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Expression Levels of Hormone Receptor and Vitellogenin mRNAs in Livers of Thai Medaka, *Oryzias minutillus*, Inhabiting the Suburbs of Bangkok, Thailand

[1]Arin Ngamniyom and [2]Busaba Panyarachun
[1]Institute of Ecotourism, Srinakharinwirot University, Bangkok, 10110, Thailand
[2]Department of Anatomy, Faculty of Medical Sciences, Srinakharinwirot University, Bangkok, 10110, Thailand

Corresponding Author: Arin Ngamniyom, Institute of Ecotourism, Srinakharinwirot University, Bangkok, 10110, Thailand

ABSTRACT

The aim of this study was to examine the expression levels of steroid hormone receptors and vitellogenin in liver of Thai Medaka, *Oryzias minutillus* from natural environment. Adult Thai Medaka were collected from the suburbs of Bangkok, Thailand. Fish were identified as either to male or female by fin morphology and the sex was further confirmed by histological examination of the gonads. Fish whose sex was initially undeterminable (sex-undeterminable individual) but upon histological examination were determined to contain testicular or ovarian tissues were sorted into group 1 and 2, respectively. Some individuals had both testicular and oocyte tissue in the gonads and were classified into the intersex group. The level of Androgen Receptor (AR) in liver was higher in males and the group 1 than in females and the group 2. Estrogen Receptor (ER) expression in the liver was lower in males, the group 1 and intersex than in females and the group 2. ER levels in the group 1 and the intersex group were higher than those in males. Vitellogenin (Vtg) expression was measurable in the livers of females, in both sex-undeterminable groups and in the intersex group. Taking these results into consideration, the expression levels of AR and ER in liver supports the role of androgen and estrogen regulation as a biological marker of endocrine disruption in wild fish. AR and ER expression in the liver may be indicative of sexual dimorphism in normal male and female adult Thai medaka. In the sex-undeterminable and intersex individuals, the high levels of Vtg expression may indicate that the fish were exposed to feminizing stresses, possibly estrogenic chemicals.

Key words: Thai medaka, sexual dimorphism, vitellogenin, xenoestrogen, Estrogen Receptor (ER), Androgen Receptor (AR)

INTRODUCTION

In the wild, intersex or hermaphroditc fish have been reported in freshwater environments. Examples include barbell, *Barbus* sp., roach, *Rutilus rutilus* and shovelnose sturgeon, *Scaphirhynchus platorynchus* (Vigano *et al*., 2006; Jobling *et al*., 2006; Amberg *et al*., 2010). Williams *et al*. (2009) reported that the existence of intersex fish correlated with exposure to the steroid, oestrogen, in the rivers of England and Wales.

In vertebrate males androgens play a crucial role in several endocrine functions (Brinkmann *et al*., 1999). This hormone works on its target cells via androgen receptors

(Jenster *et al*., 1995). Estrogen is the equivalent of androgen in females; its functions are mediated through the oestrogen receptor and play an important role in several regulatory processes (Nimrod and Benson, 1998; Nilsson *et al*., 2001).

Vitellogenin is a female-specific protein that is the precursor of egg yolk proteins in the liver of oviparous vertebrates in response to oestrogen (Lazier and MacKay, 1993; Ota *et al*., 2000). It is difficult to measure vitellogenin level in normal male fish but males are capable of vitellogenin expression when exposed to exogenous estrogenic chemicals. Therefore, the detection of vitellogenin in male fish is used as a marker to indicate exposure to oestrogenic substances and has been proposed to be a bio indicator for endocrine disruption in wildlife (Purdom *et al*., 1994; Folmar *et al*., 2000; Hemmer *et al*., 2002; Ebrahimi, 2007).

Thai medaka, *Oryzias minutillus*, is the smallest species in the genus *Oryzias* which is widely distributed in Thailand (Lynne, 2008). The habitats of this species are shallow ponds, ditches and paddy fields (Magtoon *et al*., 1992). In general, the sex of this species can be determined by the secondary sex characteristics of their fins. In the genus *Oryzias*, the dorsal and anal fins of males are usually longer than those of females (Ngamniyom *et al*., 2011).

Recently, Ngamniyom *et al*. (2007) reported that sex-undeterminable fish of Thai medaka had been found, in suburbs of Bangkok, Thailand. In these fish, the secondary sex characteristics of the fins could not be used to distinguish males from females because, the morphology of the dorsal and anal fins was intermediate between males and females. No intersex gonads were observed upon histological analysis of those individuals.

In this study, the expression levels of the Androgen Receptor (AR), Estrogen Receptor (ER) and vitellogenin (Vtg) mRNA were examined in the livers of sex-undeterminable and intersex individuals of Thai medaka and compared the expression levels to normal males and females.

MATERIALS AND METHODS

Fish: Adult Thai medaka were captured in ponds and ditches in the Nakhonpathom and Ratchaburi Provinces which are suburbs of Bangkok, Thailand, during September through to October of 2010. The standard length of the captured Thai medaka was 11-14 mm. Average water temperature in those areas was 26±1°C. Males were distinguished from females by examining the secondary sex characteristics of the dorsal and anal fins. To determine the sex ratio (male to female), the captured individuals were fixed in 5% formaldehyde (Table 1) and their sex was then determined by examining fin morphology. Individuals with intermediate fin morphologies were classified as sex-undeterminable.

Fish gonads were dissected out, re-fixed in Bouin's solution (Wolf *et al*., 2004) for 12 h and stored in 70% ethanol. The specimens were dehydrated, embedded in paraffin and sectioned at 6 μm. The sections were stained with haematoxylin and eosin (Orlu and Gabriel, 2011).

Sex-undeterminable individuals, in which the gonads were determined to contain only testicular tissue by histological analysis were pooled into group 1. Likewise, sex-undeterminable fish with ovarian tissue in the gonads were classified into the group 2. Individuals with both testicular and oocyte tissue in the gonads were the intersex group (Fig. 1).

The normal male and female fish used for semi-quantitative RT-PCR were captured in localities 2, 3 and 7 where the sex ratio was almost 1:1. Sex-undeterminable and intersex individuals were captured from localities 1, 4, 5, 6 and 8 where the sex ratio was unbalanced (Table 1). The captured individuals were immediately put into a solution of RNA later for preservation (Qiagen, Japan). Microscopy was used to determine the phenotype of their gonads. Adult male and female of Thai

Table 1: Sex ratios of males to females and number of sex-undeterminable and intersex individuals in the wild population of Thai medaka

	Number of specimens				
Local	Male	Female	Sex-undeterminable	Intersex	Sex ratio male:female
1	16	28	4	14	1.0:1.8
2	20	19	-	-	1.1:1.0
3	29	33	-	-	1.0:1.1
4	15	28	11	2	1.0:1.9
5	10	22	8	3	1.0:2.2
6	6	17	5	8	1.0:2.8
7	20	22	-	-	1.0:1.0
8	7	23	6	6	1.0:3.3

Fish individuals with uncertain sex were determined to be sex-undeterminable on the basis of their fin morphology. Some individuals whose testicular and oocyte tissues were observed in gonad of the same individuals were determined as intersex

medaka were examined because the secondary sex-characteristics are sometimes obscure in immature individuals. Thai medaka were captured from September through November because they are easy to capture during the dry season (Ngamniyom *et al*., 2009) which is the non-breeding season for Thai medaka. Conducting experiments during the non-breeding season also ensured uniform conditions.

Semi-quantitative RT-PCR: For each group, two or three livers were pooled in each tube and ten tubes were collected. Therefore, one experimental group is representative of twenty to thirty individuals.

Total RNA from each fish sample was extracted using the isolation reagent Isogen (Wako, Tokyo, Japan) according to the manufacturer's protocol and then treated with DNase1 (Takara, Tokyo, Japan) for 30 min at 37°C. One hundred nanograms of RNA were reverse-transcribed with AMV reverse transcriptase XL (Takara, Tokyo, Japan) according to the manufacturer's instructions and 0.5 μL of the resulting cDNA was used as the template for PCR.

Primers were designed based on previous data from Japanese medaka and then were used in our 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 amplification of ER nucleotides were 5'-ACTCCCCTTTACAGCCAGTCC-3' and 5'-TGGACCAGCTCCTTGTCTGCC -3' (Lee *et al*., 2002). Primers of Vtg were 5'-CACCCGTCTCTGCTGAGT-3' and 5'- TGAAGTGGTGAGAGCTCAAACTC-3' (Hong *et al*., 2007). β-actin mRNA was amplified in each RT reaction as a loading control and reference. The primers use for amplification of β-actin were 5'-AGGGAGAAGATGACC-3' and 5'-CGCAGGACGCCATACCAA-3' according to the report of Scholz *et al*. (2004).

Amplification of cDNA was conducted using the following cycling conditions: 95°C for 30 sec for the denaturation step; 62°C (AR and Vtg), 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 amplication of AR, ER and Vtg and 20 cycles were conducted 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, ER and Vtg in each fin were divided by the amplification level of β-actin. Therefore, the stated expression levels are implied relative to β-actin.

Statistical analysis: One-way ANOVA with Tukey's multiple comparison test was used to determine whether differences were statistically significant ($p<0.05$, $p<0.01$ 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

Histological analysis of fish gonads: The testicular structure of the fish in the group 1 was identical to normal males. Additionally, the testicular cysts of normal males and those of from the group 1 both contained spermatozoa (Fig. 1a, b).

In ovaries of group 2 were compared histologically with those of normal females and appeared to be normal. Vitellogenin was normally filled in mature oocytes in those individuals which appeared to be similar to the vitellogenin in mature oocytes of normal females (Fig. 1c, d). However, in the group 2, intra-ovarian space was large compared to that of normal females (Fig. 1c, d).

In intersex individuals, both testicular and oocyte tissue was observed in the gonads (Fig. 1e). A few immature oocytes were found among the testicular tissue.

The level of AR mRNA expression in the liver of Thai medaka: AR mRNA expression in the liver was significantly higher in normal males and the fish of group 1 than in normal females and fish of group 2 (one-way ANOVA test, $p<0.05$). No significant difference ($p>0.05$) in AR mRNA levels was found between the intersex, normal fish and sex-undeterminable individuals (Fig. 2a).

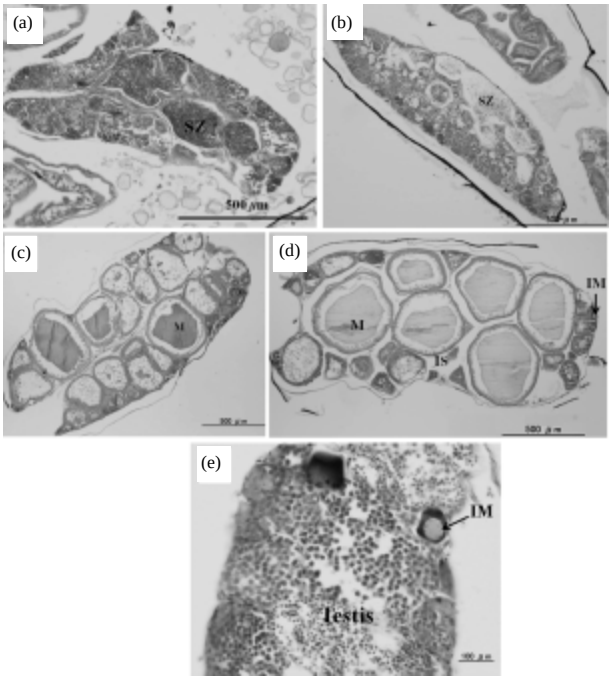

Fig. 1: (a) Histological gonads of male, (b) Group 1 of sex-undeterminable individuals, (c) Female, (d) group 2 of sex-undeterminable individuals and (e) intersex group SZ: Spermatozoa; IM: Immature oocyte; M: Mature oocyte; IS: Intra-ovarian space

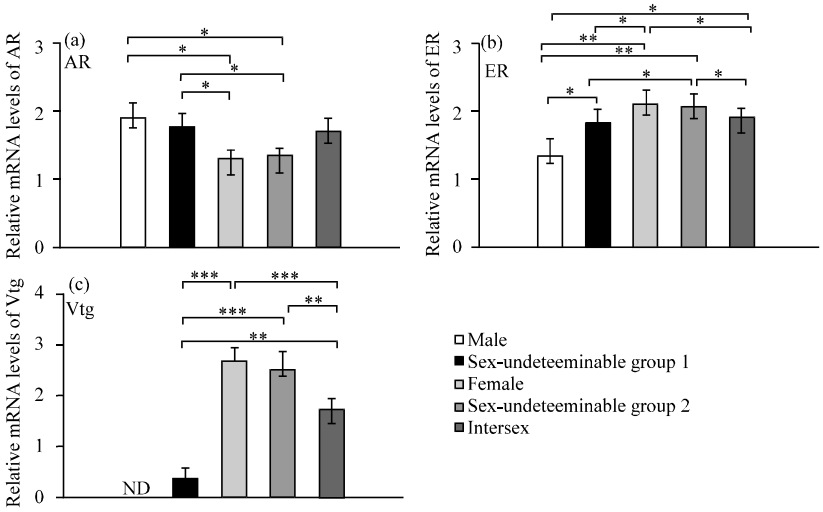

Fig. 2: Expression mRNA levels of (a) AR (b) ER and (c) Vtg in Thai medaka. Expression levels in each fish individual are relative values compared to the expression levels of β-actin mRNA (Mean±SE). Single and double-asterisk and triple-symbols show *p<0.05, **p<0.01 and ***p<0.005, respectively. Each experimental group consisted of ten samples of fish liver. ND: no detection of mRNA expression level

The level of ER mRNA expression in the liver of Thai medaka: ER mRNA expression in the liver was significantly higher I n normal female than in normal males, group 1 and intersex (one-way ANOVA test, p<0.01, p<0.05 and p<0.005, respectively) (Fig. 2b). ER mRNA expression in the liver was significantly higher in-group 2 than in normal males, group 1 and intersex (one-way ANOVA test, p<0.01, p<0.05 and p<0.005, respectively). ER mRNA levels were significantly higher in-group 1 than in normal male (Fig. 2b) (one-way ANOVA test, p<0.05).

The level of Vtg mRNA expression in the liver of Thai medaka: No expression level of Vtg mRNA was detected in the liver of normal male. In contrast, Vtg level in the liver was significantly higher in normal female and group 2 than in group 1 (one-way ANOVA test, p<0.01 and p<0.005, respectively). Vtg level was significantly higher in intersex fish than group 1 (one-way ANOVA test, p<0.01) (Fig. 2c).

DISCUSSION

Histological examination revealed that the testes and ovaries of the sex-undeterminable individuals were less developed than those normal individuals. This result corresponds with the findings of our previous report which through histological examination of the testes or ovaries of sex-undeterminable individuals were not conspicuously different histologically from those of normal males or females of Thai medaka sampling from the suburbs of Bangkok, Thailand. However, Kiparissis *et al.* (2003) reported that in Japanese medaka, *Oryzias latipes*, the increase of intra-ovarian space was evident in the individuals of which number of mature oocytes was reduced. According to these data, the presence of a large intra-ovarian space may retard the reproductive ability of medaka fish.

In contrast with our previous study, this is the first report in which intersex individuals were found among wild populations of Thai medaka in the suburbs of Bangkok. This finding is consistent

with recent reports in which the intersex shovelnose sturgeon, *Scaphirhynchus platorynchus* (Amberg *et al*., 2010) and Walleye, *Sander vitreus* (Pollock *et al*., 2010) were observed in wild freshwater environments that were contaminated with xenoestrogenic compounds. Therefore, those reports support present study that some artificial chemicals may affect the development in reproductive of Thai medaka, causing the incidence of the intersex gonads among the wild population.

AR mRNA expression in the livers of Thai medaka was found to be higher in males and fish group 1 than those in females and intersex individuals. It is well known that levels of hepatic androgens are higher in normal males than in normal females of teleost species such as Japanese medaka (Roy and Chatterjee, 1983) rainbow trout, *Salmo gairdneri* (Schulz, 1986) and fathead minnows, *Pimephales promelas* (Martyniuk *et al*., 2009). Therefore, it is clear that in Thai medaka, AR expression level in the liver is a sexually dimorphic trait in the normal development of gonads. Normally expression levels of AR mRNA were detected in the livers of females and the group 2. In teleost species androgens are converted to oestrogen by the enzyme aromatase aromatizing as part normal sexual development of females (Nakamura *et al*., 1998). Androgen activation may be required for aromatase to convert androgen to estrogen in the fish liver. There was no significant difference in the AR levels of the liver between the intersex group and any other group of fish. Iwamatsu (1999) demonstrated that oestrogens physiologically suppress some androgenic functions in Japanese medaka. Therefore, oestrogens produced from the testis-ova tissue may interfere with androgenic functions in intersex Thai medaka.

Expression level of ER mRNA in the livers was higher in the females and the group 2 than in males, group 1 and intersex fish. Similar to AR levels in males, there was a sex dependent difference in the ER expression levels in females as compared to males. Furthermore, this result suggests that oestrogens function in feminization of female fish. ER levels were also higher in the group 1 and intersex group than in normal males. ER mRNA expression increases when Olive flounder, *Paralichthys olivaceus* are exposed to 17 **β**-estradiol in (Choi, 2007) or when Japanese medaka are exposed to exogenous estrogenic bisphenol A (Hayashi *et al*., 2007). It is thus possible that some chemicals may affect endogenous oestrogen function via oestrogen receptor. Mowa and Iwanaga (2001) demonstrated that ERs are expressed in the male reproductive organs of rats which suggest a role for oestrogen in regulating tissue development and reproduction. Therefore, it supports that oestrogen may be important to some physiological role in male hepatic and reproductive tissues of Thai medaka.

Similarly to the report of Pousis *et al*. (2011), the high levels of Vtg were measured in the liver and ovaries of female Atlantic bluefin tuna, *Thunnus thynnus*. In Thai medaka, Vtg mRNA was highly expressed in the liver of normal females and in the group 2. This suggests that the vitellogenin is required for a hepatic vitellogenesis during the normal development of the ovarian gonads.

Vitellogenin is synthesized under oestrogenic control in the liver and transported to the ovary (Wahli *et al*., 1981). In male fish, the induction of vitellogenin mRNA transcription by exposure to endocrine disrupting chemicals has been examined in the livers of various fish species including Japanese medaka, *Oryzias latipes* (Inui *et al*., 2003); cunner, *Tautogolabrus adspersus* (Mills *et al*., 2003); mummichog and tilapia, *Oreochromis mossambicus* (Davis *et al*., 2009). In Thailand, the levels of xenoestrogenic DDT and endosulfan were detectable in the sediments of paddy fields and ponds from the suburbs of Bangkok (Boonyatumanond *et al*., 2002; Thapinta and Hudak, 2003). Recently, Duong *et al*. (2010) reported high concentration levels of nonylphenol, bisphenol A and genistein were found in rivers from the northeast region of Thailand. Thus, it

confirms the hypothesis that Thai medaka, sex-undeterminable fish whose gonads are contain testicular tissue, may have been affected by xenoestrogenic pollutants which caused an increase of vitellogenin level in the liver.

Vtg mRNA level was highly expressed in the liver of intersex wild Thai medaka. This finding is consistent with the reported by Bowley *et al*. (2010) that measured high Vtg levels in intersex round goby, Neogobius melanostomus from Lake Ontario, Canada, suggesting that intersex round goby were feminized by oestrogenic contaminations. Therefore, xenoestrogenic chemicals may interfere with an endocrine and reproductive systems causing immature oocyte development in testicular gonads of intersex Thai medaka.

CONCLUSION

It is surmised that some feminizing stresses may triggered in males of the Thai medaka, in the delveropment of gonads probably by activity of xenoestrogenic pollutants via AR, ER and Vtg expression level. This study used molecular biology techniques to show that AR, ER and Vtg mRNA expression in the liver is dependent on the sex of adult fish from natural environment. In addition, it supports that present data increase the knowledge regarding endocrine disruption in fresh water teleosts, although the precise physiological processes and regulatory mechanisms involved remain to be elucidated.

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