

EXPRESSION LEVELS OF SEX HORMONE RECEPTORS IN BRAINS OF JAPANESE MEDAKA, *ORYZIAS LATIPES* (ACTINOPTERYGII: BELONIFORMES: ADRIANICHTHYIDAE)

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Background. In teleost fishes, the brain is the target organ for sex steroid hormones. The actions of sex steroid hormones are mediated by their receptors and play an important role in the regulation of endocrine function in the brain. Japanese medaka, *Oryzias latipes*, is a species widely used in many fields of experimental biology, including neurobiology. In this study, we examined the mRNA expression levels of androgen and estrogen receptors in medaka brains.

Materials and methods. The brains of adult fish were separated into three parts (forebrain, midbrain and hindbrain). The expression levels of androgen receptor (*AR*) and estrogen receptor (*ER*) β from each part of the brain were determined using a semi-quantitative RT-PCR analysis.

Results. *AR* and *ER* β levels in males were higher in the forebrain and midbrain than in the hindbrain. In females, *AR* and *ER* β levels were higher in the forebrain than in the midbrain and hindbrain. *AR* levels in the forebrain and midbrain of males were higher than those of females. Conversely, there was no difference in *ER* β level between males and females.

Conclusion. These data on hormone receptors provide the foundation for understanding the molecular basis of *AR* and *ER* β mRNA expression levels in medaka brains. In addition, our results suggest that, in Japanese medaka, *AR*, but not *ER* β , expression may exhibit sexual dimorphisms between males and females in the forebrains and midbrains.

Keywords: androgen receptor, estrogen receptor β , brain, Japanese medaka, *Oryzias latipes*

INTRODUCTION

In many teleosts, sex steroid hormones play a crucial role in the physiological processes of many tissues, such as liver, gonad, kidney and bone (Jakobsson et al. 1996, Socorro et al. 2000, Amer et al. 2001, Riley et al. 2002, Ngamniyom et al. 2009).

It is known that, the brain is also the target tissue for the actions of sex steroid hormones, suggesting that these hormones are important in modulating differences in brain functions (Bernadi and Genazzani 1999).

Weltzien et al. (2004) and Munakata and Kobayashi (2010) have reported that sex steroid hormones, androgen and estrogen, play an important role in the sex behaviours and the action of gonadotropin secretion in the central nervous system of teleost fish. Androgen signals their target cells via androgen receptors (*ARs*) as the sex steroid controlling male sexual differentiation (Jenster et al. 1995, Oliveira et al. 2002). Paralleling androgen function, estrogen functions

in females via estrogen receptors (*ERs*) (Nilsson et al. 2001). However, three isoforms in *ERs*, designated as *ER* α , *ER* β , and *ER* γ , have also been reported in vertebrates (Chang et al. 1999, Sabo-Attwood et al. 2004). Especially, in teleosts, *ER* β is abundantly expressed in several organs, such as the liver, gonads and brain (Menuet et al. 2002, Hawkins and Thomas 2004). This fact suggests that, among *ERs*, *ER* β is the main *ER* in fish.

The Japanese medaka (also known as the Japanese rice fish), *Oryzias latipes*, is a model organism widely utilized for experiments in various fields such as neurobiology, developmental biology and endocrinology (Ishikawa 1997, Zhang et al. 2008, Mezhoud et al. 2009). Attributes of the medaka that make it an advantageous as an experimental animal are its small size, it is easy to keep, and its sexual dimorphism is external (Parenti 2008). Therefore, we believed that elucidation of *AR* and *ER* β expression in medaka brains might contribute to increasing our knowl-

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edge of the regulation of sex steroid hormones in teleost.

In this study, we provided the molecular-biological background of the forebrain, midbrain and hindbrain of male and female adult Japanese medaka by examining mRNA expression levels of the androgen receptor (*AR*) and estrogen receptor (*ER*) β .

MATERIAL AND METHODS

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 : 10 h light/dark photoperiod cycle at $26 \pm 1^\circ\text{C}$ for 2 weeks, 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). This experiment was conducted from August 2009 through the end of February 2010.

Adult males and females were anesthetized with $200 \text{ mg} \cdot \text{L}^{-1}$ of an ethyl-3-aminobenzoate methanesulphonate (MS-222) solution (Sigma, St. Louis, MO) and placed in a Petri dish. Brain fish was precisely separated to three parts, according to the criteria of Ishikawa et al. (1999). The part of telencephalon to the part of diencephalon was distinguished from midbrain as the forebrain. The part of nervus opticus until the posterior end of tectum opticum was indentified such the midbrain. The hindbrain was composed of the metencephalon and myelencephalon. The forebrains, midbrains, and hindbrains were dissected out from 10 male and 10 female fish (Fig. 1). Each part of brain was contained into each tube, and a sample of 10 tubes was collected.

Total RNA from each sample of medaka brains was extracted by using RNeasy Fibrous Tissue Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol and treated with *DNase*1 (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 for amplification of *AR* (5' CAGGAG--GAGTTC--CTGTGCAT-3' and 5'-GGTGGTGGTAAG-GTGAAG--GA-3') were designed from a sequence (accession number AB076399). *ER* β primers were 5'-CTGTTAGATGC--CTCGGACCTT-3' and 5'-GATTG-GCTGGTTTCGTG-3' (Inui et al., 2003). As a loading control and reference, *β -actin* mRNA was amplified for each RT reaction; primers used were 5'-AGGGAGAA-GATGACC-3' and 5'-CGCAGGACGCCATACCAA-3' (Scholz et al. 2004). PCR conditions for the amplification of cDNA were 95°C for 30 s for denaturation; 62°C (*AR*), 64°C (*ER* β), or 58°C (*β -actin*) for 45 s for annealing; and 72°C for 1 min for extension. The RT-PCRs of linear phases were determined on 20–32 cycles for *β -actin* and 32–44 cycles for hormone receptors to allow semi-quantitative comparisons of cDNAs which were developed for the optimum reactions (Figs. 2–4). Therefore, cycle numbers for *AR* and *ER* β were 36 cycles, and for *β -actin* were 26 cycles. PCR products were electrophoresed on 2% agarose gel, stained with ethidium bromide, and visualized on a UVtransilluminator (Figs. 2–4). Amplification levels were quantified by using Scion Image software for Windows (Scion, Maryland, USA). Amplification levels of *AR* and *ER* β for each brain type were divided by the corresponding amplification level of *β -actin* to obtain relative expression levels (Fig 5).

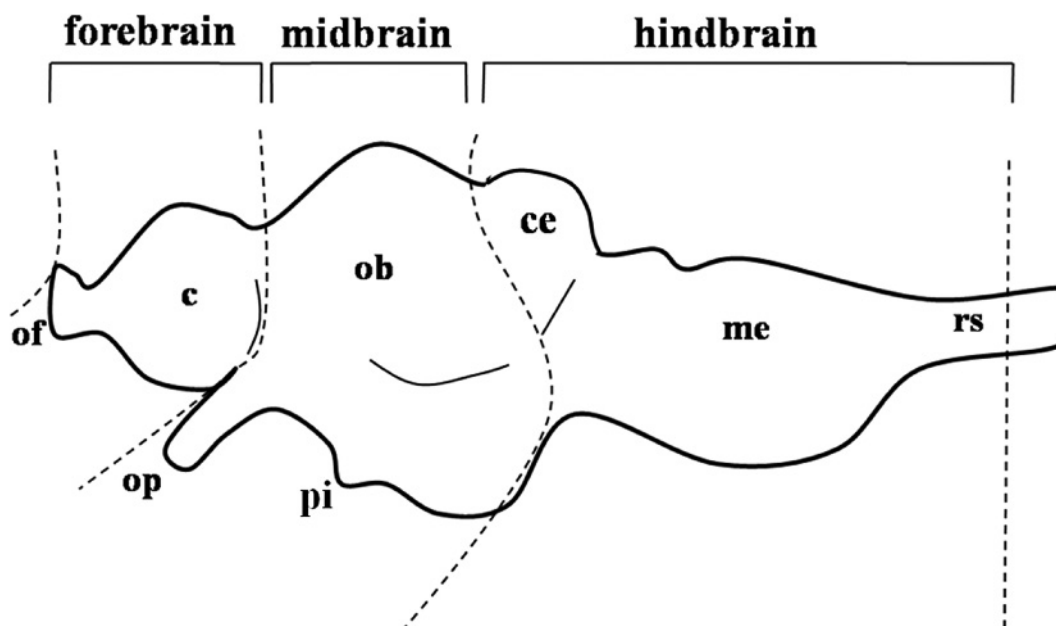
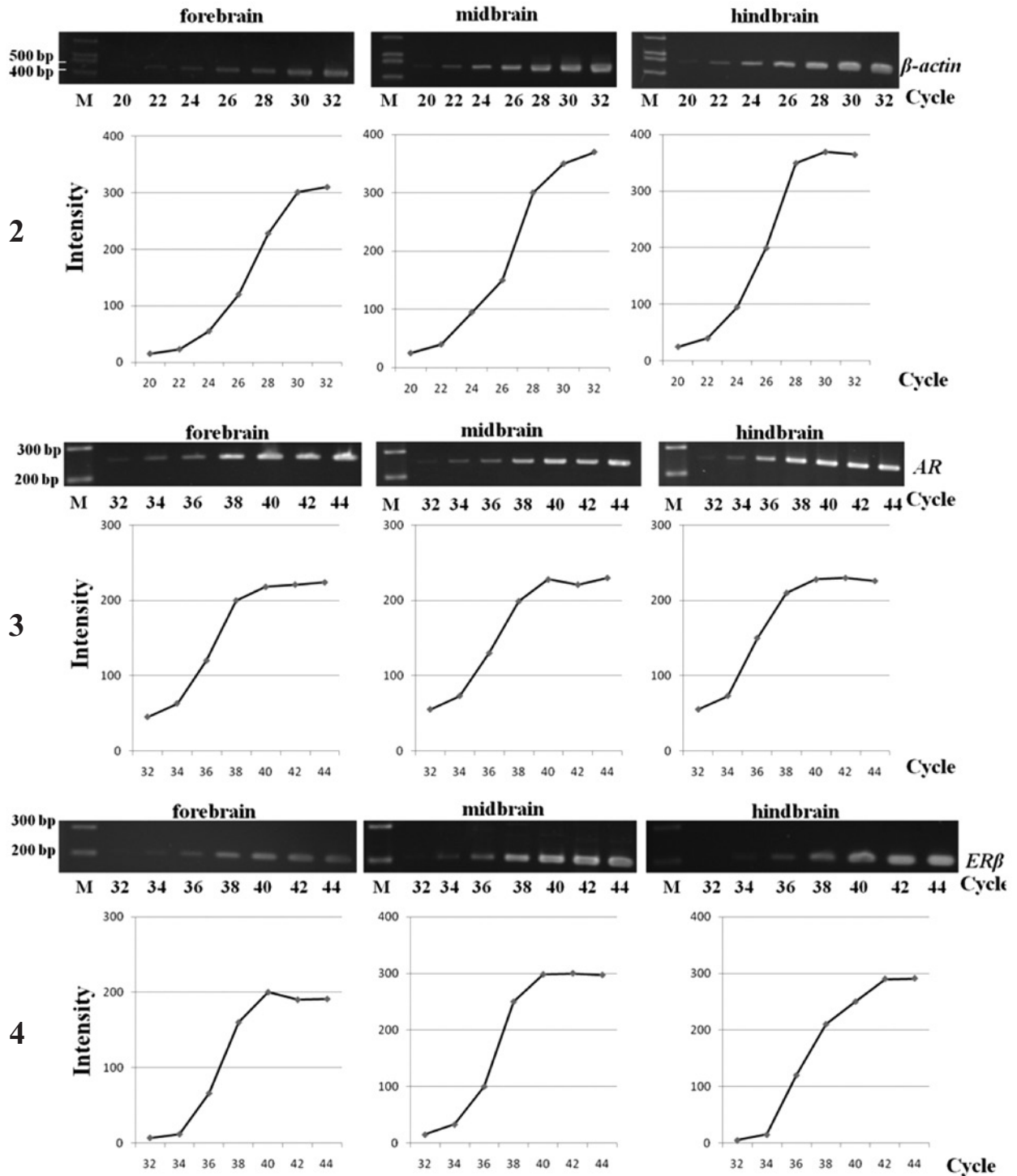


Fig. 1. Lateral view of diagrammatic illustration of brain of Japanese medaka, *Oryzias latipes*; The whole brain was separated to the forebrain, midbrain and hindbrain; Abbreviations: of, olfactory bulb; c, cerebrum; op, optic nerve; ob, optic lobe; pi, pituitary; ce, cerebellum; me, medulla; rs, rostral spinal cord

One-way ANOVA with Tukey's multiple comparison test and the unpaired Student's *t*-test were used to examine differences statistically. Data were analyzed by using the statistical package for the social sciences (SPSS) for Windows, version 14 (SPSS, Chicago, USA).

This experiment design was approved by the ethics committee of the Kanazawa University, Institute of Nature and Environmental Technology, Japan, in accordance with the recommendations of The Littlewood Declaration (Lane-Petter 1976).



Figs. 2–4. Gel electrophoresis of RT-PCR analysis and graphs of linear phase in various cycles of (Fig. 2) β -actin, (Fig. 3) AR and (Fig. 4) ER β mRNA expression in Japanese medaka, *Oryzias latipes*; The optimal numbers of cycle condition were identified at a slope of linear graph; Cycle numbers for β -actin were 26 cycles, and for AR and ER β were 36 cycles; M, molecular marker

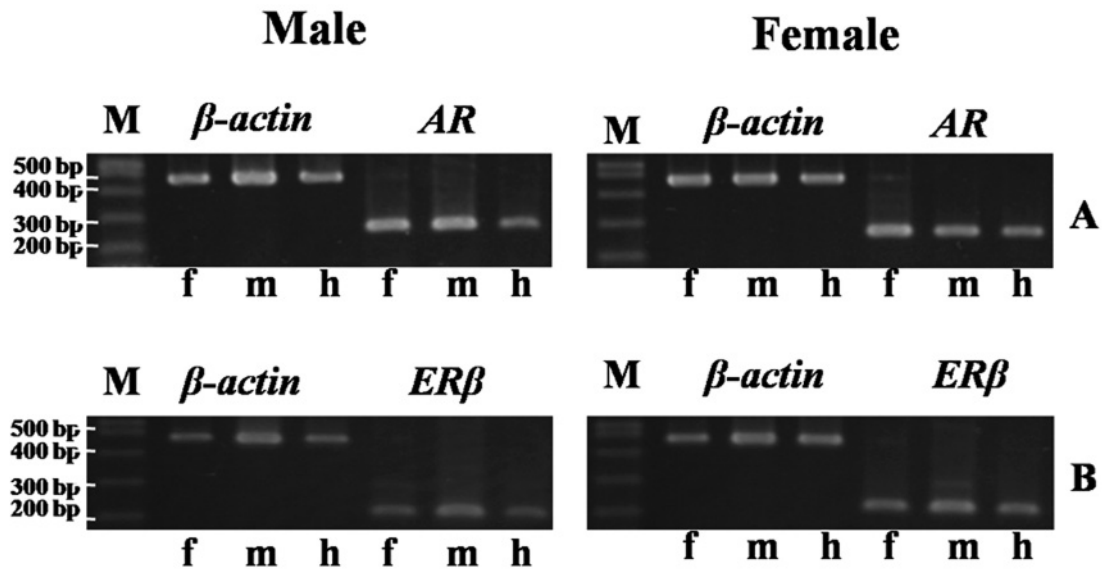


Fig. 5. Gel electrophoresis of semi-quantitative RT-PCR analysis of β -actin, *AR* and *ER β* mRNA expression in male and female medaka. M, molecular marker; f, forebrain; m, midbrain; h, hindbrain

RESULTS

Levels of *AR* mRNA expression in the medaka brain. In males, the levels of *AR* mRNA expression were significantly higher in the forebrain and midbrain than in the hindbrain (Fig. 6). In females, levels of *AR* mRNA expression were significantly higher in the forebrain than in the midbrain and hindbrain (Fig. 7). The expression levels of *AR* mRNA in the forebrain and midbrain were significantly higher in males than in females (Fig. 8). In contrast, no significant difference in *AR* mRNA expression level was found in the hindbrain between males and females (Fig. 8).

Levels of *ER β* mRNA expression in the medaka brain. In males, the levels of *ER β* mRNA expression were significantly higher in the forebrain and midbrain than in the hindbrain (Fig. 9). In females, levels of *ER β* mRNA expression were also significantly higher in the forebrain than in the midbrain and hindbrain (Fig. 10). No significant difference in *ER β* mRNA expression level was found between males and females for any of the brain regions (Fig. 11).

DISCUSSION

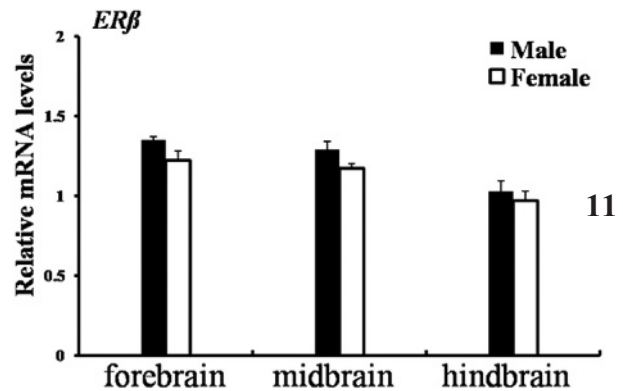
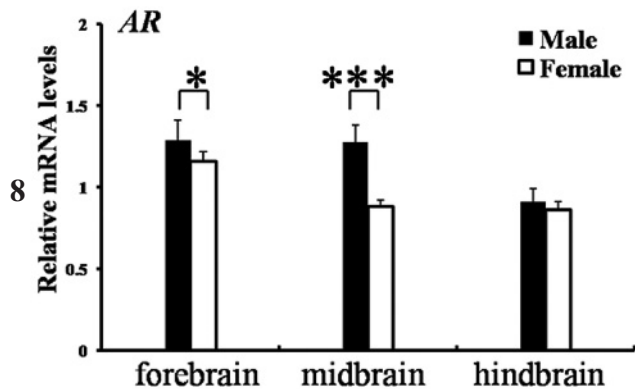
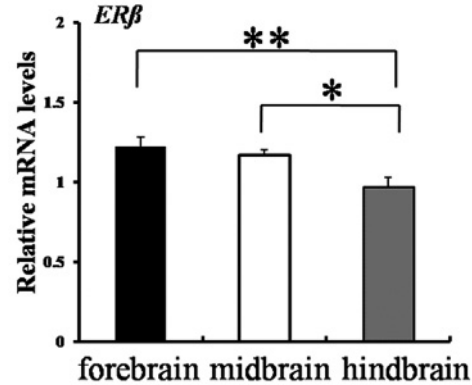
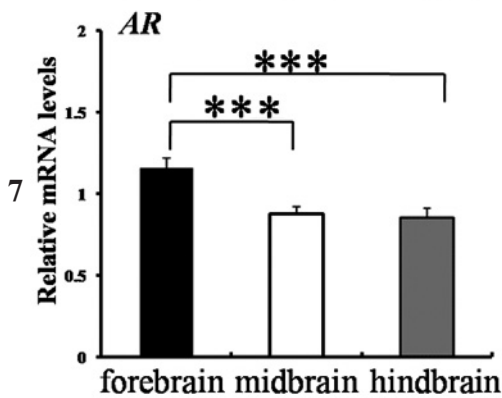
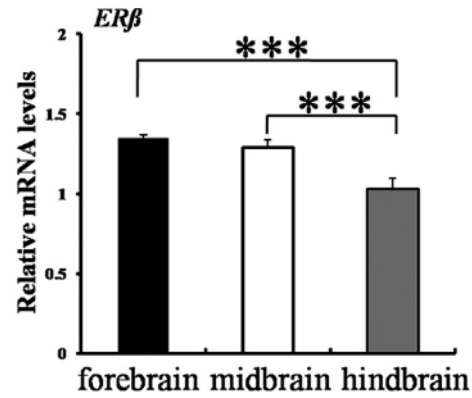
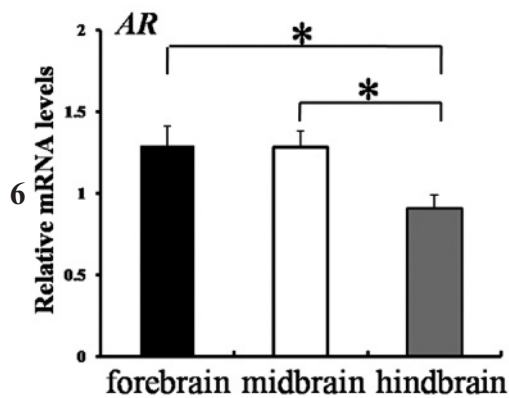
In both sexes of medaka, *AR* mRNA expression levels were higher in the forebrain and midbrain than in the hindbrain. This finding is consistent with the report of Forlano et al. (2010) that *AR* mRNA expressions was higher in the forebrain and midbrain region than in the hindbrain region of midshipman fish, *Porichthys notatus*. It is known that, in teleost fish, *AR* expression is high in the anterior and mid-regions of the brain, including the pituitary region, suggesting that androgen may act on those target regions to control the sexual behaviour of males (Burmeister et al. 2007). Thus, the strong expression of *AR* mRNA in the forebrain and midbrain may support the role of androgen as a regulator of physiological

behaviour of male fish. However, it is too soon to hypothesise that the brain contains the specific target tissues of androgen since *AR* expression *in situ* has not been elucidated in the medaka brain.

It is well known that androgen exerts its action in the brain directly via androgen receptors or via estrogen receptors after aromatisation to estrogen (Pasmanik and Callard 1988). In female fish, estrogen synthesis from androgen may be required to activate estrogen receptors. When the expression levels of sex steroid hormones were compared between males and females, *AR* mRNA in the forebrain and midbrain was found to be more abundant in males than females. This result suggests that *AR* mRNA expression level in the forebrain and midbrain may be a sexual dimorphism between male and female fish.

Similarly, the *ER β* mRNA levels in males and females were higher in the forebrain and the midbrain than in the hindbrain. This result is consistent with the reports of Anglade et al. (1994) and Muriach et al. (2008) that *ER* expression was observed in the forebrain and midbrain areas of rainbow trout, *Oncorhynchus mykiss*, and European sea bass, *Dicentrarchus labrax*. Our results suggests that, in males, the forebrain and midbrain are also targets for estrogen, even though these parts of brain have thus far not been regarded as being deeply related to brain functions of male fish. In females, the expression levels of *ER β* were higher in the forebrain and midbrain than in the hindbrain. This confirms that estrogen functions on these brain regions for the physiological processes of feminising in female teleosts.

This finding is consistent with the reports of Anglade et al. (1994) and Trudeau et al. (2005) that the *ER β* levels were not different in the brains of male and female rainbow trout, *Oncorhynchus mykiss*, or goldfish, *Carassius auratus*. Therefore, *ER β* mRNA expression level may not exhibit any sexual difference in the brain regions between



Figs. 6–11. Expression levels of *AR* mRNA (Figs. 6–7) and *ERβ* mRNA (Figs. 9–11) in the forebrain, midbrain and hindbrain of Japanese medaka, *Oryzias latipes*; **Fig. 6.** *AR* mRNA in male; **Fig. 7.** *AR* mRNA in female; **Fig. 8.** Comparison between *AR* mRNA in male and female; **Fig. 9.** *ERβ* mRNA in male; **Fig. 10.** *ERβ* mRNA in female; **Fig. 11.** Comparison between *ERβ* mRNA in male and female; The expression levels in each part of brains are values relative to the expression level of β -actin mRNA (mean \pm SE); One-way ANOVA followed by Tukey's multiple comparison test in Figs. 6, 7, 9 and 11 and by the unpaired Student's *t*-test in Figs. 8 and 11; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$

male and female fish. Furthermore, it may be worthwhile to note that estrogenic control in the forebrain and midbrain of males is relatively similar to that of females because androgenic control of the forebrains and midbrains is abundant.

In many teleost fish, it is known that levels of sex hormone receptors between males and females are different in the gonads, liver, brains and blood serum depending on the season (Owen 1936, Iguchi et al. 1991, Du et al. 2004, Desjardins et al. 2006). In the breeding season, the levels of androgens were high in male oyster toadfish, *Opsanus tau*, and showed obvious sexual dimorphism (Fine et al. 2004). Schreck and Hopwood (1974) reported that estrogen lev-

els in the female adult goldfish, *Carassius auratus*, were highest during the spawning season, indicating sexual dimorphism between males and females. In this study, the experiment was conducted from late August through February. This period was the non-breeding season of Japanese medaka (Shima and Mitani 2004). However, androgen levels were not different between males and females in the hindbrain, and no sexual dimorphism of estrogen level was found in the forebrain, midbrain or hindbrain. These results suggest that androgenic and estrogenic hormones are not necessary for acting and functioning on those target tissues.

This study provides preliminary data of AR and *ERβ* expression in the brain of the Japanese medaka. To our knowledge, this is the first study on the genus *Oryzias* to examine the AR and *ERβ* mRNA expression levels across three regions of the brain.

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