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Morphometry and Hormone Receptor Expressions in Dorsal and Anal Fins of Thai Medaka, *Oryzias minutillus*, between Breeding and Non-Breeding Seasons

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ABSTRACT

This study was conducted to examine the morphometrical changes in the dorsal and anal fins of adult Thai medaka (*Oryzias minutillus*) during the non-breeding and breeding seasons. The mRNA expression levels of hormone receptors were investigated in those fins between both seasons. The fin morphometry were measured by the values (%) of dorsal fin height (HD) divided by Standard Length (SL) and anal fin height (HA) divided with SL. The expression levels were analyzed by semi-quantitative RT-PCR. The HD/SL% and HA/SL values of males were higher in the breeding seasons than in the non-breeding seasons. In contrast, there was no difference in those values of females between two seasons. In males and females, the Androgen Receptor (AR) expression levels in dorsal fin and anterior part of anal fin were higher in the breeding season than in the non-breeding season. In males, no significant difference in Estrogen Receptor (ER) expression was found between two seasons for any fin type. In females, however, ER levels were higher in the breeding season than in the non-breeding season for dorsal fin and anterior part of anal fin. In males and females, AR and ER levels were not different between two seasons in the posterior part of anal fin. In dorsal fin and anterior part of the anal fin, there was sexual dimorphism in the AR and ER levels between two seasons. Present results suggest that sex steroid hormones regulate the season- (non-breeding or breeding) dependent characters of fin morphology with sex difference in Thai medaka.

Key words: Morphometrical changes androgen receptor (AR), estrogen receptor (ER), breeding season, Thai medaka fins

INTRODUCTION

Fish of the genus *Oryzias* are used as model organisms for experiments in various fields such as, the study of developmental biology (Carlson *et al*., 2002) and endocrinology (Paul-Prasanth *et al*., 2011). In this genus, the dorsal and anal fins are typical secondary sex characters. The dorsal and anal fins of males are usually longer than those of females (Porazinski *et al*., 2010). The male anal fin has an important role in stimulating the female for a successful fertilization, even though this fin is not a copulatory organ (Jenster *et al*., 1995; Koseki *et al*., 2000).

In vertebrate males androgen plays a crucial role in the development and maintenance of the male phenotype (Brinkmann *et al*., 1999; Marjani *et al*., 2009). This hormone acts on target cells through androgen receptors (Jenster *et al*., 1995). Estrogen functions by mediating estrogen receptor to play an important role in maturation of the reproductive organs in females (Nilsson *et al*., 2001).

Seasonal changes from the non-breeding period to breeding period can influence the levels of sex steroid hormones responsible for reproductive success in many teleost fish. Examples include three-spined stickleback (*Gasterosteus aculeatus*), rainbow trout (*Oncorhynchus mykiss*), plainfin midshipman (*Porichthys notatus*) and flying fish (*Chalcalburnus tarichi*) (Mayer *et al*., 1990; Hou *et al*., 2001; Sisneros *et al*., 2004; Unal *et al*., 2005).

The Thai medaka (*Oryzias minutillus*) is the smallest species in the genus *Oryzias* which is widely distributed in Southeast Asia. The habitats of this species are shallow ponds and paddy fields (Magtoon *et al*., 1992). In this egg-laying fish, the sex can be determined by the secondary sex characteristics of their fins. The dorsal and anal fins of male Thai medaka are larger than those of females (Ngamniyom *et al*., 2007). However, in Thai medaka, the mechanisms of sex steroid hormone regulation of the sex-dependent characters of fin morphologies remain to be elucidated.

In this study, the morphological features of the dorsal and anal fins were determined for Thai medaka. The mRNA expression levels of the Androgen Receptor (AR) and Estrogen Receptor (ER) were examined to determine the expression patterns in the fins of Thai medaka in the natural non-breeding and breeding seasons.

MATERIALS AND METHODS

Fish: Adult Thai medakas were collected from ponds and ditches in the suburbs of Bangkok, Thailand, from May to October 2009. Their standard length was 13-14 mm. The 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. To determine the sex ratio (male to female) of Thai medaka, captured individuals were fixed in 70% ethanol (Table 1). Individuals with uncertain sex were labelled as sex-undeterminable on the basis of their fin morphology. In this study, typical males and females were captured from localities 1, 3 and 4, where the sex ratio was approximately 1:1 (Table 1).

Morphometrical evaluation of fins: Adult Thai medaka were captured in natural habitats from April to May 2010. This period was the breeding season for Thai medaka. In the condition of non-breeding season, fish were collected from October to November 2010 (Ngamniyom *et al*., 2009).

The lengths of the dorsal and anal fin were measured using a digital caliper. The measurement of the fins was conducted according to the method of Khan *et al*. (2002) and Ngamniyom *et al*. (2007).

Table 1: Sex ratios of males to females and percentages of sex-undeterminable individuals relative to the population in Thai medaka

	Number of specimens			Sex ratio	
Local	Male	Female	Sex-undeter	Male: Female	Percentage of sex-undeter
1	22	23	-	1.0 : 1.0	-
2	12	28	14	1.0 : 2.3	26
3	39	33	-	1.2 : 1.0	-
4	15	13	-	1.2 : 1.0	-
5	16	22	18	1.0 : 1.4	32

Semi-quantitative RT-PCR analysis of fins: Males and females were kept in separate aquariums with a controlled 12:12 h light/dark photoperiod cycle at 26±1°C for 1 week and were fed *ad libitum* with TetraMin (Tokyo, Japan).

In the anal fins, the anterior part which corresponds to the 12th to the 18th fin ray counted from the posterior end, was separated from the posterior part (Kamsri *et al.*, 2010). The whole dorsal fin, the anterior part of the anal fin and the posterior part of the anal fin were dissected out from 70 males and 70 females. Seven fins were pooled in each tube and 10 tubes were collected. Total RNA from each sample was extracted using the Isogen reagent and treated with *DNase*1 for 30 min at 37°C. Total RNA (100 ng) was reverse-transcribed with AMV reverse transcriptase XL according to the method of Ngamniyom and Sasayama (2011). The cDNA solution of 0.5 μL was used as the template for PCR.

Primers were designed based on previous data for Japanese medaka and were used in this analysis of Thai medaka. The primers used to amplify the Androgen Receptor (AR) were 5'- CTCCTCACCAGCCTTAACGA-3' and 5'- AGACCATCACTCCCACCCAA-3', as previously reported by Inui *et al.* (2003). The primers for the amplification of ER nucleotides were 5'-ACTCCCCTTTACAGCCAGTCC-3' and 5'-TGGACCAGCTCCTTGTCTGCC -3' (Lee *et al.*, 2002). β-actin mRNA was amplified in each RT reaction as a loading control and reference. The primers used for the amplification of β-actin were 5'-AGGGAGAAGATGACC-3' and 5'-CGCAGGACGCCATACCAA-3' according to the report of Scholz *et al.* (2004).

The amplification of cDNA was conducted using the following cycling conditions: 95°C for 30 sec for the denaturation step; 62°C (AR), 64°C (ER) or 58°C (β-actin) for 1 min for the annealing step; and 72°C for 1 min for the extension step. Thirty cycles were used for the amplification of AR and ER and 20 cycles were used for β-actin. The PCR products were electrophoresed on a 2% agarose gel immersed in ethidium bromide and visualized on a UV-transilluminator. The amplification level was quantitated using Scion Image software for Windows (Scion, Maryland, USA). The amplification levels of AR and ER in each fin were divided by the amplification level of β-actin. Therefore, the stated expression levels are relative to the level of β-actin.

Statistical analysis: An unpaired Student's t-test was used to determine whether differences were statistically significant ($p<0.05$ and $p<0.005$). The data were analyzed using the Statistical Package for the Social Sciences (SPSS) for Windows version 13 (SPSS, Chicago, USA).

RESULTS

Fin morphometry of Thai medaka: In the non-breeding season, the values of HD/SL and HA/SL% for males were 19.7±0.4 (Mean±SE) and 22.4±0.3, respectively (Table 2). The values of HD/SL and HA/SL% for females were 14.0±0.4 and 16.1±0.2, respectively. In the breeding season,

Table 2: HD/SL% and HA/SL% of Thai medaka between non-breeding and breeding season

Sex	Non-breeding season	Breeding season
Male		
HD/SL%	19.2±0.4	20.8±0.4
HA/SL%	22.1±0.3	23.7±0.2
Female		
HD/SL%	14.0±0.4	14.3±0.3
HA/SL%	16.1±0.2	15.8±0.5

Significantly different from the values of Thai medaka between non-breeding and breeding season. Using unpaired Student's t-test ($p<0.05$). Values are Mean±SE. Each experiment group contained fourty Thai medaka

the HD/SL and HA/SL% values for males were 20.1±0.5 and 23.6±0.2, respectively. In females, the HD/SL and HA/SL% values were 14.3±0.3 and 15.8±0.4, respectively.

In males, the values of HD/SL% in the breeding season were significantly higher than the values in the non-breeding season (unpaired Student's t-test, $p<0.05$). Similarly to the HA/SL%, the values of HA/SL% were significantly higher in the breeding season than in the non-breeding season for male (unpaired Student t-test, $p<0.05$). In females, however, the values of HD/SL and HA/SL% were not different between the two seasons.

Semi-quantitative RT-PCR

AR and ER expression levels in male Thai medaka between the non-breeding and breeding seasons: In males, the AR mRNA expression levels in the dorsal fin and the anterior part of the anal fin were significantly higher in the breeding season than in the non-breeding season (unpaired Student's t-test, $p<0.05$) (Fig. 1a). No significant difference in the AR mRNA

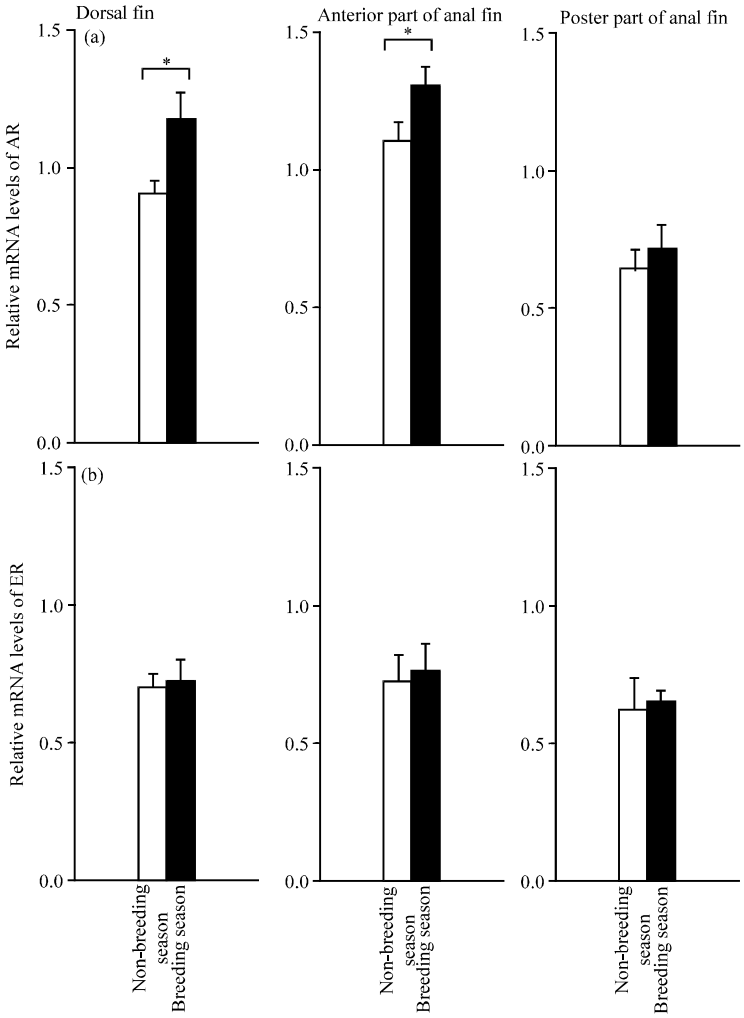

Fig. 1(a-b): Expression mRNA levels of AR (a), ER (b) in male Thai medaka between non-breeding and breeding season. Expression levels in each fish individual are relative values compared to the expression levels of β-actin mRNA (Mean±SE). *Show $p<0.05$ with unpaired Student's t-test. Each experimental group consisted of ten samples of fish fin

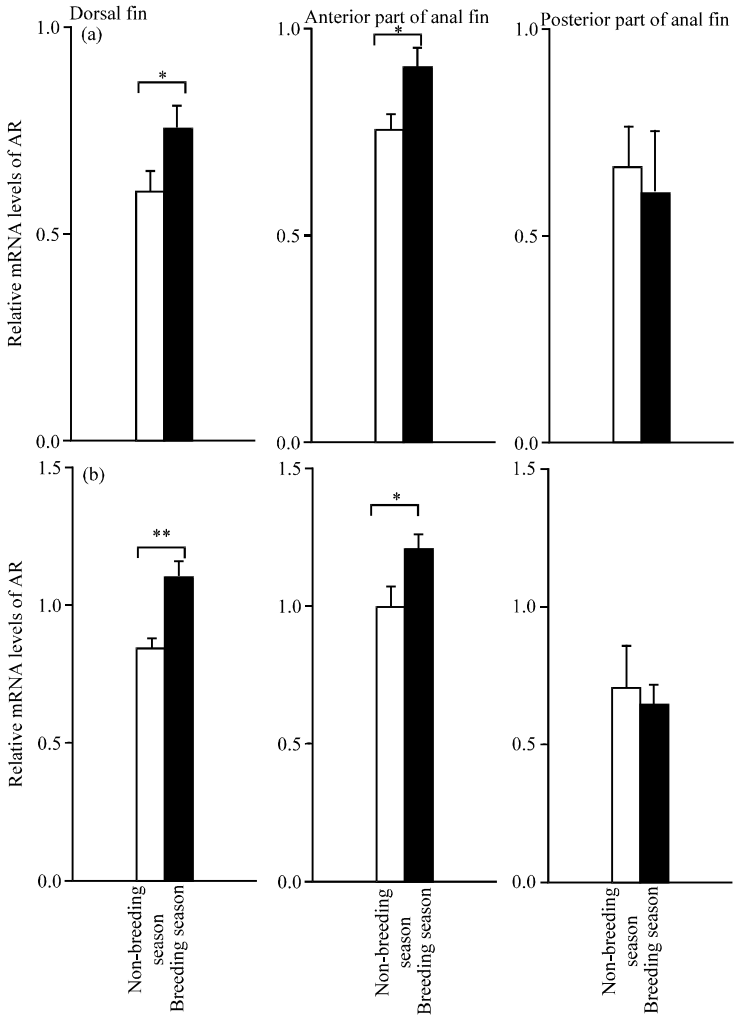

Fig. 2(a-b): Expression mRNA levels of AR (a), ER (B) in female Thai medaka between non-breeding and breeding season. Expression levels in each fish individual are relative values compared to the expression levels of β-actin mRNA (Mean±SE). *, **Show $p<0.05$ and $p<0.005$, respectively with unpaired Student's t-test. Each experimental group consisted of ten samples of fish fin

expression levels was found between the non-breeding and breeding seasons for posterior part of the anal fin. The expression levels of ER mRNA in the dorsal fin, the anterior part of the anal fin and the posterior part of the anal fin were not different between the non-breeding and breeding seasons (Fig. 1b).

AR and ER expression levels in female Thai medaka between the non-breeding and breeding seasons: In females, the expression levels of ER mRNA in the dorsal fin and the anterior part of anal fin were significantly higher in the breeding season than in the non-breeding season (unpaired Student's t-test, $p<0.05$) (Fig. 2a). In contrast, no significant difference in the expression level of AR mRNA was found in the posterior parts of the anal fin between the non-breeding and breeding seasons.

The expression levels of ER mRNA in the dorsal fin and the anterior part of the anal fin were significantly higher in the breeding season than in the non-breeding season (unpaired Student's t-test, $p<0.005$ and $p<0.05$, respectively) (Fig. 2b). No significant difference in the ER mRNA expression levels was found in the posterior part of the anal fins between the two seasons.

AR and ERβ mRNA expression levels of Thai medaka in the non-breeding seasons: In the non-breeding season, the AR mRNA expression levels in the dorsal fin and the anterior part of the anal fin were significantly higher in males than in females (unpaired Student's t-test, $p<0.005$) (Fig. 3a). The expression levels of ER mRNA in the dorsal fin and the anterior part of the anal fin were significantly lower in males than in females (unpaired Student's t-test, $p<0.05$). In contrast, no significant difference in the AR and ER mRNA expression levels was found in the posterior part of the anal fin for males and females (Fig. 3b).

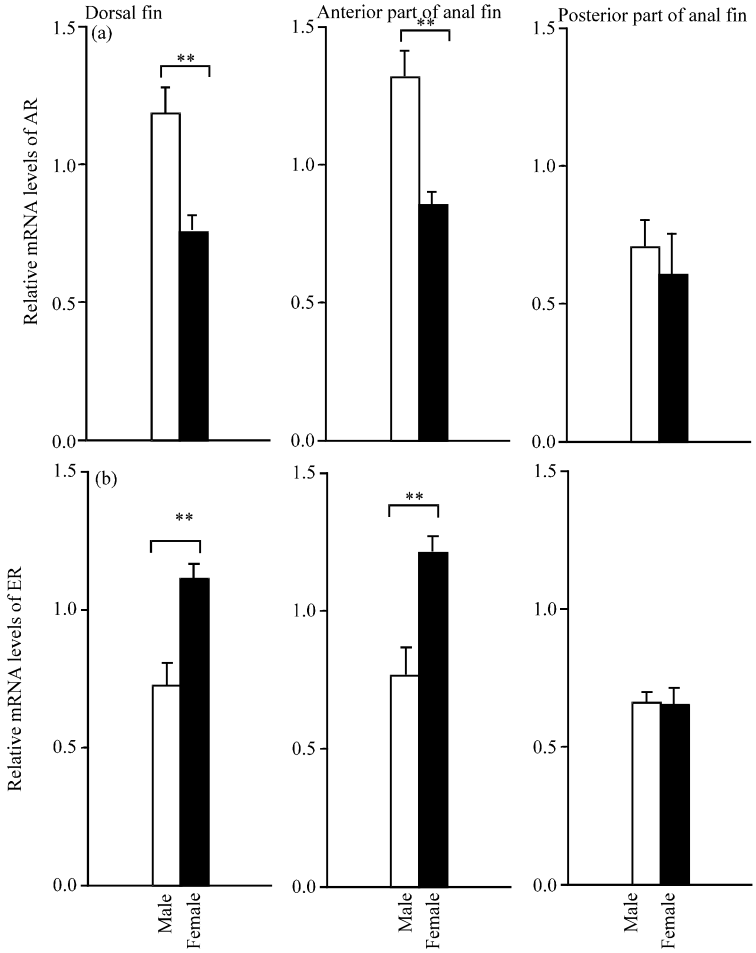

Fig. 3(a-b): Comparison mRNA expression levels of AR (a), ER (b) between male and female Thai medaka in non-breeding season. Expression levels in each fish individual are relative values compared to the expression levels of β-actin mRNA (Mean±SE). *, **Show $p<0.05$ and $p<0.005$, respectively with unpaired Student's t-test. Each experimental group consisted of ten samples of fish fin

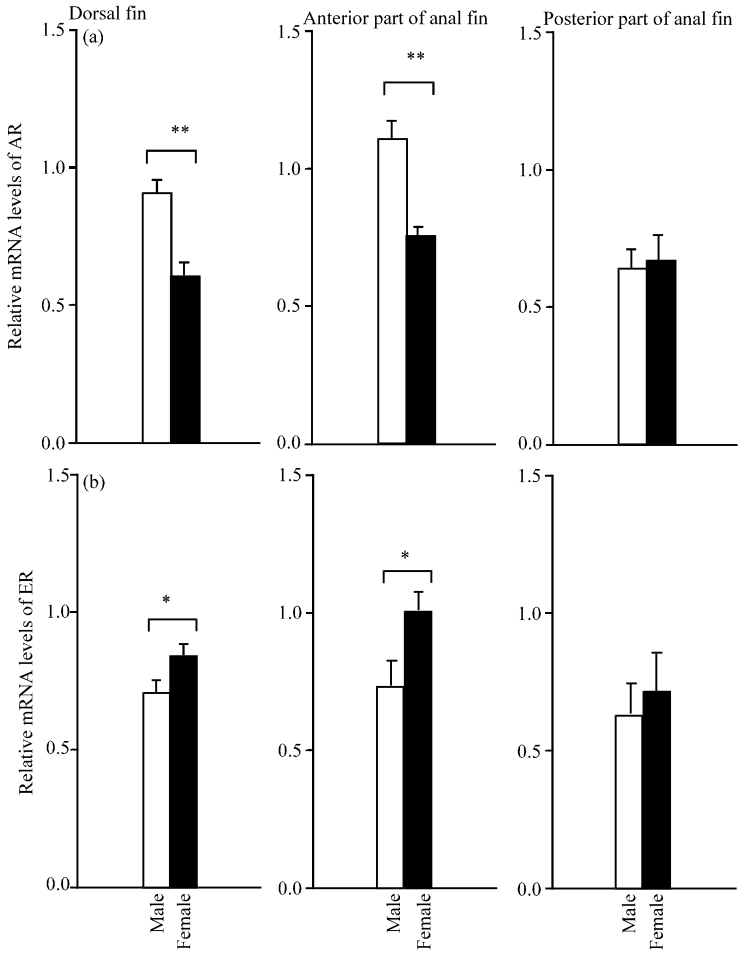

Fig. 4(a-b): Comparison mRNA expression levels of AR (a), ER (b) between male and female Thai medaka in breeding season. Expression levels in each fish individual are relative values compared to the expression levels of β-actin mRNA (Mean±SE). *, **Show $p<0.05$ and $p<0.005$, respectively with unpaired Student's t-test. Each experimental group consisted of ten samples of fish fin

AR and ER expression levels of Thai medaka in the breeding seasons: In the breeding season, the AR mRNA expression levels in the dorsal fin and the anterior part of the anal fin were significantly higher in males than in females (unpaired Student's t-test, $p<0.005$) (Fig. 4a). In contrast, no significant difference in AR mRNA expression levels was found in the posterior part of the anal fin between males and females. The expression levels of ER mRNA in the dorsal fin and the anterior part of the anal fin were significantly lower in males than in females (unpaired Student's t-test, $p<0.005$). Conversely, the expression levels of ER mRNA were not different between males and females (Fig. 4b).

DISCUSSION

In the dorsal fin and the anterior part of the anal fin in males, AR was highly expressed and analysis of the fin morphology revealed an increase in length from the non-breeding to breeding season. According to the reports of Bagra *et al.* (2009), the tubercles of assamese kingfish

(*Semiplotus semiplotus*) were dominant in anal fins of males during the breeding period. In Japanese medaka, the papillar processes of males are well developed on anal fins during the breeding period related as the result of androgenic control (Iwamatsu *et al*., 2003). Moreover, the male sex hormones were found at the highest levels during the annual breeding seasons for male rainbow trout (*Oncorhynchus mykiss*) (Hou *et al*., 2001) and male Japanese dace (*Tribolodon hakonensis*) (Ma *et al*., 2005). These results suggest that an androgen level in during the breeding season may increase to induce the development of the secondary sex characters of adult male fins to promote successful fertilization.

The estrogenic levels of dorsal fin and the anterior part of anal fin were unchanged between the two seasons in males of adult Thai medaka. Ngamniyom *et al*. (2011) reported that ER levels were not respond to an estrogenic action in any fin types of adult male Japanese medaka. Therefore, an estrogenic control may not act on the dorsal fin and anterior part of anal fin in the breeding season to promote fin growth in adult male.

In females, the ER expression levels of the dorsal and the anterior part of the anal fin were higher in breeding season than in non-breeding. This result suggests that estrogen functions to feminize these two fins to maintain the female phenotype and promote breeding success. The AR levels of the dorsal fin and the anterior part of the anal fin were detected in the non-breeding season and were high in the breeding season. It is known that, in female fish androgen is a major precursor molecule in estrogen biosynthesis which is catalyzed by an aromatase enzyme (Melo and Ramsdell, 2001; Ngamniyom and Panyarachun, 2011). Androgen may be required for estrogen to act on these fins in both seasons.

When the anal fin of Thai medaka was separated into the anterior and posterior part, the anterior part significantly exhibited sexual dimorphism in the AR and ER levels for both seasons. It is similar to the report by Ngamniyom *et al*. (2009) that the high expression levels of AR and ER were related to the secondary sex characters of fins in medaka fish. These results thus suggest that androgen and estrogen regulated the fins morphology depending on the secondary sex characters and the season (non-breeding or breeding) in adult Thai medaka.

Covertly, in adult Thai medaka, the posterior part did not assume any sex difference in AR and ER expression levels. It is known that in Thai medaka, the posterior parts of anal fins did not show secondary sex characters between adult male and female (Parenti, 2008). It is thus clear that the androgen or estrogens are not necessary for mating success with those fins, since they do not exhibit secondary sex characters.

It may be worth noting that the season (non-breeding or breeding) influences the expression levels of steroid hormone receptors with respect to their sexual difference. The present study is the first study of teleost fish to examine the expression levels of sex steroid hormone receptors depending on environmental factors and sex-dependent characters of fin morphology across three fin tissues. However, the understanding of molecular-biological mechanisms remains to be elucidated.

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