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Effects of the Insecticide Bromophos-ethyl and -Methyl on the Expression of Oestrogen Receptor α and β in the Anal Fins of Thai Medaka, *Oryzias minutillus* (Beloniformes: Adrianichthyidae)

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ABSTRACT

Bromophos-ethyl and bromophos-methyl are agricultural insecticides that occur in natural environment. The *Oryzias* fish is the genus of tiny fish that is known to be one of model organism in the biological fields such as endocrinology. The anal fins exhibit a secondary sex character controlled by sex steroid hormones and assume a sensitive indicator. Therefore, the aim of this study was to investigate the effects of the insecticide bromophos-ethyl and methyl on the expression levels of the Estrogen Receptors (ER) α and β in the anal fins of Thai medaka (*O. minutillus*). Adult fish which were exposed to 0.01, 0.1 and 1 $\mu\text{g mL}^{-1}$ of each insecticide were measured the ER levels by using a semi-quantitative PCR analysis. In males, the ER α and β expression levels in the anal fins were increased in response to treatment with both insecticides. In females, the ER α levels were also increased in response to bromophos-methyl. In contrast, the ER β levels in females were unaffected by exposure to bromophos-ethyl. Based on these results, it suggests that the effects of xenoestrogenic bromophos-ethyl and -methyl may alter in the patterns of gene expression mediated by ER α and β and depending on the sex-specific characteristics of the anal fin in Thai medaka.

Key words: Xenoestrogenic insecticides, female hormone receptors, secondary sex character, fish bioindicator

INTRODUCTION

Bromophos-ethyl and bromophos-methyl are commonly used in agricultural fields as insecticides that have been found to be contaminants in the natural environment (Lambropoulou *et al.*, 2000). Moreover, they have been shown to exhibit oestrogenic activity by inducing an increase in the oestrogen receptor levels in hamster ovary cells and these insecticides are therefore considered xenoestrogenic chemicals (Kojima *et al.*, 2004). Xenoestrogens interfere with endocrine function and the reproductive system by mimicking endogenous oestrogens via the oestrogen receptors (ERs) in humans and animals caused endocrine disruptions (Colborn, 1995). Xenoestrogenic effects have been studied in many teleosts by monitoring the levels of ER gene transcription, which are regulated in a ligand-dependent manner (Ankley *et al.*, 2010). The ERs are a hormone nuclear receptor superfamily of ligand-inducible transcription factors (Choi, 2007). Fish of the

genus *Oryzias* are a model organism that is widely used for experiments in biological fields, such as endocrinology and toxicology (Huang *et al.*, 2012). Hayashi *et al.* (2007) reported that the anal fins of Japanese medaka (*O. latipes*) were utilised to screen estrogenic compounds as a sensitive bio-indicator. Thai medaka (*O. minutillus*), the smallest in the genus *Oryzias*, is widely distributed throughout Thailand. These small fish inhabit the natural environments such as paddy field and shallow pond (Magtoon *et al.*, 1992). The anal fins of males are usually longer than those of females (Ngamniyom *et al.*, 2009). Recently, it was shown that ER β expression in the anal fins of Thai medaka was affected by exposure to oestrogenic mestranol (Ngamniyom *et al.*, 2012). Taking these reviews, it considered that anal fin assumed to be potential tool for studying the effects of xenoestrogen exposure to endocrine disruptions.

In this study, the ER α and β expression levels in the anal fins of Thai medaka were investigated after exposure to bromophos-ethyl and -methyl *in vivo*. It believed that this study contributes to the knowledge of endocrine disruption by screening xenoestrogens in a bio-indicator species.

MATERIALS AND METHODS

Fish and chemical preparation: Adult males and females of Thai medaka were maintained in separate aquariums with a 14:10 h (light: dark) cycle at $26\pm 1^{\circ}\text{C}$ for 2 weeks and fed with Tetra-KilliMin (Tetra, Tokyo, Japan). This experiment was conducted from January 2012-February 2013.

For the insecticides, 0.1, 1 and 10 mg of bromophos-ethyl (O-4-bromo-2,5-dichlorophenyl O,O-diethyl phosphorothioate) or bromophos-methyl (O-4-bromo-2, 5-dichlorophenyl O,O-dimethyl phosphorothioate) (Sigma, St. Louis, MO) were dissolved in 1 mL of dimethyl sulfoxide (DMSO) (Wako, Osaka, Japan) for the stock solutions (0.1, 1 and 10 mg mL⁻¹, respectively). A volume of 0.1 mL of each of the stock solutions was diluted with 1 L of freshwater to 0.01, 0.1 and 1 $\mu\text{g mL}^{-1}$. In the time-group experiments, males or females were kept in aquarium water containing 1 $\mu\text{g mL}^{-1}$ of bromophos-ethyl or -methyl for 3, 7 and 14 days (d) under the above conditions. In the concentration-group experiments, the medaka were treated with 0.01, 0.1 and 1 $\mu\text{g mL}^{-1}$ of each insecticide solution for 14 day. For the control group, the fish were immersed in 0.1 mL of DMSO in aquarium water without insecticide solutions for 14 day. The water in each experiment was refreshed with the same concentration every 2 day.

Semi-quantitative PCR analysis: Fish were anaesthetised with 50 $\mu\text{g mL}^{-1}$ of ethyl-3-aminobenzoate methanesulphonate (Sigma, St. Louis, MO) and the anal fins were dissected from the body using a scalpel. For each experiment, 1 tube was pooled with 4 anal fin tissues per sample and then 10 samples were prepared from 40 fin tissues. Total RNA from each sample was extracted using the RNeasy Mini Kit (Qiagen) and treated with Dnase I (Takara, Tokyo, Japan). Total RNA (80 ng) was reverse transcribed with Avian Myeloblastosis Virus (AMV) reverse transcriptase (Takara, Tokyo, Japan) according to the manufacturer's protocol. For semi-quantitative PCR, the cDNA templates (0.5 μL) were used. The primers for ER α were 5'-CACCGAGGTCACCATGATGACC-3' and 5'-GTTGCTCATGTGCCTGATGTGGG-3'. The primers for ER β were 5'-CTGTTAGATGCCTCGGACCTT-3' and 5'-GATTGGCTGTTTTCTTG-3'.

(Inui *et al.*, 2003). The primers for ribosomal protein L7 (RPL-7) were 5'-CTCCGTCTGCGCCAGATCTTC-3' and 5'-GGCCACAGGAAGTTGTTGGC-3'. RPL-7 was used as a reference gene. The optimum of PCR conditions were denaturation at 95°C for 30 sec; annealing at 57°C (ER α , ER β and RPL-7) for 1 min and extension at 72 °C for 1 min. The number of cycles for ER α and ER β was 30 and 20 cycles were used for RPL-7. The PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide and viewed under a UV transilluminator. The band density was quantified using Scion Image analysis software (version 4.0) (Scion, Maryland, USA). In the ER bands, the density of each sample was divided by the density of RPL-7 to obtain the relative expression levels.

Statistic analysis: The significant differences were analysed by one-way ANOVA with Tukey's test using SPSS, version 20. Our experimental design was approved by the ethics committee of Srinakharinwirot University, Thailand.

RESULTS AND DISCUSSION

In the male anal fins, the ER α expression levels were significantly increased in response to treatment with 1 and 0.1 $\mu\text{g mL}^{-1}$ of bromophos-ethyl at 14 day (one-way ANOVA; $p < 0.05$) (Fig. 1a, b). ER β levels were significantly increased by treatment with 1 $\mu\text{g mL}^{-1}$ of bromophos-ethyl ($p < 0.05$) (Fig. 1c, d). Significant increase was found in ER α levels by treatment of 1 $\mu\text{g mL}^{-1}$ bromophos-methyl (Fig. 1e, f) and in ER β levels by 1 and 0.1 $\mu\text{g mL}^{-1}$ at 14 day ($p < 0.05$) (Fig. 1g, h). This result confirmed the estrogenic activity of these insecticides and corresponded to the report of Kojima *et al.* (2004) that the expression of ER α and β was upregulated by bromophos-ethyl and -methyl in ovarian mammals. Therefore, the male anal fins of Thai medaka may be an alternative as a sensitive bio-indicator for estrogenic assessments.

In females, the ER α levels were significantly increased by treatment with 1 $\mu\text{g mL}^{-1}$ bromophos-ethyl for 7 and 14 day, and 0.1 $\mu\text{g mL}^{-1}$ for 14 day ($p < 0.05$) (Fig. 2a, b), but the ER β levels were not altered by chemical treatment (Fig. 2c, d). Significant increase was found in ER α levels by treatment of 1 $\mu\text{g mL}^{-1}$ bromophos-methyl (Fig. 2e, f) and in ER β by 0.1 $\mu\text{g mL}^{-1}$ for 14 day ($p < 0.05$) (Fig. 2g, h). Ngamniyom *et al.* (2012) and Ngamniyom and Panyarachun (2012) reported that the ER β levels were dramatically increased in the fins of Thai medaka following exposure to xenoestrogenic mestranol and pendimethalin, respectively. However, only the levels of the ER α subtype were stimulated by pesticides, such as methoxychlor, aldrin, chlornitrofen and dieldrin (Kojima *et al.*, 2004) in hamster ovaries. In addition, Choi (2007) reported that ER expression through oestrogen-mediated regulation was tissue-specific during the reproduction of olive flounder (*Paralichthys olivaceus*). Therefore, the result suggests that the ER expression patterns affected by xenoestrogens may include sex-specific effects on the anal fin tissue in Thai medaka. Furthermore, RPL-7 gene expression levels of Thai medaka anal fins were unaffected by exposure to those agricultural chemicals. This result also corresponded to the report of Zhang and Hu (2007), in which RPL-7 was provided as endogenous reference gene in nonresponse to an exposure of exogenous estrogens in Japanese medaka.

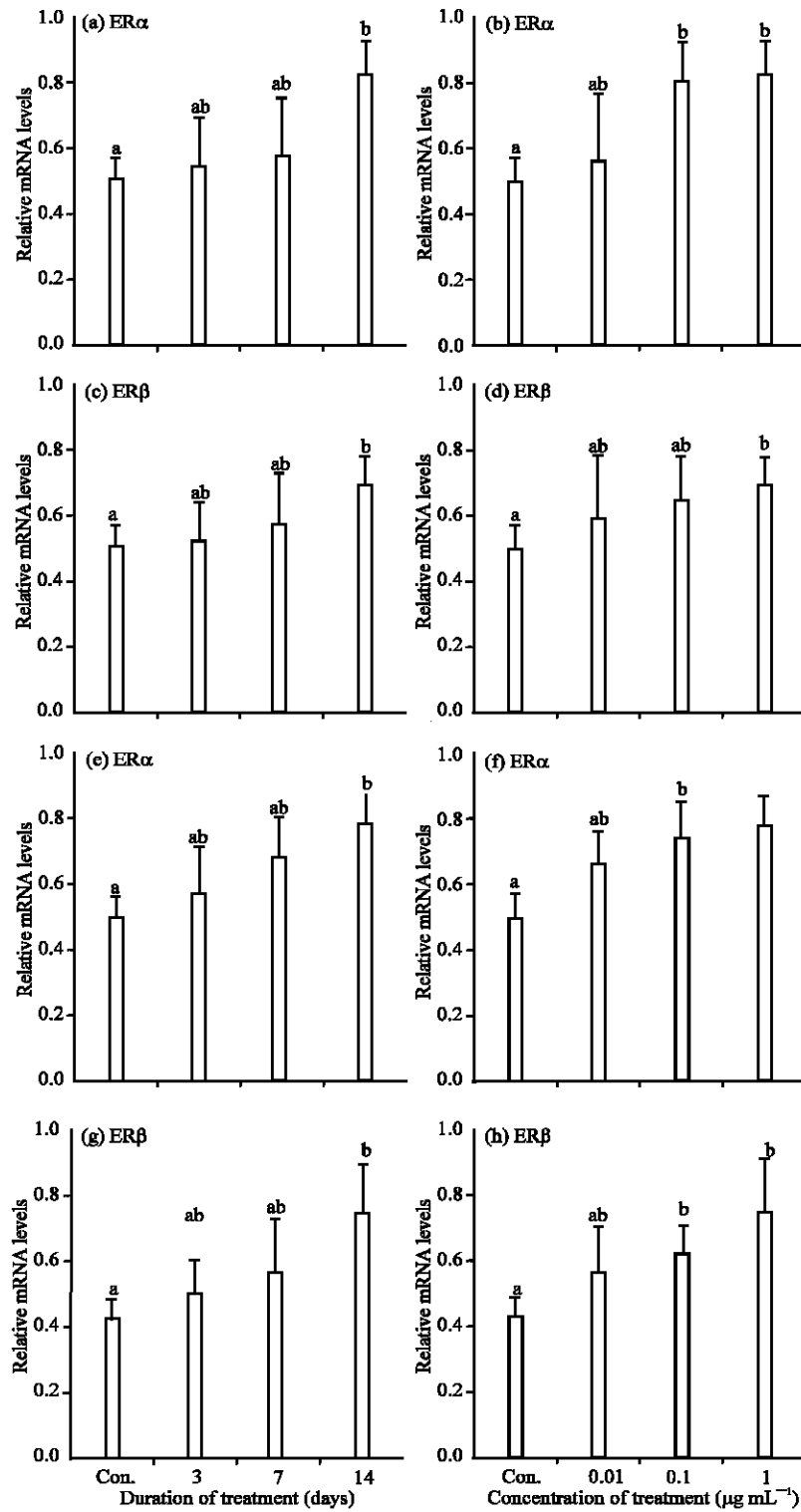


Fig. 1(a-h): Expression levels of ER α and β in anal fins of male Thai medaka exposed to bromophos-ethyl (a-d) Bromophos-methyl, (e-h) Various concentrations and durations, Con.: Control groups, expression levels in Bars indicate Mean+SE, Dissimilar alphabets indicate significant differences (one-way ANOVA; p<0.05)

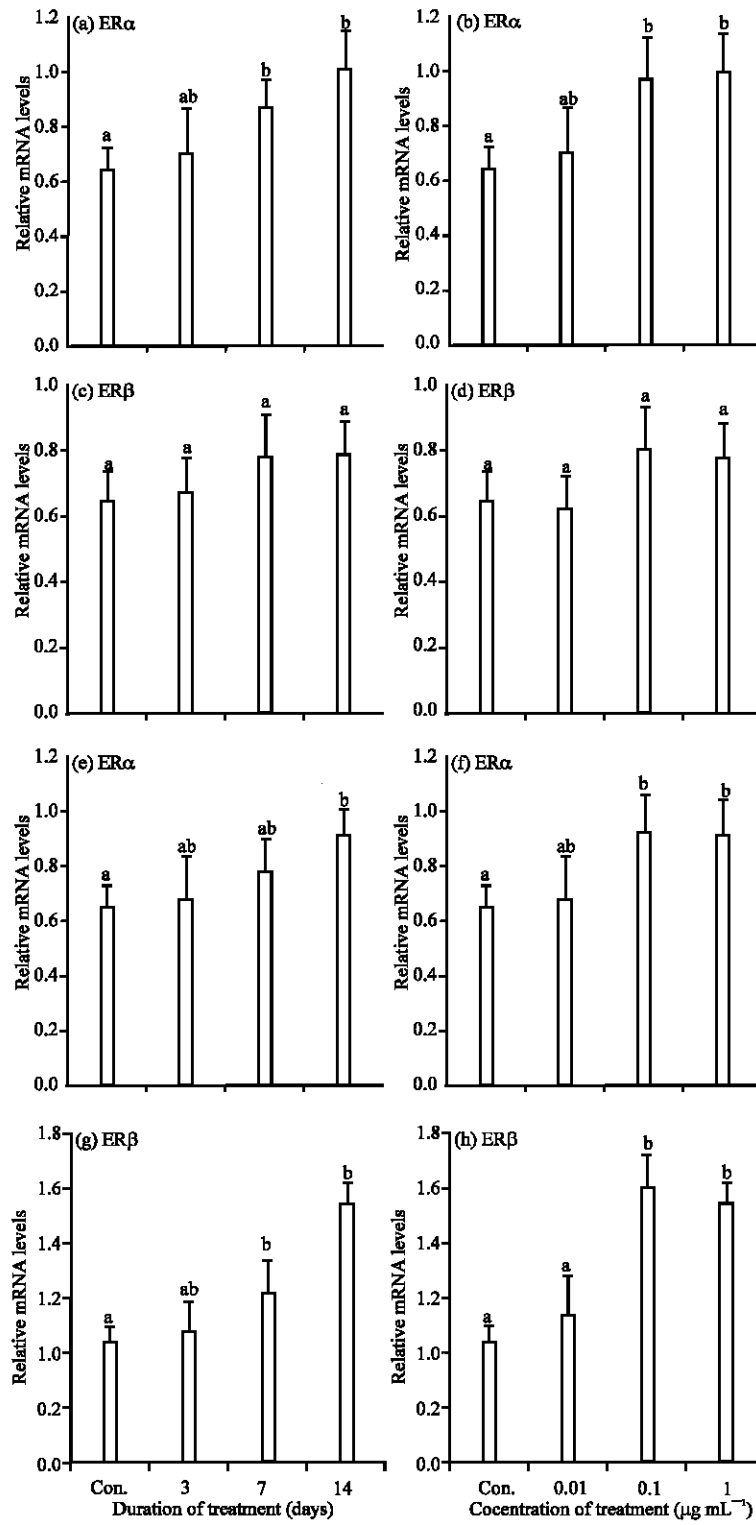


Fig. 2(a-h): Expression levels of ER α and β in anal fins of female Thai medaka exposed to bromophos-ethyl (a-d) and to bromophos-methyl (e-h) in various concentrations and durations, Con.: Control groups, Bars indicate Mean $\pm$ SE; Dissimilar alphabets indicate significant differences (one-way ANOVA; $p < 0.05$)

CONCLUSION

In summary, the present study sought to provide a sensitive bio-indicator by examining the effects of bromophos-ethyl and -methyl on the expression levels of two ER subtypes across the anal fin tissues of adult Thai medaka. This finding suggests that bromophos-ethyl and -methyl may interfere with the regulation of the levels of ER α and β by altering the gene expression patterns and the sex-dependent morphology of the anal fin in this species.

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