บทความวิจัย

อิทธิพลของโบโมฟอสเอททิลต่อการฟักไข่ การอยู่รอดของตัวเต็มวัย ความยาวครีบ และอัตราส่วนเพศของปลาซิวข้าวสารเดซี่ (*Oryzias Woworae,* Parenti and Hadiaty, 2010) (Teleostei)

อรินทม์ งามนิยม[1*] บุษบา ปันยารชุน[2] ทายาท ศรียาภัย[1] และ กัญจน์ ศิลป์ประสิทธิ์[1]

บทคัดย่อ

โบโมฟอสเอททิลเป็นยาฆ่าแมลงซึ่งส่งผลกระทบต่อการทำงานของระบบต่อไร้ท่อของสัตว์น้ำจืด และสามารถปนเปื้อนในสิ่งแวดล้อม ปลาสกุล *Oryzias* เป็นสกุลของปลาขนาดเล็กที่นิยมใช้เป็นสัตว์ทดลองต้นแบบในหลายๆ ด้านของชีววิทยาสัตว์มีกระดูกสันหลัง ปลาซิวข้าวสารเดซี่ *(O. woworae)* เป็นปลาซิวที่อาศัยอยู่ทั่วไปในประเทศอินโดนีเซีย โดยลักษณะของครีบหลังและครีบก้นมีลักษณะพิเศษในการบ่งบอกเพศซึ่งถูกควบคุมด้วยฮอร์โมนเพศ ครีบทั้งสองของตัวผู้จะยาวกว่าตัวเมียเสมอ งานวิจัยนี้มีจุดประสงค์ศึกษาอิทธิพลของโบโมฟอสเอททิลต่อการฟักไข่ การอยู่รอดของตัวเต็มวัย ความยาวครีบ และอัตราส่วนเพศของ *O. woworae* หลังจากไข่ที่ถูกเลี้ยงใน 1 $\mu g\text{-}mL^{-1}$ ของโบโมฟอสเอททิล จำนวนการฟักของตัวอ่อนและการอยู่รอดถึงระยะตัวเต็มวัยจะลดลงอย่างเป็นนัยสำคัญ ความยาวของครีบหลังและครีบก้นจะสั้นลงเมื่อเทียบกับกลุ่มปลาที่ไม่ได้รับอิทธิพลของสารนี้ อย่างไรก็ตามไม่เกิดการเปลี่ยนแปลงใดๆ ในปลาเพศเมีย ในอัตราส่วนเพศหลังได้รับอิทธิพลของ 1 $\mu g\text{-}mL^{-1}$ โบโมฟอสเอททิล เป็นเวลา 8 เดือน พบว่าจำนวนเพศเมียมากกว่าเพศผู้อย่างเป็นนัยสำคัญ จากผลการทดลองสรุปได้ว่า *O. woworae* สามารถนำมาใช้เป็นตัวบ่งชี้ทางชีวภาพที่มีความไวในการประเมินการตอบสนองต่อสารรบกวนระบบต่อไร้ท่อที่ปนเปื้อนในแหล่งน้ำธรรมชาติ สารโบโมฟอสเอททิลน่าจะเลียนแบบการทำงานของฮอร์โมนเอสโตรเจน เป็นผลให้เกิดความผิดปกติของอัตราส่วนเพศ และลักษณะคล้ายเพศเมียในครีบหลังและครีบก้นของปลาเพศผู้

คำสำคัญ: โบโมฟอสเอททิล ปลาซิวข้าวสารเดซี่ ความยาวครีบ ครีบหลัง ครีบก้น

[1]คณะวัฒนธรรมสิ่งแวดล้อมและการท่องเที่ยวเชิงนิเวศ มหาวิทยาลัยศรีนครินทรวิโรฒ

[2]ภาควิชากายวิภาคศาสตร์ คณะแพทยศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ

*ผู้นิพนธ์ประสานงาน, e-mail: arin@swu.ac.th

Effects of the Bromophos-Ethyl on Egg Hatching, Adult Survival, Fin morphometrics and Sex Ratio of Daisy's Ricefish (*Oryzias Woworae,* Parenti and Hadiaty, 2010) (Teleostei)

Arin Ngamniyom[1*], Busaba Panyarachun[2], Thayat Sriyapai[1] and Kun Silprasit[1]

ABSTRACT

Bromophos-ethyl is an insecticide that causes a negative physiological response in the endocrine functions of freshwater organisms. This insecticide is present in the natural environment as a chemical contaminant. *Oryzias* is genus of tiny fish and is a popular animal model in many fields of vertebrate biology. *Oryzias woworae* (also known as Daisy's ricefish) inhabit natural freshwater of Indonesia. The dorsal and anal fins exhibit sexual dimorphism, and the length is controlled by sex steroid hormones. The aim of this study is to investigate the effects of bromophos-ethyl on egg hatching, adult survival, fin morphometrics and the sex ratio of *Oryzias woworae,* a sensitive bioindicator. Fin morphometrics was measured as the value (%) of the dorsal fin length (DFL) or anal fin length (AFL) divided by the standard length (SL). The number of hatched fish and surviving adults decreased after the eggs were treated with 1 μg-mL^{-1} bromophos-ethyl. In adult fish, the DFL/SL% and AFL/SL% values of males were decreased, and the sex ratio was biased towards females following treatment with 1 μg-mL^{-1} of insecticide at 8 months post-hatching. These results suggest that *O. woworae* can useful in examining endocrine disruptions as a potential bioindicator. Bromophos-ethyl affected hatching and the survival rate of *O. woworae* and may exert xenoestrogenic activity by feminising the fin development of males.

Keywords: bromophos-ethyl, *O. woworae,* fin morphometrics, dorsal fin, anal fin

[1]Faculty of Environmental Culture and Ecotourism, Srinakharinwirot University

[2]Department of Anatomy, Faculty of Medicine, Srinakharinwirot University

*Corresponding author, email: arin@swu.ac.th

Introduction

Xenoestrogens are chemicals that can mimic oestrogenic hormone activity and interfere with the endocrine system, leading to abnormal reproductive physiological processes in the wild [1-3]. Bromophos-ethyl is an insecticide used against herbivorous insects in agricultural fields. This insecticide causes a negative physiological response in the endocrine systems of freshwater animals and is found in the natural environment as a chemical contaminant [4]. Kojima et al. [5] reported that this insecticide also exerts an oestrogenic action by increasing the expression levels of oestrogen receptors in Chinese hamster ovarian cells.

Oryzias (Beloniformes: Adrianichthyidae) is a genus of a small ricefish that broadly inhabit both fresh and brackish water throughout East, Central and Southeast Asia. Twenty two species have been recognised in this genus [6]. *Oryzias* are an advantageous model organism for various investigations in developmental biology, environmental biology, endocrinology and toxicology [8-10]. In this genus, the dorsal and anal fins exhibit secondary sex characteristics in the length of the fins, which is controlled by sex steroid hormones. Both of the fins in adult males are longer than those of females [11, 12]. The male anal fin is known to play a mating-related function and appears to be important in stimulating the female into oviposition, although it is not a copulatory organ [13]. In addition, the dorsal and anal fins are considered to be sensitive bioindicators for monitoring the effects of oestrogenic chemical activity [12, 14]. *O. woworae* (also known as Daisy's ricefish or Daisy's medaka) exhibit an autapomorphic colour pattern and are widely distributed in the freshwater regions of Sulawesi, Indonesia [15].

The aim of this study was to examine the effects of bromophos-ethyl on egg hatching, adult survival, fin morphometrics and the sex ratio of *O. woworae* as an alternative bioindicator for the assessment of xenoestrogenic activity.

Materials and Methods

Fish and eggs

Adult *O. woworae* (26 mm in length) were purchased from a commercial source in Bangkok, Thailand. Males and females were kept in separate freshwater aquariums with a 14:10 h (light:dark) photoperiod cycle at 26 ± 1°C for 2 weeks and fed Tetra-KilliMin (Tetra, Tokyo, Japan). Experiments were conducted from February 2012 to May 2013. Males and females were mated in the early morning to collect eggs. Twelve fertilised eggs were selected under stereomicroscopy at stage 7 of the 32 cell stage.

Chemical preparation and treatments

For the insecticide solutions, 0.1, 1, and 10 mg samples of bromophos-ethyl (O-4-bromo-2,5-dichlorophenyl O, O-diethyl phosphorothioate) (Sigma, St. Louis, MO) powder were dissolved into 1 mL of dimethyl sulphoxide (DMSO) (Sigma, St. Louis, MO) to prepare stock solutions (0.1, 1, and 10 mg · mL^{-1}, respectively). For the final working solutions, 0.1 mL from each stock solution was diluted with 1000 mL of freshwater water to obtain final concentrations of 0.01, 0.1, and 1 μg · mL^{-1}, respectively.

In each experiment, a total of twelve eggs were immersed in aquarium water containing 0.01, 0.1, and 1 μg · mL^{-1} bromophos-ethyl solutions for 8 months. For the control group, eggs were treated with DMSO in aquarium water without bromophos-ethyl for 8 months. Each experiment was repeated 5 times.

Adult males and females were removed into separate aquaria for each group after treatment with the conditions noted above. For each group, the bromophos-ethyl solution in the experimental aquaria was refreshed with the same concentration every 2 days. Fry and adult fish were fed Tetra-KilliMin 1 and 2 times per day, respectively.

Observation of egg hatching and adult survival

Hatching larvae were observed at stage 39 (hatching stage) to stage 40 (first fry stage) using stereomicroscopy. Surviving adult fish (total length exceeding 24 mm) were examined at stage 45, which is when the secondary sex characteristics of the fins first appear.

All stages of *O. woworae* development were adapted from the criteria describing developmental stages in *O. latipes* by Iwamatsu [16].

Fin morphometric analysis

The lengths of the dorsal and anal fins were measured using a digital calliper at 8 months post-hatching. In fin measurements, the measurements of the dorsal and anal fins were evaluated by dividing the dorsal fin length (DFL) and anal fin length (AFL) by the standard length (SL) and multiplying this value by 100. The resulting value was defined as DFL/SL% and AFL/SL%.

Sex ratio evaluation

Male adult fish were distinguished from female adult fish using the secondary sex characteristics of the dorsal and anal fins [15]. The sex of the fish was confirmed by observing their gonadal histology. Gonads of males and females were dissected from the fish bodies, fixed in Bouin's solution for 12 h and stored in 70% ethanol. Next, these samples were

dehydrated using a graded ethanol series, embedded in paraffin and sectioned at 6 μm using a microtome. The tissue sections were stained with haematoxylin and eosin and examined by light microscopy.

Statistical analysis

One-way ANOVA of Tukey's test and Student's *t*-test were performed to determine statistically significant differences using the Statistical Package for the Social Sciences (SPSS) version 20 (SPSS, Chicago, USA).

Animal ethics

The current experimental design was approved by the ethics committee of Srinakharinwirot University, the Department of Anatomy Faculty of Medicine, Thailand in accordance with the Guidelines for the Care and Use of Fish in Research, Teaching and Testing (http://ccac.ca/Documents/Standards/ Guidelines/Fish.pdf) [17].

Results

Egg hatching and adult survival

Egg hatching was significantly decreased following experimental treatment with $\mu g \cdot mL^{-1}$ of bromophos-ethyl when compared to control samples (one-way ANOVA: $P < 0.05$). No significant difference was found using 0.01 and 0.1 $\mu g \cdot mL^{-1}$ bromophos-ethyl treatments (Table 1).

The survival of adult fish was significantly reduced following treatment with 1 $\mu g \cdot mL^{-1}$ of bromophos-ethyl ($P < 0.05$). In contrast, the number of surviving adults was not significantly different following treatment with 0.01 and 0.1 $\mu g \cdot mL^{-1}$ of bromophos-ethyl.

Table 1 Effects of various concentrations of bromophos-ethyl on the number of hatching eggs and adult survival. DFL/SL% and AFL/SL% values in Daisy's ricefish at 8 months post-hatching.

concentration of bromophos-ethyl ($\mu g \cdot mL^{-1}$)	number of hatching eggs (mean ± SE)	number of adult survival	DFL/SL%		AFL/SL%	
			male	female	male	female
0 (control)	11.2 ± 0.3^{a}	10.4 ± 0.6^{a}	16.8 ± 0.3^{a}	15.2 ± 0.4	22.7 ± 0.4^{a}	15.4 ± 0.3
0.01	10.2 ± 0.6^{ab}	9.4 ± 0.7^{ab}	16.5 ± 0.2^{ab}	14.7 ± 0.4	22.4 ± 0.5^{ab}	15.5 ± 0.4
0.1	9.4 ± 0.5^{ab}	8.7 ± 0.3^{ab}	16.4 ± 0.3^{ab}	14.8 ± 0.4	21.5 ± 0.2^{ab}	14.9 ± 0.6
1	$8.2 \pm 0.4^{b*}$	$7.2 \pm 0.6^{b*}$	$15.4 \pm 0.4^{b*}$	15.2 ± 0.2	$20.6 \pm 0.5^{b*}$	15.6 ± 0.3

Note: Number of hatching eggs, adult survival and DFL/SL% and AFL/SL% values were compared between experiments using both the control and treatment groups. Significant differences are indicated by different letters (one-way ANOVA with Tukey's multiple test; $^{*}P < 0.05$). Each experiment consisted of 12 eggs and was repeated 5 times.

Fin morphometry

In adult males, the DFL/SL% and AFL/SL% values were found to significantly decrease after treatment with 1 $\mu g \cdot mL^{-1}$ of bromophos-ethyl ($P < 0.05$). No significant difference was observed for either value following treatment with 0.01 and 0.1 $\mu g \cdot mL^{-1}$ of bromophos-ethyl when compared to the control group (Table 1).

In adult females, the DFL/SL% and AFL/SL% values were not significantly reduced after treatment with 0.01, 0.1 and 1 $\mu g \cdot mL^{-1}$ of bromophos-ethyl.

Regarding the morphology of the male dorsal and anal fins, both fins appear to be shorter following treatment with 1 $\mu g \cdot mL^{-1}$ bromophos-ethyl when compared to the control group (Figure 1). In contrast, the female dorsal and anal fins in all treatment groups were not noticeably morphologically different when compared to the control group.

Figure 1 Fin morphology of male and female *Oryzias woworae* following exposure to various concentrations of bromophos-ethyl treatments of male dorsal fins (A-D), male anal fins (E-H), female dorsal fins (I-L) and female anal fins (M-P). Arrowheads indicate the fin edge, and bars represent 4 mm.

Sex ratio

The sex ratio (male to female) was $5.4 \pm 0.6 : 5 \pm 0.3$, $4.8 \pm 0.4 : 5.4 \pm 0.5$ and $4.6 \pm 0.5 : 4.8 \pm 0.5$ in the control group and in the experimental groups treated with 0.01 and 0.1 $\mu g \cdot mL^{-1}$ bromophos-ethyl, respectively (Figure 2). In contrast, the number of females (4.8 ± 0.4) was significantly higher in males (3.4 ± 0.2) after treatments of with 1 $\mu g \cdot mL^{-1}$ of bromophos-ethyl (Student's *t*-test: $P < 0.05$). Hermaphrodite gonads were not detected by histological analysis in any of the experimental groups for 8 months (Figure 3).

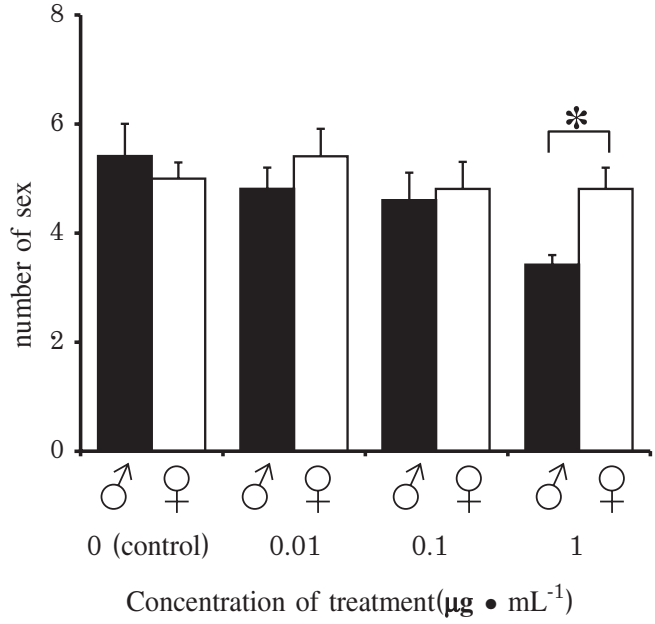

Figure 2 Sex ratio (male to female) of *Oryzias woworae* following exposure to various concentrations of bromophos-ethyl. (Student's *t*-test, $P < 0.05$). Bars indicate the mean ± SE.

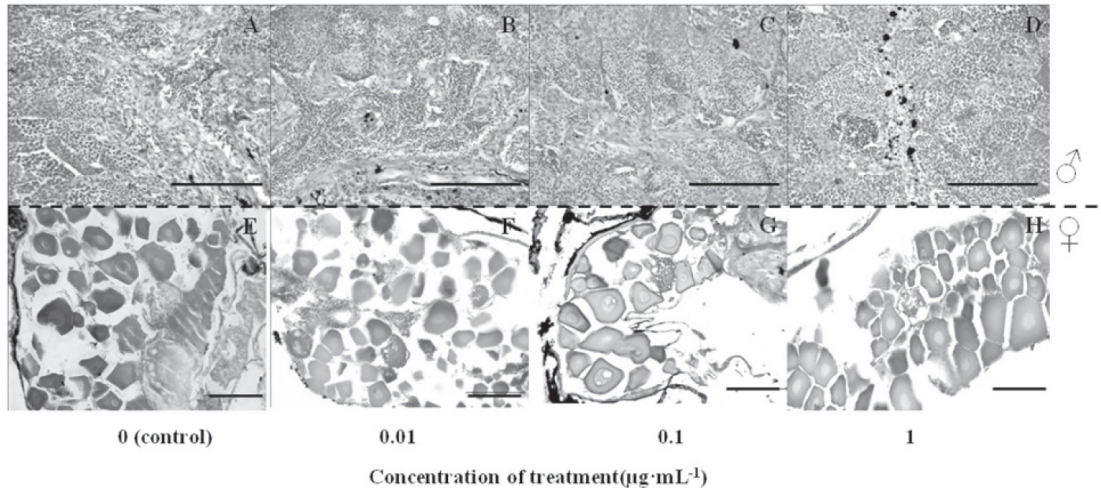

Figure 3 Sex evaluation of histological analysis stained with haematoxylin and eosin in control group and various concentrations of bromophos-ethyl treatments of males (A-D) and females (E-H)

Discussion

Recently, many studies of endocrine disruptions have been reported in which xenoestrogenic chemicals have been found to cause abnormal development in the reproductive systems of freshwater fish [18, 19]. Farmer and Orlando [20] reported that 17α-ethynylestradiol at concentrations of parts per trillion disrupts normal endocrine function, including egg viability and the sexual and gonadal development of teleosts. Reproductive success was affected as a result of demasculinisation through exposure to monocrotophos pesticide in male guppies (*Poecilia reticulata*) [21]. In this study, the insecticide bromophos-ethyl reduced the numbers of eggs hatched and surviving adults and exerted an oestrogen-like activity on the dorsal and anal fins of males and the sex ratio of *O. woworae.* These results suggest that bromophos-ethyl treatment causes abnormal sex ratios, embryo and fin development in these fish.

In males of *O. woworae,* the dorsal and anal fins were shorter when treated with bromophos-ethyl. These data are in agreement with previous reports from Ngamniyom and Panyarachun [12] and Ngamniyom et al. [22] demonstrating that pendimethalin and pharmaceutical mestranol can decrease dorsal and anal fin biometrics, respectively, in Thai ricefish (*Oryzias minutillus*). Gray et al. [23] demonstrated that the optimal development of the testis-ova was prolonged following exposure to octylphenol during gonadal differentiation in Japanese medaka. In this study, however, changes in the development of the testis-ova were not observed in adults when exposure to bromophos-ethyl was initiated close to the start of the embryonic stage. These data suggest that the concentrations of bromophos-ethyl used in this study may be not adequate to allow this insecticide to act as an oestrogenic agonist in the development of the intersex gonads in *O. woworae.* However, why bromophos-ethyl exerts a unique oestrogenic-like activity in certain physiological processes compared to that of other xenoestrogens remains unclear.

Mikula et al. [24] reported that propylparaben influenced sex differentiation processes, as indicated by a sex ratio biased towards females at 20 days post-hatching. This finding supports our result that bromophos-ethyl affects the secondary sex characteristics of male *O. woworae* and also suggests that this xenoestrogen may disrupt the endocrine system and induce feminisation in fins.

Kruger et al. [25] showed that the urogenital papillae of the male African sharptooth catfish *(Clarias gariepinus)* could be utilised as an indicator for monitoring endocrine disrupting chemicals in polluted environments in Gauteng, South Africa. This finding also supports the results of our study, suggesting that the secondary sex morphology of fins and the sex ratio in *O. woworae* may be alternative bioindicators for assessing xenoestrogenic activity.

Conclusion

The present study investigated the effects of the insecticide bromophos-ethyl using *O. woworae* as an *in vivo* model system. Our results showed that bromophos-ethyl negatively influenced the development of fish from embryo to adult. This insecticide interfered with the endocrine system and therefore has caused the feminisation or demasculinisation of the dorsal and anal fins in *O. woworae.* However, the detail of mechanism action of bromophos-ethyl in fish remains to be evaluated with regard to bioindicator tests.

Acknowledgements

The present study was supported in part by a grant from the Graduate School of Srinakharinwirot University.

References

1. Choi, S. M., Yoo, S. D., and Lee, B. M. 2004. Toxicological Characteristics of Endocrine-Disrupting Chemicals: Developmental Toxicity, Carcinogenicity, and Mutagenicity. *Journal of Toxicology and Environmental Health Part B: Critical Reviews* 7: 1-23.
2. Fusani, L., Della Seta, D., Dessì-Fulgheri, F., and Farabollini, F. 2007. Altered reproductive success in Rat Pairs after Environmental-Like Exposure to Xenoestrogen. *Proceedings of the Royal Society B: Biological Sciences* 274 (1618): 1631-1636.
3. Prado, P. S., Souza, C. C., Bazzoli, N., and Rizzo, E. 2011. Reproductive Disruption in Lambari *Astyanax fasciatus* from a Southeastern Brazilian Reservoir. *Ecotoxicology and Environmental Safety* 74 (7): 1879-87.
4. Lambropoulou, D., Sakellarides, T., and Albanis, T. 2000. Determination of Organophosphorus Insecticides in Natural Waters Using SPE-Disks and SPME Followed by GC/FTD and GC/MS. *Fresenius' Journal of Analytical Chemistry* 368: 616-623.
5. Kojima, H., Katsura, E. Takeuchi, S. Niiyama, K., and Kobayashi, K. 2004. Screening for Estrogen and Androgen Receptor Activities in 200 Pesticides by in vitro Reporter Gene Assays Using Chinese Hamster Ovary Cells. *Environmental Health Perspectives* 112: 524-531.
6. Parenti, L. R. 2008. A Phylogenetic Analysis and Taxonomic Revision of Ricefishes, *Oryzias* and Relatives (Beloniformes, Adrianichthyidae). *Zoological Journal of the Linnean Society* 154: 494-610.
7. Carlson, A., Li, Y., and Zelikoff, J. 2002. The Japanese Medaka *(Oryzias latipes)* Model: Applicability for Investigating the Immunosuppressive Effects of the Aquatic Pollutant

Benzo[a]pyrene (BaP). *Marine Environmental Science* 54: 565-568.

8. Karim, M., Daniele, P., Arul, M., Simone, P.D., and Marc, E. 2009. Medaka Proteome Study: Use of Cross-Species Matching and Expressed Sequence Tag Data for Protein Identification. *Journal of Proteomics and Bioinformatics* 2: 67-77.
9. Zhang, Z. B., Hu, J. Y., Sai, S. X., Zhao, Y. B., Huang, C., and Tian, X. J. 2008. Gene Cloning, Sequence Analysis and Tissue Expression of Estrogen-Related Receptor Alpha (Erralpha) in Japanese Medaka and its Transcriptional Responses after Differential EDCs Exposure. *Chinese Journal of Environmental Science* 29: 3153-3158.
10. Ngamniyom, A., and Sasayama, Y. 2011. Expression Levels of Sex Hormone Receptors in Brains of Japanese Medaka, *Oryzias latipes* (Actinopterygii: Beloniformes: Adrianichthyidae) *Acta Ichthyologica Et Piscatoria* 41: 29-35.
11. Ngamniyom, A., Magtoon, W., Nagahama, Y., and Sasayama, Y. 2009. Expression levels of Hormone Receptors and Bone Morphogenic Protein in Fins of Medaka. *Zoological Science* 26: 74-79.
12. Ngamniyom, A., and Panyarachun, B. 2012. Effects of the Herbicide Pendimethalin on Hormone Receptor Expressions and Dorsal Fin Biometrics in Thai Medaka, *Oryzias minutillus* (Actinopterygii: Beloniformes: Adrianichthyidae). *Acta Ichthyologica Et Piscatoria* 42: 239-246.
13. Koseki, Y., Takata, K., and Maekawa, K 2000. The Role of the Anal Fin in Fertilization Success in Male Medaka, *Oryzias latipes. Fisheries Science* 66: 633-635.
14. Hayashi, H., Nishimoto, A., Oshima, N., and Iwamuro, S. 2007. Expression of the Estrogen Receptor Alpha Gene in the Anal Fin of Japanese Medaka *(Oryzias latipes)* by Environmental Concentrations of Bisphenol A. *Journal of Toxicological Sciences* 32: 91-96.
15. Parenti, L. R., and Hadiaty, R. K. 2010. New, Remarkably Colorful, Small Ricefish of the Genus *Oryzias* (Beloniformes, Adrianichthyidae) from Sulawesi, Indonesia. *Copeia* 2010: 268-273.
16. Iwamatsu, T. 2004. Stages of normal Development in the Medaka *Oryzias latipes. Mechanisms of Development* 121: 605-618.
17. Guidelines on: The Care and Use of Fish in Research, Teaching and Testing. 2005. Canadian Council on Animal Care Available from URL: http://www.ccac.ca/Documents/Standards/Guidelines/Fish.pdf
18. Flynn, K., Swintek, J., and Johnson R. 2013. Use of Gene Expression Data to Determine Effects on Gonad Phenotype in Japanese Medaka after Exposure to Trenbolone or Estradiol. *Environmental Toxicology and Chemistry* 32: 1344-1353.

19. Saaristo, M., Tomkins, P., Allinson, M., Allinson, G., and Wong, B.B. 2013. An Androgenic Agricultural Contaminant Impairs Female Reproductive Behaviour in a Freshwater Fish. *PLoS One* 8: e62782.
20. Farmer, J., and Orlando, F. 2012. Creating Females? Developmental Effects of 17α-Ethynylestradiol on the Mangrove Rivulus Ovotestis. *Integrative and comparative biology* 52(6):769-80.
21. Tian, H., Li, Y., Wang, W., Wu, P., and Ru, S. 2012. Exposure to Monocrotophos Pesticide during Sexual Development Causes the Feminization/Demasculinization of the Reproductive Traits and a Reduction in the Reproductive Success of Male Guppies *(Poecilia reticulata). Toxicology and Applied Pharmacology* 263:163-70.
22. Ngamniyom, A., Silprasit, K., and Panyarachun, B. 2012. Effects of Pharmaceutical Mestranol on Estrogen Receptor β mRNA Expression Levels and Morphometry in the Anal Fins of Adult Thai ricefish *(Oryzias minutillus). Kasetsart Journal (Natural Science)* 43: 80-90.
23. Gray, A., Niimi, A., and Metcalfe, D. 1999. Factors Affecting the Development of Testis-Ova in Medaka, *Oryzias latipes,* Exposed to Octylphenol. *Environmental Toxicology and Chemistry* 18: 1835-1842.
24. Mikula, P., Kruíková, K., Dobšíková, R., Haruštiaková, D., and Svobodová, Z. 2009. Influence of Propylparaben on Vitellogenesis and Sex Ratio in Juvenile Zebrafish *(Danio rerio). Acta Veterinaria Brno* 78: 319-326.
25. Kruger, T., Barnhoorn, I. Jansen van Vuren, J., and Bornman R. 2013. The Use of the Urogenital Papillae of Male Feral African Sharptooth Catfish *(Clarias gariepinus)* as Indicator of Exposure to Estrogenic Chemicals in Two Polluted Dams in an Urban Nature Reserve, Gauteng, South Africa. *Ecotoxicology and Environmental Safety* 87: 98-107.

ได้รับบทความวันที่ 12 กรกฎาคม 2556
ยอมรับตีพิมพ์วันที่ 4 กันยายน 2556