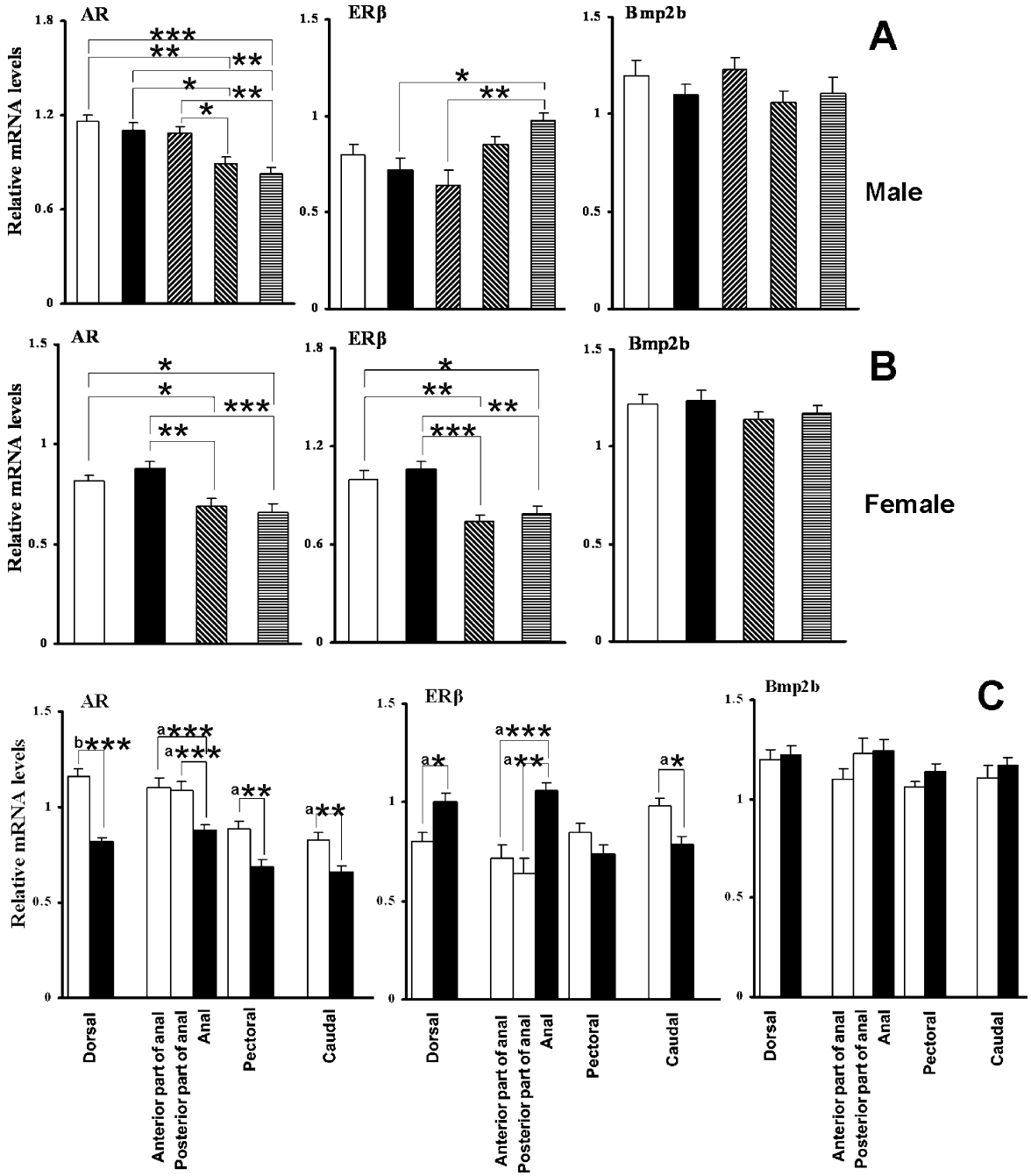

Fig. 1. **(A, B)** Expression levels of AR, ERβ, and Bmp2b mRNA in **(A)** male and **(B)** female Japanese medaka. Bars indicate the dorsal fin (□), anterior part of the anal fin (■), posterior part of the anal fin (▨), pectoral fin (▧), and caudal fin (▤). **(C)** Comparisons between adult male (□) and female (■) Japanese medaka. The expression levels in each fin are values relative to the expression level of β-actin mRNA (mean±SE). One-way ANOVA followed by Tukey's multiple comparison test in (A, B), and by the [a]unpaired Student *t*-test and [b]Mann Whitney *U* test in (C); **P*<0.05, ***P*<0.005, ****P*<0.001.

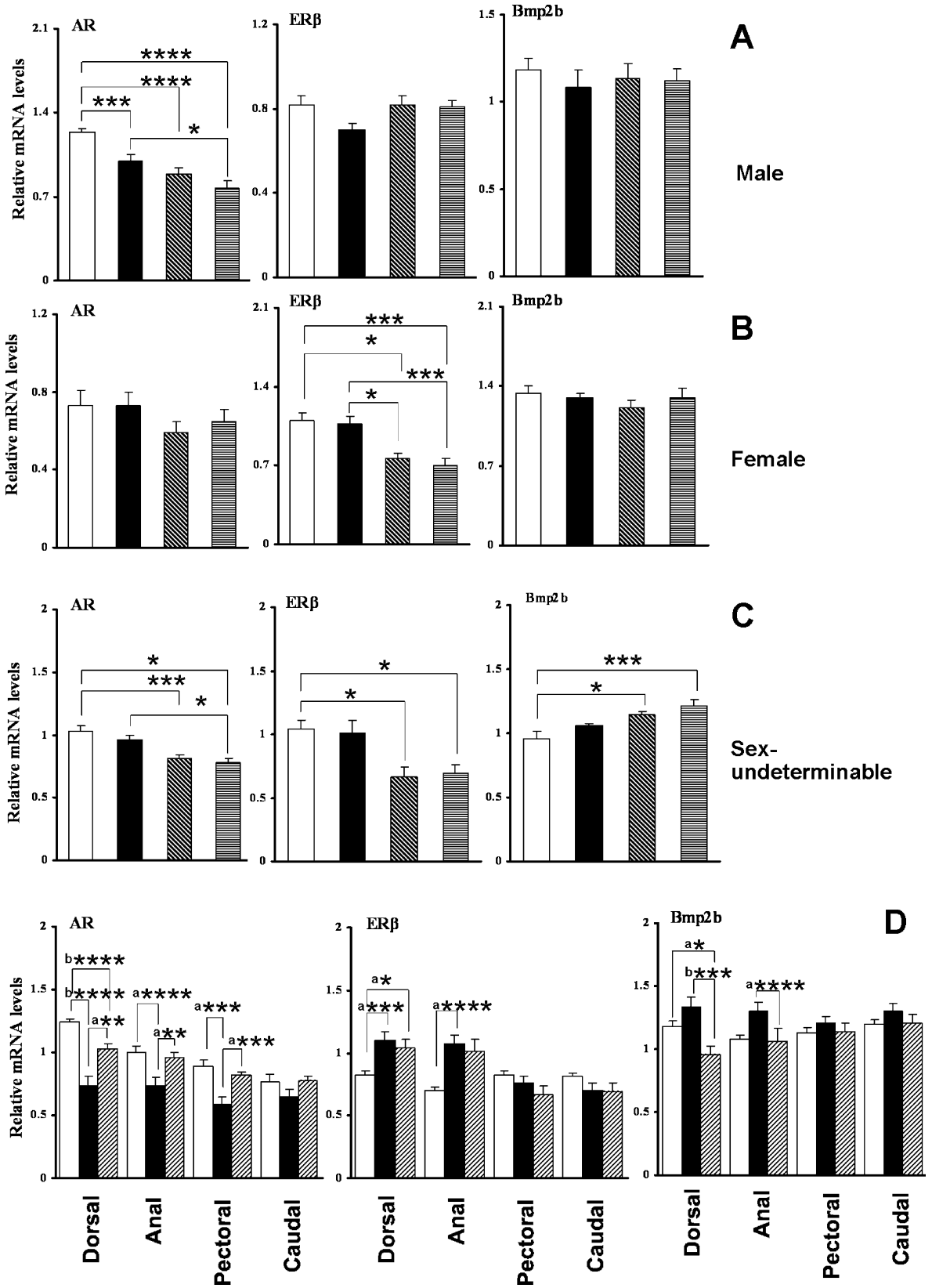

Fig. 2. **(A–C)** Expression levels of AR, ERβ, and Bmp2b mRNA in **(A)** male, **(B)** female, and **(C)** sex-undeterminable Thai medaka. Bars indicate the dorsal fin (□), anal fin (■), pectoral fin (▨), and caudal fin (▤). **(D)** Comparisons between males (□), females (■), and sex-undeterminable individuals (▨) in Thai medaka. The expression levels in each fin are values relative to the expression level of β-actin mRNA (mean±SE). One-way ANOVA followed by Tukey's multiple comparison test in (A–C), and by the [a]unpaired Student *t*-test and [b]Mann Whitney *U* test in (D); **P*<0.05, ***P*<0.005, ****P*<0.001.

in the Bmp2b mRNA expression level was found among the fins (Fig. 1B).

AR mRNA expression levels in all fins were significantly higher in males than in females (Fig. 1C). ERβ mRNA expression levels in the dorsal and anal fins were significantly higher in females than in males (Fig. 1C). Conversely, ERβ expression in the caudal fin was significantly higher in males than in females (Fig. 1C). No significant difference in Bmp2b mRNA expression was found between males and females for any of the fin types (Fig. 1C).

Levels of AR, ERβ, and Bmp2b mRNA expression in Thai medaka

In males, AR mRNA expression was significantly higher in the dorsal fin than in all other fins (Fig. 2A), and significantly higher in the anal fin than in the caudal fin. No significant differences were found in ERβ or Bmp2b mRNA expression levels among the fin types (Fig. 2A).

In females, the mRNA expression level of ERβ was significantly higher in the dorsal and anal fins than in the other fins (Fig.2B). No significant difference was found in AR or Bmp2b expression among the fin types (Fig. 2B).

In sex-undeterminable individuals, the AR mRNA expression level was significantly higher in the dorsal fin than in the pectoral and caudal fins (Fig. 2C), and significantly higher in the anal fin than in the caudal fin (Fig. 2C). The ERβ mRNA expression level was significantly higher in the dorsal fin than in the pectoral and caudal fins (Fig. 2C). Bmp2b expression was significantly lower in the dorsal fin than in the pectoral and caudal fins (Fig. 2C).

AR mRNA expression levels in the dorsal, anal, and pectoral fins were significantly higher in males than in females and sex-undeterminable individuals (Fig. 2D), and significantly higher in sex-undeterminable individuals than in females (Fig. 2D). The ERβ mRNA expression level in the dorsal fin was significantly lower in males than in females and sex-undeterminable individuals (Fig. 2D). ERβ expression in the anal fin was significantly lower in males than in females (Fig. 2D). Bmp2b expression showed no significant differences between males and females for any of the fin types (Fig. 2D). In contrast, Bmp2b expression in dorsal fins was significantly lower in sex-undeterminable individuals than in either sex (Fig. 2D), and expression in the anal fin was significantly lower in sex-undeterminable individuals than in females (Fig. 2D).

DISCUSSION

In Japanese male medaka, AR mRNA expression levels were higher in the dorsal fin and the anterior and posterior parts of the anal fin than in the pectoral and caudal fins. This result explains, from the viewpoint of molecular biology, why the dorsal and anal fins are longer in males than in females, and corresponds exactly to the report by Ogino et al. (2004), in which AR was highly expressed in the long gonopodium originating from the anal fin as a secondary sex character in male mosquitofish (*Gambusia holbrooki*). On the other hand, it is known that, in males, the expression level of ERβ is higher in the caudal fin than in other fins. Estrogenic control of the dorsal and anal fins is relatively weak compared with that of the caudal fin, since androgenic control of the dorsal and anal fins is intense.

In females, expression levels of ERβ were higher in the dorsal and anal fins than in the pectoral and caudal fins. This suggests that estrogen function has been feminized in the former two fins. Estrogen is known to suppress the development of the dorsal and anal fins (Iwamatsu, 1999; Melo and Ramsdell, 2001); however, the expression level of AR was similar to that observed in males, although the relative level was low. Androgen may be necessary to some extent for these fins to grow, or may be required for estrogen to act on them (Piferrer et al., 1993; Nakamura et al., 1998).

When the expression levels of steroid receptors were compared between males and females, AR mRNA expression levels in all fins were found to be higher in males than in females. This result suggests that the pectoral and caudal fins are targets for androgens, even though these fins have not so far been regarded as being deeply related to sex characters. The reason for high ERβ mRNA expression in the dorsal and anal fins in females was discussed above. No significant difference in the Bmp2b mRNA expression level in fins was detected between males and females. Therefore, Bmp2b expression levels may not be related to the adult secondary sex characters of fins.

In the Thai medaka, AR mRNA was highly expressed in the dorsal, anal, and pectoral fins in males. This result is consistent with that obtained in the Japanese medaka. In males, however, there was no difference in the expression level of ERβ in any of the fins. This suggests that estrogenic control in Thai medaka males is not as strong as in the Japanese medaka. In females, there was no difference in the AR expression level among all fins, which suggests that there are no androgenic effects. These two points are not congruent with the results obtained for the Japanese medaka. On the other hand, ERβ mRNA was highly expressed in the dorsal and anal fins. This suggests that the morphology of the small dorsal and anal fins is strongly affected by estrogen. Similarly to the Japanese medaka, in the Thai medaka no significant difference was detected in Bmp2b mRNA expression levels in the fins between males and females.

In sex-undeterminable individuals of Thai medaka, lengths of the dorsal and anal fins were intermediate between those in males and females. Nevertheless, the expression levels of AR appeared similar to that in males in each fin. However, the expression level of ERβ seemed similar to that in the female in each fin. Therefore, from the viewpoint of molecular biology, these expression patterns of AR and ERβ create fin morphologies that make it difficult to distinguish males from females. The expression levels of Bmp2b in dorsal and/or anal fins were lower in sex-undeterminable individuals than in normal males and females. This strongly suggests that these fins are in a state of arrested growth. Hayashi et al. (2007) reported that, in Japanese medaka, the level of ERα mRNA in anal fins increased after exposure to xenoestrogenic bisphenol A. In addition, Ngamniyom et al. (2007) found a low level of DDT in the sediment of ponds at some localities in the suburbs of Bangkok, Thailand. It has been reported that the administration of estrogen to Japanese medaka males can transform the phenotypes of the dorsal and anal fins and genital papillae from male to female (Iwamatsu, 1999; Melo and Ramsdell, 2001). Therefore, some chemicals may affect the function of estrogen,

which suppresses the growth of the dorsal fin via Bmp2b expression levels.

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